Biosynthese und Funktionen von Jasmonaten – eine zellbiologische Analyse

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1 EINLEITUNG

In den letzten Jahrzehnten hat die Zellbiologie einen enormen Aufschwung genommen. Die Kombination von klassischen zytologischen Techniken mit biochemischen und molekularbiologischen Methoden sowie die Etablierung neuer Verfahren zur Analyse von Molekülinteraktionen und zur verbesserten Strukturauflösung machten es möglich, in der funktionellen Analyse von biologischen Prozessen ein neues Niveau zu erreichen. Die Detektion des räumlichen und zeitabhängigen Vorkommens von Molekülen in Zellen und Geweben gibt Hinweise auf ihre Funktion und hilft komplexe Prozesse *in vivo* zu analysieren. Dazu trugen sowohl die Weiterentwicklung mikroskopischer Verfahren als auch die Entwicklung von spezifischen Markierungstechniken bei. Hierbei sind auf Seite der Mikroskopie sowohl neue lichtmikroskopische Verfahren, wie die konfokale Laser-Scanning-Mikroskopie (CLSM), als auch elektronenmikroskopische Techniken, wie die Elektronenmikroskopie unter atmosphärischen Bedingungen (ESEM) zu nennen. Zum Nachweis von spezifischen Molekülen in Zellen wurde ein breites Arsenal von Nachweisreaktionen und Markern entwickelt. Besonders hervorzuheben sind dabei Fluoreszenzfarbstoffe, die organellspezifisch oder als Reportermoleküle in biochemischen Reaktionen eingesetzt werden können (siehe Molecular Probes, http://www.probes.com). Außerdem erweiterte die Einführung von fluoreszierenden Proteinen die Nutzung der Fluoreszenz auch für Genexpressionsstudien bzw. die Analyse der Lokalisierung und/oder Interaktion von Makromolekülen in lebenden Zellen (zur Übersicht siehe Sullivan and Kay 1999; Zhang et al. 2002). Prominentestes Beispiel ist hierfür das Grün-fluoreszierende Protein (GFP*) aus der Qualle *Aequorea victoria*, das inzwischen mehrfach mutiert wurde und damit in einer ganzen Farbpalette anwendbar ist (Gadella et al. 1999). Parallel zu diesen Nachweismethoden kommen zur Erfassung zell- und gewebespezifischer Verteilungsmuster von Proteinen und Nukleinsäuren immunologische Methoden zum Einsatz. Dabei bezeichnet der Begriff Immunzytologie die Detektion von Proteinen mittels spezifischer Antikörper, während die *in situ*-Hybridisierung den Nachweis von Nukleinsäuren umfasst. Die Detektion kann hierbei durch Enzyme oder Farbstoffe erfolgen, die an die Antikörper oder Sonden gekoppelt wurden.

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verwendete Abkürzungen:
AOC, Alleneoxidcyclase; AOS, Allenoxidsynthase; GFP, Grün-fluoreszierendes Protein; JA, Jasmonsäure; JIP, Jasmonsäure-induziertes Protein; JM, Jasmonsäure-Methylester; JRG, Jasmonat-responsives Gen; LOX, Lipoxygenase; MtPT4, Phosphat-Transporter 4 aus *Medicago truncatula*, OPDA, oxo-Phytodiensäure; OPR, OPDA-Reduktase; PIN, Proteinase-Inhibitor; RIP, Ribosomen-inaktivierendes Protein

Im Gegensatz zum Nachweis von Makromolekülen gestaltet sich bisher der Nachweis von niedermolekularen, wasserlöslichen Substanzen, wie z.B. von Phytohormonen, noch sehr schwierig oder ist derzeit noch gar nicht möglich. Um die Wirkungsweise von Phytohormonen auf zellulärer und molekularer Ebene zu verstehen, muss auch hier ihr räumliches und zeitliches Vorkommen detektiert werden. In wenigen Fällen konnten zum Nachweis von Phytohormonen monoklonale Antikörper erfolgreich eingesetzt werden (Dewitte and van Onckelen 2001). Hauptproblem dieser Methode ist jedoch die Immobilisierung der Phytohormone in der Zelle bzw. im Gewebe. Hierfür müssen Fixierungsmethoden etabliert werden, die sowohl die Bindung der Hormone an zelluläre Komponenten gewährleisten, gleichzeitig aber auch die zellulären Strukturen und die Antigenizität der Epitope erhalten. Aufgrund der Wasserlöslichkeit von Phytohormonen kommen dafür nur sehr zeit- und apparativ aufwendige, nichtwässrige Methoden in Frage. Dies ist bisher für den Nachweis von Abscisinsäure (ABA), Indol-3-Essigsäure und Cytokininen angewendet worden (Dewitte and van Onckelen 2001). Andere Hormone, wie z.B. die Jasmonate, liegen entweder in einer zu geringen Menge vor oder sind schwer zu fixieren, so dass ein immunzytologischer Nachweis bisher nicht gelang.

In den Fällen, in denen ein direkter Nachweis eines Phytohormons derzeit nicht möglich ist, kommen „indirekte“ Methoden zum Einsatz. Durch die zeitliche und räumliche Analyse der Expression derjenigen Gene, die für Biosyntheseenzyme des jeweiligen Phytohormons kodieren, können Rückschlüsse auf den Ort der Biosynthese gezogen werden. Gleichzeitig kann durch den Vergleich dieser Daten mit der räumlichen und zeitlichen Expression von der durch das jeweilige Hormon induzierten Genexpression auf die zell- und gewebespezifische Akkumulation und Wirkung geschlossen werden. Im Fall der Auxine gelang es dabei, das zellspezifische Vorkommen dieses Hormons mittels eines Auxin-regulierten Promotors über ein Reportergen zu detektieren (Friml and Palme 2002).

Ein Teil dieser sich ergänzenden Ansätze wurde in unseren Arbeiten genutzt, um die Wirkungsweise von Jasmonaten zu analysieren. Nach der subzellulären Lokalisierung der Enzyme für die ersten Schritte der Jasmonatbiosynthese (Kapitel 2) und der Analyse der Expression von Jasmonat-induzierten Proteinen als „Marker“ für eine Jasmonatwirkung (Kapitel 3), waren die Werkzeuge vorhanden, um eine zeitliche und räumliche Analyse der Jasmonatbiosynthese und -wirkung durchzuführen. Hierbei führte die Kombination der zellbiologischen Daten mit den Ergebnissen aus molekularbiologischen und biochemischen Untersuchungen, wie z.B. die Bestimmungen des endogenen Gehalts an Jas-

monaten, zu Rückschlüssen auf die Funktionen dieses Hormons in der pflanzlichen Entwicklung (Kapitel 4) und Stressantwort (Kapitel 5).

2 DIE BIOSYNTHESE DER JASMONATE – SUBZELLULÄRE LOKALISIERUNG DER ENZYME

In den letzten Jahrzehnten wurden neben den sogenannten „klassischen“ Pflanzenhormonen Ethylen, Gibberelline, Cytokinine und Abscisinsäure weitere pflanzliche Wachstumsregulatoren aufgefunden. Dazu gehören die Brassinosteroide und die Jasmonate. Beide Substanzklassen sind ubiquitär im Pflanzenreich verbreitet und besitzen pflanzenspezifische Signaleigenschaften. Die Jasmonate sind dabei den tierischen Prostaglandinen ähnlich, die bei Entzündungsreaktionen nach Verwundung gebildet werden und dort zur Entstehung von Entzündungssymptomen beitragen.

Nach der Aufklärung der Biosynthese der Jasmonsäure (JA) durch Vick and Zimmerman (1983) und dem Nachweis von JA-Verbindungen in allen höheren Pflanzen (Meyer et al. 1984) wurde deutlich, dass Jasmonate zentrale Signale in der Antwort von Pflanzen auf biotischen und abiotischen Stress darstellen (Wasternack and Parthier 1997). So führt die Wirkung von Elizitoren oder osmotischem Stress zu einem Anstieg des endogenen Jasmonatgehaltes, der seinerseits die Expression verschiedener Gene, den Jasmonat-responsiven Genen (*JRGs*) oder die Jasmonat-spezifische Herunterregulierung von „house keeping“ Proteinen bewirkt (zur Übersicht siehe Wasternack and Hause 2002).

JA ist eine von der Linolensäure abstammende Cyclopentanonverbindung mit einer Pentenyl-Seitenkette (Abb. 1). Unter den vier möglichen Stereoisomeren sind die Stereoisomere 3*(R)*,7*(R)*-(-)-JA und 3*(R)*,7*(S)*-(+)-JA die nativ vorkommenden Formen und werden auch als (-)-JA bzw. (+)-7-iso-JA bezeichnet. Die Biosynthese der JA verläuft über den Octadecanoid-Stoffwechselweg (Abb. 1; Vick and Zimmerman 1983; Hamberg 1988) und geht von α–Linolensäure (α-LeA) aus. α-LeA wird durch Aktivierung von Phospholipasen aus der Membran freigesetzt (Farmer and Ryan 1992; Narváez-Vásquez et al. 1999) und ist das Substrat für Lipoxygenasen (LOXs), die die regio- und stereospezifische Insertion von molekularem Sauerstoff in die Position C-9 (9-LOX) oder in die Position C-13 (13-LOX) katalysieren (Brash 1999). Die entstehenden Hydroperoxyfettsäure-Isomere

können anschließend durch die Enzyme des sogenannten LOX-Reaktionswegs in verschiedene Fettsäurederivate umgesetzt werden (Feussner and Wasternack 2002). Die Bildung von JA erfolgt dabei jedoch nur von dem Produkt einer 13-LOX durch eine Allenoxidsynthase (AOS) und eine Allenoxidcyclase (AOC). Aus dem Intermediat *cis*(+)-12-oxo-Phytodiensäure (OPDA) entsteht nach Reduktion der Doppelbindung im Cyclopentenonring durch die OPDA-Reduktase (OPR) und drei Schritten in der β–Oxidation die (+)-7-*iso*-JA.

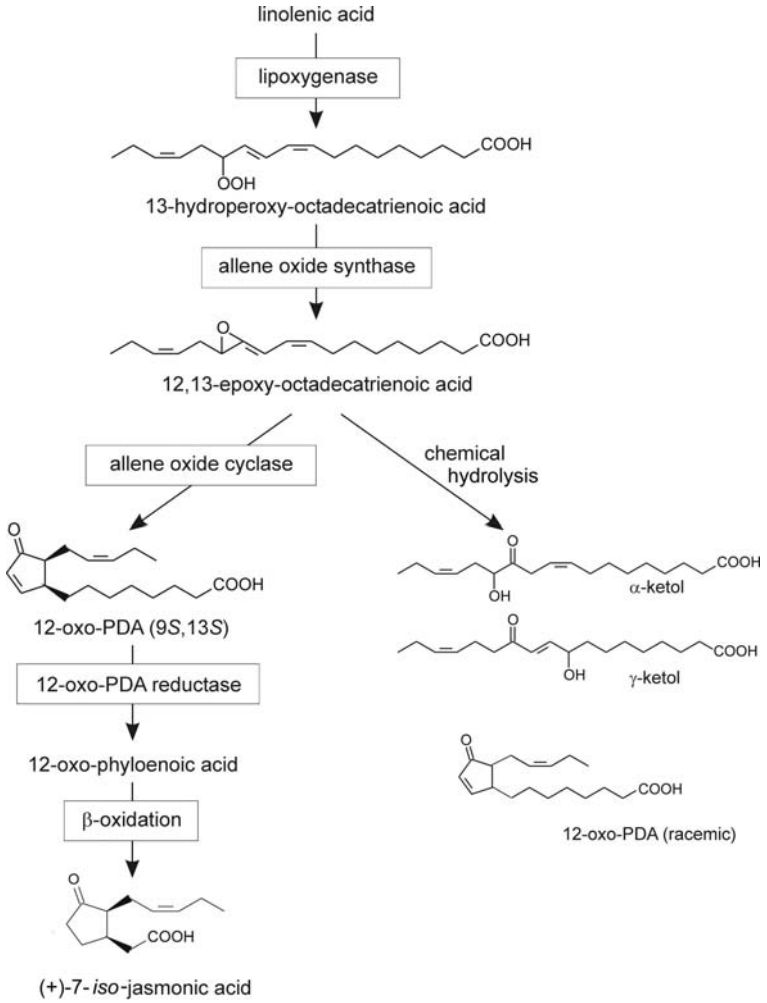

Abb. 1: Schema der JA-Biosynthese (aus Hause et al. 2000)

Mehrfach ungesättigte Fettsäuren, wie α-LeA und Linolsäure, bilden den Hauptbestandteil von plastidären Membranen (Somerville et al. 2000). Deshalb lag die Annahme nahe, dass zumindest die ersten Schritte der JA-Biosynthese in den Plastiden lokalisiert

sein könnten. In unseren Arbeiten sollte durch einen immunzytologischen Ansatz die Lokalisation von LOX, AOS und AOC geprüft werden.

In der Mehrzahl der bisher untersuchten Pflanzen wurden mehrere 13-LOXs identifiziert. Entsprechende cDNAs wurden aus Kartoffel, Tomate, Sojabohne, Arabidopsis, Gerste, Weizen und Reis isoliert (zur Übersicht siehe Feussner and Wasternack 2002). Einige dieser 13-LOXs sind transkriptionell durch Verwundung (Heitz et al. 1997), Pathogenbefall (Rancé et al. 1998) oder Behandlung mit Jasmonaten (Heitz et al. 1997; Royo et al. 1996; Vörös et al. 1998) hochreguliert, wobei bisher noch nicht gezeigt werden konnte, welche der 13-LOXs spezifisch für die JA-Biosynthese verantwortlich ist. Dabei gehören diese LOXs dem *LOX2*-Typ an, da sie eine putative Transitpeptidsequenz für den Import in Chloroplasten besitzen (Shibata et al. 1994). Ausschließlich 13-LOX-Enzymaktivität und -Reaktionsprodukte (Bleé and Joyard 1996; Bachmann et al. 2002) als auch 13-LOX-Protein (Bachmann et al. 2002; Bell et al. 1995; Feussner et al. 1995) konnten in Chloroplasten gefunden werden. Dabei ließ sich die Hauptmenge an Protein immunzytologisch nur im Stroma nachweisen (Bachmann et al. 2002), während nach biochemischer Fraktionierung Aktivitäten auch in der Chloroplastenhülle messbar waren (Bleé and Joyard 1996).

Als erstes Enzym des Jasmonat-Zweigs des LOX-Reaktionswegs setzt die 13-AOS 13*(S)*-Hydroperoxy-9*(Z)*,11*(E)*,15*(Z)*-Octadecatriensäure zur (9*Z*,11*E*,15*Z*,12*R*)-12,13-Epoxy-9,11,15-Octadecatriensäure um. Dieses Reaktionsprodukt ist in wässriger Lösung instabil und zerfällt spontan in α- und γ–Ketol oder racemische OPDA. Erst durch die AOC wird das Epoxid in das reine Enantiomer *cis*(+)-OPDA umgesetzt (Hamberg and Fahlstadius 1990). Aufgrund der kurzen Halbwertszeit des Epoxids wurde eine räumliche Nähe für die AOS und die AOC postuliert. Eine direkte Interaktion der beiden Enzyme, getestet im Hefedihybridsystem, konnten wir jedoch nicht nachweisen (Rüder 2000). Die AOS wurde zuerst aus Leinsamen gereinigt und deren cDNA kloniert (Song and Brash 1991; Song et al. 1993). Inzwischen sind die cDNAs auch aus einer Reihe anderer Pflanzenspezies bekannt, wobei die Mehrzahl ein Transitpeptid zum Chloroplastenimport enthält und im Chloroplasten lokalisiert ist (Stenzel et al. 2003a; Stenzel et al. 2003b; Wasternack and Hause 2002). Ausnahmen sind dabei die cDNAs der AOS vom Guayule-Strauch (Pan et al. 1995) und Gerste (Maucher et al. 2000). Aber auch für die Gerste konnten wir sowohl immunzytologisch als auch nach Chloroplastenisolierung zeigen, dass die AOS in diesem Organell lokalisiert ist (Maucher et al. 2000). Ein Transportmechanismus für die Gersten-AOS ist nicht bekannt, ein Import von Proteinen ohne Transitpeptid in die Plastiden ist jedoch möglich (Keegstra and Cline 1999).

Die erste Klonierung einer für eine AOC kodierenden cDNA gelang mit der Tomate (Ziegler et al. 2000). Diese und weitere AOC-cDNAs aus anderen Pflanzen weisen ebenfalls ein putatives Signalpeptid zum Chloroplastenimport auf (Stenzel et al. 2003b). Für Tomate, Arabidopsis, Kartoffel, Tabak und *Medicago truncatula* wurde die Lokalisation im Chloroplasten immunzytologisch (Stenzel et al. 2003b; Ziegler et al. 2000; unveröffentlichte Daten) oder durch Importstudien (J. E. Fröhlich, pers. Mitteilung) bestätigt.

Im nachfolgenden Schritt der JA-Biosynthese, durch die OPDA-Reduktase 3 (OPR3) katalysiert, wird nur *cis*(+)-OPDA stereospezifisch umgesetzt (Müssig et al. 2000; Sanders et al. 2000; Schaller et al. 2000). Die OPR3-cDNA enthält eine peroxisomale Targetsequenz (Stintzi and Browse 2000); das Protein ist in den Peroxisomen lokalisiert (Strassner et al. 2002). Auch die nachfolgende β-Oxidation findet in den Peroxisomen statt (Vick and Zimmerman 1983).

Damit ergibt sich das in Abb. 2 gezeigte Schema für die subzelluläre Lokalisierung der JA-Biosynthese. Nach Freisetzung von α–Linolensäure aus den Plastidenmembranen

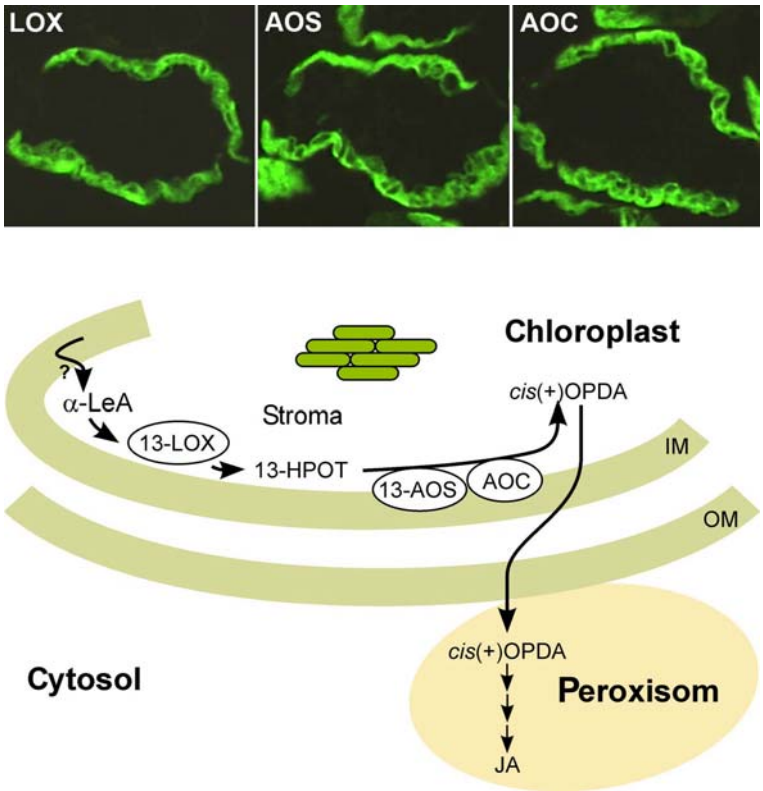

Abb. 2: Kompartimentierung der JA-Biosynthese
A. Immunzytochemischer Nachweis von LOX, AOS und AOC in den Chloroplasten der Mesophyllzellen von Arabidopsis
B. Schematische Darstellung der subzellulären Lokalisierung der JA-Biosynthese innerhalb der Chloroplasten und Peroxisomen

erfolgt die Biosynthese bis zur OPDA innerhalb der Plastiden, was in unseren Arbeiten durch die Lokalisierung der entsprechenden Biosyntheseenzyme eindeutig belegt wurde.

OPDA kann innerhalb der Plastiden in Lipiden verestert gebunden werden (Stelmach et al. 2001) und ist ebenfalls ein Signalstoff in der pflanzlichen Stressantwort, wie das anhand der Arabidopsis *opr3*-Mutante gezeigt wurde (Stintzi et al. 2001). Bei der weiteren Reaktion zur JA muss OPDA aber aus den Plastiden und in die Peroxisomen transportiert werden. Wie dieser Transport abläuft, ist bisher vollkommen ungeklärt. Die Frage, ob dabei spezifische Transportproteine involviert sind, eine Modifizierung der OPDA (z.B. als CoA-Ester) stattfindet oder ein zeitweise sehr enger räumlicher Kontakt von Plastiden und Peroxisomen eine Rolle spielt, kann derzeit noch nicht beantwortet werden.

3 JASMONAT-INDUZIERTE PROTEINE (JIPs)

Behandlung mit Jasmonaten führt zu dramatischen Änderungen in der Genexpression der meisten pflanzlichen Gewebe, die bisher analysiert wurden. Dabei ist zum Einen eine Herunterregulierung von „house keeping"-Genen zu beobachten, die z.B. für Proteine des Photosynthese-Apparates kodieren. Zum Anderen erfolgt eine transkriptionelle Hochregulierung der *JRG*s, die zur Synthese der Jasmonat-induzierten Proteine (JIPs) führt (Wasternack and Parthier 1997). Für die durch JA induzierten Proteine verschiedener Pflanzen konnten Funktionen in zahlreichen zellulären Prozessen beschrieben werden (zur Übersicht siehe Wasternack and Hause 2002). Dazu gehören u.a. die Enzyme der JA-Biosynthese und Abwehrproteine, die eine Funktion in der Reaktion der Pflanze auf Pathogene, Verwundung oder osmotischen Stress ausüben. Aber auch vegetative Speicherproteine, Proteine der Signaltransduktion, Enzyme der Sekundärstoffbiosynthese und Proteine mit einer Rolle in der Bildung von reaktiven Sauerstoffspezies konnten den JA-induzierten Proteinen zugeordnet werden.

JIPs sind erstmals in Gerstenblättern nachgewiesen worden. Hier hatte die exogene Applikation von JA neben einer stark Seneszenz-fördernden Wirkung auch die Bildung abundant auftretender Proteine zunächst unbekannter Funktion zur Folge (Weidhase et al. 1987; Hause et al. 1994). Die Akkumulation von JIPs in Gerstenblättern erfolgt außer

durch Applikation von Jasmonaten auch durch die Applikation von ABA und verschiedenen osmotische Stressoren, wie Sorbitol, Glukose und Polyethylenglykol, nicht jedoch durch NaCl und 2-Desoxyglukose (Lehmann et al. 1995). Die Reprimierung der stressinduzierten *JIP*-Expression durch Inhibitoren der JA-Synthese zeigt dabei die Abhängigkeit der JIP-Synthese von endogen gebildeter JA (Wasternack and Parthier 1997). Für einen Teil der JIPs der Gerste ließ sich bisher eine mögliche Funktion nur aus Sequenzvergleichen ableiten oder noch nicht zuordnen. Hinweise auf ihre Funktion können durch die subzelluläre Lokalisierung solcher Proteine erhalten werden. Deshalb wurden diese Untersuchungen im Rahmen unserer Arbeiten durchgeführt. Ein Schwerpunkt lag dabei neben dieser subzellulären Lokalisierung auf der Funktionsanalyse des JIP23.

3.1 Subzelluläre Lokalisation Jasmonat-induzierter Proteine der Gerste

Unter den JIPs der Gerste sind Proteine mit den Molekularmassen 6, 23, 37, 60 und 100 kDa. Sie wurden aufgrund der meist unbekannten Funktion zunächst nach ihren Molekularmassen benannt. Das JIP6 konnte als ein Thionin mit fungiziden und antibakteriellen Eigenschaften identifiziert werden (Andresen et al. 1992; Bohlmann 1994), während die Sequenz von JIP23 keinerlei Ähnlichkeiten zu bekannten Proteinen aufweist (siehe 3.2). Die Sequenz von JIP37 zeigt Ähnlichkeit zu einer Phytase (Leopold et al. 1996; Maugenest et al. 1997). Das rekombinante Protein konnte jedoch nicht enzymatisch aktiv gewonnen werden. JIP37 konnte nach JA-Behandlung des Gerstenblatts im Kern, dem Zytoplasma und der Vakuole lokalisiert werden und wurde deshalb allgemein als „Stressprotein“ eingestuft.

JIP60 ist ein Ribosomen-inaktivierendes Protein (RIP) des Typs II (Chaudhry et al. 1994; Reinbothe et al. 1994). RIPs bilden eine große Familie ubiquitär auftretender Proteine mit antiviralen und antifungalen Eigenschaften (zur Übersicht siehe Van Damme et al. 2001). Sie besitzen eine *N*-Glycosidase-Aktivität, die spezifisch ribosomale RNA schneidet. RIPs des Typs II enthalten eine Lektin-Domäne, die die Bindung an eindringende Pathogene vermitteln kann. Sie sind in der Regel vakuolär oder extrazellulär lokalisiert. JIP60 besitzt jedoch keine Lektin-Domäne. Seine intrazelluläre Lokalisierung im Kern und im Zytoplasma der Mesophyllzellen JA-behandelter Gerstenblätter spricht für eine Funktion innerhalb der Zelle nach der Induktion durch JA (Görschen et al. 1997a; Hause et al. 1994). Mit der *in vivo* nachgewiesenen *N*-Glycosidase-Aktivität könnte JIP60

zur Herunterregulierung der Translation und damit zu einer verminderten Synthese von „house keeping“-Proteinen während der Einwirkung von Stressoren beitragen (Dunaeva et al. 1999).

JIP100 ist eine der drei durch JA induzierbaren, im Chloroplasten lokalisierten Lipoxygenasen (siehe Kapitel 2). Die durch unsere Arbeiten derzeit bekannte Lokalisierung der genannten JIPs der Gerste ist in Abb. 3 zusammengefasst.

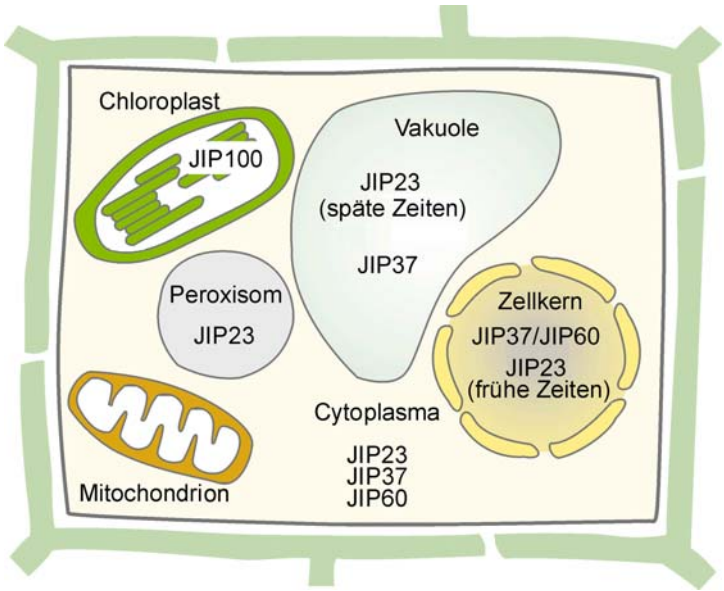

Abb. 3: Schematische Zusammenfassung der subzellulären Lokalisierung der abundanten JIPs der Gerste

3.2 JIP23

JIP23 ist das mengenmäßig am stärksten auftretende JIP der Gerstenblattsegmente. Kinetik und Dosiswirkungskurven der mRNA-Akkumulation verschiedener JIPs in Gerste belegen, dass die Akkumulation dieses JIP früher und durch niedrigere JA-Mengen als andere JIPs im Blatt induziert wird und dass schon geringe Anstiege im endogenen JA-Gehalt immer zu einer Expression von *JIP23* führen (Lehmann et al. 1995). Dabei korreliert der transiente JA-Anstieg z.B. nach Sorbitol-Stress zeitlich mit der JIP23-mRNA-Akkumulation (Kramell et al. 2000). Danach kann JIP23 bzw. seine mRNA als ein Reporter eines intrazellulären JA-Anstiegs gelten und wurde bereits mehrfach für die Analyse der JA-Wirkung bei der Entwicklung der Gerste (siehe 4.1) und ihrer Interaktion mit Mikroorganismen (siehe 5.2) genutzt.

Die Aminosäuresequenz von JIP23 (Andresen et al. 1992) weist keinerlei Ähnlichkeit zu bekannten Proteinen auf. Ähnliche Sequenzen wurden bisher nur in zwei anderen Pflanzenspezies gefunden, *Atriplex canescens* (Cairney et al. 1995) und *Mesembryanthemum crystallinum* (H. Bohnert, persönliche Mitteilung). Auch in diesen Spezies sind die entsprechenden Genprodukte Stress-induziert. Hinweise auf die Funktion von JIP23 lassen sich aus den Sequenzdaten nicht ableiten. Durch die molekularen und zellbiologischen Ansätze unserer Arbeiten sollten solche Hinweise erhalten werden.

3.2.1 Genorganisation

In der Gerste kommen mehrere Gene vor, die für JIP23 kodieren und deren Genprodukte distinkte Aufgaben erfüllen könnten. Die Analyse von 80 Gerstenkultivaren unterschiedlicher geographischer und evolutionärer Herkunft zeigte, dass alle Kultivare JIP23 während der Keimung (ohne exogene JA-Applikation) exprimieren (Hause et al. 1999). Jedoch konnte das Fehlen der JA-induzierten Expression im Blatt einiger Kultivare mit dem Fehlen eines Gens korreliert werden. Diese Zuordnung eines der JIP23-Gene zur JA-responsiven *JIP23*-Expression im Blatt wurde durch Kreuzungsexperimente und Analyse des F2-Saatguts bestätigt. Die Analyse der Promotoraktivität des JA-responsiven *JIP23* mit entsprechenden *Promotor::uidA*-Fusionen, durchgeführt im transienten Gerstenblatt-Assay, blieb jedoch ohne Ergebnis (T. Krupnova, unveröffentlicht).

3.2.2 Subzelluläre Lokalisierung und gewebespezifisches Vorkommen

JIP23 akkumuliert im JA-behandelten Gerstenblatt bis zu 25 % der Gesamtproteinmenge (Roloff et al. 1994). Es ist im Zytoplasma, in der Vakuole, den Peroxisomen und bis zu einer Inkubationsdauer von 35 h auch im granulären Bereich des Zellkerns lokalisiert (Abb. 3; Hause et al. 1994). Durch *in situ*-Hybridisierung wurde gezeigt, dass die *JIP23*-Genexpression nach JA-Applikation in allen lebenden Zellen des Gerstenblattes stattfindet (Bücking et al. 2004). Immunzytologisch konnte JIP23 in den Epidermiszellen, den Mesophyllzellen und Zellen des Phloems nachgewiesen werden (Hause et al. 1996; Hause et al. 1997).

JIP23 kommt im Gerstenblatt nach Jasmonatapplikation in sechs Isoformen vor (Hause et al. 1996). Wie die heterologe Expression der cDNA für JIP23 aus Gerste in Tabak zeigte, werden alle Isoformen von nur einer cDNA transkribiert und entstehen durch

posttranslationale Modifizierung (Görschen et al. 1997b). Interessanterweise werden alle diese Isoformen auch ohne exogene Jasmonatgabe während der Keimung zeit- und organspezifisch gebildet (Hause et al. 1996). Im Skutellum und Skutellarknoten, und hier spezifisch in den Geleitzellen, war die Akkumulation von JIP23-mRNA und -Protein nachweisbar und korrelierte mit erhöhten Jasmonat-Mengen und erhöhter Osmolalität dieser Organe. Damit sprechen die gewonnenen Daten für eine Stressprotektion durch JIP23 in Geweben erhöhter osmotischer Belastung, wie sie durch den Nährstofftransport, in diesem Fall vom Endosperm zum Keimling, auftreten kann.

3.2.3 Ansätze zur Funktionsanalyse

Eine andere Rolle von JIP23, die in der Konsequenz aber auch auf eine Stress-protektive Wirkung hinausläuft, wurde durch die heterologe Überexpression der JIP23-cDNA aus Gerste in Tabak beobachtet (Görschen et al. 1997b). Ohne signifikant veränderten Phänotyp sind „house keeping"-Proteine, wie die beiden Untereinheiten der Ribulose-1,6-bisphosphate-Carboxylase/Oxygenase (LSU und SSU), translationell herunterreguliert. Insbesondere unter Lichtstress ist dabei die Assimilationsrate erniedrigt. Die Analyse der an Ribosomen gebundenen mRNA spricht für eine Diskriminierung dieser Transkripte bei der Translationsinitiation in Anwesenheit von JIP23 aus Gerste (Görschen et al. 1997b). JIP23 scheint daher zu jener Hemmung der Translation photosynthetischer mRNA-Spezies beizutragen, die schon nach JA-Behandlung beobachtet worden war (Reinbothe et al. 1993). Eine direkte Bindung von JIP23 an mRNA oder Proteine konnte jedoch nicht nachgewiesen werden (Rüder 2000).

Ein weiterer Ansatz zur Funktionsanalyse von JIP23 in der Gerste stellt die homologe Transformation der JIP23-cDNA in sense- und antisense-Richtung dar. Sowohl die konstitutive Überexpression einerseits, als auch die Suppression der *JIP23*-Expression andererseits, sollte die Rolle dieses Proteins verdeutlichen. Besonders im Fall der konstitutiven Überexpression wurden Einflüsse auf den Translationsapparat und damit die Regulation der Expression spezifischer Gene durch eine Diskriminierung ihrer mRNAs erwartet. Es wurden Vektoren hergestellt, deren Funktionalität im transienten Protoplasten-Assay bestätigt werden konnte (Monostori et al. 2003). Trotz Etablierung des Regenerationssystems aus unreifen Skutella und Optimierung der Transformation mittels Partikelkanone konnten nur zwei putative transgene Linien nach dem Beschuss von ca. 30,000 Skutella

regeneriert werden. Die Analyse dieser Pflanzen mittels PCR ergab jedoch, dass beide nicht transgen waren (Monostori 2003).

Somit war eine direkte Analyse der Funktion von JIP23 zwar nicht möglich, aber die korrelativen Daten zur zell- und gewebespezifischen Expression bzw. zur differentiellen Expression der verschiedenen JIP23-Gene (3.2.1) lassen auf eine spezifische Funktion von JIP23 während der Keimung und in der Stressabwehr der Gerste schließen. Dabei könnte JIP23 ein allgemeines Stressprotein sein, das gewebespezifisch während der Keimung und im Blatt unter osmotischem Stress auftritt. Denkbar wäre auch, das JIP23 als „Diskriminator" für die Translationsinitiation spezifischer Gene fungiert. In beiden Fällen könnte JIP23 Funktionen der Jasmonate während der Entwicklung von monokotylen Pflanzen vermitteln (siehe 4.1).

3.3 Nictaba – ein Jasmonat-induzierbares Lektin des Tabaks

Nach Applikation von JA tritt in Tabakblättern ein Lektin auf, das spezifisch *N*-Acetylglucosamin und *N*-Acetylglucosamin-Oligomere bindet (*Nicotiana tabacum* agglutinin [Nictaba]; Chen et al. 2002). Das Auftreten dieses Proteins in allen Blattzellen und seine ausschließliche Lokalisierung im Zytoplasma und Zellkern machte eine vollkommen neue Funktion eines Hormon-induzierbaren Lektins wahrscheinlich. Proteine, die ein *N*-Acetylglucosamin- oder Chito-Oligosaccharid-Rest tragen, sind in verschiedene regulatorische Prozesse der Zelle, wie Transkription, Translation, Proteinabbau, Proteintransfer in und aus dem Kern, involviert (Wells et al. 2001). Durch die spezifische Bindung von Nictaba an diese Proteine könnten *N*-Acetylglucosamin-abhängige Signalwege moduliert werden: (i) Die Bindung von Nictaba an regulatorische Proteine, Rezeptoren und Enzyme könnte die Aktivität und/oder Stabilität dieser Proteine direkt verändern; (ii) Nictaba könnte inaktive Monomere zu aktiven Oligomeren verbinden; und (iii) Nictaba könnte den Transport von Proteinen und/oder RNA zwischen Zytoplasma und Kern beeinflussen, indem es an *N*-Acetylamin-gekoppelte Proteine des Kernporenkomplexes bindet. Für den letztgenannten Punkt sprechen neueste Daten, die zeigen, dass eine Fusion von Nictaba mit GFP nach konstitutiver Expression zunächst im Kern auftritt, später aber aus dem Kern ausgeschlossen wird und in der Nähe der Kernmembran akkumuliert (E.J.M. Van Damme, pers. Mitteilung). Damit ist dieses Protein ein Beispiel dafür, dass JIPs Funktionen in fundamentalen physiologischen Prozessen der Pflanzen besitzen können.

4 ENTWICKLUNGSABHÄNGIGE ZELL- UND GEWEBESPEZIFITÄT DER JASMONAT-BIOSYNTHESE

Kenntnisse zur Funktion von Jasmonaten in der pflanzlichen Entwicklung wurden zunächst aus den Effekten abgeleitet, die durch die Applikation von JA erzielt wurden. Dabei moduliert eine solche Applikation verschiedene Entwicklungsprozesse in Pflanzen. Die am besten beschriebenen Beispiele sind dabei die Wirkung von Jasmonaten auf die Keimung und die Entwicklung von Keimlingen, die Stimulation der Rankenkrümmung, die Ausbildung der Knollen bei Kartoffeln und die Förderung der Blattseneszenz (zur Übersicht siehe Wasternack and Hause 2002). Daneben konnte die Expression von Genen, die für Enzyme der Jasmonatbiosynthese kodieren, und die Expression von *JRG*s zeitlich mit bestimmten Entwicklungsstadien oder der pflanzlichen Organogenese korreliert werden. Besonders die Charakterisierung von Mutanten, die JA-insensitiv oder JA-defizient sind, zeigten JA-abhängige Entwicklungsprozesse auf, wie z.B. die Pollenentwicklung bei Arabidopsis (Berger 2002).

Unsere Untersuchungen hinsichtlich der Rolle von Jasmonaten in der pflanzlichen Entwicklung bezogen sich im Wesentlichen auf die Analyse der Expression von JA-Biosynthesegenen und *JRG*s in zwei unterschiedlichen Entwicklungsstadien bzw. Organen von drei Pflanzenspezies: der Keimling der Gerste (4.1) und die bis zur Blüte entwickelten Tomaten- und Arabidopsispflanzen (4.2). In beiden Fällen konnte durch die Kombination von zytologischen Techniken (Analyse der zellspezifischen Auftretens von JA-Biosyntheseenzymen bzw. JIPs) und die Bestimmung des endogenen Gehalts an Jasmonaten, Einblicke in die Zellspezifität der JA-Bildung während der Entwicklung der genannten Pflanzenorgane gewonnen werden. Durch die Diskussion unserer Ergebnisse im Vergleich mit denen anderer Arbeitsgruppen konnten neue Aspekte der Rolle von Jasmonaten in der Pflanzenentwicklung aufgezeigt werden. Es wurde deutlich, dass die Analyse von Mutanten, Ansätze zur reversen Genetik mittels transgener Pflanzen und Mutanten und der durch unsere Arbeiten verfolgte Ansatz der Analyse der zell- und gewebespezifischen JA-Bildung zu einem Gesamtbild führten.

4.1 Jasmonate in der Keimung der Gerste

Während der Keimlingsentwicklung der Gerste treten in bestimmten Geweben erhöhte Gehalte an Jasmonaten auf (Hause et al. 1996). Diese erhöhten Jasmonatmengen waren dabei hauptsächlich in photosynthetisch inaktiven Geweben messbar, wie dem Mesokotyl, dem Skutellum und der Blattbasis (Hause et al. 1996). Auch AOS-mRNA und -Protein wurden im Skutellarknoten und der Basis des Primärblattes detektiert (Maucher et al. 2000). Mittels *in situ*-Hybridisierung und Immunzytologie konnten wir erstmalig nachweisen, dass die *AOS* in den parenchymatischen Zellen, die die Leitbündel des Skutellarknotens umgeben, im jungen, noch nicht entfalteten Blatt und im Gewebe des ersten Internodiums exprimiert wird. Diese Expression der JA-Biosyntheseenzyme-Gene in den genannten Geweben könnte dann durch die Aktivität der Enzyme die Ursache des beschriebenen Anstiegs im Jasmonatgehalt sein.

Ein bekannter Effekt von Jasmonaten ist die Herunterregulation von Genen, die für Proteine des Photosyntheseapparates kodieren (Wasternack and Parthier 1997). Dies könnte im Fall von nicht-grünen Geweben einen spezifischen Schutz gegen oxidativen Stress darstellen, da diese Gewebe keine Radikalfänger wie Carotenoide besitzen. Außerdem führt der Anstieg im Jasmonatgehalt seinerseits zur Expression von *JRG*s, die dann zumindest einen Teil der Jasmonatwirkung vermitteln (Abb. 4A). So könnte JIP23, während der Keimlingsentwicklung spezifisch im Skutellum und den Geleitzellen des Phloems im Skutellarknoten exprimiert, die genannte Jasmonatwirkung verstärken und seinerseits zur Herunterregulation des Photosyntheseapparates beitragen (siehe 3.2.2).

4.2 Jasmonatbiosynthese in den Blüten von Tomate und Arabidopsis

Die Blütenentwicklung ist einer der am besten untersuchten Entwicklungsprozesse, in denen eine wesentliche Funktion von Jasmonaten und Octadecanoiden gezeigt wurde. Schon lange ist bekannt, dass relativ hohe Mengen an JA in den Reproduktionsorganen der Blüte auftreten (Creelman and Mullet 1997). Besonders hohe Mengen an Jasmonaten wurden in reifen Pollen gefunden (Miersch et al. 1998). Ein Zusammenhang dieses Auftretens von Jasmonaten und der Expression von JA-Biosynthesegenen und/oder *JRGs* konnte jedoch bis zu Beginn unserer Arbeiten nicht aufgezeigt werden. Erst unsere Untersuchungen machten deutlich, dass in der Blüte die AOC in spezifischen Geweben auftritt (Hause et al. 2000; Hause et al. 2003b). Mittels des immunzytologischen Nachweises der AOC konnten

wir zeigen, dass diese in Tomatenknospen abundant in den Samenanlagen zu finden ist. Auch die Gene für die Vorläuferenzyme in der JA-Biosynthese, die *LOX* und die *AOS*, sind in diesem Blütenorgan exprimiert (Rodríguez-Concepción and Beltrán 1995; Kubigsteltig et al. 1999).

Mit dem Auftreten der Biosyntheseenzyme korreliert eine Akkumulation von Octadecanoiden und Jasmonaten in den untersuchten Geweben – in den einzelnen Blütenorganen der Tomate konnten spezifische Oxilipinmuster nachgewiesen werden (Hause et al. 2000; Miersch et al. 2004). Damit wird eine spezifische Funktion von Jasmonaten in der Entwicklung von Blüten wahrscheinlich. Interessanterweise werden *JRG*s ebenfalls spezifisch in den Samenanlagen exprimiert. Dazu gehören solche, die für Histone, die Leucin-Aminopeptidase, PIN2 oder andere, in die pflanzliche Abwehr von biotischem und abiotischem Stress involvierte Proteine, kodieren (zur Übersicht siehe Wasternack and Hause 2002).

Da die Gene der JA-Biosyntheseenzyme durch Glukose transkriptionell regulierbar sind und Organe wie die Samenanlagen ein starkes Sink-Organ für Kohlenhydrate darstellen, lässt sich aus den Ergebnissen unserer Arbeiten das in Abb. 4B dargestellte hypothetische Schema für eine JA-Funktion in Sink-Organen ableiten: Der erhöhte Eintransport von Glukose könnte in solchen Organen die Transkription der Gene für die JA-Biosyntheseenzyme induzieren. Die Aktivität der dann synthetisierten Enzyme führt zu erhöhten Gehalten an Octadecanoiden und Jasmonaten, die dann wiederum die Expression von „Schutzproteinen“ induzieren. Für *PIN2* und *VSP1* wurde dies mittels *in situ*-Hybridisierung kürzlich gezeigt (B. McCaig, persönliche Mitteilung). Damit könnte ein Schutz gegen Pathogene entstehen, die die für die Entwicklung der Samenanlagen erforderliche Nährstoffzufuhr vermindern bzw. die Reproduktionsorgane schädigen würden.

Eine andere Funktion von Jasmonaten in der Blütenentwicklung lässt sich aus der Untersuchung von JA-defizienten oder -insensitiven Mutanten ableiten. Drastische Unterschiede zeigten sich in Abhängigkeit von der Pflanzenspezies: Während JA-Biosynthese- oder insensitive Mutanten von Arabidopsis nicht mehr in der Lage sind, fertile Pollen zu bilden (männliche Sterilität; Berger 2002; Turner et al. 2002), ist eine JA-insensitive Tomatenmutante in der Ausbildung der Samenanlagen und/oder in der Embryoentwicklung gestört (weibliche Sterilität; Li et al. 2004). Diese Befunde korrelieren mit den von uns erhaltenen Daten zur Lokalisierung der JA-Biosyntheseenzyme in den Mikrosporenmutterzellen (AOC) und Pollen (AOS) von Arabidopsis (Hause et al. 2003b) bzw. in den Samenanlagen von Tomate (Hause et al. 2000). Wie hierbei endogene Jasmonate die

einzelnen Entwicklungsphasen der reproduktiven Organe der Pflanzen regulieren, ist noch nicht geklärt. Zumindest für die Pollenentwicklung bei Arabidopsis wird ein Einfluss auf den Wassertransport und der damit verbundenen Elongation des Filaments sowie der Freisetzung der Pollen nach Abbau des Tapetums diskutiert (Ishiguro et al. 2001; Sanders et al. 2000).

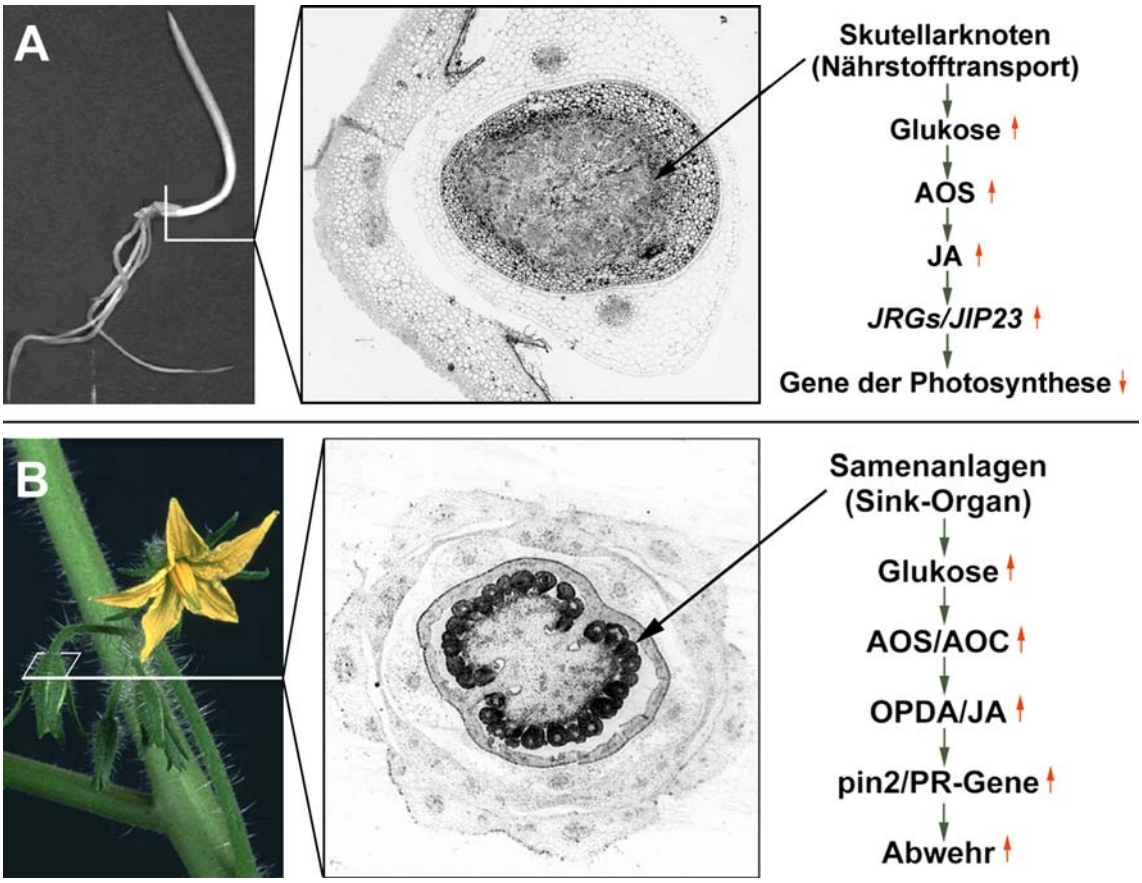

Abb.4: Hypothetisches Schema für die Induktion der JA-Biosynthese und die Wirkung von JA während der Keimung der Gerste (A) und der Blütenentwicklung der Tomate (B). Die roten Pfeile zeigen die veränderte Akkumulation von Transkripten bzw. Metaboliten. an.

5 JASMONATE IN DER PFLANZLICHEN ANTWORT AUF BIOTISCHEN STRESS

Octadecanoide und Jasmonate treten nicht nur wie in Kapitel 4 beschrieben bei pflanzlichen Entwicklungsprozessen auf, sondern akkumulieren transient in Folge von abiotischem und biotischem Stress. Dabei vermitteln sie einen Teil der Abwehrmechanismen der Pflanze zur Anpassung an wechselnde Umweltbedingungen. Frost, Kühle, Hitze, Trockenheit, Wassermangel, Sauerstoffmangel, Salzstress, Verwundung, Interaktion mit Mikroorganismen, Lichtstress, UV-Strahlung sowie osmotischer, mechanischer und oxidativer Stress sind Umweltfaktoren, die Pflanzen zur Anpassung oder Abwehr veranlassen können und bei denen eine Jasmonatakkumulation beobachtet wurde.

Unsere Arbeiten bezogen sich schwerpunktmäßig auf die Analyse der Funktion der Jasmonate in der Reaktion der Pflanzen auf zwei dieser genannten Faktoren: die Reaktion auf Verwundung und die symbiontische Interaktion mit Mykorrhizapilzen. Beide Prozesse wurden wiederum mittels zellbiologischer Methoden analysiert. In der Kombination mit molekularen, biochemischen und analytischen Techniken konnte die Funktion von Jasmonaten als Signalstoff in diesen Prozessen verifiziert und in einem Modell zusammengefasst werden.

5.1 Jasmonate als Signal in der Verwundungsreaktion der Tomate

Bekanntestes Beispiel einer Signalwirkung von Jasmonaten in der Pflanzenabwehrreaktion ist die Reaktion von Pflanzen auf Verwundung durch Herbivoren. Drei verschiedene Mechanismen, in die Jasmonate als Signalmoleküle involviert sind, wurden hierfür beschrieben (zur Übersicht siehe Wasternack and Hause 2002): (i) die Synthese von proteinogen Abwehrstoffen wie Proteinase-Inhibitoren (PINs), die die Verdauung des durch die Herbivoren aufgenommenen Blattmaterials stören; (ii) die Synthese von Phytoalexinen wie z.B. Nikotin, die direkt auf die Herbivoren wirken und (iii) die Bildung von volatilen Substanzen, die als Teil einer tritrophen Interaktion Schädlinge der Herbivoren anlocken. Durch unsere Analysen zum Auftreten der JA-Biosynthese-Enzyme im Zusammenhang mit der Bildung von JA während der Verwundung von Tomate konnten wir einen Beitrag dazu leisten, die Rolle von JA im erstgenannten Mechanismus (Bildung von PINs) aufzu-

klären. Hierbei lag der Schwerpunkt vor allem auf der lokalen Wundantwort, d.h. in der Reaktion des verwundeten Blattes selbst.

Die Gruppe von C. A. Ryan konnte zeigen, dass Jasmonate und das Peptid Systemin, bestehend aus 18 Aminosäuren, Signale der Wundantwort sind. Systemin könnte dabei eine duale Funktion besitzen und gleichzeitig als lokales und systemisches Signal wirken. Es wird aus einem Precursorprotein, dem Prosystemin, gebildet und leitet die Induktion von PINs ein. Dabei ist sowohl die Biosynthese als auch die Wirkung von JA erforderlich (Ryan 2000). Prosystemin wird konstitutiv im Leitgewebe der Tomate exprimiert (Jacinto et al. 1997). Erst die Verwundung führt zur Bildung von Systemin, das an einen Membran-lokalisierten Rezeptor bindet (Scheer and Ryan 2002) und damit zur Expression von sogenannten „frühen Genen“ führt (Stratmann 2003). Gleichzeitig konnte eine Systemin-abhängige Aktivierung der AOC, wahrscheinlich durch die Freisetzung von Linolensäure aus den Membranen, gezeigt werden. Darauf folgt ein schneller Anstieg des endogenen JA-Gehaltes im verwundeten Blatt (Ryan 2000; Stenzel et al. 2003a). Da die Bildung des Prosystemins durch Jasmonat induzierbar ist, könnte JA lokal auch zu einer Potenzierung der Systeminwirkung beitragen. Hinzu kommt, dass die AOC als Schlüsselenzym der JA-Biosynthese auch im Leitgewebe der Tomate, also im selben Gewebe wie Prosystemin, konstitutiv exprimiert wird (Hause et al. 2000). Da sich zumindest die ersten drei Enzyme der JA-Biosynthese selbst in den Siebelementen der Leitgewebe nachweisen ließen (Hause et al. 2003a), wird eine Funktion von JA auch in der systemischen Wundantwort wahrscheinlich. Dies wurde besonders durch Ergebnisse der Arbeitsgruppe von G. Howe deutlich, die durch inverse Pfropfungen von einer JA-insensitiven und einer JA-Biosynthesemutante zeigen konnten, dass im verwundeten Blatt die JA-Biosynthese und im systemischen Blatt die JA-Perzeption notwendig ist, um eine systemische Antwort zu erhalten (Li et al. 2002).

Obwohl deutlich ist, dass eine mögliche Amplifikation des Wundsignals über die Systemin-abhängige Aktivierung der AOC und die JA-induzierbare Expression des Prosystemin-Gens in den Leitgeweben erfolgt, ist jedoch noch immer unklar, wie die Signalkette weiter zur Expression der „späten“ Gene, wie z.B. *PIN2*, verläuft. Diese Expression erfolgt im Blattmesophyll und erfordert damit einen aktiven Signaltransfer oder zumindest eine Diffusion eines Signalstoffes vom Leitgewebe zum Mesophyll. Obwohl JA als Induktor der „späten“ Gene für solch einen Transfer denkbar ist, ist bisher nichts darüber bekannt, wie und wo JA akkumuliert und die JA-Perzeption stattfindet. Die Existenz eines oder mehrerer JA-Rezeptoren ist zwar wahrscheinlich, aber bisher konnte solch ein Re-

zeptor nicht identifiziert werden (Howe 2001). Erste Hinweise, dass JA vermutlich an der Plasmamembran perzipiert wird, konnten wir mittels mikroautoradiographischer Analysen nach Applikation radioaktiv-markierter JA an Tomatenblätter erhalten: Radioaktivität ließ sich nur im Apoplasten nachweisen. Die Mesophyllzellen wiesen dagegen keine intrazelluläre Markierung auf (Bücking et al. 2004). Diese Ergebnisse führten zu dem in Abb. 5 dargestellten Schema, das unsere Kenntnisse zur lokalen Wundreaktion in Tomate zusammenfasst.

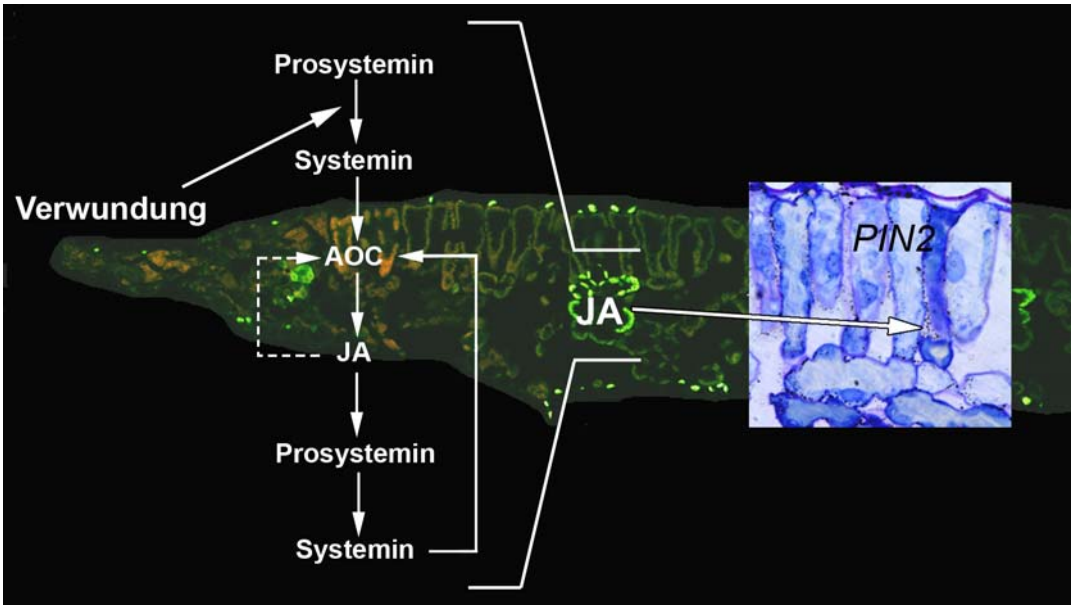

Abb. 5: Schematische Darstellung der lokalen Wundantwort in Tomatenblättern
Die Aktivierung der AOC durch Systemin (JA-Synthese) und die JA-induzierbare Expression des Prosystemin-Gens (Systemin-Bildung) führt zu einer Amplifizierung der wundinduzierten JA-Bildung innerhalb der Leitbündel. Da *PIN2* JA-abhängig im Mesophyll exprimiert wird, könnte JA auch ein interzelluläres Signal sein.

5.2 Jasmonate in der Mykorrhiza

Die arbuskuläre Mykorrhiza (AM) ist unter den mutualistisch-symbiontischen Interaktionen von Pflanzen mit Pilzen die Mykorrhizaform mit der weitesten Verbreitung. Über 80 % der Landpflanzen besitzt die Fähigkeit, mit Pilzen der Ordnung *Glomales* diese Symbiose einzugehen. Dabei wächst der Pilz in den Kortex der Pflanzenwurzel, formt dort intraradical Hyphen aus und bildet innerhalb der Kortexzellen hoch differenzierte, verzweigte Strukturen, die Arbuskeln (zur Übersicht siehe Strack et al. 2003). Über Hyphen und Ar-

buskeln erfolgt der Stoffaustausch zwischen den Partnern. Die Pflanze stellt Kohlenhydrate für den obligat heterotrophen Pilz zur Verfügung (zur Übersicht siehe Harrison 1998), die dieser wahrscheinlich über die intraradicalen Hyphen aufnimmt (Bago 2000; Gianinazzi-Pearson 1996). Die äußeren Hyphen des Pilzes bilden die Verbindung zwischen Boden und Pflanze und gewährleisten damit eine verbesserte Aufnahme von Nährstoffen, wie z.B. von Phosphat, das dann durch die Arbuskeln an die Pflanze weitergeben wird (Harrison et al. 2002; Rausch and Bucher 2002). Der gegenseitige Stoffaustausch zwischen den Partnern und die damit verbundene differentielle Genexpression ist ein wesentliches Merkmal für die Funktionalität der Symbiose.

Das Wachstum des Pilzes innerhalb der Wurzel und die Entwicklung von Arbuskeln ist nur auf den Wurzelkortex beschränkt und scheint von der Pflanze kontrolliert zu werden (zur Übersicht siehe Harrison 1999). Über mögliche Signale bei der Etablierung der Symbiose ist noch wenig bekannt. Verschiedenen Phytohormonen konnte im Zusammenhang mit bestimmten physiologischen Prozessen während der Etablierung der AM eine Rolle zugeschrieben werden (Barker and Tagu 2000; Bothe et al. 1994; García-Garrido and Ocampo 2002; Geil et al. 2001). Die Kenntnisse zur möglichen Funktion entstammen dabei vorwiegend Experimenten mit exogener Applikation des jeweiligen Hormons oder wurden aus Gehaltsänderungen abgeleitet. Funktionelle Ansätze zur Klärung der Funktion dieser Phytohormone wurden bisher nicht veröffentlicht. Ebenso wird erst die umfassende Analyse der Genexpression (Expressionskartierung mittels cDNA-Arrays) von mykorrhiziertem Gewebe eine Identifizierung der Prozesse, die durch Phytohormone gesteuert werden, möglich machen (Barker and Tagu 2000).

Eine mögliche Funktion der Jasmonate und der durch sie induzierten Gene ist auch bei der Ausbildung der Mykorrhiza wahrscheinlich: (i) Die Applikation von Jasmonaten während der Etablierung der AM in *Allium sativum* fördert die Entwicklung der Arbuskeln (Regvar et al. 1996); (ii) Hydroxyzimtsäureamide, die während der Mykorrhizierung von Gerste akkumulieren, sind in nicht-mykorrhizierten Wurzeln durch Jasmonate induzierbar (Peipp et al. 1997); und (iii) während der Mykorrhizierung der Gerste steigt der endogene Gehalt an Jasmonaten auf das Fünffache der nicht-mykorrhizierten Kontrolle an (Hause et al. 2002). Unsere Arbeiten konnten dabei eindeutig belegen, dass dieser Anstieg von einer Expression von Genen begleitet ist, die einerseits für JA-Biosyntheseenzyme und andererseits für JA-induzierte Proteine kodieren. Da die Erhöhung des JA-Gehaltes in mykorrhizierten Wurzeln erst nach den ersten sichtbaren Stadien der Interaktion erfolgt, scheint vorrangig die Entwicklung der Interaktion und nicht die gegenseitige Erkennung der Inter-

aktionspartner für die Expression der Gene und den daraus folgenden JA-Anstieg verantwortlich zu sein (Hause et al. 2002). Damit stellt sich für die Mykorrhizierung die Frage, ob Jasmonate, die als Reaktion auf die Kolonisierung durch den Pilz gebildet werden, spezifisch das Wachstum und die Ausbreitung des Pilzes in der Wurzel kontrollieren oder einen Teil der pflanzlichen Abwehr bilden. Damit könnten Jasmonate auch zu dem häufig beschriebenen, verbesserten Abwehrstatus mykorrhizierter Pflanzen beitragen. Mykorrhizierte Pflanzen weisen eine erhöhte Toleranz gegenüber Trockenstress (Augé 2001) und Sekundärinfektionen mit Pathogenen (Cordier et al. 1998) auf.

In Analogie zu Daten aus anderen Organen und Geweben von Pflanzen, in denen Jasmonate erhöht auftreten und die damit typische Sink-Gewebe bzw. -Organe darstellen, könnte folgendes Szenario für die JA-Biosynthese und JA-Wirkung in der Mykorrhiza möglich sein:

Erhöhte Sinkfunktion der mykorrhizierten Wurzel,
ausgelöst durch die Versorgung des Pilzes mit Kohlenhydraten
⇓
Verstärkte Akkumulation von löslichen Zuckern im Apoplasten
⇓
Induktion der Expression der Gene der JA-Biosynthese
⇓
Erhöhte JA-Gehalte
⇓
Schutz der Zellen vor osmotischem Stress und/oder
Erhöhung des Abwehrstatus der gesamten Pflanze gegen Pathogene

Eine Überprüfung dieses Arbeitsmodells mittels einer funktionellen Analyse der JA-Wirkung konnte im Gerstensystem nicht durchgeführt werden, da die Generierung von transgenen Pflanzen hier nicht gelang. Deshalb wurde in unseren weiteren Arbeiten die Modell-Leguminose *Medicago truncatula* genutzt, da hier sowohl die stabile Transformation mittels *Agrobacterium tumefaciens* (Trinh et al. 1998) als auch eine Wurzeltransformation mittels *A. rhizogenes* mit anschließender Mykorrhizierung möglich ist (Vieweg et al. 2004).

Auch bei der Mykorrhizierung von *M. truncatula* lässt sich ein Anstieg des endogenen JA-Gehaltes nachweisen, der ebenfalls von der erhöhten Expression eines Gens begleitet ist, das für eines der JA-Biosyntheseenzyme kodiert (Isayenkov et al., in Vorbereitung). Dass auch in der Wurzel von *M. truncatula* eine Vielzahl von Genen durch JA reguliert wird, zeigte ein Expressionsprofiling, das in Zusammenarbeit mit H. Küster (Universi-

tät Bielefeld) durchgeführt wurde. cDNA-Macro-Arrays mit ca. 6000 Wurzel-spezifischen ESTs wurden mit der RNA aus Wurzeln hybridisiert, die 2 h, 4 h und 24 h mit 50 µM JA-Methylester (JM) behandelt wurden. Im Vergleich zu unbehandeltem Material wurden starke Effekte von exogen applizierten JM auf das Expressionsmuster in Abhängigkeit von der Dauer der Behandlung deutlich. Unter den transkriptionell herunterregulierten Genen befanden sich solche, die für Metallothionine, Saccharosesynthase, Zuckertransporter und Mitglieder der Ethylen-Signalkette kodieren, während die Expression von Genen induziert wird, die z.B. für die Biosyntheseenzyme von JA (LOX, AOC), eine Lipase und wundinduzierte Proteine kodieren. Für diese Gene wurde bereits für andere Pflanzen die Induktion durch JA bzw. JM beschrieben (siehe Wasternack and Hause 2002), so dass sie als Positiv-Kontrollen für diese Methode gewertet werden konnten. Außerdem ergab die Überprüfung der am stärksten durch JM regulierten Gene im „Electronic Northern“, dass ca. 25 % dieser Gene ebenfalls Mykorrhiza-induziert sind.

Nach Transformation von *M. truncatula* mit der AOC-cDNA in sense bzw. antisense-Richtung sollten Effekte durch die Modulation des endogenen JA-Gehaltes auf die Mykorrhizierung sichtbar werden. Durch die partielle Suppression der *AOC*-Expression in Wurzeln (Wurzeltransformation mit *35S::AOCantisense*) ließen sich drastische Veränderungen im Mykorrhizierungsgrad als auch in der Expression eines mykorrhiza-spezifischen Phosphattransporters, (*MtPT4*; Harrison et al. 2002) feststellen (Isayenkov et al., in Vorbereitung). Transgene Pflanzen mit einem verminderten JA-Gehalt in den Wurzeln zeigen im Vergleich zum Wildtyp niedrigere Mykorrhizierungsraten, die mit einem erniedrigten Expressionslevel von *MtPT4* als auch mit Wachstumsdefiziten der Pflanze korrelieren. Diese Daten sprechen für eine essentielle Rolle der Jasmonate für das „Funktionieren“ der Symbiose. Ob Jasmonate dabei die Arbuskelbildung oder den Stoffaustausch zwischen den Interaktionspartnern beeinflussen, ist noch ungeklärt. Auch indirekte Effekte über eine erhöhte „Fitness“ der Pflanze und eine damit verbesserte Kohlenhydratversorgung des Pilzes wären denkbar und sollen in Zukunft auf verschiedenen Ebenen überprüft werden. Hierzu zählen beispielsweise die Analyse des Genexpressionsmusters der Wurzel und die Untersuchung der Toleranz der Pflanze gegenüber Trockenstress und Sekundärinfektionen.

6 ZUSAMMENFASSUNG/AUSBLICK

Die in der vorgelegten Arbeit vorgestellten Untersuchungen dienten der Analyse der Jasmonatbildung und -wirkung in pflanzlichen Modellsystemen. Anlass dafür war die Frage nach der Rolle dieses Phytohormons, dessen ubiquitäre Verbreitung im Pflanzenbereich und seine mögliche Funktion in der Stressantwort der Pflanze bekannt waren. Mitte der 90iger Jahre, als die Untersuchungen zu dieser Arbeit begannen, war die Chemie und Enzymatik der JA-Biosynthese weitgehend analysiert und erste JA-induzierte Gene charakterisiert. Zu diesem Zeitpunkt begann aber erst die Klonierung der für die Biosyntheseenzyme kodierenden Gene sowie die Charakterisierung der ersten JA-defizienten Mutanten. Unbekannt waren die intrazelluläre Lokalisation der JA-Biosynthese, ihre Regulation und zahlreiche Aspekte der Jasmonatwirkung bei Stress und in der Entwicklung der Pflanze einschließlich der dabei ablaufenden Signalwandlungsprozesse. Die damals erfolgte Entwicklung von neuen zellbiologischen Methoden erlaubte, Fragen zur zell- und gewebespezifischen Bildung und Funktion von Jasmonaten zu stellen. Hier setzte die vorliegende Arbeit ein.

In unseren Untersuchungen kamen folgende Pflanzen zur Anwendung: (1) Gerste als monokotyle Pflanze mit besonders abundantem Auftreten von Jasmonat-induzierten Proteinen; (2) Tomate als bestuntersuchte Pflanze für die pflanzliche Wundantwort; (3) Arabidopsis als wichtiges Modell der pflanzlichen Molekulargenetik; und (4) *Medicago truncatula* als Modellpflanze der arbuskulären Mykorrhiza. Die Analyse dieser Pflanzen wurde vorrangig mittels zytologischer Methoden durchgeführt. Dabei lag der Schwerpunkt neben der Analyse der Lokalisierung der Biosyntheseenzyme in Beiträgen zur Funktionsanalyse von JA und JA-regulierten Prozessen. Hierzu wurden Jasmonat-induzierbare Proteine (JIPs) charakterisiert, um einerseits deren Funktion zu klären und sie andererseits als Reporter einer Jasmonatwirkung nutzen zu können. Gleichzeitig wurden die zellbiologischen Arbeiten durch molekularbiologische und biochemische Ansätze ergänzt. So führte die parallele Bestimmung der Lokalisierung von JA-Biosyntheseenzymen und JIPs, des endogenen Gehalts an Jasmonsäure und ihrer Metaboliten, die Analyse von Jasmonat-induzierten Veränderungen der Genexpression sowie Ansätze zur reversen Genetik mittels transgener Pflanzen zu neuen Erkenntnissen hinsichtlich der Rolle von JA in Prozessen der pflanzlichen Entwicklung (Keimung, Blütenentwicklung) und Prozessen der pflanzlichen Stressantwort (Verwundung, Interaktion mit arbuskulären Mykorrhizapilzen).

Durch unsere Arbeiten wurde zum ersten Mal belegt, dass die ersten Schritte der JA-Biosynthese in den Plastiden lokalisiert sind. Es ist jedoch noch offen, ob die daran beteiligten Enzyme eine Protein-Protein-Interaktion eingehen, die einen effizienten Umsatz der Intermediate gewährleisten würde. Ebenso ist eine „Interaktion“ von Plastiden und Peroxisomen zu fordern, um OPDA zu transportieren. Neue zellbiologische Methoden, wie z.B. die Analyse von Proteininteraktionen mittels Fluoreszenz Resonanz Energie Transfer (FRET) oder die Analyse von Organellenbewegung mittels „live cell imaging“ könnten hier helfen, weitere Erkenntnisse zu gewinnen.

Die für die pflanzliche Entwicklung gewonnenen Daten zeigen, dass spezifische Gewebe des Keimlings (Gerste) bzw. der Blüte (Tomate und Arabidopsis) die Gene der JA-Biosyntheseenzyme exprimieren. Diese Expression führt zu einer Akkumulation von Jasmonaten, die in einem spezifischen Oxilipin-Muster des jeweiligen Organs resultiert. Untersuchungen mittels transgener Ansätze und/oder Mutanten, in denen dieses Muster verändert ist, werden in Zukunft dessen Bedeutung für die pflanzliche Entwicklung zeigen. Damit kann eine neue Funktion der Jasmonate deutlich werden, wobei das Zusammenspiel mit exogenen und endogenen Faktoren der Pflanzenentwicklung, wie z.B. mit anderen Phytohormonen, noch zu klären sein wird.

Neben der Entwicklung ist die Reaktion der Pflanze auf Stress ein Prozess, in den Jasmonate involviert sind. Hier konnte mit den vorgelegten Arbeiten zum spezifischen Vorkommen der JA-Biosyntheseenzyme und der JA-Bildung in den Leitgeweben ein Modell entworfen werden, das eine Amplifikation der Wundantwort in der Pflanze verdeutlicht. Weiterhin konnte im Kontext anderer Arbeiten JA als systemisches Signal diskutiert werden. Neue Techniken, wie z.B. die Laser-Mikrodissektion würden hier ein gänzlich neues Niveau der Analyse von zell- und gewebespezifischen Ereignissen schaffen.

Der Schwerpunkt der Arbeiten anderer Arbeitsgruppen lag aufgrund deren Untersuchungen zu Fragen der Verwundung oder Pathogenbefall vorwiegend auf der Analyse der oberirdischen Pflanzenorgane. Erst durch unsere Arbeiten wurde belegt, dass Jasmonate auch in der Stressantwort der Wurzel eine Rolle spielen. Am Beispiel der Symbiose mit Mykorrhizapilzen wird deutlich, dass Jasmonate für die Funktion dieser Interaktion wichtig sind. Dabei ergeben sich für die Jasmonatwirkung zwei Möglichkeiten, um die Interaktion zu ermöglichen: (1) Die Jasmonate werden in der Wurzel gebildet und vermitteln innerhalb der Wurzel eine Stressreaktion, z.B. auf den erhöhten Kohlenhydrat-Einstrom zur Versorgung des Pilzes; oder (2) die in der Wurzel gebildeten Jasmonate sind Signale in der Kommunikation zwischen Wurzel und Spross, um die verbesserte Versor-

gung mit Kohlenhydraten aus dem Spross zu ermöglichen. Ansätze mit transgenen Pflanzen und Mutanten in Kombination mit Methoden wie Expressions- und Metabolitenprofiling, werden in Zukunft neue Einsichten in diese Prozesse erlauben. Auch hier können die genannten neuen Methoden zur Analyse der Interaktion von Signalpartnern oder zur zell- und gewebespezifischen Analyse nach Laser-Mikrodissektion eine besondere Rolle spielen.

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Intracellular Localization of Jasmonate-Induced Proteins in Barley Leaves

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Abstract

The plant growth substance jasmonic acid and its methyl ester (JA-Me) induce a set of proteins (jasmonate-induced proteins, JIPs) when applied to leaf segments of barley (*Hordeum vulgare* L. cv. Salome). Most of these JIPs could be localized within different cell compartments by using a combination of biochemical and histochemical methods. Isolation and purification of various cell organelles of barley mesophyll cells, the separation of their proteins by one-dimensional polyacrylamide gel electrophoresis and the identification of the major abundant JIPs by Western blot analysis, as well as the immuno-gold labelling of JIPs in ultrathin sections were performed to localize JIPs intracellularly. JIP-23 was found to be in vacuoles, peroxisomes, and in the granular parts of the nucleus as well as within the cytoplasm; JIP-37 was detected in vacuoles and in the nucleoplasm; JIP-66 is a cytosolic protein. Some less abundant JIPs were also localized within different cell compartments: JIP-100 was found within the stromal fraction of chloroplasts; JIP-70 is present in the peroxisome and the nucleus; JIP-50 and JIP-6 accumulate in vacuoles. The location of JIP-66 and JIP-6 confirms their possible physiological role deduced from molecular analysis of their cDNA.

Key words

Jasmonate-induced proteins, intracellular localization, immunocytochemistry, isolated organelles, *Hordeum vulgare*.

Abbreviations and Symbols

ABA:	abscisic acid
BSA:	bovine serum albumin
JA:	jasmonic acid
JA-Me:	jasmonic acid methyl ester
JIP:	jasmonate-induced protein
MDH:	malate dehydrogenase
RuBPCase:	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS:	sodium dodecyl sulphate

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Introduction

The cyclopentanone derivative (−)-jasmonic acid and its methyl ester (JA-Me) were found to be ubiquitously distributed in higher plants (Yamane et al., 1981; Meyer et al., 1984). Both compounds are regarded as putative plant growth regulators (Parthier, 1990, 1991) exerting various inhibitory and stimulatory effects on growth and developing processes (Sembdner and Parthier, 1993). Several effects seem to be connected with the appearance of specific proteins, after expression of corresponding genes upon exogenous application of jasmonates. It was demonstrated that jasmonic acid can act as an inducer of the wound-induced proteinase inhibitors I and II of tomato and potato, being related to herbivore attack (Peña-Cortés et al., 1989; Ryan, 1992), of the vegetative storage proteins of soybean (Mason and Mullet, 1990; Staswick, 1990; Staswick et al., 1991; Creelman et al., 1992), functioning in N-partitioning or as lipoxygenase (Bell and Mullet, 1991; Tranberger et al., 1991; Grimes et al., 1992), of some alkaloid-synthesizing enzymes of elicited cell suspension cultures (Dittrich et al., 1992; Gundlach et al., 1992), and of glycine- and proline-rich cell wall proteins (Bowles, 1990; Creelman et al., 1992). Among the monocotyledons, e.g. in barley leaf segments, two specific effects have been observed which indicate the dramatic alteration of gene expression by jasmonate treatment: (i) the stimulation of senescence visualized by decreasing amount of chlorophyll as well as ribulose-1,5-bisphosphate carboxylase/oxygenase (Ueda and Kato, 1980, 1981; Weidhase et al., 1987b; Roloff et al., 1994); (ii) the accumulation of novel abundant polypeptides (jasmonate-induced proteins, JIPs) and their respective mRNAs (Weidhase et al., 1987a; Müller-Uri et al., 1988; Herrmann et al., 1989; Reinbothe et al., 1992).

Jasmonate-induced proteins in barley leaf tissue consist of a number of polypeptides with molecular masses of 6, 10/12, 23, 30, 37, 66/68 kDa (JIP-6, JIP-10/12, JIP-23, JIP-30, JIP-37, JIP-66/68). They accumulate organ- and species-specifically as indicated by the lack of immunological cross-reaction of the antibodies raised against JIP-23, JIP-37, JIP-66 of barley leaves with polypeptides of barley roots, and the polypeptides from leaves of other monocotyledonous and dicotyledonous plants, respectively (Herrmann et al., 1989).

[1] Dedicated to Professor Dr. Hubert Ziegler on occasion of his 70th birthday.

The synthesis of abundant JIPs takes place on cytoplasmic ribosomes (Weidhase et al., 1987a). In addition, appearance of the respective mRNAs is accompanied by the suppression of mRNAs for control (water-treated) proteins (Müller-Uri et al., 1988). Recently, the amino acid sequences of JIP-6 and JIP-23 could be deduced from the corresponding cDNA sequences (Andresen et al., 1992). The most abundant barley JIP, JIP-23 did not show sequence homology to any known protein, but JIP-6 was identified by sequence homology as leaf thionin (Andresen et al., 1992), a protein class which is known to be related to plant defense (Bohlmann and Apel, 1991).

JIP transcripts start to accumulate after about 4 h of JA-Me treatment, thus appearing clearly before senescence-related events as the decrease of the amount of chlorophyll and RuBPCase. Moreover, JIPs of leaf segments are also inducible by ABA treatment as well as by osmotic stress and desiccation (Weidhase et al., 1987a; Lehmann et al., personal communication). A similar induction by jasmonate and ABA was found for the proteinase inhibitor II of tomato (Peña-Cortés et al., 1989) and the vegetative storage proteins of soybean (Mason et al., 1992) suggesting a possible link between JIP synthesis and stress response (see Parthier, 1990).

With the exception of JIP-6, a thionin, none of the biological functions of barley JIPs are identified. Some of them may be putative "stress-protecting" molecules. Therefore, the intracellular location of JIPs might help to get some insight into their physiological roles or their biological functions. The present paper describes the localization of abundant JIP-23, JIP-37, JIP-66, and some less abundant JIPs within different cell compartments. This is demonstrated by immunological identification of JIPs by Western blot analysis after extraction of proteins from isolated organelles and their gel electrophoretic separation. The results obtained are confirmed by immuno-electron microscopy.

Materials and Methods

Plant material and treatment of leaf segments

Seedlings of *Hordeum vulgare* cv. Salome were grown under continuous light (3000 lx) at 24 °C. After 7 days leaf segments of 5 cm length, starting 1 cm below the tip, were cut and floated on water for 48 h (control, −) or 45 μM JA-Me for 48 h ("long", +) or for 6 h followed by gentle washing and further incubation on water for 42 h ("short", +).

Antisera

Monospecific, polyclonal antibodies raised against the major abundant JIP-23, JIP-37, and JIP-66 were prepared and characterized as described previously (Herrmann et al., 1989).

Isolation of organelles

Mitochondria were isolated and purified as described by Hause and Wasternack (1988). Nuclei were prepared according to Willmitzer and Wagner (1981), modified by Mösinger and Schäfer (1984). In order to isolate chloroplasts, vacuoles and peroxisomes, protoplasts were isolated as described by Kaiser et al. (1982): Briefly, barley leaf segments were treated with 0.5 M sorbitol, 20 mM citrate buffer, pH 5.5, 1 % cellulase Y-C and 0.2 % pectolyase Y-23 (both from Seishin Ltd., Tokyo, Japan) at 30 °C for 3 h. The lysis of protoplasts was performed either in buffer A (5 mM EDTA, 5 % Percoll [Pharmacia, Uppsala, Sweden], 25 mM Tricine-HCl, pH 8.0, 0.5 M sorbitol. 0.1 % BSA) for the isolation of chloroplasts and vacuoles, or in buffer B (5 mM EDTA, 2.5 % Ficoll [Pharmacia, Uppsala, Sweden], 50 mM Tricine-HCl, pH 7.5, 0.5 M sorbitol, 1 mM $MgCl_2$, 10 mM KCl [Feierabend and Beevers, 1972]) for the isolation of peroxisomes. Afterwards, chloroplasts and vacuoles were purified by centrifugations in a discontinuous Percoll gradient as described by Kaiser and Heber (1984). Thylakoids and stroma were separated after gentle osmotic shock of chloroplasts followed by differential centrifugation at 10,000 × g. To isolate peroxisomes, the lysate from 2×10^7 protoplasts was layered onto the top of a continuous 30 to 60 % sucrose density gradient. After centrifugation at 22,000 rpm (Beckman rotor SW 25) for 2 h, non-cytosolic fractions containing catalase activity (see Fig. 1) were harvested and used directly as peroxisomal fraction for extraction of proteins.

All organellar fractions were characterized by specific markers (Tab. 1). Except chlorophyll (Arnon, 1949), the following enzyme assays were used: Malate dehydrogenase (EC 1.1.1.37), catalase (EC 1.11.1.6) and glucose-6-phosphate de-

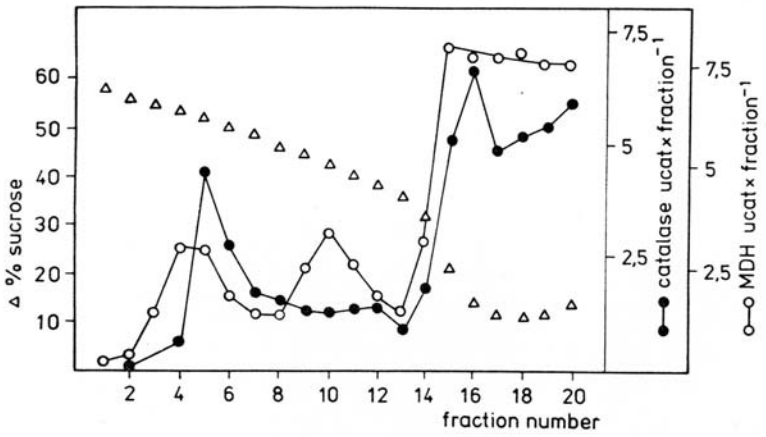

Fig. 1 Isolation of peroxisomes by sucrose density gradient centrifugations. Non-cytosolic fractions at 55 % sucrose containing catalase activity (No. 4–6) were used for isolation of peroxisomal proteins.

Table 1 Amount, intactness and cross-contamination of organelles isolated from barley leaf segments or its protoplasts.

Kind of organelles	Amount of isolated organelles		Intactness	Contamination	
	determined by marker	amount found	determined by	determined by	amount (in % of content in cell extract)
Mitochondria	sedimentation of MDH activity	30 %	latency of cytochrome c oxidase	glucose-6-phosphate dehydrogenase	1
				chlorophyll	10
Nuclei	number	$10^6 \times g^{-1}$ FW	phase contrast microscopy	n.d.	
Chloroplasts	chlorophyll content	54 %	phase contrast microscopy; amount of RuBP-Case in SDS gels	MDH	1
Peroxisomes	catalase	17 %	catalase activity at 55 % sucrose in the density gradient	MDH	12
Vacuoles	α-D-manno-sidase	34 %	phase contrast microscopy	MDH	1
				chlorophyll	< 1

hydrogenase (EC 1.1.1.49) (Bergmeyer, 1970), cytochrome-c-oxidase (EC 1.9.3.1) (Yonetami, 1967), α-D-mannosidase (EC 3.2.1.24) (Boller and Kende, 1979).

Extraction of proteins and separation by gel electrophoresis

Proteins from leaf segments, leaf homogenates, protoplasts as well as organellar fractions were extracted according to Meyer et al. (1988), solubilized in an SDS-containing buffer, and separated by SDS polyacrylamide gradient gel electrophoresis (7.5 to 25 % polyacrylamide; Laemmli, 1970). The gels were stained by the silver method reported by Blum et al. (1987). The transfer of proteins onto nitrocellulose (BA85, Schleicher and Schüll) was done as described by Towbin et al. (1979). Immunological detection of JIPs was already detailed (Herrmann et al., 1989).

Preparation of leaves for transmission- and immuno-electron microscopy

Small pieces of primary leaves, treated for various times with JA-Me, were fixed in 3 % glutaraldehyde in phosphate buffer (pH 7.4) for 2 h, post-fixed in 1 % OsO_4 in Palade buffer, dehydrated in acetone, and embedded in Araldite.

For immunolabelling leaf pieces were fixed in 4 % (w/v) formaldehyde for 2 h, dehydrated with ethanol and embedded in Lowicryl K4M (Chemische Werke Lowi GmbH, Waldkraiburg, Germany) at − 20 °C according to Carlemalm et al. (1981). Protein A gold was prepared as described by Roth et al. (1978), yielding particles with a diameter of about 17 nm. Immunolabelling of ultrathin sections was carried out with the antisera raised against JIP-23, JIP-37, and JIP-66 (diluted 1/100 in phosphate buffer [pH 7.4] containing 1 % Tween 20 and 0.5 M sodium chloride) followed by the incubation with diluted protein A gold (E = 0.06 at 520 nm). All washing procedures were performed with physiologically buffered salt containing 0.05 % Tween 20. Finally, sections were stained with 5 % aqueous uranyl acetate for 20 min at 60 °C and visualized with a transmission electron microscope BS 500 (TESLA, BRNO, CFSR).

Results

Identification of JIPs in isolated, purified cell organelles by immunoblotting of their separated proteins

A method to localize JIPs in various cell compartments is the isolation of organelles and their subsequent extraction and identification of proteins. The following organelles were isolated from JA-Me-treated leaf segments: nuclei, mitochondria, vacuoles, chloroplasts and peroxisomes. Mitochondria and nuclei were isolated after mechanical cell disruption of the tissues. The isolation of chloroplasts, vacuoles and peroxisomes was more successful by the lysis of protoplasts prepared from leaf segments.

In studying JIPs of isolated protoplasts we encountered the difficulty of being unable to isolate sufficient amounts of intact protoplasts from barley leaf segments treated with JA-Me for 24 h or longer. Only 5 % of protoplasts could be isolated compared to the water control. Therefore, the period of JA-Me-treatment was shortened to 6 h followed by floating of leaf segments on water for an additional 42 h. In comparison to the water control, about 50 % of intact protoplasts could be isolated in this way. In terms of quality the gel electrophoretic pattern of JIPs was identical to that from tissues treated for 48 h with JA-Me (Figs. **2** and **3**). Chloroplasts, vacuoles and peroxisomes could be prepared from the isolated protoplasts in sufficient quantity. Purity and intactness of the isolated organelles were checked by specific markers as indicated in Table **1**. The organellar fractions were taken for the extraction of their proteins, which were separated by one-dimensional polyacrylamide gel electrophoresis. Marked differences can be noted if comparing the protein patterns and the corresponding Western blot analyses between samples of water control and those of JA-Me-treated barley leaf segments and their organelles (Fig. **2** and **3**):

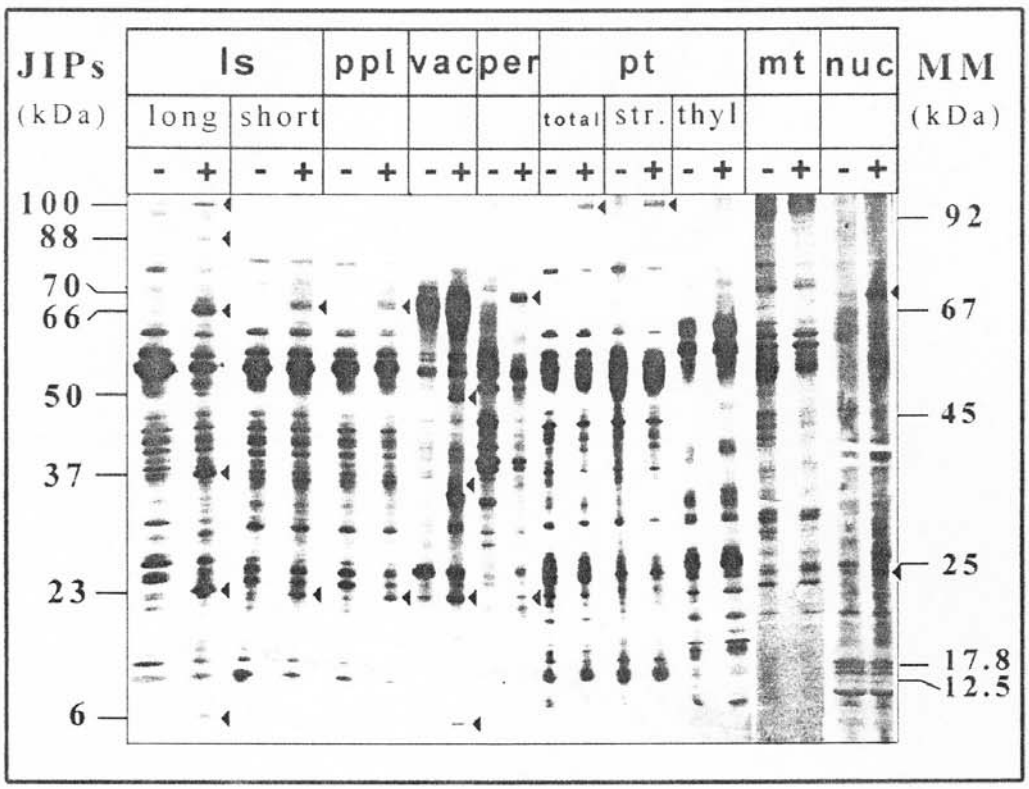

Fig. 2 One-dimensional polypeptide patterns of water- (−) or JA-Me-treated (+) barley leaf segments as well as protoplasts and organelles isolated from them.
Barley leaf segments were incubated with water or 45 μM JA-Me for 48 h (ls, long) or for 6 h followed by 42 h water-treatment (ls, short) with water or 45 μM JA-Me. Protoplasts (ppl), vacuoles (vac), peroxisomes (per), chloroplasts (pt, total) and their stromal (str) and thylakoid (thyl) fractions, mitochondria (mt), and nuclei (nuc) were isolated, purified and their proteins separated as described in "Materials and Methods". JIPs are marked by (◀), the position of the most abundant JIPs is given on the left side, molecular markers (MM) on the right side were: phosphatase (92 kDa), BSA (67 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa), myoglobin (17.8 kDa), and cytochrome (12.5 kDa).

(i) JA-Me-treated leaf segments (48 h and shortened incubation) or protoplasts isolated therefrom contain three abundant JIP classes of molecular masses of 23, 37, and 66 kDa. Additionally, less abundant JIPs of molecular masses 6, 70, 88 and 100 kDa were visible. Since no antisera were available for these JIPs, they could not be characterized by immunological methods.

(ii) Among the organellar fractions isolated, the JIP-23 appeared in the nuclear extract, in the vacuoles as well as in the peroxisomes, and JIP-37 could be detected within the nuclear and vacuolar fractions. JIP-66 could not be confined to any organellar fraction.

(iii) The organellar fractions contain "minor" JIPs besides the three most abundant JIP classes: in the vacuoles JIP-6 and JIP-50, in the nuclei and peroxisomes JIPs of 70 kDa. One of the minor-JIPs, JIP-100, was found to occur in the chloroplasts, exclusively within the soluble stroma fraction. No JIPs could be detected in the thylakoid membranes or mitochondria.

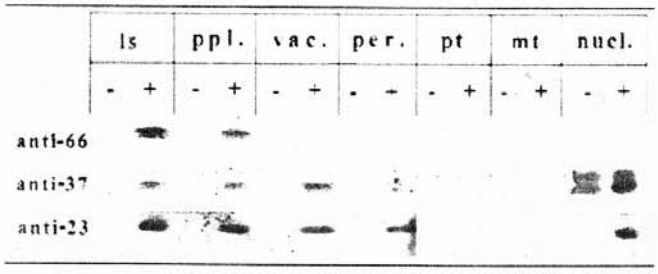

Fig. 3 Western blot analysis of protein separations from barley leaf segments, protoplasts and the isolated organelles. Areas of blots labelled with the antisera raised against JIP-66 (anti-66), JIP-37 (anti-37) as well as JIP-23 (anti-23) are selected to demonstrate the localization of JIPs. For abbreviations see Fig. **2**.

Ultrastructural changes in JA-Me-treated leaf tissues and the immunological localization of JIPs

Changes in the ultrastructure of barley leaves treated with JA-Me for one day are obvious if compared to water-treated leaves (Fig. **4**). Mainly the morphology of chloroplasts seems dramatically altered (compare Fig. **4 a** with **b**). Thus, the number of thylakoid stacks is diminished, and the stroma appears more dense. The envelope of plastids cannot be visualized within JA-Me-treated cells indicating changed composition of membrane constituents. Additionally, the chloroplasts of JA-Me-treated, parenchymatic cells contain regularly shaped, fibril-like structures (Fig. **4 b** and **e**), which are never detectable in water- or untreated tissues (Fig. **4 a** and **f**). These fibril-like structures, located within the stroma, are only detectable after fixation of the tissues with glutaraldehyde. They are stable against various enzymatic treatments, if ultrathin sections were treated with pronase K, deoxyribonuclease or ribonuclease. Moreover, these fibril structures failed to be labelled by feeding leaf-discs with (4,5-^{3}H)-leucine or (5-^{3}H)-uridine before or during the application of JA-Me, as proved by microautoradiography (results not shown). In order to check whether RuBPCase is a constituent of these structures, the ultrathin sections were treated with antisera raised against this protein (Fig. **4 d**). As a result, the tested antiserum was unable to label the stromal fibrils. After 72 h of incubation with JA-Me the fibrils within the chloroplasts disappeared (Fig. **4 c**). Within the same time, large osmiophilic granula occurred and the inner membrane system seemed to be destroyed. At that time spherical chloroplasts contain a dense stroma and the envelopes cannot be visualized anymore.

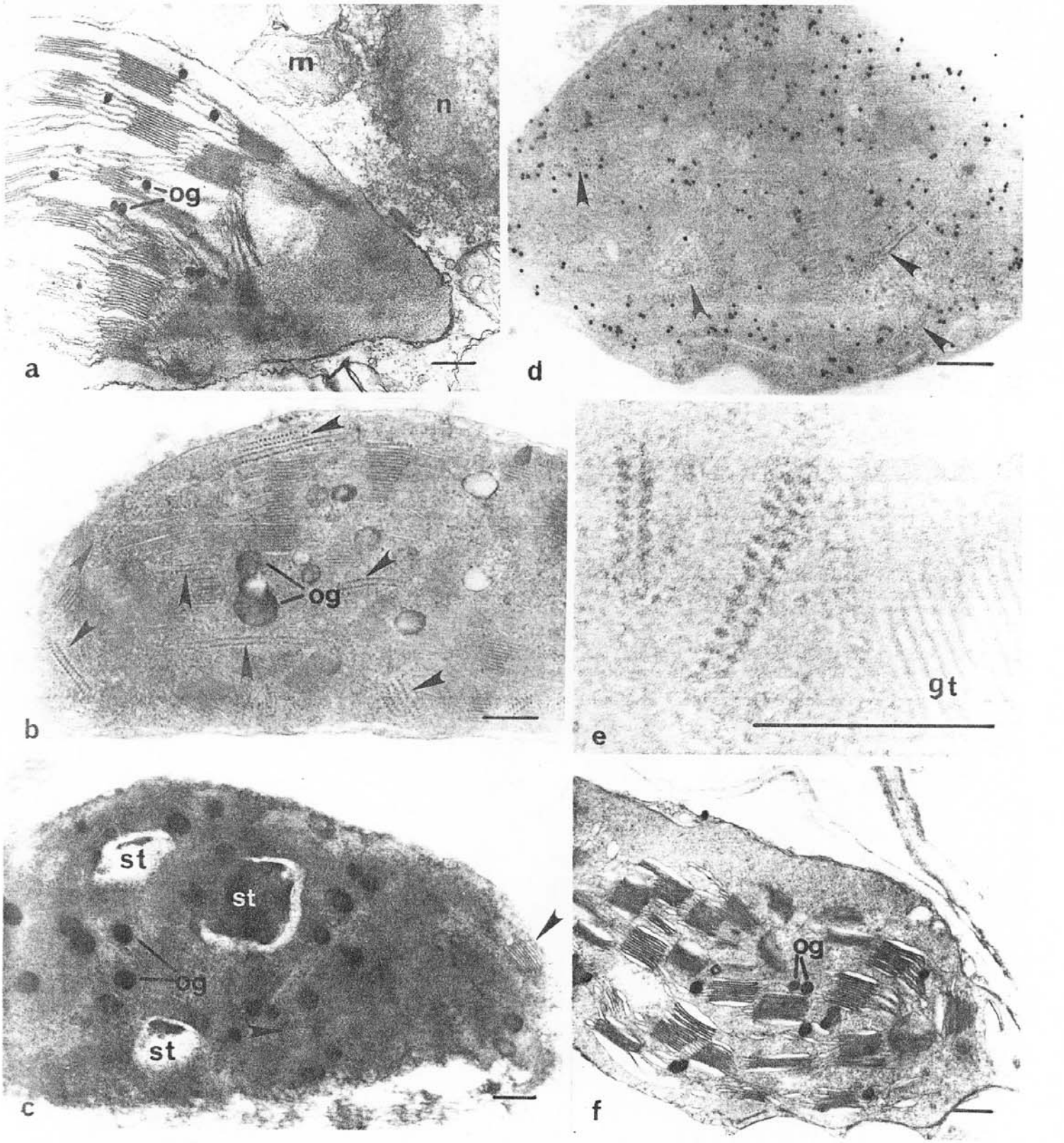

Fig. 4 Survey of the ultrastructural changes of barley leaf segments treated for 24 h with 45 μM JA-Me.

a Chloroplast of a control leaf treated with water for 24 h. Note the plastoglobuli and the typical arrangement of thylakoid stacks. Inclusions within the stroma are not detectable.

b Chloroplast of a leaf treated with JA-Me for 24 h. Note the fibril-like structures within the plastid stroma (arrows).

c Chloroplast of a leaf treated with JA-Me for 72 h. Only few lamellar stacks are visible within the dense stroma (arrows). The fibril-like structures demonstrated in Fig. **b** and **e**, had disappeared. Note the large osmiophilic globuli.

d Immuno-gold labelling of the RuBPCase in chloroplasts of JA-Me-treated leaves fixed with 3 % glutaraldehyde and embedded in Lowicryl K4M. The fibril-like structures do not exit label (arrows).

e Higher magnification of the fibril-like structures within the plastid stroma.

f Chloroplast of a leaf treated with water for 72 h (control for **c**). Grana and intergranal lamellae are swollen. The lamellar system is disorientated. Bars represent 0.25 μm in all figures. Abbreviations used: gt, granal thylakoids; m, mitochondrion; n, nucleus; og, osmiophilic globuli; p, chloroplast; ps, peroxisome; st, starch; v, vacuole; w, cell wall.

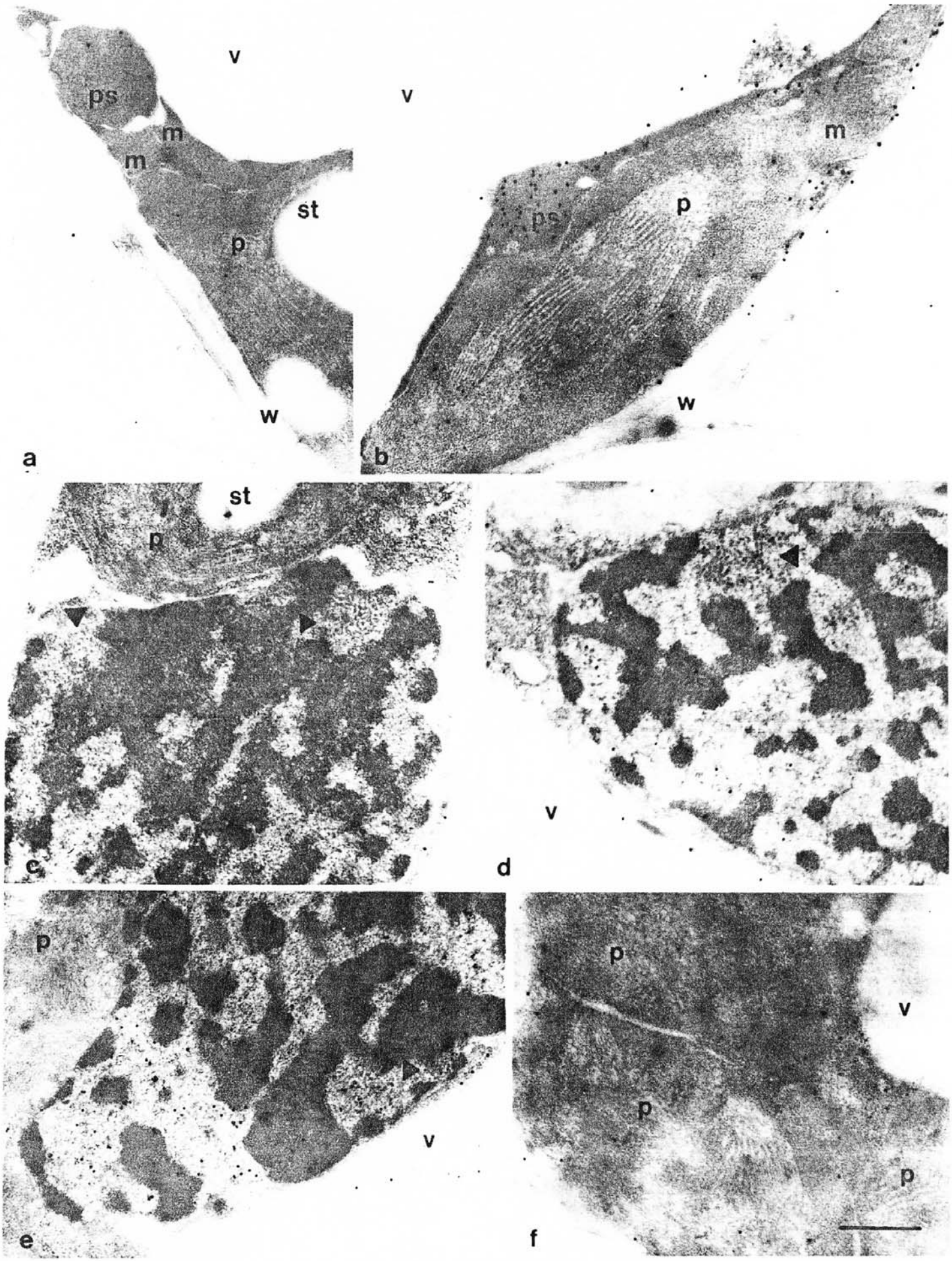

Fig. 5

To localize JIPs by histochemical methods, the time of JA-Me-treatment of leaf segments had to be varied. JIP-23 and JIP-66 were immunocytochemically detectable after 24 h of incubation, whereas JIP-37 were found to be labelled only after 48 h JA-Me-treatment. The control (water-treatment for 24 h) did not exhibit label after incubation with the antisera against the three major classes of JIPs (Fig. **5 a**). As shown in Figs. **5 b** and **d–f**, the most abundant JIPs were detected in different compartments. JIP-23 occurred in the cytoplasm as well as in the peroxisomes (Fig. **5 b**), and it was identified within the nucleus with preferential occurrence in the nucleoplasm (Fig. **5 d**). The label was found mainly in granules (arrow in Fig. **5 d**) exhibiting a diameter from approximately 20 – 25 nm. The granules are arranged in clusters located within the diffuse chromatin. The control, performed by the incubation of JA-Me-treated tissues with preimmunserum, showed unlabelled granular structures (Fig. **5 c**).

In contrast to JIP-23, JIP-37 was found in the nucleoplasm only. Here, JIP-37 was distributed equally. However, JIP-37 were not detectable in other compartments by the immunocytochemical methods. The JIP-66 seemed to be restricted to the cytoplasm (Fig. **5 f**).

Discussion

Barley leaf segments treated with jasmonic acid or its methyl ester (JA-Me) respond with altered gene expression leading to synthesis of specific proteins (JIPs) and their respective mRNAs (Müller-Uri et al., 1988; Herrmann et al., 1989; Reinbothe et al., 1992). Additionally, ultrastructural changes of the mesophyll cells occur. As documented in Fig. **4** these changes were found mainly within the chloroplasts. After 24 h of incubation with JA-Me the chloroplasts show very similar structures to that of senescent wheat leaves (Hurkman, 1979). Remarkably, and different to naturally senescent chloroplasts, JA-Me-induced senescence leads to fibril-like structures within the chloroplasts (Fig. **4 b**). Similar structures are already described as a stress-specific response of chloroplasts (Wellburn, 1984). Designated as "stromacentres" (Gunning et al., 1965) they were considered to be crystals of the large subunit of RuBPCase (Gunning et al., 1968). In our hands, these fibrils did not exhibit a labelling with an antiserum raised against RuBPCase of cucumber (Fig. **4 d**). However, this might be caused by a high degree of packaging within these crystals. It cannot be ruled out that JIP-100 appearing in the stroma of the chloroplasts (Fig. **2**) of leaves treated with jasmonate is related to these structures. After 3 days of JA-Me treatment the chloroplast exhibited an almost total loss of the inner membranes, indicating an increased breakdown of lipids, then accumulated in osmiophilic globuli.

The localization of JIPs within different compartments of barley mesophyll cells is summarized in Table **2**. The gel electrophoretic separation of proteins of isolated cell organelles and their immunological identification by Western blot analysis, as well as the histochemical localization by immuno-gold electron microscopy were used as two independent procedures. Both methods lead to corresponding results, except for some minor differences. It was not possible to localize JIPs within the vacuole by immunocytochemical methods. This might be attributed to the probability that the amount of JIPs occurring in vacuoles is below the limit of detection by this method. Furthermore, JIPs, which are not characterized immunologically, could be visualized only by the gel electrophoretic pattern of isolated organelles. Cross-contamination of isolated organelles have to be negligible for the latter method. As indicated by corresponding markers (Tab. **1**), each compartment showed sufficient purity and structural integrity for the question under study. Although a remarkable amount of the MDH activity, a mitochondrial marker, was found in peroxisomal fractions (Fig. **1**), this result does not interfere with our interpretation of localization in peroxisomes or mitochondria, since mitochondria seem to be free of JIPs.

The occurrence of JIP-23 within three organelles as well as in the cytoplasm, and of JIP-37 within two organelles, could be explained by specific locations of isoforms known from two-dimensional PAGE, especially, for JIP-23 (Müller-Uri et al., 1988). The observation that the most abundant barley JIPs are synthesized on cyto-

Table 2 Intracellular location of JIPs (barley leaf tissue).

JIP (kDa)	Location	Method: prep. of organelles 1 D	Method: prep. of organelles Immunoblot	Method: Immunogold *in situ*
23	– nucleus, granular region	+	+	+
	– peroxisomes	+	+	+
	– vacuoles	+	+	n.d.
	– cytoplasm			+
37	– nucleus	+	+	+
	– vacuoles	+	+	n.d.
66	– cytoplasm	+	+	+
100	– chloroplasts (stroma)	+		
70	– nuclei, peroxisomes	+		
50	– vacuoles	+		
6	– vacuoles	+		

n.d., not detectable

Fig. 5 Immuno-electron microscopical localization of jasmonate-induced proteins (JIPs) in parenchymatic cells of barley leaves. Water-treated for 24 h (**a**) or treated for 24 h (**b**, **c**, **d**, **f**) or 48 h (**e**) with 45 μM JA-Me.

a Control leaves (water-treated) incubated with a mixture of antisera raised against JIP-23, JIP-37 and JIP-66.

b Section of a cell of JA-Me-treated barley leaves incubated with the antiserum against JIP-23. Note the remarkable label within the cytoplasm and the peroxisome.

c Control of the immunological reaction by incubation of JA-Me-treated leaves with the preimmune serum. Note the absence of label within the granular structures of the nucleus (arrows).

d Nucleus of the JA-Me-treated cell containing interchromatin granules (ICG). Immunogold labelling with the antiserum against JIP-23 shows the main label within the ICG (arrow).

e Part of the JA-Me-treated cell incubated with the antiserum against JIP-37. Note the equally distributed label within the nucleus.

f Section of a cell of JA-Me-treated barley leaves incubated with the antiserum against JIP-66. The label is restricted to the cytoplasm. Bar represents 0.5 μm for all figures. For abbreviations see Fig. **4**.

plasmic ribosomes (inhibition by cycloheximide, cf. Weidhase et al., 1987a) raises the question of intracellular targeting of JIPs. However, the sequence data known for the cDNA of JIP-23 (Andresen et al., 1992) and for the cDNA of JIP-37 (Leopold et al., pers. comm.) do not support the occurrence of a target sequence. Still open as a problem, different genes for the JIP-23 isoforms or message heterogeneity might be attributed to it. It is interesting to note that JIP-23 was found preferentially within the nucleus in the granular regions. Such interchromatine granules were already described by Bouteille (1972) and could contain both RNA and protein. The possible role of JIPs within these granules is completely unclear.

Localization of JIP-66 within the cytoplasm accords with recent results of Chaudry et al. (pers. comm.). They observed a 100 % homology of the N-terminal half of the cDNA of JIP-60 (sequenced by Becker and Apel, 1992) with that coding for a ribosome inactivating protein (RIP) known from barley. This JIP was identified as RIP by several functional approaches, thus shedding some light on the translational control of protein synthesis by jasmonate (cf. also Reinbothe et al., 1993a, b).

The location of the JIP-6 within the vacuole is in agreement with results reported previously by Reimann-Philipp et al. (1989) and Andresen et al. (1992) who showed that barley leaf thionins are cell wall and vacuolar proteins with antifungal and antibacterial properties, and that JIP-6 is a thionin. Their possible role in plant-pathogenic interactions was recently stressed by the induction of leaf thionins in barley during appearance of systemic acquired resistance (Wasternack et al., 1994).

The functions of the various less abundant JIPs, located within the peroxisome, vacuole and nucleus are still completely unknown. Whether these inducible proteins are related to general stress responses of barley leaf or act as specific regulatory elements during plant-pathogen interactions, leaf senescence and other developmental as well as stress-mediated processes remains to be answered experimentally.

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Jasmonate-induced lipoxygenase forms are localized in chloroplasts of barley leaves (*Hordeum vulgare* cv. Salome)

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Summary

Barley leaves respond to application of (–)-jasmonic acid (JA), or its methylester (JM) with the synthesis of abundant proteins, so-called jasmonate induced proteins (JIPs). Here Western blot analysis is used to show a remarkable increase upon JM treatment of a 100 kDa lipoxygenase (LOX), and the appearance of two new LOX forms of 98 and 92 kDa. The temporal increase of LOX-100 protein upon JM treatment was clearly distinguishable from the additionally detectable LOX forms. JM-induced LOX forms in barley leaves were compared with those of *Arabidopsis* and soybean leaves. Both dicot species showed a similar increase of one LOX upon JM induction, whereas, leaves from soybean responded with additional synthesis of a newly formed LOX of 94 kDa.

Using immunofluorescence analysis and isolation of intact chloroplasts, it is demonstrated that JM-induced LOX forms of barley leaves are exclusively located in the chloroplasts of all chloroplast-containing cells. Analogous experiments carried out with *Arabidopsis* and soybean revealed a similar plastidic location of JM-induced LOX forms in *Arabidopsis* but a different situation for soybean. In untreated soybean leaves the LOX protein was mainly restricted to vacuoles of paraveinal mesophyll cells. Additionally, LOX forms could be detected in cytoplasm and nuclei of bundle sheath cells. Upon JM treatment cytosolic LOX was detectable in spongy mesophyll cells, too. The intracellular location of JM-induced LOX is discussed in terms of stress-related phenomena mediated by JM.

Introduction

Lipoxygenase (lineolate:oxygen oxidoreductase, EC 1.13.11.12) is a non-haem iron-containing dioxygenase. This enzyme catalyses the regioselective C-H insertion of oxygen into 1,4-pentadiene *cis*-polyunsaturated fatty acids resulting in a fatty acid hydroperoxide (Gardner, 1991; Vick and Zimmerman, 1987). Lipoxygenase (LOX) is ubiquitous in eukaryotes (Siedow, 1991). With C_{18} unsaturated fatty acids, such as linoleic and linolenic acid, which are common in plants, either 9- or 13-hydroperoxide derivatives may be formed. A LOX, that inserts molecular oxygen into linoleic acid at the position of carbon atoms 9 or 13 is termed 9-LOX or 13-LOX, respectively. But a LOX from potato has 9-LOX activity using linoleic acid as substrate, and additionally 5-LOX activity metabolizing arachidonic acid (Mulliez *et al.*, 1987). Different enzymatic pathways lead from products of LOX activity to a wide range of oxo-compounds, the so-called oxylipins (Hamberg, 1993). Subsequent heterologous or homologous fragmentation directs to C_9 or C_6 compounds, which are discussed as phytoalexins (Hatanaka, 1993). Among the C_{18} oxo-compounds the hormone-like substance jasmonic acid is the most important (Sembdner and Parthier, 1993).

At the biochemical level LOX forms are distinguished by comparing product formation, pH optimum, substrate specificity, molecular mass, and enzymatic stability (Axelrod *et al.*, 1981). In soybean seeds one can distinguish three soluble forms by taking into account these distinct properties. LOX-1 (Hildebrand and Hymowitz, 1982; Shibata *et al.*, 1987), the smallest in size, exhibits maximal activity at pH 9.0 and is a 13-LOX. LOX-2 (Shibata *et al.*, 1988) is the isoform of the highest molecular mass, shows a distinct pH optimum at 6.2, and is characterized by metabolizing linoleic acid to the 9-hydroperoxide compound. At least, LOX-3 (Kato *et al.*, 1992; Shibata *et al.*, 1991) exhibit maximal activity around pH 6 and generates a mixture of 9- and 13-hydroperoxides. According to work done with soybean seeds, LOX forms can be generally grouped into two classes: those with basic pH optima at pH 8–9 (type 1) and those most active near neutral pH (type 2; Vick and Zimmerman, 1987).

Despite considerable work that has been done on the elucidation of the catalytic mechanism and biochemical properties of lipoxygenases (Gardner, 1991), studies concerned with intracellular localization, tissue-specific distribution, and possible physiological functions have not been addressed extensively. Recent evidence points to the involvement of lipoxygenases in growth regulation, senescence promotion, and plant responses to stress and pathogen attack (Bell and Mullet, 1991; Hildebrand, 1989). Additionally, high LOX protein concentrations and activities are found in germinating tissues (Siedow, 1991), leading

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to the recent implication of a role in mobilization of lipid reserves (Feussner and Kindl, 1992). Furthermore, LOX activity or its transcript are enhanced when plants are wounded (Bell and Mullet, 1991; Hildebrand, 1988; Melan *et al.*, 1994) or challenged by pathogens (Bostock *et al.*, 1992; Slusarenko *et al.*, 1993). In addition, jasmonate as one of the final products of LOX activity was suggested to be part of a lipid-based signalling pathway in wounded leaves of tomato (Farmer and Ryan, 1992; Peña-Cortés *et al.*, 1993) or soybean (Creelman *et al.*, 1992), in coiling tendrils (Weiler *et al.*, 1993), or in stressed barley leaves (Wasternack *et al.*, 1994). Since endogenous jasmonates increase upon elicitation of cell suspension cultures parallel with phytoalexin synthesis, a general role of jasmonate and its derivatives has been claimed for plant pathogen interaction (Gundlach *et al.*, 1992; Müller *et al.*, 1993). But, evidence is still lacking, that in homologous plant pathogen systems jasmonate rises in response to pathogenic infections, and a detailed inspection of the resistance mechanism in barley against the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*) revealed no hints on enhanced levels of endogenous jasmonates (Kogel *et al.*, 1995). Furthermore, an increase of LOX activity occurring during hypersensitive response (Bostock *et al.*, 1992) leads to products with antifungal (Ohta *et al.*, 1990) or antibacterial activities (Croft *et al.*, 1993) thus by-passing jasmonate as a signal.

To elucidate the functional significance of any LOX form, knowledge of its intracellular location is necessary. Here, we focus on intracellular location of LOX protein in barley leaves in relation to jasmonate treatment.

Barley leaves respond to jasmonate treatment with the synthesis of abundant proteins, so-called jasmonate-induced proteins (JIPs). At least eight polypeptides with different molecular masses of 6, 10/12, 23, 30, 37, 66/68, 88 and 100 kDa were found to be abundant in the polypeptide pattern of 7-day-old leaves (Hause *et al.*, 1994; Weidhase *et al.*, 1987). On the basis of sequence homology JIP-6 was identified as thionin (Andresen *et al.*, 1992) and a JIP-60 was recently found to be a ribosome inactivating protein (RIP) being identical with JIP-66 (Chaudhry *et al.*, 1994; Reinbothe *et al.*, 1994). Intracellular localization of abundant JIPs by Western blot analysis of isolated organelles and immunogold electron microscopy on ultrathin sections revealed the occurrence of certain JIPs within the nucleus (JIP-23, JIP-37), the peroxisomes (JIP-23), the vacuoles (JIP-23, JIP-37), and the cytoplasm (JIP-60). JIP-100 was localized in the chloroplasts (Hause *et al.*, 1994). Here we describe further JIPs of barley leaves, as a set of LOX forms induced by jasmonate, and we present evidence for their location within the chloroplasts. For comparison, data are presented for green leaves of *Arabidopsis thaliana* and *Glycine max* L., and results are discussed in terms of the possible function of LOX in plant stress response.

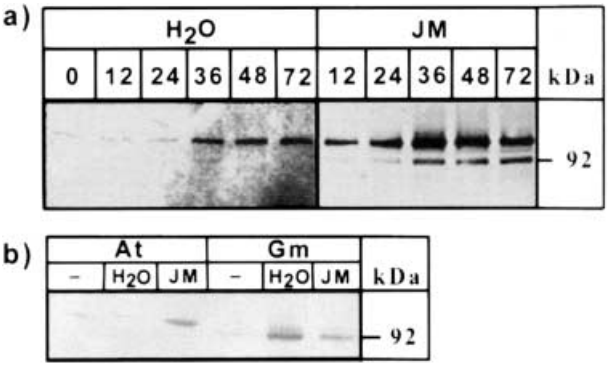

Figure 1. Immunoblot analysis illustrating LOX induction in barley leaf segments (a), *Arabidopsis* leaves (b, At), and soybean leaves (b, Gm) upon treatment with water or JM.
(a) Time course of total protein extracts of barley leaf segments, untreated (H_2O, 0), treated with water (H_2O, 12–72) for different numbers of hours, as indicated, or floated for different numbers of hours, as indicated, on 45 μM JM (JM, 12–72).
(b) Total protein extracts of *Arabidopsis* (At) leaves or soybean (Gm) leaves, untreated (–), treated for 42 h with water (H_2O), or with 45 μM JM (JM).
Protein samples were separated by SDS-PAGE, subsequently electroblotted on to nitrocellulose, probed with LOX-specific antibodies and visualized by peroxidase conjugated secondary antibodies.

Results

Methyl jasmonate (JM)-induced LOX at protein level

LOX protein abundance was compared in untreated, water- or JM-treated barley leaves. Therefore crude leaf extracts were analysed by immunoblotting using antibodies generated against cucumber LOX (Feussner and Kindl, 1992). The antibodies cross-reacted strongly and specifically with LOX forms of barley leaves. Utilizing modified SDS–PAGE (Feussner and Kindl, 1994) for the visualization of minor differences in molecular weight of LOX forms, we were able to separate LOX forms distinctly by size.

Analysis of water-treated leaves revealed a weak immunoreactive signal between 0 and 24 h (Figure 1a, H_2O). Thereafter the LOX content increased significantly up to 72 h of water treatment. The detected LOX form showed a relative molecular mass of 100 kDa. Upon JM treatment (Figure 1a, right) this isoform increased dramatically, and to a smaller degree two less abundant LOX forms appeared with relative mobilities related to 98 and 92 kDa. Whereas the LOX form of 100 kDa accumulated transiently with a maximum at 36 h, the less abundant forms increased steadily. The total amount of the isoform of 100 kDa was markedly increased compared with the amount in non- and water-treated leaves.

Similar experiments were carried out to analyse JM-induced LOX forms in *Arabidopsis* (*A. thaliana*) and soybean (*G. max* L.) leaves. Leaves from 5-week-old plants from *Arabidopsis* and soybean were extracted immediately (Figure 1b, –), or after incubation in water or 45 μM JM for 42 h. Immunoblot analysis revealed a complex response

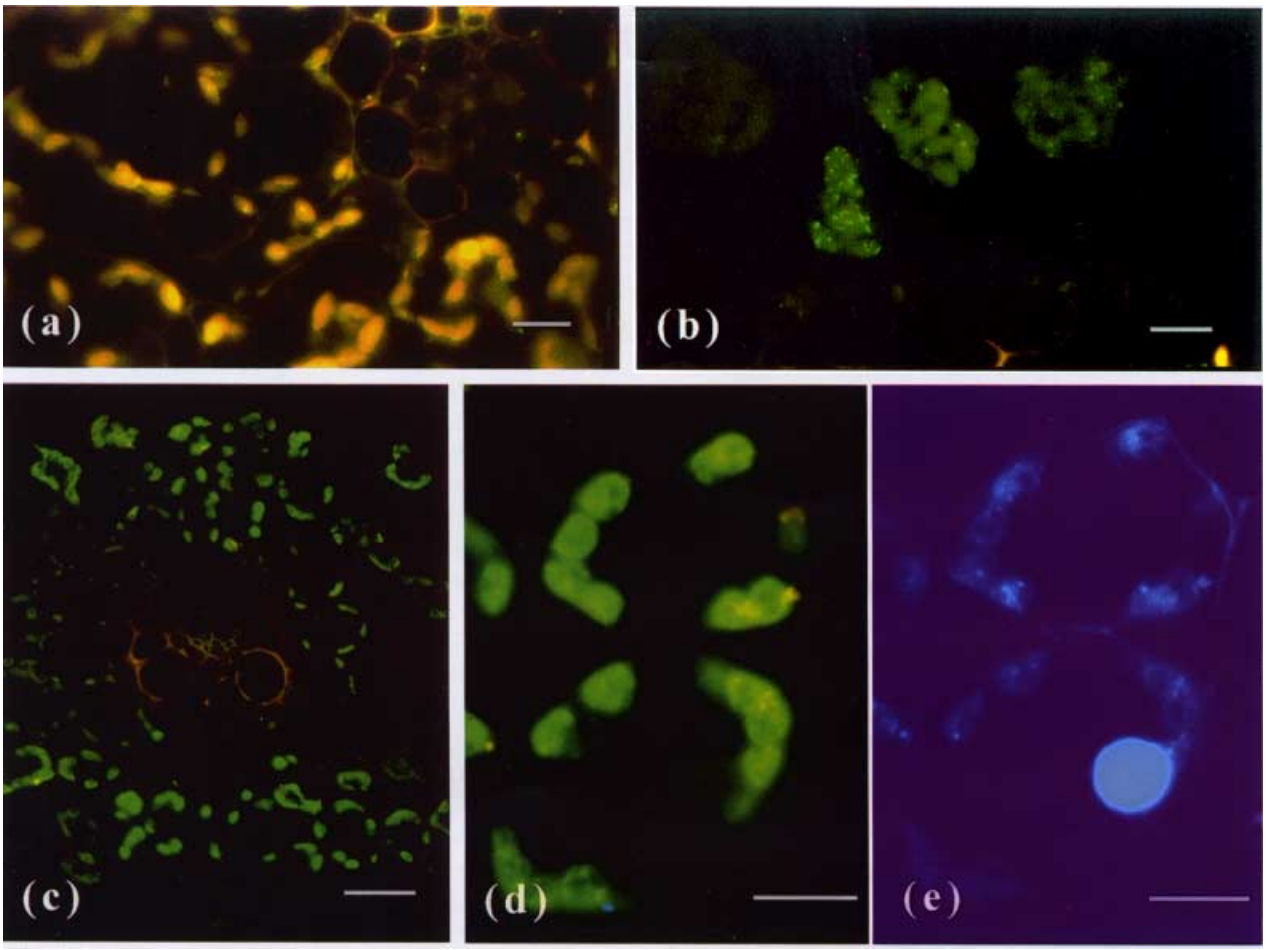

Figure 2. Cross-sections of barley leaf segments after immunofluorescence staining.
Survey of the localization of LOX protein in untreated, water-treated, or JM-treated barley leaf segments (a–e). (a–d) Show the localization of LOX protein visualized immunocytochemically by FITC conjugated secondary antibodies.
(a) Cross-section of an untreated leaf. Chloroplasts show only autofluorescence. Bar represents 10 μm.
(b) Cross-sectioned mesophyll cells from a water-treated leaf after 24 h. The bundle sheath exhibits a low amount of label within the chloroplasts. Bar represents 10 μm.
(c) Cross-section of a leaf treated with 45 μM JM for 24 h. Note, the label within all chloroplast-containing cells. Bar represents 25 μm.
(d and e) Detail of (c). LOX protein is detectable exclusively within the chloroplasts (d) as shown by the illumination of the section for DAPI staining to visualize the nucleus and the DNA-containing organelles (e). Bars represent 10 μm.

(Figure 1b) of these plants to water or JM treatment. *Arabidopsis* LOX (Figure 1b, At) increased similarly to barley after JM treatment, whereas leaves from soybean responded differently (Figure 1b, Gm): in control leaves one LOX form of 92 kDa was detectable. After 42 h water or JM treatment the protein content of the LOX-92 increased. Additionally, in water- and JM-treated leaves synthesis of a new LOX form of 94 kDa was detectable to a higher extent in water-treated as compared with JM-treated leaves.

JM-induced LOX is located within chloroplasts

The specific changes in the pattern of LOX forms in water- or JM-treated barley leaves led us to compare the intracellular distribution of LOX forms in leaf segments by indirect immunofluorescence with LOX antibodies (described above). The situation in untreated leaf segments is shown in Figure 2(a). The sensitivity of the staining procedure used in this approach does not allow one to detect the rather low amount of LOX protein in these leaves (Figure 1a, left). After 24 h, water-treated leaves displayed slight fluorescence in chloroplasts of cells near to the bundle sheaths (Figure 2b). In leaves treated for 24 h with JM, LOX protein was visible exclusively in chloroplasts of all chloroplast-containing cells (Figure 2c–e), in higher amounts as compared with LOX levels in water-treated cells. Additionally, the parallel DAPI staining (Figure 2e) of the section shown in Figure 2(d), illustrates the *de novo* synthesized LOX forms to be solely located in DNA-containing chloroplasts.

To confirm the chloroplastic localization of JM-induced

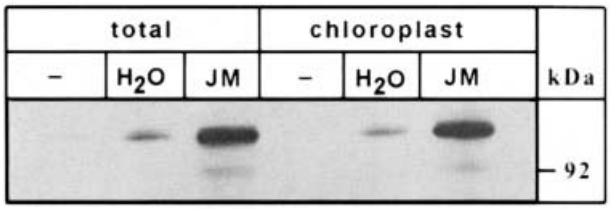

Figure 3. Immunoblot analysis comparing crude protein extracts with chloroplast preparations from barley leaf segments.
Crude protein extracts from protoplasts (total) or protein preparations of intact chloroplasts (chloroplast), untreated (-), incubated for 43 h with water (H_2O), or floated for 3 h with JM and subsequent incubation for 40 h with water (JM) as pointed out in Experimental procedures. Samples were separated by SDS-PAGE, electroblotted on to nitrocellulose, assayed with LOX-specific antibodies and visualized by peroxidase conjugated secondary antibodies.

LOX protein, intact chloroplasts were isolated from untreated leaves and from leaf segments treated with water or jasmonate, according to Hause *et al.* (1994). Proteins of crude extracts and purified chloroplasts of the same preparation were analysed by SDS-PAGE and subsequent immunoblot analysis (Figure 3). The three lanes on the left-hand side visualizing crude extracts illustrate the situation for LOX induction as already described in Figure 1(a); the right-hand lanes describe the set of LOX forms which were located in purified chloroplasts. In contrast to the situation shown in Figure 1(a), LOX-98 was not detectable in crude extracts. It was apparent, as already shown in the immunofluorescence analysis, (Figure 2c and d), that in barley leaves the bulk of LOX forms were located within the chloroplasts.

Finally, we asked the question, whether the chloroplastic localization of JM-induced LOX protein in barley leaves could be a more general phenomenon. Therefore, the intracellular distribution was investigated also in leaves from soybean and *Arabidopsis*. In soybean the situation is quite different and rather complex. LOX protein was restricted to paraveinal mesophyll cell layers and to cells of the bundle sheath (Figure 4a–c). In the paraveinal mesophyll cell layers, LOX was mainly located within the vacuole (Figure 4a). But in the cells of the bundle sheath the LOX protein occurred additionally within the cytoplasm (Figure 4b). Considerable amounts could also be seen in the nucleus (Figure 4b), which was defined by parallel DAPI staining (Figure 4c). When leaves were treated with water no additional labelling was detectable as shown for spongy mesophyll cells (Figure 4d), in contrast to the situation illustrated on immunoblots (Figure 1b). But upon JM treatment LOX protein was also detectable in spongy mesophyll cells of soybean leaves (Figure 4e). In untreated- and JM-treated leaves of *Arabidopsis* (Figure 4f and g) a situation was found similar to that of barley leaves. In untreated leaves LOX was not detectable, whereas in JM-treated leaves the chloroplasts of spongy mesophyll cells exhibited remarkable amounts of LOX protein.

Discussion

There are at least four different aspects in plant biology where lipoxygenase has distinct functions: (i) senescence; (ii) growth and development; (iii) response to wounding and pathogen attack; and (iv) N-partitioning (Grimes *et al.*, 1993; Hildebrand, 1988; Siedow, 1991). It is striking that all of these aspects show jasmonate-responsive events. These data, and the reports that elicitation of plant cell suspension cultures responds with increasing jasmonate and synthesis of phytoalexins, suggest that jasmonate is a general signal of plant defence response (Gundlach *et al.*, 1992; Müller *et al.*, 1993). However, as it has been pointed out that LOX products correlate with antifungal (Ohta *et al.*, 1990) or antibacterial activities (Croft *et al.*, 1993) as a response to LOX increase during a hypersensitive response, we have to keep in mind that there are at least three different pathways involving LOX activity, those related to jasmonic acid, to C_6 or C_9 aldehydes and traumatic acid, or to oxylipins. In this context it is important to differentiate between the various LOX forms and to evaluate their role in the event under study.

As a first step, we have investigated the patterns of LOX forms in barley leaves after jasmonate treatment and we provide evidence for their chloroplast location. Among diverse functions of different LOX forms in plants (Siedow, 1991) some might catalyse the first step in the synthesis of the putative signal compound jasmonic acid (Farmer and Ryan, 1992). The induction of LOX forms by jasmonate presented here is to our knowledge the first example of JM-induced LOX forms in monocots, whereas it has been found repeatedly in dicots. Among them soybean and *Arabidopsis*, have been studied in more detail. Beside JM-induced LOX transcripts in soybean suspension cultures (Bell and Mullet, 1991), JM also induces a LOX transcript homologous to a coding region of a LOX cDNA clone in seedlings (Grimes *et al.*, 1992) that persisted in maturing plants (Park *et al.*, 1994). In all cases described, the authors presented evidence for the identification of LOX forms homologous to the vsp-94 family, as well as for their vacuolar localization. In *Arabidopsis* JM-responsive LOX forms were found to occur in roots, germinating seedlings and leaves (Bell and Mullet, 1993; Melan *et al.*, 1993), and LOX forms seem to function not only in plant stress responses but also during seed germination (Melan *et al.*, 1994).

The scenario of different LOX forms is much more obvious having determined LOX proteins via immunoblot analysis. At least three different aspects of occurrence and levels of LOX forms have to be regarded with respect

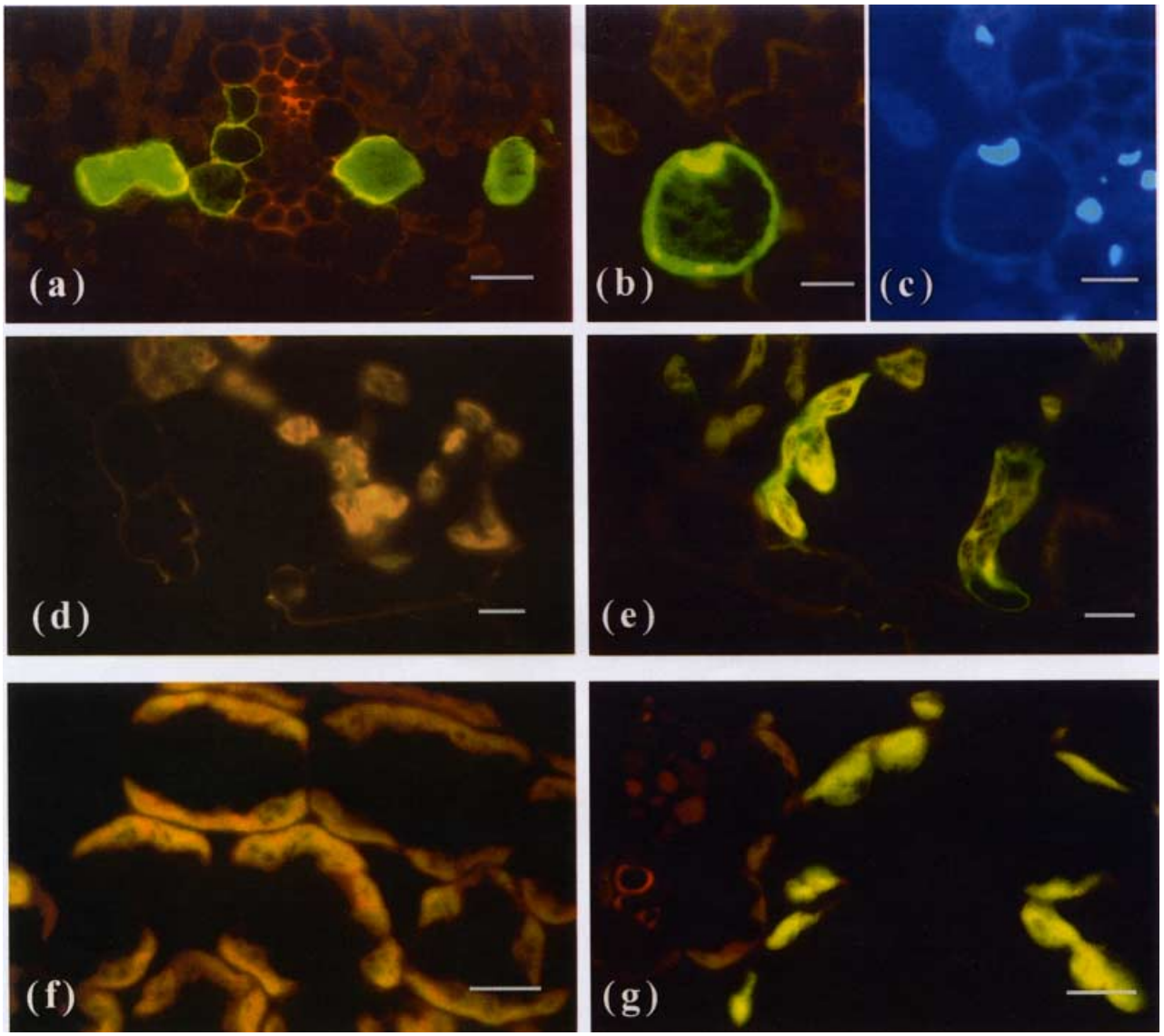

Figure 4. Immunofluorescence staining of cross-sections of leaf tissue from soybean and *Arabidopsis*.
Survey about localization of LOX protein in green leaf tissue of soybean (a–e) and *Arabidopsis* (f and g). (a, b, d–g) reveal the immunolabelling of LOX forms visualized by FITC.
(a) Cross-section of an untreated soybean leaf. Note, the strong label within the cells of the bundle sheath and within the paraveinal mesophyll cells. Bar represents 25 μm.
(b and c) Cell of the bundle sheath of an untreated soybean leaf. In addition to the label in the cytoplasm and the vacuole, LOX protein is detected also within the nucleus, which is visualized by DAPI staining in (c). Bars represent 10 μm.
(d) Cross-section of a soybean leaf disc treated for 42 h with water. The area around a stomatal apparatus is shown. Note, the orange autofluorescence of the chloroplasts of the mesophyll cells. Bar represents 10 μm.
(e) Cross-section of a soybean leaf disc treated with 45 μM JM for 42 h. The area around a stomatal apparatus is shown. Note, the label within the cytoplasm of the mesophyll cells. Bar represents 10 μm.
(f) Cross-section of an untreated *Arabidopsis* leaf. The mesophyll cells exhibit only autofluorescence. Bar represents 10 μm.
(g) Cross-section of an *Arabidopsis* leaf treated with 45 μM JM for 42 h. The chloroplasts of mesophyll cells show label. Bar represents 10 μm.

to results presented here: (i) constitutive expression; (ii) induction by JM; and (iii) intracellular location.

(i) Analogous to green leaves of rice (Ohta *et al.*, 1986), *Arabidopsis* (Melan *et al.*, 1994) or French bean (Eiben and Slusarenko, 1994), untreated green barley leaves contain a constitutively expressed low level of a 100 kDa LOX (Figure 1a, H_2O) which increases in response to water treatment. This rise might be caused by wounding and/or by disturbing sink/source relationships in the excised leaves.

(ii) Most interestingly, analogous to dicots such as *Arabidopsis* (Melan *et al.*, 1994), soybean (Bell and Mullet, 1993; Grimes *et al.*, 1992; Staswick, 1994), and potato

(Geerts *et al.*, 1994), green barley leaves respond upon JM treatment with a dramatic increase of a pre-existing LOX form of 100 kDa (Figure 1a, JM). Concerning the data presented so far it has to be assumed, that LOX-100 is not identical to JIP-100. LOX-100 is detectable in untreated, water-treated, and JM-treated leaves, whereas JIP-100 could not be detected in untreated leaves or in water-treated leaves (Hause *et al.*, 1994). This transient increase and the different temporal appearance of further JM-inducible isoforms of 92 and 98 kDa suggest a complex regulation of LOX forms in barley.

(iii) Subcellular localization of LOX forms has not been addressed satisfactorily (Mack *et al.*, 1987). Soluble LOX have been found at high amounts in the cytosol of manifold seeds (Gallaird and Chan, 1980). Thus, LOX activity has been described in vacuoles of roots and fruits of cucumbers (Wardale and Gallaird, 1977), but in potato LOX is a cytosolic enzyme (Berkeley and Gallaird, 1976). In the case of senescent tissues such as tomato fruits LOX seems to be located in microsomal fractions (Rouet-Mayer *et al.*, 1992; Todd *et al.*, 1990), and beside cytosolic LOX forms, particulate LOX forms occurred in the membranes of lipid bodies and microsomes in 4-day-old mesophyll cells of etiolated cucumber cotyledons (Feussner and Kindl, 1994). For green leaves, LOX activity was correlated with chloroplastic pellets in wheat leaves (Douillard and Bergeron, 1981; Wardale and Gallaird, 1975). Until now, analysis of intracellular compartmentation of LOX forms using immunocytochemical approaches (as the more reliable methods) has been restricted to soybean cotyledons. In soybean leaves and cotyledons LOX was found in the vacuole of paraveinal mesophyll cells and in the cytoplasm of storage tissues (Tranbarger *et al.*, 1991; Vernooy-Gerritsen *et al.*, 1983). In response to JM treatment the majority of LOX was accumulated in vacuoles of paraveinal mesophyll cells. Furthermore, newly synthesized LOX was detectable at low amounts in vacuoles and in the inclusion bodies of plastids of epidermal and cortical cells (Grimes *et al.*, 1992).

From determination of chloroplastic signal sequences in JM- and pathogen-induced LOX cDNAs, recently, it was suggested that the gene products were located within the chloroplasts (Bell and Mullet, 1993; Peng *et al.*, 1994). Here we provide direct evidence for a plastid location in the case of JM induction. Using immunofluorescence analysis in combination with Western blot analysis of the proteins of isolated chloroplasts LOX was clearly localized within this organelle (Figures 2 and 3). The observation that LOX-98 was not induced by JM under the conditions used for isolating intact chloroplasts could only be explained by the different incubation protocol (see Experimental procedures). Comparing the data presented for barley with the results described for other plants, one may suggest that *Arabidopsis* displays a simplified system which is analogous to the situation found in barley. Observations for soybean indicate a different regulation and localization of LOX forms. As well as the well-known vacuolar forms (Grimes *et al.*, 1992; Tranbarger *et al.*, 1991) we describe a localization of LOX protein in the nucleus of the bundle sheath cells (Figure 4b).

From the biochemical and genetic data known so far it seems that discrete plant tissues contain LOX enzymes as small and individual sets of LOX isoforms, which are specifically regulated by developmental and environmental conditions (Eiben and Slusarenko, 1994; Grayburn *et al.*, 1991; Melan *et al.*, 1994; Park *et al.*, 1994). This might be reflected at the level of LOX activity-derived compounds, such as jasmonate, or C_6 and C_8 aldehydes, respectively, thus leading to altered signalling as well as to stress/defence responses. Analysis of individual LOX forms and their substrate specificity as well as products formed by them will help to describe distinct functions intracellularly and during developmental and environmental reprogramming.

The intracellular locations of LOX protein described so far are in good correlation with their substrate pools of different developmental stages of the corresponding tissues. In germinating cotyledons, tissues without fatty acid synthesis, LOX forms are mainly soluble or are located in lipid bodies next to bulk amounts of unsaturated fatty acids as their substrates (Feussner and Kindl, 1992; Siedow, 1991). In contrast, in mature leaves fatty acid synthesis occurs mainly in chloroplasts (Somerville and Browse, 1991). Here LOX forms as demonstrated in this paper and other enzymes of the hydroperoxide metabolism (Vick and Zimmerman, 1987) are found next to immediately available substrate. Furthermore, in senescent plant organs, LOX is located in microsomal membranes where desaturation of fatty acids also occurs (Rouet-Mayer *et al.*, 1992; Todd *et al.*, 1990).

Experimental procedures

Plant material and treatment of leaf segments

Primary leaves of 7-day-old seedlings of barley (*Hordeum vulgare* cv. Salome) grown in soil under greenhouse conditions with continuous light (3000 lux) at 24 °C were used in all experiments. Soybean (*Glycine max* cv. Maple Arrow) and *Arabidopsis thaliana* (cv. Blanes) were grown for 5 weeks under the same conditions.

Leaf segments from barley (5 cm length, starting 1 cm below the tip) were floated for distinct periods of time (as indicated in the Results) on water or 45 μM JM. Similarly, leaf discs from soybean (diameter 2 cm) and leaves from *A. thaliana* were incubated for 42 h on water or 45 μM JM. In order to isolate chloroplasts (see below) the incubation of barley leaf segments with JM was shortened to 3 h followed by H_2O treatment for the final 40 h,

because we were unable to isolate sufficient amounts of intact protoplasts from barley leaves treated for 24 h or longer with 45 μM JM. As shown recently by Hause *et al.* (1994) the detectable pattern shown on silver-stained SDS-gels of JIPs seems to be identical for both treatments with JM.

Immunoblot analysis

Proteins from leaves, protoplasts, as well as isolated chloroplasts were extracted according to Meyer *et al.* (1988), solubilized in SDS-sample buffer and subjected to SDS–PAGE.

SDS–PAGE and immunoblot analysis were performed as described by Feussner and Kindl (1994). Protein samples (3 μg lane^{-1}) were separated on modified SDS–PAGE gels and transferred on to nitrocellulose membranes. Prior immunodecoration membranes were stained with fast green in order to control the transfer quality. After removal of the protein stain, membranes were blocked with 3% BSA in PBS (20 mM phosphate buffer, pH 7.4, 120 mM NaCl), incubated with rabbit anti-LOX antibodies (dilution 1:500; in PBS, 3% BSA) prepared according to Feussner and Kindl (1992), and finally immunodecorated with peroxidase-coupled sheep anti-rabbit IgG antibodies (dilution 1:500; in PBS, 3% BSA; Boehringer), and visualized by applying a mixture of 2 mM chloronaphthol and 0.05% hydrogen peroxide. On immunoblots the antibodies reacted specifically with lipoxygenase bands.

Immunofluorescence microscopy

Fixation of tissues in PBS containing 3% paraformaldehyde/0.1% Triton X-100, embedding in polyethyleneglycol (PEG, PEG 1500:PEG 4000 = 2:1) and cutting into 2 μm sections were performed as described by Van Lammeren *et al.* (1985). Immunolabelling was carried out with rabbit anti-LOX antibodies (see above), and a goat anti-rabbit-IgG-FITC conjugate (Sigma) as secondary antibody. After immunolabelling, sections were stained with 0.1 μg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI; Sigma) for 30 min and mounted in para-phenylenediamine (0.2 % in glycerol). Control experiments were performed by omitting the first antibody and revealed no signals. The fluorescence of DNA and immunolabelled LOX was visualized with a Nikon Optiphot epifluorescence microscope using the following filters: for DAPI: EX 365/DM 400/BA 420 and for FITC: EX 470–490/DM 510/BA 520.

Isolation of intact chloroplasts

Chloroplasts were isolated as described for barley leaf segments by Hause *et al.* (1994). Leaf segments were treated with 20 mM citrate buffer, pH 5.5, 0.5 M sorbitol, 1% cellulase Y-C and 0.2% pectolyase Y-23 (Seishin) at 30°C for 3 h. The lysis of protoplasts was performed in 25 mM Tricin–HCl, pH 8.0, 0.5 M sorbitol, 5 mM EDTA, 5 % Percoll (Pharmacia), 0.1% BSA and afterwards chloroplasts were purified by various centrifugation steps in discontinuous Percoll gradients according to Kaiser and Heber (1984). The chloroplast fraction was characterized by specific markers: (i) amount, by chlorophyll content (Arnon, 1949); (ii) intactness, by amount of ribulose-1,5-bisphosphate carboxylase/oxygenase in the gelelectrophoretic separation of proteins; (iii) contaminations, by activity of malate dehydrogenase (EC 1.1.1.37, Bergmeyer, 1970).

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Isolation, characterization and expression of a cDNA coding for a jasmonate-inducible protein of 37 kDa in barley leaves

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ABSTRACT

In barley leaves, there is a dramatic alteration of gene expression upon treatment with jasmonates leading to the accumulation of newly formed proteins, designated as jasmonate-inducible proteins (JIPs). In the present study, a new jasmonate-inducible cDNA, designated pHvJS37, has been isolated by differential screening of a λgt10 cDNA library constructed from mRNA of jasmonate-treated barley leaf segments. The open reading frame (ORF) encodes a 39·9 kDa polypeptide which cross-reacts with antibodies raised against the *in vivo* JIP-37. The hydropathic plot suggests that the protein is mainly hydrophilic, containing two hydrophobic domains near the C-terminus. Database searches did not show any sequence homology of pHvJS37 to known sequences. Southern analysis revealed at least two genes coding for JIP-37 which map to the distal portion of the long arm of chromosome 3 and are closely related to genes coding for JIP-23. The expression pattern of the JIP-37 genes over time shows differential responses to jasmonate, abscisic acid (ABA), osmotic stress (such as sorbitol treatment) and desiccation stress. No expression was found under salt stress. From experiments using an inhibitor and intermediates of jasmonate synthesis such as α-linolenic acid and 12-oxophytodienoic acid, we hypothesize that there is a stress-induced lipid-based signalling pathway in which an endogenous rise of jasmonate switches on JIP-37 gene expression. Using immunocytochemical techniques, JIP-37 was found to be simultaneously located in the nucleus, the cytoplasm and the vacuoles.

Key-words: *Hordeum vulgare* L.; Gramineae; cDNA; intracellular location; jasmonate-induced 37 kDa protein; mapping; stress-induced expression.

INTRODUCTION

An increasing number of genes are known to be expressed by the ubiquitously occurring plant growth regulator jasmonic acid (JA) or its methyl ester (JM) (for a review, see Sembdner & Parthier 1993). In dicotyledonous plants, at least five different classes of JIPs can be distinguished: (i) proteinase inhibitors such as proteinase inhibitors I and II of tomato (Peña-Cortés *et al.* 1989; Ryan 1992) or potato (Peña-Cortés *et al.* 1989; Xu *et al.* 1993); (ii) enzymes such as leucine aminopeptidase (Herbers *et al.* 1994), threonine deaminase in tomato (Hildmann *et al.* 1992), some of the alkaloid-synthesizing enzymes in elicitated cell suspension cultures (Dittrich *et al.* 1992; Gundlach *et al.* 1992) and some of the lipoxygenases (LOX) in *Arabidopsis thaliana* (Melan *et al.* 1993), barley (Feussner *et al.* 1995) and soybean (Bell & Mullet 1991); (iii) vegetative storage proteins in soybean (Creelman *et al.* 1992; Mason & Mullet 1990; Staswick 1990); (iv) seed storage proteins such as napin and cruciferin in oil seed rape (Wilen *et al.* 1991), and (v) glycine- and hydroxyproline-rich proteins in soybean (Creelman *et al.* 1992).

The diversity of jasmonate-responsive genes and the functions of the proteins they encode suggests that this plant growth regulator might be a signal in various growth and developmental processes such as the plant defence response (Melan *et al.* 1993), the response to herbivore attack including wounding (Ryan 1992), N storage (Wilen *et al.* 1991), N partitioning (Grimes *et al.* 1992; Mason & Mullet 1990; Staswick 1994), cell wall loosening during growth (Creelman *et al.* 1992), alkaloid synthesis (Gundlach *et al.* 1992), tendril coiling (Falkenstein *et al.* 1991; Weiler *et al.* 1993) and water deficit (Bell & Mullet 1991; Lehmann *et al.* 1995). Such a role for jasmonate in a lipid-based signalling pathway was first proposed for wounding (Farmer 1994). This is now substantiated by the fact that environmental conditions leading to elicitation, wounding or water deficit are accompanied by an endogenous rise in jasmonates followed by the expression of specific genes (Gundlach *et al.* 1992; Hildmann *et al.* 1992; Lehmann *et al.* 1995; Ryan 1992).

JIPs of different plant species differ with respect to their molecular mass and immunological properties (Herrmann *et al.* 1989). In addition to this species-specificity, the pleiotropic response to jasmonate is also reflected in various functions of the proteins induced within one species upon jasmonate treatment. For example, in barley, where JIPs were observed for the first time (Weidhase *et al.* 1987), there were at least eight abundantly induced proteins of which a 6 kDa thionin (Andresen *et al.* 1992), a 23 kDa protein (function unknown) (Andresen *et al.* 1992), a 60 kDa ribosomal inactivating protein (RIP) (Becker & Apel 1992; Chaudhry *et al.* 1994), and three LOX forms

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(Feussner *et al.* 1995) have so far been identified, although their functions are still under discussion. A role for JIP-60 as a plant defence protein via its ribosome-inactivating properties, which was hypothesized recently in the absence of testing in a plant–pathogen system (Reinbothe *et al.* 1994), is highly improbable because chemically induced or genetically based resistance in barley was not accompanied by the expression of JIP-60 (Kogel *et al.* 1995). To understand jasmonate-induced alteration of gene expression in barley leaves, the identification of as yet unknown genes coding for JIPs may be helpful. The work presented here was undertaken to isolate and to characterize a new jasmonate-responsive cDNA clone, designated pHvJS37. After determination of its sequence and gene number, mapping and bacterial overexpression were performed. The protein encoded by this cDNA was identified and subsequently localized intracellularly using an immunocytochemical approach. The patterns of expression in response to jasmonate and ABA treatment, as well as various stress conditions, were studied.

MATERIALS AND METHODS

Plant material and growth and incubation conditions

Seedlings of barley (*Hordeum vulgare* L. cv. Salome as well as cultivars mentioned in the legend of Fig. 3) were grown in soil under greenhouse conditions in continuous light (130 μmol m^{-2} s^{-1}) at 24 °C. In all experiments, 5 cm leaf segments were cut starting 1 cm below the tip of 7-d-old primary leaves. Segments were floated in petri dishes either on water or on aqueous solutions containing 45 mmol m^{-3} of freshly prepared (±)-JM (Firmenich, Geneve, Switzerland), 90 mmol m^{-3} (±)-*cis,trans*-ABA (Sigma Chemical Co., St. Louis, MO), 1 kmol m^{-3} sorbitol, or 1 kmol m^{-3} sodium chloride, and kept at 25 °C in continuous white light (120 μmol m^{-2} s^{-1}) provided by fluorescent lamps (NARVA, Berlin, NC 250/01) or treated as specified in the legend of Fig. 5. For desiccation stress, leaf segments were wilted in open petri dishes under the above-mentioned light conditions until 30% fresh weight had been lost. The fairly high stresses used compared to those for dicots were necessary to obtain an endogenous rise in the level of jasmonates which was able to induce JIP-37. Subsequently, petri dishes were covered with parafilm to keep the leaves at the desired humidity for the periods indicated in Fig. 5.

Extraction of DNA and RNA

High-molecular-weight barley DNA was isolated according to the protocol of Cone (1989). Plasmid DNA was isolated using the QUIAGEN Plasmid Kit (DIAGEN) for maxipreparation, and the Magic™ Miniprep DNA Purification System (Promega) for minipreparations, according to the supplier's instructions. Total RNA was extracted by phenol/chloroform/iso-amylalcohol treatment as described by Chirgwin *et al.* (1979) with the modifications by Andresen *et al.* (1992). Poly(A^+)-RNA was isolated by oligo-dT chromatography according to Aviv & Leder (1972).

Northern blot and Southern blot analysis

RNA electrophoresis (20 μg total RNA per lane) and Northern blot analysis with JIP-specific cDNA probes were performed according to Maniatis *et al.* (1989). After the transfer of RNA onto nitrocellulose BA 85 filters (Schleicher & Schüll), the filters were hybridized with a [^{32}P]-α-dATP-labelled cDNA insert prepared from the plasmid pHvJS37. Labelling was achieved by Nick translation using the system of GIBCO BRL. Hybridization was carried out in a solution containing: 6 × sodium salt citrate buffer (SSC), 5 × Denhardt's solution, 1% (w/v) sodium dodecyl sulphate (SDS), and 100 μg cm^{-3} sheared salmon sperm DNA at 65 °C for 20 h. The filters were washed once in a solution of 6 × SSC and 0·1% (w/v) SDS, three times in a solution of 2 × SSC and 0·1% (w/v) SDS, and once in a solution of 0·2 × SSC and 0·1% (w/v) SDS at 65 °C for 15 min each. Finally, the filters were exposed to AGFA X-ray film with an intensifying screen at –70 °C. Estimation of RNA sizes was based on the 0·24–9·5 kb RNA ladder of GIBCO BRL. For Southern blot analysis, 10 μg of genomic DNA was digested with the restriction enzyme *Eco*RI. Agarose gel electrophoresis, Southern blotting and hybridization were performed according to Maniatis *et al.* (1989).

Genetic mapping

DNA extraction, digestion and hybridization were performed according to Graner *et al.* (1991). For restriction fragment length polymorphism (RFLP) mapping, a population comprising 108 F3 progeny lines of a cross between the cultivar *Vada* and the *Hordeum spontaneum* line 1B-87 was used. The JIP-37 gene was placed onto the map relative to a set of previously mapped RFLP markers (Graner *et al.* 1991). Linkage analysis was performed using Mapmaker computer software (Lander *et al.* 1987). The MacIntosh version (1·0) was kindly provided by Dr S.V. Tingey (DuPont, Wilmington). Crossover units were converted into map distances (centi Morgans, cM) by applying the Kosambi function.

Construction and screening of a cDNA library

Poly(A^+) containing RNA (5 μg) from leaf segments treated with JM for 48 h was transcribed into double-stranded cDNA using the cDNA synthesis system Plus (Amersham-Buchler) according to the supplier's instructions. The resulting cDNA was ligated into λgt10-arms by a cDNA cloning system (Amersham-Buchler) and packaged *in vitro* using Gigapack II plus packaging extract (Stratagene) according to the supplier's protocol. The cDNA library was screened by differential hybridization

using [^{32}P]-γ-ATP-labelled poly(A$^+$) RNA from leaves floated on water or JM for 48 h. Positive plaques were purified.

cDNA sequencing

DNA of positive clones was isolated according to Benson & Taylor (1984) and cloned into the pBluescript SK$^-$ (Stratagene) by standard methods (Maniatis *et al.* 1989). Internal restriction sites were used to generate subclones for sequencing. Plasmid DNA was purified with StrataClean^{TM} Resin (Stratagene) and then sequenced according to Sanger *et al.* (1977) with the ^{T7}sequencing^{TM}-Kit (Pharmacia).

Overexpression in *Escherichia coli*

The vector pJC40 (Clos & Brandon 1994) and the host strain *E. coli* HMS 174 pLysE were used to overexpress the cDNA insert of the clone pHvJS37. The insert was fused to a sequence coding for 10 histidine residues (His-tag) by making a *Bst*EII-*Bam*HI fragment of pHvJS37 with a blunt end and cloning it into the *Xho*I-*Bam*HI digested vector pJC40. The correct construct was confirmed by sequencing. The pJC40, both with and without the cDNA insert, was transformed into *E. coli* HMS174 pLysE. Total proteins of isopropyl-β-thiogalactopyranoside(IPTG)-induced or non-induced cultures were isolated by sonification in cracking buffer [60 mol m^{-3} Tris-HCl, pH 6·8, 1% (w/v) 2-mercaptoethanol, 1% (w/v) SDS, 10% (w/v) glycerol and 0·1% (w/v) bromophenol blue] according to Ausubel *et al.* (1987).

After heating the samples at 95 °C for 5 min, the insoluble cell debris was removed by centrifugation at 10000 × *g* for 5 min. Determination of proteins, their electrophoretic separation and transfer onto nitrocellulose were performed as described recently by Lehmann *et al.* (1995). For immunoblot analysis, rabbit polyclonal antibodies directed against barley JIP-37 were used as primary antibodies. The antibody–protein complexes were visualized using the Western light^{TM} Chemiluminescent Detection System (Tropix, Inc. Bedford, MA).

Immunolabelling and fluorescence microscopy

Leaf segments from barley treated with water or 45 mmol m^{-3} JM for 12, 24, 36 and 48 h were prepared and immunolabelled according to Van Lammeren *et al.* (1985). Small pieces of leaves were fixed with 3% paraformaldehyde and 0·1% Triton X-100 in phosphate-buffered saline (PBS). After rinsing in PBS and dehydration in a graded series of ethanol concentrations, samples were embedded in polyethylene glycol. Sections of 2 μm thickness were immunolabelled with anti-JIP-37 (see above) as the primary antibody, and a goat-anti-rabbit-IgG-FITC (fluorescein-5-isothiocyanate) conjugate (SIGMA Chemical Co., St. Louis, MO) as the secondary antibody. Subsequently, sections were stained with 0·1 μg cm^{-3} 4,6-diamidino-2-phenylindole (DAPI) (SIGMA) and mounted in para-diphenylenamine. Control experiments were performed using the pre-immuneserum and by omitting the first antibody. The fluorescence of immunolabelled JIP-37 and of DAPI-stained nuclei was visualized with a Zeiss 'Axioplan' epifluorescence microscope using the appropriate filters for FITC and DAPI.

RESULTS

Isolation, sequencing and identification of a new jasmonate-induced cDNA of barley

Double-stranded cDNA was synthesized from 5 μg poly(A$^+$) containing RNA of barley leaf segments treated with JM for 48 h and was inserted into the vector phage λgt10, and approximately 1 × 10^6 recombinants were obtained. The library was screened with [^{32}P]-γ-ATP-labelled poly(A)-RNA extracted from water-floated and JM-floated leaf segments. Out of 30000 recombinant phages, 50 independent positive clones were isolated. From these clones, 23 clones cross-hybridized to the known cDNA pHvJ256 coding for JIP-6, and 26 clones cross-hybridized to the cDNA pHvJ3015 known to code for JIP-23 (Andresen *et al.* 1992). Only one clone carrying an insert of 1300 bp was unique, and was designated pHvJS37. Northern blot analysis (see below) showed that this clone was JM-responsive and hybridized to a transcript of 1·35 kb based on a comparison of its mobility to a ladder of RNA size standards.

This new cDNA insert was subcloned into the vector pBluescript KS$^-$ (Stratagene). Both strands were sequenced, giving a sequence of 1334 bp (Fig. 1). Only one of the possible open reading frames was not interrupted by a stop codon, and was found to code for 357 amino acids. This corresponds to a molecular mass of 39·4 kDa, which is in the range of the molecular mass of the known JIP of 37 kDa in SDS-PAGE (Weidhase *et al.* 1987). A search in database (HUSAR-EMBL Heidelberg, Swiss-Prot, using software BLASTP, release of 25/9/95) revealed no significant homology either to the barley protein sequences JIP-6, JIP-23 and JIP-60 (Andresen *et al.* 1992; Becker & Apel 1992) or to any other protein sequences. The hydropathic plot of the amino acid sequence deduced from the nucleotide sequence of clone pHvJS37 was determined according to Kyte & Doolittle (1982). The plot indicates that the protein is mainly hydrophilic with two hydrophobic domains near the C-terminal end (data not shown).

For the identification of the protein coded by pHvJS37 its overexpression in the His-tag expression vector pJC40 transformed into *E. coli* HMS 174 was used, since a coupled *in vitro* transcription/translation experiment was not successful. In addition to pHvJS37, the clone pHvJ3015 coding for JIP-23 was subcloned into pJC40 as a control for cloning and overexpression.

Immunoblot analyses with polyclonal antibodies raised against JIP-23 and JIP-37 from barley were performed

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1                                    CCCTTACCTTGTGCTCGTGGTGAAGCTGATCCTTCCATTGCTTGGTGCCCCCAAGCTACCCCAAGGCTTACATC

75   ATG TCA GGT GAC CTA GTT GAT TTA AGT CCA GGG CAG TTG CAT AAG CTA GCC GAT CTG ATC CAT CGA CAA GAA GTC CAG AAG CTT CAA GAG
      M   S   G   D   L   V   D   L   S   P   G   Q   L   H   K   L   A   D   L   I   H   R   Q   E   V   Q   K   L   Q   E   30

165  CTC GAG TTC AAC TCG TAT GCA GAG CAA CAA AAG TAT CTC CGT GAC GCC AAT GAT GCC CGC GAT AAG GTG TAT CAC ATC CTT GAC AGT GCA
      L   E   F   N   S   Y   A   E   Q   Q   K   Y   L   R   D   A   N   D   A   R   D   K   V   Y   H   I   L   D   S   A   60

255  CGG GAC ATG ATA GCA CAA ACC GAG GCT GAG AAG GAT GCC ACC AAG CAA GAT ATT GGC AAG GAT GTC TAT GAC TAC TGC ACC AAG GCC ATC
      R   D   M   I   A   Q   T   E   A   E   K   D   A   T   K   Q   D   I   G   K   D   V   Y   D   Y   C   T   K   A   I   90

345  GGA ATA TCT CTC CAA TTC ATC CGG AGT TAC AAC ACC CGT CTC ACC TAC CTT GAC AAG CTC AAG ACC CAC AGT GAT GAT CTC ATC AAG CAG
      G   I   S   L   Q   F   I   R   S   Y   N   T   R   L   T   Y   L   D   K   L   K   T   H   S   D   D   L   I   K   Q  120

435  CTA AAG TTC CTC AAT CTA GCC ACC CAA CAG AAG GAG GCC CAG CGC CTT GCC CTT GAG GCT GGC ATG TAT AAG AAA GCC ACG CTG GAG AAT
      L   K   F   L   N   L   A   T   Q   Q   K   E   A   Q   R   L   A   L   E   A   G   M   Y   K   K   A   T   L   E   N  150

525  GCC AAA AAG TTT CAA CAC TTT GCA CCA AAT CAA TTC TCA AAA TGG CTC AAG GAA AAC AAA ATC ATG TTT GAG GAT CTT GTG CAA GAA AAT
      A   K   K   F   Q   H   F   A   P   N   Q   F   S   K   W   L   K   E   N   K   I   M   F   E   D   L   V   Q   E   N  180

615  ATG TCA AAG CTT GGT TTT AGG GGG GCT TTC AAA AAC TTG GAT GAT ATC CAA AAG CTA CAG GTA TAT GAC AAT ATT ATT GCG GAA GCG GGA
      M   S   K   L   G   F   R   G   A   F   K   N   L   D   D   I   Q   K   L   Q   V   Y   D   N   I   I   A   E   A   G  210

705  CAA GGA AAA TCT GTG GTG ACT TAC TCA TTT GAA GCT TTG GGA AAA GTC GGG GTG GCA GTT TTA GTT TTT ACA GCA GCT GCA ATG GTG TGG
      Q   G   K   S   V   V   T   Y   S   F   E   A   L   G   K   V   G   V   A   V   L   V   F   T   A   A   A   M   V   W  240

795  GAC ATA TAC ACA GCA GAA GAT AAG CTG GAG GCA GCG GTT AGG GAT TCA GTA AAT GCG TTA ACT GCA GTA GTT AAC CTT GAG GTG GGA GAA
      D   I   Y   T   A   E   D   K   L   E   A   A   V   R   D   S   V   N   A   L   T   A   V   V   N   L   E   V   G   E  270

885  ATA GTT ACT ACT GCT GTA GAA GCT GGA TTC GTA GCA CTA GAC ATC GAA ATT GCT TCT GCG GCT GTC ACG GTA ATT GGA GGA GTC GTC GGT
      I   V   T   T   A   V   E   A   G   F   V   A   L   D   I   E   I   A   S   A   A   V   T   V   I   G   G   V   V   G  300

975  TTT GGA ATT GGT GCG CTC ATT GGG ATA GCT GCA GGT GCG CTG CTT GAC TTG ATT TTC AGC AGT GGA ACT AGC AAG GTG AAG ATA ACA GAC
      F   G   I   G   A   L   I   G   I   A   A   G   A   L   L   D   L   I   F   S   S   G   T   S   K   V   K   I   T   D  330

1065 GGG CTT ACA GTT TGC CGT GTG GCG CCT ATG CCT CAT GGT CTC GAA CTC GCT CGT CTA GTT AAG CAC AAT TAC CCT GAA CTTTAAACTGATCAT
      G   L   T   V   C   R   V   A   P   M   P   H   G   L   E   L   A   R   L   V   K   H   N   Y   P   E   L ***            357

1158 GCTACTACTATGATCTTTGCTATGCTATCTTCTGCTCTTAGTGKTAGCTACTACTGTCGTGAGTATGTAAGTGCCTAGTGTGCACTCCTAAAATAATACCCCCCGCCCCTGTCGGGAAA

1277 CTATTGTATTCATGATGCCATGATGAGGAATAAAAGTCATGTTGATTCAAAAAAAAAA
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Figure 1. The nucleotide sequence and the derived amino acid sequence of the pHvJS37 cDNA which encodes the JM-induced protein of M_r 37 kDa. Nucleotides and amino acid residues are numbered on the left and right, respectively. The possible polyadenylation signal is underlined. The stop codon is marked by asterisks.

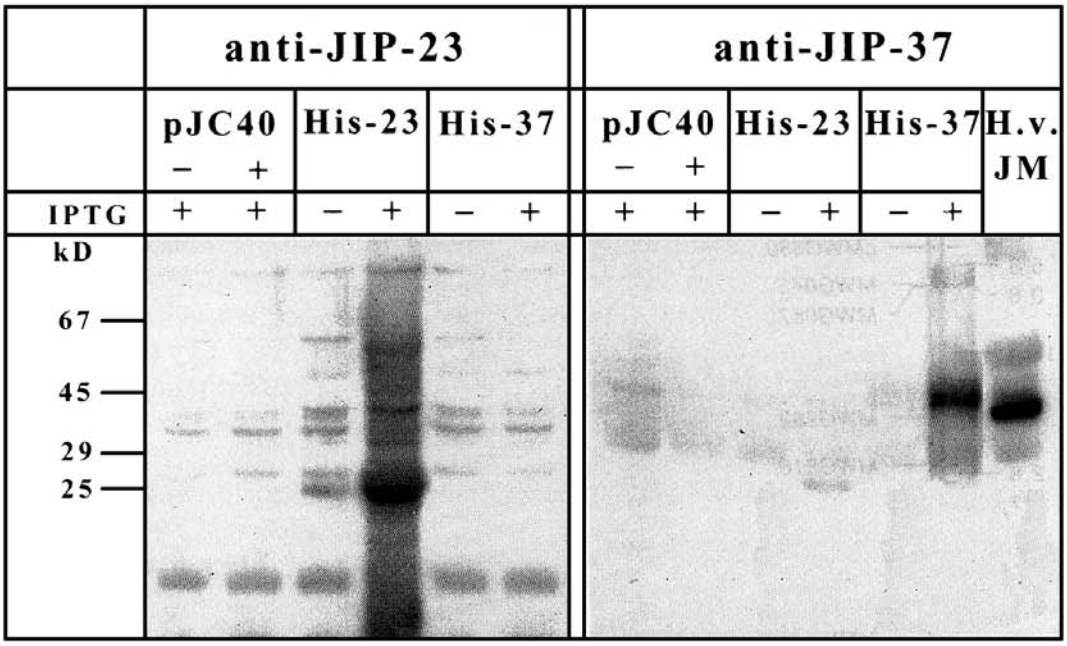

Figure 2. Identification of the translation product of the cDNA clone pHvJS37. After the expression of pHvJS37, and as a control the expression of pHvJ3015, in the His-tag expression vector pJC40 (transformed into *E. coli* HMS 174), total protein extracts were analysed by immunoblot analysis using rabbit polyclonal antibodies raised against JIP-23 and JIP-37. As controls, extracts of cells without the plasmid (pJC40–), of cells with plasmids lacking any insert (pJC40+), or of cells cultured without IPTG (IPTG–; JM–) were tested. For comparison, immunodetection is shown (H.v. JM) of JIP-37 in total protein extracts of barley leaves treated with 45 mmol m^{-3} JM for 48 h. Protein markers are indicated on the left margin.

using total protein of *E. coli* extracts (Fig. 2). IPTG treatment of cells containing the cDNA inserts from clone pHvJ3015 and from clone pHvJS37 resulted in the overexpression of two new proteins with masses of 25 kDa (His-23+) and 40 kDa (His-37+), respectively. On the other hand, in extracts of cells containing only the vector pJC40 (JC40+), or of cells not treated with IPTG (IPTG–; JM–), JIP-23 and JIP-37 were not detectable. Some cross-reactivity, mainly with anti-JIP-23 (lanes 1–6), occurred independently of the presence of the vector or treatment with IPTG. *E. coli* contaminations in the rabbit during antibody formation might be the source of this error, since no cross-reactivity was found with total protein extracts of barley leaves (H.v. JM). With respect to the molecular masses, it should be taken into account that the original translation products of both cDNA inserts were about 2 kDa larger due to the addition of the His-tag tail of the vector pJC40.

Southern analysis for various barley cultivars

To determine the number, the location and complexity of genes coding for JIP-37, genomic DNA fragments obtained by restriction digests with *Eco*RI were separated electrophoretically, and Southern blots were hybridized with the [^{32}P]-α-dATP-labelled cDNA insert of pHvJS37 (Fig. 3). In different cultivars, one major band of 9 kb and one minor band of 6 kb could be determined. This suggests that barley contains two genes which code for JIP-37. Interestingly, some cultivars, such as Gerbel, Atlas S and Atlas 46, do not contain the JIP-37 gene (Fig. 3, lanes 6–8).

Genomic mapping of the JIP-37 gene

In the mapping population, no crossover was observed between the two DNA fragments determined in the Southern analysis. These two DNA fragments are therefore considered to form a single gene, although the possibility that the JIP-37 locus consists of two closely linked genes cannot be ruled out. The JIP-37 gene maps to the distal portion of the long arm of chromosome 3 (Fig. 4). Here, it shows tight linkage with MWG630, a locus detected by an anonymous RFLP marker, and one out of two loci carrying JIP-23 genes (a second JIP-23 locus maps to the distal portion of chromosome 5S).

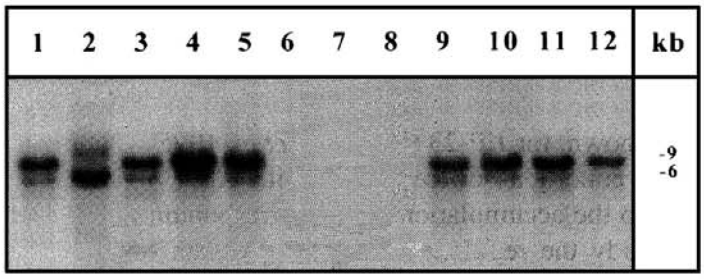

Figure 3. Southern blot analysis of the total DNA of *Hordeum vulgare* cv. California mariout (lane 1); cv. Himalaja (lane 2); cv. Alfa (lane 3); cv. Morex (lane 4); cv. Pallas containing the mlo gene (lane 5); cv. Gerbel (lane 6); cv. Atlas S (lane 7); cv. Atlas 46 (lane 8); cv. Bomi (lane 9); cv. Steptoe (lane 10); cv. Gula (lane 11); and cv. Salome (lane 12). The DNA was digested with *Eco*RI and the resulting DNA fragments were separated by agarose gel electrophoresis, transferred onto nitrocellulose filters and hybridized to the labelled pHvJS37 probe.

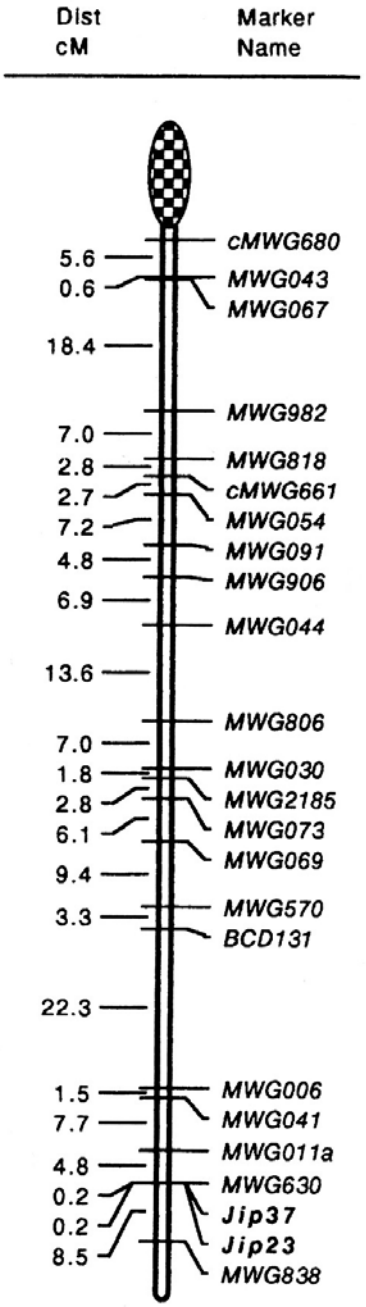

Figure 4. RFLP map of the long arm of chromosome 3. Together with JIP-23, the JIP-37 gene is located in the distal portion.

The JIP-37 gene is differentially regulated by jasmonates, ABA and stress such as desiccation or treatment by salt or osmotica

It is known, for JIP-23 (Lehmann *et al.* 1995), that various stress conditions or treatments with ABA or jasmonates lead to the accumulation of the corresponding transcripts. To study the regulation of the expression of the JIP-37 genes, the temporal accumulation of the transcripts was recorded by Northern blot analysis (Fig. 5A). JIP-37 mRNA continued to accumulate between 6 and 72 h after the onset of JM treatment. A slower accumulation of these transcripts was observed in response to desiccation stress. The responses to both treatments differ clearly from the accumulation of this mRNA in response to ABA treatment. Here these transcripts were observed only after 48 h and increased up to 72 h.

Osmotic stress by sorbitol treatment led to a fast and transient accumulation of JIP-37 transcripts, whereas no accumulation occurred upon salt stress. These data support the idea that the JIP-37 genes are differentially regulated under different stress conditions or hormone treatments. Endogenous jasmonates accumulate upon desiccation stress or osmotic stress but not upon treatment with ABA or NaCl (Lehmann *et al.* 1995; Wasternack *et al.* 1995). To test whether a stress-induced increase of jasmonates is a sufficient signal for accumulation of JIP-37 transcripts, the level of JIP-37 transcript was determined in leaves stressed osmotically in the presence of the growth retardant tetcyclacis (Fig. 5B). Tetcyclacis, which is known to be an inhibitor of jasmonate synthesis at concentrations of 10^{-4} kmol m^{-3}, is able to decrease the jasmonate content of a sorbitol-treated leaf from 800 ng g^{-1} FW down to 300 ng g^{-1} FW (Wasternack *et al.* 1995). At this low jasmonate content, JIP-37 mRNA accumulation ceased (Fig. 5B). 12-oxo-phytodienoic acid, an intermediate metabolite of jasmonic acid biosynthesis which is synthesized in a reaction subsequent to that inhibited by tetcyclacis treatment

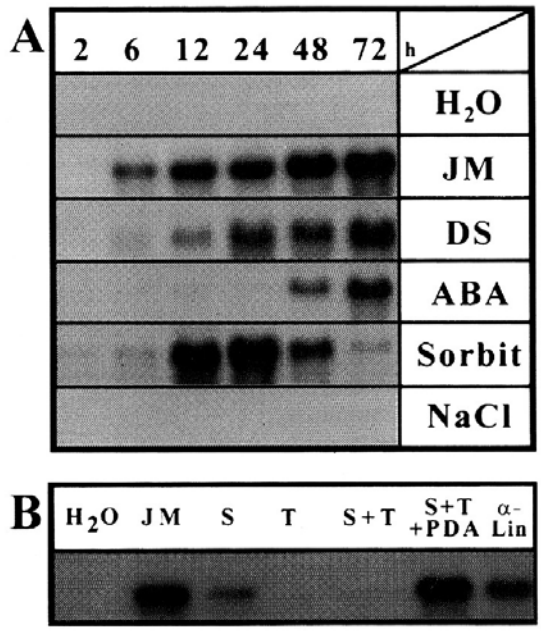

Figure 5. Northern blot analysis of (A) stress- or hormone-induced JIP-37 transcript accumulation in leaves of *Hordeum vulgare* and (B) transcript accumulation after inhibition of stress-induced jasmonate synthesis. For (A), 20 μg of total RNA prepared from leaf segments was loaded per lane; the leaves were floated on water, or solutions of 45 mmol m^{-3} JM, 90 mmol m^{-3} ABA, 1 kmol m^{-3} sorbitol or 1 kmol m^{-3} NaCl, or were desiccated down to 70% of the original fresh weight (DS) for the indicated periods. For (B), 20 μg of total RNA prepared from leaf segments was loaded in each lane; the leaves were floated on water or solutions of 45 mmol m^{-3} JM, 5×10^{-5} kmol m^{-3} tetcyclacis (T), 1 kmol m^{-3} sorbitol (S), 1 kmol m^{-3} sorbitol containing 5×10^{-5} kmol m^{-3} tetcyclacis (S+T) in the absence or presence of 45 mmol m^{-3} 12-oxo-phytodienoic acid (PDA), or 45 mmol m^{-3} α-linolenic acid (Lin) for 24 h. The RNA was fractionated electrophoretically on 1·5% agarose gels, transferred onto nitrocellulose and hybridized with the ^{32}P-labelled cDNA insert of clone pHvJS37. The approximate size of the transcript is 1·35 kb.

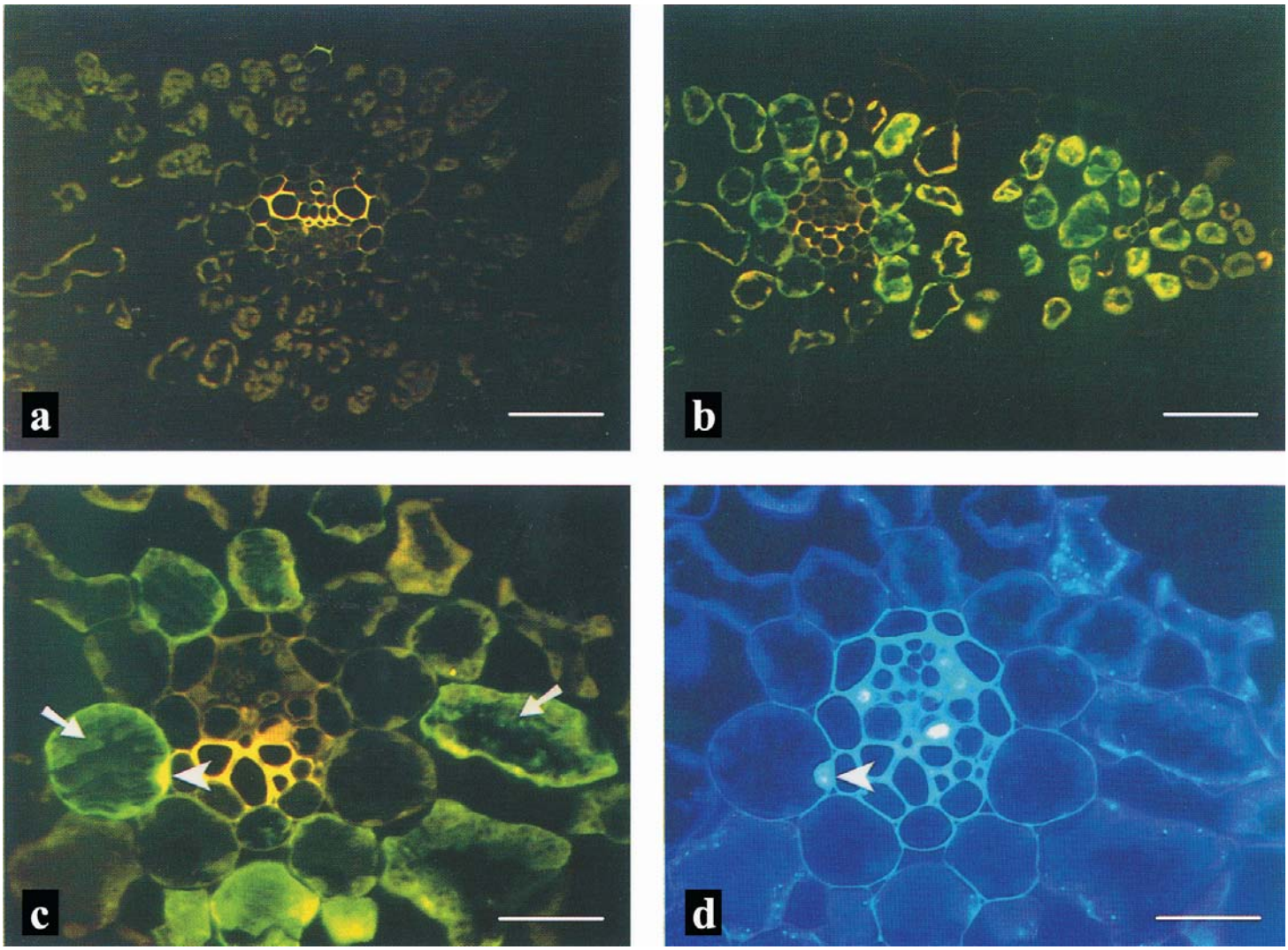

Figure 6. Survey of the localization of JIP-37 visualized by FITC-conjugated antibodies in barley leaf segments treated with JM. (a) Cross-section of an untreated barley leaf showing the brown and yellow autofluorescence of the plastids and vascular bundle, respectively. Bar = 50 μM. (b) Cross-section of a barley leaf treated for 24 h with JM. Note the label within some mesophyll cells. Bar = 50 μm. (c) Detail of (b) showing the subcellular distribution of JIP-37 within the labelled mesophyll cells. Note the label within the vacuoles (arrows) and the nucleus (arrow head). Bar = 20 μm. (d) The same section as in c illuminated for DAPI staining to show the DNA-containing nucleus (arrow head). Bar = 20 μm.

(Wasternack *et al.* 1995), can restore the accumulation of JIP-37 transcripts (Fig. 5B). Additionally, treatment with α-linolenic acid, the precursor of jasmonate synthesis, led to JIP-37 mRNA accumulation (Fig. 5B).

Tissue and intracellular location of JIP-37

Earlier reports from our group on the intracellular localization of JIPs, including JIP-37 in barley leaves, revealed by immunoblot analysis of isolated organelles and immunogold electron microscopy an intracellular distribution of JIP-37 upon JM treatment for 48 h in three compartments: the nucleus, the cytoplasm and the vacuole (Hause *et al.* 1994). Here, we confirm these data by the fluorescence-immunological detection of JIP-37 after different periods of JM treatment. Therefore, cross-sections of barley leaves treated with JM for 12, 24, 36 and 48 h were labelled with anti-JIP-37 followed by treatment with FITC-conjugated secondary antibodies (Fig. 6). As shown by a green fluorescence, JIP-37 was simultaneously detectable in the nuclei, cytoplasm and vacuoles of barley leaves treated with jasmonate for 24 h (Figs 6c&d). The label can be clearly distinguished from the brown autofluorescence of non-treated tissues (Fig. 6a). After 24 h of treatment, JIP-37 could be detected specifically in some cells of the barley leaf tissue (Figs 6b&c).

DISCUSSION

Among hormone-induced alterations of gene expression in plants, the ABA responsiveness of genes has been well studied (Dure 1993; Skriver & Mundy 1990; Voesenet & Van der Veen 1994). The *rab/Lea/dehydrin* genes (responsive to ABA/late embryogenesis abundant/dehydration-induced) have been sequenced from many species and are postulated to have a function in dehydration survival and cold acclimation (Dure 1993; Skriver & Mundy 1990; Wilen *et al.* 1991). More recently, it has been suggested that jasmonates are hormone-like compounds exerting similar effects to ABA on plant growth and development

as well as being involved in signalling environmental stress (Sembdner & Parthier 1993). In contrast to ABA, there are low or no homologies among the genes induced by jasmonates in different plant species (Farmer 1994). Furthermore, within species, for example *Hordeum vulgare*, a variety of functionally independent genes have been found to be expressed upon jasmonate treatment.

One way to elucidate this pleiotropic effect of jasmonate is to carry out the identification and functional analysis of further genes expressed by jasmonate. Here, we describe a new cDNA coding for JIP-37 of barley leaves. The identification was based on the following criteria. A possible open reading frame of 1021 bp was found which codes for a protein of an apparent molecular mass of 39·4 kDa; this corresponds in size approximately to the JIP-37 identified in two-dimensional SDS-PAGE of proteins derived from jasmonate-treated leaf segments (Müller-Uri *et al.* 1988; Weidhase *et al.* 1987). Overexpression of the recombinant protein in *E. coli* HMS174 pLysE yielded a protein of 40 kDa which cross-reacted with an antibody raised against JIP-37 of barley leaves (Herrmann *et al.* 1989). Like other barley JIPs and LEA proteins or dehydrins, JIP-37 was found to be hydrophilic. However, JIP-37 showed no sequence homology to these and other proteins available in the databases. This again demonstrates the diversity of the JIP genes in contrast to genes for dehydrins or LEA proteins. Furthermore, the lack of sequence homology among barley JIPs identified so far (JIP-6, JIP-23, JIP-37 and JIP-60) is in agreement with the fact that an antibody raised against a specific JIP does not react with other JIPs of barley (Herrmann *et al.* 1989). The only exception is the lipoxygenase which was found to be jasmonate-inducible in soybean (Bell & Mullet 1991), *Arabidopsis thaliana* (Melan *et al.* 1993), potato (Geerts *et al.* 1994) and barley (Feussner *et al.* 1995).

Southern analysis revealed that two fragments hybridized to the JIP-37 cDNA probe indicating that most barley cultivars tested contain at least two JIP-37 genes. By screening a genomic library we isolated two different clones. One of them differed significantly from JIP-37 cDNA in the nucleotide sequence of the coding region (data not shown). Therefore, it is possible that the minor band results from lower homology between the second gene and pHvJS37. Another possible interpretation of the hybridization pattern is that the major band contains two copies of the JIP-37 gene.

In contrast to the multi-gene family of thionins in barley, genetic analysis in the present study revealed that JIP-37 is encoded by at last two genes which are located on the long arm of chromosome 3. Together with a series of cultivars not related by descent, one parent of the mapping population (Vada) displayed a null allele at the JIP-37 locus. Since Vada does not show null alleles at any of the neighbouring loci, the deletion must be confined to the close proximity of the JIP-37 gene. Genetic analysis of a much larger progeny will be required, however, to provide a more detailed picture of this chromosomal region, since differences in the likelihood of different orders within the MWG630, JIP-37, JIP-23 cluster are close to zero in the present mapping population.

Due to its dominant inheritance, the application of the JIP-37 gene probe will facilitate the rapid marker-assisted generation of near-isogenic lines. These in turn will provide the basis of a further functional analysis of the gene. The observed absence of the JIP-37 gene in the cultivars Gerbel, Atlas S and Atlas 46 does not lead to a changed phenotype upon various stress treatments if compared with cultivars which express this gene. Transformation of this gene is therefore under study as a part of its functional analysis.

To understand which factors control the expression of the JIP-37 gene, the response to various environmental and hormonal signals was analysed. Northern blot analyses revealed that the JIP-37 gene is differentially regulated upon environmental stress or treatment by jasmonate and ABA. In contrast to LEA and dehydrin genes, JIP-37 transcripts do not accumulate upon salt stress but are transiently synthesized in response to osmotic stress. This strengthens the recently published hypothesis (Lehmann *et al.* 1995) that JIP gene expression in barley triggered by osmotic stress is not dependent on the osmolality of the stressor. Although ABA accumulated at a lower threshold than jasmonate, it is not a primary signal. A detailed inspection of JIP gene expression at elevated and down-regulated levels of ABA and/or jasmonates based on the use of inhibitors and mutants revealed that jasmonate is the primary signal to turn on these genes (Wasternack *et al.* 1994).

The accumulation of the JIP-37 transcript was strongly reduced when leaves were stressed in the presence of tetcyclacis, an inhibitor of jasmonate synthesis (Wasternack *et al.* 1995). This influence of tetcyclacis, however, can be overcome by 12-oxophytodienoic acid, an intermediate in jasmonate biosynthesis. Furthermore, α-linolenic acid, a precursor of jasmonate, leads to JIP-37 gene expression. These facts, and the observation that an endogenous rise of jasmonate is a stringent step of stress-induced JIP gene expression, strongly suggest that a lipid-based signalling pathway exists in barley, in which an elevated jasmonate level is a signal transducer switching on gene expression upon environmental stress. This accords with data on leaves of tomato, potato, soybean and other plants (Farmer 1994). Recently, the conclusion was drawn from studies with cell suspension cultures that 12-oxophytodienoic acid and related compounds might be the primary signals (Blechert *et al.* 1995). So far, there is no proof that these compounds accumulate *in vivo* up to a threshold level at which genes would be turned on, as is known for jasmonates (Wasternack *et al.* 1995). Furthermore, one has to keep in mind that different responses were determined for jasmonates and 12-oxophytodienoic acid derivatives in different biological assays, suggesting different receptor systems (Blechert *et al.* 1995).

In leaf tissues, immunocytochemical labelling of JIP-37 reveals its preferential location in only some mesophyll cells. In contrast, another JIP identified as LOX was found

in all cells under equivalent conditions (Feussner *et al.* 1995). This indicates that the preferential occurrence of JIP-37 was not due to insufficient supply of methyl jasmonate. At the intracellular level, the localization of JIP-37 was clearly detected in the nucleus, the vacuoles and cytoplasm after 24 h of jasmonate treatment. These findings substantiate previous reports on intracellular localization of JIP-37 in barley leaves (Hause *et al.* 1994). Although at the cDNA level no obvious signal sequence could be determined, one can not exclude signal-mediated transport. The question of whether the protein conformation is a sufficient signal for the subcellular localization, as was repeatedly observed for vacuolar proteins (Chrispeels & Raikhel 1992), will be addressed by heterologous expression of the JIP-37 genes.

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Developmental and Tissue-Specific Expression of JIP-23, a Jasmonate-Inducible Protein of Barley

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Developmental expression of a 23 kDa jasmonate-induced protein (JIP-23) of barley leaves (*Hordeum vulgare* cv. Salome) was studied by measuring the time-dependent accumulation of transcript and protein during germination. Tissue-specific expression of JIP-23 was analyzed immunocytochemically and by in situ hybridizations, respectively. During seed germination JIP-23 mRNA was found to accumulate transiently with a maximum at 32 h, whereas the protein was steadily detectable after the onset of expression. The occurrence of new isoforms of JIP-23 during germination in comparison to jasmonate-treated leaves suggests, that the JIP-23 gene family of barley is able to express different subsets of isoforms dependent on the developmental stage.

JIP-23 and its transcript were found mainly in the scutellum, the scutellar nodule and in lower parts of the primary leaf of 6 days old seedlings. All these tissues exhibited high levels of endogenous jasmonates. In situ hybridization revealed specific accumulation of JIP-23 mRNA in companion cells of the phloem in the nodule plate of the scutellum. In accordance with that, JIP-23 was detected immunocytochemically in phloem cells of the root as well as of the scutellar nodule and in parenchymatic cells of the scutellum. The cell type-specific occurrence of JIP-23 was restricted to cells, which are known to be highly stressed osmotically by active solute transport. This observation suggests, that the expression of this protein might be a response to osmotic stress during development.

Key-words: Endogenous jasmonate level — Germination — *Hordeum vulgare* L. — Immunofluorescence analysis — In situ hybridization — Scutellar nodule.

Among jasmonates, a class of cyclopentanone compounds, mainly (−)-jasmonic acid (JA), its methyl ester (JM) and its amino acid conjugates are regarded to be putative regulators of plant growth and development (Sembdner and Parthier 1993). (−)-JA occurs ubiquitously in higher plants (Yamane et al. 1981, Meyer et al. 1984). Similar to abscisic acid (ABA), JA was suggested to be a signal functioning upon environmental stress. To elucidate signalling via JA and its function in altering expression of distinct genes, JA-inducible genes have been identified. Among them there are genes which express the wound-induced proteinase inhibitors I and II of tomato and potato, implicating a role of JA in the plant defence related to herbivore attack (Peña-Cortés et al. 1989, Ryan 1992). In contrast, N-partitioning in soybean via vegetative storage proteins is triggered also by JA (for review see Staswick 1994). Among the enzymes induced by jasmonate there are lipoxygenases (Bell and Mullet 1991, Melan et al. 1993, Feussner et al. 1995) and the alkaloid-synthesizing enzymes of elicitated cell suspension cultures (Dittrich et al. 1992, Gundlach et al. 1992).

In barley leaves JA exerts two different processes: (i) a down-regulation of mainly plastid proteins such as ribulose bisphosphate carboxylase/oxygenase caused by a translational control (Reinbothe et al. 1993), (ii) synthesis of abundant proteins, so-called jasmonate-induced proteins (JIPs) of different molecular masses as 6, 23, 37, 66, 68, 100 kDa (JIP-6, JIP-23, JIP-37, JIP-66, JIP-68, JIP-100) (Weidhase et al. 1987, Hause et al. 1994). On the basis of sequence homology, JIP-6 was identified as a leaf thionin (Andresen et al. 1992) and JIP-60, which could be shown to be identical with JIP-66 (Demus, unpublished), was found to be a ribosome inactivating protein (RIP) (Chaudhry et al. 1994, Reinbothe et al. 1994). In contrast, no indication of function could be drawn from sequence comparisons in the data bases for cDNA clones coding for JIP-23 (Andresen et al. 1992) or JIP-37 (Leopold et al. 1996). Another group of JIPs in barley was immunologically characterized as a family of lipoxygenases (Feussner et al. 1995).

All these proteins are synthesized upon exogenous application of jasmonate or its endogenous rise, which occurs due to environmental stress, e.g. desiccation or osmotic stress (Lehmann et al. 1995). In any case of JIP gene expression observed so far in barley leaves, jasmonate was found to be the most effective inducer (Wasternack et al. 1995). The question remains, as to whether JIP gene expression can be developmentally, regardless of the JA content of the respective tissue. As known for a number of so-called stress related proteins, these are expressed during distinct developmental stages of a plant life. As described for heat shock

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumine; DIG, digoxigenine; FITC, fluorescein-5-isothiocyanate; JA, jasmonic acid; JIP, jasmonate-induced protein; JM, jasmonic acid methyl ester; NBT, nitroblue tetrazolium chloride; PBS, phosphate buffered saline; PEG, polyethylene glycol; SSC, sodium salt citrate.

[1] The first two authors contributed equally to this work.

proteins, gene expression is differentially regulated during embryogenesis (Domoney et al. 1991, DeRocher and Vierling 1995, Zur Nieden et al. 1995) as well as during morphogenesis (Duck et al. 1989). Some of the pathogenesis-related (PR) proteins, which are mainly expressed in response to pathogenic attack, are constitutively expressed like β-1,3-glucanases in different tissues of unstressed potato (Garcia-Garcia et al. 1994), chitinases in reproductive organs of rice (Zhu et al. 1993) as well as during somatic embryogenesis in carrot (De Jong et al. 1992) or PR10 in the stigma of potato plants (Constabel and Brisson 1995).

These data prompted us to investigate developmental and tissue-specific expression of JIP-23, the most abundant jasmonate responsive protein of barley leaves. Using immunocytochemical techniques and in situ hybridization, cell- and tissue-specific expression in 6 days old plants was determined. By Western and Northern blot analysis, expression of JIP-23 gene(s) during germination and seedling growth was recorded in comparison with the jasmonate content of the respective tissues.

Materials and Methods

Plant material—Barley (*Hordeum vulgare* cv. Salome) grains were germinated on wet filter paper in the dark at 24°C up to 3 days. At selected time periods, seedlings were dissected, frozen in liquid nitrogen and used for isolation of total RNA and protein as well as for the extraction of endogenous jasmonates, or they were used directly for the histochemical analyses. Light grown seedlings of barley were grown for 6 days in soil under greenhouse conditions in continuous light (300 μE m^{-2}) at 24°C and 60% relative humidity. Treatments with the racemic (±)-jasmonic acid methyl ester (Firmenich Comp., Geneva, Switzerland) was performed by flotation of five cm-segments (cut 1 cm below the tip) of primary leaves of seven days old seedlings on a 45 μM solution (freshly prepared) in a petri dish under continuous light (120 μE m^{-2} s^{-1}) at 25°C.

Extraction of RNA and proteins—Total RNA and protein were isolated simultaneously from each sample. The dissected seedlings as well as the various tissues of the light grown seedlings were homogenized in liquid nitrogen and extracted by Tris-EDTA-buffered phenol. RNAs were precipitated by addition of isopropanol to the aqueous phase (modified according to Chirgwin et al. 1979). Proteins were isolated from the phenolic phase by precipitation with methanol/ammonium acetate according to Meyer et al. (1988).

RNA electrophoresis and Northern blot analysis—RNA electrophoresis (10 μg total RNA per lane), and Northern blot analysis with the cDNA probe pHvJ3015, which codes for JIP-23 (Andresen et al. 1992), were carried out according to Sambrook et al. (1989). Plasmid DNA of pHvJ3015 was isolated using the QIAGEN Plasmid Kit (QIAGEN). The DIG labeling of the insert of pHvJ3015 as well as the hybridization at 65°C were performed according to the supplier's instructions (Boehringer, Mannheim, Germany).

Protein electrophoresis and Western blot analysis—Precipitated proteins (see above) were solubilized in a sodium dodecyl sulphate (SDS) containing buffer and were then separated by SDS polyacrylamide gel electrophoresis (12% polyacrylamide; Laemmli 1970). Two-dimensional separations included isoelectric focussing gels (Weidhase et al. 1987) in the first dimension in combination with SDS-polyacrylamide gels (12%) in the second dimension. After transfer of proteins onto nitrocellulose BA85 (Schleicher & Schüll, Darmstadt, Germany), immunological detection of JIP-23 was carried out with a rabbit polyclonal monospecific antibody raised against the most abundant isoform of JIP-23 of JM-treated barley leaves (arrow in Fig. 2c). The antibody was used at a dilution of 1 : 800 in the Western Light™ Chemiluminescent Detection System (Tropix Inc. Bedford, MA, U.S.A.) according to the supplier's instructions.

Extraction and analysis of endogenous jasmonates—(−)-Jasmonic acid (JA) was extracted, methylated and determined from the dissected seedlings as well as from the various tissues of the light grown seedlings as described by Wasternack et al. (1994). The enzyme immunoassay used followed the protocol of Weiler (1986). The properties of the antiserum are described by Knöfel et al. (1990). The assay is highly specific for (−)-JM and the amino acid conjugates of (−)-JA. The measuring range of standard curves lies between 0.05 and 25 pmol of (−)-JA with a reliable detection limit of 0.1 pmol. Although the detection limit for this assay lies at least three orders of magnitude lower than the average amount found in seedlings, care has been taken with respect to fresh weight determination. By collecting more than 60 different samples per time point or organ, we tried to minimize biological differences as well as deviations of fresh weight. Data of Fig. 1c and 3d were obtained by three independent experiments, each of them done in duplicates. Data of one representative experiment are presented.

Osmolytic analysis of different organs—To determine osmolytic values of different organs during germination, roots, leaves, mesocotyl and scutellum were collected at 14, 25, 39, 49 and 57 h of germination or were taken from 6 days old light grown seedlings. The tissues immediately frozen in liquid nitrogen were processed by three subsequently performed freeze-thaw steps followed by centrifugation at 20,000 × g for 30 min using a nylone sieve (10 μm) to collect the cell sap. At least 25 μl of cell sap were used directly or after appropriate dilution for measurement of osmolytic value with a cryoscopic osmometer from Roebling (Berlin, Germany).

Preparation of material for microscopical analyses—Microscopical analysis was performed with (i) seedlings, dissected 48 h after imbibition and (ii) with tissue around the scutellar nodule of 6 days old light grown seedlings (see Fig. 4). Equivalent tissues were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS), supplied with 0.1% Triton X-100 for 2 h at room temperature. After dehydration by graded series of ethanol, samples were embedded in polyethylene glycol (PEG, PEG 1500 : PEG 4000 = 2 : 1) and cut into 5 μm sections (in situ hybridization) or into 2 μm sections (immunolabelling) according to Van Lammeren et al. (1985).

In situ hybridization—Longitudinal as well as cross sections were mounted on poly-L-lysine coated slides, rinsed in 0.01 M Tris-HCl, pH 8.0, and incubated in 1% bovine serum albumine (BSA) in the same buffer for 1 h. The sections were acetylated by incubation with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min and washed briefly in 2 × sodium salt citrate (SSC). Prehybridization was performed for 1 h at 45°C in the hybridization buffer [50% formamide, 4 × SSC, 150 μg ml^{-1} tRNA, 0.5% blocking reagent (Boehringer, Germany), 40 units ml^{-1} of RNase inhibitor (Boehringer, Germany)]. Hybridization was carried out with the digoxigenine (DIG)-labelled insert of pHvJ3015

(see above) in the same buffer at 45°C for 16 h. After hybridization washes were performed at 45°C firstly with 50% formamide in 4×SSC for 10 min, 4×SSC for 10 min, and finally with 0.1× SSC for 5 min. Slides were immunolabelled with an anti-DIG-fab-fragment conjugated with alkaline phosphatase (Boehringer, Germany) according to the supplier's protocol. The staining procedure was performed with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Finally, the slides were enclosed with glycergen (DAKO, Germany) and analyzed by bright field microscopy and differential interference contrast microscopy with a Zeiss "Axioplan" microscope (Zeiss, Germany). Controls done by omitting the DNA probe revealed no signal.

Immunocytochemistry—Sections of the PEG-embedded material were collected on poly-L-lysine coated slides, rinsed with PBS and then incubated with 1% BSA in PBS for 30 min. For immunolabelling rabbit polyclonal antibodies raised against JIP-23 were used (see above) in a dilution of 1 : 400, and the V-type H^+-ATPase-holoenzyme from *Kalanchoë daigremontiana*, in a dilution of 1 : 1,000, respectively. The anti-H^+-ATPase antibody was kindly provided by Prof. U. Lüttge, Darmstadt (cf. Haschke et al. 1989). Goat anti-rabbit-IgG-fluorescein-5-isothiocyanate (FITC) conjugate (Sigma) was used as secondary antibody. After immunolabelling, sections were mounted in para-phenylendiamine (0.2% in glycerol). Control experiments were performed with preimmuneserum.

The staining of sections with aniline blue was done according to Gerlach (1984). The fluorescence of the immunolabelled JIP-23 and H^+-ATPase as well as of callose, stained by aniline blue, was visualized with a Zeiss "Axioplan" epifluorescence microscope using the filter combination: BP 485/20//FT510// LP520.

Results

Expression of the JIP-23 gene(s) during germination of barley seeds—Northern blot analysis for JIP-23 during seed germination revealed first transcript accumulation at 14 h and a transient rise which peaked sharply at 32 h (Fig. 1a). The different amounts of mRNA of seedlings 32 h after imbibition, and of leaves JM-treated for 24 h were measured by densitometric tracing of the hybridization signals (data not shown). The amount of JIP-23 transcript of the seedlings was found to be approximately 10% of the total amount of transcript accumulated by JM-treated leaves. Western blot analysis was used to investigate the occurrence of JIP-23. Figure 1b depicts the appearance of JIP-23 following 25 h of imbibition. Unlike the course of its respective mRNA, the amount of JIP-23 increased up to 39 h but remained constant up to 57 h.

The pattern of the isoforms of JIP-23 occurring during germination, was analysed by two-dimensional gel electrophoresis followed by immunological detection (Fig. 2). At least six different isoforms were recognized among the polypeptides of JM-treated leaves (Fig. 2c). Three of these isoforms, appearing upon JM treatment, were also detectable in seedlings, germinated for 28 h (arrowheads at Fig. 2a, c). Interestingly, the most basic isoform in seedlings did not occur during JM-treatment of leaf segments (arrow at Fig. 2a). Otherwise, three acidic isoforms appeared only in JM-treated leaves (arrows at Fig. 2c). The identity of isoforms, occurring either in seedlings or of JM-treated leaves, was determined by separating a mixture of the two polypeptide extracts (Fig. 2b).

Summarizing, germinating seedlings revealed the synthesis of several isoforms of JIP-23 without exogenous jasmonate application. This was substantiated by inspection whether or not JIP-23 and its transcript occurred in different organs of 6 days old seedlings (cf. Fig. 3a). The mRNA of JIP-23 was detectable only within three parts of the seedling (Fig. 3b): (i) the tissue around the scutellar

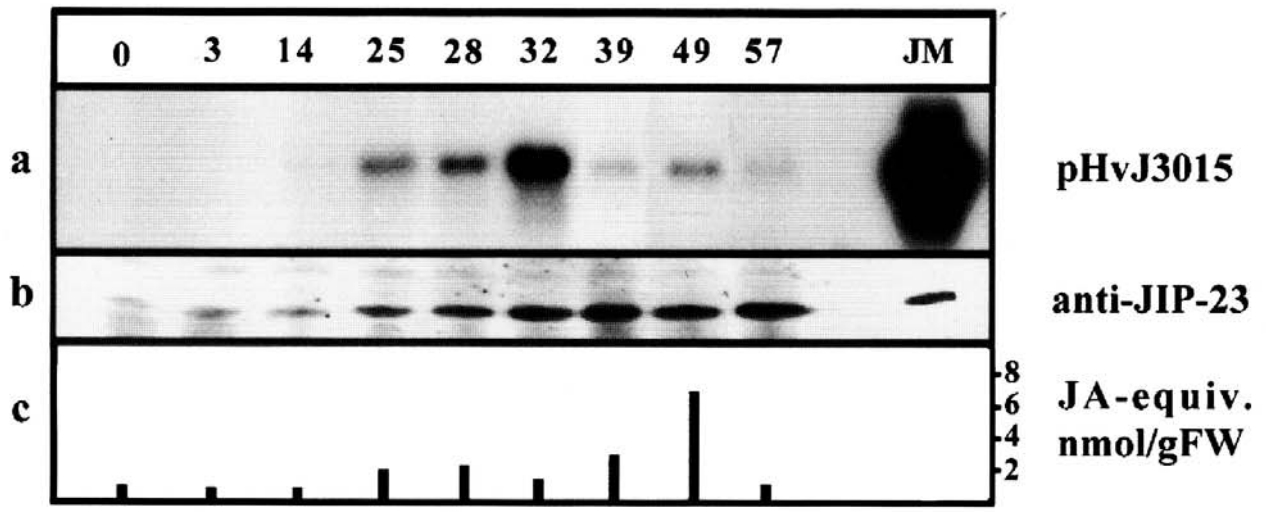


Fig. 1 Accumulation of JIP-23 mRNA (a), JIP-23 (b) and jasmonates (c) in seeds and germinating seeds of barley. Total RNA (10 μg per lane) or total protein (7 μg per lane) were used from different stages of germination. The accumulation of JIP-23 mRNA and JIP-23, respectively, were compared with that occurring in barley leaf segments upon JM treatment. To present the data of JM treatment in a corresponding signal intensity, total RNA (a) and total protein (b) from leaves, 72 h treated with JM, were diluted 1 : 10 before Northern blot and Western blot analyses, respectively. Amount of endogenous jasmonate equivalents (JA-equiv., c) represents the sum of (−)-jasmonic acid methylated after extraction, and its amino acid conjugates. The JA-equivalents are given in nmol per g FW.

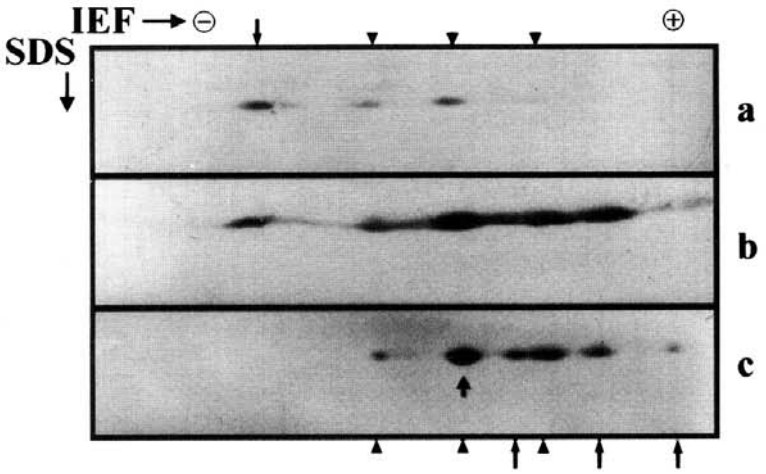


Fig. 2 Isoforms of a 23 kDa protein of developing barley seedlings (a) are partially identical with isoforms of JIP-23 occurring in barley leaves upon JM treatment for 72 h (c). Total protein, in (a) 250 μg and in (c) 25 μg, was separated by two-dimensional gel electrophoresis. Proteins in the area of the 23 kDa isoforms were electroblotted to cellulose nitrate membranes and immunodetected by the Western light ™ Chemiluminescence detection system. The most abundant isoform of JIP-23, marked by an arrow in (c), was originally isolated from JM-treated barley leaves and used for antibody production. At (a), the arrow indicates a developmentally expressed basic isoform not appearing in JM-treated leaves. At (c), acidic isoforms induced exclusively upon JM treatment are marked by arrows, and JM-inducible isoforms being identical to developmentally expressed isoforms in (a) are indicated by arrowheads (also at (a)). To check identity of some isoforms shown in (a) and (c), a mixture of both total protein extracts (250 μg of seedling protein and 25 μg of JM treated leaf protein) was separated and immunodetected in (b).

nodule (mesocotyl), (ii) the scutellum, and (iii) the youngest part of the primary leaf. JIP-23 was found mainly in these three tissues, but a low amount of protein was clearly detectable in roots and the primary leaf, too (Fig. 3c). Moreover, in the seedlings this amount decreased gradually from the scutellar nodule to the tip of the primary leaf.

JIP-23 mRNA is expressed within tissues exhibiting an increased level of endogenous jasmonates—In order to prove whether or not a threshold of endogenous jasmonates is needed to trigger the synthesis of JIP-23, we have analyzed the total amount of jasmonates by an ELISA specific for (−)-jasmonic acid methyl ester and the amino acid conjugates of (−)-JA. Therefore, free (−)-JA extracted from the tissues was methylated. During seed germination the level of jasmonates (Fig. 1c) was found to be higher than that of untreated primary leaves of 7 days old plants (0.6 nmol (g FW)$^{-1}$, cf. Lehmann et al. 1995): Between 25 h and 39 h of germination the amount was up to 5-fold and at 49 h of germination about 12-fold higher than that of untreated leaves. Only during the first increase of the endogenous jasmonates an accumulation of JIP-23 mRNA occurred, whereas at 49 h of germination JIP-23 mRNA was hardly detectable.

In 6 days old seedlings a correlation exists for some tissues between the endogenous content of jasmonates and the expression of JIP genes (Fig. 3d versus b). On the one hand, high levels of jasmonates were found in the scutellar nodule, which exhibits JIP-23 mRNA accumulation (lane 2 in Fig. 3b). On the other hand, in the lower part of the primary leaf a remarkable amount of jasmonates appeared, although JIP-23 mRNA was not detectable (lanes 6 and 7 in Fig. 3b versus d). But, the accumulation of JIP-23 protein (lane 6 in Fig. 3c) suggests that its synthesis was already switched on during an earlier stage of development. Within the scutellum the jasmonate content is only slightly higher

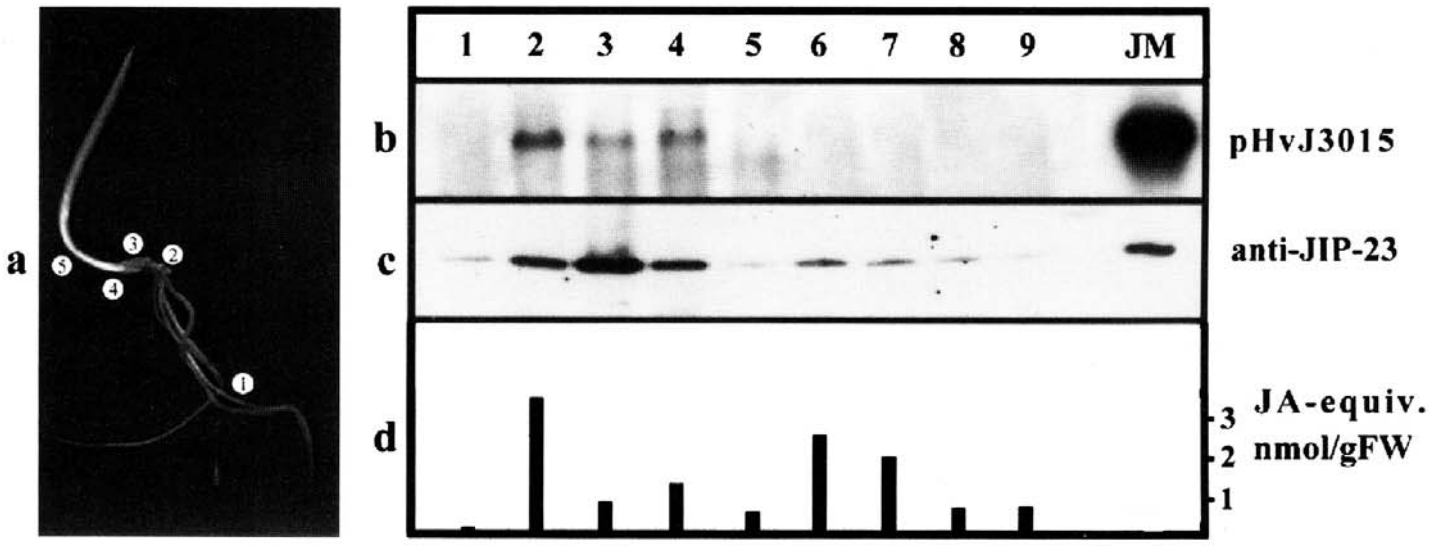


Fig. 3 Accumulation of JIP-23 mRNA (b), JIP-23 (c) and jasmonate content (d) of different organs of a 6 days old barley seedling. The organs are indicated in (a) showing a 3 days old seedling. Total RNA and protein extracts were from roots (1); the transition region between roots and the cotyledons, including the nodal plate of the scutellum (2); scutellum (3); 1 cm of primary leaf below the coleoptile (4); coleoptile (5); or from four 2 cm primary leaf segments starting above the coleoptile (6–9). JM controls are presented identical to that of Fig. 1. Amount of jasmonate equivalents (JA-equiv., d) is given as indicated for Fig. 1.

than that of an unstressed leaf, and the accumulation of JIP-23 mRNA is rather low (lane 3 in Fig. 3). However, the scutellum exhibited high amounts of JIP-23 protein. Since the scutellum contains similarly high amount of jasmonates as the scutellar nodule at 24 h of germination (data not shown), JIP-23 protein formed at this time might be still persisting within the cells after 6 days of seedling development.

Interestingly, the high level of jasmonates coincided with a high osmotic potential in different organs and during germination. Between 14 and 36 h of germination an average osmotic potential of −1.4 MPa occurred in the scutellum, which was clearly higher than that of roots, leaves and mesocotyl (−0.98 MPa). After 24 h of germination there was a continuous decline in the osmotic potential of roots and leaves leading to −0.65 MPa in the primary leaves of 6 days old seedlings, whereas the osmotic potentials of mesocotyl and scutellum remained high, at −1.1 MPa and −1.38 MPa, respectively.

Tissue-specific occurrence of JIP-23 mRNA—The specific occurrence of JIP-23 gene expression in the region of the scutellar nodule of 6 days old seedlings encouraged us to undertake a more detailed inspection by cytological methods. As indicated in the schematic drawing of a 6 days old seedling (Fig. 4), we analysed this region by longitudinal and cross-sections (Fig. 5). We could identify the different tissues within the scutellar nodule: the xylem, the phloem and the parenchymatic cells (Fig. 5b–g) as well as the shoot apex (Fig. 5a).

By in situ hybridization of longitudinal sections (Fig. 5a–c), JIP-23-mRNA became clearly detectable only within the vascular bundles of the scutellar nodule (Fig. 5b, c). The shoot apex of the seedling was free of any label (Fig. 5a). All labelled cells were connected with the phloem. The unlabelled xylem was clearly defined by the typical shape of the tracheids (arrows in Fig. 5c). The phloem-specific occurrence of JIP-23 mRNA was substantiated by in situ hybridizations of cross-sections through the upper and the lower part of the nodal plate of the scutellum (Fig. 5d, e, f, g, for the location of sections within the seedling see Fig. 4). Again, the label was found exclusively within cells connected with the vascular bundle, especially with the phloem. The scutellum as well as the vascular cylinder of the root did not show any label (data not shown).

Tissue-specific localization of JIP-23 protein—The cell type-specific expression of the JIP-23 gene(s) was confirmed by localization of JIP-23 via immunocytochemical techniques. In cross-sections of a 6 days old seedling (indicated in Fig. 4) the location of JIP-23 is visualized by the green fluorescence of the FITC-labeled antibody. JIP-23 was located in the vascular bundles of the scutellar nodule (Fig. 6d, d′), in the parenchymatic cells of the scutellum (Fig. 6b) as well as in the vascular cylinder of the root (Fig. 6c). The primary and secondary leaves seemed to be free of JIP-23 (Fig. 6a). The label within the vascular cylinder of the root (Fig. 6c) points to the location of JIP-23 within the phloem. Here, the tissue is characterized by the typical anatomy of an exarch xylem and an alternative arrangement of xylem and phloem. Moreover, immuno-labeling of cross sections through the scutellar nodule of seedlings germinated for 48 h exhibited the occurrence of JIP-23 within the phloem, too (Fig. 5f, f′).

In order to determine which of the phloem cells contain JIP-23, specific staining was used (i) for callose by aniline blue, thus visualizing the sieve plates of the sieve tubes, and (ii) for H^+-ATPase to visualize immunologically the companion cells of the sieve tube complex. As shown in Fig. 6e, the sieve tubes are characterized by their typical shape and the presence of the stained sieve plates (arrows in Fig. 6e). It is well established, that companion cells contain a high amount of H^+-ATPase (cf. Michelet and Boutry 1995) and so this cell-type could be defined by its preferential staining with a FITC-labelled-H^+-ATPase antibody (Fig. 6g). Comparing all these cells, which could be labelled by anti-JIP-23 antibodies, by DIG-labelled cDNA coding for JIP-23 and by anti-H^+-ATPase antibodies, it is obvious, that JIP-23 gene expression in 6 days old seedlings is restricted to the companion cells of the vascular bundles of the scutellar nodule.

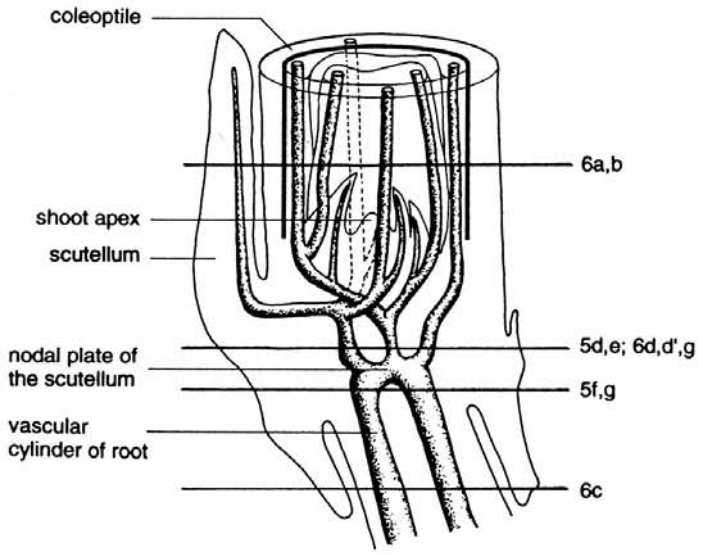
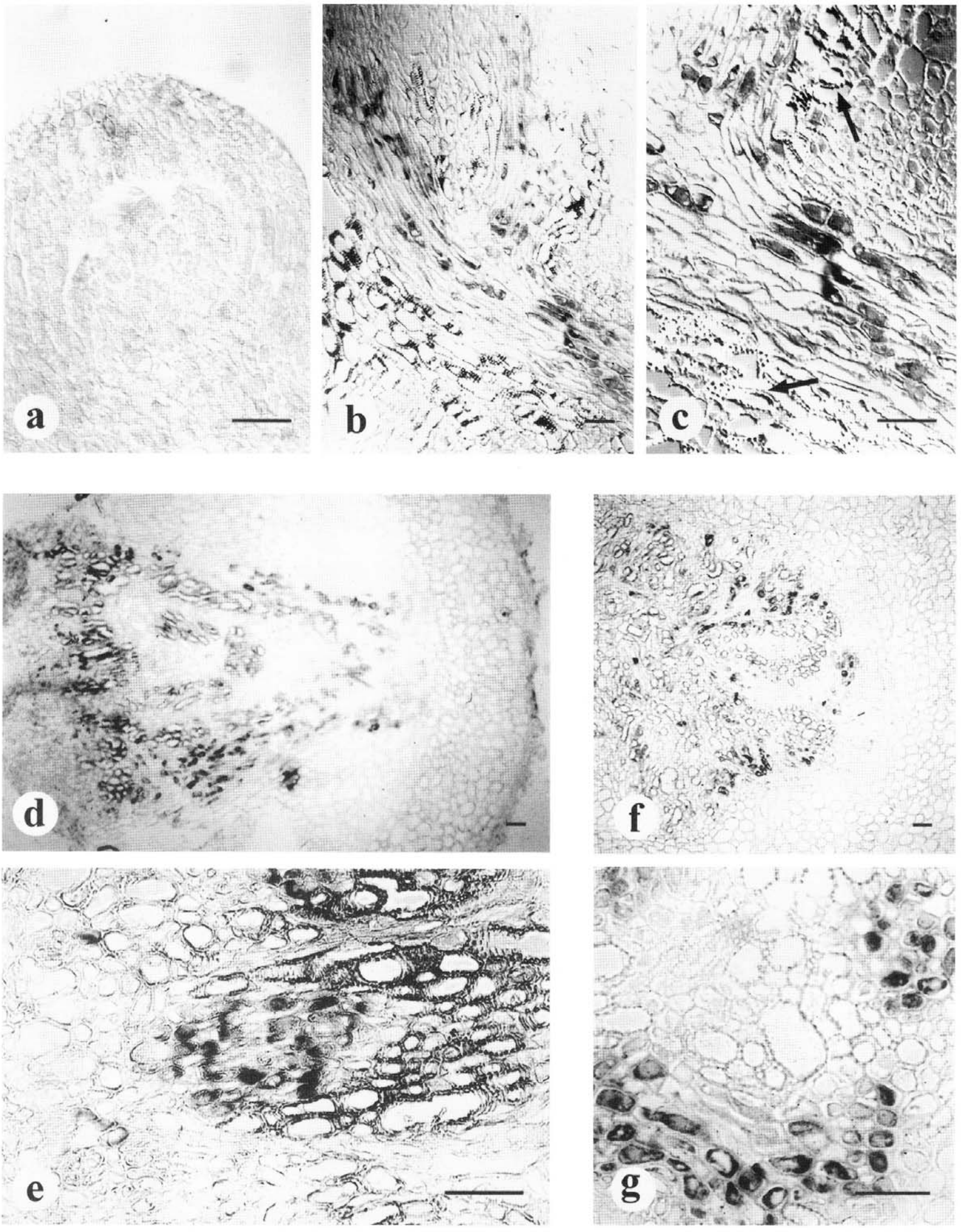


Fig. 4 Schematically longitudinal view of the transition region of a barley seedling (adapted from Esau 1965). The vascular bundles are shown by stippled areas. The positions of cross-sections used for in situ hybridization presented in Fig. 5 or for immunocytochemistry shown in Fig. 6 are indicated by the corresponding numbers and letters used in both figures.

Discussion

Developmental expression of various stress-related proteins prompted us to investigate, whether or not jasmonate responsive proteins of barley leaves are expressed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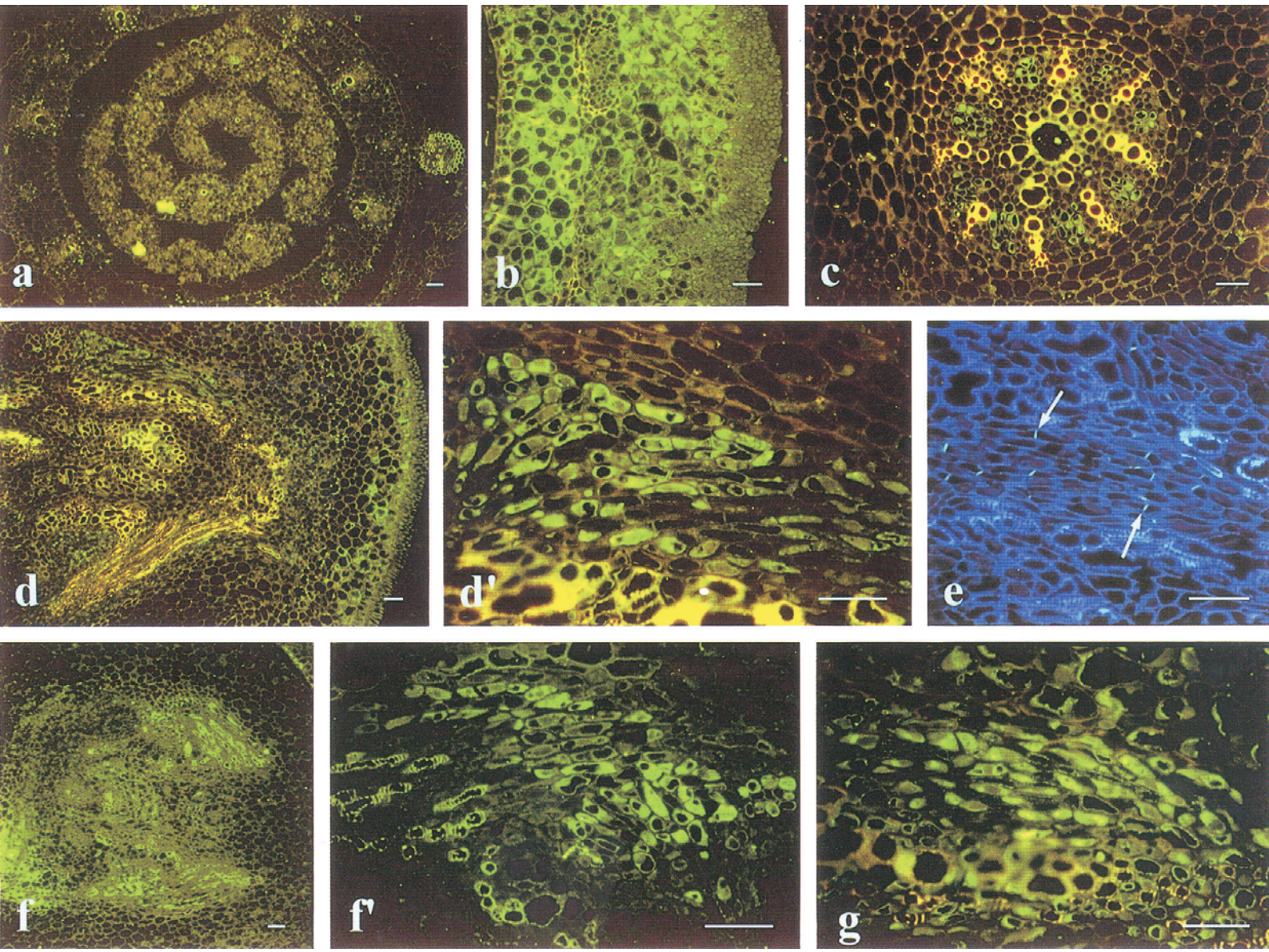

Fig. 6 Survey about the localization of JIP-23 (a)–(d′) as well as H^+-ATPase (g) in 6 days old seedlings and of JIP-23 in seedlings 48 h after imbibition (f, f′) visualized by indirect immunofluorescence with FITC-conjugated antibodies. (a) Cross section through a lower part of the coleoptile and the primary as well as secondary leaf of the seedling. Note the absence of any specific label, the tracheids are visible by the yellow autofluorescence of lignin. (b) Cross section through the scutellum at the same level as in (a) (cf. Fig. 4). The parenchymatic cells are strong labelled as visible by green fluorescence. (c) Cross section of a vascular cylinder of the root. Note the typical anatomy of the exarch xylem and the alternative arrangement of phloem and xylem. Only the phloem exhibits label. (d) Cross section above the nodal plate of the scutellum. The label is restricted to the epidermal layer which is in contact with the endosperm and the vascular bundles. The higher magnification shown in (d′) shows the label within the phloem. By comparison with (e) and (g) the labelled cells can be characterized as companion cells. (e) Parallel section as in (d′) stained with aniline blue to visualize callose. The sieve plates of the sieve tubes are indicated by yellow fluorescence (arrows). (f) Cross section below the nodal plate of the scutellum of a seedling 48 h after imbibition. (f′) shows the higher magnification of (f). Note the comparable distribution of the label as in (d), (d′). (g) Parallel section as in (d′) immunolabelled with antibodies raised against H^+-ATPase to visualize the companion cells. Bars represent 50 μm in all figures.

Fig. 5 Accumulation of JIP-23-mRNA within a 6 days old seedling of barley visualized by in situ hybridization of longitudinal (a)–(c) as well as cross-sections (d)–(g). (a) Longitudinal section through the shoot apex. The cytoplasm-rich cells are unlabelled. (b) Longitudinal section through the nodal plate of the scutellum. Note the label within cells related to the vascular bundles. (c) Higher magnification of (b). The occurrence of JIP-23 mRNA is restricted to cells of the phloem. The xylem is characterized by the typical shape of the tracheids (arrows). (d) Cross section above the nodal plate of the scutellum. Note the label within the regions of the vascular bundles. (e) Higher magnification of a section from the same region as in (d). The label is obviously visible within cells of the phloem. (f) Cross section below the nodal plate of the scutellum. An area of the transition from the endarch to the exarch xylem is visible. (g) Higher magnification of a section from the same region as in (f). The label is visible within the cytoplasm of the phloem-related cells. Bars represent 50 μm in all figures.

developmentally regulated during germination as well as growth of young seedlings. JIP-23, the most abundant JIP of barley, is known to be expressed in leaves upon treatment with exogenous jasmonate or various stresses (Lehmann et al. 1995). To date, there is no suggestion of function by sequence homology (Andresen et al. 1992) or specific expression pattern.

Interestingly, JIP-23 is synthesized upon JM treatment in several immunological related isoforms. During germination this pattern of JIP-23 isoforms is altered in comparison with that of JM-treated leaves (Fig. 2a versus c) by the appearance of a newly synthesized basic isoform and disappearance of acidic isoforms. But it is unclear, whether these polypeptides are distinct gene products or whether they are post-transcriptionally or post-translationally modified forms. Preliminary studies gave no indications for post-translational modifications such as phosphorylation or glycosylation (unpublished results). Southern blot analysis revealed the presence of at least two different genes for JIP-23 (unpublished results). If two genes exist indeed, and if both of them have a function, then they are indistinguishable in the size of their transcripts (Fig. 1, 3). Possible minor differences in the nucleotide sequences among the JIP-23 genes may result in the different pattern of isoforms. Nevertheless, it is noticeable, that at least three isoforms, which appear upon JM treatment (Fig. 1c), are identical to those occurring upon osmotic stress by sorbitol treatment (Lehmann et al. 1995, Wasternack et al. 1995) or during germination (Fig. 1a). This may suggest a similar function of the proteins, both in response to stress and in the germination process.

In a wide range of conditions tested so far (Wasternack et al. 1994, 1995, Lehmann et al. 1995) JIP-23 was synthesized only in response of a preceding rise of endogenous jasmonates. Therefore, the developmental expression of JIP-23 was tested parallel with the determination of jasmonates. During seed germination a specific appearance of JIP-23 transcript after 25 h was weakly correlated with an enhanced content of jasmonates in the whole seedling (2.5 nmol $(g\ FW)^{-1}$) (Fig. 1). This amount is nearly in the range as that found for stressed leaves (4 nmol $(g\ FW)^{-1}$ in 24 h sorbitol treated leaves, Wasternack et al. 1995) capable to induce JIP-23 gene expression (Lehmann et al. 1995). Compared to non-stressed leaf segments of 7 days old seedlings, which contain 0.6 nmol jasmonates $(g\ FW)^{-1}$ (Lehmann et al. 1995), the level determined at 25 h of germination might function as threshold level able to switch on JIP-23 gene expression. However, a second increase of the amount in endogenous jasmonates, e.g. at 49 h of germination, did not induce the expression of JIP-23 (Fig. 1), suggesting an altered threshold necessary for the induction or a sequestration of jasmonates as proposed recently for transgenic potato overexpressing an enzyme of the biosynthetic pathway of JA (Harms et al. 1995).

In 6 days old seedlings only in the scutellar nodule the accumulation of JIP-23 mRNA coincides with an increased level of jasmonates compared to unstressed leaf segments (Fig. 3, lane 2). However, remarkable accumulation of JIP-23 in the scutellum lacking high jasmonate content (Fig. 3, lane 3) or in the lower parts of the primary leaf lacking JIP-23 mRNA (Fig. 3, lanes 4 and 6) may indicate persistance of JIP-23 from a preceding JIP-23 gene expression. Indeed, after 24 h of germination high amount of jasmonates in the scutellum coincides with JIP-23 mRNA accumulation (Fig. 1a). Once synthesized JIP-23 might occur in the parenchymatic cells of the scutellum due to its negligible turnover known for barley leaf segments (Böhling, pers. communication).

The scutellum is known to challenge a tremendous solute transport from the endosperm into the growing seedling. In a more specific manner, companion cells being the physiologically active part of the sieve tube complex of the phloem, function in feeding the neighbouring cells/tissues. Preferentially those cells, which were found to synthesize JIP-23 (Fig. 6), are highly stressed osmotically via carbohydrate breakdown products (cf. Thorne 1985, Briggs 1992). Thus, a speculative scheme can be envisaged: Cells which are highly stressed during a distinct stage of development may respond with jasmonate synthesis, which enable them to form proteins like JIP-23. Indeed, at 24 h of germination the scutellum showed the high osmolytic value of -1.4 MPa and a high jasmonate level, both of them corresponding to values found in osmotically stressed leaves (Lehmann et al. 1995). The detailed function of JIP-23 is unknown. But the concomitant occurrence of cellular stress with the synthesis of JIP-23 up to its abundant occurrence suggest possible protective effects of JIP-23 on cell constituents during stress periods. Whether these effects may occur directly by protein-protein-interactions or indirectly by preserving cell water or by another mechanism, remains to be answered.

Although we cannot exclude, that a developmentally regulated JIP gene expression exists separately, the data presented here and published elsewhere (Lehmann et al. 1995) indicate jasmonate to be the ultimate signal in turning on expression of certain genes upon environmentally or developmentally caused stress.

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Expression of the ribosome-inactivating protein JIP60 from barley in transgenic tobacco leads to an abnormal phenotype and alterations on the level of translation

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Abstract. In this paper we report the in-planta activity of the ribosome-inactivating protein JIP60, a 60-kDa jasmonate-induced protein from barley (*Hordeum vulgare* L.), in transgenic tobacco (*Nicotiana tabacum* L.) plants. All plants expressing the complete JIP60 cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter exhibited conspicuous and similar phenotypic alterations, such as slower growth, shorter internodes, lanceolate leaves, reduced root development, and premature senescence of leaves. Microscopic inspection of developing leaves showed a loss of residual meristems and higher degree of vacuolation of mesophyll cells as compared to the wild type. When probed with an antiserum which was immunoreactive against both the N- and the C-terminal half of JIP60, a polypeptide with a molecular mass of about 30 kDa, most probably a processed JIP60 product, could be detected. Phenotypic alterations could be correlated with the differences in the detectable amount of the JIP60 mRNA and processed JIP60 protein. The protein biosynthesis of the transformants was characterized by an increased polysome/monosome ratio but a decreased in-vivo translation activity. These findings suggest that JIP60 perturbs the translation machinery in planta. An immunohistological analysis using the JIP60 antiserum indicated that the immunoreactive polypeptide(s) are located mainly in the nucleus of transgenic tobacco leaf cells and to a minor extent in the cytoplasm.

Key words: Jasmonate-induced protein JIP60 – *Nicotiana* – Protein biosynthesis – Ribosome-inactivating protein – Transformation

Abbreviations: CaMV = cauliflower mosaic virus; DAPI = 4,6-diamidino-2-phenylindole; FITC = fluorescein-5-isothiocyanate; JIP = jasmonate-induced protein; RIP = ribosome-inactivating protein; WT = wild type

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Introduction

Barley leaf segments respond to methyl jasmonate with the de-novo synthesis of a variety of proteins of different molecular weights, so-called jasmonate induced proteins (JIPs; Weidhase et al. 1987; Hause et al. 1994; Lee et al. 1996). A JIP with a molecular mass of 60 kDa has been identified recently as a ribosome-inactivating protein (RIP) by sequence homology of the 30-kDa N-terminal region and in-vitro activity studies (Chaudhry et al. 1994; Reinbothe et al. 1994b). The RIPs occur in a number of plant species of diverse genera and are divided according to their structure into two main groups: (i) the single-chained Type I RIPs with a molecular mass of about 30 kDa, and (ii) the double-chained Type II RIPs with a molecular mass of about 60 kDa. Both types of RIP are potent inhibitors of translation, cleaving the N-glycosidic bond of adenine$_{4324}$ from mammalian 28S rRNA, as well as modifying ribosomes of higher plants and fungi in an analogous manner (Ferreras et al. 1994, 1995). This was shown using in-vitro translation systems and mammalian cell cultures. The N-glycosidase activity leads to loss of binding of the eukaryotic elongation factor 2 (eEF-2) and consequently to attenuation of the translation (for review, see Barbieri et al. 1993). Because of this activity, both RIP types are thought to be involved in plant defense against pathogens, such as fungi or viruses, and their vectors (Logemann et al. 1992; Lodge et al. 1993; Jach et al. 1995). The structural differences between Type I and Type II RIPs are thought to contribute to their different modes of action. Type I RIPs exist mainly in an active form in the cell wall matrix and have been found to act on homologous ribosomes. Disruption of the cell wall leads to release of Type I RIPs into the cell lumen, where they can inactivate host ribosomes and may prevent propagation of viruses or fungi (Lodge et al. 1993; Taylor et al. 1994). Type II RIPs act only on heterologous ribosomes. They commonly consist of a N-terminal A-chain (the catalytic domain; Endo and Tsurugi 1987) and a C-terminal B-chain (the galactose-

binding lectin site; Zentz et al. 1978; Baenziger and Fiete 1979), which are linked via a disulfide bond. The mature toxin is stored in cytoplasmic protein bodies (Barbieri et al. 1993). When the plant cell is damaged by fungi or animals, the liberated Type II RIP binds via its B-chain to the cell membrane of the attacking organism and is internalized. Once in the cell, the A-chain attacks the ribosomes and blocks protein biosynthesis irreversibly. In contrast to the common 60-kDa RIPs (double-chained Type II RIPs) described above, the 30-kDa C-terminal region of JIP60 has no sequence homology to lectins. Instead, this domain shares some sequence homologies with regions of the eukaryotic initiation factor 4γ (eIF-4γ) subunits (Chaudhry et al. 1994). Due to the lack of the lectin site, JIP60 is most likely unable to bind to and subsequently to cross cell membranes. Because of these properties, it is hard to assume that JIP60 functions in plant defense like the Type II RIPs outlined before. Furthermore, its cytosolic (Hause et al. 1994) and nuclear location (this paper) and its inability to depurinate conspecific (barley and wheat) ribosomes (Chaudhry et al. 1994) exclude an antiviral or antifungal mechanism as has been proposed for most of the Type I RIPs (Taylor et al. 1994). This apparent lack of a defense function and the partial sequence homologies of JIP60 to eIF- 4γ and to ribonucleoprotein motifs led to the proposal that JIP60 may play a regulatory role during protein biosynthesis of barley cells, occurring in response to jasmonate (Chaudhry et al. 1994).

To investigate in planta the biological role of JIP60 we transformed tobacco plants with a T-DNA construct, which carried the JIP60 cDNA under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. Here, we provide evidence that JIP60 interferes with the translational machinery, possibly by hindering the elongation step in transgenic tobacco. This accords with the reduced regeneration capability, reduced growth and perturbed cellular development observed in the transformants expressing JIP60. By the means of in-situ immunostaining we show an unexpected nuclear location of JIP60 or one of its domains in transgenic tobacco.

Materials and methods

Construction of the Agrobacterium vectors. All manipulations were done according to standard techniques (Sambrook et al. 1989). In brief, the JIP60 cDNA containing the entire coding region (Becker and Apel 1992) was inserted as a *Kpn*I/*Xba*I fragment in sense and inverted orientation into a pBi121 derivative between the CaMV 35S promoter and the nopaline synthase polyadenylation region (Jefferson et al. 1987; S. Melzer, ETH Zürich, Switzerland, personal communication). The resulting plasmids, pBi*jip60*s and pBi*jip60*inv, were transformed into the *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw technique described by Höfgen and Willmitzer (1988).

Transformation of Nicotiana tabacum L. cv. Samsun NN. Transformation of tobacco (seeds obtained from U. Sonnewald, IPK Gatersleben, Germany) was performed according to Horsch et al. (1985). Transformed plants were grown axenically or in soil in the growth chamber at 25 °C and 16 h/8 h light/dark cycle with an irradiance of 95 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The F_1 progeny were grown for further investigations under the conditions described above.

Molecular analysis of the transformed plants. The DNA extraction procedure was adapted from the method of Löbler and Hirsch (1990) to isolate DNA and RNA simultaneously. In brief, the leaf material was homogenized in liquid nitrogen and suspended in lysis buffer (7 M urea; 0.35 M NaCl; 0.05 M EDTA; 0.01 M Tris-HCl, pH 7.0; 2% sodium lauroylsarcosine). The suspension was extracted twice with an equal volume of phenol (equilibrated with lysis buffer)/chloroform/*iso*amyl alcohol (25:24:1, by vol.). Precipitation of nucleic acids was obtained by adding sodium acetate to a final concentration of 0.3 M and 0.55 vol. of 2-propanol. The nucleic acids were dissolved in TE (1 mM Tris, 10 mM EDTA, pH 8.0), precipitated with 0.2 vol. of 10 M ammonium acetate and 1 vol. of 100% ethanol and re-dissolved in TE. The RNA was precipitated overnight with 0.5 vol. of 4 M LiCl at 4 °C. The DNA was precipitated from the supernatant by adding 0.1 vol. of 3 M sodium acetate and 2.5 vol. ethanol, whilst the RNA was dissolved in TE and precipitated as above and re-dissolved in TE.

The copy number of the integrated cDNA was determined by genomic Southern blots. High-molecular-weight DNA was digested with appropriate restriction enzymes (Gibco BRL Life Technologies, Eggenstein, Germany) according to the manufacturer's protocol, separated on a 0.8% agarose gel and blotted according to Sambrook et al. (1989) using charged nylon membranes (Qiabrane; Qiagen, Hilden, Germany).

The expression of introduced genes was investigated using the Northern blot technique (Sambrook et al. 1989). Total RNA (10–20 μg) was applied to a denaturing 1.2% agarose gel, separated and transferred onto uncharged nylon membranes (Qiabrane) via capillary blot. To control the uniform loading of the gel, it was stained with ethidium bromide prior to blotting.

Probes labeled with digoxigenin (DIG), specific for the full-length JIP60 cDNA, were synthesized using the DIG labeling and detection kit (Boehringer, Mannheim, Germany). Hybridization and detection with 3-(4-methoxyspiro{1,2-dioxetan-3,2′-(5′chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) (CSPD; Tropix, Juc. Bedford, Mass., USA) followed the protocol of Engler-Blum et al. (1993).

Total proteins were extracted, separated by electrophoresis using a 7.5–25% gradient SDS-polyacrylamide gel and blotted onto nitrocellulose (BA 85; Schleicher and Schuell, Dassel, Germany) as described previously (Lehmann et al. 1995). Rabbit polyclonal antibodies directed against JIP60 were taken as primary antibodies in a dilution of 1:2400 in the Western Light Chemiluminescent Detection Kit (Tropix) following the manufacturer's instructions.

Immunofluorescence microscopy. For microscopical analyses of the transgenic tobacco plants, pieces of leaves and the apical region, both of the sense as well as of the control plants, were used. Moreover, segments of primary leaves of barley floated for 24 h on water or 45 μM methyl jasmonate (for conditions see Leopold et al. 1996) were used as controls. Tissues were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) supplied with 0.1% Triton X-100 for 2 h at room temperature. After dehydration by graded series of ethanol, samples were embedded in polyethylene glycol (PEG; PEG 1500:PEG 4000 = 2:1) and cut into 2-μm sections according to Van Lammeren et al. (1985). The sections were collected on poly-L-lysine-coated slides, rinsed with PBS and then stained with toluidine blue (apical regions) according to Gerlach (1984) or used for immunolabelling (leaves). Therefore, sections were incubated with 1% bovine serum albumin (BSA) in PBS for 30 min and immunolabelled with rabbit polyclonal antibodies raised against the JIP60 (see above). Goat anti-rabbit-IgG-fluorescein-5-isothiocyanate (FITC) conjugate (Sigma, München, Germany) was used as secondary antibody. After immunolabelling, sections were stained with 0.1 $\mu g \cdot ml^{-1}$ 4,6-diamidino-2-phenylindole (DAPI; Sigma) and mounted in *p*-phenylendiamine (0.2% in glycerol). Control experiments were performed by ommitting the first antibody and revealed no signals.

The fluorescence of the immunolabelled JIP60 and DNA was visualized with an "Axioplan" epifluorescence microscope (Zeiss, Oberkochen, Germany) using the filter combinations: BP485/20//FT510//LP520 for FITC and G365//FT395//LP420 for DAPI.

Isolation of polysomes. Frozen leaves of tobacco were ground in liquid nitrogen and homogenized in 5 vol. of 250 mM sucrose containing 200 mM Tris-HCl (pH 8.9), 60 mM KCl, 25 mM $MgCl_2$, and 4 mM 2-mercaptoethanol. After two subsequent low-speed centrifugations (10 min at 8 000 · *g* and 20 min at 15 000 · *g*), the homogenate was filtered through Miracloth (Calbiochem-Novabiochem, Bad Soden, Germany). Subsequently, Triton X-100 was added to a final concentration of 2% (w/v). Polysomes were pelleted by centrifugation at 160 000 · *g* for 100 min through 1.75M sucrose in 40 mM Tris-HCl (pH 8.9), 20 mM KCl, 10 mM $MgCl_2$, and 4 mM 2-mercaptoethanol. The polysomes were resuspended in 10 mM Tris-HCl (pH 8.9), 25 mM KCl, 5 mM $MgCl_2$, frozen in liquid nitrogen and stored at −70 °C until use or were immediately fractionated on a linear sucrose gradient (15–55% w/v) by centrifugation at 260 000 · *g* for 100 min. Gradients were monitored at 254 nm and polysome and monosome percentages were estimated from the corresponding peak areas. All operations were carried out at 4 °C.

Determination of in-vivo translation activity. Leaf discs (6 mm diameter) were placed on 3.7 MBq L-[^{35}S] methionine (3.7 TBq · mmol^{-1}, Amersham, Bucks., UK) and incubated under growth conditions for 3 h. The segments were then rinsed with distilled water, frozen in liquid nitrogen and stored at −70 °C until use. Frozen material was ground in liquid nitrogen, and homogenated in 4 vol. of buffer containing 50 mM Tris-HCl (pH 6.5) and 2% SDS and centrifuged at 8 000 · *g* for 10 min. The supernatant was loaded on glass filter discs and treated according to Mans and Novelli (1961). Radioactivity was measured in a scintillation counter (TLS 1000, Beckman, Palo Alto, Cal., USA) using a toluene scintillant.

Results

Regeneration of transgenic tobacco plants expressing JIP60 cDNA in sense and inverted orientation. The complete cDNA of JIP60 was transcriptionally fused in sense and inverted orientation to the CaMV 35S promoter. As the JIP60 cDNA does not cross-hybridize with genes or RNAs of tobacco under high-stringency conditions (data not shown), we used the construct pBi*jip60*inv as an internal control of transformation efficiency. The resulting constructs were introduced into the genome of *Nicotiana tabacum* cv. Samsun NN plants using the *Agrobacterium*-mediated leaf disc transformation procedure. Three independent transformation experiments with a total of 80 leaf discs were carried out using both the sense construct pBi*jip60*s as well as the control construct pBi*jip60*inv. Using the sense construct pBi*jip60*s, we could only regenerate a total of twelve independent lines, referred to as sense transgenic plants (lines 30/3, 30/4, 30/6, 30/9; 37/1, 37/4, 37/6; 39/1, 39/2, 39/5, 39/7, 39/9). These lines were subcloned in vitro and proven to be independent transformants by Southern blot assays with a *jip60*-specific probe (data not shown). In transformation experiments with the control construct, however, shootlets could be regenerated with high frequencies. From each transformation experiment, ten of these shootlets were randomly chosen to be grown into mature plants, referred to as control plants.

Plants transformed with pBijip60s have abnormal phenotypes. The phenotypes of the transgenic tobacco plants expressing the sense construct pBi*jip60*s were compared to those of the wild type (WT) plants and the control plants. All sense transgenic plants exhibited similar and clearly altered phenotypes. In general, the phenotype was characterized by slower growth, shorter internodes, reduced development of the roots in terms of number and length, chlorotic leaves, and premature senescence. Eight lines grew very poorly (lines 37/1, 37/4, 37/6; 39/1, 39/2, 39/5, 39/7, 39/9; Fig. 1a), whereas four lines could be transferred into soil (lines 30/3, 30/4, 30/6 and 30/9). During the first six weeks after transfer, the development of these plants was nearly identical to control and WT plants which were treated identically. However, severe impairment of development of the apical meristem and the young leaves of the sense transgenic plants became visible, when flowering was induced in the control and WT plants. The lines 30/4, 30/9, and to a lesser extent line 30/6, showed an impeded elongation of the internodes and smaller leaves with undulatory growth during the vegetative phase. Line 30/3 had an almost WT appearance, despite a somewhat reduced stem elongation and lanceolate leaves (Fig. 1b). Older leaves of the sense transgenic plants showed symptoms of senescence, such as loss of turgor, chlorosis and premature abscission. Furthermore, the sense transgenic plants had only small inflorescences and, after selfing, produced no (lines 30/3, 30/9) or only a few (lines 30/4, 30/6) seeds.

The abnormal phenotype of young leaves in sense transgenic lines formed after flower induction prompted us to study developing leaves from the sense transgenic line 30/4 and a control plant at the cellular level. Cross-sections of the fourth leaf (counted from the apex) were made and stained with toluidine blue (Fig. 2a,b). The mesophyll cells of the sense transgenic line (Fig. 2b) contained larger vacuoles than the mesophyll cells of the control plant (Fig. 2a). Interestingly, the number of cell layers in the cross-section of the leaflet of the line 30/4 was identical to that of the control but the cells of the sense transgenic line itself were remarkably larger. Moreover, the residual meristem and the procambial cells, which are responsible for the development of the leaf blade and the vascular tissue were lost completely in the sense transgenic line (Fig. 2, arrowheads). The cross-section of developing leaves of line 30/4 resembled that of a leaf stalk of a fully differentiated leaf. Leaves exhibiting this growth pattern did not develop any further.

The stable integration and the inheritibility of the construct was proven by germination under selective conditions. The germination of seeds and the seedling development from selfed lines 30/4 and 30/6 which contained six and two T-DNA inserts, respectively, were delayed by 3–5 d compared to control and WT plants.

The kanamycin-resistant progeny of the lines 30/4 and 30/6 segregated into nearly identical phenotypes as exemplified for one phenotypic class in Fig. 3. The phenotypes of the F_1 progeny could be divided into four classes which were clearly distinguishable from each other. Out of 24 kanamycin-resistant seedlings of line

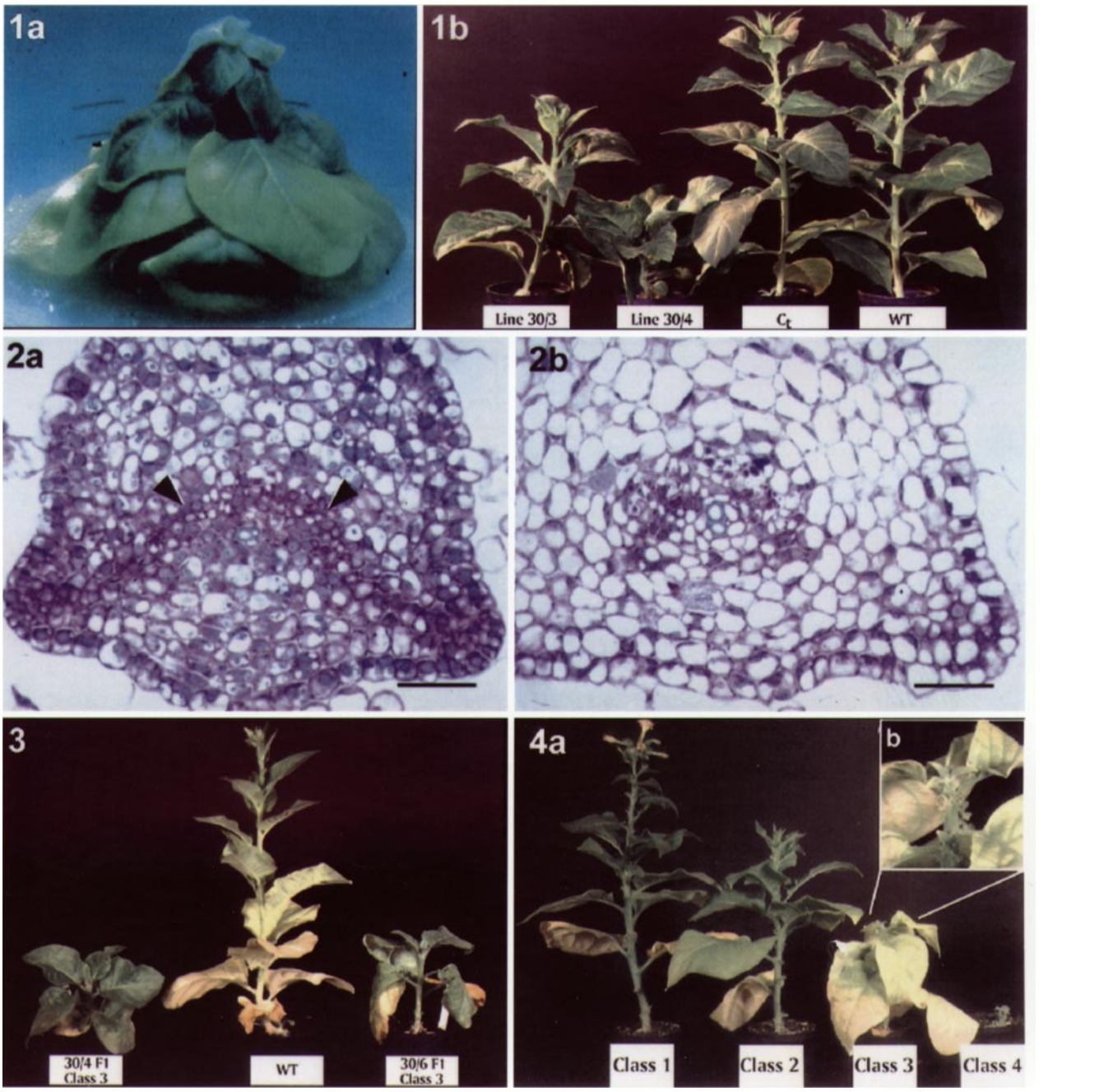

Fig. 1a,b. Phenotypes of transgenic tobacco plants expressing JIP60. **a** Phenotype of the line 39/2, representing the most severely affected transgenic lines (lines 37/6, 39/2, 39/5 and 39/9) approx. nine months after regeneration. **b** Phenotypes of lines 30/3 and 30/4 ten weeks after transfer to soil compared to a control (C_t) and a wild-type (WT) plant. Shoots were rooted in vitro and transferred into the greenhouse simultaneously

Fig. 2a,b. Cross-sections of the fourth leaf of a control plant (**a**) and of a plant of the sense transgenic line 30/4 (**b**). Sections were stained with toluidine blue to visualize cellular structures. The *arrowheads* in **a** mark residual meristems and procambium cells which are absent in the leaf of the transgenic line 30/4 (**b**). Bars = 50 μm

Fig. 3. Class 3 phenotypes of progeny of the independent transgenic lines 30/4 and 30/6 compared to the WT, twelve weeks after germination. Note the retarded growth of the progeny of independent transformant lines

Fig. 4a,b. Segregation of different phenotypes in the F_1 progeny of the transformant line 30/6. Southern blots revealed for this line two loci of JIP60 cDNA. **a** The *numbers* indicate the size-classes in which the tested population of 25 kanamycin-resistant plants segregated. Class 1 contained one plant (expected: 1.67 plants with four loci), class 2 contained six plants (expected: 6.67 plants with three loci), class 3 contained eleven plants (expected: 10 plants with two loci) and class 4 contained seven plants (expected: 6.67 plants with one locus). This segregation accords with the Mendelian laws assuming two independent loci. **b** Close up of the typical appearance of the apical stem from a class 3 plant

30/6, seven plantlets were almost identical in vegetative and generative phenotypes to that of the WT (Fig. 4, class 1). Ten plantlets showed retarded growth. However, they preserved the ability to produce fertile inflorescences and resembled the parent (Fig. 4, class 2). Six plants exhibited shorter internodes and abnormal apices six weeks after transfer to soil (Fig. 4, class 3), and one plant grew with hardly visible internodes (Fig. 4, class 4). This represented a 4 (one locus):6 (two loci):4 (three loci):1 (four loci) segregation, omitting kanamycin-sensitive seedlings. This indicated two independent active T-DNA inserts in the parent, which is in agreement with data obtained by Southern blot analysis. Moreover, a correlation of the phenotype with gene dosage was obvious. Similar phenotypic classes were observed in the progeny of line 30/4, but due to the higher number of T-DNA inserts, the segregation pattern was less clear.

Expression of JIP60 in the transgenic plants. To investigate the expression of the JIP60 cDNA in the primary transformant lines and selected progeny of the lines 30/4 and 30/6, these plants were subjected to Northern and Western blot analysis, respectively. In all of the transgenic lines we detected a transcription product of the predicted size of about 1.9 kb (Fig. 5). The amount of mRNA differed between the lines but correlated mostly with the severity of the observed alterations.

Western blot analysis of SDS-PAGE-separated total protein extracts of the twelve lines was performed with a polyclonal antiserum raised against JIP60. Thus both 30-kDa domains were expected to be immunoreactive. In four lines out of the twelve transformant lines we could detect an immunoreactive polypeptide with a molecular mass of about 30 kDa. Neither an unprocessed 60-kDa protein nor further subfragments (which would represent other processing products) were detectable (Fig. 6). The amount of 30-kDa immunoreactive polypeptide could be correlated with the degree of phenotypic alterations but only partially with the amount of JIP60 mRNA of the corresponding lines. The lines 37/6, 39/5 and 39/9, for which the highest amount of JIP60 was indicated, exhibited the most severe alterations and did not survive. In constrast, line 30/4 showing only a very weak signal in the Western analysis was able to grow in soil. It exhibited a conspicious phenotype and a high transcription level of the transgene. Therefore, progeny of line 30/4, which showed a class 2 and class 3 phenotype, respectively, were chosen in the further experiments to investigate the influence of JIP60 on protein synthesis.

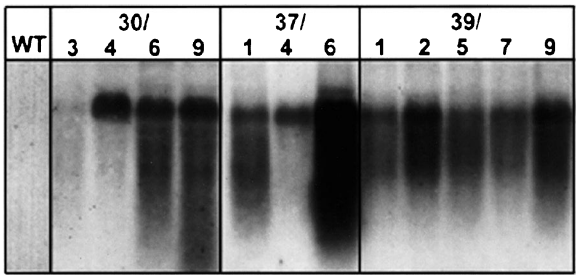

Fig. 5. Analysis of the JIP60 mRNA of WT and transgenic tobacco lines 30/3, 4, 6, 9; 37/1, 4, 6; 39/1, 2, 5, 7, 9 (20 μg of total RNA from leaves per lane was applied). The *arrowhead* indicates the JIP60 mRNA with the size of 1.9 nucleotides. The blot was strongly overexposed to show the expression of JIP60 mRNA in line 30/3, leading to an increased detection of degradation products

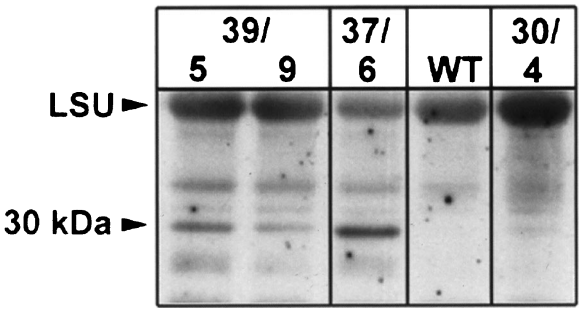

Fig. 6. Western blot analysis of the transgenic lines 30/4, 37/6, 39/5 and 39/9. Total proteins of leaves (5 μg per lane) were separated in an SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. The JIP60 was detected using polyclonal antiserum raised against JIP60 of barley (Herrmann et al. 1989). The 30-kDa immunoreactive polypeptide is indicated. The weaker additional signals in the transformants as well as in the WT are due to the long exposure of the blot

Translation is altered in the presence of JIP60. To determine whether or not the presence of JIP60 in tobacco plants leads to altered translation, we measured the polysome/monosome ratio and the in-vivo translational activity of detached leaves of selected progeny of line 30/4. The ratio of monosomes to polysomes depends on the relative rates of initiation, elongation and termination. The comparison of the polysome profiles with the rate of in-vivo protein synthesis in the sense transformed and the WT plants answers unambiguously the question of whether the initiation or the elongation is affected by JIP60. Since there is general agreement that RIPs inhibit the elongation cycle of protein synthesis, rather than the step of initiation, one may assume only minor changes of the rate of initiation. Given this situation, the action of JIP60 as an RIP would lead to an accumulation of differently sized polysomes due to depression of elongation. This would be accompanied by depletion of monosomes. Indeed these changes occurred in the progeny of the investigated line 30/4, representing the phenotypic classes 2 and 3, compared to WT plants (Fig. 7a). To prove that these changes are in fact a consequence of decreased elongation, rather than increased initiation we determined the in-vivo translational activity of young leaves taken from identical growth stages of WT, class 2 and class 3 plants. The amount of radioactivity incorporated into total proteins per gram fresh weight was significantly decreased in the sense transgenic plants to 17% and 10% of the incorporated radioactivity obtained by using leaf discs of WT plants, respectively (Fig. 7b). The degree of these changes correlated with the JIP60 mRNA amount as well as

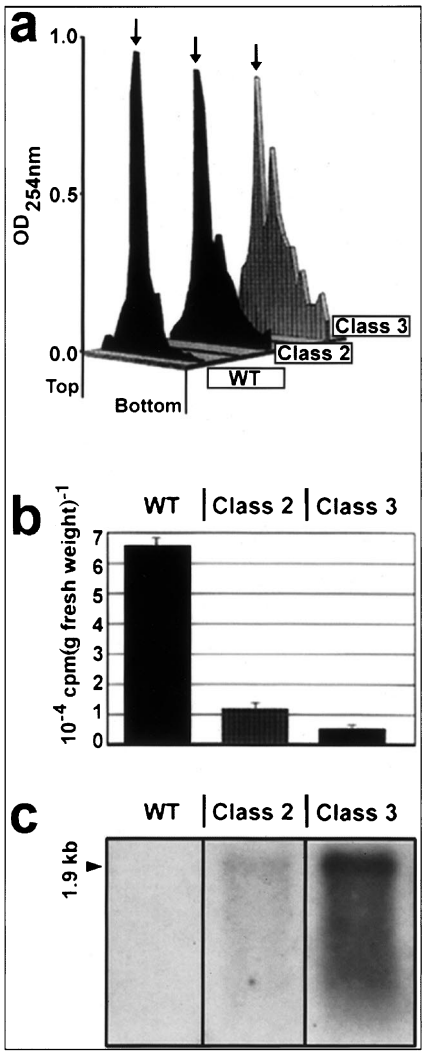

Fig. 7a–c. Analysis of the translational machinery of WT and transgenic tobacco plants with the phenotype of class 2 and class 3 plants. Data in **a** and **b** are obtained from the same plants. **a** Sedimentation profile of ribosomes isolated from young leaves. Centrifugation was performed in 15–55% sucrose gradients. The fractions were harvested from the bottom to the top and monitored at 254 nm. Monosomal peaks are marked by an *arrow*. **b** Estimation of the incorporation of radioactivity into newly synthesized proteins from leaf discs fed in vivo with [^{35}S] methionine as label during 3 h before harvest. The measured radioactivity was corrected for the fresh weight in grams. Data represent three replicates. **c** JIP60 mRNA amounts in the WT and the class 2 and class 3 plants of line 30/4 (20 μg total RNA was applied per lane). Note the correlation between the increase in polysomes (**a**), the decrease in in-vivo translation activity (**b**) and the amount of JIP60 mRNA (**c**) in WT, class 2 and class 3 plants

with the degree of phenotypical alteration (Figs. 7c, 4a). However, in contrast to the primary transformant line 30/4, no JIP60 protein was detectable in the investigated progeny.

The Jasmonate-induced protein is mainly located in the nucleus. The ribosome-inactivating property of JIP60 can occur only in the cytoplasm. In fact, in barley leaves JIP60 was shown by immuno-gold electron microscopy to be located in the cytoplasm (Hause et al. 1994). In addition, a targeting sequence could not be identified for JIP60 (Chaudhry et al. 1994). To test whether or not JIP60 is located in the same compartments in transgenic tobacco, we inspected cross-sections of leaves of line 37/6 and of a control plant by means of indirect immunofluorescence using a JIP60-specific antiserum. The line 37/6 was selected because this line contained the highest amount of immunoreactive polypeptide (Fig. 6). Cross-sections of the control plant showed no label, indicating that no unspecific binding of the antibody probe occurred (Fig. 8a). Cross-sections of the sense transgenic lines revealed label in the cytoplasm but, interestingly, the nuclei were even more strongly labeled (Fig. 8b). To prove that this result is not caused by the heterologous tobacco background, we analysed cross-sections of methyl jasmonate-treated (45 μM, 24 h) barley leaf segments with the same approach. Here, label was also detected in both the cytoplasmic and the nuclear compartments. But in contrast to the transgenic tobacco line, JIP60 seemed to be equally distributed between the cytoplasm and the nucleus of barley (Fig. 8c).

Discussion

Ribosome-inactivating proteins (RIPs) are able to block protein biosynthesis by cleaving the N-glycosidic bond of adenine$_{4324}$ located in the highly conserved region of the 28S/25S/23S rRNA of eukaryotic ribosomes (Endo and Tsurugi 1987; Hartley et al. 1991).

The toxic effect may protect host plants against plant pathogens such as viruses and fungi (Logemann et al. 1992; Lodge et al. 1993; Taylor et al. 1994; Jach et al. 1995). However, the question is still open as to whether RIPs are only involved in plant defense mechanisms or take part in regulatory processes during protein biosynthesis. This is particularly true for the recently identified JIP60. Chaudhry et al. (1994) showed that the 30-kDa N-terminal region of this nonsecreted protein shares high sequence homology to the conserved catalytic A-chain of known Type II RIPs and was able to inactivate mammalian ribosomes in vitro. Interestingly, the C-terminal region of JIP60 displays no homology to lectins, as in the case of Type II RIPs, but it shares significant homology to certain regions of the eukaryotic initiation factor eIF-4γ. The RIP features of the N-terminal part of JIP60 led to the speculation that it might function as a defense protein against pathogens of barley (Reinbothe et al. 1994a,b), as has also been proposed for other RIPs, e.g. pokeweed antiviral protein, Dianthin 32 and Ricin A-chain (Barbieri et al. 1993 and references therein; Taylor et al. 1994). However, JIP60 lacks the binding domain which would be necessary to cross cell membranes of pathogens or predators. In addition, no induction of JIP synthesis (including JIP60) in genetically based resistance against powdery mildew was observed (Kogel et al. 1995) when whole leaf extracts and attacked cells were analysed (Hause et al. 1997). These findings did not support a function of JIP60 in pathogen defense in barley.

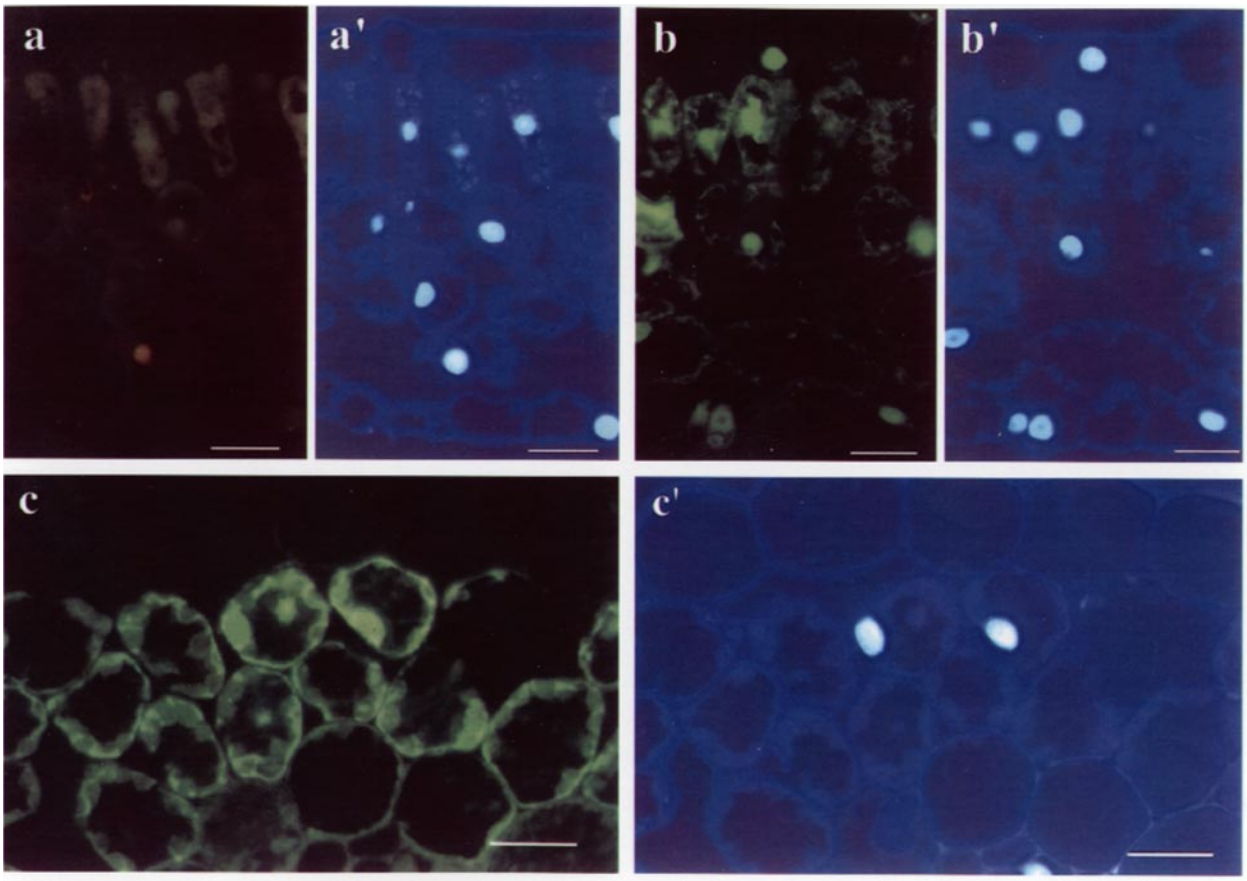

Fig. 8a–c′. Survey of the localization of JIP60 in cross-sectioned leaves of transgenic tobacco (**a, a′, b, b′**), as well as of barley treated with methyl jasmonate for 24 h (**c, c′**). JIP60 is visualized in **a**, **b** and **c** by FITC-conjugated antibodies exhibiting green fluorescence, whereas **a′**, **b′** and **c′** show the identical sections illuminated for the DAPI-stained nuclei. **a, a′** Control plants. Note the low level of autofluorescence which is clearly distinguishable from the typical green fluorescence of FITC. **b, b′** Transgenic line 37/6. The JIP60 is located preferentially within the nuclei. A lower level is detectable within the thin cytoplasmic layer around the chloroplasts. **c**, **c′** The mesophyll cells of the barley leaf treated with methyl jasmonate exhibit similar label within the nucleus and the cytoplasm. Bars = 10 μm

Parts of the C-terminal region showed unusual homologies to eIF-4γ. Also, jasmonate treatment, senescence or stress-like desiccation led to the preferred translation of JIP mRNAs (Reinbothe et al. 1994b). Both observations support the suggestion of Chaudhry et al. (1994) that JIP60 might regulate protein synthesis.

Following this suggestion we expected phenotypic alterations and a disturbed translation in tobacco lines transformed with JIP60 cDNA. Indeed, severe impairments of developement occurred, mainly in leaves, roots and flowers (Fig. 1a,b). This indicates that JIP60 is predominantly active in meristems, a region in which cells are highly active in protein biosynthesis. As substantiated by microscopic inspection, affected leaves showed the almost complete absence of residual meristems even in young leaves (Fig. 2b versus 2a). This loss of cell division activity may be the reason for the lower regeneration capability of the transformed tobacco cells as well as for most of the detectable growth abnormalities.

Only in four out of the twelve primary transformant lines could an immunoreactive polypeptide of about 30 kDa be observed in total protein extracts (Fig. 6), despite the presence of JIP60 mRNA (1.9 kb) in all lines (Fig. 5). The lack of JIP60 protein in transformants carrying JIP60 mRNA may be the result of either a low rate of translation and/or a rapid degradation of JIP60, allowing only a low accumulation of the toxin within the cells. Since we were able to regenerate only transformants that expressed no or very little JIP60, it is very likely that this protein is indeed toxic. With tobacco expressing the very active Type I RIP pokeweed antiviral protein (PAP) at high levels, poor regeneration efficiency and similar growth abnormalities were also found (Lodge et al. 1993), indicating that at least JIP60 and PAP may act via a similar target.

Type II RIPs need to be processed into two separate 30-kDa chains in order to become fully active (A- and B-chain; Lord 1985; Barbieri et al. 1993). Chaudhry et al. (1994) have demonstrated that JIP60 of barley needs at least two steps of maturation to achieve full activity in destruction of mammalian ribosomes in vitro, too. In our experiments with the JIP60 antiserum the sense transgenic tobacco lines show an immunoreactive signal appearing at 30 kDa (Fig. 6). Thus, JIP60 was probably processed into one or two peptides of 30 kDa. Since the

antiserum used is directed against the two halves of JIP60, we are unable at present to determine whether the signal belongs to only one or to both of them. The lack of additional degradation products and the distinct intracellular distribution of the immunoreactive polypeptide(s) (see below) suggest, however, that the signal is indicative of both the N- and the C-terminal parts.

The plants transformed with the sense construct were viable at least for a short period of time, suggesting that if JIP60 does indeed exhibit RIP activity, then it produces only a moderate amount of ribosomal damage. This prevents the direct detection of RIP activity, for instance by the occurrence of the liberated 400- to 450-nucleotide large rRNA fragment (Chaudhry et al. 1994). Therefore, we tested for the activity of JIP60 on ribosomes indirectly by measuring the alteration in their functions in terms of the ribosome profile (polysome/monosome ratio) and in-vivo protein synthesis. In the choosen progeny of line 30/4 (class 2 and class 3 plants), a shift in the ratio of monosomes to polysomes occurred compared with WT plants. This phenomenon correlated with a reduction of in-vivo protein synthesis, the amount of JIP60 mRNA and the phenotype of the plants (Figs. 4, 7a,b,c). The decreased in-vivo translation activity in spite of the increased amount of polysomes suggests that JIP60 might interfere with the translational machinery, most probably at the level of elongation rather than initiation. The JIP60-containing cells are likely to have difficulties in liberating the mRNA from the ribosomes, since they contain more translationally inactive polysomes (Fig. 7a versus 7b). The resulting decrease in the in-vivo translation activity might be attributable to the growth abnormalities of the transformed plants.

Interestingly, similar alterations of the polysome/monosome profile and the in-vivo translation activity have been detected in long-term (48 h) jasmonate-treated barley leaf segments (data not shown). This implies that the sense transgenic lines could reflect the response of the homologous system.

To summarize, the data presented here indicate that JIP60 may exhibit a ribosome-inactivating property in planta. The previously reported data about JIP60 are of limited value for discussing the biological functions of this protein, because (i) only the catalytic N-terminal region (Chaudhry et al. 1994) or the unprocessed in-vitro-synthesized protein (Reinbothe et al. 1994b) was monitored, and (ii) differences in the concentration of cofactors such as Mg^{2+} and K^{+} between the in-vitro and in-planta situation may cause differences in the susceptibility of the ribosomal target, and the presence or absence of distinct tRNAs may influence RIP activity (Rhodes III and Irvin 1981; Brigotti et al. 1995; Ferreras et al. 1995). Such differences could be the reason that the JIP60 N-terminal region was found to be inactive in the in-vitro wheat-germ translation system (Chaudhry et al. 1994), whereas in our hands this protein shows inactivation of protein biosynthesis in planta.

Using an immunohistological approach, JIP60 was found to be located preferentially in the nucleus and to a lesser extent in the cytoplasm (Fig. 8b). This fact was quite unexpected because all known RIPs so far have been found in protein bodies (e.g. ricin), in the cell wall matrix (e.g. PAP, saporins), in vacuoles (e.g. saporins) or in the cytoplasm (RIP30) only. Using electron-microscopical methods, JIP60 was detected only within the cytoplasm in jasmonate-treated barley leaf segments (Hause et al. 1994). However, with the more-sensitive technique of fluorescence immunocytochemistry the nuclear location of JIP60 was confirmed in jasmonate-treated barley leaf segments (Fig. 8c, c′). This can be taken as an indication that the compartmentalization described above is a general feature of JIP60.

Since no nuclear localization sequence has been identified in JIP60 so far, cotransport or diffusion/retention processes might be involved in its localization (Harootunian et al. 1993; Osborne and Silver 1993). Many nuclear proteins which are involved in mRNA synthesis and splicing contain RNA-binding motifs (Burd and Dreyfuss 1994). Therefore, the ribonucleoprotein motifs of JIP60 may be possible candidates for the localization via a diffusion/retention process.

A nuclear function of JIP60, or parts of it, is lacking at present. However, as recently reported, Type I as well as Type II RIPs cleaved not only rRNA and other RNA substrates but also DNA, releasing adenine residues from plasmid and herring sperm in vitro (Li et al. 1991; Barbieri et al. 1994; Ling et al. 1994; Stirpe et al. 1996). So far, neither the natural target of this activity is known nor is the question resolved whether this unexpected activity is common to all RIPs including JIP60.

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Cultivar-Specific Expression of the Jasmonate-Induced Protein of 23 kDa (JIP-23) Occurs in *Hordeum vulgare* L. by Jasmonates but not During Seed Germination

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Abstract: Treatment of barley leaf segments with jasmonic acid methyl ester (JM) leads to the accumulation of a set of newly formed abundant proteins. Among them, the most abundant protein exhibits a molecular mass of 23 kDa (JIP-23). Here, data are presented on the occurrence and expression of the JIP-23 genes in different cultivars of *Hordeum vulgare*. Southern blot analysis of 80 cultivars revealed the occurrence of 2 to 4 genes coding for JIP-23 in all cultivars. By means of Northern blot and immunoblot analysis it is shown that some cultivars lack the expression of *jip-23* upon treatment of primary leaves with JM as well as upon stress performed by incubation with 1 M sorbitol solution. During germination, however, all tested cultivars exhibited developmental expression of *jip-23*. The results are discussed in terms of possible functions of JIP-23 in barley.

Key words: *Hordeum vulgare*, cultivar-specific expression, jasmonate-induced protein, germination, stress by sorbitol treatment.

Abbreviations:

COMT:	caffeic acid *O*-methyl transferase
JA:	jasmonic acid
JIP:	jasmonate-induced protein
JM:	jasmonic acid methyl ester
jrg:	jasmonate responsive gene
LOX:	lipoxygenase
PAGE:	polyacrylamide gel electrophoresis
SDS:	sodium dodecyl sulfate

Introduction

Jasmonic acid (JA) and its methyl ester (JM) are ubiquitously occurring plant growth regulators (Sembdner and Parthier, 1993[16]), which were proposed to be signals in plant responses to different environmental stresses such as wounding, desiccation or elicitation during a pathogen attack (Creelman and Mullet, 1997[4]; Wasternack and Parthier, 1997[18]). In barley leaves several abundant proteins are synthesized upon treatment with both compounds. Among them are a thionin of 6 kDa (JIP-6) (Andresen et al., 1992[1]), a 23 kDa protein (JIP-23) and a 37 kDa protein (JIP-37), the latter two of unknown function (Andresen et al., 1992[1]; Leopold et al., 1996[12]), a 60 kDa protein (JIP-60) which exhibits ribosome inactivating properties *in vitro* (Reinbothe et al., 1994[14]; Chaudhry et al., 1994[3]) and *in planta* (Görschen et al., 1997a[7]), and finally several lipoxygenase forms with the predominant occurrence of a 100 kDa form (Feussner et al., 1995[5]; Vörös et al., 1998[17]). Furthermore, by a differential display several genes were identified that are exclusively responsive to jasmonate (*jrg*). Among them there is a gene coding for a caffeic acid-*O*-methyl transferase (Lee et al., 1996[9], 1997[10]).

In leaves of the barley cultivar Salome, JIP-23 genes are expressed upon various stresses such as sorbitol treatment and drought (Lehmann et al., 1995[11]). However, the expression of JIP-23 genes was also found to occur in distinct stages of seedling development (Hause et al., 1996[8]). Interestingly, JIP-23 occurs specifically within parenchymatic cells of the scutellum and within companion cells of the phloem of the scutellar nodule, suggesting a relationship to osmotic adjustment of these tissue/cells (Hause et al., 1996[8]). It remains to be elucidated whether JIP-23 may protect cells from environmental or developmental stresses.

Preliminary studies revealed that the gene(s) coding for JIP-23 occurs only in cereal plants such as barley, wheat, and oats (U. Demus, E. Görschen, pers. commun.), whereas other genes, coding for jasmonate-induced proteins such as thionins, LOX forms, COMT or PAL, occur in numerous species of monocotyledonous and dicotyledonous plants (Bohlmann, 1994[2]; Feussner et al., 1995[5]; Lee et al., 1996[10]; Creelman and Mullet, 1997[4]). This restricted occurrence of JIP-23 in some cereal plant species and its apparently stress-related expression within the life cycle of barley plants prompted us to assume differences in the occurrence and expression of *jip-23* among cultivars of different evolutionary and geographical origin.

In the present study we have analysed this question by means of Southern blot, Northern blot, and immunoblot analyses in 80 cultivars of *Hordeum vulgare* L.

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Material and Methods

Plant material, growth and incubation conditions

Seeds of the different cultivars were kindly provided by the Genebank, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany. To obtain seedlings, seeds were germinated on wet filter paper in the dark for 48 h at 25 °C. Before RNA and protein extraction the endosperm was removed according to Hause et al. (1996[8]).

Primary leaves of 7-day-old barley plants grown in a greenhouse were used for analysing jasmonate- and stress-induced expression of *jip-23*. Segments of 5 cm (cut 1 cm below the tip) were floated on aqueous solutions of 45 µM (±) JM or 1 M sorbitol under continuous light (130 µmol m^{-2} s^{-1}) at 25 °C. Leaf segments floated on water were used as control.

For DNA extraction young leaves of barley plants grown under greenhouse conditions were used.

Extraction of DNA and electrophoresis

Extraction of genomic DNA was carried out according to Löbler and Hirsch (1990[13]) by treatment of leaf homogenates with phenol/chloroform/isoamyl alcohol. DNA precipitation was achieved by addition of sodium acetate (final concentration 0.2 M) and 0.55 vol. isopropanol. DNA was resolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and precipitated by addition of ammonium acetate (final concentration 2 M) and 1 vol. 100% ethanol. After washing the pellet with 70% ethanol and drying, the DNA was finally dissolved in TE and stored at 4 °C.

Six µg of genomic DNA were digested overnight by incubation with 10 U enzyme/1 µg DNA of the restriction enzymes Eco RI, Hind III or Bam HI. Electrophoretic separation of the resulting DNA fragments on 1% agarose gel and blotting onto a nytran membrane (NY 13N Nytran, 0.45 µm, Schleicher and Schuell, Darmstadt, Germany) using capillary transfer in 20 × sodium salt citrate buffer (SSC) overnight were carried out according to Sambrook et al. (1989[15]). After blotting, DNA fragments were fixed to the membrane by UV cross-linking in a UV-Stratalinker^{R} 1800 (Stratagene, Heidelberg, Germany) and baked for 2 h at 80 °C. Hind III digested DNA of the bacteriophage λ served as a marker for estimation of fragment size.

Extraction and electrophoresis of RNA and proteins

Total RNA and proteins were extracted simultaneously from homogenised plant material as described by Hause et al. (1996[8]). After extraction of the plant material by Tris-EDTA-buffered phenol, total RNA was precipitated by addition of isopropanol to the aqueous phase, and proteins were precipitated by addition of methanol/ammonium acetate to the phenolic phase.

RNA electrophoresis with 10 µg total RNA per lane and blotting onto a nytran membrane (NY 13N Nytran, 0.45 µm, Schleicher and Schuell, Darmstadt, Germany) were performed according to Sambrook et al. (1989[15]). RNA size was estimated using the RNA ladder of 0.24 – 9.5 kb (Gibco BRL Life Technologies, Eggenstein, Germany).

After solubilization in SDS containing buffer, proteins were electrophoretically separated using a 12% SDS-polyacrylamide gel and blotted onto nitrocellulose BA 85 (Schleicher and Schuell, Darmstadt, Germany).

Southern and Northern blot analyses

Southern and Northern blot analysis were carried out using a cDNA probe pHvJ3015 encoding JIP-23 (Andresen et al., 1992[1]). The DIG-labelling of the insert of pHvJ3015 as well as the hybridisations at 42 °C (Southern blot analysis) and 65 °C (Northern blot analysis) were performed according to the supplier's instructions (Boehringer, Mannheim, Germany).

Alternatively, ^{32}P-labeled probes were used for hybridisation according to Sambrook et al. (1989[15]) at 65 °C overnight. Labelling was achieved using the Nick translation system of Gibco BRL Life Technologies (Eggenstein, Germany). Finally, the filters were exposed to AGFA X-ray film with an intensifying screen at – 70 °C.

Immunoblot analysis

Immunological detection of JIP-23 was carried out with rabbit polyclonal, monospecific antibodies raised against the most abundant isoform of JIP-23 of JM-treated barley leaves (cv. Salome). The antibodies were used at a dilution of 1 : 800 in the Western Light™ Chemiluminescent Detection System (Tropix Inc. Bedford, MA, USA) according to the manufacturer's instructions.

Results and Discussion

The expression of JIP-23 is inducible after stress-related treatments such as sorbitol treatment and drought (Lehmann et al. 1995[11]). Since there are barley cultivars of different geographic origin which are evolutionary-adapted to survive under varying degrees of environmental stresses, it might be interesting to study the possible variations of barley cultivars in the expression patterns of stress-related genes such as *jip-23*. Therefore, we inspected 80 cultivars covering this aspect, for the occurrence of *jip-23* and its expression upon leaf treatment with jasmonates as well as during germination. First, we analysed *jip-23* expression upon jasmonate treatment by Northern blot analysis as well as by immunoblot analysis after separation of protein extracts by one-dimensional PAGE. As summarized in Table **1**, the relative amount of mRNA of JIP-23 differed remarkably between cultivars. Fig. **1** shows examples of these differences in the level of JIP-23 mRNA as well as accumulated protein. For several cultivars, including var. *hybernum*, Gerbel (No 77, lane 8 in Fig. **1**), and var. *densum* (No 49, lane 9 in Fig. **1**), no JIP-23 mRNA could be detected. Among the 80 cultivars, a total of 8 were found lacking detectable JIP-23 mRNA accumulation if leaves were treated with jasmonate. Correspondingly, the JIP-23 protein was also not detected in jasmonate-treated leaves of these cultivars. The ability of a cultivar to express *jip-23* upon jasmonate treatment, however, lacks any relationship to the geographic origin of the cultivar, of the convariety and of the barley group (for classification see Zhang et al., 1992[19]).

Table 1 *Jip-23* expression in *Hordeum vulgare* cultivars treated with 45 µM JM for 24 h. Total RNA (10 µg per lane) was separated by agarose gel electrophoresis, and Northern blot analysis was performed by hybridising with a DIG-labelled cDNA insert of pHvJ3015 which encodes JIP-23

No	species	cv.	var.	geographic origin	mRNA accumulation
12	*H. vulgare*	hexastichon Alef	tibetanoides	Tibet	–
18	*H. vulgare*	hexastichon Alef	rikotense	Hindukush	–
70	*H. vulgare*	hexastichon Alef	subviolaceum	Libya	–
49	*H. vulgare*	hexastichon Alef	densum	Nepal	–
55	*H. vulgare*	hexastichon Alef	densum	China	–
67	*H. vulgare*	intermedium	atterbergii	Libya	–
69	*H. vulgare*	hexastichon Alef	nudipyramidatum	China	–
77 = Gerbel	*H. vulgare*	hexastichon Alef	hybernum	France	–
1	*H. vulgare*	hexastichon Alef	hybernum	Middle America	+
2	*H. spontaneum*		spontaneum	Iran	+
4	*H. agriocrithon*		dawoense Aberg	Tibet	+
5	*H. agriocrithon*		agriocrithon	Tibet	+
6	*H. vulgare*	hexastichon Alef	subparallelum	Japan	+
7	*H. vulgare*	hexastichon Alef	subparallelum	Japan	+
9	*H. vulgare*	intermedium	abergii hort	Tibet	+
10	*H. vulgare*	hexastichon Alef	himalayense	Tibet	+
11	*H. vulgare*	hexastichon Alef	subparallelum	Hindukush	+
14	*H. vulgare*	distichon Alef	nigrescens	Hindukush	+
15	*H. vulgare*	hexastichon Alef	hybernum	Hindukush	+
16	*H. vulgare*	hexastichon Alef	violaceum	Tibet	+
17	*H. vulgare*	hexastichon Alef	violaceum	Tibet	+
20	*H. vulgare*	hexastichon Alef	hybernum	Anatolia	+
22	*H. vulgare*	hexastichon Alef	subpyramidatum	Japan	+
26	*H. vulgare*	hexastichon Alef	subparallelum	Japan	+
27	*H. vulgare*	distichon Alef	vizide	Middle America	+
29	*H. vulgare*	hexastichon Alef	subpyramidatum	Hindukush	+
30	*H. vulgare*	intermedium Mansf.	japonicum	Japan	+
31	*H. vulgare*	hexastichon Alef	hybernum	Sweden	+
33	*H. vulgare*	hexastichon Alef	tibetanoides	Tibet	+
34	*H. vulgare*	hexastichon Alef	trifurcatum	Nepal	+
35	*H. agriocrithon*		agriocrithon	Israel	+
36	*H. vulgare*	distichon Alef	medicum	Anatolia	+
37	*H. vulgare*	intermedium	viridihaxtoni	Nepal	+
38	*H. vulgare*	deficiens	abyssinicum	Ethiopia	+
47	*H. vulgare*	intermedium	haxtoni	Nepal	+
48	*H. vulgare*	intermedium	nudijaponicum	Nepal	+
58	*H. vulgare*	hexastichon Alef	violaceum	Libya	+
63	*H. vulgare*	hexastichon Alef	hybernum	Libya	+
64	*H. vulgare*	hexastichon Alef	hybernum "California Mariout"	USA	+
65	*H. vulgare*	hexastichon Alef	lockere addisabebae	Libya	+
66	*H. vulgare*	distichon Alef	neogenes	USA, Ethiopia	+
72	*H. vulgare*	"Himalaya"			+
13	*H. vulgare*	distichon Alef	freislebenii	Hindukush	++
19	*H. vulgare*	distichon Alef	nigrescens	Anatolia	++
28	*H. vulgare*	hexastichon Alef	violaceum	Ethiopia	++
32	*H. vulgare*	hexastichon Alef	revelatum	Ethiopia	++
43	*H. vulgare*	intermedium	haxtoni	USA, Ethiopia	++
46	*H. vulgare*	intermedium	atratum	Nepal	++
62	*H. vulgare*	hexastichon Alef	rikolense	Libya	++
71	*H. vulgare*	hexastichon Alef	hybernum	Libya	++
94	*H. vulgare*	"Igri"			++

continued next page

Table 1 continued

No	species	cv.	var.	geographic origin	mRNA accumulation
95	*H. vulgare*				++
96	*H. vulgare*				++
3	*H. vulgare*	distichon Alef	gymnocrithon	China	+++
8	*H. vulgare*	distichon Alef	glabrierectum	China	+++
21	*H. vulgare*	hexastichon Alef	erectum	Anatolia	+++
23	*H. vulgare*	hexastichon Alef	hybernum	Anatolia	+++
24	*H. vulgare*	distichon Alef	nutans	Anatolia	+++
25	*H. vulgare*	hexastichon Alef	hybernum	Japan	+++
39	*H. vulgare*	distichon	neogenes	China	+++
41	*H. vulgare*	distichon Alef	breve Alef	China	+++
42	*H. vulgare*	distichon Alef	erectum	China	+++
44	*H. vulgare*	intermedium	mortoni	USA, Ethiopia	+++
45	*H. vulgare*	intermedium	atterbergii	Ethiopia	+++
53	*H. vulgare*	distichon Alef	nutans	Bolivia	+++
56	*H. agriocrithon*		agriocrithon	China	+++
57	*H. vulgare*	hexastichon Alef	atrum	Peru	+++
59	*H. vulgare*	deficiens	daghestanicum	Libya	+++
60	*H. vulgare*	hexastichon Alef	atrum	Peru	+++
61	*H. vulgare*	distichon Alef	nutans	Libya	+++
68	*H. vulgare*	distichon Alef	nutans	China	+++
93	*H. vulgare*	"Bigo"			+++
97	*H. vulgare*				+++
98	*H. vulgare*	Sommergerste S 3170			+++
99	*H. vulgare*	"Lenka"			+++
100	*H. vulgare*	"Nebi"			+++
104	*H. vulgare*				+++
90a	*H. vulgare*	"Trumpf"			+++
91	*H. vulgare*	"Gimpel"			+++
92	*H. vulgare*	"Salome"			+++

"–": JIP-23 mRNA was not detectable; "+": weak; "++": intermediate expression of JIP-23 gene(s); "+++": strong

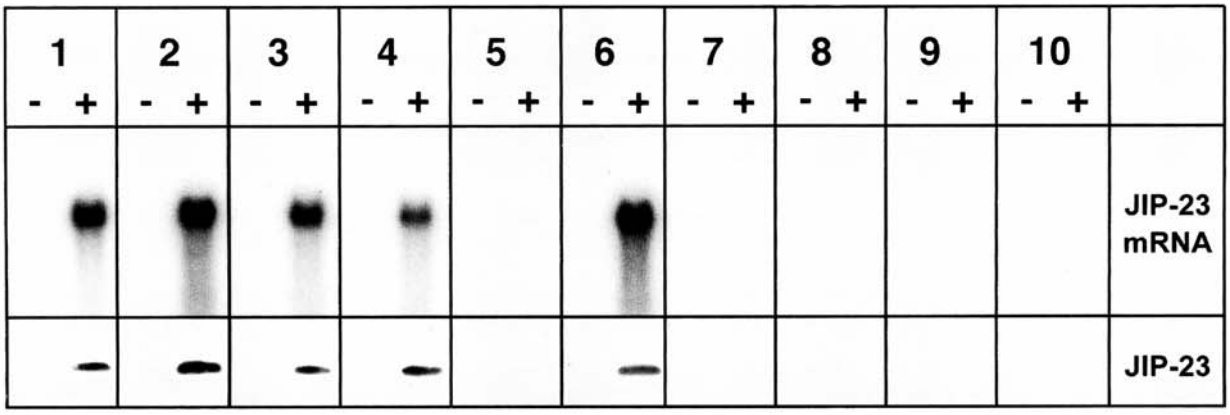

Fig. 1 Northern blot and immunoblot analysis of the accumulation of JIP-23 mRNA and JIP-23 protein in leaves of different barley varieties upon floating on water (–) or 45 µM JM (+) for 24 h. For Northern blot analysis 10 µg total RNA, and for immunoblot analysis 0.5 µg total protein of leaves of each cultivar were loaded. RNA was fractionated electrophoretically on 1.5% agarose gels, transferred onto a nytran membrane and hybridised with ^{32}P-labelled cDNA insert of the clone pHvJ3015. Immunoblot analysis was performed as described in Material and Methods. Lane 1: No 92 (cv. Salome); lane 2: No 41 (var. Breve Alef); lane 3: No 61 (var. nutans); lane 4: No 43 (var. haxtoni); lane 5: No 22 (var. subpyramidatum); lane 6: No 64 (var. hybernum, "California Mariout"); lane 7: No 69 (var. nudipyramidatum); lane 8: No 77 (var. hybernum, cv. Gerbel); lane 9: No 49 (var. densum); lane 10: No 12 (var. tibetanoides).

Interestingly, JIP-37, a further jasmonate-induced protein in barley leaves, is also absent after treatment with jasmonates in some barley cultivars such as Gerbel (Leopold et al., 1996[12]). Here, the absence of JIP-37 is caused by the absence of its gene(s). These data prompted us to screen for the occurrence of gene(s) coding for JIP-23 in all 80 different cultivars listed in Table **1**. Based on fragment comparison obtained by Southern blot analysis of genomic DNA restricted with Eco RI, Hind III or Bam HI, 2 to 4 genes seem to occur in each cultivar (examples are shown in Fig. **2**). Several cultivars exhibited similar if not identical restriction patterns (e.g., lanes 2 and 4 in Figs. **2A,B**). However, the signal intensity for the restriction fragments differed, and after restriction with Eco RI, at least one additional hybridising band occurs in those cultivars which expressed *jip-23* upon exogenous treatment with JM (see lanes 1–4 and 6 in Fig. **2B** versus Fig. **1**). This suggests an additional gene which could be responsible for expression in leaves upon treatment with JM.

The question rises whether the other gene(s) are functional and whether the cultivars lacking expression upon jasmonate treatment are able to express *jip-23* under other conditions. Because of the developmentally induced expression of *jip-23* shown for cv. Salome (Hause et al., 1996[8]), we inspected its expression in some cultivars during seed germination. Surprisingly, expression of *jip-23* was found by detecting its mRNA as well as protein in all cultivars tested so far (Fig. **3A**). The significant differences in steady-state levels of mRNA and protein which were detected (Fig. **3A**) might be caused by different timing of germination of the various cultivars.

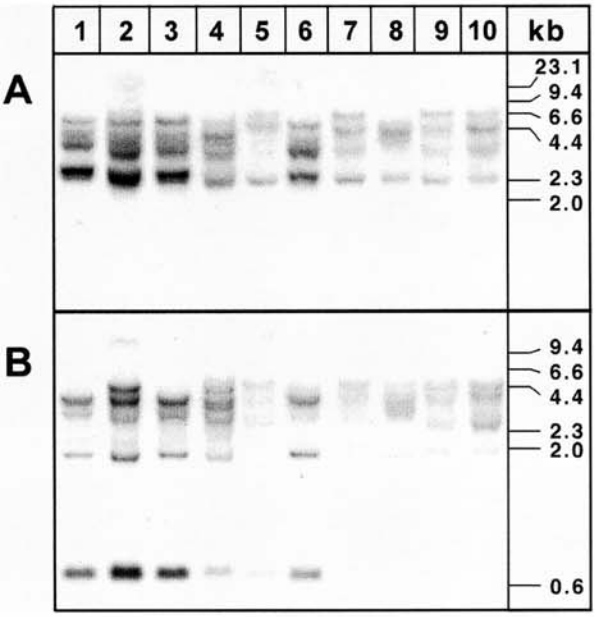

Fig. 2 Southern blot analysis of genomic DNA of different varieties digested with Bam HI (**A**) or Eco RI (**B**). 6 µg genomic DNA of each cultivar were digested, and the resulting DNA fragments were separated by agarose gel electrophoresis, transferred onto a nytran membrane and hybridised to the ^{32}P-labelled pHvJ3015 probe. Numbers of lanes are explained in the legend of Fig. **1**.

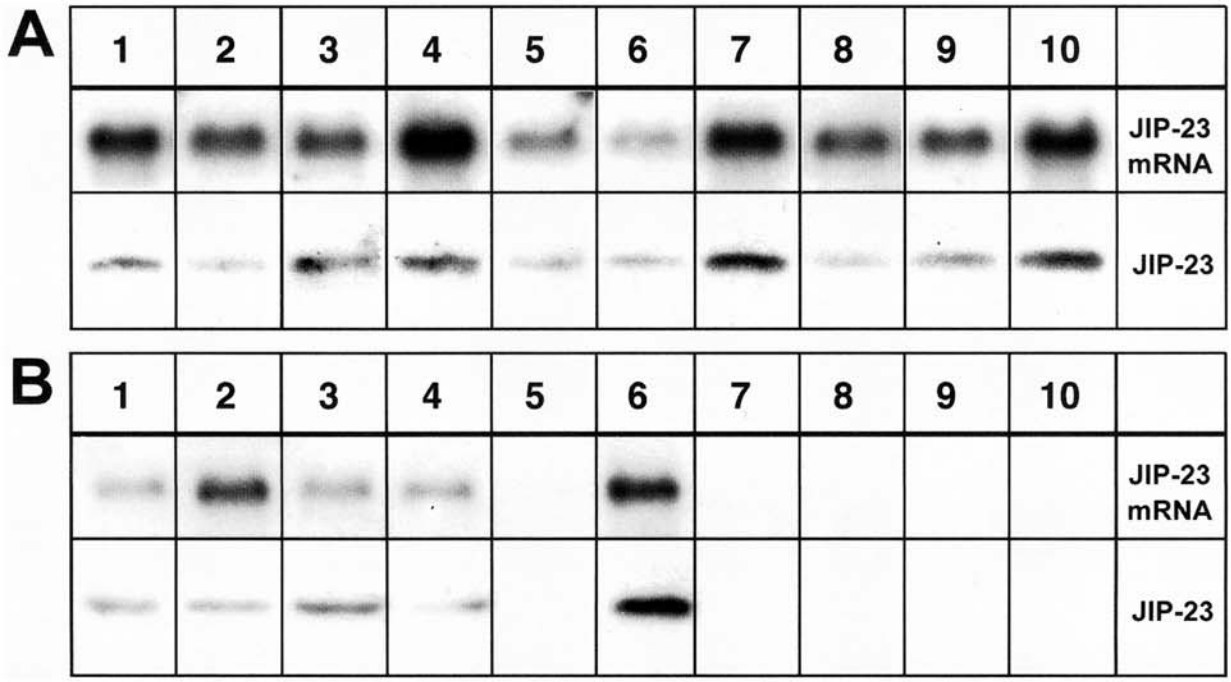

Fig. 3 Northern blot and immunoblot analysis of the accumulation of JIP-23 mRNA and JIP-23 protein in seedlings (**A**) and in leaves treated with 1 M sorbitol for 24 h (**B**) of different barley varieties. For Northern blot analysis 10 µg total RNA and for immunoblot analysis 5 µg total protein were loaded. RNA was fractionated electrophoretically on 1.5% agarose gels, transferred onto a nytran membrane and hybridized with a DIG-labeled cDNA insert of clone pHvJ3015. Immunoblot analysis was performed as described in Material and Methods. Numbers of lanes are explained in the legend of Fig. **1**.

This constitutive expression in distinct developmental stages and the lack of expression of *jip-23* within JM-treated leaf segments of some cultivars suggest differences in the induction pathway for the various JIP-23 genes, e.g., some cultivars might have lost a *jip-23* which is able to respond to exogenous jasmonates. Indeed, signal transduction *via* jasmonate differs in cv. Salome with respect to expression of different genes: Three jasmonate-regulated genes (*jrg5, jrg10* and *jrg12*, Lee et al., 1996[9]) as well as of *LOX:2:Hv:1* (Vörös et al., 1998[17]) were all expressed exclusively upon exogenous treatment with jasmonates. However, other genes, such as *jrg1* and those coding for JIP-23 or JIP-37 are inducible upon exogenous application and endogenous rise of jasmonates (Lee et al., 1996[9], 1997[10]; Lehmann et al., 1995[11]; Leopold et al., 1996[12]). These data led to the suggestion that different signalling pathways may exist for exogenously given and endogenous raised jasmonates (Lee et al., 1997[10]; Vörös et al., 1998[17]).

To check whether similar differences were responsible for the cultivar-specific expression of *jip-23* shown here, we analysed the accumulation of JIP-23 and its mRNA upon treatment of leaf segments with 1 M sorbitol, which leads to an endogenous rise of jasmonates (Lehmann et al., 1995[11]). Cultivars which were able to express *jip-23* upon JM treatment (Fig. **1**), were also able to express *jip-23* upon sorbitol treatment (Fig. **3B**), and all those cultivars lacking expression by JM treatment did not respond to endogenous rise of jasmonates with the expression of *jip-23*.

Summarising, all cultivars exhibited *jip-23* expression during seed germination, whereas some cultivars, which lack at least one JIP-23 gene, are unable to respond to exogenously given as well as to endogenously raised jasmonates with the expression of *jip-23*. This suggests a distinct role of JIP-23 during the development of barley seedlings. Beside its proposed protective role during osmotic stress assumed from its tissue-specific expression in early seed germination (Hause et al., 1996[8]), JIP-23 might be involved in the down-regulation of "house-keeping" proteins: In transgenic tobacco overexpressing JIP-23, mRNAs of photosynthesis-related proteins are less translated than in wild type plants (Görschen et al., 1997b[6]). During the early seed germination of barley, JIP-23 might act in a similar manner by "repressing" the amount of photosynthesis-related and nuclear-encoded proteins in cells which have yet to form functional chloroplasts.

The data presented here indicate the existence of several JIP-23 genes. All these genes seem to be clustered, since *jip-23* mapped only at 0.2 cM in the distal portion of the long arm of chromosome 3 (Leopold et al., 1996[12]). While it is not possible to rule out that some hybridising bands represent pseudogenes of the *jip-23* gene family, the results suggest that different genes might be responsible for jasmonate-induced expression in a fully differentiated leaf and the developmentally regulated expression. In contrast, the 8 cultivars, which do not express *jip-23* upon jasmonate treatment, are able to respond to jasmonate treatment when tested, since *jrg1* is expressed upon application of jasmonate (data not shown). This excludes the possibility that these 8 cultivars lack an element of the jasmonate reception or signal transduction within differentiated leaves.

The lack of jasmonate-inducible expression of *jip-23* could be correlated with the absence of a hybridising band in the Southern blot. This suggest that the missing band corresponds to the jasmonate-responsive *jip-23*. To clarify this point analysis of the corresponding promoters will be done.

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Allene oxide synthases of barley (*Hordeum vulgare* cv. Salome): tissue specific regulation in seedling development

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Summary

Allene oxide synthase (AOS) is the first enzyme in the lipoxygenase (LOX) pathway which leads to formation of jasmonic acid (JA). Two full-length cDNAs of AOS designated as AOS1 and AOS2, respectively, were isolated from barley (*H. vulgare* cv. Salome) leaves, which represent the first AOS clones from a monocotyledonous species. For AOS1, the open reading frame encompasses 1461 bp encoding a polypeptide of 487 amino acids with calculated molecular mass of 53.4 kDa and an isoelectric point of 9.3, whereas the corresponding data of AOS2 are 1443 bp, 480 amino acids, 52.7 kDa and 7.9. Southern blot analysis revealed at least two genes. Despite the lack of a putative chloroplast signal peptide in both sequences, the protein co-purified with chloroplasts and was localized within chloroplasts by immunocytochemical analysis. The barley AOSs, expressed in bacteria as active enzymes, catalyze the dehydration of LOX-derived 9- as well as 13-hydroperoxides of polyenoic fatty acids to the unstable allene oxides. In leaves, AOS mRNA accumulated upon treatment with jasmonates, octadecanoids and metabolizable carbohydrates, but not upon floating on abscisic acid, NaCl, Na-salicylate or infection with powdery mildew. In developing seedlings, AOS mRNA strongly accumulated in the scutellar nodule, but less in the leaf base. Both tissues exhibited elevated JA levels. *In situ* hybridizations revealed the preferential occurrence of AOS mRNA in parenchymatic cells surrounding the vascular bundles of the scutellar nodule and in the young convoluted leaves as well as within the first internode. The properties of both barley AOSs, their up-regulation of their mRNAs and their tissue specific expression suggest a role during seedling development and jasmonate biosynthesis.

Introduction

Among compounds which act as signals in various environmentally and developmentally regulated processes, jasmonates are discussed to function as a 'master switch' (Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Weiler, 1997). Jasmonic acid (JA) and its methyl ester (JAME) collectively named 'jasmonates' are ubiquitously occurring plant growth regulators. Increases in endogenous JA levels at distinct developmental stages (Creelman and Mullet, 1995; Hause *et al.*, 1996), tissue specific JA responses (Hause *et al.*, 1996), as well as the male sterility of jasmonate-deficient mutants (McConn and Browse, 1996) revealed a function of jasmonates in developmental processes such as seedling growth or pollen formation. Accumulation of JA and its precursor 12-oxo-phytodienoic acid (OPDA) is also observed in response to biotic and abiotic stress such as pathogen attack (Thomma *et al.*, 1998), wounding (Conconi *et al.*, 1996a; O'Donnell *et al.*, 1996; Peña-Cortés *et al.*, 1995), tendril coiling (Stelmach *et al.*, 1998; Weiler *et al.*, 1993), water deficit (Lehmann *et al.*, 1995), elicitation (Gundlach *et al.*, 1992; Parchmann *et al.*, 1997), UV light (Conconi *et al.*, 1996b), burning and electric current application (Herde *et al.*, 1996), chitosan and oligogalacturonide treatment (Bowles, 1990; Doares *et al.*, 1995), or imbalance of nitrogen supply (Creelman and Mullet, 1997). This induced formation of jasmonates is followed by the expression of distinct sets of genes such as those coding for proteinase inhibitors, enzymes of phytoalexin synthesis, vegetative storage proteins, thionins and defensins. Many of these proteins attribute to an increased defense status of the plants (see review of Creelman and Mullet, 1997; Farmer *et al.*, 1998; Weiler *et al.*, 1998).

Therefore, jasmonate signalling has attracted increasing attention which led to the cloning and characterization of cDNAs coding for enzymes of jasmonate biosynthesis. According to the biosynthetic route proposed by Vick and Zimmerman (1984), jasmonates originate from α-linolenic acid via a linoleneate 13-lipoxygenase-(13-LOX)-catalyzed oxygenation which forms (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT). 13-HPOT is the substrate of several enzymes (see Blée, 1998 and references therein), such as hydroperoxide lyase (HPL), reductase, divinyl ether synthase and peroxygenase. The dehydration of the 13-HPOT to an unstable allene oxide by allene oxide synthase (AOS) (E.C.4.2.1.92, formerly hydroperoxide dehydratase) represents the first specific reaction in the so-called LOX pathway leading to jasmonate synthesis. The allene oxide is either chemically hydrolyzed to the dominating α-ketol (about 80%), γ-ketols (about 10%), and racemic *cis*-12-oxo-phytodienoic acid (OPDA) (about 10%) (Figure 1), or enzymatically converted by allene oxide cyclase (AOC) to enantiomeric pure *cis*-(+)-OPDA (Hamberg and Fahlstadius, 1990), the immediate precursor of JA. The final steps of JA biosynthesis consist of a reduction of OPDA followed by three β-oxidation steps. Whereas nothing is known about localization or the enzymatic nature of the β-oxidation steps, two OPDA reductases, OPR 1 and OPR 2, were purified (Schaller and Weiler, 1997a; Schaller *et al.*, 1998). Two genes, *OPR1* and *OPR2*, with 90% similarity were identified on a genomic fragment from *Arabidopsis thaliana* (Biesgen and Weiler, 1999). OPR 1 shows a significant similarity to the sequence and properties of O. WARBURG's yellow enzyme of yeast which reduces the double bound in α,β-unsaturated ketones (Schaller and Weiler, 1997b). This, together with the almost exclusive activity towards those OPDA-isomers which are not formed in plants, suggests a less specific function of OPR1 for JA biosynthesis. In contrast, OPR2 exhibits a high specificity towards the (9*S*,13*S*)-enantiomer of OPDA (Schaller *et al.*, 1998). Furthermore, the AOC reaction is highly specific for JA biosynthesis in that it produces the cyclic core structure of the jasmonates with the correct stereochemistry of the side chains (Hamberg and Fahlstadius, 1990). This enzyme only converts the AOS product formed from 13-HPOT, which further supports its specificity for the biosynthesis of jasmonates (Ziegler *et al.*, 1999). AOC was purified recently to homogeneity from corn (Ziegler *et al.*, 1997), but physiological data on the importance of that enzyme for the regulation of JA levels are not yet available.

More is known about AOS, which was purified and cloned, respectively, from flax seeds (Song and Brash, 1991; Song *et al.*, 1993), *A. thaliana* (Laudert *et al.*, 1996) and guayule (Pan *et al.*, 1995), a rubber-producing species from North America. AOS is a cytochrome P450 enzyme of the CYP74A family and carries, at least in the case for the

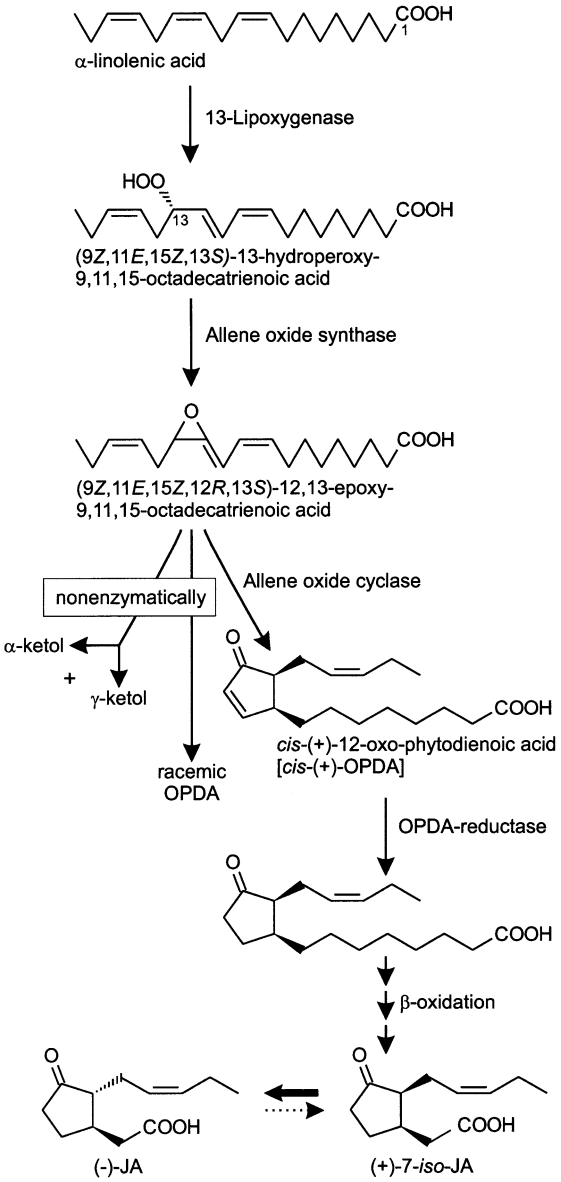


Figure 1. LOX and AOS reactions with subsequent hydrolysis or enzymatic conversion of the allene oxide by AOC.

Arabidopsis and flax enzymes, a putative chloroplast transit peptide and was suggested to be imported into chloroplasts (Harms *et al.*, 1995; Laudert *et al.*, 1996).

Expression of **AOS** was shown to be tightly linked with elevated JA content during the wound response in *A. thaliana* (Laudert and Weiler, 1998), suggesting an important regulatory role for AOS in this process. Furthermore, the **AOS** promoter of *A. thaliana* was found to be activated preferentially in the abscission zones of floral organs (Kubigsteltig *et al.*, 1999). If one assumes that such **AOS** expression attributes to elevated JA levels, a role of jasmonates in floral organ abscission may occur as suggested recently (Kubigsteltig *et al.*, 1999). Taken to-

gether, the present data on AOS point to a decisive role of this enzyme in JA-dependent stress responses and developmental processes.

Here, we describe two AOSs from barley which are regulated in seedling development. These AOSs are (i) located in chloroplasts; (ii) preferentially expressed in parenchymatic cells of the scutellar nodule and of the leaf base; and (iii) their appearance is correlated with elevated levels of jasmonates. The isolated and characterized AOS cDNAs were also found to be up-regulated by treatments with jasmonates, octadecanoids, glucose or sorbitol, and were unaffected by treatments with abscisic acid (ABA), Na-salicylate (SA), NaCl or pathogen infection.

Results

Cloning and characterization of full-length cDNAs and Southern blot analysis

A cDNA library prepared from barley leaves treated for 24 h and 48 h with JAME was screened with the flax AOS cDNA. Among the isolated and sequenced clones, two full-length cDNAs revealed sequence homology to the flax AOS. Both clones exhibited stop codons before the open reading frames. These clones (designated as pHvAOS1 and pHvAOS2) contained an open reading frame of 1461 bp and 1443 bp, respectively, which encode polypeptides of 487 and 480, respectively, amino acids with a calculated molecular mass of 53.4 kDa and 52.7 kDa, respectively, and an isoelectric point of 9.3 and 7.9, respectively (Figure 2). Comparison with published sequences revealed for the HvAOS1 identity to the flax AOS of 53.9% (Song *et al.*, 1993), to the guayule AOS of 52.8% (Pan *et al.*, 1995) and to the *Arabidopsis* AOS of 53.5% (Kubigsteltig *et al.*, 1999) on the protein level, whereas the identity to the barley AOS2 is 89.6%. The corresponding data for HvAOS2 were 54.1%, 52.5% and 53.7%. Similar to the guayule AOS, both barley AOSs lack a putative chloroplast peptide, thus contrasting from both the flax AOS as well as the *Arabidopsis* AOS (Figure 2).

Whereas the consensus sequence **P-V-NKQCAG** of the heme binding domain of the CYP74A enzymes, being a variant of the **FxxGxxxCxG** motif known from most cytochrome P450 enzymes, is largely preserved among all known AOS, the barley AOS1 and AOS2 exhibit an **A** → **P** transition. Furthermore, both AOS exhibit a **T** → **S** transition in the heme binding domain compared to the *Arabidopsis* and guayule AOS. Also in the highly conserved motif **–G-KIL** (Chapple, 1998), both barley AOSs differ from the three other known AOS sequences by a **I** → **V** transition (Figure 2).

Southern blot analysis of barley DNA performed with a labeled full-length cDNA probe of AOS1 revealed two bands if restricted with *Hin*dIII, *Eco*RV or *Dra*I. Three bands could be detected if the DNA was restricted with *Xba*I or *Bam*HI (Figure 3), which might be due to internal restriction sites for both enzymes. This banding pattern suggests the existence of two genes in the barley genome coding for AOSs.

Overexpression of AOS and identification of the reaction products

The whole coding regions of pHvAOS1 and pHvAOS2 were cloned in the bacterial expression vector pQE31 and transformed into the host cells SG13009. After induction with IPTG, an additional and prominent band of the expected size of 55 kDa (calculated size 53.4 kDa) could be detected in the total protein patterns of both bacterial extracts upon SDS-PAGE, when compared to the protein pattern of non-induced host cells shown for AOS1 in Figure 4(a). Immunoblot analysis with a rabbit polyclonal antibody raised against the recombinant protein of pHvAOS1 revealed one band at a similar kDa range (Figure 4b).

To detect AOS activity, enzyme assays were performed with [1-^{14}C]-13-HPOT as substrate. When the affinity-purified recombinant AOS1 and AOS2 from barley were used in this assay, the formation of γ- and α-ketol and OPDA could be detected either by GC–MS analysis (data not shown) or by radio HPLC analysis (Figure 4d,e). These products are known to derive from the hydrolysis of the unstable allene oxide, thus indicating that the recombinant enzymes are able to perform the AOS reaction. Within Figure 4(c) a control incubation with 13-HPOT is shown.

Enzymatic properties of the recombinant AOS1

The substrate specificity of the recombinant AOS1 from barley was determined for six fatty acid hydroperoxides. For substrates we used the 9- and 13-hydroperoxides of linoleic as well as linolenic acid, and hydroperoxides of two C_{20} fatty acids, both containing the hydroperoxy group in position n-6, but one derived from arachidonic acid and one containing one more double bond at n-3. As shown in Table 1, all substrates were converted by AOS with rather different kinetics. The lowest affinity was observed with 13-HPOD followed by the 9-hydroperoxides. Particularly remarkable was the almost fivefold increased affinity toward the 13-hydroperoxides from linolenic acid when compared to the linoleic acid derivatives. Furthermore, conversion of the 9-hydroperoxides of linolenic acid and linoleic acid is surprising, and was added to our knowledge of this type of AOS activity for the first time. It will be interesting to test the substrate specificity occurring *in vivo* by analyzing the endogenous occurrence of the corresponding ketols. The hydroperoxides of the C_{20} fatty acids had the highest affinity toward AOS, especially when the

```
                1                                                          60
Hordeum vulgare 1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~M
Hordeum vulgare 2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Linum usitatissimum MASSALNNLV AVNPNTLSPS PKSTPLPNTF SNLRRVSAFR PIKASLFGDS PIKIPGITSQ
Parthenium argentatum ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Arabidopsis thaliana ~~~~~~~~~~ ~~~~~~~~~~ MASISTPFPI SLHPKTVRSK PLKFRVL.TR PIKASGSETP
Consensus           ---------- ---------- ---------- ---------- ---------- ----------
                                        signal peptide

                61                                                         120
Hordeum vulgare 1   NQSGMARSDE GSLVPREVPG SYGLPFVSAI RDRLDFYYFQ .GQDKYFESR VEKYGSTVVR
Hordeum vulgare 2   ~~~~MNQSAI GSLVPRQAPG SYGLPFVSAI RDRLDFYYFQ .GEAKYFESR VEKHGSTVLR
Linum usitatissimum PPPSSDET.. .TLPIRQIPG DYGLPGIGPI QDRLDYFYNQ .GREEFFKSR LQKYKSTVYR
Parthenium argentatum ~~~~MDPS.. .SKPLREIPG SYGIPFFQPI KDRLEYFYGT GGRDEYFRSR MQKYQSTVFR
Arabidopsis thaliana DLTVATRTGS KDLPIRNIPG NYGLPIVGPI KDRWDYFYDQ .GAEEFFKSR IRKYNSTVYR
Consensus           ---------- -----R--PG -YG-P----I -DR----Y-- -G----F-SR --K--STV-R

                121                                                        180
Hordeum vulgare 1   INVPPGPFMA RDPRVVAVLD AKSFPVLFDV TKVEKKNLFT GTYMPSTSLT GGFPVCSYLD
Hordeum vulgare 2   INVPPGPFMA RDPRVVAVLD AKSFPVLFDV DKVEKKNLFT GTYMPSTSLT GGFRVCAYLD
Linum usitatissimum ANMPPGPFIA SNPRVIVLLD AKSFPVLFDM SKVEKKDLFT GTYMPSTELT GGYRILSYLD
Parthenium argentatum ANMPPGPFVS SNPKVIVLLD AKSFPILFDV SKVEKKDLFT GTYMPSTKLT GAYRVLSYLD
Arabidopsis thaliana VNMPPGAFIA ENPQVVALLD GKSFPVLFDV DKVEKKDLFT GTYMPSTELT GGYRILSYLD
Consensus           -N-PPG-F-- --P-V---LD -KSFP-LFD- -KVEKK-LFT GTYMPST-LT G------YLD

                181                                                        240
Hordeum vulgare 1   PSEPTHTKVK QLLFSLLASR KDAFIPAFRS HFSSLLATVE SQLLLSGKSN FNTLNDATSF
Hordeum vulgare 2   PSEPTHTKVK QLLFSLLASR KDAVIPAFRS HFSSLLATVE SQLVLSGKSN FNTLNDFTSF
Linum usitatissimum PSEPNHTKLK QLLFNLIKNR RDYVIPEFSS SFTDLCEVVE YDLATKGKAA FNDPAEQAAF
Parthenium argentatum PSEPRHAQLK NLLFFMLKNS SNRVIPQFET TYTELFEGLE AELAKNGKAA FNDVGEQAAF
Arabidopsis thaliana PSEPKHEKLK NLLFFLLKSS RNRIFPEFQA TYSELFDSLE KELSLKGKAD FGGSSDGTAF
Consensus           PSEP-H---K -LLF------ -----P-F-- ----L----E --L---GK-- F--------F

                241                                                        300
Hordeum vulgare 1   EFIGDGYFGV LPSASDLGTT GPAKAAKWLI FQLHPLVTLG LPMILEEPLL HTVHLPPFLV
Hordeum vulgare 2   EFIADTYFGV LPSASDLGTT GPAKAAKWLI FQLHPLVTFG LPMILEEPLL HTVLLPPIFV
Linum usitatissimum NFLSRAFFGV KPIDTPLGKD APSLISKWVL FNLAPILSVG LPKEVEEATL HSVRLPPLLV
Parthenium argentatum RFLGRAYFNS NPEETKLGTS APTLISSWVL FNLAPTLDLG LPWFLQEPLL HTFRLPAFLI
Arabidopsis thaliana NFLARAFYGT NPADTKLKAD APGLITKWVL FNLHPLLSIG LPRVIEEPLI HTFSLPPALV
Consensus           -F-------- -P----L--- -P-----W-- F-L-P----G LP----E--- H---LP----

                301                                                        360
Hordeum vulgare 1   SGDYKALYKY FFAAATKALD TAEGLGLKRD EACHNLLFAT VFNSYG**GLKV LL**PGILARIA
Hordeum vulgare 2   SGDYKALYKY FYAAATKALD MAESLGLNRD EACHNLLFAT VFNSYG**GLKV ML**PGFLGRIA
Linum usitatissimum QNDYHRLYEF FTSAAGSVLD EAEQSGISRD EACHNILFAV CFNSWG**GFKI LF**PSLMKWIG
Parthenium argentatum KSTYNKLYDY FQSVATPVME QAEKLGVPKD EAVHNILFAV CFNTFG**GVKI LF**PNTLKWIG
Arabidopsis thaliana KSDYQRLYEF FLESAGEILV EADKLGISRE EATHNLLFAT CFNTWG**GMKI LF**PNMVKRIG
Consensus           ---Y--LY-- F---A----- -A---G---- EA-HN-LFA- -FN--GG-K- --P-----I-

                361                                                        420
Hordeum vulgare 1   DSGEKFHKKL VTEIRAAVAE AG.GKVTIEA LEKMELTKSA VWEALRLDPA VKFQYGRAKA
Hordeum vulgare 2   EAGEKFHQRL AAEVRTAVAD AG.GKVTIEA LEKMELTKSA VWEALRLEPP VKFQYGRAKV
Linum usitatissimum RAGLELHTKL AQEIRSAIQS TGGGKVTMAA MEQMPLMKSV VYETLRIEPP VALQYGKAKK
Parthenium argentatum VAGENLHTQL AEEIRGAIKS YGDGNVTLEA IEQMPLTKSV VYESLRIEPP VPPQYGKAKS
Arabidopsis thaliana RAGHQVHNRL AEEIRSVIKS NG.GELTMGA IEKMELTKSV VYECLRFEPP VTAQYGRAKK
Consensus           --G---H--L --E-R----- -G-G--T--A -E-M-L-KS- V-E-LR--P- V--QYG-AK-

                421                                                        480
Hordeum vulgare 1   DMNIESHDAV FAVKKGEMLF GYQPCATKDP RVFGPTAREF VGDRFVGKEG SKLLKYVYWS
Hordeum vulgare 2   DMNIESHDAV FAVQKGEMLF GYQPCATKDP RVFGSTAREF VGDRFVG.EG SKLLQYVYWS
Linum usitatissimum DFILESHEAA YQVKEGEMLF GYQPFATKDP KIF.DRPEEF VADRFVG.EG VKLMEYVMWS
Parthenium argentatum NFTIESHDAT FEVKKGEMLF GYQPFATKDP KVF.DRPEEF VPDRFVG.DG EALLKYVWWS
Arabidopsis thaliana DLVIESHDAA FKVKAGEMLY GYQPLATRDP KIF.DRADEF VPERFVGEEG EKLLRHVLWS
Consensus           ----ESH-A- --V--GEML- GYQP-AT-DP --F-----EF V--RFVG--G --L---V-WS

                481                                                        540
Hordeum vulgare 1   NGRETES**PSV HNKQCPG**KNL VVLVGRLLVV ELFLRYDTFT AKVGLDLLGT KVEFTGVTKA
Hordeum vulgare 2   NGRETES**PSV DNKQCPG**KNL VVLVGRLLVV ELFLRYDTFT ADVGVDLLGP KVEFTGVTKA
Linum usitatissimum NGPETET**PSV ANKQCAG**KDF VVMAARLFVV ELFKRYDSFD IEVGTSSLGA SITLTSLKRS
Parthenium argentatum NGPETES**PTV ENKQCAG**KDF VVLITRLFVI ELFRRYDSFE IELGESPLGA AVTLTFLKRA
Arabidopsis thaliana NGPETET**PTV GNKQCAG**KDF VVLVARLFVI EIFRRYDSFD IEVGTSPLGS SVNFSSLRKA
Consensus           NG-ETE-P-V -NKQC-GK-- VV---RL-V- E-F-RYD-F- ---G---LG- ----------
                          heme binding

                541
Hordeum vulgare 1   TSGVADAV
Hordeum vulgare 2   TSG.PGAV
Linum usitatissimum TF*~~~~~
Parthenium argentatum SI*~~~~~
Arabidopsis thaliana SF*~~~~~
Consensus           --------
```

Figure 2. Amino acid sequence comparison of the isolated AOS1 and AOS2 from barley (*Hordeum vulgare* cv. Salome) with that of *Arabidopsis* (*A. thaliana*) (revised sequence published by Kubigsteltig *et al.*, 1999), flax *(Linum usitatissimum)* (Song *et al.*, 1993) and guayule (*Parthenium argentatum*) (Pan *et al.*, 1995).
Putative chloroplast transit peptides are underlined. The consensus sequence **P-V-NK QCAG** of the heme binding domain and the highly conserved motif **-G-KIL** of the CYP74A enzymes are indicated by bold letters.

number of double bonds is increased. With the exception of 9-HPOD, which showed the highest rate of conversion, the reaction velocity decreased as the affinity increased.

Accumulation of AOS mRNA upon various treatments

Barley leaves respond to treatment with sorbitol or metabolizable carbohydrates with the endogenous accumulation of JA (Lehmann *et al.*, 1995), JA amino acid conjugates (Kramell *et al.*, 1995) and octadecanoids (Kramell *et al.*, 2000). This accumulation might be accompanied with an up-regulation of AOS mRNAs. Kinetic analysis of AOS mRNA accumulation was performed with the full-length cDNA coding for AOS1. As expected with a high degree of sequence similarity, the use of the full-length cDNA coding for AOS2 led to identical results in this type of Northern analysis (data not shown). Upon continuous treatments, mRNA accumulations revealed that there was no detectable constitutive expression in water-treated controls. Desiccated leaves and leaves floated on 1 M sorbitol showed a weak, but significant up-regulation, whereas 0.5 M glucose strongly induced AOS mRNA (Figure 5a). Exogenous application of JAME, JA, the conjugate of JA with L-isoleucine (JA-L-Ile), OPDA or OPDAME led to an earlier and transient rise of AOS mRNA accumulation which peaked about 4 h after onset of

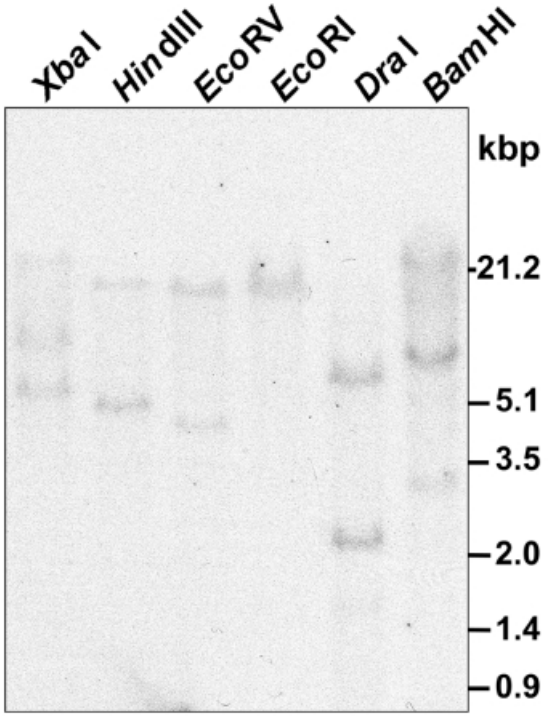


Figure 3. Southern blot analysis of the total DNA of *Hordeum vulgare* cv. Salome.
The DNA was digested with *Xba*1, *Hin*dIII, *Eco*RII, *Eco*RI, *Dra*I and *Bam*HI, and the resulting DNA fragments were separated by 0.8% agarose gel electrophoresis, transferred onto nitrocellulose filters and hybridized with ^{32}P-labeled AOS1 cDNA as described in Experimental procedures.

treatment (Figure 5a). However, no AOS mRNA accumulation occurred upon treatment with 90 μM ABA, 50 μM SA (Figure 5a), 1 M NaCl, 2 mM aspirin, 1 M 6-deoxyglucose or infection of leaves with powdery mildew (data not shown). The AOS mRNA accumulation was followed by a delayed but constant accumulation of the AOS protein up to 72 h of JAME treatment. The same accumulation, but with a further delay and less abundantly, was observed during sorbitol treatment (Figure 5b).

Intracellular localization of AOS protein

AOS proteins localized intracellularly thus far were found within chloroplasts, and the corresponding cDNAs were shown to code for a putative chloroplast transit peptide (Harms *et al.*, 1995; Laudert *et al.*, 1996; Song *et al.*, 1993). Due to the apparent lack of a putative chloroplast transit peptide within both barley AOSs (Figure 2), we were interested in showing their intracellular location. Due to the sequence similarity of AOS1 and AOS2, the antibody raised against the recombinant AOS1 recognized both AOSs equally in each immunological technique. Taking advantage of its up-regulation upon JAME treatment, immunoblot analysis was performed for total protein extracts of protoplasts and chloroplasts isolated from barley leaves treated or non-treated with JAME. The AOS proteins occurred at elevated levels in leaf segments treated with JAME for 24 h (Figure 6a, LS). After purification of chloroplasts from protoplasts isolated from JAME-treated leaf segments, the protein ratio of protoplasts to chloroplasts calculated via equal amounts of chlorophyll was found to be about 7 : 5. Therefore, the weak elevated amount of AOS protein in chloroplasts compared to protoplasts (Figure 6a, CP versus PP) reflects a preferential if not exclusive occurrence in the chloroplast. In contrast, the cytoplasmatically located JIP-23 is dramatically reduced after purification of chloroplasts (Figure 6a). Upon treatment with a proteinase, the AOS protein was detected in chloroplast containing fractions at a similar amount as before the treatment (Figure 6a, CP versus CP*). Due to the fact that JIP-23 is proteinase K resistant, activity of this proteinase was checked by degradation of Rubisco in a crude extract (data not shown). The apparent location of AOS protein in chloroplasts could be confirmed by immunocytochemical analysis (Figure 6b–d). There was some cross-reactivity of the anti-AOS antibody with cell walls in water- as well as JAME-treated tissues (Figure 6b,c). Due to the cross-reactivity of anti-AOS antibody to an 18 kDa protein in extracts of leaf segments (Figure 6a, LS) appearing identically in extracts of water-treated and JAME-treated tissues, it is highly probable that the cell wall fluorescence was caused by this cross-reactivity. However, a significant fluorescence label was found in chloroplasts of JAME-treated leaf tissues (Figure 6c), whereas in water-treated leaf segments no significant fluorescence was detectable (Figure 6b). Since there was only one band recognized by the anti-AOS antibody in total protein extracts of isolated protoplasts (Figure 6a), the fluorescence is indicative for AOS protein suggesting its location in chloroplasts. The autofluorescence of chloroplasts is shown in cross-sections which were performed for immunolabeling without the first antibody (Figure 6d).

Organ- and tissue-specific accumulation of AOS mRNA correlates with elevated JA levels

The most abundant protein of barley leaves accumulating upon JA treatment is a 23 kDa protein which is also formed in distinct tissues such as the scutellar nodule during seedling development (Hause *et al.*, 1996). This might be linked to JA biosynthesis. Therefore, and with respect to the unique role of AOS in the formation of JA, we analyzed JA levels and tissue-specific expression of AOS during seedling development. In a 6-day-old seedling, AOS mRNA accumulated weakly in the root tip but abundantly in the tissues around the scutellar nodule (Figure 7). Moreover, the AOS mRNA accumulated in the base of the primary

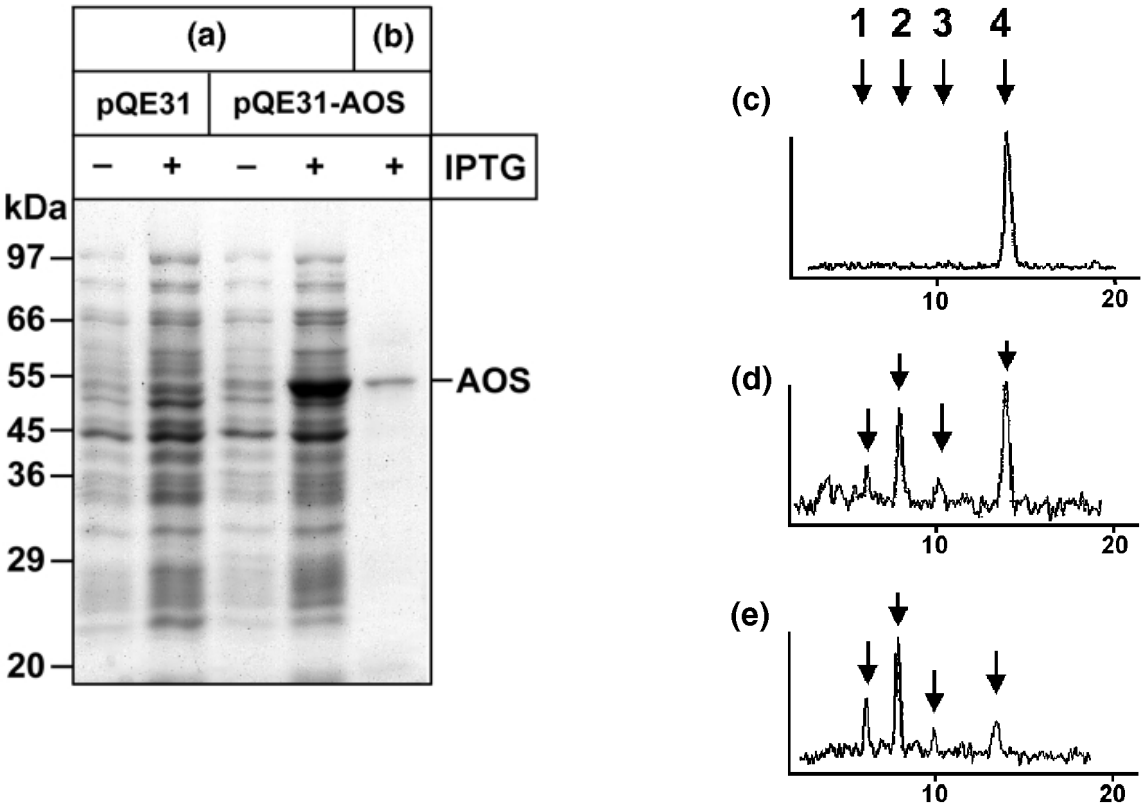


Figure 4. Bacterial overexpression of the barley AOS (a,b) and radiochromatograms of reaction products of control incubation with [1–^{14}C]-13-HPOT (c) or with AOS1 and AOS2 (d,e).

The cDNA clones pHvAOS1 and pHvAOS2 were subcloned into the vector pQE31 and transfected into *E. coli* strain SG13009. Extracts from bacteria carrying the vector alone (pQE31) or with the AOS cDNAs (pQE31 AOS) grown in the absence (–) or presence of IPTG (+), were separated by SDS-PAGE and stained with Coomassie blue (a) or probed with an anti-AOS antibody (b). Due to the identical results for AOS1 and AOS2 data for AOS1 are presented. The position of the AOS protein is indicated. (c) Control incubation with [1–^{14}C]-13-HPOT alone; (d,e) analysis of products formed after incubation of [1–^{14}C]-13-HPOT in the presence of 2 μg affinity-purified recombinant barley AOS1 (d) or recombinant barley AOS2 (e). The incubation mixture contained 100 nmol of [1–^{14}C]-13-HPOT (86 000 d.p.m.). The reaction products were extracted after a 15 min incubation and analyzed by radio RP-HPLC. The numbers on the chromatograms refer to the following compounds: 1, γ-ketol; 2, α-ketol; 3, OPDA; 4, 13-HPOT.

leaf, and the signal decreased up to the leaf blade to undetectable levels. This is in agreement with the undetectable level of AOS mRNA in water-floated leaf segments (Figure 5a). To inspect the tissue-specific accumulation of AOS-protein and mRNA in more detail, immunolocalization and *in situ* hybridization were done with different cross-sections of the mesocotyl of a 6-day-old barley seedling (Figure 8). AOS protein was clearly detectable in parenchymatic cells of the first internode (Figure 8b, showing section III in (a)), whereas the vascular bundles of the coleoptile, the shoot apex and the scutellum were free of label. This result could be confirmed by *in situ* hybridization. Using different cross-sections below and above the nodal plate of the scutellum (Figure 8a), AOS mRNA was detectable in young leaves, in the second shoot, in the first internode as well as in the parenchymatic cells surrounding the vascular bundles of the scutellar nodule. The coleoptile and the scutellum were free of label. This tissue-specific expression suggests a specific role of AOSs in seedling development. Interestingly, tissues with strong AOS mRNA accumulation such as the scutellar nodule and the leaf base (Figures 7 and 8) exhibited significantly elevated levels of JA. Furthermore, a gradual decrease of AOS mRNA accumulation from the leaf base up to the leaf tip correlated with a decrease in the JA level. These data support the assumption that the AOS described here functions in JA biosynthesis.

Discussion

Among the numerous LOX-derived compounds, the allene oxide is dominant since it is the first intermediate of the JA-forming branch of the LOX pathway, and AOS expression seems to be subject to complex control (Laudert and Weiler, 1998).

Although AOS belongs to the family of the cytochrome P450 enzymes (Song and Brash, 1991), there is no requirement for oxygen, NADPH and a P450 reductase. The enzyme also exhibits an unusually high turnover number when compared to other P450s (Chapple, 1998).

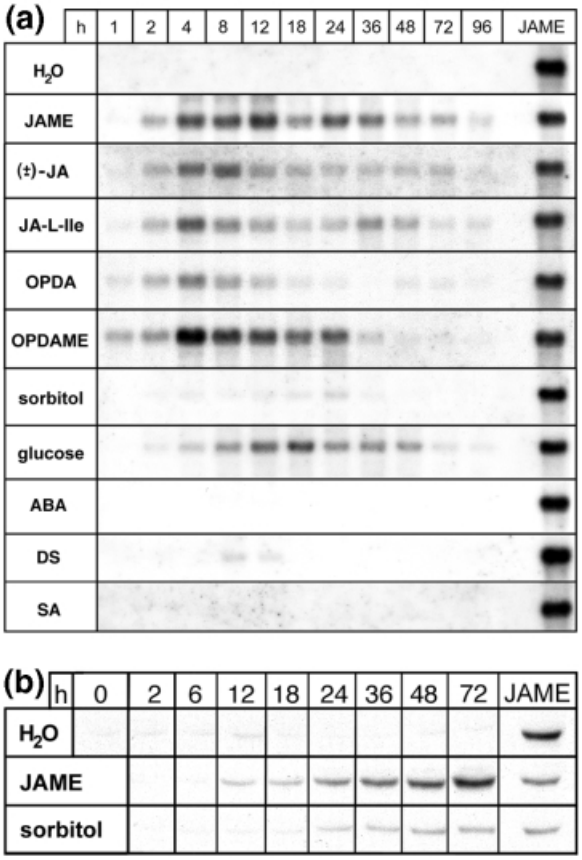


Figure 5. Northern blot analysis of AOS mRNAs accumulation (a) and immunoblot analysis of AOS protein (b).
Barley leaf segments were floated on water, 45 μM JAME 50 μM JA, 50 μM JA-L-Ile, 50 μM OPDA, 50 μM OPDAME, 1 M sorbitol, 0.5 M glucose, 90 μM ABA, or 50 μM SA or were subjected to desiccation stress (DS). For (a), 10 μg total RNA was loaded per lane. Loading was inspected by recording the ethidium bromide staining of rRNA. For comparison of different filters, AOS mRNA accumulating during treatment with 45 μM JAME for 24 h was used as internal control. Northern blot analysis was performed with a ^{32}P-labeled insert of the full-length AOS1 cDNA as described in Experimental procedures. For (b), total proteins were extracted in parallel to RNA extraction from leaf segments floated on water, 45 μM JAME or 1 M sorbitol solutions for the indicated times. Immunoblot analysis was performed with 10 μg protein per lane and a purified rabbit anti-AOS antibody (diluted 1:50) raised against the recombinant barley AOS1 as described in Experimental procedures.

Due to notable differences in the heme-binding domain **FxxGxxxCxG** of most P450 enzymes, there are two families of the CYP74 type. The CYP74A family contains all AOS, whereas the CYP74B family thus far contains the HPL of pepper (Matsui *et al.*, 1996) as well as of *Arabidopsis* (Bate *et al.*, 1998). Even among the three AOS sequences known to date, there are differences with respect to the occurrence of a putative chloroplast transit peptide. Whereas AOS from *A. thaliana* (Laudert *et al.*, 1996) and flax (Song *et al.*, 1993) carry this sequence, it is not present in the AOS of rubber particles of the desert shrub guayule (Pan *et al.*, 1995). The possible regulatory role of AOS in JA biosynthesis as well as the apparent heterogeneity among the known dicotyledoneous AOS prompted us to isolate one or more AOS cDNA(s) from a monocotyledonous plant to inspect the expression and to investigate a possible further diversification between AOS from the two classes of angiosperms.

The first monocotyledonous AOSs are different from other AOSs

Both full-length cDNAs, isolated from JA-treated barley leaves, could be identified as AOS via its sequence identity to known AOS as well as by activity tests upon bacterial overexpression. The barley enzymes exhibit two notable features: (i) as the guayule AOS, both barley AOSs lack properties indicative for a chloroplast leader such as the absence of D, I and E, MA at the start, or S-enrichment (Von Heijne *et al.*, 1989); and (ii) the consensus sequence **P-V-NKQCAG**, which represents the heme binding domain in CYP74A enzymes, carries an **A** → **P** transition (Figure 2). This might be of consequence for the enzymatic properties due to the assumed role of isoleucine in oxygen activation (Van Wachenfeldt and Johnson, 1995).

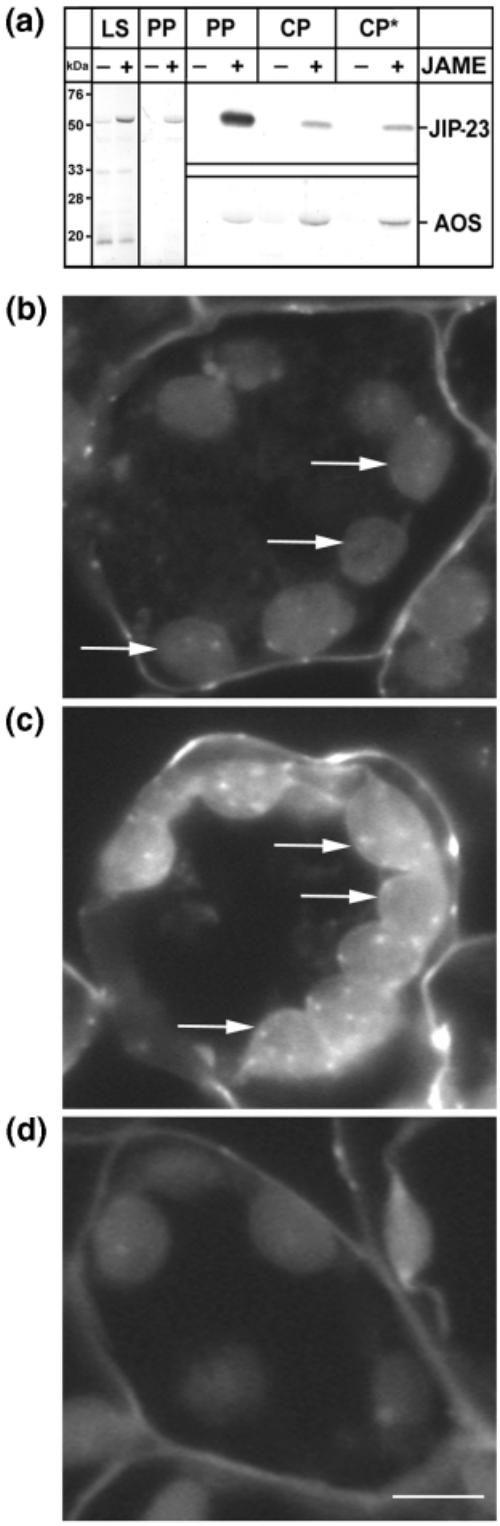
The lack of a putative chloroplast leader sequence in the guayule AOS is not that unexpected due to its association with rubber particles (Pan *et al.*, 1995), but the function of AOS in rubber particles is not clear. However, the absence of a chloroplast target sequence in both barley AOS was

Table 1. Substrate specificity of barley AOS1

Substrate		K_M μM	V_{max} nkat 2 μl^{-1}
(9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid	(9-HPOD)	24.6 ± 6.1	3.2 ± 0.4
(13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid	(13-HPOD)	46.6 ± 3.5	0.96 ± 0.04
(9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid	(9-HPOT)	33.1 ± 2.4	0.58 ± 0.02
(13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid	(13-HPOT)	9.1 ± 0.8	0.32 ± 0.008
(15*S*,5*Z*,8*Z*,11*Z*,13*E*)-15-hydroperoxy-5, 8,11,13-eicosatetraenoic acid	(15-HPET)	8.3 ± 1.5	0.1 ± 0.005
(15*S*,5*Z*,8*Z*,11*Z*,13*E*,17*Z*)-15-hydroperoxy-5, 8,11,13,17-eicosapentaenoic acid	(15-HPEP)	3.1 ± 0.6	0.05 ± 0.031

The mean values ± SD of at least four replicates are shown.

somewhat surprising since in leaf tissues, enzymes of JA biosynthesis catalyzing reactions upstream of OPDA were found in chloroplasts, such as LOX (Bell *et al.*, 1995; Feussner *et al.*, 1995; Heitz *et al.*, 1997), AOS (Blée and Joyard, 1996; Harms *et al.*, 1995) or AOC (B. Hause, unpublished results). Therefore, we also expected a chloroplast location for barley AOS. Both methods used in this study, cell fractionation and immunocytochemical analysis, revealed the occurrence of AOS protein in barley mesophyll chloroplasts. This is consistent with (i) biochemical data on the occurrence of AOS activity in envelope membrane fractions from pea (Blée and Joyard, 1996); (ii) the putative chloroplast signal peptide in the flax AOS (Song *et al.*, 1993) and the *Arabidopsis* AOS (Laudert *et al.*, 1996); and (iii) the chloroplastic location of the flax AOS upon overexpression in transgenic potato (Harms *et al.*, 1995). In contrast, transgenic tobacco plants, expressing a flax AOS in the cytoplasm by lack of its chloroplast signal peptide, showed elevated JA and OPDA



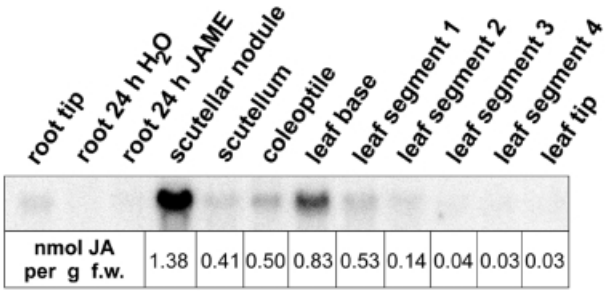


Figure 7. Northern blot analysis of AOS mRNA levels in different tissues of 6-day-old barley seedlings and their JA levels.
Each lane was loaded with 30 μg total RNA. JA determinations were performed by GC/MS analysis as described in Experimental procedures. Four different extractions and analysis were performed giving identical tendency of data. One series of data is given.

Figure 6. Intracellular localization of AOS protein within barley leaves.
(a) Co-purification of chloroplasts and AOS protein. Barley leaf segments floated on water or 45 μM JAME for 6 h followed by floating on water for an additional 42 h were subjected to isolation of protoplasts (PP) and chloroplasts (CP). Chloroplasts were purified and partially treated with proteinase (CP*) as indicated in Experimental procedures. As a positive control, leaf segments were used, which were floated on water or 45 μM JAME for 24 h (LS). Total proteins (7 μg per lane) were subjected to immunoblot analysis with a purified rabbit anti-AOS antibody raised against the recombinant barley AOS1 at a dilution of 1 : 10. To check the enrichment of plastidic proteins in the chloroplast fraction compared to the protoplast fraction, immunoblot analysis was performed with the rabbit polyclonal antibody raised against JIP-23, a non-plastidic protein of barley leaves, at the dilution of 1 : 5000. For proteinase K treatment (CP*) activity of digestion was checked by degradation of Rubisco in total protein extracts since JIP-23 is proteinase K-resistant (data not shown).
(b–d) Immunolocalization of AOSs in cross-sections of barley leaf segments. AOSs were visualized by immunodecoration with a purified anti-AOS antibody (see above) followed by treatments according to the TSA detection kit (NEN). (b) Mesophyll cell of a barley leaf segment floated on water in the dark for 48 h. The cell wall as well as the chloroplasts (arrows) exhibit some autofluorescence. (c) Mesophyll cell of a barley leaf segment floated on 45 μM JAME in the dark for 48 h. Chloroplasts exhibit a specific fluorescence signal which accumulates partially at their border (arrows). (d) Concomitant section to (c) but immunolabelled by omitting the first antibody. The bar represents 5 μm for all figures.

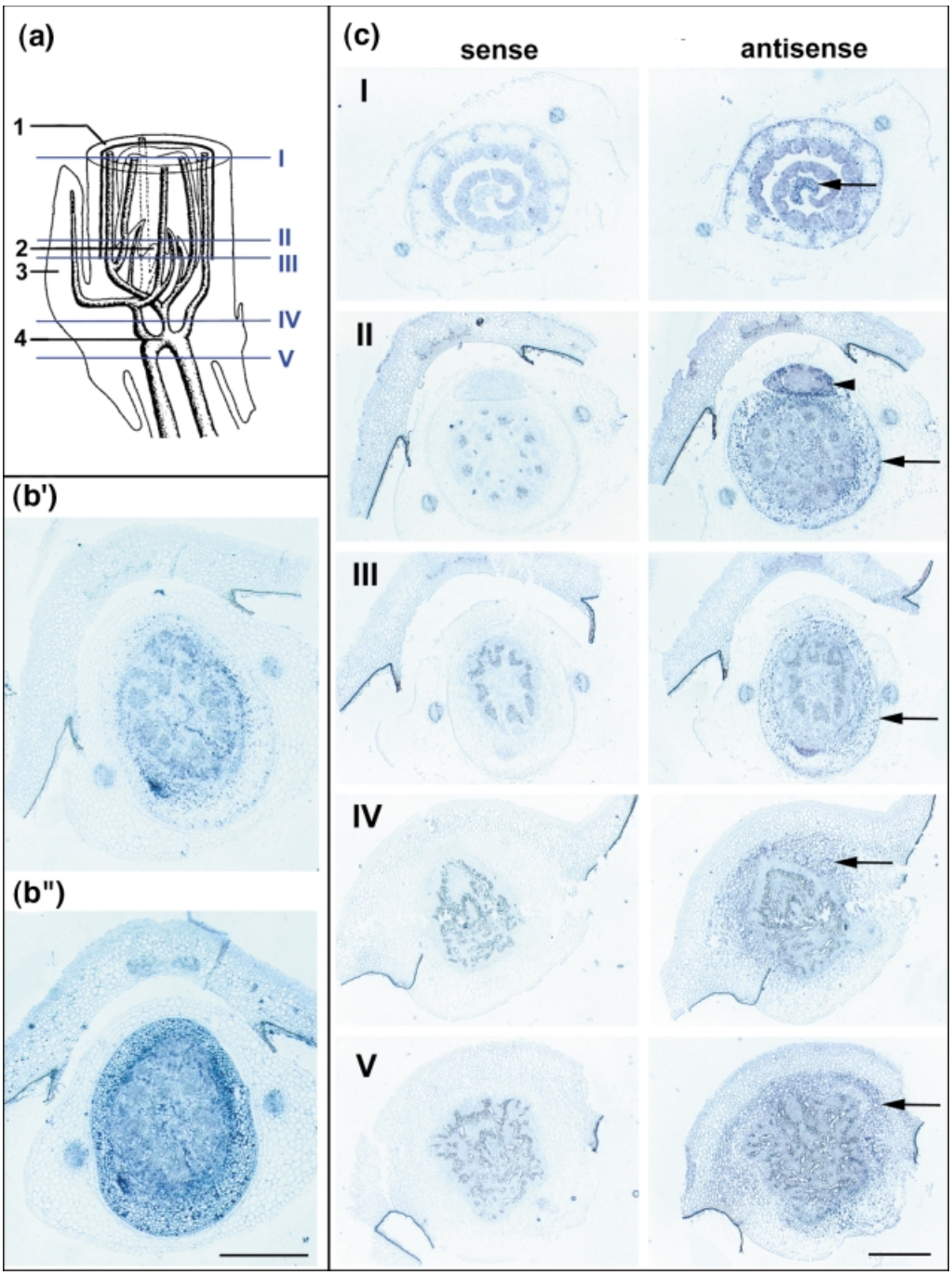


Figure 8. Expression of AOS in the mesocotyl of 6-day-old barley seedlings.
(a) Schematic presentation of the morphology of the mesocotyl region of barley seedlings (adapted from Esau, 1965). (1) coleoptile, (2) shoot apex, (3) scutellum, (4) nodal plate of the scutellum (transition region between vascular bundles of leaves and roots). I–V indicate the positions where cross-sections were performed for immunocytology shown in (b) and *in situ* hybridization shown in (c).
(b′–b″) Localization of AOS protein in cross-sections of the mesocotyl of a barley seedling (level III in (a)). AOS protein was visualized by immunodecoration with a purified rabbit anti-AOS antibody (see Figure 6) followed by a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase. Staining was performed as described in the Experimental procedures. (b′) Control performed by omitting the first antibody. Note some unspecific staining within the cell layers at the border between the first internode and the shoot apex. (b″) Immunostain with anti-AOS antibody. In comparison to (b′), label is clearly visible within the first internode, whereas the vascular bundles of coleoptile, shoot apex and scutellum are not labeled. The bar represents 500 μm for both figures.
(c) *In situ* hybridization of different cross-sections, indicated in (a), of the mesocotyl of a barley seedling. Parallel cross-sections were hybridized with sense or antisense DIG-labeled RNA, respectively. AOS mRNAs could be detected in young leaves (arrow in I), in the second shoot (arrow head in II), in the first internode (arrows in II and III) and in parenchymatic cells surrounding vascular bundles of the scutellar nodule (arrows in IV and V). The coleoptile as well as the scutellum did not exhibited label. The bar represents 500 μm for all figures.

levels upon wounding only (Wang *et al.*, 1999). This points, together with the recently observed location of AOC within chloroplasts (B. Hause, unpublished results), to a common location of AOS and AOC in wild-type conditions. It remains to be elucidated whether the barley AOS occurs in the outer envelope membrane which is the location where chloroplast proteins lacking a corresponding signal sequence are imported via a transit peptide-independent sorting route (Keegstra and Cline, 1999).

The enzymatic properties of the recombinant barley AOS1 revealed a decreasing K_M in the following order of substrates: 9-HPOD > 13-HPOD > 9-HPOT > 13-HPOT. It remains to be elucidated whether the interesting activity with 9-hydroperoxides as substrates occurs *in vivo*. Very similar kinetic parameters were found recently for the AOS of the 100 000 g membrane pellet of the corn seed homogenate (Ziegler *et al.*, 1999). This preference of the AOS1 for 13-HPOT and the dominant occurrence of 13-LOX forms catalyzing formation of 13-HPOT within the barley chloroplasts (Feussner *et al.*, 1995; Kohlmann *et al.*, 1999), suggest that the AOS characterized here may function in JA biosynthesis. Furthermore, all treatments which do not lead to endogenous rise of jasmonates failed to give AOS mRNA accumulation. Among them are 1 M NaCl, 6-deoxyglucose, aspirin, SA, ABA and pathogen infection. The inability of ABA to cause AOS mRNA accumulation accords with the fact that in barley there is no synergistic effect of ABA and JA (Ortel *et al.*, 1999).

Up-regulation of barley AOS mRNAs support that these AOSs are part of a complex regulation of LOX-derived compounds

Up-regulation of mRNAs of barley AOSs support that at least one of these AOS forms are related to JA biosynthesis. Floating of leaf segments on sorbitol or glucose solution led to AOS mRNA accumulation (Figure 5). Both compounds are known to lead to the accumulation of jasmonates (Lehmann *et al.*, 1995), JA amino acid conjugates (Kramell *et al.*, 1995) or octadecanoids (Kramell *et al.*, 2000). Like the Arabidopsis AOS (Laudert and Weiler, 1998) and the flax AOS (Harms *et al.*, 1998), the expression of the barley AOSs is induced by JA and OPDA as well as by their methyl esters. This points to an increased capacity of the octadecanoid pathway, as also shown by elevated JA levels in transgenic potato plants overexpressing flax AOS cDNA (Harms *et al.*, 1995). However, the lack of complete correlation of AOS mRNA accumulation, AOS protein accumulation, AOS activity and JA/OPDA accumulation observed in Arabidopsis leaves upon JA treatment or wounding (Laudert and Weiler, 1998), suggest a more complex regulation than detected so far. Other hydroperoxide converting enzymes in leaves, mainly HPL, might compete with the AOS for the common substrate 13-HPOT and have to be regarded as a further determinant in the regulation of JA biosynthesis. Furthermore, (9*Z*,12*R*,13*S*)-12,13-epoxy-9-octadecenoic acid, which might be formed via the peroxygenase branch of the LOX pathway (Blée, 1998), is a strong inhibitor of the AOC (Ziegler *et al.*, 1997), indicating that JA biosynthesis might also be regulated by compounds formed in other branches of the LOX pathway.

Interestingly, SA, which was suggested to block the wound-induced JA biosynthesis in tomato leaves upstream of OPDA (Peña-Cortés *et al.*, 1993) and to act down-stream of JA (Doares *et al.*, 1995), increased the accumulation of AOS mRNA, AOS protein and, most surprisingly, OPDA levels in *Arabidopsis* leaves (Laudert and Weiler, 1998). These data led to the suggestion that SA may inhibit the release of OPDA from the chloroplast. Here, a further difference of a monocotyledonous AOS from that of a dicotyledonous plant is obvious: in barley leaves, SA as well as aspirin did not induce accumulation of AOS mRNA (Figure 5). However, a remarkable shift of LOX-derived compounds into products of the reductase was shown for barley leaf segments which were floated on SA compared to those which were floated on JA or sorbitol (Weichert *et al.*, 1999). Furthermore, SA induces 13-LOX (Hause *et al.*, 1999), which is not linked to the JA formation (Vörös *et al.*, 1998). As a consequence, a remarkable flux of LOX-derived metabolites upon SA treatment might be possible which bypasses the AOS reaction and leads to compounds putatively regulating JA biosynthesis. Such a link of SA and JA might explain their independent action in separate signaling pathways which were repeatedly observed (Niki *et al.*, 1998; Thomma *et al.*, 1998).

AOSs are expressed tissue-specifically in seedling development and correlate to JA levels

A role of jasmonates is suggested for pollen development in *Arabidopsis* since both the JA insensitive mutant *coi1* (Feys *et al.*, 1994) and the JA-deficient triple mutant *fad3-2 fad7-2, fad8* (McConn and Browse, 1996) are male sterile. The recently shown AOS promoter activity of *A. thaliana* in maturing pollen and bases of petioles and stipules correspond to these data, thus suggesting a role of JA in floral organ abscission (Kubigsteltig *et al.*, 1999). Organ-specific analysis of JA levels will substantiate this suggestion.

During seedling development of soybean (Creelman and Mullet, 1995) and barley (Hause *et al.*, 1996), there are elevated levels of JA preferentially in photosynthetically inactive tissues. Interestingly, the AOS forms described here were found to be expressed preferentially in parenchymatic cells around the vascular bundles of the scutellar nodule and leaf base (Figure 8). According to previous data (Hause *et al.*, 1996) and that shown here, the JA levels are also remarkably higher in these tissues.

As revealed by ^{13}C-NMR spectroscopy, the mesocotyl and its scutellar nodule are known to facilitate transport of metabolizable sugars (Ishida *et al.*, 1996) released from the endosperm into the leaf base, the growth zone of a monocotyledonous leaf. This may cause osmotic stress in these tissues according to the higher osmolality detected (Hause *et al.*, 1996). One may envisage the following scenario: carbohydrates degraded in the endosperm lead to osmotic stress in such cells mainly used for their transport into growing tissues. As a consequence, a stress-induced rise of JA may occur, which might be at least partially caused by a transcriptional up-regulation of AOS in these tissues (Figure 8). This could explain the preferential accumulation of jasmonates in photosynthetically inactive tissues during seedling development. As suggested by Creelman and Mullet (1997), the down-regulation of genes coding for the photosynthetic apparatus, a well-known effect of JA (Wasternack and Parthier, 1997; Weiler *et al.*, 1998), may attribute to protection of oxidative stress in tissues still lacking radical scavengers like chlorophyll. Interestingly, the JA-induced JIP-23 of barley leaves if heterologous overexpressed in tobacco also attributes to the down-regulation of photosynthetic genes (Görschen *et al.*, 1997). Since JIP-23 is expressed during seedling development in the companion cells of the vascular bundle of the scutellar nodule and leaf base (Hause *et al.*, 1996), JIP-23 may function as a developmentally regulated factor which is able to potentiate the down-regulation caused by jasmonate. It will be interesting to analyze whether the tissue-specific occurrence of AOS and the overall elevation of JA levels shown here accords with a tissue-specific rise of JA and down-regulation of photosynthetic genes.

Experimental procedures

Reagents

cis-(+)-OPDA was purified from flax seeds and checked for purity by GC–MS. C-18 fatty acids and C_{20} fatty acids were obtained from NuChek Prep (Elysian, MN, USA) and Sigma (St Louis, MO, USA), respectively.

Plant material and treatment of leaf segments

Seedlings of barley (*Hordeum vulgare* cv. Salome) were grown under greenhouse conditions with 16 h light (with a minimum intensity of 130 μmol m^{-2} s^{-1}) at 25°C and 70% relative humidity for 7 days if not otherwise indicated. Primary leaves were harvested and cut into 5 cm segments starting 1 cm below the tip. Subsequently, they were floated on water or in an aqueous solution containing either 45 μM (±) JAME, 45 μM JA, 50 μM JA-L-Ile, 50 μM OPDA, 50 μM OPDA ME, 1 M sorbitol, 0.5 M glucose, 1 M 6-deoxyglucose, 1 M mannitol, 90 μM (±-ABA, 1 M NaCl, 50 μM SA or 2 mM aspirin for the times indicated. Floating was performed in Petri dishes containing 25 ml solution per 6 segments at 25°C under continuous white light (120 μmol m^{-2} s^{-1}) provided by fluorescent lamps (Narva, Berlin, NC, USA, 250/01). Desiccation stress (DS) was performed to a loss of 30% original fresh weight followed by keeping the leaf segments in a plastic bag up to the indicated times. Inoculations with powdery mildew conidia were performed as described previously (Hause *et al.*, 1999). Plant material was shock frozen in liquid nitrogen and stored at –80°C until use.

Isolation of AOS cDNAs and its expression in Escherichia coli

A cDNA library prepared from barley leaves treated with 45 μM JAME for 24 h and 48 h was used (Lee *et al.*, 1996). 5×10^5 phages of the cDNA library were screened with the flax AOS cDNA (Song *et al.*, 1993). Pre-hybridzation was performed at 50°C in 6 × SSC, 5 × Denhardt's reagent, 1% SDS, 100 μg ml^{-1} salmon sperm DNA for 1.5 h, followed by hybridization at 50°C for 20 h with 5×10^7 dpm ml^{-1} of labeled flax-AOS cDNA in the pre-hybidization solution. Filters were washed at 50°C in 2 × SSC + 0.1% SDS, 1 × SSC + 1 × 0.1% SDS and 0.5 × 0.1% SDS, for 30 min each. Positive plaques were purified and converted in phagemids by *in vivo* excision.

Sequence analysis of plasmid and phagemid clones was performed with the dideoxynucleotide chain termination method using the Thermo Sequenase Cycle Sequencing Kit (Amersham). Complete sequencing of both strands were achieved by means of primer walking. The cDNA inserts homologous to AOS were PCR amplified and cloned into the *Hin*dIII–*Bam*HI digested histidine-tagged expression vector pQE31 (Quiagen, Hilden, Germany). The pQE31, both with and without the cDNA inserts, were transformed into the host strain *E. coli* SG 13009. Total proteins of isopropyl-β-thiogalactopyranoside (IPTG)-induced or non-induced cultures were isolated by sonification in a buffer containing 100 mM Tris–HCl, pH 8.0, 10% (w/v) glycerol, 300 mM NaCl, and 0.1% (w/v) Tween 20. Subsequently, samples were centrifuged at 25 000 ***g*** for 15 min to remove cell debris. The recombinant proteins were purified by affinity chromatography on Ni-NTA-agarose in cracking buffer. First, AOSs were bound in the presence of 10 mM imidazol, followed by subsequent washings with 20 mM, 60 mM and 100 mM imidazol, respectively, to remove slightly bound protein. Finally, AOSs were eluted with 500 mM imidazol. To raise rabbit polyclonal antibodies, recombinant AOS1 was isolated and purified in a buffer containing 8 M urea. Determination of proteins, their electrophoretic separation and transfer onto nitrocellulose were performed as described previously (Lehmann *et al.*, 1995).

Extractions of RNA and Northern blot analysis

Total RNA of frozen tissues was extracted by phenol: chloroform: isoamyl alcohol 90 : 30 : 6 (v/v) using the modifications of Andresen *et al.* (1992). Electrophoresis of 10 μg total RNA per lane (if not indicated otherwise) and Northern blot analysis was performed according to Maniatis *et al.* (1989). Blots were hybridized at 65°C for 16 h with ^{32}P-labelled fragments of the barley AOS1 cDNA encompassing the full-length cDNA sequence. Gel loading was checked by comparing ethidium bromide-stained rRNA.

Southern blot analysis

For Southern blot analysis, 10 μg of genomic DNA was digested with the restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, *Eco*RV, *Dra*I

and *Xba*I and were separated by agarose gel electrophoresis. After vacuum transfer onto a nylon membrane, hybridization was performed with the ^{32}P-labelled fragment of the full-length AOS1 cDNA according to Maniatis *et al.* (1989).

Analysis of reaction products of AOSs

Two μg affinity-purified recombinant barley AOSs diluted in 0.9 ml 100 mM potassium phosphate buffer, pH 7.0, were incubated with 100 nmol of [1–^{14}C]-13-HPOT (86 000 dpm) for 30 min at room temperature. The reaction was stopped by adding 100 μl of glacial acetic acid and extracted twice with diethyl ether. The combined organic phases were evaporated under a stream of nitrogen and lipids were reconstituted in 100 μl of HPLC solvent (acetonitrile: water: acetic acid, 60 : 40 : 0.1 v/v/v). The HPLC analysis was essentially performed as described previously (Blée and Joyard, 1996) with a Jasco HPLC system (Gross Zimmern, Germany).

Immunoblot analysis

Proteins from leaves, protoplasts, isolated chloroplasts or bacterial extracts were extracted according to Meyer *et al.* (1988), solubilized in SDS-sample buffer, subjected to SDS-PAGE and used in immunoblot analysis. Immunodetection of AOSs was performed by using an affinity-purified antibody raised against the recombinant AOS1 as primary antibody and anti-rabbit-IgG conjugated with alkaline phosphatase (Boehringer, Mannheim) as secondary antibody. Staining of immunodecorated AOSs was undertaken with p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Isolation of intact chloroplasts

Chloroplasts were isolated as described for barley leaf segments by Hause *et al.* (1994). Briefly, barley leaf segments, floated on distilled water for 48 h or on 45 μM JAME for 6 h followed by H_2O treatment for the final 42 h, were treated with 20 mM citrate buffer, pH 5.5; 0.5 M sorbitol, 1% cellulase Y-C and 0.1% pectolyase Y-23 (Seishin, Japan) at 30°C for 3 h. Protoplasts purified in a discontinuous Percoll gradient were lysed in 25 mM Tricin-HCl, pH 8.0 and 0.5 M sorbitol, and chloroplasts were purified by various centrifugation steps in discontinuous Percoll gradients. Treatment of purified chloroplasts with proteinase was performed by incubation with 0.2 mg ml^{-1} proteinase K for 30 min at room temperature following Percoll gradient centrifugation. The chloroplast fractions were characterized using the following criteria: (i) amount, by chlorophyll content; (ii) intactness, inspected by loss of ribulose-1,5-bisphosphate carboxylase/oxygenase using gelelectrophoretic separation of proteins; and (iii) contaminations, by immunodetection of JIP-23, a non-plastidic protein of barley leaves (Hause *et al.*, 1994).

In situ *hybridization and immunocytochemistry*

Leaf segments treated for 48 h with water or 45 μM JAME in the dark and tissue around the scutellar nodule of 6-day-old light grown seedlings (see Figure 8a) were fixed, embedded in PEG and cut as described previously (Hause *et al.*, 1996).

For *in situ* hybridization, 5 μm thick cross-sections were mounted on poly L-lysine-coated slides, rinsed in Tris–HCl, pH 8.0, and incubated in 1% bovine serum albumin (BSA) in the same buffer for 1 h. After acetylation, sections were dehydrated by a graded series of ethanol and air-dried. For hybridization, a solution of 0.3 M NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 × Denhardt's solution, 50% formamide, 2 mg ml^{-1} tRNA, and 200 U ml^{-1} RNase inhibitor containing denaturated DIG-labeled sense or antisense AOS1-RNA was applied and slides were incubated in a humid box at 45°C overnight. After two washing steps with 0.2 × SSC at 55°C for 30 min each, slides were incubated with 20 μg ml^{-1} RNase A at 37°C for 30 min, followed by washing with 0.2 × SSC at 55°C for 1 h. Immunological detection of DIG-labeled RNA-hybrids was performed with anti-DIG-fab fragment conjugated with alkaline phosphatase (Boehringer, Mannheim, Germany) according to the supplier's protocol. For localization of AOS protein within the mesocotyl of barley seedlings, 2 μm thick sections of the same tissues used for *in situ* hybridization were immunolabeled with the purified rabbit-anti-AOS antibody (diluted 1 : 50 in PBS containing 5% (w/v) (BSA) followed by anti-rabbit-IgG antibody conjugated with alkaline phosphatase as described previously (Hause *et al.*, 1996). Staining procedure was performed with NBT and BCIP. Finally, slides were analyzed by bright field microscopy with a Zeiss 'Axioskop' microscope (Zeiss, Germany) equipped with a CCD camera (Sony, Japan).

Cross-sections of leaves (2 μm thickness) were immunolabelled by incubation with purified rabbit anti-AOS antibody (diluted as mentioned above) followed by Tyramide Signal Amplification (NEN) according to the supplier's instructions. Due to the use of a purified antibody it was not suitable to check the specificity via pre-immune serum. The specificity of the purified anti-AOS antibody is indicated by one band detected in total protein extracts of protoplasts (Figure 6a). After immunolabeling, sections were mounted in citifluor/glycerol. Control experiments were performed by omitting the first antibody. The fluorescence of immunolabeled AOSs was visualized with a Zeiss 'Axioskop' epifluorescence microscope using the proper filter combination.

Extraction and quantification of jasmonates and octadecanoids

Barley leaf segments and other barley tissues (1 g f.w.) were frozen in liquid nitrogen, homogenized in a mortar and extracted with 5 ml 80% (v/v) methanol. For quantification of JA and JAME, appropriate amounts of ($^2H_6$)JA were supplemented to the extract. Ion exchange chromatography on DEAE Sephadex A-25 cartridges, RP-HPLC and GC–MS/SIM was performed according to Kramell *et al.* (1999).

Fatty acid hydroperoxides

The n-6 hydroperoxides (13-HPOT, 13-HPOD, 15-HPET and 15-HPEP) were prepared by incubation of the corresponding fatty acids with soybean lipoxygenase (Sigma) (Hamberg and Gotthammar, 1973), whereas the n-10 hydroperoxides (9-HPOD and 9-HPOT) were obtained by incubation of the appropriate precursor acids with tomato fruit homogenates (Matthew *et al.*, 1977).

AOS activity assay

AOS activity was measured at pH 7 (50 mM potassium phosphate) in a total volume of 1 ml. The reaction was initiated by the addition of the fatty acid hydroperoxide and the consumption of the substrates was followed by the decrease in the absorbance at 235 nm, which reflects the disappearance of the conjugated double bond of the hydroperoxides. For the determination of

the kinetic parameters of the recombinant barley AOSs, the initial reaction velocity was determined at 14 concentrations in the range of 5–90 μM for each substrate. The K_M and V_{max} values were calculated directly using the Michaelis Menten equation and by plotting the data according to Eadie–Hofstee. Both methods gave similar results.

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Molecular Cloning of Allene Oxide Cyclase

THE ENZYME ESTABLISHING THE STEREOCHEMISTRY OF OCTADECANOIDS AND JASMONATES*

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Allene oxide cyclase (EC 5.3.99.6) catalyzes the stereospecific cyclization of an unstable allene oxide to (9*S*,13*S*)-12-oxo-(10,15*Z*)-phytodienoic acid, the ultimate precursor of jasmonic acid. This dimeric enzyme has previously been purified, and two almost identical N-terminal peptides were found, suggesting allene oxide cyclase to be a homodimeric protein. Furthermore, the native protein was N-terminally processed. Using degenerate primers, a polymerase chain reaction fragment could be generated from tomato, which was further used to isolate a full-length cDNA clone of 1 kilobase pair coding for a protein of 245 amino acids with a molecular mass of 26 kDa. Whereas expression of the whole coding region failed to detect allene oxide cyclase activity, a 5′-truncated protein showed high activity, suggesting that additional amino acids impair the enzymatic function. Steric analysis of the 12-oxophytodienoic acid formed by the recombinant enzyme revealed exclusive (>99%) formation of the 9*S*,13*S* enantiomer. Exclusive formation of this enantiomer was also found in wounded tomato leaves. Southern analysis and genetic mapping revealed the existence of a single gene for allene oxide cyclase located on chromosome 2 of tomato. Inspection of the N terminus revealed the presence of a chloroplastic transit peptide, and the location of allene oxide cyclase protein in that compartment could be shown by immunohistochemical methods. Concomitant with the jasmonate levels, the accumulation of allene oxide cyclase mRNA was transiently induced after wounding of tomato leaves.

Jasmonic acid (JA)[1] and its methyl ester, collectively named jasmonates, consist of a cyclopentanoic ring where an acetic acid and a pentenyl side chain are attached (Fig. 1). These side chains are either oriented in the *cis* (3*R*/7*S*) or the *trans* form (3*R*/7*R*), and a number of structurally related compounds have been described and found to occur ubiquitously in plants (1). The first physiological role of JA found in 1971 was inhibition of plant growth (2). Since then, jasmonates were identified as signals of altered gene expression in various plant responses to biotic and abiotic stresses as well as of distinct stages of plant development (3, 4). In tomato leaves, JA was recognized as an essential intermediate in the wound-induced signaling cascade following herbivore attack (5), and for numerous cell suspension cultures JA was described as a signal of elicitor-induced phytoalexin synthesis (6). Beside expression of defense genes such as proteinase inhibitors (5), defensins (7), or thionines (8), the syntheses of phytoalexins (9), alkaloids (10), and volatiles such as terpenoids (11) are the most intriguing JA responses, caused in most cases by up-regulation of specific enzymes (9, 12). JA responses were identified by means of altered expression of specific genes, by JA-insensitive and JA-deficient mutants, by JA-deficient transgenes, or by corresponding endogenous rises of jasmonates including inhibitor studies. More recently, the JA precursor (9*S*,13*S*)-12-oxo-(10,15*Z*)-phytodienoic acid (OPDA) was suggested to be the preferential signal of JA-mediated responses such as tendril coiling (13) or terpenoid biosynthesis (11). Among developmental processes, pollen maturation (14) and seedling growth (15) are JA-dependent.

The biosynthesis of JA proceeds via an oxylipin pathway (Fig. 1), starting with the lipoxygenase-catalyzed insertion of molecular oxygen into position 13 of linolenic acid followed by the dehydration of the resulting fatty acid hydroperoxide ((13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid) by allene oxide synthase (AOS; EC 4.2.1.92) to an allene oxide (1). This allene oxide is then cyclized by allene oxide cyclase (AOC; EC 5.3.99.6) to OPDA. After reduction of the ring double bond by a reductase and three rounds of β-oxidation, (+)-7-*iso*-JA, *i.e.* (3*R*,7*S*)-JA, is formed. Vick and Zimmerman (16) already proposed a similar biosynthetic scheme in 1983, but the formation of OPDA from (13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid was believed to be the result of a single enzyme called hydroperoxide cyclase. In 1988, Hamberg (17) showed that this step was performed by two enzyme activities. One of them, a membrane-bound activity purified later as AOS (18), catalyzed the formation of an unstable allene oxide, which rapidly decays by chemical hydrolysis with a half-life of 25 s to α- and γ-ketol and OPDA (Fig. 1). OPDA only amounts to 10–15% of the total degradation products and is racemic, con-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ 272026.

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[1] The abbreviations used are: JA, jasmonic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; α-ketol, 12-oxo-13-hydroxy-(9*Z*,15*Z*)-octadecadienoic acid; γ-ketol, 12-oxo-9-hydroxy-(10*E*,15*Z*)-octadecadienoic acid; OPDA, 12-oxo-(10,15*Z*)-phytodienoic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

sisting of the *cis* isomers 9*S*,13*S* and 9*R*,13*R*. However, in the presence of a second, soluble enzyme activity (AOC) that was purified recently (19), the amount of α- and γ-ketols decreased, and the 9*S*,13*S* enantiomer of OPDA was formed exclusively. This specificity of AOC determines the stereochemistry of the side chains in the naturally occurring jasmonate structure.

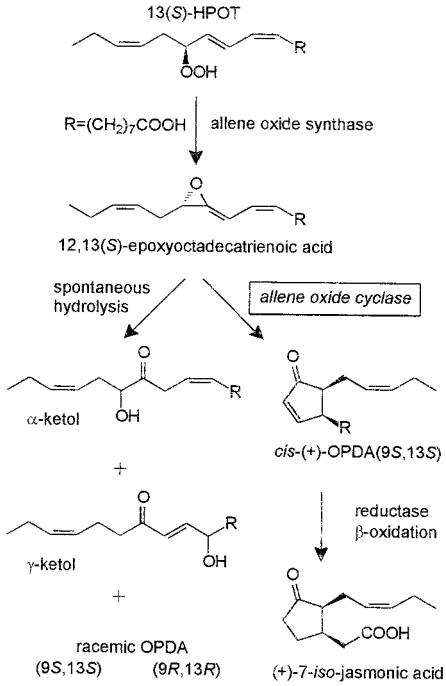

FIG. 1. **Scheme of jasmonate biosynthesis.** The reaction sequence after the lipoxygenase-catalyzed formation of the linolenic acid hydroperoxide is shown. This scheme includes the enzymatic conversion of the allene oxide (12,13*S*)-epoxyoctadecatrienoic acid to enantiomeric OPDA and later to jasmonic acid as well as the chemical decomposition to α- and γ-ketol and racemic OPDA, which has, up to now, only been shown to occur *in vitro*. *13(S)-HPOT*, (13*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid.

In addition, the lipoxygenase-derived products can be converted by a divinyl ether synthase, a reductase, a peroxygenase, and a hydroperoxide lyase (20). Due to these facts and the unspecific ketol formation following the AOS step, the AOC can be regarded as the first enzyme specific for JA synthesis. Interestingly, correct isomeric structure of OPDA formed by AOC is kept only by one of two reductases isolated so far (21).

Several forms of lipoxygenases, AOSs, and OPDA reductases have been cloned from different plant species and have been characterized biochemically, leading to hints of their physiological significance (21–25). So far, for AOC only biochemical data of the purified enzyme including substrate specificity are available (19, 26). In order to analyze the physiological importance of that step of JA biosynthesis, we cloned AOC. Here, we describe a full-length cDNA isolated from tomato leaves coding for a protein of 26 kDa that was localized in chloroplasts and whose 5′-truncated version exhibited AOC activity.

EXPERIMENTAL PROCEDURES

Plant Material—- Corn (*Zea mays* L. cv. Boss), barley (*Hordeum vulgare* L. cv. Salome) and tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) were grown in soil under greenhouse conditions with 16 h light (with a minimum intensity of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 °C. Primary barley leaves were harvested 7 days after germination, cut into 5-cm segments starting 1 cm below the leaf tip, and floated in Petri dishes on a 1 M sorbitol solution. Developing corn seeds were harvested 2 weeks after anthesis. Tomato plants were grown for 8 weeks, and the second leaf was excised and wounded using a fabric pattern wheel and subsequently floated on distilled water under continuous white light (120 $\mu mol \cdot m^{-2} \cdot s^{1}$) for the indicated times.

Analysis of Endogenous JA and OPDA—Quantitative determination of levels of nonesterified OPDA and linolenic acid and steric analysis of OPDA were performed in unwounded leaves of tomato (15–20 g) or leaves (3–5 g) that had been wounded *in situ* for 0.5 h (OPDA) and 3 h (linolenic acid), respectively, by use of a hemostat. The tissue was shock-frozen in liquid nitrogen, and the powder was extracted with ethanol. [$^2H_5$]OPDA and [$^2H_5$]linolenic acid were added to a small part of the extracts, and the levels of OPDA and linolenic acid were determined by mass spectrometry. The remaining parts of the extracts were subjected to solid phase extraction and reversed-phase high pressure liquid chromatography, and the isolated OPDA was subjected to steric analysis as described by Ziegler *et al.* (26). JA levels were determined as described by Kramell *et al.* (27).

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TTTAAGTCTTCTTTTTTGACCGCTAGAACCAACAAATTGTTCAGCAATGGCCACTGTTTCCTCAGCCTCTGCTGCTCTTAGAACCATTTCTTCTTCCTCATCC   103
                                                M  A  T  V  S  S  A  S  A  A  L  R  T  I  S  S  S  S  S    19

AAGCTATCTTCTGCCTTCCAAACTAAAAAGATCCAATCTTTTAAACTACCTAACCCTCTCATTTCTCAGAATCATAAACTTACTACCACCTCTACTACTGCTTCC   208
 K  L  S  S  A  F  Q  T  K  K  I  Q  S  F  K  L  P  N  P  L  I  S  Q  N  H  K  L  T  T  T  S  T  T  A  S    54

AGATCATTTTCCTGCAAGAGCCAGAGCACCTCAACAGATTCAACTAACACTGAAGTTCAAGAACTTAGTGTCTATGAGATCAATGAACGTGATCGTGGAAGCCCT   313
 R  S  F  S  C  K  S  Q  S  T  S  T  D  S  T  N  T  E  V  Q  E  L  S  V  Y  E  I  N  E  R  D  R  G  S  P    89

GCTTATCTTCGATTGAGCCAAAAGACTGTTAATTCACTCGGAGATCTTGTCCCCTTTAGCAACAAGCTATATACTGCAGATCTAAAGAAGAGAATTGGAATAACA   418
 A  Y  L  R  L  S  Q  K  T  V  N  S  L  G  D  L  V  P  F  S  N  K  L  Y  T  A  D  L  K  K  R  I  G  I  T   124

GCAGGACTCTGCATTCTGATCAAGCACGAAGAAGAGAAGAAAGGAGATCGCTATGAAGCTGTTTACAGCTTCTACTTCGGCGATTACGGTCATATCGCCGTTCAG   523
 A  G  L  C  I  L  I  K  H  E  E  E  K  K  G  D  R  Y  E  A  V  Y  S  F  Y  F  G  D  Y  G  H  I  A  V  Q   159

GGAGCGTACTTAACCTATGAAGAAACTTACCTAGCCGTCACCGGTGGATCCGGCATATTTGCAGGGGTTTCCGGTCAAGTGAAATTGCAGCAACTCATTTTCCCT   628
 G  A  Y  L  T  Y  E  E  T  Y  L  A  V  T  G  G  S  G  I  F  A  G  V  S  G  Q  V  K  L  Q  Q  L  I  F  P   194

TTCAAGCTATTTTACACTTTTTACTTGAAGGGGATCCCCGGTCTGCCATCTGAATTGTTGTGTACGGCGGTTCCTCCGTCGCCGACGGTGGAGCCAACACCTGAA   733
 F  K  L  F  Y  T  F  Y  L  K  G  I  P  G  L  P  S  E  L  L  C  T  A  V  P  P  S  P  T  V  E  P  T  P  E   229

GCTAAAGCTTGTGAGGAAGGGGCCGCACTGAAAAATTACACTAATTAAGTGGGGGTGTTGATGTGCAAATCACAACTTTTGTTGGGGTCGTTCTGAAATAATCAA   838
 A  K  A  C  E  E  G  A  A  L  K  N  Y  T  N  *                                                           244

TAACTACGTGCTCTAGAATTAAAAAAAAAAGTAACATCAACGTCCTTTTATTTTGCAATTTTTGGTATGTTATTTGTCGTTTTGTTGGGTAAAAGTTTAACTGTT   943
TTGTTAGTACTTTGTATGTATGCCTATTATCTGAATTGTATCCCTATGTGATACGTTCCATCTTTATTATAAAGTCCAGAAAAAGATAAAAATGAAAAAATTATT   1048
TGGTAATGAATTCAAGGAAAAAAAAAAAAAAAAA                                                                        1083
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FIG. 2. **Nucleotide sequence of tomato allene oxide cyclase cDNA and deduced amino acid sequence.** Sequence stretches obtained by amino acid sequencing of the purified corn protein are *underlined*. *Solid arrows*, sequences used for reverse transcriptase-PCR; *broken arrows*, sequences used to amplify a fragment encoding a truncated version of allene oxide cyclase used for overexpression. *Double underlines*, putative cleavage sites for chloroplastic signal peptide as predicted by the ChloroP program, version 1.1 (available on the World Wide Web).

Determination of Peptide Sequences and PCR Cloning—AOC was purified from corn seeds as described (19). The purified protein was subjected to SDS-PAGE and electroblotted on a polyvinylidene difluoride membrane. The protein band was excised and subjected to N-terminal sequencing as well as to internal sequencing after Lys C digestion by automated Edman degradation.

For PCR cloning total RNA was extracted from developing corn seeds, barley leaves, and tomato leaves according to standard protocols (28). The RNA of all three tissues was used in a reverse transcriptase-PCR kit (Titan TM, Roche Molecular Biochemicals) with the following primers: upstream, 5′-CAA GAA CTT TAC GT(A/C/G/T) TA(T/C) GA(A/G)-3′; downstream, 5′-dT_{23}(C/G)(A/C/G/T)-3′. The following temperature program was used: 30 min at 42 °C for the reverse transcription reaction followed by 30 cycles of 94 °C for 1 min, 48 °C for 1.5 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were blunt end-ligated into pBSK Bluescript (Stratagene) and sequenced. The clone pTomAOC4 was used as a probe to screen 5×10^5 plaque-forming units of a cDNA library made of 5 μg of mRNA from a tomato cell culture using the λ-ZAP cDNA library kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. Prehybridization was performed at 65 °C in 6× SSC, 5× Denhardt's reagent, 1% SDS, 100 μg/ml salmon sperm DNA for 3 h, followed by hybridization at 65 °C overnight with the ^{32}P-labeled insert from clone pTomAOC4 in the prehybridization solution. Filters were washed three times at 65 °C in 2× SSC plus 0,1% SDS for 15 min each. Positive plaques were purified and converted into phagemids by *in vivo* excision. Sequence analysis of plasmid and phagemid clones was performed with the dideoxynucleotide chain termination method using the Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Overexpression of AOC—Either the whole coding region or a 5′-deletion starting at nucleotide 235 as seen in Fig. 2 was amplified by PCR with a 5′ *Nde*I and a 3′ *Eco*RI restriction site, respectively, and cloned into the cloning sites of the expression vector pJC20 and pJC40 (29). The resulting plasmids were introduced into the host strain BL21DE3(pLysS). The bacteria were grown in LB medium up to an A_{600} of 0.5 absorbance units and induced by 1 mM isopropyl-β-thiogalactopyranoside for 4 h. The bacteria were collected by centrifugation, subjected to two freeze thaw cycles, and lysed by sonication in 20 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.1% Tween 20, and 10% glycerol. The extracts were centrifuged, and the supernatant was used for the determination of AOC activity. The enzyme activity was either determined using the radioactivity assay as described by Ziegler *et al.* (19) or by estimating the enantiomeric composition of OPDA according to Ref. 26. The recombinant protein expressed in pJC40 was purified by affinity chromatography on Ni^{2+}-nitrilotriacetic acid-agarose (Quiagen), and the purity was checked by SDS-PAGE. The purified recombinant AOC was used to raise rabbit polyclonal antibodies.

Southern Analysis and Genetic Mapping—5–10 μg of genomic DNA were digested with restriction enzymes and separated by agarose gel electrophoresis. Southern blotting onto nylon membranes was performed as described by the manufacturer. For genetic mapping, 47 F2 plants were used from the mapping population described (30). Onto this interspecific population (*L. esculentum* × *Lycopersicon pennellii*) more than 1000 markers have been mapped previously. Since DNA from the same plants was used, the mapping data could be integrated into this map. The hybridization of the AOC gene and the integration into the mapping framework were done according to standard procedures (30).

Immunocytochemistry—Small pieces of young leaves were fixed, embedded in polyethylene glycol, and cut as described (31). Cross-sections (2-μm thickness) were immunolabeled by incubation with rabbit anti-AOC antibodies (diluted 1:2000) followed by a goat anti-rabbit-IgG antibody conjugated with BODIPY (Molecular Probes, Inc., Eugene, OR). After immunolabeling, sections were mounted in citifluor/glycerol. Control experiments were performed by using preimmune serum. The fluorescence of immunolabeled AOC was visualized with a Zeiss "Axioskop" epifluorescence microscope using the proper filter combination.

RESULTS AND DISCUSSION

Protein Sequencing and Cloning of AOC—From the sequencing of the purified corn AOC protein (19), one internal sequence of 6 amino acids (SPAYLR) and two N-terminal peptides (AKDARPTKVQELYVYEIN and KAKDARPTKVQELYVYEI) were obtained. The latter were almost identical and only differed by one additional Lys residue at the N-terminal end. Previously, a dimeric nature of AOC was suggested by the molecular size of about 48 kDa as estimated by gel filtration compared with the migration of the AOC protein as a 22-kDa band on SDS-PAGE (19). The finding of two almost identical N-terminal peptides supports the suggestion that AOC is a homodimeric protein. Additionally, both N-terminal sequences lack the start methionine residue. This could be caused by post-translational processing, which might be necessary for dimer formation or for the transport across intracellular membranes.

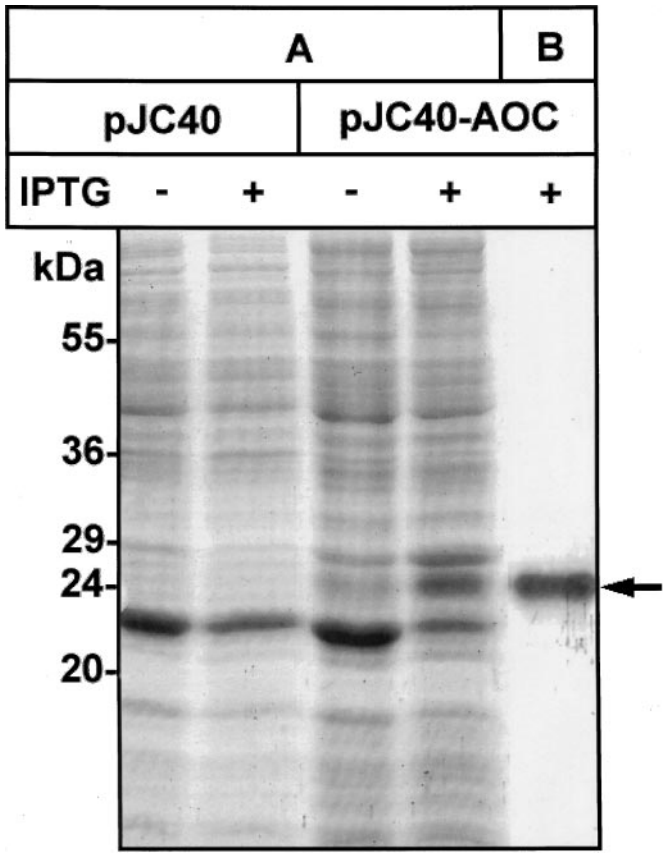

FIG. 3. **Bacterial overexpression of tomato allene oxide cyclase.** For overexpression, an N-terminal truncated protein starting at amino acid residue 64 as shown in Fig. 2 was chosen. The corresponding partial cDNA was subcloned into the His tag vector pJC40 and transfected into *Escherichia coli* strain BL21DE3(pLysS). Extracts from bacteria carrying the vector alone (pJC40) or with the partial AOC-cDNA (pJC40-AOC) grown in the absence (−) or presence (+) of isopropyl-β-D-thiogalactoside (*IPTG*) were separated by SDS-PAGE and stained with Coomassie Blue (*A*) or probed with an anti-AOC antibody (*B*). The AOC protein is indicated by an *arrow*.

The obtained peptide sequences served as templates for the generation of oligonucleotide primers to perform a reverse transcriptase-PCR-based cloning approach for AOC. As upstream primers, degenerate oligonucleotides directed against different regions of the N-terminal sequence were used, whereas for the downstream primer, an oligo(dT) anchor was chosen. Since developing corn seeds showed a high AOC activity, we used the RNA from that tissue as a template. Irrespective of the primer combinations and PCR conditions, no specific PCR fragment could be amplified, suggesting that either the peptide information is not specific enough to amplify the desired product or that the mRNA is of very low abundance or even absent in this tissue. In order to exclude the second possibility as far as possible, we focused on tissues, where an accumulation of endogenous JA levels can be induced, presumably preceded by an increase in AOC expression. Therefore,

sorbitol-stressed barley leaf segments (32) and wounded tomato leaves (33) were used as a source for RNA. No specific PCR products were obtained with the barley RNA, but reverse transcriptase-PCR with RNA from wounded tomato leaves resulted in a weak band of about 850 bp. Sequencing of this PCR fragment revealed that it also encodes the internal sequence of six amino acids obtained from the purified corn protein. This PCR product was used as a probe to screen a tomato cDNA library, resulting in the isolation of a 1-kilobase pair clone. This size approximately corresponds to the size of the signal detected on the Northern blots, suggesting that a full-length cDNA clone was obtained. The first start codon in frame with the peptide sequences from the purified corn AOC is located at position 47 and is preceded by a stop codon at position 16. The protein coding region encompasses 732 bp encoding a protein of 244 amino acids with a calculated molecular mass of 26 kDa (Fig. 2). The difference of about 4 kDa between the deduced molecular mass of the tomato protein and that from the subunit of the purified corn enzyme as determined by SDS-PAGE could, in part, be due to the post-translational removal of amino acids at the N terminus.

Overexpression of AOC—In order to identify the protein encoded by the cDNA clone as an AOC, we performed overexpression to measure AOC activity. At first, the whole coding region was cloned into the expression vector pJC20 and, for purification, into the His tag vector pJC40. After induction by isopropyl-β-thiogalactopyranoside, only a low expression of the recombinant protein was observed on SDS-PAGE, but after Ni^{2+}-nitrilotriacetic acid-agarose chromatography of the His-tagged protein, one band of the expected size of 26 kDa could be detected. However, neither the crude bacterial extracts nor the purified protein showed AOC activity. Considering that the purified protein from corn was N-terminally processed, the lack of enzymatic activity in the full-length tomato protein could be due to the failure of the right post-translational modification in the bacteria. To explore this possibility, we amplified a fragment from the tomato sequence, which produces a truncated protein starting at amino acid residue 64. This corresponds in length to the processed N terminus of the purified corn protein. In that case, the start codon was introduced by a *Nde*I restriction site. After induction of the bacteria, SDS-PAGE revealed a prominent band of 22 kDa that was absent in control bacteria transformed only with the empty vector (Fig. 3). The same band was also observed in noninduced bacteria, which might be due to an insufficient repression of the bacterial expression system in the absence of inducer. The bacterial extracts were then examined for AOC activity. As shown in Table I, the control bacteria showed no activity at all, whereas in induced, transformed bacteria a high specific activity of about 15,000 nmol of OPDA/mg of protein was detected. As expected from the protein pattern on SDS-PAGE, the noninduced bacteria also showed AOC activity, but in this case a 10-fold lower specific activity was obtained. The AOC activity of the recombinant protein was sensitive to proteinase K digestion and was inhibited by 12,13-epoxyoctadecenoic acid, a specific AOC inhibitor (19, 26), which further supports the specificity of the recombinant protein. One special feature of the AOC reaction is the competition between the chemical decomposition of the unstable allene oxide substrate, giving rise to racemic OPDA and the enzymatic conversion to enantiomeric OPDA. Therefore, the ultimate identification for a protein as AOC can only be achieved by the proof that the enzyme specifically forms (9*S*,13*S*)-OPDA. The steric analysis of the reaction products showed that the recombinant protein almost exclusively forms the (9*S*,13*S*)-OPDA enantiomer. Proteinase K digestion and the addition of 12,13-epoxyoctadecenoic acid decreased the amount of that OPDA enantiomer to levels seen after chemical decomposition. Altogether, the results on the AOC activity showed that the isolated clone from tomato codes for AOC. Interestingly, the specific activity of the N-terminal, His-tagged protein expressed in the pJC40 vector was about 10-fold lower than that of the untagged protein. Together with the observation that

TABLE I

Allene oxide cyclase activity and enantiomeric composition of 12-oxophytodienoic acid produced by the recombinant truncated allene oxide cyclase

The cDNA coding for an N-terminal truncated AOC as described in the legend to Fig. 2 was cloned into the expression vector pJC20 and in the His-tagged vector pJC40. The expression of the recombinant proteins in *E. coli* was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG), and the crude bacterial extracts were examined for AOC activity. The calculation of the specific enzyme activity was based on the α-ketol to OPDA ratio as described in Ref. 19. For the determination of the enantiomeric composition as a mean to prove the stereoselectivity of the enzyme, the reaction products were isolated and subjected to steric analysis according to Ref. 26. As controls, the enzyme extracts were incubated for 6 h with 400 μg of proteinase K, or the enzyme assay was performed in the presence of a 100 μM concentration of the specific AOC inhibitor 12,13-epoxyoctadecenoic acid. ND, not determined.

	Specific AOC activity		Percentage (S/S) of total OPDA
	nmol of OPDA × mg^{-1} protein[a]	Percentage	
	nmol/mg	%	%
pJC20/pJC40	Only chemical	0	49
pJC20 + insert (no IPTG)	1202	7	55
pJC20 + insert + IPTG	15,741	100	96
with proteinase K	944	6	51
with inhibitor (100 μM)	1259	8	56
pJC40 + insert + IPTG	1302	8	ND

[a] Due to the assay design, AOC activity could only be calculated by end point measurements of OPDA.

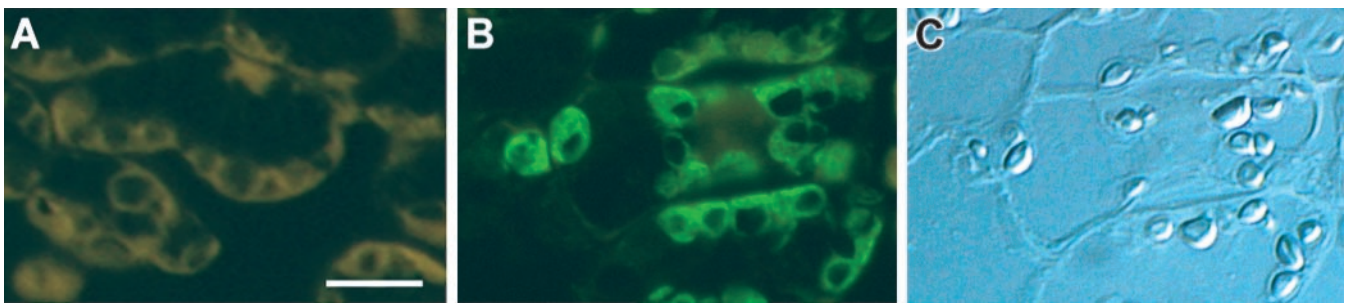

FIG. 4. **Immunocytochemical localization of allene oxide cyclase protein in tomato leaves.** Cross-section of leaves floated on 50 μM jasmonic acid methyl ester for 24 h were probed with preimmune serum (*A*) or with anti-AOC-antibody raised against purified recombinant AOC of tomato (*B*), followed by a labeled secondary antibody. In contrast to the yellow-brown label upon treatment with preimmune serum, a strong green fluorescence label within chloroplasts in B is indicative of the AOC protein. Starch granules within chloroplasts seen in *B* as nonfluorescent areas were visualized by differential interference contrast image (*C*). *Bar*, 10 μm.

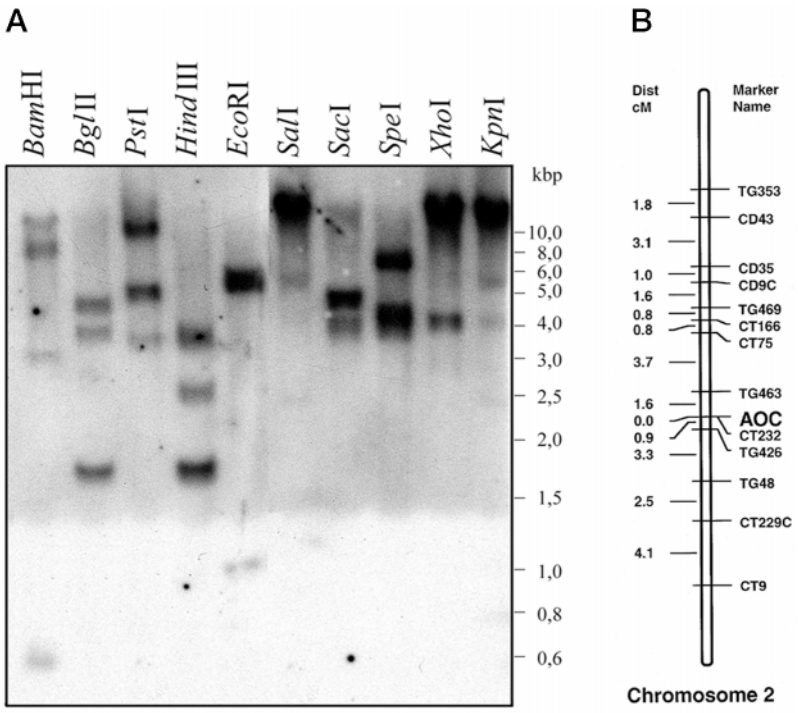

FIG. 5. **Southern blot analysis of genomic tomato DNA (*A*) and genetic mapping of allene oxide cyclase (*B*).** Restriction with the various enzymes revealed a single AOC gene substantiated by one mapping position on chromosome 2. For clarity, the map position of AOC is only indicated relative to chromosomal framework markers in vicinity of the AOC mapping position on tomato chromosome 2. The map position of AOC relative to all other mapped restriction fragment length polymorphism markers of chromosome 2 can be deduced from the data in Ref. 30. *kbp*, kilobase pairs.

only the truncated protein was active, it seems possible that additional amino acids at the N terminus might somehow impair dimer formation.

Intracellular Localization of AOC—Most enzymes of the oxylipin pathway have been reported to reside in the chloroplast. N-terminal amino acid sequence analysis of cloned lipoxygenase from barley (34), *Arabidopsis* (35), and tomato (36) as well as partially performed import studies (35, 36) indicated the occurrence and function of putative chloroplast signal peptides. Also, the AOS from flax (23) and *Arabidopsis* (37) carry a putative chloroplast signal peptide, and the barley AOS co-purified with chloroplasts (25). Biochemical data revealed the presence of the enzyme activities of lipoxygenase, hydroperoxide lyase, and AOS in the outer envelope membrane (38). In case of AOS and lipoxygenase, their chloroplastic location was also shown immunocytochemically (12, 25). Inspection of the N-terminal region of the tomato AOC also revealed structural features for a chloroplastic signal peptide. It is highly enriched in Ser residues (26% in the first 50 amino acids); the start Met is followed by an Ala residue, and the first 10 amino acids are devoid of any charged residue (39). Computer analysis of the first 100 amino acids using the ChloroP version 1.1 program (available on the World Wide Web) predicts a chloroplastic localization with a putative cleavage site between position 93 and 94. However, this cleavage site is highly unlikely, since the purified mature protein from corn starts at amino acid residue 64 in the tomato sequence. Other predicted possible cleavage sites would be at residues 41, 52, and 60. To establish the localization of AOC experimentally, we performed an immunocytological approach using an antibody directed against the recombinant AOC. Cross-sections of tomato leaf tissues probed with that antibody showed a significant fluorescence label in the chloroplasts (Fig. 4). The autofluorescence of chloroplasts is shown by cross-section of tissues that were treated with preimmune serum. This confirms the data from the sequence analysis indicating that AOC is a chloroplastic protein. In contrast to AOS, which was co-purified with outer envelope membranes (38), AOC is a soluble protein (17, 19). Since its substrate is highly unstable, it seems reasonable to expect AOC to be in close proximity to AOS in order to convert the substrate efficiently to (9*S*,13*S*)-OPDA. In order to study this point further, the levels and stereoconfiguration of endogenous OPDA were determined in tomato leaves. In a typical experiment using unwounded leaves, the levels of OPDA and nonesterified linolenic acid were 2 and 206 ng/g, fresh weight, respectively. Levels of OPDA and linolenic acid increased to 187 ng/g (90-fold) and 1813 ng/g (9-fold), respectively, within 30 and 180 min, respectively, upon mechanical wounding, which was in the range observed previously (40). Steric analysis of the wound-induced OPDA showed it to be due exclusively (>99%) to the (9*S*,13*S*)-stereoisomer. Additionally, no ketols or racemic OPDA have been detected in plants up to now (41), suggesting that chemical degradation of the allene oxide is improbable *in vivo*. One may suggest, therefore, that AOS and AOC are either localized close to each other or even loosely associated. This putative interaction of AOS and AOC is now under study using the corresponding clones in a yeast two-hybrid system.

Genomic Analysis and Mapping of the AOC Gene—Southern blot analysis with genomic DNA from tomato revealed a hybridization pattern that is in agreement with a single gene for AOC in the tomato genome (Fig. 5*A*). Clear polymorphism was detected with several restriction enzymes in the parents of the standard tomato mapping population (30). All segregating fragments could be mapped to a single locus on chromosome 2 of tomato (Fig. 5*B*).

Physiology of AOC Expression—The accumulation of JA has been shown to be an important part of a signal transduction cascade in response to wounding (33, 40, 42, 43). In tomato, a chloroplastic lipoxygenase is up-regulated upon wounding (36), and *Arabidopsis* plants co-suppressed with a specific chloroplastic lipoxygenase failed to respond with increased JA levels upon wounding (35). Moreover, the strict spatial and temporal regulation of the second enzyme of the pathway, AOS, during the wound response in *Arabidopsis* underscores the importance of the activation of JA biosynthetic enzymes in the accumulation of JA (24, 44). Because AOC is the enzyme that catalyzes

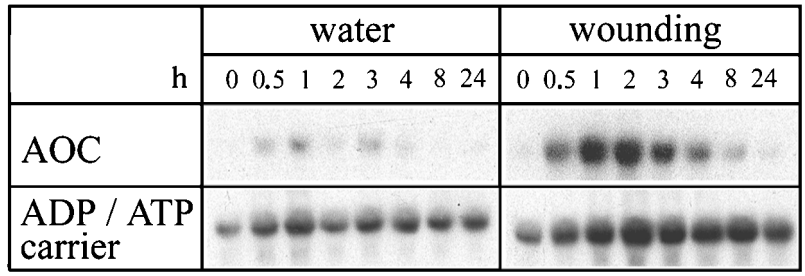

FIG. 6. **Wound-induced accumulation of allene oxide cyclase mRNA in tomato leaves.** Water-treated or wounded leaves kept for different times were subjected to Northern blot analysis. About 10 μg of total RNA was loaded per lane, and loading was checked by hybridization with a tomato cDNA probe coding for ADP/ATP carrier.

the first committed step in the sequence leading to JA, establishing the stereochemistry of the naturally occurring enantiomer, we were interested in knowing whether the expression of AOC coincides in time with the increase in JA levels after wounding. As seen in Fig. 6, AOC mRNA levels start to increase 30 min after wounding of tomato leaves. The maximum induction was observed after 2 h, and at 8 h the control level was reached again. This correlated well with the JA levels measured after wounding, which also showed a transient accumulation with a peak at 1 h (40, 43, 45). Moreover, this corresponds to the exclusive formation of the (9*S*,13*S*)-stereoisomer 30 min after wounding mentioned above. This result suggests an important physiological function also for AOC in the regulation of JA levels during the wound response in tomato.

Another, highly attractive function for AOC might be in the development of floral organs. A data base search with the cloned tomato AOC revealed its identity with the tomato clone TPP15 (46). This yet unidentified clone was isolated by differential hybridization of tomato pistil cDNA libraries and was found to be highly expressed in pistils, mature petals, red fruits, and, to a lower level, in stamens. In young, developing flower buds, no expression was detected. *Arabidopsis* mutants shown to be defective in JA signaling, such as the *coi1* mutant, or JA-deficient, such as the *fad3–2 fad7–2 fad8* mutant, are both male sterile (14, 47), indicating the importance of JA in flower development. It was also shown that the AOS gene is highly transcribed in floral organs of *Arabidopsis thaliana*, suggesting that JA might be produced in flowers (44). This corresponds to elevated levels of JA repeatedly found in flowers (48). It will be interesting to analyze the expression of AOC in floral organs as well as during development and its correlation to the corresponding levels of jasmonates and octadecanoids.

Conclusion—Through the pioneering work of Vick and Zimmermann (16) and Hamberg (17), the enzymes for JA biosynthesis were elucidated. In the last decade, the characterization and cloning of these enzymes have been greatly advanced, and clones of lipoxygenase (22), AOS (23, 25, 37), and OPDA reductases (49, 50) are already available. With the described isolation of a cDNA clone coding for AOC, all enzymes leading to the first physiologically active cyclopentenone, OPDA, are now cloned. Additionally, this enzyme may be of major importance, since it determines the stereochemistry of the cyclopentanones and has a pivotal role in directing one of the oxylipin metabolic pathways to the biosynthesis of the jasmonates. Using the AOC clone and those for the other enzymes, extensive physiological studies and biotechnological applications are now possible to reveal the participation of each of the biosynthetic enzymes in the stress-induced or developmentally regulated levels of JA.

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Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles

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Summary

A crucial step in the biosynthesis of jasmonic acid (JA) is the formation of its correct stereoisomeric precursor, *cis*(+)12-oxophytodienoic acid (OPDA). This step is catalysed by allene oxide cyclase (AOC), which has been recently cloned from tomato. In stems, young leaves and young flowers, AOC mRNA accumulates to a low level, contrasting with a high accumulation in flower buds, flower stalks and roots. The high levels of AOC mRNA and AOC protein in distinct flower organs correlate with high AOC activity, and with elevated levels of JA, OPDA and JA isoleucine conjugate. These compounds accumulate in flowers to levels of about 20 nmol g^{-1} fresh weight, which is two orders of magnitude higher than in leaves. In pistils, the level of OPDA is much higher than that of JA, whereas in flower stalks, the level of JA exceeds that of OPDA. In other flower tissues, the ratios among JA, OPDA and JA isoleucine conjugate differ remarkably, suggesting a tissue-specific oxylipin signature. Immunocytochemical analysis revealed the specific occurrence of the AOC protein in ovules, the transmission tissue of the style and in vascular bundles of receptacles, flower stalks, stems, petioles and roots. Based on the tissue-specific AOC expression and formation of JA, OPDA and JA amino acid conjugates, a possible role for these compounds in flower development is discussed in terms of their effect on sink–source relationships and plant defence reactions. Furthermore, the AOC expression in vascular bundles might play a role in the systemin-mediated wound response of tomato.

Keywords: tomato (*Lycopersicon esculentum*), allene oxide cyclase, jasmonates, oxylipin signature, vascular bundles, ovules.

Introduction

Jasmonic acid (JA) and its methyl ester (JAME), collectively named jasmonates, are ubiquitously occurring plant growth regulators. They act as signals in various plant responses to biotic and abiotic stresses, as well as in distinct stages of plant development (reviewed in Creelman and Mullet, 1997; Wasternack and Parthier, 1997). An endogenous rise of jasmonates occurs upon wounding (Conconi *et al.*, 1996a; O'Donnell *et al.*, 1996; Peña-Cortés *et al.*, 1995), elicitation of cell suspension cultures (Gundlach *et al.*, 1992), osmotic stress (Kramell *et al.*, 2000), pathogen attack (Penninckx *et al.*, 1996), burning and electric current application (Herde *et al.*, 1996), chitosan and oligogalacturonide treatment (Bowles, 1990; Doares *et al.*, 1995) or UV stress (Conconi *et al.*, 1996b). Recent GC–MS analysis revealed that the rise in JA is preceded by a more pronounced rise in octadecanoids such as 12-oxophytodienoic acid (OPDA), as shown for elicitation of cell suspension cultures (Parchmann *et al.*, 1997), osmotic stress (Kramell *et al.*, 2000), wounding (Ziegler *et al.*, 2000) or touch of tendrils (Stelmach *et al.*, 1998). The rise in the levels of jasmonates and OPDA is followed by expression of specific genes. These up-regulated genes include those coding for proteinase inhibitors (reviewed by Ryan, 1992), thionins (Bohlmann *et al.*, 1998) and defensins (Thomma *et al.*, 1998), as well as for enzymes involved in the biosynthesis

of phytoalexins (Blechert *et al.*, 1995), alkaloids (Baldwin *et al.*, 1997) and monoterpenes (Koch *et al.*, 1999). Enzymes involved in the biosynthesis of JA are also induced by exogenous application of JA (Feussner *et al.*, 1995; Heitz *et al.*, 1997; Laudert and Weiler, 1998; Maucher *et al.*, 2000). Another interesting aspect of JA action is the down-regulation of house-keeping proteins such as Rubisco (Reinbothe *et al.*, 1993; Roloff *et al.*, 1994), which may be part of its senescence-promoting effect.

Apart from jasmonates, OPDA has also been shown to possess signalling properties, e.g. in volatile formation (Koch *et al.*, 1999), in tendril coiling (Blechert *et al.*, 1999; Stelmach *et al.*, 1998), or in the expression of specific genes (Kramell *et al.*, 2000). The profiles of various oxylipins including octadecanoids were found to be different in various plants. This 'oxylipin signature' was suggested to mediate plant-specific JA/OPDA-related responses (Farmer *et al.*, 1998; Weber *et al.*, 1997).

There is currently great interest in cloning all genes coding for enzymes of JA biosynthesis. The biosynthesis of JA elucidated by Vick and Zimmerman (1983) originates from α-linolenic acid (α-LeA), thus representing one branch within the oxylipin pathway (reviewed in Blée, 1998; Hamberg and Gardner, 1992). The α-LeA might be released from membrane-located phospholipids by phospholipase A_2 activity as shown recently (Narváez-Vásquez *et al.*, 1999). In the subsequent step, a 13-lipoxygenase (LOX) catalyses the regio-selective and stereo-specific dioxygenation of α-LeA to (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-(9,11,15)-octadecatrienoic acid (13-HPOT) which may be metabolized into leaf aldehydes, divinyl ether, or products of the peroxygenase as well as of the reductase branch of the LOX pathway. However, 13-HPOT is also the substrate for the JA-specific branch of the oxylipin pathway, leading in a first step to the formation of an unstable allene oxide, catalysed by allene oxide synthase (AOS). In aqueous media, the allene oxide is converted non-enzymatically into α- and γ-ketols and a minor amount of a cyclopentenone compound, racemic OPDA, whereas the enzymatic formation of OPDA by allene oxide cyclase (AOC) leads to the exclusive formation of its *cis*-(9*S*,13*S*) enantiomer. Finally, the reduction of OPDA and three β-oxidation steps lead to (+)-7-*iso*-(3*R*,7*S*)-JA, which isomerizes into the more stable (–)-(3*R*,7*R*)-JA (Figure 1).

With the exception of the enzymes catalysing the β-oxidation steps, all the enzymes of JA biosynthesis have been cloned in recent years. For 13-lipoxygenases, several cDNAs coding for different isoenzymes were characterized for tomato (Heitz *et al.*, 1997), potato (Royo *et al.*, 1996), soybean (Bunker *et al.*, 1995), *Arabidopsis* (Bell and Mullet, 1993), rice (Peng *et al.*, 1994), tobacco (Véronési *et al.*, 1996) and barley (Holtman *et al.*, 1997; Vörös *et al.*, 1998). However, the first clues as to which isoform functions

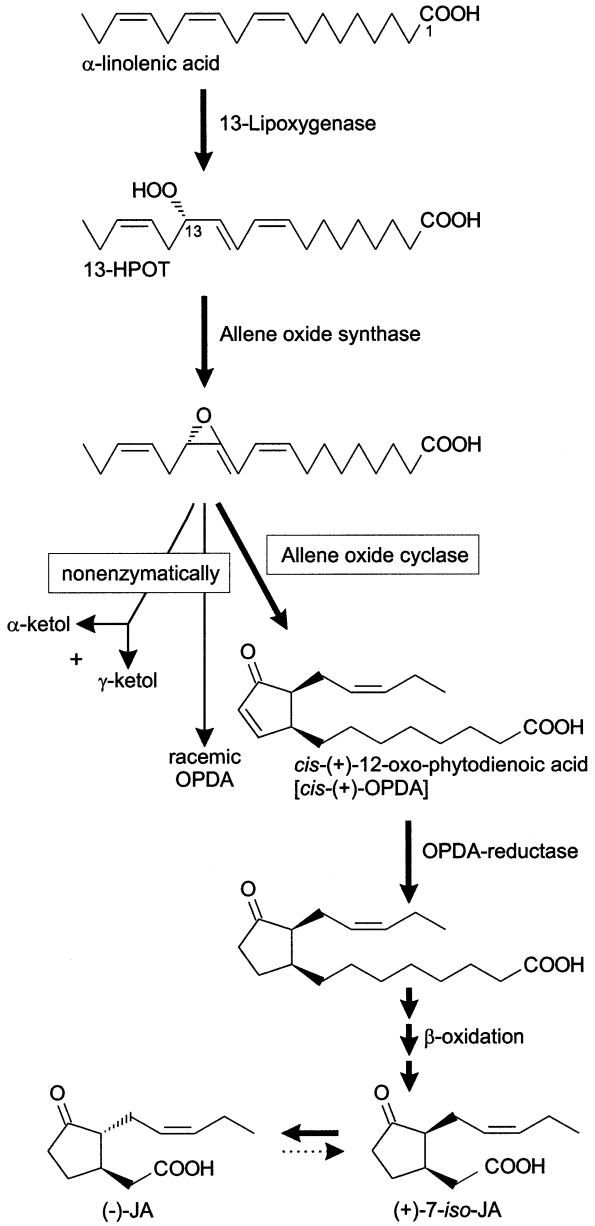


Figure 1. Metabolic scheme of JA biosynthesis showing the stereo-specific reaction in the AOC catalysis.

specifically in JA biosynthesis were obtained only recently (Bell *et al.*, 1995; Heitz *et al.*, 1997; Royo *et al.*, 1999). Most of the 13-LOXs are wound- and JA-inducible and are located within the chloroplasts (Bell *et al.*, 1995; Feussner *et al.*, 1995). Some of them were found to be expressed specifically in flower development (Rodriguez-Concepción and Beltrán, 1995). cDNAs coding for the AOS have been characterized from flax (Song *et al.*, 1993), *Arabidopsis* (Laudert *et al.*, 1996), guayule (Pan *et al.*, 1995), tomato (Howe *et al.*, 2000; Sivasankar *et al.*, 2000) and barley (Maucher *et al.*, 2000), and the AOS protein has been

shown to reside in the chloroplast (Harms *et al.*, 1995; Maucher *et al.*, 2000). AOS has been suggested to be a control point in JA biosynthesis (Laudert *et al.*, 1998). However, it is not yet known how much racemic OPDA and α- and γ-ketols, which were detected recently in *Lemna paucicostata* (Yokoyama *et al.*, 2000) and barley (O. Miersch, unpublished data), could bypass a regulatory role of AOS. Furthermore, the recently purified AOS of corn is irreversibly inactivated by its substrate 13-HPOD (Utsunomiya *et al.*, 2000). If this effect occurred *in vivo*, the positive feedback regulation of JA biosynthesis would be hampered. A recent isotopic dilution experiment including GC–MS analysis suggested that there is no positive feedback of JA biosynthesis in tomato leaves (Miersch and Wasternack, 2000). The AOC-catalysed reaction produces *cis*(9*S*,13*S*)-OPDA, which is, according to present knowledge, the unique precursor of the naturally occurring stereoisomer of JA. AOC was first purified to apparent homogeneity from corn (Ziegler *et al.*, 1997). Its importance in JA biosynthesis is reflected by its exclusive specificity for fatty acid derivatives carrying an epoxide group in the *n*-6,7 position and a double bond in the *n*-3 position, whose cyclization only leads to JA-like structures (Ziegler *et al.*, 1999). Two OPDA reductases (OPR1, OPR2) were purified recently which differed in their substrate specificity (Schaller *et al.*, 1998). OPR1 was cloned from *Arabidopsis* and did not use the 13-*S*-configurated isomer of OPDA which is the intermediate in JA biosynthesis (Schaller and Weiler, 1997). Among the OPRs known so far, only the recently characterized OPR3 was shown to act in JA biosynthesis due to its exclusive formation of the naturally occurring *cis*(+)-stereoisomer (Schaller *et al.*, 2000).

The increased knowledge on JA biosynthesis and the availability of cDNAs coding for its enzymes allow analysis of the physiological effects of JA on senescence, root growth or germination. For AOS, a specific up-regulation was found in distinct tissues of developing barley seedlings and was correlated with elevated JA levels (Maucher *et al.*, 2000). In *A. thaliana*, the AOS promoter has been shown to be preferentially active in distinct tissues such as the abscission zone of flowers (Kubigsteltig *et al.*, 1999). Although it remains to be determined whether this activity leads to increased JA levels, these data on AOS point to a tissue-specific function for JA biosynthesis. Due to instability of the AOS product, tissues and even organelles with AOS activity should contain AOC activity.

Recently, a full-length cDNA clone coding for AOC was isolated from tomato (Ziegler *et al.*, 2000). Over-expression of a truncated version of the protein showed that it catalysed the exclusive formation of *cis*(9*S*,13*S*)-OPDA. In this paper, we have analysed AOC expression during development. Most surprisingly, organ- and tissue-specific expression was detected within vascular bundles of roots, stems and leaves. Expression also occurred within flower stalks and ovules of flower buds and of young flowers. This tissue-specific expression was tightly correlated with elevated levels of JA, JAME, JA amino acid conjugates and octadecanoids, and the various flower tissues contained unique ratios of the various jasmonates and octadecanoids. This suggests a specific role of AOC and octadecanoid/jasmonate profiles during flower development and stress responses.

Results

The AOC mRNA accumulates tissue-specifically during flower development

Analysis of the organ-specific expression of AOC showed a weak constitutive accumulation of AOC mRNA in the stem, young leaves and young flowers (Figure 2). A detailed inspection of the various parts of flowers revealed a significant accumulation in sepals and the pistil. The most abundant AOC mRNA accumulation was in the flower stalks and flower buds (Figure 2). Also, a remarkable amount of AOC mRNA accumulated in roots. No accumulation was detected in fruits, seeds or later leaf stages including senescent (yellow) leaves. The occurrence and amount of AOC protein correlated only partially with that of AOC mRNA. Most interestingly, a high level of AOC protein was detected in the pistil. Even red fruits, old flowers and senescent leaves, which were devoid of detectable amounts of AOC mRNA, contained significant amounts of AOC protein.

The AOC protein accumulates in ovules, and in parenchymatic cells of vascular bundles of flower stalks, petioles, stems and roots, and is accompanied by high AOC activity

The different levels of AOC expression in various flower organs and its specific occurrence in flower buds prompted us to examine the presence of the AOC protein in different floral tissues and in tissues with vascular bundles using an immunocytochemical approach. For this we chose flower buds which allowed inspection of several flower organs in the same section. If probed with a preimmune serum, transverse sections of flower buds (6–9 mm in length) exhibited very faint staining due to some non-specific cross-reactions (Figure 3a–f). However, specific labelling could be shown with an anti-AOC antibody. The strongest signal was found within the pistil (Figure 3a′,a″,b′). Here, the transmission tissue of the style (Figure 3a′,a″,a‴) as well as the ovules within the ovary (Figure 3b′,b″) exhibited strong labelling. In contrast, the petals showed only a weak staining, whereas sepals and stamens were free of label (Figure 3a′).

Moreover, the parenchymatic cells of vascular bundles of a receptacle (Figure 3c′,c″) as well as of the flower stalk above (Figure 3e′,e″) or below (Figure 3d′) the abscission region were also labelled. Longitudinal sections of the flower stalk indicated that, with the exception of the vascular bundles, no label occurred even in the abscission region (Figure 3f′). The tissue-specific occurrence of AOC protein in flowers probed immunocytochemically corresponds to immunoblot analysis and Northern blot analysis (Figure 2).

Of the flower tissues exhibiting high AOC protein levels, flower stalks were chosen for AOC activity testing. In crude extracts of this tissue, AOC activity of about 8 nmol OPDA formed per mg protein was measured, whereas in crude extracts of leaves no AOC activity could be detected. In AOC expression studies involving different treatments of leaves, AOC mRNA was found to accumulate abundantly upon glucose treatment (data not shown). Together with the fact that ovule tissues are regarded to be sink tissues, this prompted us to determine whether levels of the AOC transcript can be up-regulated in flower buds by glucose. As shown in Figure 4, freshly harvested or water-floated flower buds exhibited AOC mRNA levels comparable to those in Figure 2. However, AOS mRNA accumulated strongly upon glucose treatment and weakly upon JAME treatment, but not upon treatment with 6-deoxyglucose, indicating that AOC gene expression in flower buds is glucose-responsive.

In addition to the analysis of flower tissues, the high expression of AOC in flower stalks, stems and roots prompted us to conduct immunocytochemical analysis on these tissues, as well as on petioles. Immunostaining of hand sections of roots, stems and petioles revealed the presence of AOC protein in parenchymatic cells of all vascular bundles (Figure 5a–c″). Only hand sections that were treated with the anti-AOC antibody exhibited the characteristic colour given by the product of the alkaline phosphatase reaction (Figure 5a′–c′, a″–c″), whereas hand sections treated with the pre-immune serum showed only a green colour due to chlorophyll (Figure 5a,b,c). Even the bicollateral organization of the tomato vascular bundles can be seen by this staining procedure, preferentially in hand sections of the petiole (arrows in Figure 5c″), and specific labelling of parenchymatic cells of the vascular bundles is clear. To inspect the obviously cell-specific occurrence of AOC protein in the vascular bundles more clearly, we compared semi-thin cross-sections of the petiole (Figure 5d–d″), a middle vein of a leaflet (Figure 5e–e″) and the intercostal region of a leaflet

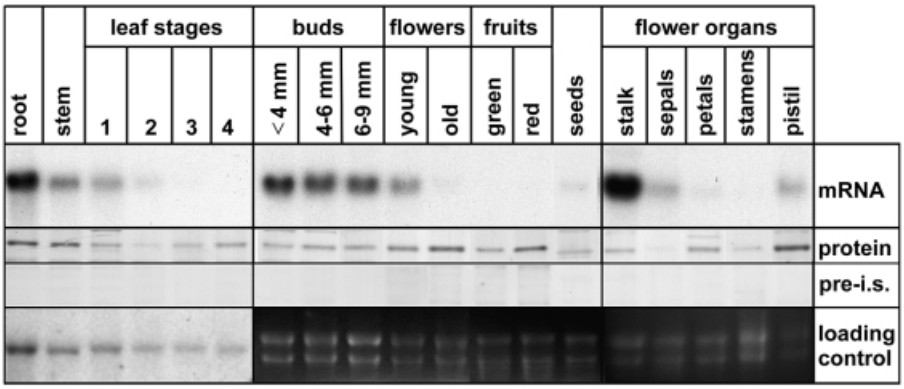
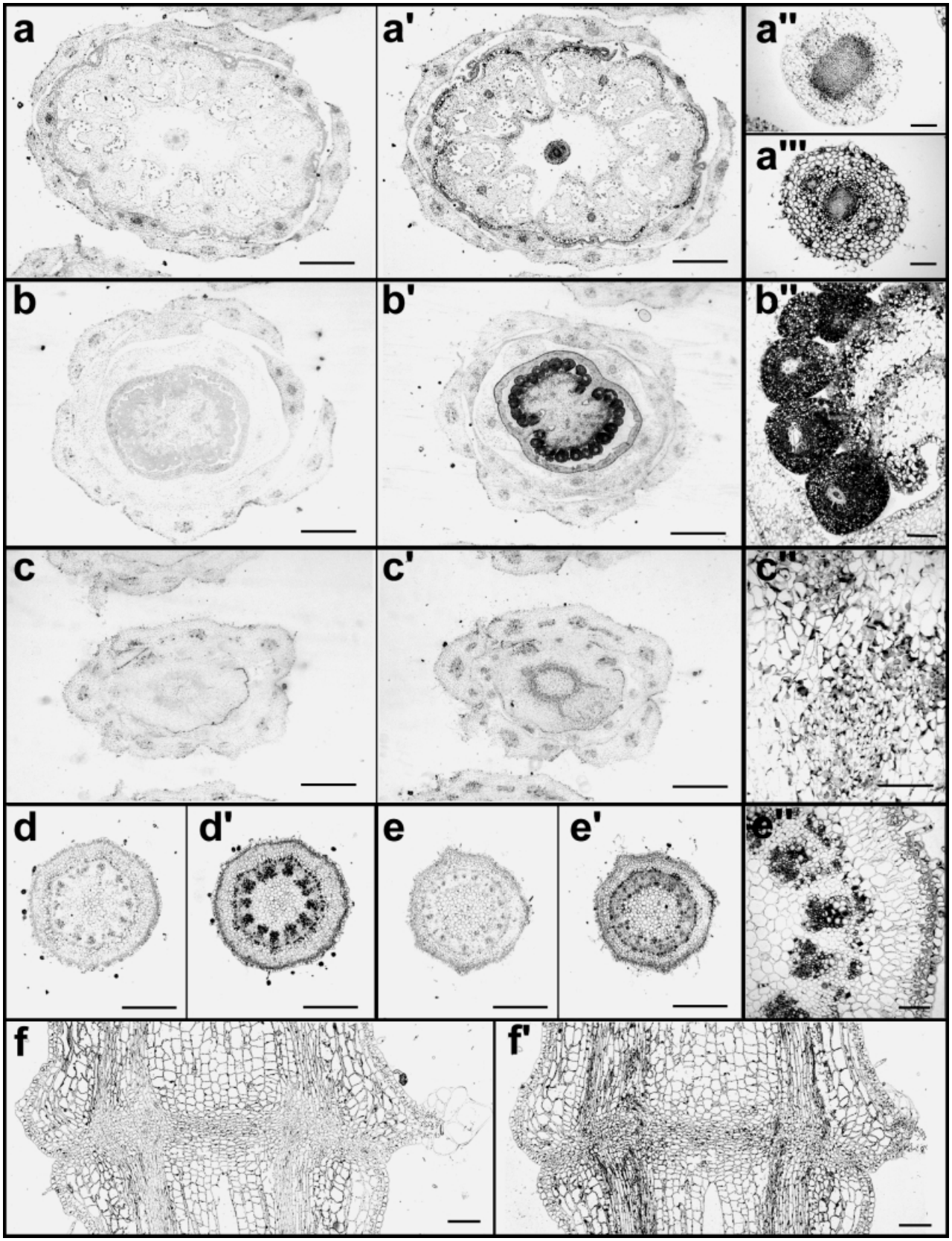


Figure 2. Accumulation of AOC mRNA and AOC protein in different organs and various developmental stages of tomato.
For Northern blot analysis, 20 μg total RNA were loaded per lane. Loading was inspected by recording the ethidium bromide staining of rRNA (right) or hybridizing with a ^{32}P-labelled cDNA fragment homologous to the ADP/ATP carrier nucleotide sequence (left). Northern blot analysis was performed with a full-length AOC cDNA probe as described in Experimental procedures. Immunoblot analysis was performed using 10 μg total protein per lane and with a purified rabbit anti-AOC antibody (diluted 1:5000) raised against the recombinant tomato AOC or with the corresponding pre-immune serum (pre-i.s., diluted 1:5000) as described in Experimental procedures.

Figure 3. Tissue-specific expression of AOC in flowers of 6-week-old tomato plants.
For immunocytochemical analysis, transverse (a–e, a′–e′, a″–e″, a‴) or longitudinal sections (f,f′) were probed with pre-immune serum (a–f) or the AOC protein was visualized by immunodecoration with rabbit anti-AOC antibody (a′–c′, a″–c″, a‴), both followed by a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase and staining as described in Experimental procedures. (a,a′) Transverse section of the middle part of a flower bud of 6 mm length. (a″,a‴) Details of a′, showing the style in the upper region (a″) and the lower region (a‴) of the middle part of the flower bud. (b,b′) Transverse section of the ovary-containing part of a 6 mm flower bud. (b″) Detail of b′. (c,c′) Transverse section of the receptacle of a flower bud. (c″) Detail of c′. (d,d′) Cross-section below the abscission zone of the stalk of a young flower. (e,e′) As d′, but a cross-section above the abscission zone. (e″) Detail of e′. (f,f′) Longitudinal section through the abscission zone of a flower stalk. Other than some label in petals (a′), most AOC protein was detected in the style (a′,a″,a‴), ovules (b′,b″) and parenchymatic cells of vascular bundles of the receptacle (c′,c″) as well as of the flower stalk (d′,e′). The abscission zone of the flower stalk was free of label (f′).The bars represent 500 μm (a–e,a′–e′), 100 μm (f,f′) or 50 μm (a″,a‴,b″,c″,e″).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(Figure 5f–f″). In all three tissues, AOC protein was clearly detectable in parenchymatic cells of the vascular bundles (arrows in d″ and e″). In the leaf cross-section, AOC protein was found in the chloroplasts of cells of the bundle sheath (Figure 5f″).

Jasmonates and octadecanoids accumulate tissue-specifically during flower development

To determine whether the tissue-specific accumulation of AOC mRNA and AOC protein is accompanied by elevated levels of octadecanoids and jasmonates, these compounds were analysed quantitatively by GC–MS analysis (Figure 6).

With the exception of ripe flowers, the accumulation of AOC mRNA and AOC protein was accompanied by a remarkable elevation of jasmonates/octadecanoids (Figure 6d versus Figure 2). In all tissues, the JA content was about one order of magnitude higher than the methyl ester, reaching 5–7 nmol g^{-1} fresh weight in buds (< 6 mm), stalks and sepals (Figure 6a).

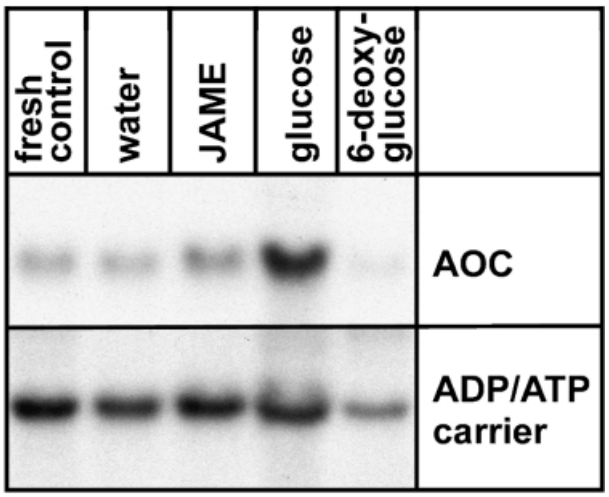


Figure 4. Accumulation of AOC mRNA in flower buds.
Flower buds (6–9 mm length) were freshly harvested (fresh control) or were floated on water, 50 μM JAME, 0.5 M glucose or 0.5 M deoxyglucose for 4 h. Northern blot analysis was performed with 20 μg total RNA using a full-length AOC cDNA probe as described in Experimental procedures. Loading was controlled by hybridization with a ^{32}P-labelled cDNA fragment homologous to the ADP/ATP carrier nucleotide sequence.

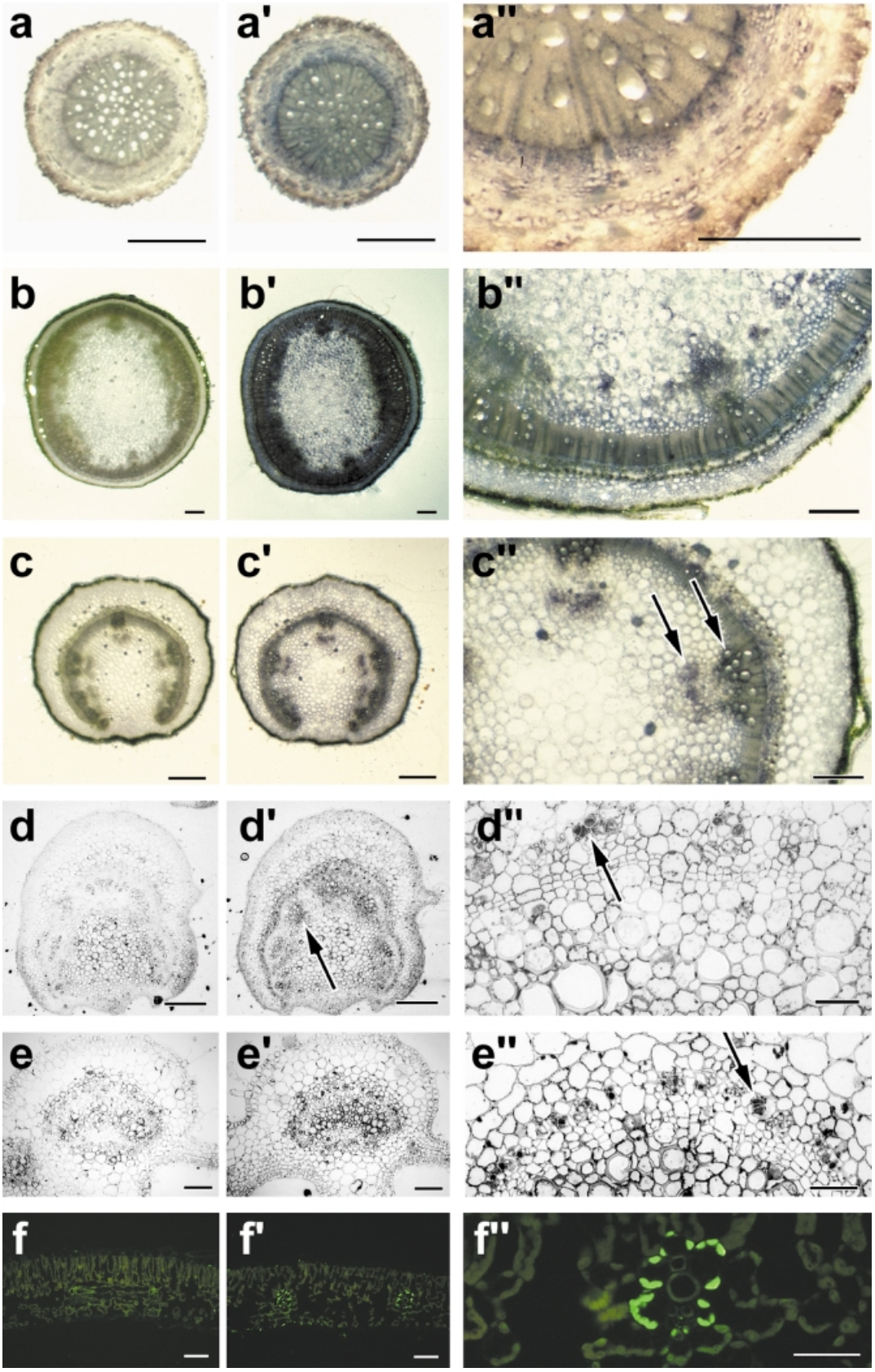
Remarkably high amounts of the isoleucine conjugate of JA were found in buds (< 6 mm), ripe flowers, stalks and pistils (Figure 6b). Interestingly, in pistils, the methyl ester of the isoleucine conjugate of JA accumulated in similar amounts as the free acid of the conjugate, whereas in buds a greater than fivefold higher level of the methyl ester of the isoleucine conjugate of JA was found. Buds, ripe flowers and pistils contained more conjugates than JA. Surprisingly, in the various organs, the OPDA levels are not reflected in the levels of JA. In pistils, OPDA accumulated up to 7 nmol g^{-1} fresh weight, which is about fourfold higher than the JA level in this tissue. However, in sepals, for example, up to 5 nmol JA g^{-1} fresh weight and almost no OPDA could be detected. Despite the metabolic link between OPDA, JA and the isoleucine conjugate of JA, the ratios of their levels are significantly different in different tissues.

Discussion

In contrast to the altered jasmonate levels that occur in response to external stimuli, little is known about jasmonate levels during development. Such levels might be sustained by substrate availability in the JA pathway, and/or activity of the enzymes of the pathway. The first possibility was suggested by the linolenic acid (18:3) deficiency of the mutant *fad3–2 fad7–2 fad8* of *Arabidopsis thaliana* (McConn and Browse, 1996). The unique deficiency of linolenic acid in the tapetum, the pollen-feeding tissue of flowers, led to male sterility (McConn and Browse, 1996), thereby demonstrating the essential role of JA in flower development. Furthermore, this mutant exhibits JA deficiency in the leaves (McConn *et al.*, 1997).

Jasmonate levels may be taken as an indication of the activity of the JA pathway. In soybean seedlings, a gradient of jasmonates was found, with higher levels in young rapidly dividing tissues (Creelman and Mullet, 1995). This agrees with the observation that in barley seedlings a gradient of jasmonate was found in the root, increasing towards the tip (Hause *et al.*, unpublished data), and in the leaf, increasing towards the base (Maucher *et al.*, 2000). Flowers of dicotyledonous plants are known to

Figure 5. Tissue-specific expression of AOC in roots (a′,a″), stems (b′,b″), and petioles (c′,c″), analysed in hand sections, and in petioles (d′,d″), middle vein of the leaflet (e′,e″), and bundle sheath of the intercostal region of the leaflet (f′,f″) analysed in semi-thin cross-sections of embedded tissues.
For immunocytochemical analysis, sections were probed with pre-immune serum (a–f), or the AOC protein was visualized by immunodecoration with purified rabbit anti-AOC antibody (a′–f′), both followed by a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase and staining as described in Experimental procedures. Enlargements of the immunodecorated sections are given in a″–f″. In hand sections (a–c″), the AOC protein was detected by dark purple staining in parenchymatic cells of the vascular bundles (arrows in c″), whereas sections treated with pre-immune serum exhibit chlorophyll and other pigments only. In semi-thin cross-sections (d–f″), AOC protein was detectable in parenchymatic cells of the bicollateral vascular bundles (arrows in d′,d″,e″). AOC protein was defined in chloroplasts of the parenchymatic cells of a bundle sheath (f″).The bars represent 1 mm (a,a′,b,b′,c,c′), 500 μm (a″,b″,c″,d,d′), 100 μm in (d″,e,e′), 50 μm in (e″,f,f′) or 20 μm (f″).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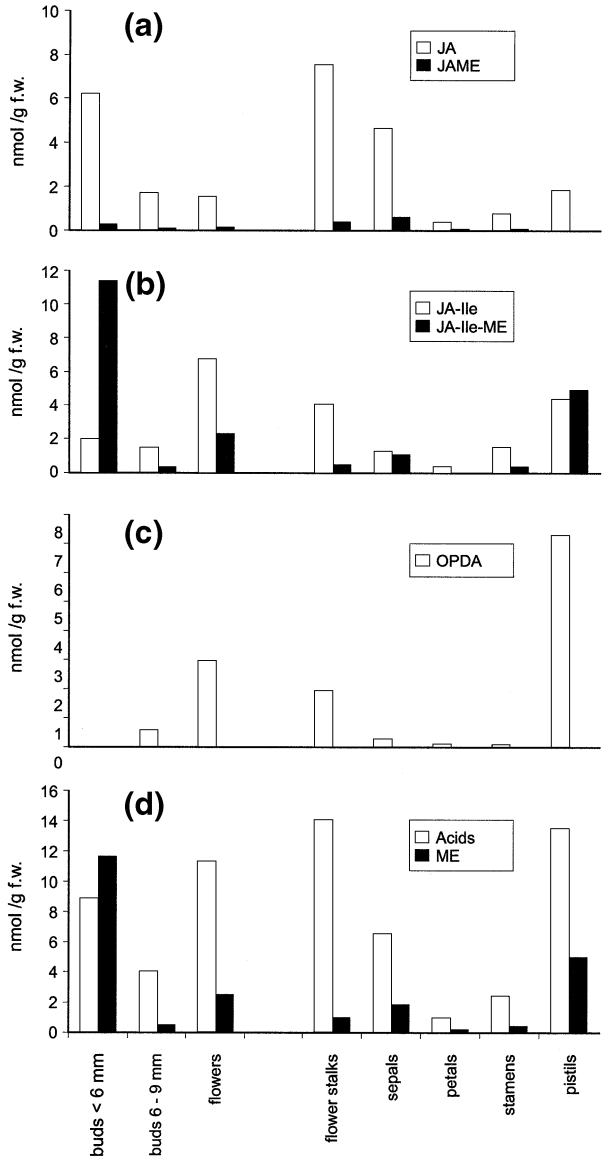

Figure 6. Accumulation of free acids and the corresponding methyl esters of JA (a), the isoleucine conjugate of JA (b), OPDA (c) and the sum of all of them (d) at different stages of tomato flower development and in different flower organs.
Jasmonates, octadecanoids and the conjugates were extracted and quantified from 1 g fresh weight tissue as indicated in Experimental procedures. Four different extractions and analyses were performed, giving identical ratios between all compounds with a deviation of about 15%. One series of data is given.

contain high levels of jasmonates including amino acid conjugates or amides (Miersch *et al.*, 1997; Schmidt *et al.*, 1990). However, the correlation of jasmonate content in distinct plant tissues with the corresponding occurrence and activity of JA biosynthetic enzymes is not yet known, but a correlation between the levels of AOS mRNA, AOS protein and JA was recently observed during barley seedling development in specific tissues such as the scutellar nodule and the leaf base (Maucher *et al.*, 2000).

AOC is expressed preferentially in vascular bundles and in ovule tissues, and its occurrence correlates with elevated levels of jasmonates and octadecanoids

The tissue-specific AOC mRNA accumulation occurring abundantly in roots, flower buds and flower stalks is not completely reflected in AOC protein accumulation, which could be observed in tissues with undetectable amount of AOC mRNA. This might be caused by instability of AOC mRNA in some tissues and/or post-transcriptional control of AOC protein levels. In general, tissues with high AOC expression such as flower buds, flower stalks and pistils (Figure 2) also exhibited elevated levels of octadecanoids and jasmonates (Figure 6). Furthermore, high AOC activity was detectable in crude extracts of flower stalks but not in crude extracts of leaf tissue. The activity in flower stalks is about twofold higher than in corn seeds, in which the highest AOC activity so far has been measured (Ziegler *et al.*, 1997). This correlation between AOC expression and jasmonate/octadecanoid levels points to a regulatory link between both events. Also, AOC expression and accumulation correlate temporally upon external stimuli such as wounding (Ziegler *et al.*, 2000). A similar link was found between wound-induced AOS expression and JA levels in *Arabidopsis* (Laudert and Weiler, 1998), as well as transgenic potato and tobacco plants over-expressing the flax AOS (Harms *et al.*, 1995; Wang *et al.*, 1999). Moreover, the recently cloned tomato AOS is highly expressed in flower buds and mature unopened flowers as well as in leaves upon wounding or treatment with systemin, OPDA or JAME (Howe *et al.*, 2000; Sivasankar *et al.*, 2000). These data correspond with the high AOC expression shown here for flower buds and flower stalks and upon treatment of tomato leaves by wounding (Ziegler *et al.*, 2000) or with systemin, OPDA or JAME (Stenzel *et al.*, unpublished data). The expression of AOS and AOC might be coordinately regulated. However, the intriguing role of AOC is due to its ability to catalyse the exclusive formation of *cis*(+)OPDA, as shown recently with recombinant AOC as well as *in vivo* upon wounding of tomato leaves (Ziegler *et al.*, 2000).

Two types of tissues exhibited the presence of AOC protein: (i) the parenchymatic cells of vascular bundles of various organs such as root, stem, petiole or flower stalk, and (ii) the ovules and the transmission tissue of the style. Interestingly, the prosystemin gene coding for the polypeptide precursor of the systemic wound signal systemin (McGurl *et al.*, 1992) is specifically expressed in vascular bundles of stems, petioles or petiolules, the region of attachment of the leaflet to the petiole (Jacinto *et al.*, 1997). Furthermore, systemin is transported in vascular bundles (Narváez-Vásquez *et al.*, 1995). Recently, the following scenario was suggested (Jacinto *et al.*, 1997; Ryan, 2000). Prosystemin is expressed when a vein is wounded,

followed by its processing to systemin. Systemin may activate wound signal pathway genes in the vascular bundle and is rapidly loaded into the phloem, where it can be distributed throughout the plant, thereby functioning systemically in wound-responsive gene expression in the mesophyll cells of a leaf. The data presented here extend this scenario: The tissue-specific expression of AOC in the vascular bundles (Figure 5) is reinforced by up-regulation of AOC upon wounding (Ziegler *et al.*, 2000), and in the wounded leaf, the levels of *cis*(+)OPDA and the corresponding enantiomer of JA will rise, presumably mainly in the veins (Ziegler *et al.*, 2000). This local rise of octadecanoids and jasmonates may potentiate the JA-responsive prosystemin expression (Jacinto *et al.*, 1997), leading to an increased systemic response. Furthermore, octadecanoids and jasmonates may diffuse into the surrounding tissues, thus leading to the local wound response seen for many wound-inducible genes.

The specific AOC expression in certain flower tissues and the accumulation of octadecanoids and jasmonates shown here suggest a specific function in development. Interestingly, a number of JA-responsive genes are also specifically expressed in ovules and flower stalks. Among them are those coding for histones (Kim *et al.*, 1998). As evidenced by *in situ* hybridization with a leucine amino peptidase (LAPA) antisense RNA probe or promoter activity tests in *LapA*1:*GUS* tomato plants (Chao *et al.*, 1999) and *pin2:GUS* tomato plants (Peña-Cortés *et al.*, 1991), both genes are highly expressed in ovules. These data correspond to the high trypsin inhibitory activity of ovaries of *Nicotiana tabacum* and *N. plumbaginifolia*, where *pin2* is highly expressed (Atkinson *et al.*, 1993; Ausloos *et al.*, 1995). Another JA-responsive gene specifically expressed in the pistils of potato is a dioxygenase suggested to be involved in the biosynthesis of deterrent alkaloids (Lantin *et al.*, 1999). Pathogenesis-related (PR) proteins including chitinase, β-1,3-glucanases and osmotin are also developmentally up-regulated in flower tissue (Lotan *et al.*, 1989; Neale *et al.*, 1990), and are at least partially JA-responsive (Chao *et al.*, 1999). So far, the signals that contribute to the tissue-specific expression of all these plant defence proteins are unknown. It is tempting to speculate that JA might be such a signal, since AOC expression and elevated JA levels occur in these tissues exhibiting JA-responsive gene expression.

Another known effect of exogenously applied JA/JAME is the redistribution of nutrients (Creelman and Mullet, 1997). This effect suggests that an analysis of the JA responsiveness of genes coding for enzymes with a function in sink–source relationships such as invertases would be worthwhile. Interestingly, one of the extracellular tomato invertases (LIN6) known to supply a sink tissue with glucose is specifically expressed in flower buds (Godt and Roitsch, 1997). Such a tissue-specific increase in glucose could supply energy to a non-photosynthetic tissue such as the ovule. Consequently, ovule-specific AOC expression may occur due to its high glucose responsiveness as shown in Figure 4. This in turn may elevate OPDA/JA levels which may contribute to the protection of these tissues against pathogens by expression of various defence proteins such as pin2, LAPA or PR proteins.

An equivalent scenario was recently described in which glucose was shown to simultaneously induce sink-specific genes and defence-related genes, accompanied by down-regulation of photosynthetic genes (Ehness *et al.*, 1997). The elevation of jasmonates in a sink tissue such as ovules following the glucose-inducible AOC expression shown here would synergistically potentiate the effect of glucose. The down-regulation of photosynthetic genes by jasmonate is a well-known phenomenon (cf. Creelman and Mullet, 1997).

An oxylipin signature occurs tissue-specifically in tomato flowers

JA and JAME are considered to be signals in various stress responses. This is based on the observation that an external stimulus leads to an endogenous rise of jasmonates followed by the expression of a distinct set of genes which is similar or even identical to that expressed upon JA treatment of a plant tissue. Also JA amino acid conjugates (Kramell *et al.*, 1997) and structurally related indanone derivatives (Krumm *et al.*, 1995) were shown to be independent signals. Furthermore, the JA precursor OPDA was found to accumulate upon touch (Stelmach *et al.*, 1998), osmotic stress (Kramell *et al.*, 2000), elicitation of cell cultures (Parchmann *et al.*, 1997) or wounding of leaves (Parchmann *et al.*, 1997; Ziegler *et al.*, 2000), and was identified as JA-independent signal in JA-related responses (Blechert *et al.*, 1995; Blechert *et al.*, 1999; Kramell *et al.*, 2000). The term 'oxylipin signature' was proposed after the observation that different plants exhibit distinct oxylipin profiles upon wounding (Weber *et al.*, 1997). Such an individual oxylipin pattern upon external stimuli may be caused by a shift in the various branches of the LOX pathway as was recently shown for barley leaves treated with salicylate (Weichert *et al.*, 1999).

Much less is known about oxylipin patterns during development. Here, we detected for the first time the occurrence of OPDA, JA isoleucine conjugate and its methyl ester in flowers. Most interestingly, during flower development, the different tissues exhibited different ratios of JA, JAME, OPDA and JA isoleucine conjugates. For leaves, JA, OPDA, the structural related coronatine, isoleucine conjugates of JA, and structural analogues such as indanone derivatives were shown to induce gene expression *per se*, if applied exogenously (Blechert *et al.*,

1995; Blechert *et al.*, 1999; Kramell *et al.*, 1997; Kramell *et al.*, 2000; Wasternack *et al.*, 1998; Koch *et al.*, 1999). Due to the preferential accumulation of one or more jasmonate and octadecanoid compound(s) in the various tissues of tomato flowers (Figure 6), it is tempting to speculate that this tissue-specific oxylipin signature might contribute to the expression of individual sets of genes.

Furthermore, JA amino acid conjugates seem to possess a specific function in pollen development. These conjugates are the unique JA compounds accumulating in pollen (Knöfel and Sembdner, 1995), sometimes existing as special derivatives such as [(–)-jasmonoyl]-tyramine (Miersch *et al.*, 1997). The ultimate role of jasmonates in flower development is also supported by three *Arabidopsis* mutants which are disturbed in fatty acid synthesis and degradation as well as in biosynthesis of jasmonates. The triple mutant *fad3-2 fad7-2 fad8* is unable to form linolenic acid, the essential precursor of JA biosynthesis (McConn and Browse, 1996). Male sterility and impairment of pollen development were the strongest alterations of the phenotype. The recently identified *aim1* mutant of *Arabidopsis* was shown to be deficient in the enoyl-CoA hydratase, an essential enzyme of β-oxidation. As a consequence, abnormal inflorescence development and altered fatty acid composition occurred, which led to the suggestion that lipid-derived signals including jasmonates might be altered in *aim1* (Richmond and Blecker, 2000). Mutants in biosynthesis of jasmonates in *Arabidopsis* found recently exhibit a block in OPR3 and are male-sterile (Sanders *et al.*, 2000; Stintzi and Browse, 2000).

So far we have no data on the preferential expression of AOC and elevated levels of jasmonates and octadecanoids in tomato anthers, but the data discussed above suggest expression of plant defence genes via facilitation of jasmonate biosynthesis in another tissue of the tomato flower, the ovules.

Experimental procedures

Chemicals

(±)-JAME ([3*R*,7*R*]/[3*S*,7*S*]-JAME) was purchased from Firmenich (Geneva, Switzerland) and used to prepare JA by saponification. (±)-JAME contained less than 10% of (±)-7-*iso*-JAME ([3*R*/7*S*]/[3*S*/7*R*]-JAME) as determined by GC–MS analysis. [$^2H_6$]-JA was synthesized as described previously (Miersch, 1991). JA-[$^2H_3$]-leucine was synthesized chemically from (±)-JA and [$^2H_3$]-leucine as described for the isoleucine conjugate of JA (Kramell *et al.*, 1997).

Plant materials and treatments

Lycopersicon esculentum Mill. cv. Moneymaker was grown as described previously (Wasternack *et al.*, 1998). Whole flowers before opening were floated on water (control), 0.5 M glucose, 0.5 M 6-deoxyglucose or 50 μM JA, for 4 h. Plant material was frozen in liquid nitrogen and stored at –80°C until use.

Extraction of RNA and Northern blot analysis

Total RNA was extracted from frozen tissues by the use of phenol:chloroform:isoamyl alcohol 25:24:1 (v/v/v) according to the method described by Sambrook *et al.* (1989). If not otherwise indicated, 20 μg total RNA per lane was subjected to electrophoresis, and Northern blot analysis was performed according to the method described by Sambrook *et al.* (1989). Blots were hybridized at 65°C for 16 h with a ^{32}P-labelled fragment of tomato AOC cDNA encompassing the full-length cDNA sequence. Gel loading was checked by probing with a 600 bp fragment of a cDNA homologous tomato ADP/ATP carrier nucleotide sequence. In cases of different levels of occurrence of ADP/ATP carrier mRNA in flower organs, ethidium bromide staining of the rRNA was used as a control.

Extraction of proteins, immunoblot analysis and assays of AOC activity

Proteins extracted according to the method described by Meyer *et al.* (1988) were solubilized in SDS sample buffer, subjected to SDS–PAGE and used in immunoblot analysis. A rabbit polyclonal antibody raised against the recombinant tomato AOC (Ziegler *et al.*, 2000) was used as primary antibody at a dilution of 1:5000, and anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim) was used as secondary antibody. Staining of immunodecorated AOC was done with *p*-nitroblue tetrazoline chloride (NBT) and 5-bromo-4-chloro-3-indolylphos-phate (BCIP). The anti-AOC antibody was free of cross-reactivity in an immunoblot analysis of a total protein extract of tomato leaves. AOC activity was measured in 0.5 g flower stalks or 0.5 g leaf tissues as described previously (Ziegler *et al.*, 1997; Ziegler *et al.*, 1999).

Immunocytochemistry

Tissues were fixed, embedded in PEG and cut as described previously (Hause *et al.*, 1996). Sections of 2 μm thickness were immunolabelled with the rabbit anti-AOC antibody raised against recombinant tomato AOC (diluted 1:2000 in PBS containing 5% w/v BSA). Subsequently, an anti-rabbit IgG antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Germany) was used according to the supplier's instructions. After staining with NBT and BCIP, the slides were analysed by bright-field microscopy with a Zeiss 'Axioskop' microscope (Zeiss, Jena, Germany) equipped with a CCD camera (Sony, Japan).

Hand sections (0.5 mm thickness) from roots, stems and petioles were fixed with 4% paraformaldehyde in PBS at room temperature for 4 h. After washing with PBS and blocking with 5% BSA in PBS, sections were immediately incubated with the rabbit anti-AOC antibody (diluted 1:5000 in PBS containing 5% BSA) at 4°C overnight. Subsequently, incubations were performed as described above with the secondary antibody and staining with NBT and BCIP. Sections were analysed with a stereomicroscope (Stemi 2000, Zeiss) equipped with a CCD camera. In all experiments, controls were performed by using the pre-immune serum.

Extraction, isolation and quantification of jasmonates, JA amino acid conjugates and octadecanoids

Tissues (1 g fresh weight) were frozen in liquid nitrogen, homogenized in a mortar and extracted with 5 ml 80% v/v

methanol. For quantification of JA and JAME, appropriate amounts of ($^2H_6$) JA were added to the extract, whereas in the case of the isoleucine conjugate of JA and its methyl ester, JA-[$^2H_3$]-Leu was used as the internal standard.

The methanolic extracts were purified by chromatographic steps as described for the isolation of JA and JAME (Kramell *et al*., 2000). The final separation was performed by RP–HPLC (column: LiChrospher 100, RP-18, 250 mm × 4 mm, length 5 m; flow rate: 1 ml min^{-1}; UV detection at 210 nm) using a 70:30 v/v mixture of methanol and water (containing 0.2% v/v acetic acid) as the mobile phase. The fractions corresponding to authentic JA (4–5 min) and the leucine and isoleucine conjugates of JA (6–8 min) were concentrated *in vacuo*. The content of the isoleucine conjugate of JA and its methyl ester was calculated on the basis of a calibration curve recorded with methylated JA-[$^2H_3$]-Leu. The intensities of the molecular ions at *m/z* 340 for the deuterated compound and *m/z* 337 for the non-labelled compounds were compared. For quantification of OPDA, 0.5 g plant material was extracted and prepared for GC–MS analysis according to the method described by Müller and Brodschelm (1994). GC–MS was performed with a Finnigan GCQ: 70 eV, NCI, ionization gas NH_3, source temperature 140°C, column Rtx-5 (30 m × 0.25 mm, 0.25 μm film thickness), injection temperature 250°C, interface temperature 275°C, helium 40 cm sec^{-1}; splitless injection; column temperature programme: 1 min at 60°C, 25°C per min to 180°C, 5°C per min to 270°C, 1 min at 270°C, 10°C per min to 300°C, 25 min at 300°C. Retention times were 9,10,dihydro-JA-pentafluorobenzyl ester: 15.13 min; OPDA-pentafluorobenzyl ester: 25.21 min and 25.88 min using fragments *m/z* 211 (standard) and *m/z* 291 for quantification.

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Jasmonic acid methyl ester induces the synthesis of a cytoplasmic/nuclear chito-oligosaccharide binding lectin in tobacco leaves[1]

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SPECIFIC AIMS

The physiological role of plant lectins is controversial because there is no unambiguous evidence that these carbohydrate binding proteins are synthesized in response to specific exogenous or endogenous stimuli and are capable of interacting with endogenous receptor glycans. Here we report the specific induction of a lectin in *Nicotiana tabacum* leaves, which by virtue of its exclusive specificity toward oligomers of N-acetylglucosamine (GlcNAc) and its location in the cytoplasm and the nucleus may be involved in regulating gene expression in stressed plants through modulation of O-linked N-acetylglucosamine-dependent cell signaling.

PRINCIPAL FINDINGS

1. Treatment of tobacco plants with jasmonic acid methyl ester (JAME) induces the synthesis of a lectin that is undetectable in untreated plants

Extracts of untreated tobacco (*Nicotiana tabacum*, var. Samsun NN) plants contain no detectable agglutination activity. However, after exposure to JAME for 3 or 4 days, a strong agglutinating activity is found in the leaves. Determination of a dose-response curve indicated that Nictaba is induced in floating leaves at a physiologically relevant concentration of JAME (25–125 μM). At this dose, Nictaba becomes detectable 24 h after exposure to JAME and rapidly increases for ~48 h. mRNAs encoding Nictaba were also detected 24 h after the application of JAME (**Fig. 1**).

2. Characterization of the *Nicotiana tabacum* agglutinin (Nictaba)

The tobacco lectin, called Nictaba, was isolated using a combination of affinity chromatography on crude chitin and immobilized thyroglobulin. Native Nictaba is a homodimeric protein consisting of two identical unglycosylated subunits of 19,104 ± 2 Da that are amino-terminally blocked. Sequence analysis of peptides generated by endoproteinase Lys-C digestion and cDNA clones encoding Nictaba showed that the primary translation products correspond to mature lectin polypeptides. The apparent absence of a signal peptide indicates that Nictaba is synthesized on free polysomes and the presence of a typical nuclear localization signal (NLS) (^{102}KKKK^{105}) suggests that the lectin may be targeted into the nucleus.

3. Biological activities of Nictaba

Nictaba readily agglutinates red blood cells from human and animal origin. Hapten inhibition assays of the agglutination of rabbit erythrocytes revealed that purified Nictaba is inhibited only by GlcNAc and GlcNAc oligomers, $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ being ~50-, 1600-, and 3000-fold more potent inhibitors, respectively. Surface plasmon resonance analysis of the interaction between Nictaba and animal/plant glycoproteins not only confirmed that $(GlcNAc)_3$ and $(GlcNAc)_4$ are far more potent inhibitors than GlcNAc and $(GlcNAc)_2$, but also demonstrated that $(GlcNAc)_3$ and $(GlcNAc)_4$ are virtually equally active, in turn indicating that the binding site of Nictaba is most complementary to $(GlcNAc)_3$.

4. Nictaba occurs in all leaf cells and is exclusively found in the cytoplasm and nucleus

Immunocytochemical localization of Nictaba demonstrated that the lectin occurs in all leaf cells and is

1 To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.01–0598fje; to cite this article, use *FASEB J.* (April 23, 2002) 10.1096/fj.01–0598fje.

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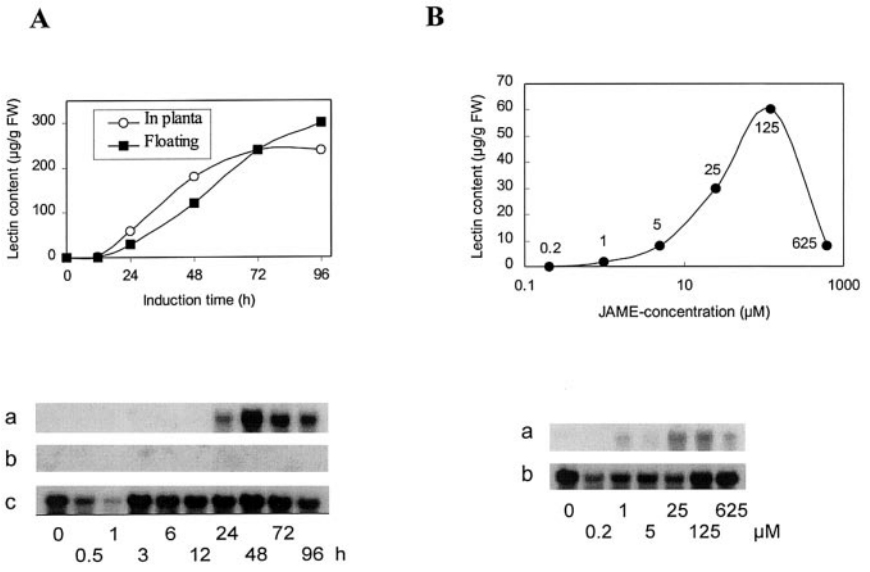

Figure 1. *A)* Time course of the induction of Nictaba by JAME in intact plants induced through the gas phase (in planta) and in excised leaves floated on a 25 µM solution of JAME (floating) for increasing time periods. After incubation, the lectin content was quantified (upper panel). The presence of mRNA encoding Nictaba was checked in floated leaves (lower panel). The Northern blot was hybridized with a specific probe for lectin mRNA (*a*), proteinase inhibitor (*b*), and rRNA (*c*). *B)* Dose-response curve of the induction of Nictaba by JAME. Leaves were floated on solutions of different concentrations of JAME for 24 h. At the end of the incubation, lectin content was determined by agglutination assays (upper panel) and the presence of mRNA encoding Nictaba was checked by Northern blot analysis (lower panel). The Northern blot was hybridized with a specific probe for lectin mRNA (*a*) and rRNA (*b*).

detected only in the cytoplasm and nucleus (**Fig. 2**). Sections of leaves from uninduced plants do not react with antibodies against Nictaba, confirming that the lectin is absent from untreated plants. These results are in good agreement with the absence of a signal peptide and the presence of a NLS in the sequence of Nictaba. Despite the presence of the NLS, however, it is evident that part of Nictaba remains in the cytoplasm.

5. Nictaba is evolutionary related to the *Cucurbitaceae phloem* lectins

A BLAST search revealed that Nictaba exhibits sequence similarity with protein sequences annotated as (putative) phloem-specific lectins or lectin-like proteins. However, since these annotations are based solely on sequence similarities with the so-called Cucurbitaceae phloem lectins (which are a small group of chitin binding lectins found in the phloem exudate of Cucurbitaceae species), it remains to be demonstrated that the corresponding proteins are active lectins. The striking similarities in sequence and specificity strongly suggest that Nictaba and the Cucurbitaceae phloem lectins belong to the same protein family and are, at least for what concerns the sugar binding domain, closely related structurally. However, a closer examination of the sequences reveals two major differences. First, the Cucurbitaceae phloem lectins lack the NLS present in Nictaba. Second, the cysteine-rich carboxyl-terminal domain of the Cucurbitaceae phloem lectins, which is involved in the formation of intermolecular disulfide bridges between these lectins and the phloem protein 1 in the phloem exudate, is absent from Nictaba.

6. Nictaba is the prototype of a widespread/ubiquitous family of plant lectins

Sequences encoding (putative) proteins with the same length and overall structure as Nictaba have been identified in numerous species from different taxonomic groups of flowering plants. Besides arabidopsis, expressed sequence tag sequences encoding putative Nictaba homologues have been found in *Solanum tuberosum, Lycopersicon esculentum, L. hirsutum, Glycine max, Medicago truncatula, Lotus japonicus, Gossypium hirsutum, Hordeum vulgare, Oryza sativa, Secale cereale, Sorghum bicolor,* and *Mesembryanthemum crystallinum.* It can be concluded therefore that Nictaba is the prototype of a widespread (or ubiquitous?) family of cytoplasmic chitin binding proteins.

7. Physiological role and mode of action of Nictaba

Experiments with different chemical or biotic/abiotic factors indicated that Nictaba is induced exclusively by JAME. To understand the role of Nictaba, the question

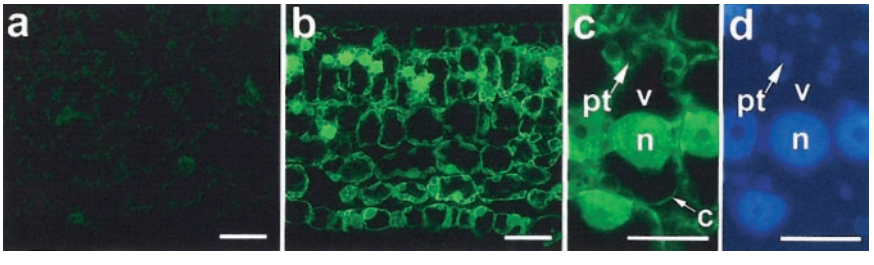

Figure 2. Immunolocalization of Nictaba in tobacco leaves. Cross section of a tobacco leaf of a control plant (*a*) and a plant treated with JAME for 5 days (*b*). Cytoplasm (c) surrounding the plastids, nucleus (n), plastids (pt), and vacuole (v) are indicated in a close-up (zoom-out) of panel *b* (see panels *c* and *d*. *d)* DAPI staining of the section shown in panel *c*. Bars represent 25 µm in panels *a, b,* 10 µm in panels *c, d.*

why tobacco plants express a cytoplasmic/nuclear lectin with an exclusive specificity toward chito-oligosaccharides in response to JAME must be addressed. The most logical explanation is that the plant tries to anticipate the adverse effects of a 'threatening' situation through a mechanism that involves the binding of a newly synthesized lectin to constitutively expressed chito-oligosaccharides or chito-oligosaccharide-containing glycoconjugates. According to its specificity and subcellular location, Nictaba is destined to bind GlcNAc or GlcNAc oligomers present in the cytoplasmic and/or nuclear compartment(s) of the cell. Free chito-oligosaccharides, which are important signaling molecules in plants, are not suitable candidates because they are located extracellularly. Therefore, cytoplasmic and/or nuclear proteins carrying O-linked GlcNAc are the most likely receptor molecules for Nictaba. Many cytoplasmic and nuclear proteins contain GlcNAc O-linked to serine and threonine residues. O-linked GlcNAc oligomers consisting of >five residues have been identified on the nuclear pore complex proteins of plants (whereas in animals, the serine and threonine residues are substituted by a single GlcNAc-residue). Similar O-linked GlcNAc oligomers may also occur on O-glycosylated cytoplasmic and nucleoplasmic proteins in the plant cell.

CONCLUSIONS AND SIGNIFICANCE

The finding that tobacco plants react upon exogenous application of the plant hormone JAME by the expression of a cytoplasmic/nuclear lectin with an exclusive specificity toward GlcNAc and GlcNAc oligomers puts the physiological role of plant lectins in a new perspective. First, convincing evidence has been obtained for the first time that a plant expresses a specific lectin in response to a specific plant hormone. Second, the (partial) nuclear location of Nictaba provides a strong indication that a plant lectin is involved in a specific regulatory process in the nucleus. Third, the specificity and subcellular location of Nictaba demonstrate that tobacco plants react on a chemical triggering by the synthesis of a lectin that is capable of interacting with cytoplasmic and nuclear glycoproteins carrying O-linked GlcNAc or chito-oligosaccharides. This suggests that expression of Nictaba and the subsequent binding of the lectin to regulatory cytoplasmic and/or nuclear proteins provide the plant with a unique mechanism to modulate O-linked N-acetylglucosamine-dependent cell signaling. Along with the compelling evidence that O-GlcNAc modifications of cytoplasmic and nuclear proteins play an important role in key cellular events such as transcription, translation, protein degradation, nuclear export/import, and cell signaling, the discovery of an additional regulatory mechanism is of broad biological significance. The most direct evidence for the involvement of O-GlcNAc modification of proteins in signal transduction comes from studies with plants. Genetic studies with arabidopsis suggested that a recessive mutation called Spindly (Spy), which leads to a constitutive activation of the gibberellin signaling pathway, is affected in a gene encoding a homologue of the O-linked GlcNAc-transferase from humans, rats, and *Caenorhabditis elegans*. Wild-type Spy presumably encodes an O-linked GlcNAc-transferase that acts as a negative regulator early in the pathway of the gibberellin signal transduction cascade.

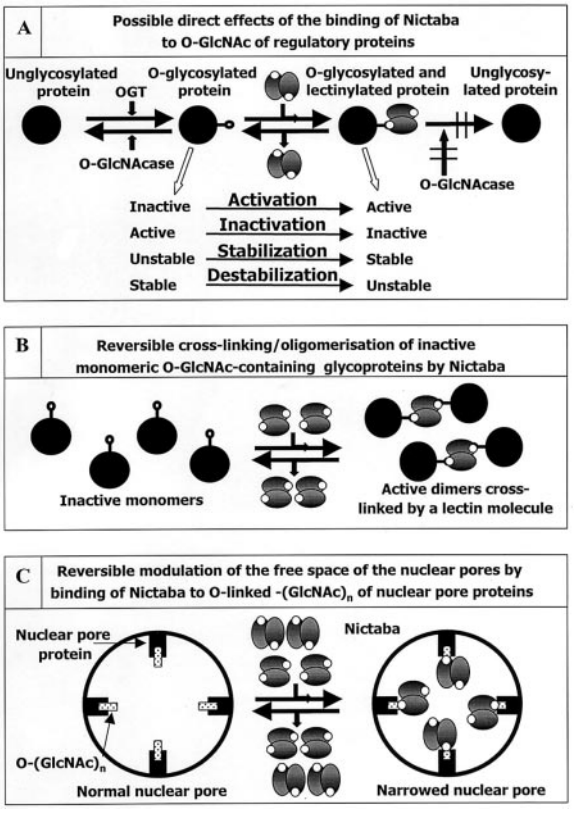

Figure 3. Schematic representation of the mode of action of Nictaba. *A)* Regulatory proteins are glycosylated by an O-linked GlcNAc transferase (OGT). Binding of a lectin molecule to the O-GlcNAc may directly activate or inactivate these O-GlcNAc-containing regulatory proteins. Alternatively, bound Nictaba may protect the O-GlcNAc from removal by O-GlcNAcase. *B)* Nictaba may cross-link inactive monomeric O-GlcNAc-containing proteins into physiologically active oligomeric forms. *C)* Binding of Nictaba to O-linked GlcNAc oligomers of nuclear pore proteins may change the accessible size of the nuclear pores and modulate the transport of proteins and/or RNA between the nucleoplasm and the cytoplasm.

Nictaba can modulate O-linked N-acetylglucosamine-dependent cell signaling in three different ways (**Fig. 3**). *1)* Binding of Nictaba to O-linked GlcNAc of soluble cytoplasmic/nucleoplasmic regulatory proteins (e.g., transcription and translation factors), receptors, and enzymes (e.g., protein kinases) can directly alter the activity or stability of these proteins. *2)* Nictaba may cross-link inactive monomeric proteins into physiologically active oligomers. *3)* Nictaba may modulate the transport of proteins and/or RNA between the nucleus and the cytoplasm through a mechanism based on interaction of the lectin with O-GlcNAc glycosylated proteins of the nuclear pore complex. Whatever the mode of action is, the jasmonate-induced synthesis of a cytoplasmic/nuclear lectin with an exclusive specificity toward GlcNAc/GlcNAc oligomers illustrates that protein—carbohydrate interactions may play an important role in some fundamental physiological processes of flowering plants. FJ

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Jasmonate-Induced Lipid Peroxidation in Barley Leaves Initiated by Distinct 13-LOX Forms of Chloroplasts*

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* Dedicated to Professor Benno Parthier on the occasion of his 70th birthday

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In addition to a previously characterized 13-lipoxygenase of 100 kDa encoded by *LOX2:Hv:1* [Vörös *et al.,* Eur. J. Biochem. *251* (1998), 36–44], two full-length cDNAs (*LOX2:Hv:2*, *LOX2:Hv:3*) were isolated from barley leaves (*Hordeum vulgare* cv. Salome) and characterized. Both of them encode 13-lipoxygenases with putative target sequences for chloroplast import. Immunogold labeling revealed preferential, if not exclusive, localization of lipoxygenase proteins in the stroma. The ultrastructure of the chloroplast was dramatically altered following methyl jasmonate treatment, indicated by a loss of thylakoid membranes, decreased number of stacks and appearance of numerous osmiophilic globuli. The three 13-lipoxygenases are differentially expressed during treatment with jasmonate, salicylate, glucose or sorbitol. Metabolite profiling of free linolenic acid and free linoleic acid, the substrates of lipoxygenases, in water floated or jasmonate-treated leaves revealed preferential accumulation of linolenic acid. Remarkable amounts of free 9- as well as 13-hydroperoxy linolenic acid were found. In addition, metabolites of these hydroperoxides, such as the hydroxy derivatives and the respective aldehydes, appeared following methyl jasmonate treatment. These findings were substantiated by metabolite profiling of isolated chloroplasts, and subfractions including the envelope, the stroma and the thylakoids, indicating a preferential occurrence of lipoxygenase-derived products in the stroma and in the envelope. These data revealed jasmonate-induced activation of the hydroperoxide lyase and reductase branch within the lipoxygenase pathway and suggest differential activity of the three 13-lipoxygenases under different stress conditions.

Key words: *Hordeum vulgare* L. / Immunogold localization / Oxylipin profiling / Senescence / Stroma.

Introduction

Senescence is one of the most complex processes within the plant life cycle. It can be developmentally regulated during the annual life cycle of a plant or be induced by environmental factors such as darkness, pathogen attack and nutrient reallocation (Smart, 1994; Quirino *et al.*, 2000). In leaf development, senescence appears as the terminal stage and is regarded to be a type of programmed cell death (PCD). Degradation and partial recycling of cellular constituents occur, including a dramatic reprogramming of the expression of so-called senescence associated genes (SAGs). Numerous SAGs were characterized indicating a complex network of different pathways, which contribute collectively to leaf senescence (He *et al.,* 2001). Among them there is a large group of genes induced by jasmonates or conditions leading to endogenous rise in jasmonates. Jasmonates, a group of ubiquitously occurring plant growth regulators, were initially analyzed to be among the strongest effectors of promotion of leaf senescence (Parthier, 1990). Their biosynthesis originates from lipid membrane constituents, the α-linolenic acid (α-LeA) and is initiated by lipoxygenase (LOX)-catalyzed lipid peroxidation which can occur either with esterified or with free fatty acids.

Since degradation of membrane constituents by lipid peroxidation is a characteristic feature of leaf senescence, and jasmonates promote senescence preferentially in monocotyledonous plants, we have chosen jasmonic acid methyl ester (JAME)-treated barley leaves as a model system to analyze the initial reactions preceding senescence. One of the most obvious and dramatic events appearing in both, JAME-treated tissues as well as naturally senescing leaves, is the degradation of chloroplast constituents such as chlorophyll and thylakoid membranes (Smart, 1994; Matile and Hortensteiner, 1999). Among the main constituents of chloroplast membranes there are polyunsaturated fatty acids (PUFAs) (Somerville *et al.,* 2000), such as α-LeA and linoleic acid (LA), which occur in chloroplasts in the ratio of about 10:1 (Gunstone *et al.,* 1994). Both are substrates for LOXs, which catalyze the regioselective and stereospecific insertion of molecular oxygen into position C-9 (9-LOX) or position C-13 (13-LOX) of the PUFAs (Brash, 1999). Another more comprehensive classification of plant LOXs is based on their structural features (Shibata *et al.,* 1994). The members of the so-called LOX1-type group exhibit remarkable degree of sequence similarity, whereas LOX2-type enzymes have moderate sequence similarity, but carry a putative transient peptide sequence for chloroplast import. In case of 13-LOX the resulting prod-

ucts are (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOTrE) or (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), respectively. These compounds can then be further converted by different branches of the so-called LOX pathway (Feussner and Wasternack, 2002) (Figure 1). Most of these reactions were found to occur in subfractions derived from chloroplasts (Blée and Joyard, 1996).

In case of the AOS branch 12-oxo-phytodienoic acid (OPDA) and jasmonates such as the free acid (JA), its methyl ester JAME, or its conjugates with amino acids are formed. For the OPDA-forming enzymes 13-LOX, AOS and AOC a chloroplast localization was shown (Feussner and Wasternack, 2002). The reduction of OPDA is catalyzed by a specific OPDA reductase (OPR3) presumably located in peroxisomes (Schaller *et al.,* 2000; Stintzi and Browse, 2000), where also the subsequent β-oxidation steps are expected to occur. However, it is still unclear how OPDA is channeled into the peroxisome (Laudert and Weiler, 1998; Froehlich *et al.*, 2001).

A characteristic feature of the AOS branch is its activa-

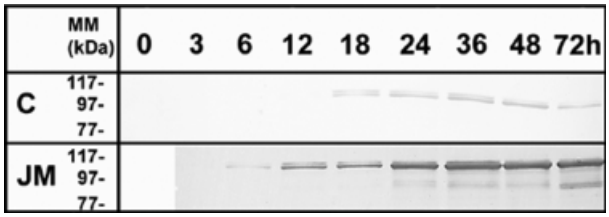

Fig. 2 Immunoblot Analysis of LOX Forms Appearing in Barley Leaf Segments upon Water (C) and JAME Treatment (JM).

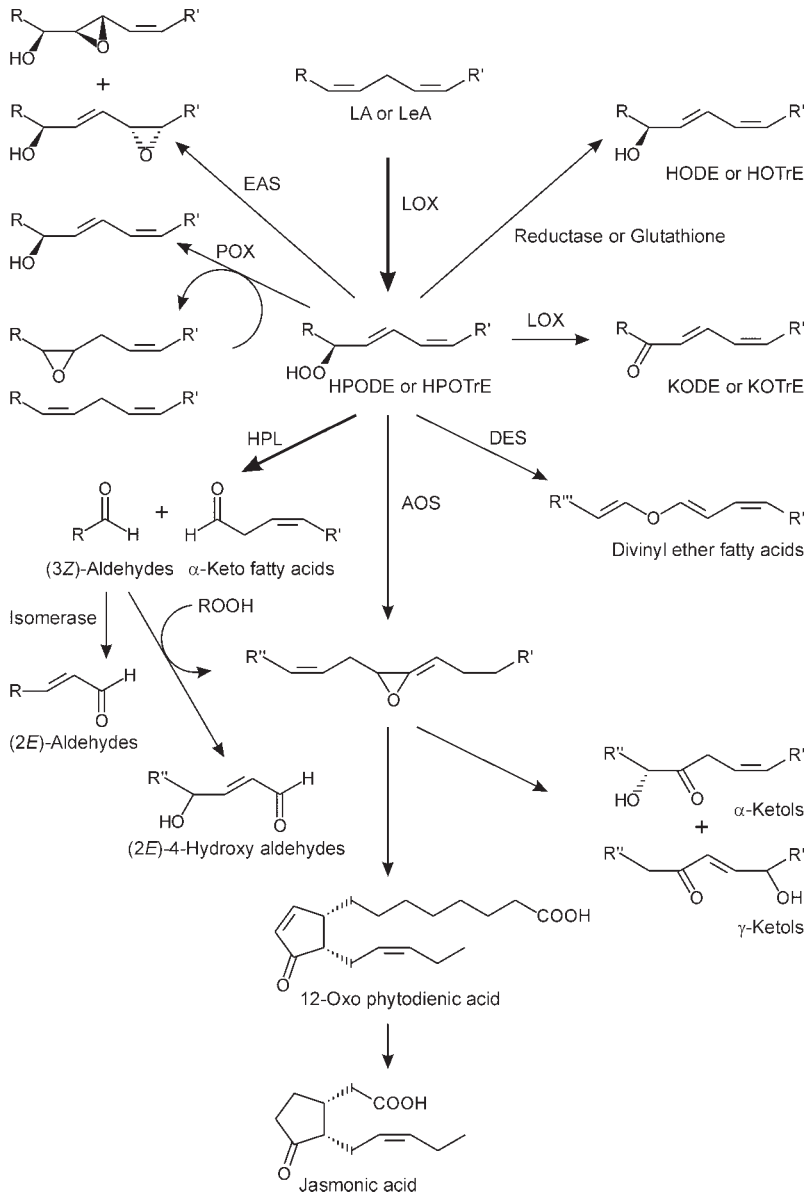

Fig. 1 Metabolic Routes for LOX-Dependent Catabolism of PUFAs in Plants.
The preferential route described here for JAME-treated barley leaves is indicated by bold arrows (EAS, epoxy alcohol synthase; POX, peroxygenase; HPL, hydroperoxide lyase; AOS, allene oxide synthase; DES, divinyl ether synthase).

tion in response to stress appearing at distinct developmental stages, or being caused by the plant environment. In barley seedling development a simultaneous increase of *AOS* expression and JA levels was found in the scutellar node and leaf base (Maucher *et al.,* 2000), correlating spatially and temporally with JA-responsive gene expression (Hause *et al.,* 1996). Also, stress-induced activation of the AOS branch, *e.g*. sorbitol treatment of leaf segments, results in a simultaneous elevation of octadecanoid and JA levels as well as in JA-responsive gene expression (Kramell *et al.*, 2000).

One 13-LOX from barley chloroplasts has so far been characterized (Vörös *et al.,* 1998). This protein of 100 kDa (LOX-100) accumulates most abundantly in JA-treated leaves and is encoded by the *LOX2:Hv:1* gene (Vörös *et al.,* 1998), whose expression was also induced by salicylic acid (SA) treatment (Hause *et al.,* 1999). Initial inspection of LOX forms detectable upon JAME and sorbitol treatment (Feussner *et al.*, 1995a,b) and SA treatment (Weichert *et al.,* 1999) revealed (i) a distinct set of LOX forms showing (ii) remarkable differences in the amount and ratio of these isoforms, accompanied by changes in the type and quantities of LOX-derived products (Weichert *et al.*, 1999, 2000).

These results prompted us (i) to analyze the isoform pattern of LOXs in JAME-treated leaves, (ii) to inspect ultrastructural changes within chloroplasts upon JAME treatment of leaves including LOX localization by immunogold labeling, (iii) to isolate further cDNAs coding for LOXs of barley leaves, (iv) to analyze the expression pattern of the different LOX forms upon various stimuli, and (v) to record quantitative metabolite profiles of LOX-derived products in leaves and chloroplast subfractions of JAME-treated leaves. Our data reveal JAME-induced lipid peroxidation of chloroplasts as an initial event preceding senescence. Furthermore, a preferential occur-

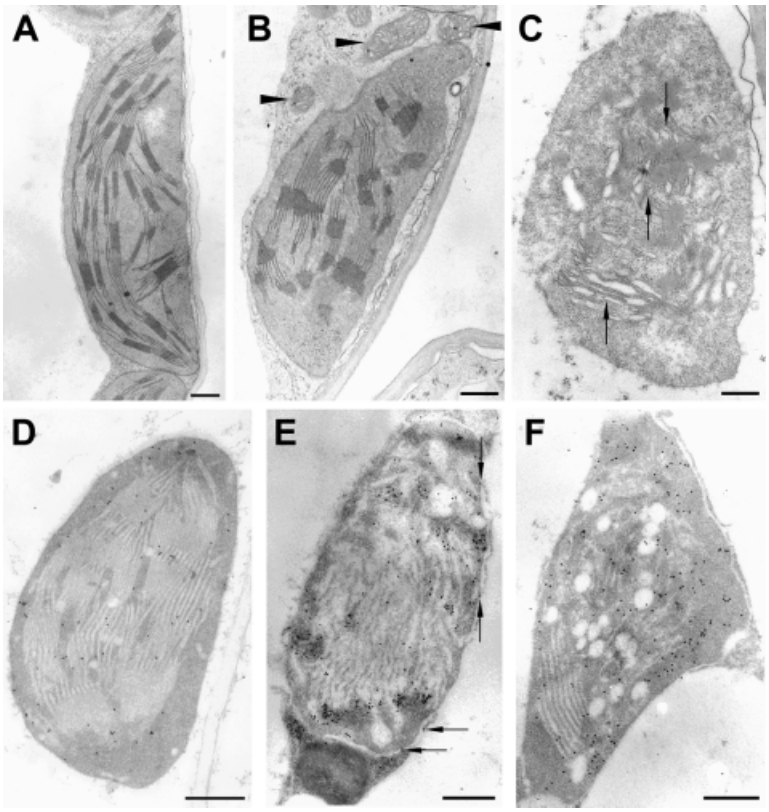

Fig. 3 Analysis of the Ultrastructure of Chloroplasts (A–C) and the Localization of LOX Proteins within Chloroplasts (D–F) upon Treatment with JAME (B, C, E, F).
(A) Chloroplast of a mesophyll cell of an untreated primary leaf.
(B) Chloroplast of a mesophyll cell of barley leaf segment treated with 45 μM JAME for 48 h. Note the loss of thylakoid stacks in comparison to chloroplasts of untreated leaves (A). The ultrastructure of mitochondria (arrowheads) seems not to be altered by the treatment with JAME.
(C) Chloroplast of a mesophyll cell of barley leaf segment treated with 45 μM JAME for 72 h. Only few thylakoid membranes are visible (arrows). Note the occurrence of osmiophilic globuli within the stroma.
(D) Immunolocalization of LOX protein within the chloroplast of an untreated leaf. Only a few gold particles are visible due to the low amount of LOX protein.
(E) Immunolocalization of LOX protein within the chloroplast of a leaf treated with JAME for 24 h. Note the high amount of label within the stroma. Only few gold particles are visible near the chloroplast envelope (arrows).
(F) Immunolocalization of LOX within a chloroplast of a leaf treated with JAME for 72 h. The label is mainly restricted to the stroma. Bars represent 0.5 μm in all micrographs.

rence of 13-LOXs in the stroma fraction of chloroplasts and a preferential activity of the HPL branch within the LOX pathway of total leaf extracts were found.

Results

JAME-Induced Changes in the Pattern of Barley LOX Forms

Barley leaves contain at least 3 LOXs with molecular masses of 92, 98 and 100 kDa (LOX-92, LOX-98, LOX-100; Feussner *et al.*, 1995a). As shown recently (Weichert *et al.,* 1999) and documented here for comparison, these LOX forms are differentially induced by JAME treatment (Figure 2). One of these LOX forms, LOX-100, is encoded by the previously identified *LOX2:Hv:1* gene (Vörös *et al.,* 1998).

Subcellular Localization of LOX Proteins

In order to localize LOX protein within the chloroplast, immunogold labeling and electron microscopic analysis of chloroplasts was performed. Since monospecific antisera against the different isoforms are not yet available, a polyclonal antiserum was used which immunodecorates several LOX isoforms. Thus, a differential analysis of the three LOX forms could not be expected.

Based on the observation that JAME treatment led to accumulation of LOX proteins (Figure 2) and the hypothesis that these LOXs might be involved in the degradation of chloroplast membranes (Feussner and Wasternack, 1998), untreated leaves and leaves treated for various times with JAME were investigated. JAME treatment led to a dramatically altered ultrastructure of chloroplasts. Whereas chloroplasts of untreated leaves exhibited large numbers of well-defined thylakoid stacks, a significant loss of well-structured thylakoid membranes and the number of stacks can be seen after 24 h of JAME treatment (Figure 3A *versus* B). Mitochondria seem to be unchanged in ultrastructure under these conditions. The most dramatic changes following JAME treatment appear after 72 h. Only few thylakoid membranes and numerous osmophilic globuli are visible (Figure 3C). Corresponding to the low amount of LOX protein in untreated leaves (Figure 2), immunogold labeling revealed only a few gold particles (Figure 3D). In contrast, in chloroplasts of leaves treated with JAME for 24 h, high intensity of label appeared in the stroma accompanied by only few label near the envelope (arrow in Figure 3E). Although the ultrastructure of chloroplasts of leaves treated with JAME for 72 h was dramatically altered, LOX protein was detectable mainly in the stroma (Figure 3F). These data revealed the occurrence of LOX protein preferentially, if not exclusively, in the stroma of chloroplasts.

Isolation and Characterization of Further cDNAs Coding for LOX Forms

In order to differentiate at the molecular as well as on the biochemical level between different LOX forms, further cDNAs coding for LOXs were isolated. Initially, a cDNA library was prepared with poly(A) RNA isolated from barley leaf segments treated for 12 h with 1 M sorbitol and was differentially screened with the radiolabeled probe of *LOX2:Hv:1* as described in the Material and Methods section. Thirty eight positive clones hybridizing exclusively with the *LOX2:Hv:1* probe were chosen for *in vivo* excision and sequence analysis. Among all clones only one full-length cDNA was isolated and designated as pLOX2/10. In addition, another full-length cDNA clone encoding a putative LOX was isolated from the previously described cDNA library prepared from barley leaves treated for 24 and 48 h with JAME (Vörös *et al.,* 1998) and was designated as pLOX3/15.

For identification and biochemical analysis of gene products encoded by the two isolated full-length cDNAs bacterial overexpression was performed in *E. coli*. Using the bacterial lysates, LOX enzyme activity tests with LA or arachidonic acid revealed for both enzymes formation

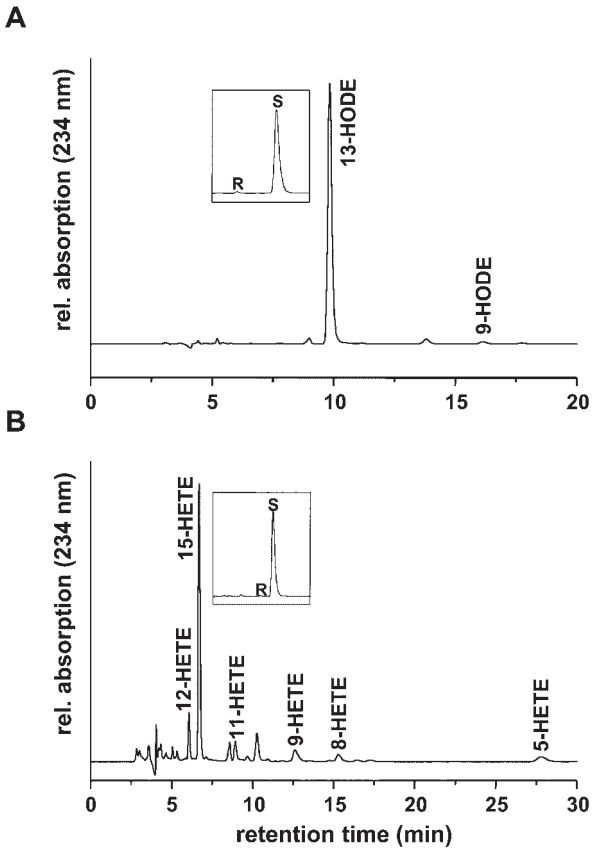

Fig. 4 HPLC Analysis of LA (A) and Arachidonic Acid (B) Oxydation Products Generated by Recombinant *LOX2:Hv2*.
Crude extracts of *E. coli* expressing *LOX2:Hv2* enzyme were incubated with 300 mM LA (A) or 300 mM arachidonic acid (B) in 0.1 M phosphate buffer, pH 7.3, for 15 min. The hydroperoxy derivatives were reduced with sodium borohydride. Subsequently, the hydroxy derivatives of LA (HODEs) and the corresponding hydroxy eicosatetraenoic acids (HETEs), were extracted, isolated by RP-HPLC and analyzed on SP-HPLC as described in Materials and Methods. The insets show separation of positional isomers of 13-HODE and 15-HETE, respectively, by chiral phase HPLC.

of 13-H(P)ODE and the corresponding (15*S*)-hydroperoxy derivative of arachidonic acid, which were identified after acidification and HPLC analysis as the corresponding LOX-derived hydroxy compounds shown for *LOX2:Hv:2* in Figure 4. These data indicate that both isolated full-length clones code for a 13-LOX. Due to their structural features and the sequence similarities to other members of the LOX2-type group described below, both new 13-LOXs are regarded to be of LOX2-type according the nomenclature of Shibata *et al.* (1994). Therefore, the clone pLOX2/10 was designated as *LOX2:Hv:2* and the clone pLOX3/15 was named *LOX2:Hv:3*. Recombinant *LOX2:Hv:2* and *LOX2:Hv:3* exhibited a pH optimum of 6.5, whereas for the recombinant *LOX2:Hv:1* a pH optimum of 7.2 had been determined (Vörös *et al.,* 1998).

The open reading frame of *LOX2:Hv:2* covered 2799 bp and that of *LOX2:Hv:3* 2691 bp, coding for polypeptides of 932 and 896, amino acids, respectively. The completeness of both cDNAs was indicated by the fact that both start codons were preceded by a stop codon in frame. The amino acid sequences deduced from the nucleotide sequence of *LOX2:Hv:2* and *LOX2:Hv:3* were aligned and compared with that of *LOX2:Hv:1* (Figure 5). *LOX2:Hv:2* and *LOX2:Hv:3* revealed calculated molecular masses of 106.5 kDa and 101.2 kDa, respectively, and IEPs of 6.73 and 6.26, respectively. The overall amino acid identity be-

```
          1                                                                                                  100
Lox2:Hv:1 MLTATKPLVG GACAAPSSSA RRRTFVVPEA RRKPG.NGRR TSVSKVGSTS TSTTTTTTTT LSADSNGAAV GTVTRPDVHV QDRTHATEMK ATVTVHM...
Lox2:Hv:2 MQTATKPLV. GARAVPLS.. RRASFLVAEA RRKPSTNARR T...RVGSTS ...TTTTTTT ILTDVNGPAL TTVAKPG.HQ YDLKQTVEMK ATVSVHMKSF
Lox2:Hv:3 MIHLKQPLVL SA...QSSNV ASPLFVAGGQ QRRASGAGRT CSGRRLSARR ISCAST.... ..EEAVGVST SVTTK..... .......ERA LTVTAIVTAQ
Consensus M-----PLV- -A-----S-- ----F----- -R------R- ---------- -----T---- ------G--- ---------- -------E-- -TV-------

          101                                                                                                200
Lox2:Hv:1 ....SKAAGV RDFLYDLILK TWLHVDLVSS ELDPQTGQER EPISGAVKHS GRVDDEWDMY EATFKVPASF GPIGAVQVTN YHHSEMLLGD IEVFPTGQE.
Lox2:Hv:2 WWSDEKKERA RDWAYDLILG SWLTLELVSS ELDPKTGQEH DVISGKLKHS RETEKDYDLY EAIFTCRHRL APSGAVRLVN YHHTEMLLGE VKIFPAGEDP
Lox2:Hv:3 VPTSVYVARG LDDIQDLFGK T.LLLELVSS ELDPKTGRER ERVKG.FAHM TLKE...GTY EAKMSVPASF GPVGAVLVEN EHHREMFIKD IKLITGGDE.
Consensus ---------- -D---DL--- --L---LVSS ELDP-TG-E- --I-G---H- ---------Y EA-------- -P-GAV---N -HH-EM---- I-----G---

          201                                                                                                300
Lox2:Hv:1 ..ESAVT.FH CKSWIDPSHC TPDKRVFFPA H.SYLPSQTP KGVEGLRKRE LEILRGTGCG ERKEHDRIYD YDVYNDLGNP DDDNNPTTRP VLGGKEHPYP
Lox2:Hv:2 TKSSAVTLFH CQSWIDPSHC SPDKRTFFPV EKSYIPSQTP KGVEKLRKSE LEALRGNGCG ERKKHDRIYD YDVYNDLGKP E.....SKRP VLGGKEHPYP
Lox2:Hv:3 ..STAIT.FD VASWVHSKFD DPEPRAFFTV .KSYLPSQTP PGIEALRKKE LETLRGDGHS ERKFHERVYD YDTYNDLGDP D.KNIDHKRP VLGTKEHPYP
Consensus ----AVT-F- --SWI----- -P--R-FF-- --SY-PSQTP -GVE-LRK-E LE-LRG-G-- ERK-H-RIYD YD-YNDLG-P --------RP VLG-KEHPYP

          301                                                                                                400
Lox2:Hv:1 RRCRTGRPRS KKDPFSEERS HKE.HIYVPR DEAFTERKMG AFDTKKFMSQ LHALTTGLKT AKHKSQSFPS LSAIDQLYDD NFRNQPVQPE GGKLRFVIDL
Lox2:Hv:2 RRCRTGRPRS KTDPSSEEES HKKGEMYVPR DETFTERKEQ AFLTKQLLSQ LHGLCTGLKV NKDILPSFPT LASIDALYDD DFRNQPVQPE GGKVRLILDL
Lox2:Hv:3 RRCRTGRPKT LYDPETETRS ...SPVYVPR DEQFSDVKGR TFSATTLRSG LHAILPAVAP LLNNSHGFSH FPAIDALYSD GI.PLPVDGH GGNSFNVIND
Consensus RRCRTGRP-- --DP--E--S ------YVPR DE-F---K-- -F------S- LH-------- -------F-- ---ID-LY-D -----PV--- GG----V---

          401                                                                                                500
Lox2:Hv:1 LETELLHLFK LEGAAFLEGI RRVFKFETPE IHDRDKFAWF RDEEFARQTI AGMNPMSIQL VTEFPIKSNL DEATYGPADS LITKEVVEEQ IRRVMTADEA
Lox2:Hv:2 LAKELVHLVK LEGAEFVEGI RRVFKFETPE IHDMDKLAWF RDEEFARQTL AGMNPLSIQL VTELPIVSKL DELKYGPADS LITKELIEKQ INRIMTAEEA
Lox2:Hv:3 VIPRVVQMIE .......DTT EHVLRFEVPE MLERDRFSWF RDEEFARQTL AGLNPICIRR LTEFPIVSKL DPAVYGPAES ALSKEILEKM MNGRMTVEEA
Consensus ---------- ---------- --V--FE-PE ----D---WF RDEEFARQT- AG-NP--I-- -TE-PI-S-L D---YGPA-S ---KE--E-- ----MT--EA

          501                                                                                                600
Lox2:Hv:1 VQNKKLFMLD YHDLLLPYVH KVRKLDGTTL YGSRALFFLT ADGTLRPIAI ELTRPKSKKK PQWRQVFTPG C..DGSVTGS WLWQLAKAHI LAHDAGVHQL
Lox2:Hv:2 VAQKKLFMLD YHDLLLPYVH RVRKLDNKTM YGSRTLFFLA DDGTLRPIAI ELTRPKSPHK QQWRKVFTPG SGYSGSVTGS WEWQLAKIHV LSHDTGYHQL
Lox2:Hv:3 MEKKRLFLLD YHDVFLPYVH RVRELPDTTL YGSRTVFFLS DEGTLMPLAI ELTRPQSPTK PQWKRAFTHG S....DATES WLWKLAKAHV LTHDTGYHQL
Consensus ---K-LF-LD YHD--LPYVH -VR-L---T- YGSR--FFL- --GTL-P-AI ELTRP-S--K -QW---FT-G -------T-S W-W-LAK-HV L-HD-G-HQL

          601                                                                                                700
Lox2:Hv:1 VSHWLRTHAC TEPYIIAANR QLSQMHPVYR LLHPHFRFTM EINAQARAML INAGGIIEGS FVPGEYSLEL SSVAYDQQWR FDMEALPEDL IRRGMAVRNP
Lox2:Hv:2 VSHWLRTHCC VEPYVIAANR QLSQMHPIYR LLHPHFRFTM EINAQARGML ICADGIIEKT FSPGEFSMEI SSAAYDKQWR FDMEALPEDL IRRGMAVRGE
Lox2:Hv:3 VSHWLRTHAC VEPYIIATNR QLSRMHPVYR LLHPHFRYTM EINALAREAL INADGIIEEA FLAGKYSIEL SSVAYGAAWQ FNTEALPEDL INRGLAVRRD
Consensus VSHWLRTH-C -EPYIIA-NR QLS-MHPVYR LLHPHFRFTM EINA-AR--L I-A-GIIE-- F--G-YS-E- SS-AY---W- F--EALPEDL I-RG-AVR--

          701                                                                                                800
Lox2:Hv:1 NGELELAIED YPYANDGLLV WDAIKQWALT YVQHYYPCAA DIVDDEELQA WWTEVRTKGH ADKQDEPWWP ELDSHENLAQ TLATIMWVTS GHHAAVNFGQ
Lox2:Hv:2 DGKLELAIED YPYANDGLLV WDAIKQWASD YVAHYYPCAV DIVDDEELQD WWTEVRTKGH PDKQDEPWWP ELDCHESLVQ VLATIMWVTS AHHAAVNFGQ
Lox2:Hv:3 DGELELAIKD YPYADDGLLI WGSIKQWASD YVDFYYKSDG DVAGDEELRA WWEEVRTKGH ADKKDEPWWP VCDTKENLVQ ILTIIMWVTS GHHAAVNFGQ
Consensus -G-LELAI-D YPYA-DGLLV W--IKQWA-- YV--YY---- DI--DEEL-- WW-EVRTKGH -DK-DEPWWP --D--E-L-Q -L--IMWVTS -HHAAVNFGQ

          801                                                                                                900
Lox2:Hv:1 YPMAGYIPNR PTMARRNMPT EIG.GDDMRD FVEAPEKVLL DTFPSQYQSA IVLAILDLLS THSSDEEYMG THEEPAWTKD GVINQAFEEF KESTRKIVEQ
Lox2:Hv:2 YPMAGYVPNH PSIARRNMPC EMG.PEEMLA FKAAPEKVWL DTLPSQLQTV MVMATLDLLS SHASDEEYMG THQEPAWQRD GEVDKAFQVF QKKMRDIAEQ
Lox2:Hv:3 YHYAGYFPNR PTVVRRNIPV EENRDDEMKK FMARPEEVLL QSLPSQMQAI KVMATLDILS SHSPDEEYMG EYAEPAWLAE PMVKAAFEKF SGRLKEAEGT
Consensus Y--AGY-PN- P---RRN-P- E------M-- F---PE-V-L ---PSQ-Q-- -V-A-LD-LS -H--DEEYMG ---EPAW--- --V--AF--F ----------

          901                                  953
Lox2:Hv:1 VDEWNNDPDR KNRHGAGMVP YVLLRPSDGD PTDGDPTDEK MVMEMGIPNS ISI
Lox2:Hv:2 VEEWNKDDSR RNRHGAGVVP YVLLR..... PLNGNPMDAK TVMEMGIPNS ISI
Lox2:Hv:3 IDMRNNNPEN KNRCGAGIVP YELLK..... .....PFSEP GVTGRGIPNS ISH
Consensus V---N----- -NR-GAG-VP Y-LL------ -----P---- -V---GIPNS ISI
```

Fig. 5 Amino Acid Sequence Alignment of LOX Forms Found in Barley Leaves.
Putative chloroplast target peptides are underlined, conserved histidine residues for iron binding and the highly conserved C-terminus are boxed in black.

tween both sequences is 53.2%. Whereas *LOX2:Hv:3* showed 55.7% homology to the previously isolated *LOX2:Hv:1* (Vörös *et al.,* 1998), *LOX2:Hv:2* exhibited 73.5% homology. Using the program ChloroPV 1.1 (http://www.dtn.dk/services/ChloroP) a chloroplast target peptide was predicted for both polypeptides. Valine 40 was predicted to be the first amino acid of the mature peptide in case of *LOX2:Hv:2*, whereas in case of *LOX2:Hv:3* this was cysteine 50. A histidine triad (His 603, His 608, His 793), common to all plant LOX2-type amino acid sequences and suggested to be related to iron binding (Siedow, 1991), was found in *LOX2:Hv:2* and *LOX2:Hv:3* (Figure 5). In addition, corresponding asparagine and isoleucine residues, suggested to function in iron binding, were found at position 797 and 953, respectively

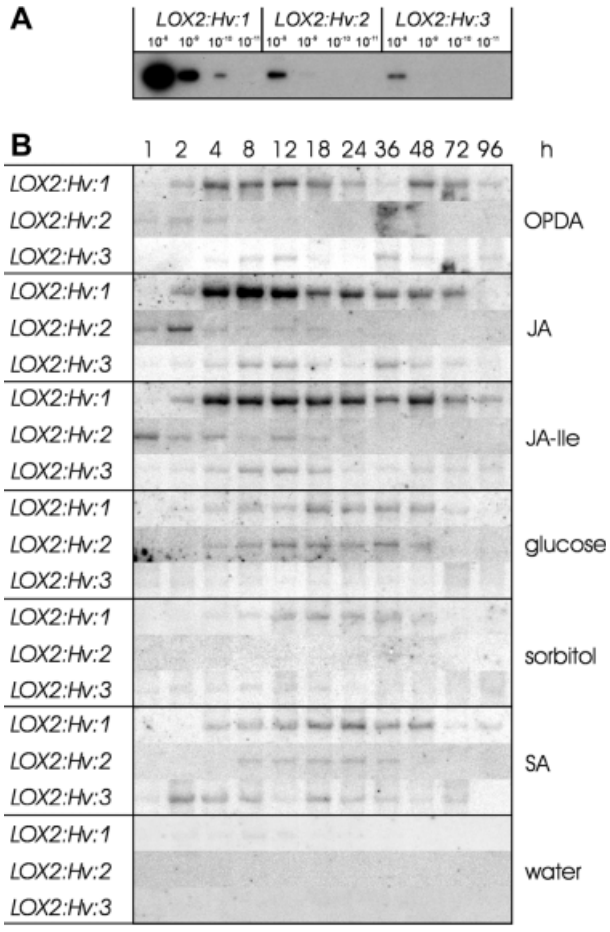

Fig. 6 Differential Induction of Different LOX Forms in Barley Leaves.
(A) To check the specificity of each LOX probe as well as the hybridization and washing conditions in each experiment, a membrane was added with serial dilutions of all LOX-containing plasmids from 10^{-8} to 10^{-11} g. In the example shown here the filter was hybridized with the *LOX2:Hv:1* probe, and cross-hybridization was 5% and 2%, respectively. (B) For analysis of differential induction of LOX forms, barley leaves were treated for indicated times with 50 μM OPDA, 50 μM JA, 50 μM JA-Ile, 0.5 M glucose, 1 M sorbitol or 50 μM SA. Identical Northern blots were probed with N-terminal fragments from *LOX2:Hv:1* (135 bp), *LOX2:Hv:2* (126 bp) and *LOX3:Hv:3* (114 bp).

(Siedow, 1991). The C-terminal motif GIPNSISI found in both new LOX sequences was identical to C-termini of *LOX1*-type polypeptides, whereas all other *LOX2*-type sequences known so far exhibit one or two amino acid substitutions in this region.

Comparative Expression Analysis of *LOX2:Hv:1*, *LOX2:Hv:2* and *LOX2:Hv:3*

The expression of the previously characterized *LOX2:Hv:1* was found to be strongly induced by JAME treatment and weakly induced by sorbitol treatment (Vörös *et al.,* 1998). In order to analyze differential expression of *LOX2:Hv:1*, *LOX:Hv:2*, and *LOX:Hv:3*, gene-specific probes were generated and used in Northern blot analyses. As indicated by dilution analysis shown in Figure 6A, all probes were specific, and *LOX2:Hv:1* mRNA appeared most abundantly in JA-treated leaves. Also with other treatments, mRNA of *LOX1:Hv:1* accumulated to higher levels than the two other LOX forms, and all accumulations of the different mRNAs appeared transiently (Figure 6B). Three main features are obvious:

(i) similar mRNA accumulation occurs for each LOX form in response to OPDA, JA and JA-Ile conjugate, suggesting common signaling properties of these compounds;
(ii) *LOX2:Hv:2* mRNA accumulated within 1 h clearly before *LOX2:Hv:3* mRNA and *LOX2:Hv:1* mRNA;
(iii) only weak mRNA accumulation occurred for *LOX2:Hv:1* and *LOX2:Hv:2* following glucose treatment as well as for *LOX2:Hv:1* following sorbitol treatment. All these accumulations started between 12 and 18 h and appeared later than those in response to OPDA, JA and JA-Ile;
(iv) SA treatment led to a slightly delayed *LOX2:Hv:1* mRNA accumulation in comparison to that following JA treatment. *LOX2:Hv:2* mRNA accumulated significantly earlier than *LOX2:Hv:1* mRNA following SA treatment.

Metabolite Profiling of the LOX Pathway in JAME-Treated Leaves and Subfractions of Chloroplasts

In order to follow the question of a physiological function of the JAME-induced LOXs, we recorded metabolite profiles of oxylipins upon JAME treatment of total lipid extracts of leaves. This serves as an indicator of the *in vivo* action of the different enzymes within the LOX pathway (Figure 1). In initial experiments we detected only 5% of the total lipid extract to be esterified LOX products. Therefore, for the work presented here only free fatty acid-derived LOX products were analyzed. Both possible substrates of the LOX reaction in plants, α-LeA and LA, accumulated transiently up to 72 h, when leaf segments were floated on JAME (Figure 7A, B), whereas controls (water treatment) remained unchanged. Both fatty acids exhibited maximal accumulation at about 18 h. α-LeA ac-

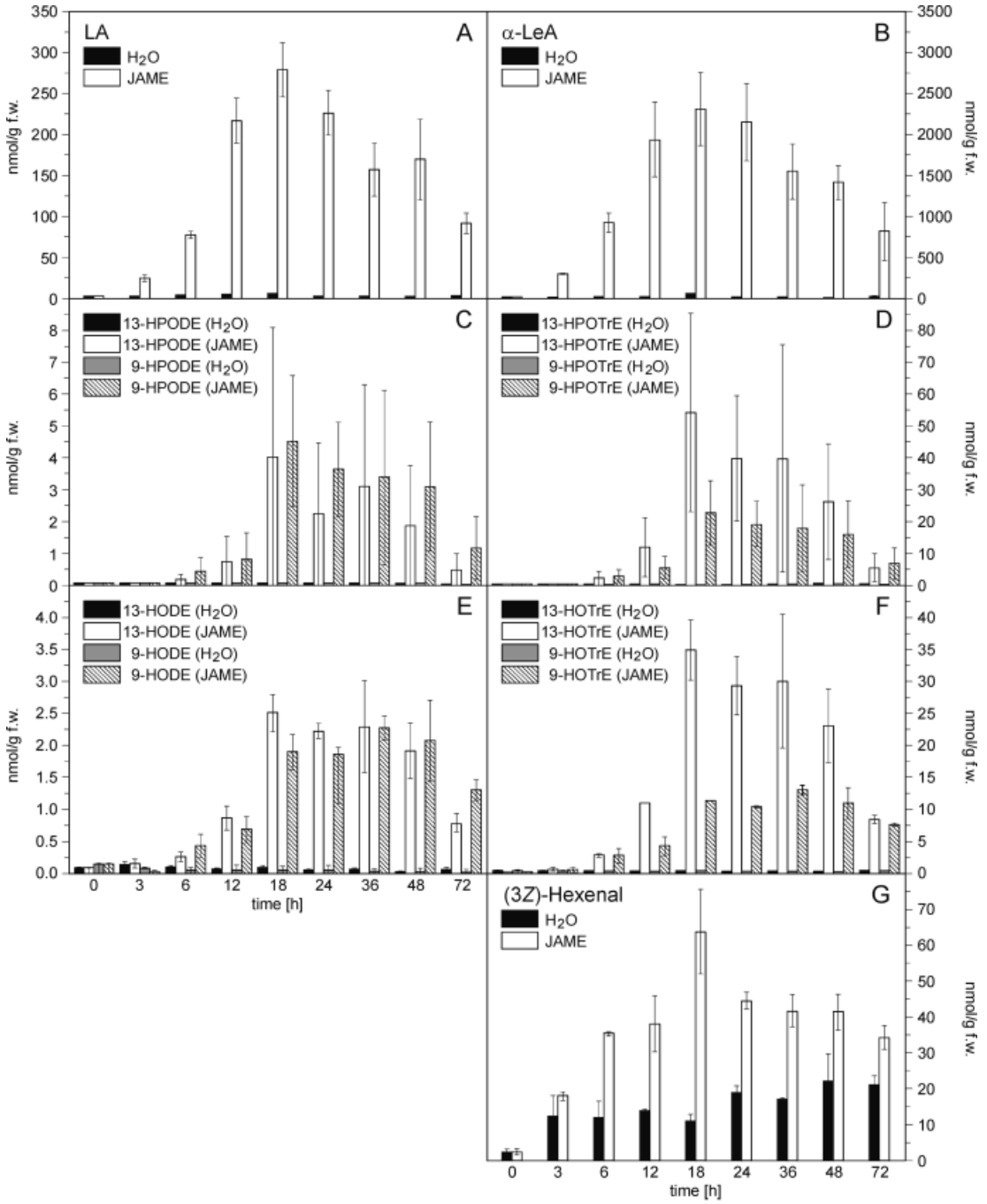

Fig. 7 Oxylipin Profiling of Barley Leaves Treated with JAME for Different Time Periods.
Metabolite profiling of LA (A), α-LeA (B) and oxylipins derived there from (C–G) in barley leaf segments floated on water or 50 μM JAME. As shown in (C), upon JAME treatment the prevailing LA-derived LOX product is 13-HPODE (open columns), whereas 9-HPODE (grey columns) accumulates much less. As shown in (D), upon JAME treatment the prevailing α-LeA-derived LOX product is 13-HPOTrE (open columns), whereas 9-HPOTrE (grey columns) accumulates much less. As shown in (E), upon JAME treatment the prevailing LA-derived reductase product is 13-HODE (open columns), whereas 9-HODE (grey columns) accumulates much less. As shown in (F), upon JAME treatment the prevailing α-LeA-derived reductase product is 13-HOTrE (open columns), whereas 9-HOTrE (grey columns) accumulates much less. As indicated in (G), JAME treatment led to an increase in endogenous amounts of leaf aldehydes such as (3*Z*)-hexenal (white columns) compared to the water control. The results represent the mean of two measurements performed with two independent flotation experiments.

cumulated up to 10-fold higher levels than LA, thereby reflecting the naturally existing ratio of about 10:1 between both free fatty acids in leaf tissues (Gunstone *et al.,* 1994).

Interestingly, JAME treatment led to the accumulation of the initial LOX products, the HPODE derived from LA and the HPOTrE derived from α-LeA (Figure 7C, D). In case of LA-derived LOX products both positional isomers, 13-HPODE and the 9-HPODE, accumulated to a

similar amount throughout the time of JAME treatment (Figure 7C), indicating either the lack of positional specificity of the induced LOXs with LA as substrate or the concerted action of a 13-LOX and a 9-LOX. However, a significantly higher amount of HPOTrEs was found and, moreover, a lower amount of 9-HPOTrE than 13-HPOTrE was detected (Figure 7D), indicating that α-LeA is the preferred substrate of the induced 13-LOXs. All LOX products accumulated up to a detectable level upon JAME treatment, but not by water treatment. Moreover, no positional isomers such as 12- or 16-H(P)OTrE which are indicative for autoxidation (Rusterucci *et al.*, 1999; Berger *et al.*, 2001) had been observed in JAME-treated barley leaves. All accumulation profiles were transiently within 72 h of JAME treatment peaking at about 18 h (Figure 7C, D). Similar to the accumulation of LA and α-LeA, the accumulated amounts of the HPODEs and the HPOTrEs, respectively, were in a ratio of 1:10, thus reflecting the ratio of LA:α-LeA levels in leaf tissues. As reported previously, lipid hydroperoxides are unstable compounds, and therefore they may not be stable completely during the work-up and analysis procedure used here (Feussner *et al.,* 1997). This is reflected by the high variation within all measured amounts of these derivatives.

The intermediate accumulation of free LOX-derived lipid hydroperoxides raises the question in which of the various branches of the LOX pathway (Figure 1) these hydroperoxy compounds are used as a preferential substrate. For all time periods of JAME treatment no divinyl ether derivatives such as colnele(n)ic acid or etherole(n)ic acid and no OPDA and JA/JAME were found, indicating that the DES and the AOS branch is either not present at detectable levels or not activated by JAME treatment in barley. A remarkable accumulation, however, of the 9- and 13-isomers of HODE and HOTrE, respectively, could be detected (Figure 7E, F). This indicates a JAME-induced activation of the reductase branch of this metabolic pathway. Again, for all times of JAME treatment, hydroxy compounds originating from α-LeA were found at about 10-fold higher levels than that originating form LA. Similar to the LOX-derived products 13-HPODE and 9-HPODE, the reductase products 13-HODE and 9-HODE accumulated at similar levels throughout the JAME treat-

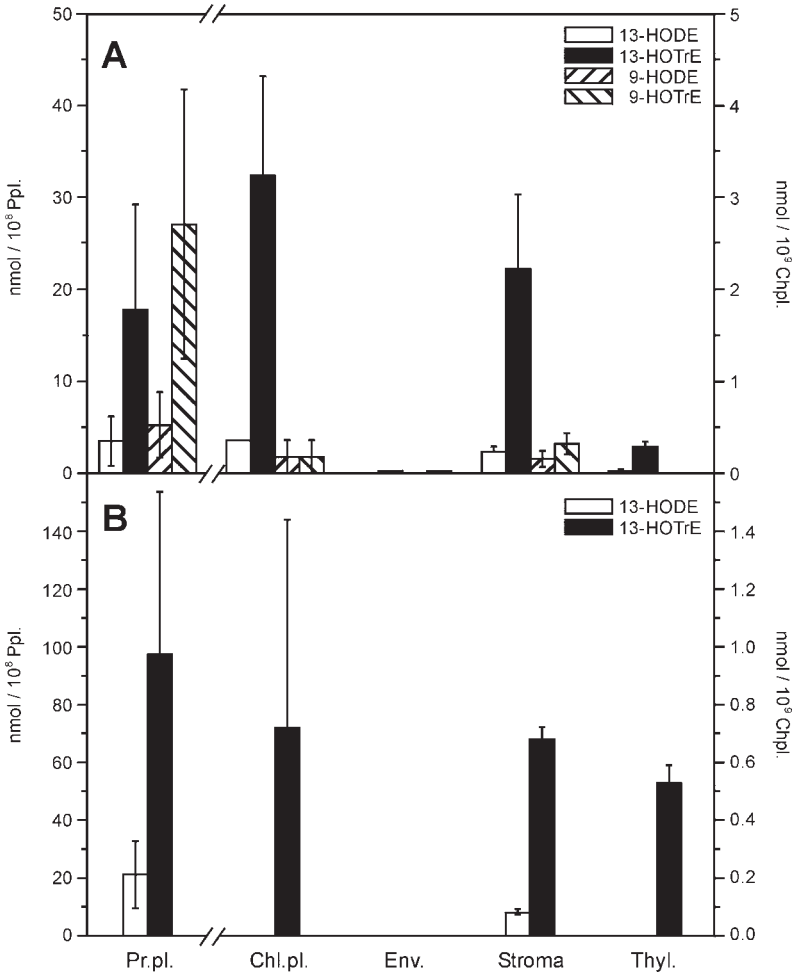

Fig. 8 Oxylipin Profiles of Protoplasts, Chloroplasts and Subfractions of Chloroplasts Isolated from Barley Leaves Freshly Harvested (A) or Treated with 45 μM JAME for 24 h (B).
9-HODE and 9-HOTrE were below the detection limit in the experiment shown in (B). The results represent the mean of two independent experiments.

ment, starting to be maximal at about 18 h (Figure 7E). In contrast, among the reductase products 13-HOTrE and 9-HOTrE originating from α-LeA, 13-HOTrE accumulated transiently with a peak at about 18 h and accumulated to clearly higher levels than 9-HOTrE (Figure 7F), as it was the case for the LOX-derived hydroperoxy fatty acids (Figure 7D).

For both, the LA- and the α-LeA-derived compounds, the overall accumulation of reductase products was about half of that occurring for LOX-derived hydroperoxy compounds (Figure 7E, F *vs*. C, D). Interestingly, HPL-derived aldehydes such as (3*Z*)-hexenal accumulated to a similar level as the HPL-substrate 13-HPOTrE (Figure 7G *vs*. D). Although there is a higher basal level of aldehydes by water treatment of leaf segments compared to that of the hydroxy and the hydroperoxy compounds, the accumulation of (3*Z*)-hexenal upon JAME treatment clearly indicates the activation of the HPL branch of the LOX pathway exceeding that of the reductase branch. Due to the earlier accumulation of (3*Z*)-hexenal, first detected at 3 h compared to that of the 13-HPOTrE (Figure 7G *vs*. D), there seems to be a remarkable flux into the HPL branch before 18 h of treatment.

The activity of different LOX forms indicated by these metabolic profiling data from whole leaf extracts was further investigated by analyzing protoplasts, chloroplasts and subfractions of chloroplasts all of them isolated either from freshly harvested leaves (Figure 8A) or from 24 h-JAME-treated leaf segments (Figure 8B). Since (3*Z*)-hexenal is a volatile compound that most likely escapes from the samples during the whole work-up procedure, in this experiment only the LOX-derived fatty acid derivatives were analyzed. The pattern of LOX products formed by chloroplast subfractions showed again a preferential accumulation of 13-LOX-derived 13-HOTrE with a specific localization in the stroma fraction. This may indicate again a preferred activity/amount of 13-LOXs in this subcompartment. Also upon 24 h JAME treatment 13-HOT accumulates slightly more in the stroma fraction than in the thylakoid fraction. The profiling data suggest high 13-LOX activity against free α-LeA as substrate in these chloroplast subfractions. In contrast, the preferential appearance of 9-HOTrE in protoplasts of untreated leaves compared to residual amount in chloroplasts is indicative for cytosolic location of 9-LOX protein. Taken together, the metabolic profiling data revealed individual functions of LOX forms following JAME treatment including a preferential activity in the stroma fraction of chloroplasts.

Discussion

Leaf senescence is accompanied by a dramatic alteration of gene expression. Transcript abundance during senescence has been described for more than 100 genes including nucleases, lipases, chlorophyllases, proteinases and other enzymes involved in nutrient salvage. The complex nature of senescence has been recently demonstrated by isolation of *Arabidopsis* senescence-associated genes which exhibit in the expression pattern a hierarchical link to external stimuli such as jasmonate, ethylene, desiccation or darkness (He *et al.,* 2001). Promotion of leaf senescence by application of jasmonates has been known for the last decade (Parthier, 1990) and has been recently substantiated by data on a concomitant activation of genes encoding enzymes of jasmonate biosynthesis (He *et al.,* 2002), or lipid degradation (He and Gan, 2002).

In barley leaves the senescence-promoting effect of jasmonates is much stronger than in other plants (Parthier, 1990). This is indicated by an early loss of chlorophyll, by immediate down-regulation of genes encoding proteins of the photosynthetic apparatus, and by the rapid formation of a specific subset of proteins, the jasmonate-induced proteins (JIPs) (Wasternack and Parthier, 1997; Wasternack and Hause, 2002).

One of these JIPs was identified as a chloroplast-located 13-LOX (Feussner *et al.*, 1995a; Vörös *et al.*, 1998) suggesting a causal link between JA, LOX and senescence. Therefore, we used JAME-treated barley leaf segments as a model system to analyze the LOX-mediated lipid peroxidation during JAME-induced senescence in molecular, biochemical and ultrastructural parameters. In addition, we wanted to know whether there might be a contribution of autoxidative lipid peroxidation during the senescence process in barley as was found recently for *Arabidopsis* leaves (Berger *et al.,* 2001).

Multiple LOX forms seem to be a common feature of many plants to alter free fatty acids and their esterified counterparts during developmental and environmental processes (Feussner and Wasternack, 2002). For germinating barley seedlings two LOX forms were characterized to be a 9-LOX (LOX-1) and a 13-LOX (LOX-2), respectively (van Aarle *et al.*, 1991; Doderer *et al.*, 1992). LOX-2 is newly synthesized in embryo development and is thought to be involved in degradation of storage lipids during germination (Holtman *et al.,* 1997), whereas LOX-1 appeared in quiescent grains as well. In contrast, barley leaves contain at least three LOX forms (Feussner *et al.*,1995a). Here we showed their concomitant induction by JA treatment and isolation of two further LOX cDNAs. Both of them exhibit a putative target sequence for chloroplast-import, 13-LOX activity as recombinant protein and sequence characteristics common to all plant *LOX2*-type LOX forms.

The comparative expression analysis for *LOX2:Hv:1*, *LOX2:Hv:2* and *LOX2:Hv:3* revealed common activation in response to JA, OPDA and JA-Ile, which is indicative for common signaling properties of these compounds. *LOX2:Hv:2*-mRNA accumulated already within 1 h of treatment contrasting from the maximal mRNA accumulation of *LOX2:Hv:1* and *LOX2:Hv:3* between 12 and 18 hours. Such a temporal difference in the expression of various LOX forms was repeatedly observed, *e.g*. in potato (Royo *et al.,* 1996) and tomato (Heitz *et al.,* 1997).

The differential activation by JAME of three 13-LOX

forms, presumably all of them located within chloroplasts, suggests that lipid degradation of chloroplast membranes is an early event in JA-induced senescence. Membrane degradation is visible as a feature of dramatically altered ultrastructure within the chloroplast. The number of well-structured thylakoid membranes and stacks decreased already within 24 h of JAME treatment (Figure 3). The JAME-induced activation of 13-LOX forms and the ultrastructural changes within the chloroplast following JA treatment are reflected by an enhanced activity level (Feussner *et al.*, 1995b) and in the metabolite profiling data (Figures 7 and 8).

Free α-LeA and LA accumulate dramatically after several hours of JAME treatment. Among the various phospholipases (PLA) known to be JA-inducible, a leaf homolog of the recently cloned chloroplast-located PLA_1 (Ishiguro *et al.,* 2001) might the responsible for release of α-LeA and LA. Also senescence-related acyl hydrolases may attribute to this rise (Hong *et al.*, 2000; He and Gan, 2002).

Most interestingly, the hydroperoxy compounds HPODE (derived from LA) and HPOTrE (derived from α-LeA) were detectable upon JAME treatment preferentially as the 13-isomer of HPOTrE (Figures 7 and 8). This (i) reflects the preferential occurrence of α-LeA in chloroplast membranes, and (ii) illustrates the activity of the JAME-induced 13-LOX forms characterized before and in this report. Other abundant oxylipins are 13-HOTrE, the product of the reductase branch, and (3*Z*)-hexenal, a product of the HPL branch. The additional formation of other oxylipins including jasmonates was not observed in barley leaves following JAME treatment (Kramell *et al.,* 2000). In contrast to the natural senescence in trees and *Arabidopsis* leaves (Berger *et al.,* 2001), oxylipins indicative for autoxidative processes have not been observed.

Hydroperoxide derivatives are highly toxic compounds, but lipid hydroperoxides have been found at substantial amounts in lipid bodies of cucumber and sunflower cotyledons under physiological conditions without any toxic effects (Feussner *et al.,* 1997, 1998a,b; Weichert *et al.,* 2002). This may be due to the complete separation of toxic lipid peroxides from sensitive metabolic reactions, *i.e*. glycolysis in the cytosol during germination. The data presented here for barley leaves show for the first time the occurrence of 13-LOX-derived lipid hydroperoxides as non-esterified constituents even in the chloroplast. There is no hint that these hydroperoxide compounds are separated from other chloroplast constituents. It is tempting to assume that the preferential accumulation of 13-HPOTrE may be a main reason for the senescence-promoting effect of JAME in this system. In non-treated barley leaves only HOTrEs are found in the stroma of chloroplasts, but following JAME treatment an increased accumulation of 13-H(P)OTrE is found in the stroma and most importantly in thylakoid membranes. This massive increase in the accumulation of specific peroxides is most likely caused by a loss of autoxidative capacity of chloroplast membranes known to occur during senescence (Munné-Bosch *et al.,* 2001). Moreover, a misallocation of oxylipins toward the thylakoids may be a reason for their observed destruction and the disappearance of their stacks. Taken together, data obtained for chloroplastic 13-LOXs from barley leaves show that monocot plants as dicot plants contain a whole set of isoforms which are tightly regulated upon changes in environmental conditions. However, if they are induced to a certain threshold, their products may have toxic effects and may cause the destruction of organellar membranes as shown here and also described for the involvement of mammalian 15-LOX forms in the degradation of mitochondrial membranes (Schewe and Kühn, 1991).

Materials and Methods

Chemicals

Standards of fatty acids as well as all other chemicals were obtained from Sigma (Deisenhofen, Germany); oxylipins were from Cayman Chemicals (Ann Arbor, MI, USA), methanol, hexane, 2-propanol (all HPLC grade) were from Baker (Deventer, The Netherlands).

Plant Growth

In all experiments primary leaves of 7-day-old seedlings of barley (*Hordeum vulgare* L. cv. Salome) were used. Growth of seedlings was performed as described (Feussner *et al.,* 1995a; Hause *et al.,* 1999). Treatments were performed by floating leaf segments of 5 cm length on water (control) or on 50 μM solutions of OPDA, JAME, JA, JA-Ile, on 1 M solution of sorbitol or 0.5 M solutions of glucose at 25 °C under continuous white light (120 μmol photons $m^{-2} \cdot s^{-1}$) provided by a fluorescent lamp (NARVA) for indicated time periods. Upon sampling fresh weight (about 1 g per time point) was removed, and leaf tissue was subsequently frozen in liquid nitrogen.

Electron Microscopy

For analysis of the ultrastructure of chloroplasts, small pieces of leaf segments (untreated or treated with 45 μM JAME for the indicated time periods) were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 2 h at RT. After rinsing with buffer, samples were postfixed with 1% (w/v) osmium tetroxide in buffer for 1 h, rinsed again and dehydrated in a graded ethanol series. Uranyl acetate (1% w/v) was added to the 70% ethanol step and applied for 1 h. Ethanol was substituted by epoxy resin (Spurr, 1969) and samples were polymerized at 70 °C. Ultrathin sections were post-stained with uranyl acetate and lead citrate.

In order to localize LOX protein within chloroplasts, small pieces of leaf segments were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 2 h at RT. After rinsing with buffer, samples were dehydrated in a graded ethanol series. Ethanol was substituted by LR-White (Polysciences, Warrington, PA, USA). Immunolabeling of ultrathin sections was carried out with a rabbit anti-LOX antibody (Feussner and Kindl, 1994) and a goat anti-rabbit IgG conjugated with 10 μm colloidal gold (Biotrend, Cologne, Germany). After immunolabeling, sections were post-stained with uranyl acetate and lead citrate. Control experiments were performed by use of pre-immune serum and revealed no signals. Sections were visualized with a Zeiss TEM 900 electron microscope.

Western and Northern Blotting

Western blot analysis of LOX forms was performed as described (Feussner and Kindl, 1994). Northern blot analyses were performed according to Sambrook *et al.* (1989), by using the formaldehyde gel electrophoresis method and nylon membranes as carrier. Hybridization was performed at 65 °C followed by washing steps at 65 °C for 3 times 5 min in 2× SSC, 0.1% SDS, 15 min in 1× SSC, 0.1% SDS, 15 min in 0.5× SSC, 0.1% SDS, 15 min in 0.25× SSC, 0.1% SDS, 15 min in 0.125× SSC, 0.1% SDS. Each hybridization experiment was paralleled by a control blot with serial dilutions of the three LOX cDNA probes.

Oxylipin Profiling

The analysis of LOX-derived products was performed as described before using some modifications (Weichert *et al.,* 1999). In initial experiments we detected only 5% of the total lipid extract to be esterified LOX products. Therefore, for the work presented here only free LOX products were analyzed. Frozen leaf tissue corresponding 1 g fresh weight was added to 20 ml of extraction medium (hexane/isopropanol, 3:2 v/v with 0.0025% w/v butylated hydroxytoluene) and was immediately homogenized with an Ultra Turrax for 30 s under a stream of argon on ice. The extract was centrifuged at 4500 *g* at 4 °C for 15 min. The clear upper phase was collected, and the pellet was extracted three times with 3 ml each of the extraction medium. To the combined organic phases a 6.7% (w/v) solution of potassium sulfate was added up to a volume of 47 ml. After vigorous shaking the upper hexane-rich layer was removed. The remaining organic phase, which contained potentially oxylipins, was subsequently dried under a nitrogen stream. The remaining lipids were redissolved in 1 ml of chloroform and stored under argon atmosphere at –80 °C until use. At first, oxylipins were either isolated (hydroperoxy PUFA and hydroxy PUFA isomers) or analyzed (divinyl ethers) by reversed phase HPLC (RP-HPLC) on a Nucleosil C-18 column (Macherey-Nagel, KS-system, 250 × 4.6 mm, 5 µm particle size) with a solvent system of methanol/ water/acetic acid (85:15:0.1 v/v) and a flow rate of 1 ml/min. For detection of the hydroperoxy PUFAs and hydroxy PUFAs, the absorbance at 234 nm indicating the conjugated diene system was recorded. For analysis of PUFAs, the absorbance at 210 nm indicating the double bond of the fatty acids was recorded. For analysis of divinyl ethers, the absorbances at 250 nm and 268 nm indicating either one or two conjugated diene systems in conjugation to an ether bond of the fatty acids were recorded. The different vinyl ether isomers eluted between 7.3 min (etherolenic acid) and 8.6 min (colneleic acid). Separation of hydroperoxy PUFA and hydroxy PUFA isomers was carried out by solid phase HPLC (SP-HPLC) on a Zorbax SIL column (HP, 250 × 4.6 mm, 5 µm particle size) with a solvent system of *n*-hexane/2-propanol/acetic acid (100:1:0.1 v/v) and a flow rate of 1 ml/min. The enantiomer composition of hydroperoxy PUFAs as well as of the hydroxy PUFAs was analyzed by chiral phase-HPLC (CP-HPLC) on a Chiralcel OD column (Diacel Chem. Industries, 250 × 4.6 mm, 5 µm particle size; purchased from Baker, Deventer, The Netherlands) with a solvent system of hexane/2-propanol/acetic acid (100:5:0.1 v/v) and a flow rate of 1 ml/min. All substances were identified by their characteristic UV spectra and by co-injection of authentic standards. Calibration curves (five point measurements) for LA, α-LeA and 13-HOTrE were established. For quantification as internal standard (13*S*,6*Z*,9*Z*,11*E*)-13-hydroxy-6,9,11-octadecatrienoic acid was used.

The analysis of LOX-derived aldehydes was performed as described before using some modifications (Kohlmann *et al.,* 1999). One g of frozen leaf tissue was added to 10 ml of extraction buffer (methanol:2 mM HCl, 1:1 v/v, pH 3.0, with 0.001% w/v butylated hydroxytoluene), containing 20 ng of (2*E*)-pentenal as internal standard. The solution was immediately homogenized with an Ultra Turrax for 30 s under a stream of argon on ice. The extract was centrifuged at 4500 *g* at 4 °C for 25 min. The clear upper phase was collected, and the pellet was extracted twice with 5 ml each of extraction buffer. In order to form the 2.4-dinitrophenylhydrazone (DNPH) derivatives of aldehydes the collected upper phases were stirred in presence of 1 ml of DNPH-reagent (2.4-dinitrophenylhydrazine, 0.1% w/v in 1 M HCl) at room temperature for 1 h. The reaction mixture was extracted three times with 5 ml hexane each, and the collected organic phases were dried under a stream of N_2. DNPH derivatives were redissolved in 400 µl of acetonitrile. HPLC analysis was carried out by RP-HPLC of the DNPH derivatives on a Jupiter C-18 300A column (250 × 1.0 mm, 5 µm particle size, Phenomenex, Germany) using a binary gradient system (solvent A: acetonitrile:water, 60:40 v/v; solvent B: acetonitrile:water, 80:20 v/v) with the following gradient program: 100% solvent A for 15 min, followed by a linear increase of solvent B up to 50% solvent B within 5 min, by a linear increase of solvent B up to 100% within 13.4 min and finally by an isocratic postrun at 100% solvent B for 11.6 min. The flow rate was 0.05 ml/min. For analysis of the DNPH derivatives, the absorbance at 365 nm indicating the DNPH chromophore was recorded. Hexenal was identified by its characteristic UV spectrum and by co-injection of an authentic standard. A calibration curve (five-point measurement) was established.

Isolation of LOX cDNAs

Total RNA was isolated as described (Chirgwin *et al.,* 1979) with some modifications. The plant material was ground under liquid nitrogen and homogenized in 5 w/v buffered 4 M guanidinium thiocyanate, 100 mM Tris-HCl, 25 mM EDTA, 100 mM β-mercaptoethanol, 2% N-lauroylsarcosin, pH 7,5. Following centrifugation at 10 000 *g* for 10 min at 4 °C, the supernatant was extracted with phenol:chloroform:isoamylalcohol (25:24:1; v/v/v) and nucleic acids from the upper phase were precipitated with ethanol. RNA from solubilized pellets were precipitated overnight with 2 M LiCl. Total RNA was dissolved in water. For poly(A)-RNA isolation, Oligotex particles (Qiagen, Hilden, Germany) were used according to the manufacturer's instruction.

cDNA synthesis was performed with 5 µg of poly(A)-RNA isolated from barley leaves treated for 12 h with 1 M sorbitol using the Stratagene ZAP Express cDNA synthesis kit.

Differential library screening

First screening was done with 500 000 phage plaques and an oligonucleotide probe deduced to the 5'-end of *LOX2:Hv:1*. Prehybridization was performed at 54 °C in 6× SSC, 5× Denhardt's reagent, 1% SDS, 100 µg/ml Salmon sperm DNA followed by 16 h hybridization with the radiolabeled oligonucleotide 5'-G_{2374}ATAGCCATGAGAACCTGGCGCAGACC$_{2400}$-3' at 54 °C in the same solution. Membranes were washed at 60 °C for 3 times 5 min in 6× SSC and 0.1% SDS, followed by 2 times 15 min in 1× SSC and 0.1% SDS. For the second screening membranes were stripped at 95 °C for 2 × 5 min in 0.1% SDS. The second screening was performed with a radiolabeled probe of *LOX2:Hv:1*. The pre-hybridization, hybridization and washing steps of the membranes were done in the same solutions as mentioned above at 60 °C with a final washing step for 15 min in 0.1× SSC and 0.1% SDS. Positive plaques hybridizing to the cDNA but not to the oligonucleotide probe were purified and converted into plasmids by *in vivo* excision. Sequence analysis of plasmid clones was performed with the dideoxynucleotide

chain termination method using Thermo Sequenase® Cycle Sequencing kit (Amersham, Pharmacia Biotech Europe, Freiburg, Germany).

Bacterial Expression and Enzymatic Characterization of LOX cDNAs

For bacterial expression of the different putative LOX clones the plasmid pQE-30, pQE-31 and pQE-32 (Qiagen) was used as described (Feussner *et al.,* 1998b). pLOX2/10 was cloned into pQE-31 by using *Bam*HI and *Kpn*I as restriction sites. The missing 5'-end, was amplified by PCR with 5'-GAC$\underline{\text{GGATCC}}_{263}$AATGGGCCAGCCCTCACCACG-3´ and 5´-G_{472}TTTTT$\underline{\text{GGATCC}}$AACTCGGAGCTGAC$_{447}$-3' as primers and was introduced at the *Bam*HI site. pLOX3/15 was cloned into pQE-32 using the *Sal*I restriction site at the 5'-end and a filled *Xba*I (cDNA), *Hin*dIII (vector) at the 3'-end. During ligation a *Xba*I site was generated and the *Hin*dIII site was destroyed.

The pQE-30, pQE-31 and pQE-32 plasmids containing putative LOX-cDNAs were expressed in *E. coli* (strain SG13009 [pREP4]). At least three different bacterial clones were used for analysis of enzymatic activity. Clones were cultured at 37 °C in LB medium containing 25 µg/ml kanamycin, and 100 µg/ml carbenicillin up to an absorbance of about 0.7 at 600 nm. Expression was induced by addition of IPTG to a final concentration of 2 mM followed by culturing at 10 °C for 18 – 20 h. Cells from a 10 ml culture were spun down, resuspended in lysis-buffer (50 mM Tris-HCl, pH 7.5, containing 10% v/v glycerol, 0.1% Tween 20, 0.5 M NaCl) and sonicated. The cellular debris was pelleted. 0.1 ml of the supernatant was incubated with final concentrations of 0.9 mM LA or arachidonic acid in 100 mM Tris-HCl buffer, pH 7.5, for 30 min at room temperature. Reactions were stopped by the addition of sodium borohydride to reduce the LOX-derived hydroperoxy fatty acids to their corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (Bligh and Dyer, 1959). The lower chloroform-phase was recovered, the solvent was evaporated, the remaining lipids were reconstituted in 0.1 ml methanol, and aliquots were subjected to HPLC analysis (see above).

Enzyme Activity Test

LOX activity was measured quantitatively by using a spectrophotometric assay. Thus, the formation of the conjugated diene system within the substrate was followed by measuring the increase in absorbance at 234 nm in the appropriate buffers (pH 7.3 at 25 °C as described by Vörös *et al.,* 1998). The substrate concentration was 20 µM (α-LeA and arachidonic acid) and the reaction was started with the addition of the enzyme.

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Induction of Jasmonate Biosynthesis in Arbuscular Mycorrhizal Barley Roots[1,2]

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Colonization of barley (*Hordeum vulgare* cv Salome) roots by an arbuscular mycorrhizal fungus, *Glomus intraradices* Schenck & Smith, leads to elevated levels of endogenous jasmonic acid (JA) and its amino acid conjugate JA-isoleucine, whereas the level of the JA precursor, oxophytodienoic acid, remains constant. The rise in jasmonates is accompanied by the expression of genes coding for an enzyme of JA biosynthesis (allene oxide synthase) and of a jasmonate-induced protein (JIP23). In situ hybridization and immunocytochemical analysis revealed that expression of these genes occurred cell specifically within arbuscule-containing root cortex cells. The concomitant gene expression indicates that jasmonates are generated and act within arbuscule-containing cells. By use of a near-synchronous mycorrhization, analysis of temporal expression patterns showed the occurrence of transcript accumulation 4 to 6 d after the appearance of the first arbuscules. This suggests that the endogenous rise in jasmonates might be related to the fully established symbiosis rather than to the recognition of interacting partners or to the onset of interaction. Because the plant supplies the fungus with carbohydrates, a model is proposed in which the induction of JA biosynthesis in colonized roots is linked to the stronger sink function of mycorrhizal roots compared with nonmycorrhizal roots.

Arbuscular mycorrhizas (AMs) are the most common type of mycorrhizas (for review, see Smith and Read, 1997). AMs are formed between roots of more than 80% of all terrestrial plant species and Zygomycete fungi from the order Glomales. The fungus is able to grow into the root cortex by forming intraradical hyphae, which are subsequently differentiated into highly branched structures, the arbuscules, within cortex cells. Intraradical hyphae and arbuscules are responsible for exchange of nutrients between the plant and the fungus. The plant supplies the fungus with carbohydrates, whereas the fungus assists the plant with the acquisition of phosphate and other mineral nutrients from the soil (Harrison, 1998). The beneficial effects of the AM symbiosis result from a complex molecular dialogue between the two symbiotic partners (Harrison, 1999). Some processes occurring in this dialogue are known to be mediated by phytohormones on the plant side. However, most of these phytohormone effects were suggested from application experiments (Barker and Tagu, 2000). A possible role for abscisic acid in the establishment of mycorrhiza was suggested from the fact that the endogenous content of abscisic acid was increased in mycorrhizal roots, but not in nonmycorrhizal roots (Bothe et al., 1994). In a previous study, the establishment of AM in barley (*Hordeum vulgare*) roots was shown to be accompanied by the accumulation of putrescine and agmatine amides of 4-coumarate and ferulate, respectively, compounds that are also accumulated upon treatment of nonmycorrhizal barley roots with jasmonates (Peipp et al., 1997). This suggests a possible role of jasmonates in AM formation.

Jasmonic acid (JA) and its derivatives, commonly termed jasmonates, are hormonal regulators involved in plant responses to abiotic and biotic stresses, as well as in plant development (Creelman and Mullet, 1997; Wasternack and Parthier, 1997). The role of jasmonates is well established as part of a complex signal transduction pathway activated upon local wounding of leaves (Ryan, 2000). Levels of endogenous jasmonate increase upon wounding and are followed by activation of genes involved in plant defense responses such as those coding for proteinase inhibitors, enzymes of phytoalexin synthesis, vegetative storage proteins, thionins, and defensins (Creelman and Mullet, 1997; Farmer et al., 1998; Ryan, 2000). However, it is less well understood how the rise of jasmonates is regulated. The elevation of jasmonate levels is usually correlated with the activation of genes coding for JA biosynthetic enzymes (for review, see Wasternack and Hause, 2002).

The biosynthesis of JA (Fig. 1) starts with the insertion of oxygen at position 13 of α-linolenic acid catalyzed by 13-LOX. The resulting hydroperoxide is converted by AOS into an unstable allene oxide that can rapidly be degraded in vitro by chemical hydrolysis. Under cellular conditions, the allene oxide is

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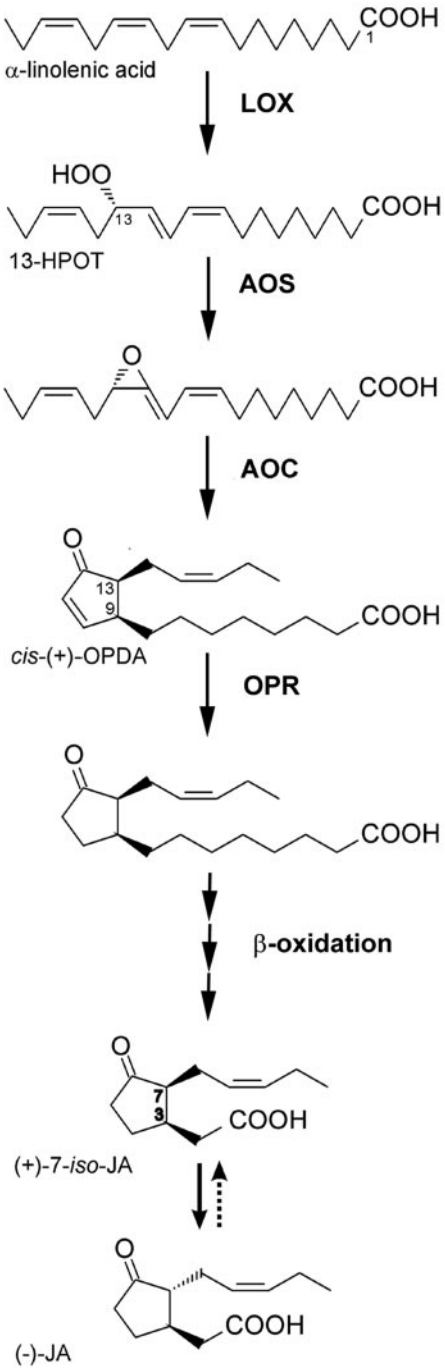

Figure 1. Metabolic scheme of JA biosynthesis. The enzymes involved in JA biosynthesis are 13-lipoxygenase (13-LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), and oxophytodienoic acid reductase (OPR).

preferentially, if not exclusively, converted by AOC into (9*S*, 13*S*)-oxophytodienoic acid (OPDA). According to the present knowledge, this enantiomer is the unique precursor for the naturally occurring (+)-7-*iso*-JA, which is formed by reduction of OPDA catalyzed by an OPDA reductase and three subsequent steps of β-oxidation. The more stable (–)-JA is then formed by spontaneous isomerization.

In barley, as in other plants, the genes coding for 13-LOX (Vörös et al., 1998), AOS (Maucher et al., 2000), and AOC (H. Maucher, personal communication) are transcriptionally activated upon treatment with jasmonates. Osmotic stress, caused experimentally by sorbitol treatment or appearing tissue specifically in seedling development, also induces the expression of *AOS* and *AOC*, and is strictly correlated with elevation of JA levels (Maucher et al., 2000; H. Maucher, personal communication). Such an endogenous rise in jasmonates is functionally relevant because jasmonate-induced gene expression occurs. The most abundant gene product occurring in barley leaves upon JA treatment or upon endogenous rise of JA is a 23-kD protein (JIP23; Andresen et al., 1992; Lehmann et al., 1995). JIP23 is always detectable after the elevation of jasmonate levels (Kramell et al., 2000). Therefore, the occurrence of JIP23 is a valuable reporter of endogenous rise of jasmonates as used for the analysis of the pathogenic interaction of barley leaves with powdery mildew (Hause et al., 1997). Also, in other tissues of the barley plant, there is a strict correlation of the expression of *JIP23* and enhanced endogenous JA levels. *JIP23* is expressed constitutively in the root tip, the scutellar node, and the leaf base, which are tissues that show enhanced JA levels (Hause et al., 1996; Maucher et al., 2000). Furthermore, the elevated JA level in these barley tissues correlates with *AOS* expression, suggesting a causal link between expression of genes coding for JA biosynthetic enzymes, elevation of JA levels, and expression of JA-induced genes (Maucher et al., 2000). Therefore, simultaneous recording of the expression of *AOS* and *JIP23* and JA levels represents a tool for asking whether an increase in JA biosynthesis is correlated with expression of JA-biosynthetic genes and JA-dependent processes.

In the present work, we show for the first time, to our knowledge, that the interaction of barley roots with a mycorrhizal fungus leads to marked increases of JA levels. To analyze the possible involvement of jasmonates in the establishment of AMs, we recorded JA levels, as well as the temporal and spatial expression patterns of genes coding for AOS and JIP23. The data revealed expression of both genes within arbuscule-containing cells after the onset of arbuscule formation. We discuss a possible link between the enhanced sink function of mycorrhizal roots compared with nonmycorrhizal roots and the induction of JA biosynthesis.

RESULTS

Elevated Jasmonate Levels in Mycorrhizal Roots Are Accompanied by Expression of a Gene Encoding a Pivotal Enzyme of JA Biosynthesis

To analyze a possible link between mycorrhization and endogenous levels of jasmonates, mycorrhization rates, and contents of JA, JA-Ile and OPDA were

determined during the time course of mycorrhization of barley roots with the AM fungus *Glomus intraradices*. Inoculation with fungal spores according to the standard protocol (Maier et al., 1995) led to an increase of the mycorrhization rate from 20% at week 3 to 60% at week 8 (Fig. 2A). The JA level increased in the mycorrhizal roots up to 4-fold between weeks 3 and 4 of cultivation, but remained constant at a low level in nonmycorrhizal roots (Fig. 2A). Also, the level of the major amino acid conjugate of JA, JA-Ile, exhibited a transient rise upon mycorrhization. The level of the JA precursor OPDA remained nearly constant at a basal level of about 0.2 nmol g^{-1} fresh weight during mycorrhization.

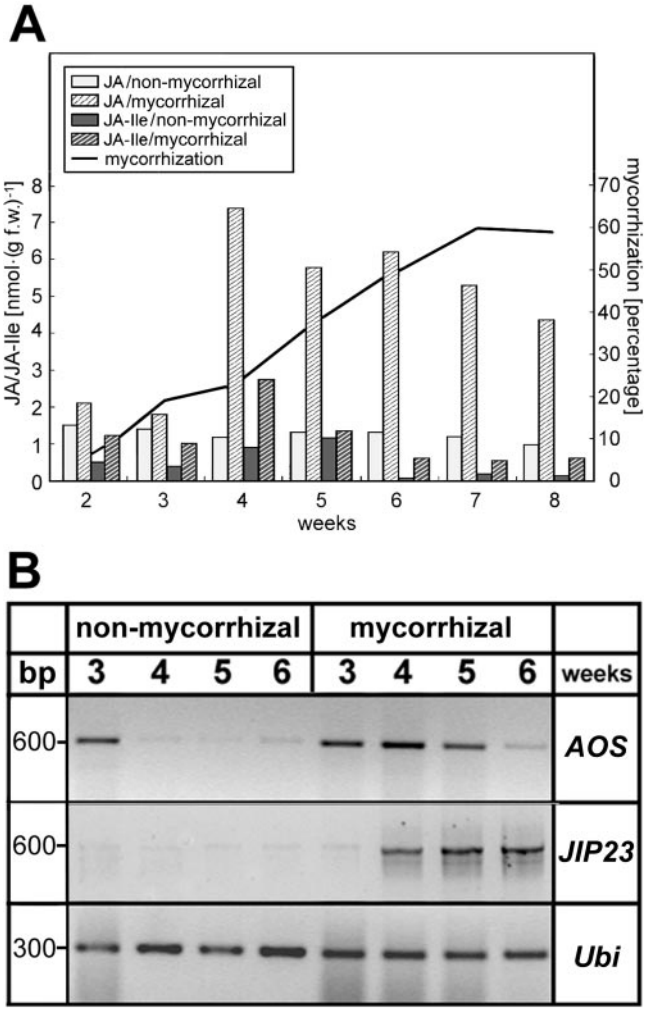

Figure 2. Accumulation of endogenous jasmonates and of transcripts of *AOS* and *JIP23* upon inoculation of barley roots with spores of *G. intraradices*. A, Accumulation of free JA and JA-Ile in nonmycorrhizal and mycorrhizal barley roots. Jasmonates and the conjugate were extracted and quantified from roots pooled from five different plants as indicated in "Materials and Methods." Three independent extractions and analyses were performed giving similar values that varied by about 15%. One series of data is given. The mycorrhization rate is given as the percentage of colonized root segments pooled from five different plants. B, Accumulation of transcripts of genes coding for AOS and JIP23 analyzed by reverse transcriptase (RT)-PCR. Ubiquitin transcripts were used as control to confirm constant levels of amplified fragments for all samples.

Figure 2B illustrates AM-induced AOS mRNA accumulation. Three-week-old nonmycorrhizal roots exhibited some AOS mRNA accumulation, presumably due to the high percentage of *AOS*-expressing root tips in young roots (Maucher et al., 2000). However, at later stages, only mycorrhizal roots exhibited AOS mRNA accumulation, suggesting an increase in capacity of JA biosynthesis. This is indicated by the rise in JA level and by the expression of the JA-responsive gene *JIP23* (Fig. 2B), both occurring exclusively in mycorrhizal roots.

AOS and JIP23 Transcripts and Protein Accumulate within Arbuscule-Containing Cells

In extraction procedures for mRNA analyses and JA measurements, mycorrhizal root cells are mixed with nonmycorrhizal cells. Therefore, we analyzed whether or not only the mycorrhizal roots cells exhibit the respective altered gene expression by performing in situ hybridization for *AOS* and *JIP23* expression with young mycorrhizal roots from plants of nurse-pot cultures at 12 d after transplantation (see below). As shown in Figure 3, A through D, hybridizations with the antisense probes revealed occurrence of AOS mRNA and JIP23 mRNA only in arbuscule-containing cortex cells. Hybridizations with the *AOS* and *JIP23* sense probe did not exhibit any label as exemplified for *AOS* (Fig. 3, E and F). This result could be confirmed by immunocytochemical detection of AOS and JIP23. Using mycorrhizal roots 8 weeks after inoculation, immunolabeling was performed with monospecific polyclonal antibodies raised against AOS and JIP23. As expected, nonmycorrhizal roots did not exhibit staining with anti-AOS antibodies (Fig. 4A) or with anti-JIP23 antibodies (Fig. 4, D, E). AOS protein was clearly and exclusively detectable in arbuscule-containing cells of the inner root cortex (Fig. 4, B, C), whereas the central cylinder and the rhizodermis were free of label. Using parallel cross sections, JIP23 was localized within the same cells (Fig. 4, F, G). However, an additional immunodecoration was found within the central cylinder (Fig. 4G, arrows). Here, the companion cells of the sieve element complex of the phloem exhibited label. The controls performed with preimmune sera showed only faint background staining as shown in Figure 4H for JIP23. These data support the assumption that JA biosynthesis (shown by expression of *AOS*) and the expression of JA-induced genes occur within cells harboring arbuscules.

Higher JA Levels Occur after the Initial Steps of the Establishment of Mycorrhiza

Because of the slow increase of mycorrhization rate upon inoculation with fungal spores, the stage of

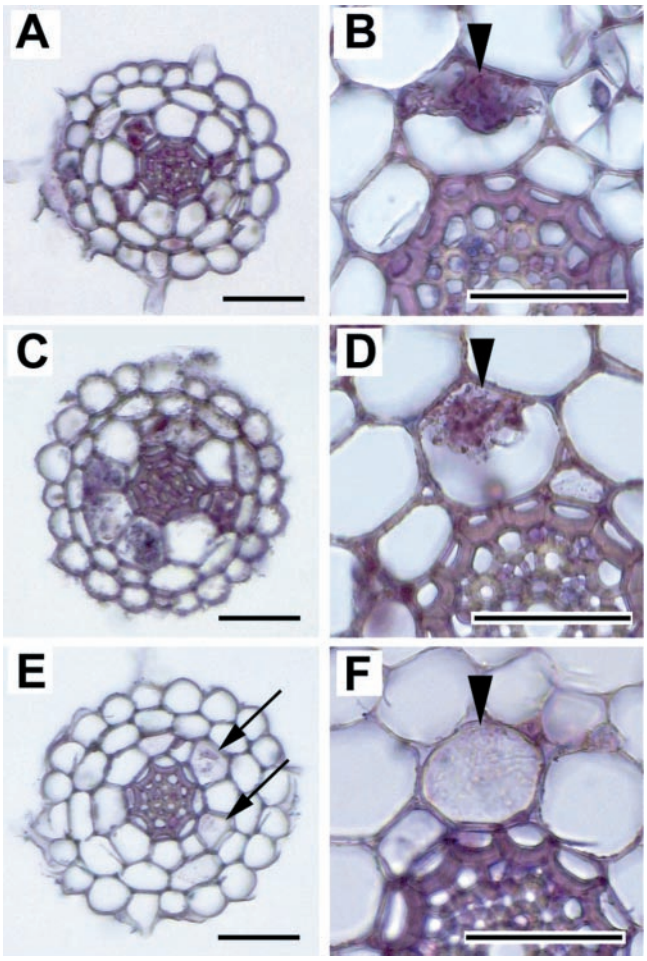

Figure 3. Localization of *AOS* and *JIP23* mRNA accumulating in mycorrhizal barley roots. Root segments from barley plants inoculated in nurse pot cultures for 12 d were processed for in situ hybridization using digoxigenin-labeled antisense RNA for *AOS* (A and B) and *JIP23* (C and D). Note the occurrence of positive staining in arbuscule-containing cells (arrowhead in B and D). Negative controls performed by using DIG-labeled sense probes do not exhibit label as shown for *AOS* (E and F). Here, arbuscules are hardly detectable due to the absence of staining (arrows in E, and arrowhead in F). Bars = 50 μm.

mycorrhization in which the initial rise in the JA level occurs cannot be analyzed precisely. Therefore, a nurse-pot culture system was used to achieve a rapid and near-synchronous mycorrhization (Roswarne et al., 1997). The first arbuscules could be detected after 6 d of cultivation (Fig. 5A), if 3-d-old barley seedlings were transplanted into the center of pots containing mycorrhizal leek (*Allium porrum*) plants. The frequency of arbuscules increased from 20% at d 6 to 45% at d 12 after transplantation. The mycorrhizal roots exhibited a drastic decrease of the arbuscule frequency at d 14. As a consequence, the time period used in the experimental setup covered the first generation of arbuscules, which usually remain active up to 14 d within the root cortex cells (Smith and Read, 1997).

In nonmycorrhizal barley roots (transplanted in between nonmycorrhizal leek plants), the JA level remains constant during the cultivation period, whereas mycorrhization led to an increase in the JA level (Fig. 5A). Here, a 3-fold increase occurs from d 8 to 14 after transplantation. The endogenous rise of JA was accompanied by AOS mRNA accumulation preferentially at d 12 after transplantation (Fig. 5B). In nonmycorrhizal roots exhibiting constant JA levels, constant AOS mRNA levels were also found. *JIP23* mRNA, indicative of elevated JA levels, accumulated at d 12 and 14 after transplantation (Fig. 5B). The data reveal that upon near-synchronous mycorrhization, the elevated JA level is accompanied by transcript accumulation of genes coding for a JA-biosynthetic enzyme and a JA-inducible protein. However, the first arbuscules appeared 4 to 6 d prior to the accumulation of AOS and JIP23 mRNAs and of jasmonates.

DISCUSSION

The role of jasmonates in plant responses to wounding and pathogen attack is well established (for review, see Wasternack and Hause, 2002). However, in symbiotic interactions, putative signals, including jasmonates, are poorly understood. To date, only a few hints for a possible involvement of JA in mycorrhization have been shown: Treatment with JA stimulated the mycorrhizal development of an ectomycorrhiza (Regvar et al., 1996, 1997), and accumulation of secondary compounds occurring in AM barley roots is inducible in nonmycorrhizal roots by jasmonates (Peipp et al., 1997). Here, we analyzed the involvement of jasmonates in the formation of an AM symbiosis established by colonization of barley roots with *G. intraradices*.

Mycorrhization of barley roots is accompanied by a 5-fold elevation of the JA level and a 2.5-fold increase in the level of JA-Ile, both suggesting a causal link to mycorrhization. These levels most likely reflect the total amount of all JA-related compounds because a loss of JA-related compounds into the gaseous phase could not be observed in barley (W. Boland, personal communication) and catabolites have been not detected so far (O. Miersch, unpublished data). In barley leaves, OPDA was among JA-related compounds accumulating upon osmotic stress (Kramell et al., 2000). In other plants, OPDA accumulates in response to touch (Stelmach et al., 1998), elicitation of cell cultures (Parchmann et al., 1997), or wounding of leaves (Parchmann et al., 1997), usually together with JA. Both compounds were shown to function independently as signals in plant stress as shown by an Arabidopsis mutant unable to convert OPDA into JA (Stintzi and Browse, 2000). However, mycorrhization does not seem to be related to OPDA because it did not accumulate.

To date, there is no approach to directly localize the site of JA accumulation within plant tissues. There-

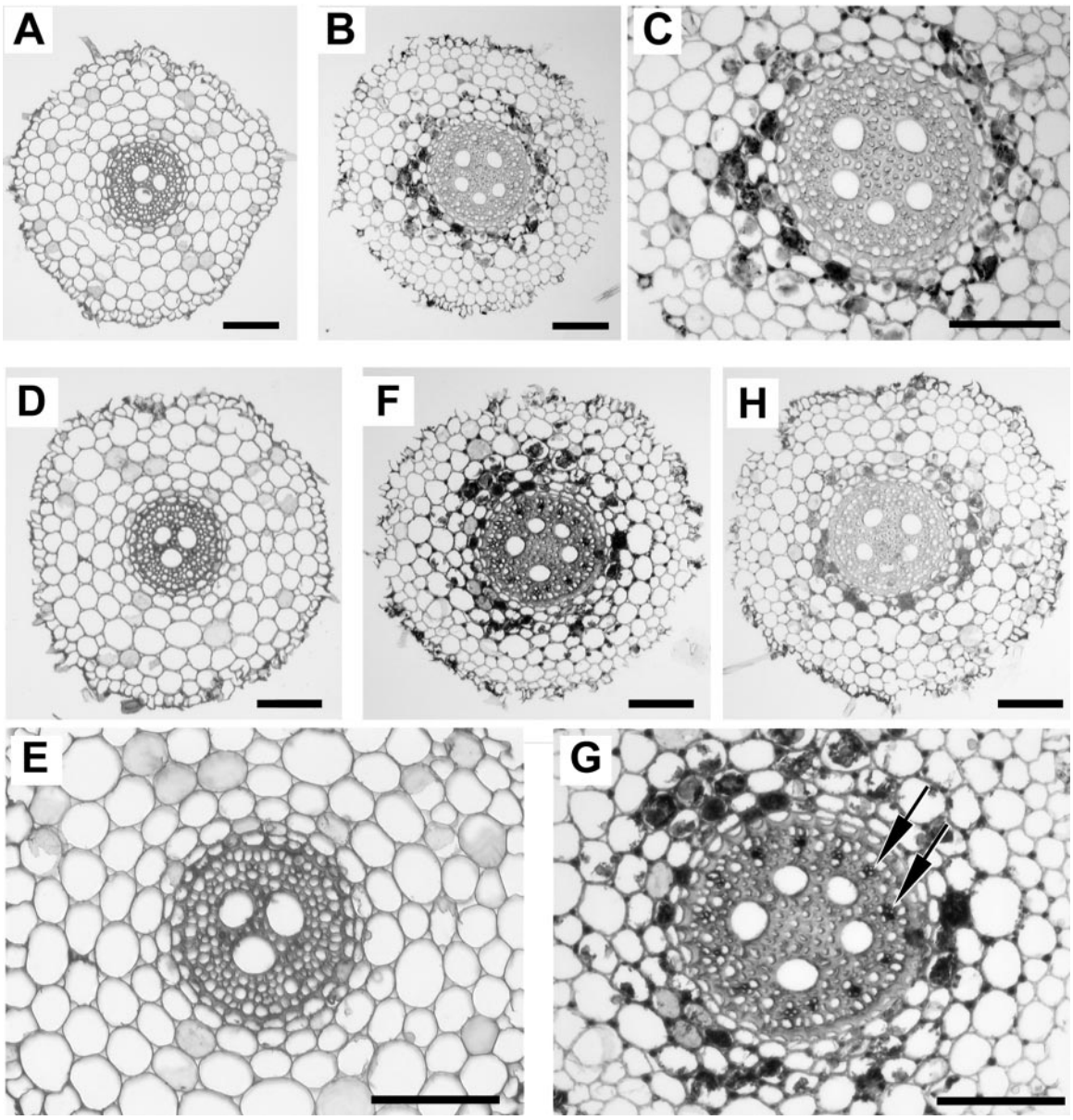

Figure 4. Localization of AOS and JIP23 in nonmycorrhizal and mycorrhizal barley roots. Immunochemical detection of AOS (A–C) and of JIP23 (D–G) in cross sections of roots of 8-week-old nonmycorrhizal (A, D, and E) or mycorrhizal (B, C, F, and G) plants, respectively. AOS protein was visualized by immunodecoration with a purified rabbit anti-AOS antibody; to visualize JIP23, polyclonal, monospecific rabbit anti-JIP23 antibodies were used. Both incubations were followed by a goat anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase. Note the occurrence of both proteins within arbuscule-containing cortex cells. The immunodecoration with anti-JIP23 exhibits an additional label within companion cells of the central cylinder in mycorrhizal roots (arrows in G). A control (H), in which cross-sectioned mycorrhizal roots were incubated with JIP23 preimmune serum followed by the same secondary antibody, does not show labeling. Bars =100 μm.

fore, we used an indirect method by analyzing the temporal and spatial expression of a JA-biosynthetic gene, *AOS*, shown to be indicative of elevated JA levels (Maucher et al., 2000). These data were compared with the spatial expression pattern of a JA-responsive gene. As a consequence, the occurrence of the encoded protein is indicative of elevated JA levels. In situ hybridization and immunolocalization, performed with mycorrhizal barley roots, revealed an expression of both genes within cells that contain the main symbiotic interface by harboring arbuscules. This spatially coordinated expression suggests that elevated JA levels may occur specifically within arbuscule-containing cells. These results also suggest that the cell-specific and local rise of jasmonates by far should exceed the 4-fold increase measured from

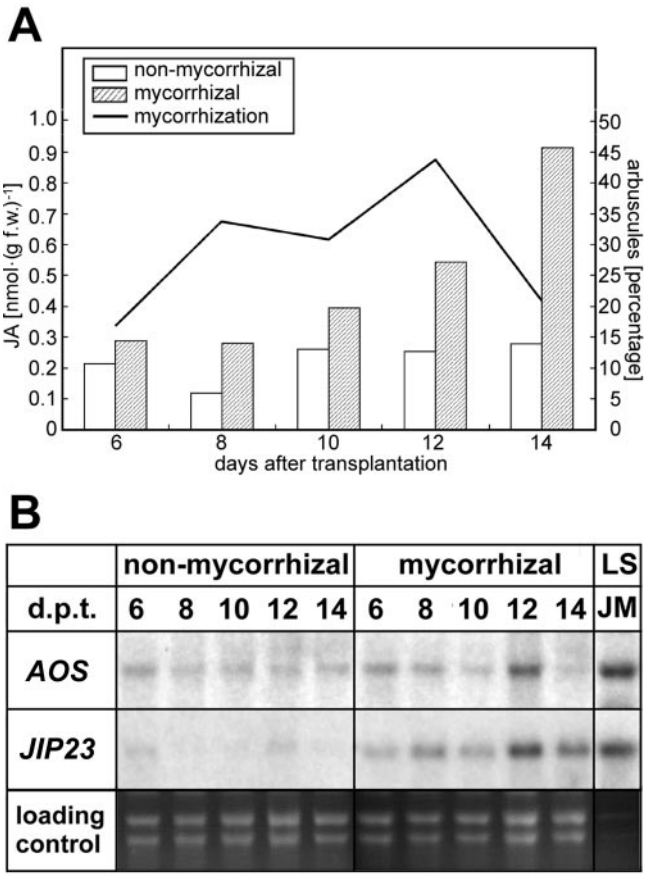

Figure 5. Accumulation of endogenous JA and of transcripts of *AOS* and *JIP23* upon near-synchronous colonization of barley roots with *G. intraradices*. A, Accumulation of free JA in nonmycorrhizal and mycorrhizal barley roots. JA was extracted and quantified from roots pooled from five different plants as indicated in "Materials and Methods." Three independent extractions and analyses were performed giving similar values that varied by about 15%. One series of data is shown. The mycorrhization rate is given as the percentage of arbuscule-containing root segments pooled from five different plants. First arbuscules occurred at the 6 d after transplantation. B, Accumulation of transcripts of genes coding for AOS and JIP23. For northern-blot analysis, 20 μg of total RNA was loaded per lane. The positive control was performed by loading 2 μg of total RNA from barley leaf segments treated with 45 μM JA methyl ester for 24 h (LS/JM). Loading was checked by recording the ethidium bromide staining of rRNA.

whole tissue extraction. The occurrence of JIP23 protein within the companion cells of mycorrhizal roots without detection of its mRNA may indicate persistence of JIP23 from a preceding *JIP23* gene expression. Once synthesized, JIP23 protein might occur in these cells due to its negligible turnover known for barley leaf segments and seedlings (Hause et al., 1996).

In addition to the spatial link between mycorrhization and JA levels, a temporal correlation was found. However, higher JA levels occurred after the onset of mycorrhization, implying that a fully established mycorrhiza rather than the recognition of the interacting partners or the establishment of the symbiotic interface might cause *AOS* expression and elevation of JA levels. In addition to stress-induced changes, jasmonate levels rise at distinct developmental stages (Wasternack and Hause, 2002). Tissues accumulating jasmonates during development are the hypocotyl hook of soybean (*Glycine max*) seedlings (Creelman and Mullet, 1995), the scutellar node of barley seedlings (Hause et al., 1996), and ovules of tomato (*Lycopersicon esculentum*) flower buds (Hause et al., 2000). These elevated JA levels were accompanied by simultaneous increase in expression of *AOS* (Maucher et al., 2000) or *AOC* (Hause et al., 2000). It is interesting that all of these tissues exhibiting simultaneous rise in JA level and *AOS/AOC* expression are sink tissues. Arbuscule-containing cells also represent sinks for carbohydrates because a main feature of the mycorrhizal symbiosis is the supply of the obligate heterotrophic fungus with carbohydrates (Harrison, 1999).

It has been clearly shown that the uptake of hexoses takes place within the host root only (Shachar-Hill et al., 1995). Root cortex cells release Suc, which is converted into hexoses by acid invertases (Blee and Anderson, 1998). Hexoses can be subsequently taken up by the fungus within the apoplastic compartment (Bago et al., 2000). Thus, by supplying the fungus with carbohydrates, mycorrhizal roots represent a much stronger sink organ than nonmycorrhizal roots (Douds et al., 2000). This is supported by the observation that in mycorrhizal barley roots, JIP23 also occurred in companion cells, which are known to be osmotically stressed by active solute transport. Until now, the occurrence of JIP23 in companion cells has been found only in the scutellar node and the leaf base of germinating seedlings, where carbohydrates were transported from the endosperm to the developing tissues (Hause et al., 1996). Thus, the enhanced transport of carbohydrates into the root, and the inducibility of *AOS* and *AOC* expression by Glc (Hause et al., 2000; Maucher et al., 2000) suggest the following scenario: Sugars supplied by source tissues are translocated into sink tissues, here, mycorrhizal roots. The resulting putative osmotic stress or induction by the sugar itself may lead to expression of genes coding for enzymes of JA biosynthesis and finally to rise of JA levels.

Sugars are the substrate for heterotrophically growing tissues, including invading organisms such as symbiotic fungi, and they are also important regulatory signals for the metabolism of source as well as sink tissues (Roitsch, 1999). Sink tissues are usually potential targets for pathogens due to their high amounts of carbohydrates. However, a characteristic feature of sink tissues is the expression of sink-specific as well as defense-related genes (Roitsch, 1999). The latter may contribute to an increased defense status. Jasmonates may modulate such a defense status because they were shown to induce ex-

pression of pathogenesis- and stress-related genes (Wasternack and Hause, 2002). It is interesting that the induced systemic resistance of Arabidopsis triggered by nonpathogenic, root-colonizing *Pseudomonas fluorescence* bacteria is associated with jasmonate-responsive gene activation (Pieterse et al., 1998).

It is tempting to speculate that elevated JA levels occurring upon mycorrhization may enhance the defense status of mycorrhizal tissues, which were shown to be less sensitive to secondary infections by pathogens (Cordier et al., 1998) or to drought and osmotic stress (Augé, 2001). As a consequence, mycorrhizal plants should have maximal benefit from the symbiotic interaction. The data described in this report now provide the basis to analyze such a role of jasmonates in mycorrhizal roots by modulating endogenous JA levels via overexpression or suppression of JA biosynthetic genes.

MATERIALS AND METHODS

Plant Material and AM Inoculation

Cultivation and inoculation of barely (*Hordeum vulgare* cv Salome) and the propagation of the AM fungus, *Glomus intraradices* Schenck & Smith, were described previously (Maier et al., 1995). Mycorrhiza formation was induced by growing the plants in expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay, Pinneberg, Germany) mixed with 10% (v/v) of the fungal inoculum. To obtain nearly synchronous mycorrhization, 3-d-old barley seedlings were transplanted into the middle of pots containing mycorrhizal leek (*Allium porrum*) plants according to Roswarne et al. (1997). Mycorrhiza formation was determined microscopically with the gridline-intersection method at a magnification of ×20 after staining with trypan blue (Phillips and Hayman, 1970).

Extraction and Measurement of OPDA, JA, and JA-Ile

Fresh roots from at least five different plants were pooled to minimize biological differences and were immediately frozen in liquid nitrogen. One gram of root material was homogenized in a mortar and was extracted with 10 mL of 80% (v/v) methanol. For quantification of JA, appropriate amounts of ($^2H_6$) JA were added to the extract, whereas in the case of JA-Ile, JA-[$^2H_3$]-Leu was used as internal standard. The methanolic extracts were purified by chromatographic steps as described for the isolation of JA and JA methyl ester (Kramell et al., 1997). The final separation was performed by reverse phase-HPLC (column: LiChrospher 100, RP-18, 250 × 4 mm, 5 m; flow rate: 1 mL min^{-1}; UV detection at 210 nm) using a 70:30 (v/v) mixture of methanol and water (containing 0.2% [v/v] acetic acid) as the mobile phase. The fractions corresponding to authentic JA (4–5 min) and JA-Ile/JA-Leu (6–8 min) were concentrated in vacuo. The content of JA-Ile was calculated on the basis of a calibration curve recorded with methylated JA-[$^2H_3$]-Leu. The intensities of the molecular ions at *m/z* 340 for the deuterated compound and *m/z* 337 for the nonlabeled compounds were compared. For quantitation of OPDA, 0.5 g of plant material was extracted and prepared for gas chromatography-mass spectrometry analysis according to Mueller and Brodschelm (1994). Gas chromatography-mass spectrometry was performed as previously described (Hause et al., 2000).

Extractions of RNA, Northern-Blot Analysis, and RT-PCR

Total RNA of frozen tissues was extracted by phenol:chloroform:isoamyl alcohol 25:24:1 (v/v) using modifications of Andresen et al. (1992). Because of the low amount of RNA isolated from several-week-old barley roots, RT-PCR was performed with 0.1 μg of total RNA by using the Titan One Tube RT-PCR System (Roche Diagnostics, Mannheim, Germany). Primers were designed according to the AOS sequences (Maucher et al., 2000), the JIP23 sequence (Andresen et al., 1992), and ubiquitin sequences (accession no. M60175/M60176) with the following combinations used: AOS: 5′-CCAGCGACCGCCTC-3′ and 5′-GGAGCGGCTCCTCGAGG-3′, resulting in a fragment of 600 bp from both AOS genes; JIP23: 5′-AATGGCCTCAGGAGTGTTTG-3′ and 5′-TTCATGGTAGTGCCTTCACC-3′, resulting in a fragment of 602 bp; and ubiquitin: 5′-CTCGCCGACTACAACATCC3′ and 5′-GGTAAAAGAGCAGAGCAAAC-3′, resulting in a fragment of 294 bp. The annealing temperature was 55°C for all reactions, and fragments were amplified by 35 PCR cycles.

In the experiments done with young barley roots from nurse-pot cultures, electrophoresis of 20 μg of total RNA per lane and northern-blot analysis were performed according to Sambrook et al. (1989). Blots were hybridized at 65°C for 16 h with ^{32}P-labeled fragments of the barley AOS1 cDNA or the JIP23 cDNA, both encompassing the full-length cDNA sequence. Gel loading was checked by comparing ethidium bromide-stained rRNA.

In Situ Hybridization and Immunocytochemistry

Small pieces of mycorrhizal and nonmycorrhizal roots were fixed with 3% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4). After dehydration in a graded series of ethanol, material was embedded in polyethylene glycol and cut as described (Hause et al., 1996). For in situ hybridization, cross sections of 10 μm thickness were collected in sieves, rinsed in Tris-HCl (pH 8.0), and incubated with 1% (w/v) bovine serum albumin (BSA) in the same buffer for 1 h. After acetylation, sections were dehydrated in graded series of ethanol and were air-dried. For hybridization, a solution of 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1× Denhardt's solution, 50% (v/v) formamide, 2 mg mL^{-1} tRNA, and 200 U mL^{-1} RNase inhibitor containing denaturated DIG-labeled sense or antisense RNA was applied and sections were incubated in a humid box at 45°C overnight. After two washing steps with 0.2× SSC at 55°C for 30 min each, sections were incubated with 20 μg mL^{-1} RNase A at 37°C for 30 min, followed by washing with 0.2× SSC at 55°C for 1 h. Immunological detection of DIG-labeled RNA hybrids was performed with an anti-DIG-fab fragment conjugated with alkaline phosphatase (Roche Diagnostics) according to the supplier's protocol. For localization of AOS protein and JIP23, sections of 5 μm thickness were immunolabeled with the purified rabbit-anti-AOS antibody (diluted 1:50 in PBS containing 5% [w/v] BSA; Maucher et al., 2000) or with the rabbit-anti-JIP23 antibody (diluted 1:5,000 in PBS containing 5% [w/v] BSA; Hause et al., 1996) followed by anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase as described (Hause et al., 2000). The staining procedure was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Sections were mounted on poly-L-Lys-coated slides and were analyzed by bright field microscopy with an Axioskop microscope (Zeiss, Jena, Germany). Pictures were taken by a CCD camera (Sony, Tokyo) and were processed through Photoshop 4.0 (Adobe Systems, Seattle).

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Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato – amplification in wound signalling

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Summary

The allene oxide cyclase (AOC)-catalyzed step in jasmonate (JA) biosynthesis is important in the wound response of tomato. As shown by treatments with systemin and its inactive analog, and by analysis of *35S::prosysteminsense* and *35S::prosysteminantisense* plants, the AOC seems to be activated by systemin (and JA) leading to elevated formation of JA. Data are presented on the local wound response following activation of AOC and generation of JA, both in vascular bundles. The tissue-specific occurrence of AOC protein and generation of JA is kept upon wounding or other stresses, but is compromised in *35S::AOC-sense* plants, whereas *35S::AOCantisense* plants exhibited residual *AOC* expression, a less than 10% rise in JA, and no detectable expression of wound response genes. The (i) activation of systemin-dependent AOC and JA biosynthesis occurring only upon substrate generation, (ii) the tissue-specific occurrence of AOC in vascular bundles, where the prosystemin gene is expressed, and (iii) the tissue-specific generation of JA suggest an amplification in the wound response of tomato leaves allowing local and rapid defense responses.

Keywords: tomato, jasmonate biosynthesis, allene oxide cyclase, wound response, vascular bundle.

Introduction

Plants have to adjust to numerous biotic and abiotic stresses, and many of the accompanied responses are mediated by jasmonates (Wasternack and Hause, 2002). The term jasmonate is used for the cyclopentanone compound jasmonic acid (JA), its methyl ester (JAME) and derivatives such as JA amino acid conjugates. In the last decade, jasmonates were recognized as central elements in a lipid- based signalling cascade, which has a pivotal role in plant defense reactions against herbivore and pathogen attack (Kessler and Baldwin, 2002; Wasternack and Hause, 2002). As well as JA, its precursor 12-oxophytodienoic acid (OPDA) and related compounds, collectively named octadecanoids, were suggested to function as independent signals (Blechert *et al.*, 1999; Kramell *et al.*, 2000; Stintzi *et al.*, 2001).

Initially, expression of defense genes such as those coding for proteinase inhibitors (PINs) by airborne JAME or wounding was observed in tomato leaves (Farmer and Ryan, 1990). Later on, numerous biotic and abiotic stimuli were found to induce expression of specific genes via an endogenous rise of octadecanoids and jasmonates. Beside wounding, such stimuli are elicitation of cell suspension cultures (Parchmann *et al.*, 1997), pathogen attack (Penninckx *et al.*, 1996), osmotic stress (Kramell *et al.*, 2000), or chitosan and oligogalacturonide treatment (Doares *et al.*, 1995). Other important aspects of JA action are its role in seedling and flower development (Hause *et al.*, 1996, 2000) and the downregulation of house-keeping proteins such as Rubisco (Reinbothe *et al.*, 1993).

The wound response in tomato is one of the best-studied jasmonate signaling pathways (Ryan and Pearce, 1998; Ryan, 2000). In tomato, local wounding leads to expression of the prosystemin gene within vascular bundles (Jacinto *et al.*, 1997; McGurl *et al.*, 1994). The encoded pro-peptide of 200 amino acids is processed into a peptide of 18 amino acid residues, called systemin (Ryan *et al.*, 2002), which can

activate the recently cloned membrane-located receptor (Scheer and Ryan, 2002), whereas its analog with alanine at position 17 (Sys-17) is inactive (Dombrowski *et al.*, 1999). Downstream events of the signal perception are activation of a MAPK, a Ca^{2+} ion flux, and rise in linolenic acid and jasmonate levels (Conconi *et al.*, 1996; O'Donnell *et al.*, 1996). Finally, expression of various defense genes such as that coding for PINs is followed by a systemic response due to loading of systemin in the phloem and transport to upper leaves (Ryan, 2000).

The biosynthesis of JA, elucidated by Vick and Zimmerman (1983), proceeds via an oxylipin pathway (Figure 1). As a first step, α-linolenic acid (α-LeA) is released from chloroplast membranes as indicated by JA deficiency of the mutant of *Arabidopsis thaliana* delayed anther dehiscence1 (*dad1*) which is defective in a chloroplast-located phospholipase of the A1-type (Ishiguro *et al.*, 2001). Subsequently, molecular oxygen is inserted by a lipoxygenase (LOX)-catalyzed reaction at position 13 of α-LeA. The resulting fatty acid hydroperoxide ((13*S*)-hydroperoxy(9*Z*,11*E*,15*Z*)-octadecatrienoic acid, 13-HPOT) is dehydrated by an allene oxide synthase (AOS) to an allene oxide. This highly unstable compound can rapidly decay by chemical hydrolysis to α- and γ-ketol and racemic OPDA (Figure 1) or, in presence of the allene oxide cyclase (AOC), can be cyclized exclusively to the *cis*-(+)-enantiomer (9*S*,13*S*) of OPDA. Only this enantiomer carries the stereochemistry of the naturally occurring (+)-7-*iso*-JA. OPDA reductase (OPR) and three β-oxidation steps lead to JA.

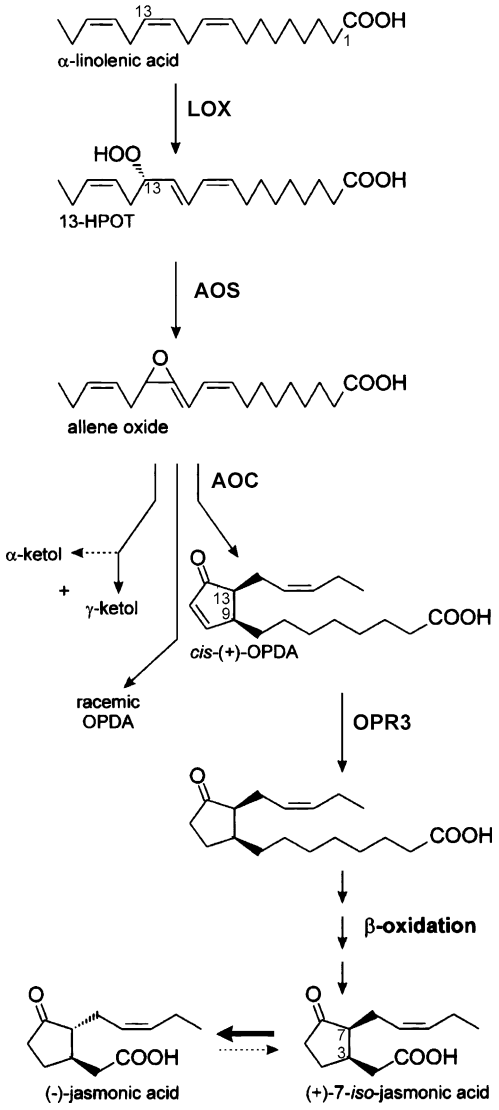

Figure 1. Scheme of jasmonate biosynthesis.

Most of the genes coding for the enzymes of JA biosynthesis are cloned. Several cDNAs coding for different isoforms of 13-LOX were isolated from various plants including tomato (Heitz *et al.*, 1997). However, there is no functional proof so far, to indicate which of the various 13-LOX forms acts in JA biosynthesis. The LOX-product 13-HPOT can serve as a substrate for various branches of the LOX pathway (Feussner and Wasternack, 2002). In the AOS-catalyzed reaction, 13-HPOT is directed to the formation of signaling compounds such as octadecanoids and jasmonates. cDNAs coding for AOS were isolated from various plants including tomato (Howe *et al.*, 2000; Sivasankar *et al.*, 2000). Recently, the first cDNA coding for AOC was isolated from tomato (Ziegler *et al.*, 2000). Finally, among three *Arabidopsis* sequences coding for OPRs, only the OPR3 is active in JA biosynthesis (Müssig *et al.*, 2000; Schaller *et al.*, 2000).

Most of the cDNAs coding for LOXs, AOSs, and AOCs carry a putative chloroplast transit peptide sequence, and the proteins were found to be imported and located in the chloroplast (Bell *et al.*, 1995; Feussner *et al.*, 1995; Froehlich *et al.*, 2001; Harms *et al.*, 1995; Stenzel *et al.*, 2003; Ziegler *et al.*, 2000). OPR3, which carries a peroxisomal target sequence (Stintzi and Browse, 2000), and the β-oxidation steps are thought to be located in peroxisomes.

Recently, two types of mutants were isolated both of which suppressing 35S::prosystemin-mediated signaling (Li *et al.*, 2001). Mutants of one group are affected in JA biosynthesis, e.g. *spr-2*, whereas another group contains JA- and systemin-insensitive mutants, such as *jai-1* (Li *et al.*, 2002). Grafting experiments with both mutants and wild type led to the final proof that a functional JA biosynthetic pathway is required for the local wound response, but not for the systemic response (Li *et al.*, 2002). It is less understood, however, how the local rise of JA is regulated. In tomato, JA biosynthetic enzymes such as LOX, AOS, and AOC are transcriptionally upregulated upon wounding (Heitz *et al.*, 1997; Howe *et al.*, 2000; Sivasankar *et al.*, 2000; Ziegler *et al.*, 2000). In case of AOC, an increase in mRNA within the first 30 min upon wounding coincided with the exclusive formation of *cis*(+)OPDA (Ziegler *et al.*,

2000), whereas in case of AOS1 and AOS2 the mRNA accumulation appeared later (Howe *et al.*, 2000; Sivasankar *et al.*, 2000; Stenzel *et al.*, 2002). In untreated tomato leaves, however, the AOC protein is specifically detectable in parenchymatic cells of vascular bundles (Hause *et al.*, 2000), the tissue of prosystemin gene expression and systemin generation (Jacinto *et al.*, 1997), suggesting a spatial and temporal role of AOC as an intermediate step of the wound response pathway downstream of systemin.

Here, we show that the constitutive occurrence of AOC in vascular bundles (Hause *et al.*, 2000) contributes to JA formation if the AOC substrate is generated upon wounding. The vascular bundle-specific occurrence of AOC is kept upon wounding or other treatments. Consequently, JA levels are preferentially elevated in main veins compared to surrounding areas. Inspection of the corresponding wild types, *35S::AOCsense* (*AOCs*), *35S::AOCantisense* (*AOCas*), *35S::prosysteminsense (PrSs)* and *35S::prosysteminantisense* (*PrSas*) plants for levels of AOC mRNA, AOC protein, AOC activity, for tissue-specific location of AOC protein as well as for levels of OPDA, JA and *PIN2* mRNA suggests a systemin-dependent AOC expression and an AOC-dependent amplification of the local wound response via vascular bundle-specific generation of jasmonates.

Results

AOC mRNA accumulates upon systemin treatment and precedes pin2 *mRNA accumulation*

The recently cloned AOC of tomato was found to be wound inducible (Ziegler *et al.*, 2000), and specifically expressed in vascular bundles (Hause *et al.*, 2000), the tissue in which the prosystemin gene is expressed (Jacinto *et al.*, 1997). This prompted us to inspect whether *AOC* expression depends on systemin. As shown in Figure 2(a), AOC mRNA accumulated upon wounding and systemin treatment with similar kinetics. Both mRNA accumulations could be decreased significantly by pre-treatment with the inactive systemin analog Sys17. These data suggest that at least locally wound-induced expression of *AOC* may occur via systemin. A final proof, however, is still missing showing that accumulation of mRNAs coding for JA biosynthesis enzymes such as AOS (Howe *et al.*, 2000) or AOC (Figure 2) is necessary and sufficient for the rise in JA known to occur in tomato leaves following wounding (Heitz *et al.*, 1997; Ziegler *et al.*, 2000). Therefore, kinetics of mRNA accumulation of AOC and the JA-inducible gene *PIN1* were recorded. Treatments such as wounding, or floating of detached leaves on 50 μM JAME led to AOC mRNA accumulation peaking at about 1–2 h which clearly preceded PIN1 mRNA accumulation (Figure 2b).

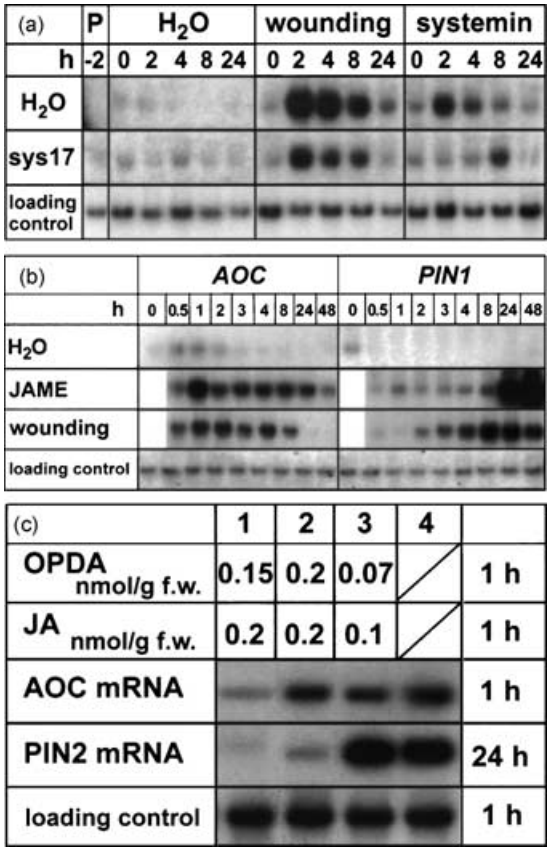

Figure 2. Expression of the AOC gene.
(a) AOC mRNA accumulation in response to wounding and systemin treatment. Leaves of 6-week-old plants cv. Moneymaker were freshly excised, pre-treated via the petiole for 2 h with distilled water or 100 nM of the inactive systemin analog Sys-17 and were subsequently supplied via the petiole with water, 100 nM systemin or were wounded and floated on water. Loading of total RNA (20 μg per lane) was checked by hybridizing with a cDNA probe coding for the ATP/ADP carrier as exemplified for one experiment.
(b) AOC mRNA and PIN1 mRNA accumulation in leaves of 6-week-old tomato plants cv. Moneymaker. Freshly excised leaves were wounded or were floated on distilled water and 50 μM of JAME, respectively. Loading of total RNA (20 μg per lane) was checked in all experiments as described in (a).
(c) Effect of 12-hydroxy-JA and ethyl-indanoyl isoleucine conjugate on the accumulation of JA, OPDA, AOC mRNA, and PIN2 mRNA. Freshly excised leaves of 6-week-old tomato plants cv. Moneymaker were supplied via the petiole with distilled water (1), 50 μM 12-hydroxy-JA (2), 50 μM ethyl-indanoyl isoleucine conjugate (3), or 50 μM JA (4) for 1 h (AOC mRNA, JA, OPDA) or 24 h (PIN2 mRNA). Total RNA (20 μg per lane) was loaded and checked as in (a). GC/MS analyses of JA and OPDA were performed with 0.5 g FW. Values of one of three independent extractions of pooled leaf material and measurements are given in nmol g^{-1} FW.

No rise in JA and OPDA, however, could be found upon treatment with 12-OH-JA, although this compound induced AOC mRNA accumulation (Figure 2c, lane 2). Also, upon treatment with the ethyl-indanoyl isoleucine conjugate, which mimics the action of JA without rise in JA (Koch *et al.*, 1999), AOC mRNA appeared and the lack of rise of JA was confirmed (Figure 2c). Obviously, this compound can

induce per se jasmonate-responsive gene expression, since PIN2 mRNA accumulated (Figure 2c). These data suggest that there is no causal link between AOC mRNA accumulation and rise in JA formation within the first hour of treatment.

AOC protein was found to be confined to vascular bundles and its localization was not altered upon wounding and JA treatment

Inspection of AOC protein level following wounding or treatment with JAME revealed constant levels until 24 h of treatment (Figure 3a). This contrasts with the strong and transient changes in AOC mRNA levels following identical treatments (Figure 2b). Immunocytochemical analysis revealed that the previously shown confinement of AOC protein to vascular bundles (Hause *et al.*, 2000) is kept upon treatment of detached wild-type leaves with water or JA for 24 h, or upon wounding for 30 min (Figure 3c). These results indicate that the AOC protein detected by Western blot analysis is preferentially, if not exclusively, confined to vascular bundles. Interestingly, wounding performed locally by puncture with a needle and indicated by autofluorescence of damaged cells (see arrow in Figure 3c), did not lead to AOC accumulation in surrounding mesophyll cells. A confinement of AOC protein in vascular bundles was also observed upon treatment of wild-type leaves with systemin and in wounded leaves of *35S::prosysteminsense* plants (data not shown). In contrast, the LOX protein was detected in all leaf tissues of an unwounded (Figure 3d) and wounded leaf (data not shown), suggesting that LOX products are generated everywhere. The AOS protein was also detected in all tissues of an unwounded leaf (Figure 3e). Immunolabeling experiments with the corresponding pre-immune serum did not show any label (Figure 3b).

Amplification of the wound response by AOC

To analyze the suggested systemin dependence of AOC expression/JA generation, we compared the wild-type Better Boy (BB) with *PrSs* and *PrSas* plants.

35S::prosysteminsense *plants.* Due to the constitutive expression of the prosystemin gene in these plants

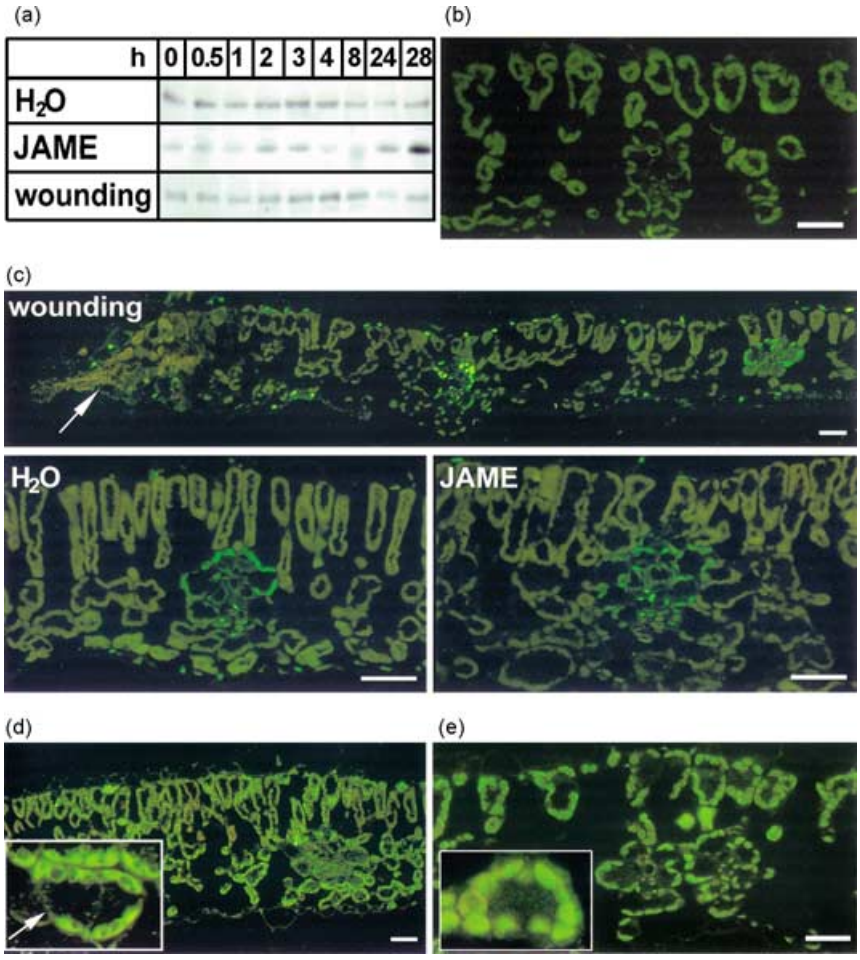

Figure 3. Accumulation of AOC, LOX, and AOS proteins in excised leaves of 6-week-old tomato plants cv. Moneymaker upon wounding or JAME treatment.
(a) Immunoblot analysis of AOC protein. Freshly excised leaves were wounded or floated on distilled water and 50 μM JAME, respectively, for indicated times, and 10 μg of total protein was subjected to analysis.
(b) Cross-section of untreated tomato leaves stained with pre-immune serum of AOS. Bar = 25 μm.
(c) Tissue-specific expression of AOC. Cross-sections are shown of excised leaves floated on distilled water and 50 μM JAME, respectively, or were wounded. Samples were taken after 30 min (wounding) or 24 h (water and JAME). All tissues were subjected to immunocytochemical analysis as described in Experimental procedures using an anti-AOC antibody in the dilution of 1 : 2000. The AOC protein is indicated by the green fluorescence and appeared specifically in parenchymatic cells of the vascular bundles. The wound site is indicated by the autofluorescence of damaged cells (arrow). Bar = 25 μm.
(d) Accumulation of LOX protein in untreated tomato leaves. Cross-section shows LOX protein in all leaf tissues. The inset shows LOX protein restricted to chloroplasts; cytoplasm does not show label (arrow). Bar = 25 μm.
(e) Accumulation of AOS protein in untreated tomato leaves. Cross-section treated with anti-AOS antibody shows AOS protein in all leaf tissues. The AOS protein is confined to chloroplasts (inset). Bar = 25 μm.

(McGurl *et al.*, 1994), constitutive elevation of AOC mRNA, AOC protein, AOC activity leading to elevated levels of JA or OPDA was expected in freshly harvested leaves. This was not the case (Figure 4d). There was, however, a higher and earlier rise of JA in wounded transgenic leaves of 6-week-old plants than that of the wild-type Better Boy (Figure 4d). Furthermore, detached unwounded leaves of 3-week-old *PrSs* plants accumulated already about 1.2 nmol g^{-1} FW JA and 1.4 nmol g^{-1} FW OPDA. Such levels are sufficient to induce *PIN2* expression, and might be the reason for the observed constitutive *PIN2* expression in 2-week-old *PrSs* plants (McGurl *et al.*, 1994). In contrast, in 6-week-old plants, the JA levels are not different in unwounded leaves (Figure 4d). However, differences can be observed between wild-type and *PrSs* or *PrSas* plants after wounding.

35S::prosysteminantisense *plants.* Presumably caused by the low differences in constitutive prosystemin mRNA levels between wild-type and *PrSas* plants (Orozco-Cardenas *et al.*, 1993), both types of leaves exhibited no difference in AOC mRNA levels without wounding. There

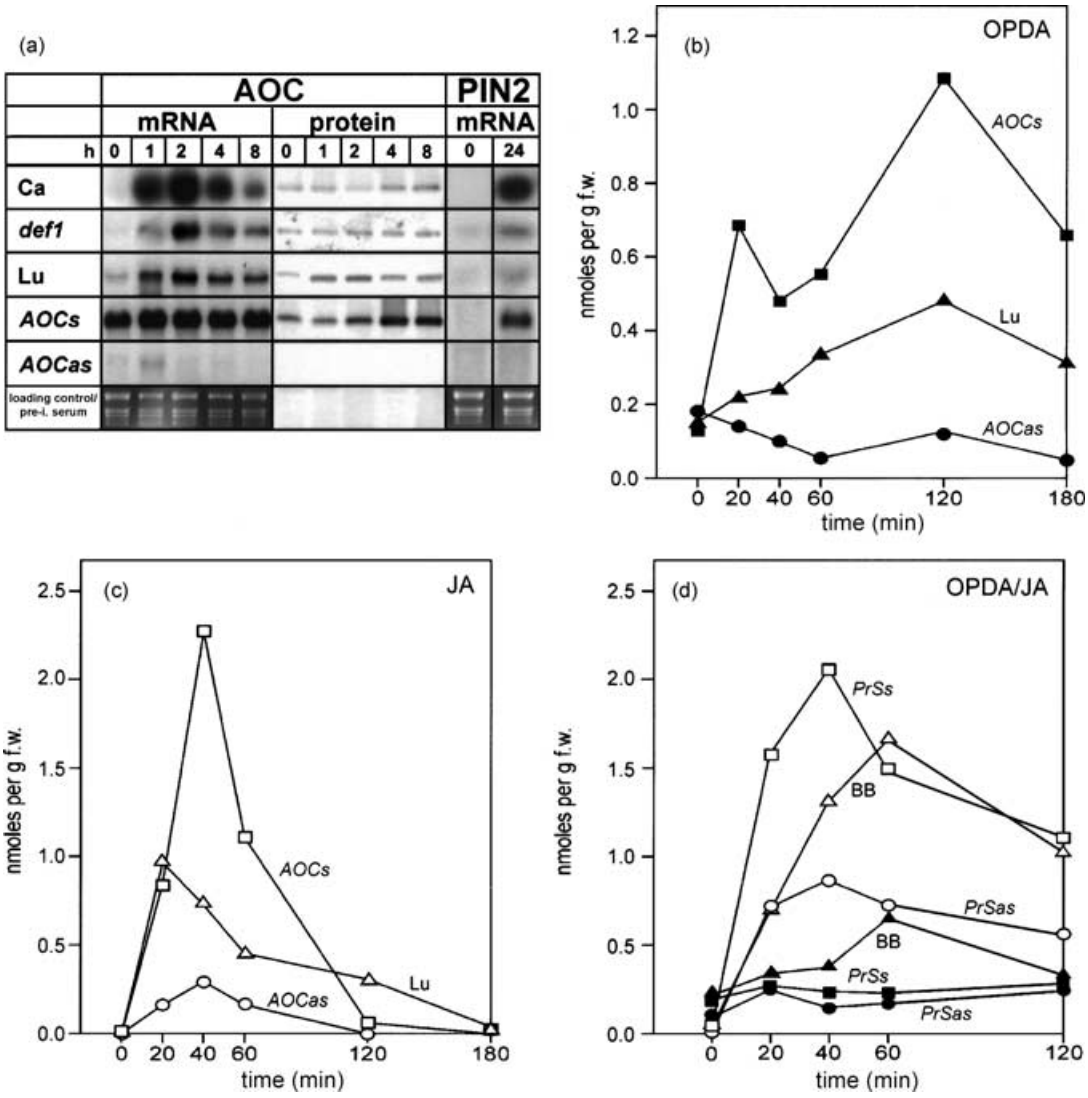

Figure 4. Wound-induced accumulation of AOC mRNA, PIN2 mRNA, and AOC protein (a), and accumulation of OPDA (b,d), JA (c,d) in excised leaves of 6-week-old tomato plants.

(a) Leaves of *def1*, *AOCs*, and *AOCas* plants as well as the corresponding wild types, Castlemart (Ca) and Lukullus (Lu), respectively, were wounded. For AOC mRNA and PIN2 mRNA analysis, 20 μg total RNA per lane and ethidium bromide-stained rRNA as a loading control were used and is exemplified for the wild type Castlemart. Immunoblot analysis was performed with total protein extracts (10 μg protein per lane) and an anti-AOC antibody in the dilution of 1 : 5000.

(b–d) Leaves were pooled from three different plants upon wounding. Plant material was identical to that of (a) or was from the wild type Better Boy (BB) and the *PrSs* as well as *PrSas* plants. One gram FW was subjected to GC/MS analysis. Three independent extractions and measurements were done for OPDA (b,d, closed symbols), and JA (c,d, open symbols) deviating by 15%. Data are shown for the wild type cv. Lukullus (b,c Lu, triangles), *AOCs* plants (b,c, squares), *AOCas* plants (b,c, circles), the wild type cv. Better Boy (d, BB, triangles), the *PrSs* plants (d, squares), and the *PrSas* plants (d, circles).

was, however, less AOC mRNA level in *PrSas* than in wild-type leaves following wounding (data not shown), and wound-induced JA levels were significantly lower in *PrSas* leaves than in wild-type leaves indicating systemin dependence (Figure 4d).

To inspect the role of AOC and its vascular bundle-specific occurrence in the local wound response of tomato, we generated *AOCs* and *AOCas* plants and compared them with the corresponding wild-type Lukullus (Lu).

35S::AOCsense *plants.* These plants exhibited a strong constitutive accumulation of AOC mRNA and AOC protein (Figure 4a). Immunocytochemical analysis revealed *AOC* expression in all leaf tissues of *AOCs* plants, thereby contrasting with the tissue-specific expression in wild-type leaves (Figures 3 and 5). JA and OPDA levels in freshly harvested (0-time) leaves of wild-type and *AOCs* plants were identical (Figure 4b,c), indicating that the high levels of AOC mRNA and AOC protein (Figure 4a) did not cause an increase in these compounds. *In vitro* AOC activity (Table 1) of freshly harvested leaves was 10-fold higher in *AOCs* plants than in wild-type plants, indicating that the capacity to form OPDA and JA is elevated. Only upon wounding, however, an increase could be detected for both compounds (Figure 4b,c). This wound-induced rise was more than twofold higher in *AOCs* leaves than in wild-type leaves.

35S::AOCantisense *plants.* AOC mRNA was undetectable in freshly harvested leaves and no AOC protein was detected by Western blot analysis (Figure 4a). A residual amount of AOC protein was found by immunocytochemical analysis (Figure 5) which might be responsible for the AOC activity detected *in vitro* (Table 1). Upon wounding, only a weak accumulation of AOC mRNA occurred, indicating that *AOC* expression is strongly diminished. Consequently, in wounded *AOCas* leaves, no increase was found for OPDA, and only a minor increase was detected for JA, indicating a loss of AOC function. The lack of *PIN2* mRNA accumulation following wounding and systemin treatment clearly indicates an AOC dependence of the wound response. Furthermore, upon systemin treatment of *AOCas* leaves for 30 min, only 0.25 nmol g^{-1} FW of JA were found, whereas the corresponding wild-type leaves accumulated 1.75 nmol g^{-1} FW.

Finally, we analyzed the JA-deficient mutant *def1* which has reduced wound-induced JA accumulation (Howe *et al.*, 1996) compared with the wild-type Castlemart.

Def1 *plants.* Upon wounding of detached *def1* leaves a similar kinetics of induction but lower AOC mRNA accumulation was observed compared to the wild-type Castlemart (Figure 4a). Again, levels of AOC protein remained unchanged in both cases. The data suggest that the diminished JA formation of *def1* in response to wounding observed previously (Howe *et al.*, 1996) is not due to an impaired AOC gene. To prove this, we sequenced AOC cDNAs isolated from *def1* (Acc. No. AJ308482), *Lycopersicon esculentum* cv. Moneymaker (Acc. No. AJ308481) and *L. pennellii* and compared the sequence with that of *L. esculentum* cv. Lukullus (Ziegler *et al.*, 2000). AOC sequences of *def1*, Moneymaker and Lukullus were identical (data not shown). Another possible explanation for the signaling defect in *def1* plants might be found in the tissue-specific expression of AOC. However, the immunocytochemical inspection of *def1* leaves revealed exclusive occurrence of AOC protein in parenchymatic cells of vascular bundles similar to that of the wild type (Figure 5). *Def1* leaf protein extracts exhibited much lower *in vitro* AOC activity compared to the wild type even upon wounding (Table 1). In *def1* leaves, the wound-induced levels of OPDA (200 pmol g^{-1} FW at 40 min) and JA (450 pmol g^{-1} FW at 40 min) were also lower than that of wounded wild-type leaves (350 pmol g^{-1} FW OPDA and 750 pmol g^{-1} FW JA). Taken together, these data indicate that *def1* plants possess an intact AOC gene expression and the AOC protein has an identical localization as that of the wild type, but the mutant exhibits a deficiency in AOC activity.

The data can be summarized as follows:

1. *PrSs* plants are apparently pre-conditioned by overexpression of the *prosystemin* which in turn may contribute to the rapid rise in JA levels upon wounding.
2. A systemin dependence was suggested by less wound-induced AOC mRNA accumulation and JA formation in *PrSas* than in wild-type leaves.
3. Overexpression of *AOC* increased AOC protein and AOC *in vitro* activity constitutively, whereas levels of JA and OPDA and the *PIN2* transcript increased only upon wounding.
4. *AOCantisense* plants lack any wound response in terms of *PIN2* expression, whereas AOC protein and OPDA were below the detection limit, and the *in vitro* AOC activity and JA levels were strongly diminished.
5. In all plants except the *AOCs* plants, AOC protein was confined to vascular bundles.

Preferential generation of jasmonates in main veins of tomato leaves

The specific occurrence of AOC protein in vascular bundles suggests that elevation of jasmonates upon wounding or other treatments might occur predominantly in veins. Indeed, in leaf areas of the wild-type Castlemart containing predominantly main veins, the JA level increased within 30 min upon wounding from 0.3 to 2.2 nmol g^{-1} FW, whereas in the remaining leaf lamina, the levels increased only from 0.2 to 1.0 nmol g^{-1} FW (Table 2). Also, OPDA

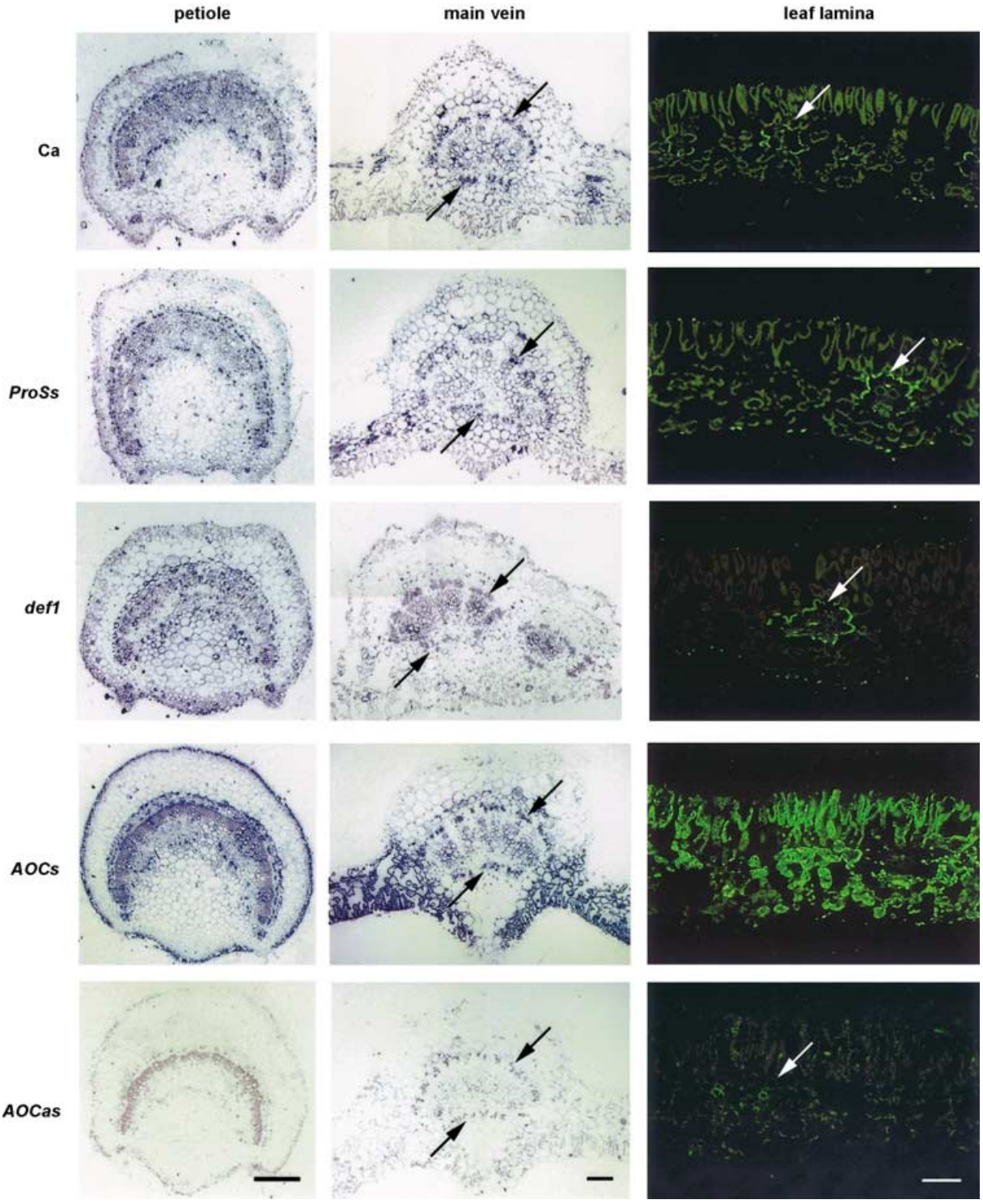

Figure 5. Tissue-specific expression of *AOC*.
Unwounded leaves of plants identical to that of Figure 4(a) were cut for immediate embedding of pieces of petioles, of the main veins or parts of the leaf lamina, respectively, and were analyzed immunohistochemically. The arrows indicate occurrence of AOC protein in parenchymatic cells of the vascular bundles of the main vein. AOC protein is also marked in the bundle sheet of the minor vein within the leaf lamina visualized by green fluorescence contrasting from a brown-green autofluorescence of mesophyll cells. In case of *AOCs* plants, AOC protein appeared also in mesophyll cells as well as in epidermal cells of the petiole. (Bars: petiole = 500 μm; main vein = 100 μm; leaf lamina = 50 μm).

Table 1 *In vitro* AOC activity in crude extracts of unwounded and wounded tomato leaves

	nmol *cis*(+)-OPDA per mg protein after wounding	
	0 min	20 min
Castlemart	25	27
def1	11	11
35S::AOCsense	203	151
35S::AOCantisense	8	7

AOC activity was detected by measuring formation of *cis*(+)-OPDA as described in Experimental procedures.

increased preferentially in main vein-containing leaf areas, whereas dinorOPDA was neglected in all measurements due to its minor occurrence. In contrast, the JA level and the OPDA level differed only slightly between main vein-containing leaf area and the remaining leaf tissues in *AOCs* plants (Table 2), thus reflecting the abundant and equal occurrence of AOC in all leaf tissues (Figure 5). A residual amount of JA in both types of wounded leaf areas were found in the *AOCas* plants.

The preferential biosynthesis of jasmonates in the main vein compared to the leaf lamina is also indicated by the amount of (+)-7-*iso*-JA, the initial product in JA biosynthesis, thereby indicating *de novo* synthesis. In the main vein-containing tissues of wounded wild-type leaves, 70% of all jasmonates were (+)-7-*iso*-JA, whereas only 15% were in the leaf lamina. In contrast, in wounded *AOCs* plants, both types of tissues contained about 60% (+)-7-*iso*-JA of total amount of jasmonates, indicating identical capacity for *de novo* synthesis of JA.

Discussion

Among the lipid-derived compounds, octadecanoids and jasmonates have a crucial role in plant responses to biotic and abiotic stresses such as wounding (León *et al.*, 2001; Wasternack and Hause, 2002; Weber, 2002). Jasmonic acid is suggested to be one of several interacting signals which, in tomato, lead to expression of *PINs* and other plant defense genes (Howe and Schilmiller, 2002; Ryan, 2000). JA can act as an intra- and intercellular as well as inter-organismic signal (Farmer, 2001). Upon wounding, JA levels increase locally and systemically (Herde *et al.*, 1996), but it is unclear how this is regulated. Recent grafting experiments (Li *et al.*, 2002) with the JA-insensitive tomato mutant *jai-1* and the *spr2* mutant, which is defective in JA biosynthesis, revealed strong evidence that JA or a derivative may act as a long-distance transmissible signal in wound signaling (Ryan and Moura, 2002). It is still unknown, however, how JA levels are elevated. Here, we show that an amplification of the local wound response may occur by activation of AOC and generation of jasmonates in vascular bundles presumably in a systemin-dependent manner.

In tomato, the AOC is encoded by a single-copy gene (Ziegler *et al.*, 2000). Thus, the immunological detection of AOC protein in vascular bundles (Hause *et al.*, 2000) indicates that all AOC is confined in this tissue. This is reminiscent of a vascular bundle-specific location of the JA-inducible vegetative storage proteins in soybean (Huang *et al.*, 1991), which is de-localized following jasmonate treatment. The confinement of AOC to vascular bundles of tomato is kept upon JA- or wound-induced *AOC* expression (Figure 3c). As a consequence, the capacity of the different leaf areas to form JA may differ, preferentially in leaf areas differing in the types of veins. In the minor veins, the AOC protein was found exclusively in the cells of the bundle sheet (Figures 3 and 5). In contrast, main veins and leaf stalks, both of them composed of many vascular bundles (Figure 5), contain AOC protein within the parenchymatic cells. Therefore, leaf stalks and main veins carry a higher total amount of AOC protein than a comparable leaf area containing minor veins and mesophyll cells. Since the LOX protein and the AOS protein were equally distributed throughout the leaf tissues (Figure 3d,e), mainly the AOC protein might be of special importance in this

Table 2 Preferential accumulation of OPDA and JA in main veins of wild type and wounded leaves of *AOCs* and *AOCas* plants

	OPDA		JA	
Line/time of wounding	Main veins	Leaf lamina	Main veins	Leaf lamina
Castlemart/0 min	0.35	0.2	0.3	0.2
Castlemart/30 min	1.4	0.9	2.2	1.0
35S::AOCsense/30 min	1.9	1.8	2.8	2.7
35S::AOCantisense/30 min	0.07	0.05	0.06	0.05

Preferential accumulation of OPDA and JA occurs in main veins of wild-type leaves following wounding, but not in wounded leaves of *AOCs* plants and *AOCas* plants. Main vein-containing leaf areas were cut off from the remaining tissues within 20 sec, and both leaf tissues were frozen by liquid nitrogen and subjected to GC/MS analysis as described in Experimental procedures. Data of three independent sampling and extractions were obtained and deviated by 15%. One representative set of data is shown in nmol g^{-1} FW.

preferential generation of jasmonates in main veins compared to surrounding leaf areas (Table 2). The immunological analysis of LOX and AOS, however, was hampered by the fact that the antibodies did not distinguish between the various LOX and AOS forms appearing in tomato leaves (Heitz *et al.*, 1997; Howe *et al.*, 2000). Thus, there is still the possibility that a LOX and/or an AOS form occurs in a tissue-specific manner. Nevertheless, leaves of *AOCs* plants exhibiting constitutively high level of AOC protein in all leaf tissues did not show such a preferential JA accumulation (Figure 5; Table 2). Thus, localized generation of OPDA and JA might be preferentially caused by the tissue specificity of AOC. Moreover (+)-7-*iso*-JA accumulated preferentially in the main vein-containing leaf area of a wounded wild-type leaf, but was equally distributed in wounded *AOCs* leaves. (+)-7-*iso*-JA is the initial product in JA biosynthesis which isomerizes after synthesis into the more stable (–)-JA. Thus, the appearance of (+)-7-*iso*-JA above the basal level indicates *de novo* synthesis.

The preferential generation of jasmonates and exclusive localization of AOC protein in the same tissue, in which systemin is generated from its precursor prosystemin, substantiate the recently proposed model on local wound signaling (Orozco-Cardenas *et al.*, 2001; Ryan, 2000). According to this model, the prosystemin gene expression occurs in vascular bundles and is JA and wound inducible (Jacinto *et al.*, 1997; Ryan and Moura, 2002). Subsequently, prosystemin is processed to systemin which is released into the vascular system. Here, systemin can be a mobile signal (Narváez-Vásquez *et al.*, 1995) and/or can be perceived locally by the systemin receptor (Scheer and Ryan, 2002), possibly in the vascular parenchyma cells (Orozco-Cardenas *et al.*, 2001; Ryan and Moura, 2002). Due to AOC expression in these cells (Figure 5), the following scenario is suggested: The preferential generation of JA in main veins occurring in the first hour following wounding is the consequence of wound-induced generation of systemin and vascular bundle-specific occurrence and activiation of AOC protein. Subsequently, the generated JA may contribute to the JA-inducible prosystemin gene expression in the same tissue early after wounding. Such spatial and temporal coincidence in the generation of cumulatively acting signals would amplify the wound response. Consistent with this model is that (i) in comparison to the corresponding wild type, young *PrSs* leaves exhibited elevated levels of JA and OPDA, constitutive occurrence of *PIN2*, and rapid generation of JA upon wounding (Figure 4d); (ii) *PrSas* leaves exhibited less AOC mRNA accumulation and JA formation upon wounding than wild-type leaves; and (iii) systemin treatment elevates *in vitro* AOC activity in wild-type leaves but much less in AOCas leaves (data not shown). The data are consistent with a systemin-dependent AOC activation, but additional triggering by JA cannot be excluded. Finally, JA is essential for the local wound response in terms of *PIN2* expression, since PIN2 is absent in wounded *AOCas* leaves (Figure 4a) and cannot be induced by systemin treatment of these leaves (C. Kutter and C. Wasternack, unpublished).

Another scenario is highlighted by the vascular bundle-specific occurrence and activation of AOC and the local generation of JA adjacent to the veins. The paraveinal cells adjacent to the veins exhibit a more rapid oxidative burst in the incompatible plant pathogen interaction of *Arabidopsis* (Alvarez *et al.*, 1998), and tomato leaves treated with race-specific elicitors of *Cladosporium* also show rapid and preferential cell death in paraveinal cells (Hammond-Kosack *et al.*, 1994). Generation of reactive oxygen species has been repeatedly proposed to be linked to JA. Indeed, such a cell type-specific generation of H_2O_2 was detected in tomato and discussed as a consequence of polygalacturonase gene expression which is induced by wounding or treatment with systemin and JA in the first 2 h (Bergey and Ryan, 1999). Non-mobile oligogalacturonides are generated by this enzyme accompanied with the formation of mobile H_2O_2 in parenchymatic cells of vascular bundles about 4 h after wounding (Orozco-Cardenas *et al.*, 2001). Accordingly, systemin potentiates the oxidative burst in cultured tomato cells (Stennis *et al.*, 1998). Thus, the early activation of AOC and generation of JA coincides spatially and temporally with the generation of H_2O_2 which is suggested to diffuse into mesophyll tissue (Orozco-Cardenas *et al.*, 2001), where *PIN2* expression is located (Narváez-Vásquez *et al.*, 1993).

Obviously, compartmentalization of prosystemin and AOC in vascular bundles keeps the tissue in a pre-activated state which allows immediate response upon wounding. In this context, regulation of JA biosynthesis by substrate availability would contribute to its rapid generation. In transgenic tobacco plants overexpressing AOS, elevated level of JA were found only upon stimulation such as wounding, suggesting that wound-induced substrate generation is necessary for JA biosynthesis (Laudert *et al.*, 2000; Wang *et al.*, 1999). Substrate-dependent JA formation was also found in non-transgenic *Arabidopsis* plants, where LOX, AOS, and AOC occur abundantly without tissue specificity (Stenzel *et al.*, 2003), and in transgenic *Arabidopsis* plants overexpressing AOS (Park *et al.*, 2002). In contrast, in transgenic potato plants overexpressing the flax AOS elevated JA levels without constitutive PIN2 expression were found (Harms *et al.*, 1995).

The data shown here confirm a control by substrate availability. The basic level of JA and OPDA in untreated tomato leaves is low even under constitutive overexpression of AOC (Figure 4b,c). Upon wounding, however, a rapid transient rise was observed, indicating that AOS is not limiting in tomato, even under constitutive overexpression of AOC. This is consistent with metabolite profiling data of 10 free oxylipins in tomato leaves, where the initial

substrate of the AOS branch, 13-HPOT, could not be detected (Stenzel *et al.*, 2003). It is possible that the activation of pre-existing JA biosynthetic enzymes by substrate generation or other mechanisms allow the leaf to generate JA immediately. The 10-fold higher *in vitro* AOC activity in total leaf extracts of *AOCs* plants and much less activity in *def1* plants are in line with such a post-translational control. Furthermore, *in situ* hybridization of AOC mRNA (data not shown) and immunocytochemical localization of AOC (Figure 3c) revealed that AOC protein, but not AOC mRNA, appeared in a tissue-specific manner, suggesting post-translational control which might be overridden in the *AOCs* leaves (Figure 5).

The vascular bundle-specific AOC expression and JA generation combined with a post-translational control in JA biosynthesis at the AOC level may allow the plant to respond locally and rapidly leading to a burst in JA and/or related compounds. Such a burst within the first hour of elicitation, wounding or other external factors is regularly found (Koch *et al.*, 1999; Ziegler *et al.*, 2001) (Figure 4c). In tomato, amplification of JA and systemin formation may lead to a level sufficient for a systemic response. Indeed, transport of JA was found (Zhang and Baldwin, 1997), and elevation of JA level was detected in systemic leaves (Herde *et al.*, 1996). Most convincingly, the recent grafting experiments with mutants in JA biosynthesis and signalling revealed a wound response in the systemic unwounded leaf with blocked JA biosynthesis but intact JA signaling (Li *et al.*, 2002; Ryan and Moura, 2002), suggesting that JA or related compounds act as systemic signal.

Experimental procedures

Plant materials, treatments and reagents

Lycopersicon esculentum Mill. cv. Moneymaker, cv. Lukullus, cv. Castlemart or Better Boy were grown as described (Wasternack *et al.*, 1998). Identical cultivation conditions were used for the homozygous *def1* plants, and transgenic lines. For treatments, leaves were cut at the petiole and were floated on 50 μM (+/–)-JAME in Petri dishes containing 50 ml solution per leaf and were incubated at 25°C under continuous white light (120 μmol m^{-2} sec^{-1}) provided by fluorescent lamps (Narva, Berlin, NC 250/01). In some experiments indicated in the legends, 100 nM systemin, 100 nM Sys17, 50 μM (+/–)-JA, 50 μM 12-hydroxy-JA, or 50 μM ethyl-indanoyl isoleucine conjugate were supplied via the petiole or applied to non-detached leaves.

All jasmonates and octadecanoids were prepared and purchased, respectively, checked on purity and used as described (Hause *et al.*, 2000; Maucher *et al.*, 2000). 13(*S*)-HPOT was prepared from α-linolenic acid by incubation with soybean LOX (Sigma Chemical Co., St. Louis, USA) (Hamberg and Gotthammar, 1973). 12-OH-JA was prepared according to Kitahara *et al.* (1984). The ethyl-indanoyl isoleucine conjugate recently described (Schüler *et al.*, 2001) was kindly provided by Prof. W. Boland (Jena). Systemin and its analog systemin-17 were synthesized and kindly provided by Dr T. Nürnberger (Halle).

Extraction of RNA and Northern blot analysis

Total RNA was purified from frozen tissues by treatments with buffered phenol:chloroform:isoamyl alcohol 25 : 24 : 1 (v/v/v) and 20 μg per lane were subjected to RNA gel blot analysis according to Sambrook *et al.* (1989). Blots were hybridized at 60°C for 16 h with ^{32}P-labeled cDNAs of tomato *AOC* (full length), tomato *AOS* (near full length), tomato *PIN1 (*1200-bp fragment containing 1 intron), tomato *PIN2* (800-bp fragment), and potato *PR*1b (300-bp fragment). Loading control was performed by ethidium bromide staining of rRNA or by hybridization with a tomato cDNA of ATP/ADP carrier (600-bp fragment), and is exemplified as shown. cDNA fragments of *PIN1* and *PIN2* were isolated by PCR with genomic tomato DNA and primers deduced from database sequences (NO. KO3290 for Le*PIN1* and NO. KO3291 for Le*PIN2*).

Extraction of proteins, immunoblot analysis and assay of AOC activity

For protein analysis, all tissues were subjected to extraction procedures described by Meyer *et al.* (1988). Proteins were solubilized in SDS sample buffer followed by SDS–PAGE and immunoblot analysis according to standard protocols. Immunodetection was performed with a polyclonal antibody raised against the recombinant AOC (Ziegler *et al.*, 2000) as primary antibody in a dilution of 1 : 5000 and antirabbit IgG conjugated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) as secondary antibody. Immunodecorated AOC was stained with *p*-nitroblue tetrazoline chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). With the anti-AOC antibody, only one band was detected in total protein extracts of tomato leaves or tomato flowers (Hause *et al.*, 2000). AOC activity tests were performed as follows: Leaf tissue (0.5 g) was ground under liquid nitrogen and the resulting powder was extracted with 50 mM Na-phosphate buffer, pH 7, containing 2% PVPP and 0.1% Triton X-100. The homogenate was centrifuged at 15 000 × ***g*** for 10 min and the supernatant was used as the enzyme extract. The enzyme assay consisted of excess of purified recombinant AOS (Maucher *et al.*, 2000) with an activity of 4 nkat, 50 mM Na-phosphate buffer, pH 7, and the enzyme extract in a total volume of 250 μl. The reaction was started by the addition of 5 μl of 10 mM 13-HPOT and was terminated by extraction of the reaction products with ether. Subsequently, the extract was incubated with 0.1 M NaOH in order to achieve *cis*→*trans* isomerization of OPDA. OPDA was purified by reversed phase HPLC on Nucleosil 120-5 C18 (Macherey-Nagel, Düren, Germany) with isocratic elution (63% v/v MeOH in water containing 0.1% acetic acid). The *trans* enantiomers were separated on Nucleodex-β-PM (250 mm × 4.6 mm, 5 μm; Macherey-Nagel, Düren, Germany) by isocratic elution with MeOH/H_2O (0.1% triethylammoniumacetate pH 4) 60/40 (v/v) at a detection wavelength of 225 nm. The peak area of (9*S*,13*R*)-OPDA, which originated from the (9*S*,13*S*)-enantiomer, exceeding 50% detected after chemical hydrolysis, was used to calculate AOC activity.

Extraction and quantitative analysis of jasmonates and octadecanoids

Plant material from three different plants was pooled to minimize biological differences and was immediately frozen in liquid nitrogen. One gram (FW) was homogenized in a mortar and extracted with 5 ml of 80% (v/v) methanol. Appropriate amounts of ($^2H_6$)-JA were added as internal standards for quantitative analysis of JA. For purification and fractionation before GC–MS analysis, all jasmonate-containing compounds were subjected to ion exchange

chromatography on DEAE Sephadex A-25 cartridges and RP-HPLC as described (Kramell *et al.*, 2000). For quantitative analysis of OPDA, 0.5 g (FW) plant tissue was extracted and prepared for GC–MS analysis as described by Mueller and Brodschelm (1994). GC–MS analysis was performed with a Finnigan GCQ as described (Hause *et al.*, 2000).

Construction of vectors and plant transformation

Transformation of tomato plants was performed with 35S::*AOC-sense* and 35S::*AOCantisense* constructs (Maucher *et al.*, in preparation). Briefly, the *Sma*I/*Sma*I–*Apa*I/*Sma*I 1.1-kb fragment of the tomato AOC-cDNA (full-length coding region; cf. Ziegler *et al.*, 2000) was introduced in *sense (s)* and *antisense (as)* orientation by blunt end ligation into the multiple cloning site of the binary vector pBin-Hyg-Tx derived from pBin19 (Bevan, 1984). Recombinant plasmids were maintained and selected in *Escherichia coli* XL1-blue MRF[1]. Plasmids pBin-*s*AOC and pBin-*as*AOC were transfected into *Agrobacterium tumefaciens* strain LBA 4404. For transformation, cotyledons of tomato plants (*L. esculentum* cv. Lukullus) were used according to standard transformation protocol (Ling *et al.*, 1998). Five independent *sense* and nine independent *antisense* plants were selected from 170 primary explants followed by isolation of homozygous lines in the T_2 generation (Maucher *et al.*, in preparation). These lines were used here.

Immunocytochemistry

Immunocytochemical analysis was performed as described (Hause *et al.*, 2000) using cross-sections (2 μm thick) from PEG-embedded material. The anti-AOC antibody raised against recombinant tomato AOC (Ziegler *et al.*, 2000) was used in a dilution of 1 : 2000. The LOX antibody raised against lipid body LOX of cucumber (Feussner *et al.*, 1995) recognizes one LOX form in the Western blot analysis of total leaf extracts of untreated tomato leaves (Wasternack *et al.*, 1998), and was used here in a dilution of 1 : 500. It cannot be excluded, however, that more than one of the chloroplast-located LOX forms of tomato were recognized in the immunocytological analysis. The tomato AOS antibody tomato AOS1 recognized in the Western blot analysis of total leaf extracts of untreated tomato leaves one of the two AOCs known to occur in leaves (Howe *et al.*, 2000; Sivasankar *et al.*, 2000) and was used in a dilution of 1 : 1000. As secondary antibody, antirabbit IgG conjugated with alkaline phosphatase and antirabbit IgG conjugated with Alexa488 (Molecular Probes Eugene, OR), respectively, were used.

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Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*

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Abstract

In biosynthesis of octadecanoids and jasmonate (JA), the naturally occurring enantiomer is established in a step catalysed by the gene cloned recently from tomato as a single-copy gene (Ziegler *et al.*, 2000). Based on sequence homology, four full-length cDNAs were isolated from *Arabidopsis thaliana* ecotype Columbia coding for proteins with AOC activity. The expression of *AOC* genes was transiently and differentially up-regulated upon wounding both locally and systemically and was induced by JA treatment. In contrast, AOC protein appeared at constitutively high basal levels and was slightly increased by the treatments. Immunohistochemical analyses revealed abundant occurrence of AOC protein as well as of the preceding enzymes in octadecanoid biosynthesis, lipoxygenase (LOX) and allene oxide synthase (AOS), in fully developed tissues, but much less so in 7-day old leaf tissues. Metabolic profiling data of free and esterified polyunsaturated fatty acids and lipid peroxidation products including JA and octadecanoids in wild-type leaves and the jasmonate-deficient mutant OPDA reductase 3 (*opr3*) revealed preferential activity of the AOS branch within the LOX pathway. 13-LOX products occurred predominantly as esterified derivatives, and all 13-hydroperoxy derivatives were below the detection limits. There was a constitutive high level of free 12-oxo-phytodienoic acid (OPDA) in untreated wild-type and *opr3* leaves, but an undetectable expression of *AOC*. Upon wounding *opr3* leaves exhibited only low expression of *AOC*, wounded wild-type leaves, however, accumulated JA and AOC mRNA. These and further data suggest regulation of JA biosynthesis by OPDA compartmentalization and a positive feedback by JA during leaf development.

Abbreviations: α-LeA, α-linolenic acid; AOC, allene oxide cyclase; *cet*, mutant with constitutive expression of thionin; *dad1*, mutant with delayed anther dehiscence1; dn-OPDA, dinor-12-oxo-phytodienoic acid; 13-HPOT, 13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid; JA, jasmonic acid; JAME, JA methyl ester; L.A, linoleic acid; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic acid; *opr3*, mutant defective in OPR3; OPR3, OPDA reductase3; PLA1, phospholipase of the A1 type; PUFA, polyunsaturated fatty acids; SA, salicylate

Introduction

Generation of lipid-derived signal molecules is a common phenomenon in higher organisms. In plants jasmonates and octadecanoids are of particular importance (Bergey *et al.*, 1996; Ryan, 2000). They originate from polyunsaturated fatty acids (PUFA) and are formed by one of the seven different branches of the LOX pathway, the AOS branch (Feussner and Wasternack, 2002). The other branches lead to leaf aldehydes and leaf alcohols as well as various derivatives of PUFAs such as epoxy-, hydroxy-, keto- or ether PUFA and epoxy hydroxy PUFA (Feussner and Wasternack, 2002).

The nucleotide sequence data reported will appear in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ308483 (*AOC1*); AJ308484 (*AOC2*); AJ308485 (*AOC3*) and AJ308486 (*AOC4*).

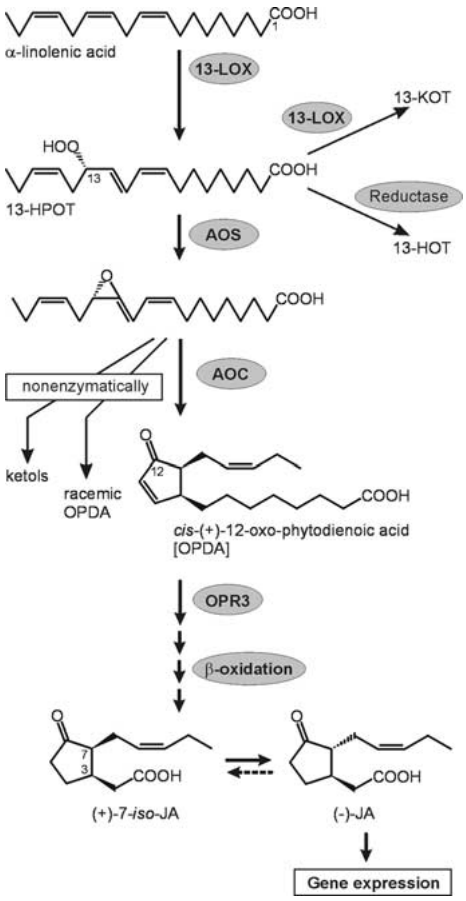

Figure 1. Scheme of JA biosynthesis and further 13-LOX products derived from α-LeA, 13-HPOT and (9*Z*,11*E*,15*Z*)-13-keto-(9,11,15)-octadecatrienoic acid (13-KOT). Identical reactions occur with LA as substrate, whereas the corresponding 9-derivatives are formed via 9-LOX catalysis.

The biosynthesis of jasmonic acid (JA) and its methyl ester (JAME) was elucidated by Vick and Zimmerman (1983) and Hamberg and Hughes (1988). With α-linolenic acid (α-LeA) as substrate, molecular oxygen is inserted by a 13-LOX at carbon atom 13 leading to the formation of a fatty acid hydroperoxide 13*S*-hydroperoxy-(*9Z*,11*E*,15)-octadecatrienoic acid, 13-HPOT (Figure 1). This compound is dehydrated by the allene oxide synthase (AOS) to an unstable allene oxide which can be either hydrolysed non-enzymatically to α- and γ-ketols or cyclized to racemic 12-oxo-phytodienoic acid (OPDA). In the presence of an allene oxide cyclase (AOC), preferential formation of the (9*S*,13*S*) enantiomer of OPDA occurs. The AOC-catalysed step is regarded as the crucial step in octadecanoid and jasmonate biosynthesis because only this enantiomeric form is the substrate for the naturally occurring (+)-7-*iso*-JA, which is formed after reduction of OPDA by a specific OPDA reductase (OPR3) and three cycles of β-oxidation (Schaller *et al.*, 2000; Ziegler *et al.*, 2000).

Several cDNAs coding for LOXs, AOSs and OPRs have been cloned from different plant species as reviewed by Schaller (2001) and Feussner and Wasternack (2002). The first AOC was recently cloned from tomato and found to be encoded by a single-copy gene (Ziegler *et al.*, 2000). Treatment of plants with products of the AOS branch, the octadecanoids and jasmonates, led to accumulation of mRNAs coding for LOX, AOS, AOC and OPR3 suggesting a feed-forward regulation in JA biosynthesis (Sasaki *et al.*, 2001). Also, biotic or abiotic stresses, which result in endogenous increases of octadecanoids and jasmonates, are usually accompanied by a transcriptional up-regulation of AOS, AOC and OPR3 (Howe *et al.*, 2000; Maucher *et al.*, 2000; Ziegler *et al.*, 2000). However, the function of this mRNA accumulation is not understood, because it was not accompanied by endogenous formation of jasmonates as shown by isotopic dilution analysis using barley and tomato leaves during the first 24 h of mRNA accumulation (Kramell *et al.*, 2000; Miersch and Wasternack, 2000). Furthermore, the rise in JA upon wounding appeared before the onset of AOS or AOC mRNA accumulation (Ziegler *et al.*, 2001; Stenzel, 2002).

A chloroplast location of the first half of biosynthetic steps to JA is generally assumed. Since chloroplast LOXs are predominantly localized within the stroma and the inner envelope membranes of chloroplasts (Feussner *et al.*, 1995; Blée and Joyard, 1996), α-LeA of chloroplast envelope membranes is suggested as the substrate for jasmonate biosynthesis. Indeed, the recently identified jasmonate-deficient mutant *dad1* (delayed anther dehiscence1) is defective in a chloroplast-located phospholipase of the A1 type (PLA1) (Ishiguro *et al.*, 2001). Furthermore, it seems ubiquitous that the location of 13-LOXs, AOSs and AOC is the chloroplast, since the proteins were detected in the chloroplast by import studies (Bell *et al.*, 1995; Froehlich *et al.*, 2001), by chloroplast isolation (Harms *et al.*, 1995; Feussner *et al.*, 1995) or by immunocytochemical analysis (Feussner *et al.*, 1995; Ziegler *et al.*, 2000). Even the barley AOS lacking a transit sequence for chloroplast import was immunocytochemically shown to be located within the chloroplast (Maucher *et al.*, 2000). By contrast, OPR3 carries a peroxisomal target sequence (Stintzi and Browse, 2000), and might thus be co-localized

with the subsequent β-oxidation steps in the peroxisomes (Kindl, 1987). It is still unclear, however, how metabolites in JA biosynthesis are channelled between the two compartments involved (Laudert and Weiler, 1998; Froehlich *et al.*, 2001).

In *Arabidopsis*, *AOS* (Kubigsteltig *et al.*, 1999) and *OPR3* (Sanders *et al.*, 2000; Stintzi and Browse, 2000) were shown to be expressed at distinct stages of flower development. Two mutants defective in OPR3 (*dde1*, Sanders *et al.*, 2000; *opr3*, Stintzi and Browse, 2000) and the *dad1* mutant are male-sterile. In leaves of the *opr3* mutant, OPDA accumulated upon wounding, and plants were resistant to fungal and insect attack (Stintzi *et al.*, 2001). Thus, this mutant is a powerful tool to dissect distinct signalling properties of OPDA and JA which have been suggested by several groups (Blechert *et al.*, 1999; Kramell *et al.*, 2000; Vollenweider *et al.*, 2000). Furthermore, *opr3* plants seem to be a useful tool to analyse regulation of JA biosynthesis.

In *Arabidopsis* cDNAs for all enzymes of JA biosynthesis have been cloned, except for AOC and those of β-oxidation. To date, two different LOX cDNAs have been described (AtLOX2, Bell and Mullet, 1993; AtLOX1, Melan *et al.*, 1993). Recently, four other LOX cDNAs were listed in the databases (AtLOX3, AAF79461; AtLOX4, AAF21176; AtLOX5, CAC19365; AtLOX6, AAG52309). Out of these six LOXs, four might be involved in JA biosynthesis (AtLOX2, 3, 4, 6) (Bell *et al.*, 1995; I. Feussner, unpublished). For AOS, a single-copy gene was found (Laudert *et al.*, 1996). Of the three different OPRs, only the recently cloned OPR3 exhibited the exclusive formation of the naturally occurring cyclopentanone compound (Müssig *et al.*, 2000; Sanders *et al.*, 2000; Schaller *et al.*, 2000).

The unique role of AOC in generating the correct enantiomeric form in JA biosynthesis, and its specific occurrence in all vascular bundles and in flower tissues of tomato (Hause *et al.*, 2000a) prompted us to analyse AOC(s) of *Arabidopsis*.

Here, we describe cloning and characterization of four cDNAs coding for proteins with AOC activity. Northern blot analysis revealed transient and differential expression upon wounding. However, AOC, LOX as well as AOS protein appeared constitutively in fully developed leaf tissues with only minor additional accumulation upon wounding. Untreated wild-type and *opr3* leaves exhibited a constitutive high level of free OPDA, but undetectable expression of *AOC*. Upon wounding *opr3* leaves exhibited only low expression of *AOC*; wounded wild-type leaves, however, accumulated JA and AOC mRNA. These and further data on profiles of free and esterified PUFAs and oxylipins suggest regulation of JA biosynthesis by OPDA compartmentalization and positive feedback during growth.

Material and methods

Materials

Opr3 mutant seeds (in the Wassilewskija background) were kindly provided by Prof. J. Browse and Dr A. Stintzi. All non-isotopic jasmonates and octadecanoids were prepared or purchased, checked on purity and used as described (Kramell *et al.*, 2000; Hause *et al.*, 2000a). (13*S*)-HPOT was prepared from α-LeA by incubation with soybean LOX (Sigma, St. Louis, MO). Other hydroperoxy and hydroxy PUFAs as well as other oxylipins were prepared or purchased as described (Kohlmann *et al.*, 1999; Weichert *et al.*, 1999).

The [$^2H_6$]-JA was synthesized as described (Miersch, 1991). [$^2H_5$]-OPDA was prepared from [17-$^2H_2$,18-$^2H_3$]-linolenic acid (Zimmerman and Feng, 1978), and both compounds were used as internal standards in GC-MS analysis. Rabbit polyclonal antibodies against the lipid body LOX of cucumber (Hause *et al.*, 2000b), the recombinant AOS of *A. thaliana* (Laudert and Weiler, 1998) and the recombinant AOC2 of *A. thaliana* were used.

Plant growth and treatments

A. thaliana ecotype Columbia and ecotype Wassilewskija were cultivated in controlled chambers (Percival, CLF) at 70% relative humidity under short-day conditions of 8 h light,

210 μE m^{-2} s^{-1} for 6 weeks. For treatments the complete rosette of a plant was cut above the roots and floated on distilled water, 50 μM JAME, 50 μM 13-HPOT, 1 M sorbitol, 0.5 M glucose, 50 μM salicylate (SA) or 1 M NaCl for indicated times. Wounding was performed by crushing the leaves with a forceps across the mid-vein.

cDNA isolation, RT-PCR, cloning and sequencing

The cDNA library prepared from 4-week old rosette leaves of ecotype Columbia treated for 24 h with 100 μM JAME and maintained in γZAP express Stratagene was kindly provided by Dr Stefan Bau

(Cologne). The cDNA library was hybridized with the full-length AOC cDNA from tomato (Ziegler *et al.*, 2000) (55 °C, 5× SSC, 5× Denhardt's reagent, 100 μg/ml shared salmon sperm DNA, 0.1% w/v SDS, 1×10^6 dpm ml^{-1} of labelled probe, 18 h). Positive clones were isolated and converted into phagemids by *in vivo* excision, and were sequenced on both strands with IRO 700 labelled primers with the Thermo Sequenase DYEnamic Direct Cycle Sequencing Kit (Amersham Pharmacia Biotech). Only one AOC homologue of 991 bp was isolated and designated as *AOC1*.

Based on three other database sequences with partial homology to the *AOC* sequence, we used RT-PCR for isolation of full-length cDNAs. mRNA isolated from rosette leaves floated on 100 μM JAME for 2 h was used with the following primer combinations: AOC2, 5′ primer CCAATCAAGTAGAGTTTCTTC, 3′ primer ACACAGCGATACGAGAAAC; AOC3, 5′ primer CCATTAAAACCAAAGATCAATC, 3′ primer CTAACCTCACACAACTCATC; AOC4, 5′ primer ATTAAACTAACTCAGGCAAG, 3′ primer CCATGACAAAGACACCCAC. Generated RT-PCR products were cloned into the vector pCR II TOPO (Invitrogen) and sequenced.

RNA and immunoblot analysis

Total RNA was extracted from frozen tissues with buffer (4 M guanidinium isothiocyanate, 50 mM sodium citrate pH 4.0, 0.5% sarcosyl, 0.1% 2-mercaptoethanol) and purified by treatments with buffered phenol/chloroform/isoamyl alcohol 25:24:1 (v/v/v). After ethanol precipitations 20 μg per lane was subjected to RNA gel blot analysis according to Sambrook *et al.* (1989). Gel loading was checked by comparing ethidium bromide-stained rRNAs. Hybridization was performed at 60 °C for 16 h with ^{32}P-labelled full-length cDNAs of *AOC1* (Figure 6) or of AOC3 (Figures 5 and 9), and *AOS* of *A. thaliana*. For the creation of gene-specific probes used in Figure 5, we amplified 150 bp of the 3′-untranslated region (3′-UTR) starting after the stop codon of each *AOC*. These PCR products were cloned into the pCR II-TOPO vector and sequenced. The specificity of each probe was tested with PCR-amplified genomic fragments which contained the complete sequence as well as 150 bp of the 5′-UTR and 3′-UTR of each AOC. Gel loading was checked by comparing ethidium bromide-stained rRNAs. Proteins were isolated from the phenolic phase and were used for separation and immunoblot analysis as described by Hause *et al.* (1996, 2000a). Immunoblot analysis was performed by using an antibody raised against recombinant AOC2 (*A. thaliana*) (1:5000 dilution) or against recombinant AOS (*A. thaliana*) (1:2500 dilution) or against cucumber lipid body LOX (Hause *et al.*, 2000b) (1:1000 dilution).

AOC expression in E. coli *and AOC activity assay*

Expression of *AOC1*, *AOC2*, *AOC3* and *AOC4* was performed after cloning of amplified PCR fragments for each AOC as follows. For over-expression of *AOC1*, a fragment covering amino acids 31–253 was generated with the 5′ primer CGC GGATCCC$_{159}$TTGGTTTCTCTAAATCCTT (*Bam*HI restriction site underlined) and the 3′ primer ACCGGT CGACC$_{835}$ACTAATTTGTAAAGTTGCTT AC (*Sal*I restriction site underlined). After cloning into the pCR II-TOPO vector and sequencing, the 680 bp fragment was subcloned into pQE30 with the *Bam*HI/*Sal*I restriction sites. For over-expression of *AOC2*, *AOC3* and *AOC4*, the same approach was used with CGCGGATCCC$_{139}$TTGGTTCCTCTAAATCCTT as 5′ primer and ACCGGTCGACA$_{812}$ATTAGTTGGTA TAgTTACTTAT as 3′ primer for *AOC2*, CGCGGAT CC$_{169}$TTGGTTTCTCAAGATCTTTC as 5′ primer and ACCGGTCGACA$_{851}$CTTAATTAGTAAAGTTA CTTAT as 3′ primer for *AOC3*, and, finally, CGCGGATCCC$_{114}$ TCGGTTTCTCTAGATCCTT as 5′ primer and ACCGGTCGACT$_{790}$TTCAATTAGTAA AGTTAGCGAT as 3′ primer for *AOC4*. pQE30, without or with these inserts, were transformed into the host strain *E. coli* M15. Total protein of isopropyl-β-thiogalactopyranoside (IPTG)-induced or non-induced cultures were isolated and purified as described (Maucher *et al.*, 2000). The resulting supernatant was used for AOC activity assay as described (Ziegler *et al.*, 1997, 1999, 2000).

Quantitative measurement of α-linolenic acid, jasmonates, octadecanoids and other LOX-derived compounds

For quantitative analysis of OPDA, dinor-12-oxo-phytodienoic acid (dn-OPDA) and JA, 1 g (fresh weight) of plant tissue was frozen with liquid nitrogen, homogenized in a mortar and extracted with 10 ml of methanol, and 100 ng of [$^2H_6$]-JA and 100 ng of [$^2H_5$]-OPDA were added as internal standards. The filtrate was loaded onto 3 ml of DEAE-Sephadex A25 columns (Ac$^-$ form, methanol), and the columns

were washed with 3 ml of methanol. After subsequent washing with 3 ml of 0.1 M acetic acid in methanol, eluents obtained with 3 ml of 1 M acetic acid in methanol and with 3 ml of 1.5 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC (column: Eurospher 100-C18, 5 μm, 250 mm $\times$ 4 mm, flow rate 1 ml/min). Separation was performed with solvent A (methanol) and solvent B (0.2% acetic acid in H_2O) with a gradient of 40% solvent A to 100% within 25 min. Fractions eluting between 12 and 13.3 min, between 18.30 and 20 min and between 20.30 and 22 min were collected and evaporated. For subsequent derivatization samples were dissolved in a mixture of 200 μl $CHCl_3$ and *N*,*N*-diisopropylethylamine (1:1, v/v) and derivatized with 10 μl of pentafluorobenzyl bromide at 20 °C overnight. The samples were evaporated, dissolved in 5 ml of *n*-hexane and passed through a SiOH column (500 mg; Machery-Nagel). The pentafluorobenzyl esters were eluted with a mixture of 7 ml of *n*-hexane and diethyl ether (2:1, v/v). After evaporation probes were dissolved in 100 μl of acetonitrile and subjected to GC-MS analysis with a GCQ Finnigan instrument in the following conditions: 70 eV, NCI, ionization gas NH_3, source temperature 140 °C, column Rtx-5w/Integra Guard (Restek, Germany), 5 m inert precolumn; 30 m $\times$ 0.25 mm, 0.25 μm film thickness, injection temperature 250 °C, interface temperature 275 °C; helium 40 cm/s; splitless injection; column temperature program: 1 min at 100 °C, 25 °C/min to 200 °C, 5 °C/min to 300 °C, 20 min at 300 °C. Retention times were for [$^2H_6$]-JA-pentafluorobenzyl ester 11.92 min, for JA-pentafluorobenzyl ester 11.98 min, for dn-OPDA-pentafluorobenzyl ester 18.59 min, for [$^2H_5$]-OPDA-pentafluorobenzyl ester 21.31 min, and for OPDA-pentafluorobenzyl ester 21.39 min. Fragments *m/z* 209 (JA), 215 (JA-standard), 291 (OPDA), 296 (OPDA-standard) and 263 (dn-OPDA) were used for quantification.

PUFA and oxylipins were extracted, derivatized and separated into the corresponding positional and stereoisomeric forms by the following methods. Oxidized fatty acids were extracted according to the method of Weichert *et al.* (1999); for esterified derivatives 0.5 g fresh weight (f.w.) and for free fatty acid derivatives 1.0 g f.w. of frozen leaf tissue was added to 10 ml of extraction solvent (isohexane/isopropanol, 3:2 v/v with 0.0025% w/v BHT) and immediately homogenized with an Ultra Turrax under a stream of argon on ice for 30 s. The extract was centrifuged at 4500 $\times$ *g* at 4 °C for 10 min. The clear upper phase was collected and the pellet extracted three times with 3 ml each of extraction solvent. To the combined organic phases a 6.7% w/v solution of potassium sulfate was added to a volume of 47 ml. After vigorous shaking the upper hexane-rich layer was removed. The upper organic phase containing the oxylipin and fatty acid derivatives was dried under nitrogen and redissolved in 1.5 ml of isohexane/2-propanol (100:5 v/v), and stored under argon at -80 °C until use.

For the analysis of esterified fatty acids, the solvent was removed, and 333 μl of a mixture of toluene and methanol (1:1 v/v) and 167 μl of 0.5 mM sodium methoxide was added. As internal standards triheptadecanoate and triricinoleate were added (100 μg per probe). After incubation of the samples for 20 min, 0.5 ml of 1 M sodium chloride and 50 μl of HCl (37% v/v) were added, and fatty acid methyl esters were extracted twice each with 0.75 ml of hexane. The combined organic phases were evaporated to dryness under a nitrogen stream and the corresponding fatty acid methyl esters were reconstituted in 50 μl of methanol/water/acetic acid (85:15:0.1, v/v).

For the analysis of non-esterified oxylipins in the isohexane/isopropanol extract, the solvent was removed and the sample was solved in 400 μl of methanol. As internal standards heptadecanoic acid and (15*S*,11*Z*,13*E*)-15-hydroxy-11,13-eicosadienoic acid were added. Then 10 μl of an EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) solution (1 mg EDAC in 10 μl methanol) was added and incubated for 2 h. After adding 200 μl of 0.1 M Tris-HCl, pH 7.5, the fatty acid methyl esters were extracted twice each with 1 ml of hexane. The combined organic phases were evaporated to dryness under a nitrogen stream and the corresponding fatty acid methyl esters were reconstituted in 50 μl of methanol/water/acetic acid (85:15:0.1, v/v).

Oxylipins were analysed by HPLC on an Agilent 1100 HPLC system coupled to a diode array detector (Weichert *et al.*, 1999). At first oxylipins were purified on reversed-phase HPLC. This was carried out on a ET250/2 Nucleosil 120-5 C18 column (Machery-Nagel, 2.1 mm $\times$ 250 mm, 5 μm particle size) with methanol/water/acetic acid (85:15:0.1, v/v) as solvent system, at a flow rate of 0.18 ml/min. Straight-phase HPLC of the hydro(pero)xy fatty acids was carried out on a Zorbax Rx-SIL column (Agilent, 2.1 mm $\times$ 150 mm, 5 μm particle size) with *n*-hexane/2-propanol/acetic acid (100:1:0.1, v/v) as solvent system, at a flow rate of 0.1 ml/min. Chiral-phase HPLC of the hydro(pero)xy fatty acids was carried out on a

Chiralcel OD-H column (Daicel, 2.1 mm × 150 mm, 5 μm particle size) with *n*-hexane/2-propanol/acetic acid (100:5:0.1, v/v) as solvent system, at a flow rate of 0.1 ml/min. Absorbance at 234 nm was monitored. All oxylipins were identified by comparison to the elution times of authentic standards.

Analysis of fatty acid derivatives was carried out by gas chromatography (GC). GC analysis was performed with an Agilent GC 6890 system coupled with a FID detector and equipped with a capillary HP INNOWAX column (0.32 mm × 30 m; 0.5 μm coating thickness; Agilent, Germany). Helium was used as carrier gas (30 cm/s). The samples were measured with a split of 60:1 at an injector temperature of 220 °C. The temperature gradient was 150 °C for 1 min, 150–200 °C at 15 °C/min, 200–250 °C at 2 °C/min, and 250 °C for 10 min. All fatty acids were identified by comparison with the retention times of authentic standards.

Immunocytochemistry

Immunocytochemical analysis was performed as described recently (Hause *et al.*, 2000a). Cross-sections of leaves (2 μm thickness) were immuno-labelled with anti-AOC2 antibody (*A. thaliana*) (diluted 1:2000), anti-LOX antibody (cucumber, diluted 1:500) or anti-AOS antibody (*A. thaliana*, diluted 1:2000). As a secondary antibody a goat anti-rabbit IgG conjugated with Alexa 488 (Molecular Probes, Eugene, OR) was used in the dilution 1:500. Each immunocytochemical staining was analysed under an epifluorescence microscope (Axioskop, Carl Zeiss, Jena, Germany) with the proper filter combination.

Results

Cloning of four cDNAs coding for allene oxide cyclase: primary sequence and gene structure

To identify the *A. thaliana* homologue(s) of the recently cloned tomato AOC (Ziegler *et al.*, 2000), sequence database information revealed existence of four homologous genes. Full-length cDNAs were isolated by screening a λZAP expression library made from mRNA of rosette leaves treated with 100 μM JAME for 24 h, or were generated via RT-PCR from mRNA of rosette leaves floated on 100 μM JAME for 2 h. For this, specific primers for the putative AOC homologues found in the sequence database were used. The four different full-length cDNAs were designated as *AOC1*, *AOC2*, *AOC3* and *AOC4*. The open reading frames ranged from 759 to 774 bp (Table 1), corresponding to proteins containing 253–258 amino acid residues. The calculated molecular masses for all AOCs were about 28 kDa. Using full-length sequences, a neutral pI was calculated for AOC2, whereas for AOC1, AOC3 and AOC4 an alkaline pI was found. In contrast, calculations with sequences lacking the putative chloroplast signal peptide revealed an acidic pI for AOC1 and AOC2, whereas for AOC3 and AOC4 an alkaline pI was calculated (Table 1).

Table 1. Molecular characteristics of AOC1, AOC2, AOC3 and AOC4 of *A. thaliana* ecotype Columbia.

	AOC1	AOC2	AOC3	AOC4
ORF (bp)	762	759	774	759
Number of amino acids	254	253	258	253
Molecular mass (kDa)	27.8	27.6	28.4	27.7
pI (full-length)	9.11	6.91	9.19	9.27
pI (without putative chloroplast target sequence)	5.94	5.4	8.7	8.37
Location on chromosome	3	3	3	1

For activity assays, 5′-truncated cDNAs were cloned into the pQE30 vector and expressed in *E. coli*. Induction of expression in bacteria led to the appearance of an additional band at 26 kDa, which was absent in control bacteria transformed with the empty vector only (data not shown). Examination of bacterial extracts on AOC activity was performed as described (Ziegler *et al.*, 1997, 1999). The assay, which included recombinant AOS protein of barley as a helper enzyme (Maucher *et al.*, 2000) revealed *cis*-(+)-OPDA formation with AOC1, AOC2, AOC3 and AOC4, with the highest activity for AOC2 (data not shown). Given the specificity of the assay conditions, the *cis*-(+)-OPDA formation is indicative of AOC activity. In each AOC sequence of *Arabidopsis*, N-glycosylation sites, phosphorylation sites for protein kinase C, tyrosine kinase and casein kinase II, as well as N-myristolation sites were identified by computional analysis, but their functionality has not been tested yet. A common feature of AOC1, AOC2, AOC3 and AOC4 is the occurrence of a putative chloroplast transit peptide. All N-terminal regions are rich in Ser residues (26–30% in the first 50 amino acids of AOC1–AOC4), the start Met is followed by an Ala residue, and among the first 10 amino acids there is no charged

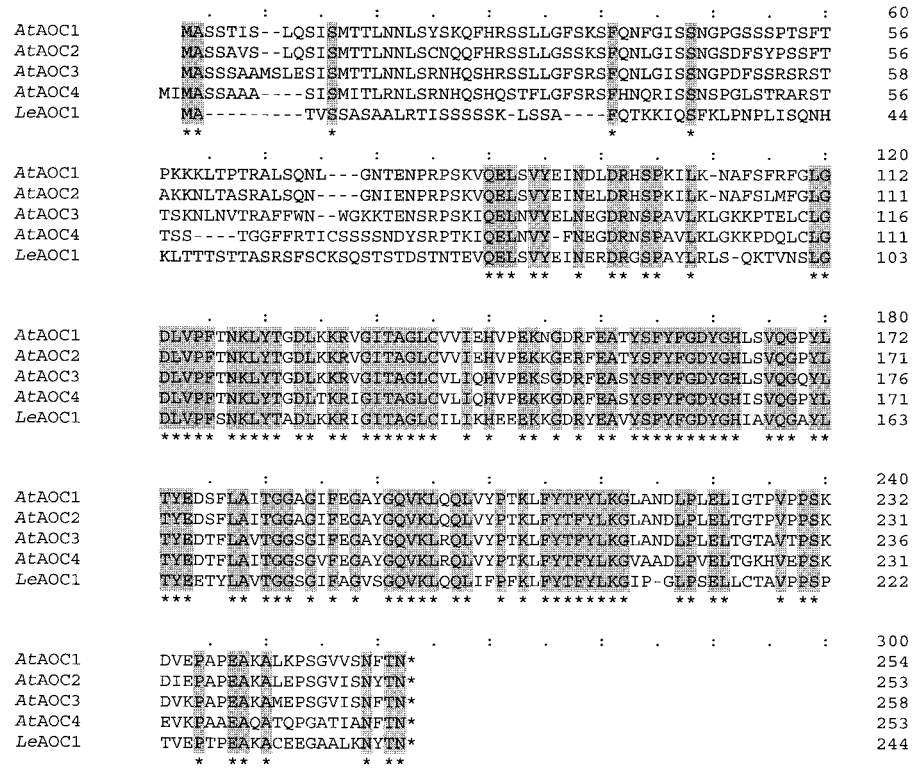

Figure 2. Amino acid sequence comparison of the four isolated AOCs from *A. thaliana* ecotype Columbia with that of tomato *Lycopersicon esculentum* cv. Lukullus published recently (Ziegler *et al.*, 2000). Putative chloroplast transit peptides are boxed in light grey. Conserved sequences are boxed in dark-grey and marked with asterisks.

amino acid. Computer analysis of the first 100 amino acids was performed with the ChloroP V 1.1 program (http://www.dtu.dk/services/ChloroP) (Emanuelsson *et al.*, 1999) and the TargetP program ver. 1.0 (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson *et al.*, 2000). In both analyses a chloroplast localization was predicted. The putative cleavage site lies between the amino acids 78 and 54 for AOC1, AOC2, AOC3 and AOC4 (Figure 2, shaded in light grey). Using an anti-AOC2 antibody (see below), we confirmed the localization of the AOC protein in chloroplasts by immunocytological analysis. Cross-sections of untreated rosette leaves incubated with anti-AOC2 antibody exhibited a strong green fluorescence label within chloroplasts (Figure 3B, D) indicating the occurrence of AOC protein. After incubation with preimmune serum only yellow-brown autofluorescence was observed (Figure 3A).

Comparison of the cDNA sequences of AOC1, AOC2, AOC3 and AOC4 with sequence database information (http://www.ucbi.nih.gov/) revealed mapping and genomic structure as follows (Figure 4A): *AOC1*, *AOC2* and *AOC3* are located on chromosome 3, whereas *AOC4* is located on chromosome 1. All four coding regions contain one intron of 369 bp, 103 bp, 271 bp and 164 bp, respectively. A search with AOC1 protein sequence with the tblastn program in the EMBL EST database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1997) and in the TIGR database (http://www.tigr.org/tab/e2K1/agi/ath1) (Altschul *et al.*, 1990) 35 putative AOC sequences could be found and reconstructed, respectively. Sequence comparison was performed which did not include putative chloroplast signal sequences in the conserved region starting at KVYEL up to the C-terminus. Among the four AOCs an identity of 74–94% was found between AOC2, AOC3 and AOC4, whereas AOC1 exhibited 60–74% identity to all other AOCs. For phylogenetic tree analysis the program Phylip 3.6 (http://www.evolution.genetics.washington.edu/phylip.html) was used (Figure 4B). The AOCs of *A. thaliana* fall into their own subgroup like solanaceous AOCs. The monocotyledonous AOCs are grouped in

a clearly different branch. Gene duplications might have occurred in the case of *A. thaliana*, *Zea mays*, *Physcomitrella patens* and *Gossypium arboretum*.

In order to record differential expression for *AOC1*, *AOC2*, *AOC3* and *AOC4*, we used specific primers amplified 150 bp after the stop codon of the corresponding 3′-UTR of each AOC. Specificity of the primers were found, with 1, 10 and 100 ng genomic PCR products of each AOC which contained the corresponding gene and 150 bp of each 5′- and 3′-UTR (Figure 5A). In the local and systemic wound response of rosette leaves AOC2 mRNA accumulated significantly more than that of AOC1, AOC3 and AOC4. Furthermore, the systemic response in terms of increase in mRNA accumulation was stronger for AOC1 than for the AOC3 and AOC4. Beside these preferential mRNA accumulations, each AOC exhibited transiently a local and systemic mRNA accumulation.

Abundant occurrence of AOC protein in leaves is independent of stress-induced AOC mRNA accumulation

Accumulation of jasmonates and octadecanoids, occurring upon sorbitol treatment (Bohlmann *et al.*, 1998) or wounding (Laudert and Weiler, 1998; Reymond *et al.*, 2000) of *A. thaliana* leaves, correlates in timing with the mRNA accumulation of 13-LOX (Bell *et al.*, 1995) and of AOS (Laudert and Weiler, 1998). We complemented these data by recording accumulation of AOC mRNA. The full-length cDNA (AOC1) which hybridized also with all other mRNAs coding for AOCs was used. Total RNA of rosette leaves treated with JAME, its precursor 13-HPOT or various stresses was probed (Figure 6A). Rapid and transient AOC mRNA accumulation occurred already in detached water-treated rosette leaves, reflecting a wound response by detachment. In contrast, untreated leaves of intact plants exhibited a negligible AOC mRNA accumulation (Figure 8C). Treatment with 50 μM JAME or 13-HPOT (Figure 6A) led to additional AOC mRNA accumulation, which was similar upon treatment with OPDA or JA-amino acid conjugates (data not shown). In case of 13-HPOT treatment, 3-fold higher levels of OPDA and JA could be detected within 30 min (data not shown), indicating endogenous formation of both compounds. Accumulation of AOC mRNA occurred also in a similar amount and time range, when leaves were floated on 1 M sorbitol or 0.5 M glucose. In contrast, with 50 μM SA and, in a more pronounced fashion, with 1 M NaCl an inhibitory effect on AOC mRNA accumulation compared to water-treated leaves was found. For comparison, AOS mRNA accumulation was analysed. Except for NaCl treatment, similar if not identical results were found.

The AOC mRNA levels were not reflected by the AOC protein levels. In most treatments, the AOC protein exhibited constitutively high level with slight rise in the first hour and a significant rise after 8 h (Figure 6B). The anti-AOC2 antibody was able to recognize all four recombinant AOCs (Figure 6C). However, the AOC2 protein was preferentially recognized (cf. legend to Figure 6C). Therefore, AOC2 might be preferentially detected in the immunoblot analysis and the immunocytochemical analysis. Immunocytological inspection revealed an abundant appearance of AOC protein in all tissues of fully developed rosette leaves of the ecotype Columbia, whereas developing rosette leaves showed less AOC protein (Figure 7). A similar distribution and abundance were detected for LOX and AOS at the protein level. This constitutive occurrence of LOX, AOS and AOC protein in untreated fully developed leaf tissues is in apparent contrast to the stress-induced transient accumulation of their mRNAs (Figure 6A) (Bell *et al.*, 1995; Laudert and Weiler, 1998).

The oxylipin profiles of untreated rosette leaves reveal constitutive LOX pathway reactions with preferential activity of the AOS branch

The abundant occurrence of LOX, AOS and AOC proteins in untreated leaf tissues prompted us to determine profiles of fatty acids and various LOX-derived metabolites including the initial JA-precursor 13-HPOT. Each of them was recorded quantitatively for the ecotype Columbia in the free and the esterified form (Table 2). The *Arabidopsis* genome contains no gene coding for divinyl ether synthase. Therefore, formation of divinyl ethers is not to be expected. The amounts of C6 volatiles as well as α- and γ-ketols were below the detection limit which is in the range of 10 pmol per gram f.w. for all compounds tested. In the case of esterified fatty acids a strong preponderance of polyenoic fatty acids was observed with preferential occurrence of α-LeA up to the micromolar range (Table 2). Among esterified compounds originating from LOX activity, substantial amounts (20 nmol/g f.w.) of 13-HOT were detected, whereas other LOX products were found esterified to less than 10-fold lower levels.

Table 2. Amounts of free and esterified fatty acids and the corresponding hydroxy derivatives as well as of free dn-OPDA, OPDA, and JA in untreated 6-week old rosette leaves of ecotype Columbia. Leaf tissue was colleted from 3 to 4 different plants and subjected to extraction and separation as described in Materials and methods.

Fatty acid	Esterified, pmol per gram f.w.	Free, pmol per gram f.w.
16:0	620 000	222 000
16:1	60 000	n.d.[a]
16:3	990 000	n.d.
18:0	50 000	280 000
18:1	180 000	4 000
18:2	900 000	7 000
18:3	2 820 000	10 000
9-HOD	100 000	30
13-HOD[d]	400 000	32
13-/9-KOD	n.d.	10
9-KOD	n.d.	12
9-HOT	300	28
13-HOT	20	179
13-/9-KOT	2 200	n.d.
12-HOT	n.d.	n.d.
16-HOT	n.d.	n.d.
OPDA	–[c]	951
dn-OPDA	–[c]	75
JA	–	43

[a]Not detectable; [b]13-KOD ((9*Z*,11*E*)-13-keto-(9,11)-octadecadienoic acid); [c]Not determined; [d](13*S*,9*Z*,11*E*,15*Z*)-hydroxy-(9,11,15)-octadecadienoic acid.

Among the free fatty acids 16:0 and 18:0 dominated, whereas linoleic acid (LA) and α-LeA were found at 7 and 10 nmol per gram f.w., respectively. The substrate for generation of dn-OPDA, the 16:3 fatty acid, could not be detected in its free form. Also products of the LOX reaction itself, HPOD originating from LA and HPOT originating from α-LeA, could not be detected, although these hydroperoxy compounds are stable at least in part in our work-up procedure (Feussner *et al.*, 1997). However, HOD and HOT derivatives, both indicative of the reductase branch of the LOX pathway, were detected with preference to 13-HOT. LA derivatives accumulated to more than 6-fold lower level without preference of a positional isomer. Among all free LOX-derived products, compounds of the AOS branch were dominant. Nearly 1 nmol per gram f.w. OPDA was found, whereas for dn-OPDA and JA 13-fold and 22-fold lower levels were detected, respectively.

Oxylipin profiles and AOC expression in opr3 mutant leaves suggest a positive feedback loop in JA biosynthesis

The abundant occurrence of OPDA and the 10-fold higher level of the LOX substrate, α-LeA, as well as constitutive high levels of LOX, AOS and AOC protein in untreated rosette leaves raise the question of how regulation of JA biosynthesis occurs. To dissect the effects of JA and OPDA, we performed metabolic profiling and expression analyses of JA biosynthetic genes with untreated and wounded leaves of the JA-deficient mutant *opr3* as well as the corresponding wild-type Wassilewskija. In the case of free and esterified fatty acids, as well as hydroxy and hydroperoxy fatty acid derivatives, most levels were similar if not identical in the *opr3* mutant compared to the wild type. Only 13-HOT levels were 5-fold lower in the mutant, suggesting decreased capacity of the LOX-catalysed step (data not shown).

In untreated leaves of the ecotype Wassilewskija about 2.9 nmol per gram f.w. OPDA was found, which is somewhat above the range of previously detected levels (Stelmach *et al.*, 1999; Reymond *et al.*, 2000; Stintzi and Browse, 2000). This amount exceeds that of JA and dn-OPDA 13- to 35-fold, respectively (Figure 8A). OPDA is also the dominant compound in untreated *opr3* mutant leaves. Despite these remarkable OPDA levels in untreated leaves of both the wild type and the mutant, and the fact that AOC expression is OPDA responsive, AOC mRNA accumulation was below the detection limit (Figure 8C). Upon wounding of wild-type leaves, no dramatic changes occurred in OPDA and dn-OPDA levels compared to a 24-fold increase in JA levels within the first 1.5 h. Even in the *opr3* mutant, OPDA levels did not increase dramatically. The JA level decreased up to 24 h upon wounding of wild-type leaves, whereas OPDA and dn-OPDA levels increased in this time. AOC mRNA and AOS mRNA accumulated abundantly within 1.5 h upon wounding of wild-type leaves (Figure 6A, Figure 8C), possibly caused by the preferential accumulation of JA. Indeed, in wounded leaves of the *opr3* mutant exhibiting the expected JA deficiency (Figure 8B), much less AOC mRNA and AOS mRNA accumulated than in the wild type during 1.5 h of wounding (Figure 8C). Most interestingly, the level of AOC protein, but not of AOS protein, was much less in the untreated *opr3* mutant leaves and increased slightly upon wounding (Figure 8C). This is also indicated by the immunocytochemical inspection, where AOC protein but not

LOX and AOS protein was less abundant in untreated *opr3* mutant leaves than in untreated leaves of the corresponding wild type Wassilewskija (Figure 7). These data suggest a lower capacity of the AOS branch in the JA-deficient mutant *opr3*.

Discussion

In Arabidopsis *four genes encode chloroplast-located AOCs*

The lipid-derived jasmonates and octadecanoids are of considerable and increasing interest by virtue of their unique role in plant stress responses and in distinct stages of development (for review, see Wasternack and Hause, 2002). In JA biosynthesis the AOC catalyses a crucial step by establishing the correct enantiomeric structure of jasmonates. Here, we identified four genes coding for AOCs of *A. thaliana*. All of them carry a chloroplast target sequence which seems to be active as revealed by immunocytochemical analysis (Figure 3). The anti-AOC2 antibody used for these experiments recognized all recombinant AOCs of *A. thaliana* (Figure 6C). Therefore, the lack of detectable AOC protein outside the chloroplast suggests that it is predominantly if not completely located in this organelle. In contrast to the existence of four different AOCs, only one gene coding for AOS exists in *A. thaliana* (Laudert *et al.*, 1996; Laudert and Weiler, 1998). The putative chloroplast target sequence detected for AOS (Laudert *et al.*, 1996) is shown here to be functionally active (Figure 7). Because of the short half-life of the AOS product in aqueous solution, a physical interaction of AOS and AOC might be assumed. However, we and others (E. Weiler, personal communication) failed to show such an interaction in a yeast two-hybrid system. It will be interesting to see whether the various AOCs differ in their location within sub-compartments of the chloroplast.

The existence of multiple genes coding for an enzyme of hormone biosynthesis is a common phenomenon. It has been reported for nitrilase in auxin biosynthesis (Bartel and Fink, 1994) and for 1-aminocyclopropane-1-carboxylate oxidase in ethylene biosynthesis (Barry *et al.*, 1996), and was found to be related to separate functions. Also for the AOC-catalysed step in JA biosynthesis, we expect non-redundant activity as suggested by preliminary data on specific expression of *AOC1*, *AOC2*, *AOC3* and *AOC4* in different organs. This is under study with promoter GUS lines of *AOC1*, *AOC2*, *AOC3* and *AOC4*. With probes specific for each *AOC*, a preferential AOC2 mRNA accumulation and a slightly higher AOC1 mRNA accumulation in the systemic response upon wounding of rosette leaves were found (Figure 5A, B), suggesting distinct roles in the wound response. Because of the systemic induction of AOS upon local wounding (Laudert and Weiler, 1998; Kubigsteltig *et al.*, 1999), it will be interesting to see whether AOS functions specifically with AOC1 in the systemic leaf. Another possibility for distinct functions of the different AOC genes is given by the occurrence of OPDA and dn-OPDA in *A. thaliana* (Figure 8) (Weber *et al.*, 1997; Reymond *et al.*, 2000). Possibly, the AOCs differ in their specificity to substrates originating from α-LeA and leading to OPDA or originating from hexadecatrienoic acid and leading to dn-OPDA. Also their pH optima might be different due to their different pIs (Table 1). However, such data are difficult to obtain, because the substrate for the AOC reaction is unstable, and to date only qualitative enzyme measurements are possible by using coupled enzyme assay.

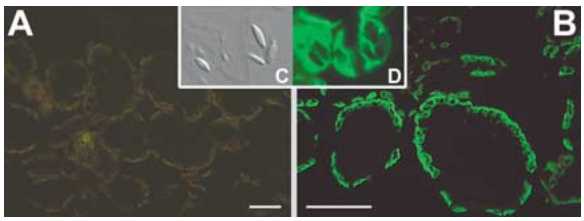

Figure 3. Immunocytological localization of AOC protein in rosette leaves of ecotype Columbia. Cross-sections of untreated leaves were probed with preimmune serum (A) or with anti-AOC-antibody raised against purified recombinant AOC2 of *A. thaliana* (B), followed by labelling with fluorescence-labelled secondary antibody. Whereas the labelling with pre-immune serum exhibits only the yellow-brown autofluorescence of chloroplasts (A), strong green fluorescence label within chloroplasts in B is indicative of the AOC protein. In C and D higher magnifications of B are shown to visualize starch granules by DIC image (C) or by the absence of fluorescence (D) (Bars: 50 μm in A and B).

JA biosynthesis is regulated by OPDA compartmentalization and a JA-mediated positive feedback loop

The stress-induced rise of jasmonates is a common phenomenon in all plants analysed so far. It is less understood, however, how this rise is regulated. A positive feedback was discussed based on data of transcriptional up-regulation of LOX, AOS, AOC and OPR3 upon treatment with jasmonates or biotic and

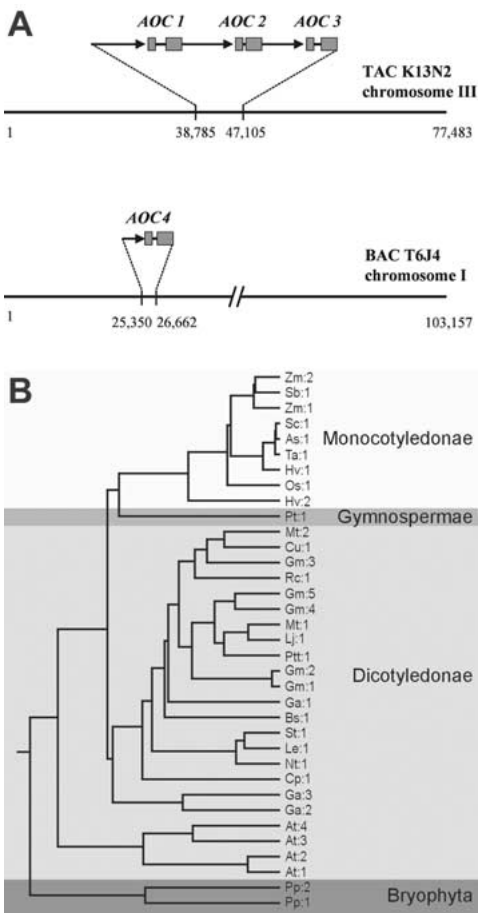

Figure 4. Mapping and genomic structure of *AOC1*, *AOC2*, *AOC3* and *AOC4* (A) and phylogenetic tree analysis (B). In A exons are denoted by closed rectangles and introns are represented by lines. *AOC1*, *AOC2* and *AOC3* map to TAC clone K 13N2 (chromosome 3, accession number AB028607) and *AOC4* maps to BAC clone T6J4 (chromosome 1, ACO11810). B shows a phylogenetic tree of 35 AOC proteins performed with the program Phylip 3.6. The AOC proteins refer to the following accession numbers in GenBank and the TIGR database (release of 7 February 2002): *Gossypium arboreum*, Ga:1 (TC926), Ga:2 (BG442722), Ga:3 (BG447327); *Medicago truncatula*, Mt:1 (AJ308489), Mt:2 (TC38027); *Citrus unshiu*, Cu:1 (C95219); *Ricinus communis*, Rc:1 (T15248); *Glycine max*, Gm:1 (TC79538), Gm:2 (TC79537), Gm:3 (TC79536), Gm:4 (TC84305), Gm:5 (TC85514); *Lotus japonicus*, Lj:1 (BI418833); *Populus tremula* and *Populus tremuloides*, Ptt:1 (BI128083 and BI069431); *Bruguiera sexangula*, Bs:1 (AB037929); *Solanum tuberosum*, St:1 (TC24555); *Lycopersicon esculentum*, Le:1 (AJ272026); *Nicotiana tabacum*, Nt:1 (AJ308487); *Citrus paradisi*, Cp:1 (BE205690); *Pinus taeda*, Pt:1 (BF609803 and BG275459); *Zea mays*, Zm:1 (TC92158), Zm:2 (TC92160); *Sorghum bicolor*, Sb:1 (TC25717); *Secale cerale*, Sc:1 (TC88); *Aegilops speltoides*, As:1 (BF291320); *Triticum aestivum*, Ta:1 (TC15431); *Hordeum vulgare*, Hv:1 (AJ308488), Hv:2 (BF256000); *Oryza sativa*, Os:1 (TC57764); *Arabidopsis thaliana*, At:1 (AJ308483), At:2 (AJ308484), At:3 (AJ308485), At:4 (AJ308486); *Physcomitrella patens*, Pp:1 (BJ182454 and AW509842), Pp:2 (BJ204710, BJ190940 and BJ168463).

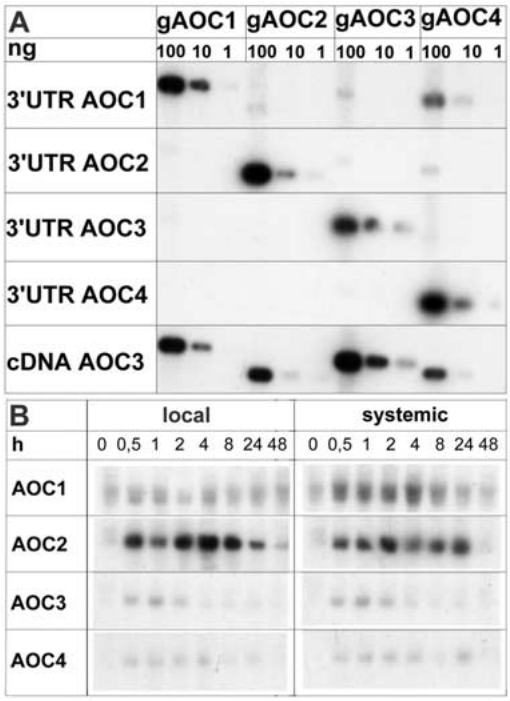

Figure 5. Expression analysis for *AOC1*, *AOC2, AOC3* and *AOC4* during local and systemic wound response. As shown in A, a 150 bp fragment of the 3′-untranslated region (3′-UTR) of each AOC hybridizes specifically with the corresponding genomic PCR fragment (100, 10, 1 ng) whereas the full-length cDNA of AOC3 hybridizes with genomic fragments of each AOC. In B the 150 bp 3′-UTR fragments were used to probe kinetics of local and systemic mRNA accumulation of AOC1, AOC2, AOC3 and AOC4 upon wounding.

abiotic stresses (Royo *et al.*, 1996; Laudert and Weiler, 1998; Howe *et al.*, 2000; Maucher *et al.*, 2000; Müssig *et al.*, 2000; Reymond *et al.*, 2000; Sivasankar *et al.*, 2000; Ziegler *et al.*, 2000). However, the following observations do not fit with a positive feedback in transcriptional regulation, at least in short-term treatments. First, despite transcriptional up-regulation of JA biosynthetic enzymes, there was no endogenous formation of jasmonates in barley or tomato leaves over 24 h (Kramell *et al.*, 2000; Miersch and Wasternack, 2000). Second, although AOS mRNA, protein and activity increased upon JA treatment in *Arabidopsis* (Laudert and Weiler, 1998), there was no corresponding OPDA accumulation (Stelmach *et al.*, 1999). Third, wounding or insect feeding led to a transient burst of JA within the first hour preceding the accumulation of AOS mRNA and AOC mRNA, respectively (Ziegler *et al.*, 2001; Stenzel *et al*., 2002).

Here, we detected basal occurrence of LOX, AOS and AOC proteins in all tissues of fully developed, un-

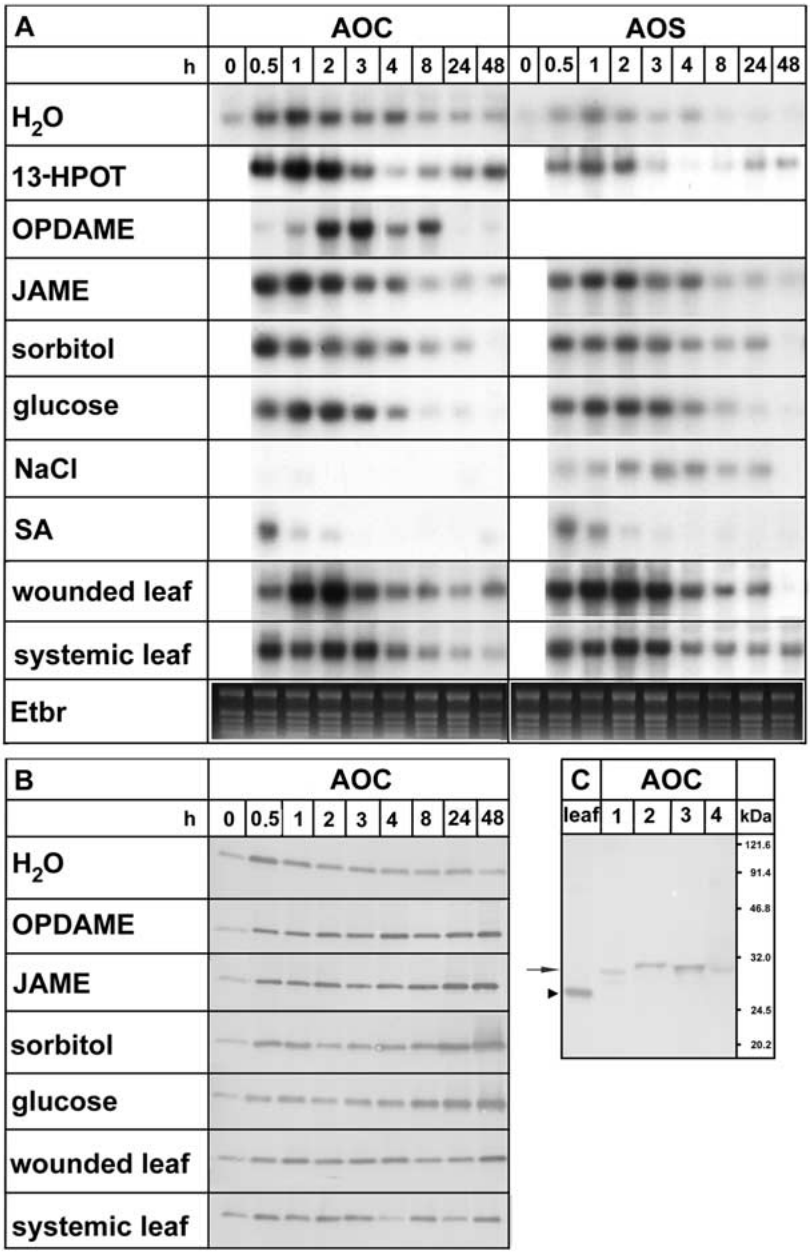

Figure 6. Expression of AOC genes in rosette leaves of *A. thaliana* in response to JAME and various other substance applications or stress conditions probed by northern blot analysis (A) and immunoblot analysis (B). For northern blot analysis rosettes were floated on distilled water, 50 μM 13-HPOT, 50 μM JAME, 1 M sorbitol, 0.5 μM glucose, 1 M NaCl or 50 μM SA for indicated times or wounded by crushing with a tailor's wheel in 3 mm distance. Per lane 20 μg of total RNA was loaded, and ethidium bromide staining served as loading control, as shown. All blots were hybridized with a ^{32}P-labelled full-length cDNA of AOC2. For immunoblot analysis (B), identical samples as in (A) were used for total protein extraction. Analysis was performed with 5 μg proteins per lane and an anti-AOC antibody diluted 1:5000. In (C) cross-reactivity is shown of the anti-AOC antibody raised against AOC2, with recombinant AOC1, AOC2, AOC3 and AOC4, indicated by an arrow as well as with AOC of a total leaf extract indicated by triangle. As a control 5 μg leaf protein was loaded. To detect cross-reactivity of the anti-AOC2 antibody with the other recombinant AOCs, the following amounts (ng per lane) was loaded: AOC1, 62.5; AOC2, 2.5; AOC3, 6.25; AOC4, 37.5.

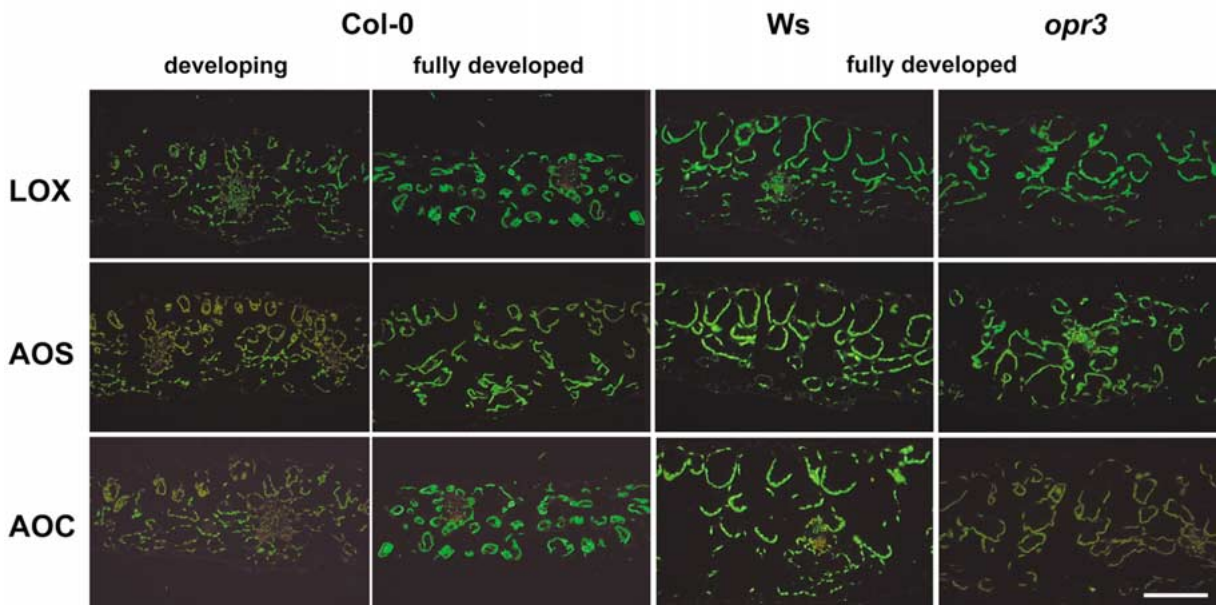

Figure 7. Immunohistochemical analysis on the occurrence of LOX, AOS, and AOC in untreated rosette leaves of the ecotypes Columbia (Col-O) and Wassilewskija (WS) and of the *opr3* mutant. Cross-sections were probed with an anti-LOX-antibody (dilution 1:500), an anti-AOS antibody (dilution 1:2000) or an anti-AOC antibody, used for Columbia in a dilution of 1:2000 and for Wassilewskija and *opr3* in a dilution 1:1000. The green fluorescence indicates occurrence of LOX, AOS and AOC protein, respectively. The fluorescence intensity is clearly more pronounced in fully developed leaves than in developing leaves of the ecotype Columbia. Whereas in the ecotype Wassilewskija and the *opr3* mutant similar label intensity is indicated for LOX and AOS proteins, the AOC protein level is significantly less in the mutant leaves than in the wild type. Yellow-brown autofluorescence appeared only upon treatment with the corresponding preimmune sera (data not shown, see also Figure 3A). Bars represent 100 μm in all figures.

treated rosette leaves (Figure 7). In contrast, tomato leaves exhibit specific expression of AOC in vascular bundles (Hause *et al.*, 2000a), accompanied by preferential accumulation of jasmonates in main veins compared to intercostal regions (Stenzel *et al.*, 2002). The constitutive high level of LOX, AOS and AOC in all leaf tissues of *A. thaliana* suggests that OPDA can be formed constitutively. Indeed, levels of free OPDA were detected of about 1 nmol per gram f.w. in the ecotype Columbia (Table 2) (Reymond *et al.*, 2000) and about 2.9 nmol/g in the ecotype Wassilewskija (Figure 8A). Furthermore, besides this remarkable amount of free OPDA, the main portion is esterified in chloroplast lipids (Stelmach *et al.*, 2001). Although its formation is unclear to date, one possibility is the action of LOX, AOS and AOC with esterified substrates, generating esterified OPDA. Upon wounding esterified OPDA is rapidly released (Stelmach *et al.*, 2001).

The remarkable level of free OPDA in untreated leaves (Figure 8) and the significant increase in LOX, AOS and AOC protein between young leaves to fully developed leaves (Figure 7) suggest a positive feed back loop during leaf development. *Arabidopsis* leaves treated with OPDA accumulate mRNA of LOX and AOS (Laudert and Weiler, 1998; Stintzi *et al.*, 2001) and of AOC (this paper). However, despite the remarkable levels of free OPDA (Figure 8) and esterified OPDA (Stelmach *et al.*, 2001) in untreated wild-type leaves, AOS RNA accumulated only very weakly and expression of AOC genes appeared only upon wounding (Figure 8C). Also in untreated *opr3* mutant leaves, AOC mRNA was not detectable despite high levels of OPDA. We suggest that over extended periods such as development of a wild-type leaf, release of OPDA from the chloroplast and generation of JA can occur at low levels. This amount of JA may induce expression of undetectable levels of mRNAs of LOX, AOS and AOC while leading to accumulation of the corresponding proteins detected by immunoblot and immunocytochemical analyses (Figures 7 and 8C). This scenario is supported by several findings. First, upon external application of JA or stress, both of which lead to an endogenous rise of jasmonates, mRNAs of LOX, AOS and AOC accumulate rapidly but transiently (Figure 6) (Bell and Mullet, 1993; Laud-

ert and Weiler, 1998). Second, the remarkable level of free and esterified OPDA might be biologically inactive in untreated developing leaves due to its confinement within the chloroplast. This is indicated by the fact, that OPDA-responsive *AOC* expression occurs in wounded but not unwounded *opr3* mutant leaves, and that wounded but not unwounded leaves of Thi2.1::*uidA* plants as well as VSP1::*uidA* plants show promoter activity (Bohlmann *et al.*, 1998; Ellis and Turner, 2001). In contrast, in untreated leaves of the *cet* (constitutive expression of thionin) mutant, which contains constitutively elevated JA levels, constitutive expression of the JA-responsive *Thi2.1* gene occurs (Hilpert *et al.*, 2001). Third, the proposed positive feed back loop seems to occur to a smaller extent in the JA-deficient OPDA-containing *opr3* mutant than in the wild type as shown by much less AOC protein in untreated mutant leaves (Figures 7 and 8C). Consequently, unwounded *opr3* mutant leaves contain less free OPDA than expected by the block in OPR3. This suggests that JA but not OPDA functions as the preferential signal of a putative feedback. Consistent with that is the significantly lower accumulation of AOS mRNA and AOC mRNA accumulation in wounded *opr3* leaves than in wild-type leaves. Interestingly, only the AOC protein but not the LOX and AOS proteins accumulated to less extent in *opr3* suggesting a preferential role of AOC in regulation of JA biosynthesis (Figures 7 and 8C). It will be interesting to see how OPR3 and β-oxidation steps, both assumed to be located within the peroxisomes, as well as a putative activation/transport of OPDA into peroxisomes attribute to overall regulation of JA biosynthesis. A positive feedback in JA biosynthesis was also suggested for senescing leaves which exhibited elevated JA levels and rise in mRNA accumulation of most genes coding for JA biosynthetic enzymes (He *et al.*, 2002).

13-HPOT, the initial substrate of the AOS branch was below the detection limit. However, JA levels are known to rise transiently upon wounding or other treatments generating α-LeA (Weber *et al.*, 1997; Bohlmann *et al.*, 1998; Reymond *et al.*, 2000). Consequently, application of 13-HPOT increases JA levels (data not shown) and induces expression of genes of AOC or other enzymes in JA biosynthesis (Figure 6) (Stenzel *et al.*, 2002). In contrast to leaves, in flowers of the mutant *dad1*, which is defective in a chloroplast-located PLA1, the JA levels were only 22% of that of the wild type suggesting limited generation of the initial substrate in JA biosynthesis (Ishiguro *et al.*, 2001).

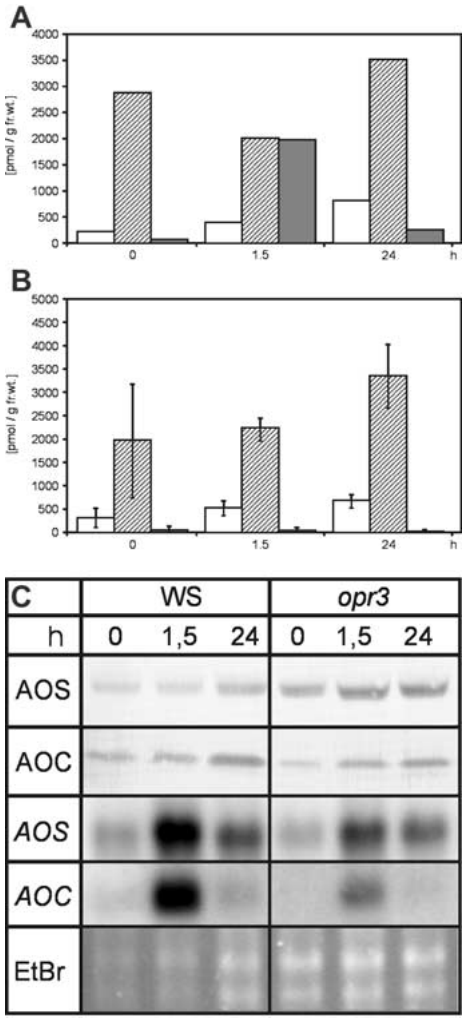

Figure 8. Levels of dn-OPDA, OPDA, and JA (A, B) and accumulation of mRNAs of AOS and AOC as well as of AOS protein and AOC protein (C) in unwounded and wounded rosette leaves of wild type (WS) and *opr3* mutant. For A and B, rosette leaves of 6-week old plants were wounded. For A leaves were pooled at least from four different plants of identical treatment and were subjected to analysis as described in Materials and methods. For B, three independent batches of leaf material were analysed for dn-OPDA (open bars), OPDA (hatched bars) and JA (filled bars). For C, an aliquot of leaf material as in A and B was subjected to northern blot and immunoblot analysis. Per lane 5 μg of total RNA was loaded, which was checked by ethidium bromide (EtBr) staining. For protein analysis 5 μg per lane was loaded and probed with anti-AOS antibody at a dilution of 1:2500 or with an anti-AOC antibody at a dilution of 1:5000.

To date the function of homologous proteins of DAD1 in rosette leaves is unknown. The undetectable amount of 13-HPOT and the high level of free and esterified α-LeA suggest the LOX reaction as another or additional control point in the substrate generation for JA biosynthesis. To date, however, there is no proof as to which of the six LOX forms found in the genome of *A. thaliana* functions in JA biosynthesis (Feussner and Wasternack, 2002). Four of them (LOX2, 3, 4, 6) are 13-LOXs and exhibit putative chloroplast target sequences. They might be detected in the immunocytochemical analysis shown in Figure 7, and they are candidates to function in JA biosynthesis possibly in different tissues of the plant. Based on previous data, it is very likely that in the case of leaves LOX2 is the major LOX involved in JA biosynthesis (Bell *et al.*, 1995).

The abundant occurrence of enzymes of JA biosynthesis within the chloroplast and rise in JA levels upon external stimuli such as wounding shown here, together with the recently found covalently linked OPDA derivatives, point to sequestration of enzymes and substrates as an important type of control in JA biosynthesis. LOX, AOS and AOC protein compartmentalized within the chloroplast (Figures 3 and 7) (Bell *et al.*, 1995; Laudert and Weiler, 1998) might function by substrate channelling during basal JA formation, and wounding may allow substrate accessibility by the relevant enzymes, thereby increasing JA formation. This model was discussed recently (Froehlich *et al.*, 2001) and is in accordance to previous data on 35S::LOX2*antisense* plants (Bell *et al.*, 1995), which exhibited a decrease in wound-induced JA formation only, but were still able to form residual levels of JA.

Another possibility in regulation of JA biosynthesis would be an activity control by post-translational modifications of the pre-existing enzymes such as AOC. Such a control would allow the plant to respond rapidly upon wounding or pathogen/insect attack by formation of JA which functions subsequently as a signal in defence gene expression. Such a rapid burst of JA and/or OPDA was found in tobacco and other plants upon insect attack (Heil *et al.*, 2001; Ziegler *et al.*, 2001) or wounding (Laudert *et al.*, 2000; Stenzel *et al.*, 2002, submitted). The functional role of subsequent transcriptional up-regulation of JA-biosynthetic enzymes observed here and by many others remains to be elucidated.

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Short Communication

Enzymes of Jasmonate Biosynthesis Occur in Tomato Sieve Elements

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The allene oxide cyclase (AOC) is a plastid-located enzyme in the biosynthesis of the signaling compound jasmonic acid (JA). In tomato, AOC occurs specifically in ovules and vascular bundles [Hause et al. (2000) *Plant J.* 24; 113]. Immunocytological analysis of longitudinal sections of petioles and flower stalks revealed the occurrence of AOC in companion cells (CC) and sieve elements (SE). Electron microscopic analysis led to the conclusion that the AOC-containing structures of SE are plastids. AOC was not detected in SE of 35S::*AOCantisense* plants. The enzymes preceding AOC in JA biosynthesis, the allene oxide synthase (AOS) and the lipoxygenase, were also detected in SE. In situ hybridization showed that the SE are free of AOC-mRNA suggesting AOC protein traffic from CC to SE via plasmodesmata. A control by in situ hybridization of AOS mRNA coding for a protein with a size above the exclusion limit of plasmodesmata indicated mRNA in CC and SE. The data suggest that SE carry the capacity to form 12-oxo-phytodienoic acid, the unique precursor of JA. Together with preferential generation of JA in vascular bundles [Stenzel et al. (2003) *Plant J.* 33: 577], the data support a role of JA in systemic wound signaling.

Keywords: Enzymes in JA biosynthesis — Immunocytochemistry — Sieve elements — Tomato.

Abbreviations: α-LeA, α-linolenic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; CC, companion cells; 13-HPOT, 13-hydroperoxy-octadecatrienoic acid; JA, jasmonic acid; JAME, JA methyl ester; 13-LOX, 13-lipoxygenase, OPDA, 12-oxo-phytodienoic acid; SE, sieve elements.

Jasmonic acid (JA) and its methyl ester (JAME) collectively called jasmonates, are members of a lipid-based signaling cascade originating from polyunsaturated fatty acids such as α-linolenic acid (α-LeA). Together with their precursor 12-oxo-phytodienoic acid (OPDA), these compounds are signals in plant responses to biotic and abiotic stresses and in plant development (Wasternack and Hause 2002). A common property of numerous plant stress responses is the elevation of OPDA and jasmonate levels followed by expression of jasmonate-responsive genes. Among them are genes that code for enzymes of JA biosynthesis thus implicating a positive feed-back loop.

The biosynthesis of JA is initiated by insertion of molecular oxygen into position 13 of α-linolenic acid (α-LeA) catalyzed by a 13-lipoxygenase (13-LOX) (Feussner and Wasternack 2002). The LOX product (13*S*)-hydroperoxy (9*Z*,11*E*,15*Z*) octadecatrienoic acid (13-HPOT) is converted by an allene oxide synthase (AOS) to highly unstable allene oxide which in turn can rapidly decay in aqueous solution by chemical hydrolysis to α- and γ-ketols and racemic OPDA. In the presence of the allene oxide cyclase (AOC), exclusive formation of the *cis*-(+)-enantiomer (9*S*,13*S*) of OPDA occurs. This enantiomer carries the structure of the naturally occurring (+)-7-*iso*-JA, which is generated from an OPDA reduction and three β-oxidative steps.

The initial substrate of JA biosynthesis is α-LeA and originates from chloroplast membranes. A recently characterized JA-deficient mutant of *Arabidopsis*, was found to be affected in a chloroplast-located phospholipase A1 (Ishiguro et al. 2001). Subsequent enzymes of the first half of JA biosynthesis including the 13-LOX, the AOS and the AOC, carry putative target sequences for plastid import, and their location within chloroplasts was shown by immunocytochemical analysis (Feussner et al. 1995, Maucher et al. 2000, Ziegler et al. 2000) or import studies (Bell et al. 1995, Froehlich et al. 2001). OPDA reduction was localized in peroxisomes (Stintzi and Browse 2000, Strassner et al. 2002), where the final β-oxidation steps are assumed to occur.

The occurrence of enzymes of JA biosynthesis and the ability to form JA and related compounds were observed in various tissues including flower organs (Hause et al. 2000), leaves (Wasternack and Hause 2002), roots (Hause et al. 2002), as well as mesocotyl (Maucher et al. 2000). The latter examples indicate that JA biosynthesis is not dependent on photosynthetically active chloroplasts. There is, however, a remarkable tissue-specificity in the occurrence of enzymes of JA biosynthesis such as AOS and AOC. In barley, AOS is expressed in young convoluted leaves, in the first internode and in the scutellar node, all exhibiting elevated JA levels

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(Maucher et al. 2000). In tomato, the AOC, a protein of 26 kDa, was detected by immunocytochemical analysis exclusively in vascular bundles of petioles, petiolules and main veins of leaves, in the bundle sheet of the minor veins and in ovules of flower buds (Hause et al. 2000).

The phloem, composed of enucleate sieve elements (SE) which are intimately connected with companion cells (CC), functions in long distance transport of signaling molecules and pathogens and for nutrient reallocation (Oparka and Turgeon 1999). More than 200 proteins were found in fully developed SE ranging from 2 to 200 kDa. Among them are the abundant P-proteins (Golecki et al. 1999), proteinases (Mehta et al. 1996), proteinase inhibitors (Xu et al. 2001) and protein kinases (Yoo et al. 2002). Only some SE-located proteins were found in plastids. Sucrose transporters could be co-localized in plasma membranes of SE and may function by oligomerization (Reinders et al. 2002). Recently, presence and activity of a complete antioxidant defense system was detected in mature SE (Walz et al. 2002). This example emphasizes that in SE circuit functions of nucleate cells are kept.

Direct analysis of phloem sap composition could be performed with only few plants due to sealing of the wounded phloem. In *Cucurbita*, *Ricinus*, *Perilla* or *Oryza*, phloem sap could be sampled directly or by use of aphids followed by complex analysis including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Haebel and Kehr 2001, Hoffmann-Benning et al. 2002, Nakamura et al. 1995). In most cases location of proteins in the SE was identified via immunocytological techniques. Here, we show by means of immunocytological analysis that enzymes of JA biosynthesis such as LOX, AOS and AOC are located within SE. Using in situ hybridizations, AOC-mRNA was found exclusively in CC, whereas AOS mRNA was found in both cell types suggesting AOC transport via plasmodesmata into SE. The data implicate the capacity of SE to form OPDA and possibly JA and suggest a role in systemic wound signaling.

Lycopersicon esculentum Mill. cv. Lukullus plants were grown as described (Wasternack et al. 1998). Identical cultivation conditions were used for the homozygous transgenic line of the T2 generation carrying the tomato cDNA for AOC in antisense orientation under the control of the 35S promoter. Field grown *Plantago major* plants were used for mechanical isolation of intact phloem from petioles. For extraction and quantitative analysis of JA and OPDA, tissues (0.5 g FW) were frozen in liquid nitrogen, homogenized in a mortar and extracted with 5 ml 80% (v/v) methanol. For quantification $[^2H_6]$JA and $[^2H_5]$OPDA were added as internal standards. Purification, fractionation and quantitative analysis of OPDA and JA by HPLC and GC-MS were performed as described (Hause et al. 2000, Kramell et al. 2000).

Small pieces of petioles or flower stalks of 6-week-old tomato plants were harvested and fixed with 3% (w/v) paraformaldehyde in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). After dehydration by a graded series of ethanol, material was embedded in paraplast (Sigma, Deisenhofen, Germany) or polyethylene glycol (PEG, Merck, Darmstadt, Germany). For in situ hybridization cross-sections of paraplast-embedded material (10 μm thick) were mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated. In situ hybridization was performed as described (Maucher et al. 2000) using DIG-labeled *antisense* and *sense* RNA probes obtained from tomato AOC cDNA or tomato AOS2 cDNA (Howe et al. 2000) by in vitro transcription.

Immunocytochemical analysis was performed as described (Hause et al. 2000) using longitudinal sections (2 μm thick) from PEG-embedded tissues. Samples were analyzed with an anti-LOX-antibody raised against the lipid body LOX of cucumber (Feussner et al. 1995) (dilution of 1 : 500), an anti-AOS-antibody raised against recombinant tomato AOS (Howe et al. 2000) (dilution 1 : 1,000) and an anti-AOC-antibody raised against recombinant tomato AOC (Ziegler et al. 2000) (dilution 1 : 2,000), followed by treatment with anti-rabbit IgG-conjugated with Alexa 488 (Molecular Probes Eugene, OR, U.S.A.) as secondary antibody. To visualize nuclei, sections were subsequently stained with the DNA dye 4,6-diamidino-2-phenylindole (DAPI, SIGMA Chemical Co., St. Louis, MO, U.S.A.). Stained sections were analyzed with an epifluorescence microscope "Axioskop" (Carl Zeiss, Jena, Germany) using the bright field mode (in situ hybridization) and fluorescence mode with the proper filter combinations (immunocytochemical analysis), respectively.

For electron microscopic analysis petioles were fixed in 3% (v/v) sodium cacodylate-buffered glutaraldehyde (pH 7.2), post-fixed with 1% (w/v) OsO_4 solution, dehydrated in an ethanol series and embedded in epoxy resin (Spurr 1969). Ultrathin-sections (90 nm) were stained with uranyl acetate/lead citrate and observed with an EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany).

The phloem-specific occurrence of AOC protein in tomato (Hause et al. 2000) and the preferential occurrence of OPDA and JA in main veins compared to leaf lamina (Stenzel et al. 2003) prompted us to inspect localization of AOC protein by immunolabeling in more detail including putative occurrence in SE. Since it is impossible to cut mechanically main vein-containing leaf areas free of the surrounding mesophyll cells, we inspected for comparison *P. major* leaf petioles from which phloem can be easily isolated. In cross-sections the isolated phloem was free of mesophyll tissue (data not shown). This corresponds to the fact that a phloem-specific cDNA library could be generated from isolated phloem of *P. major* (Stadler et al. 1995). The isolated phloem of *P. major* contained 13.6 nmol OPDA $(g\ FW)^{-1}$ and 5.8 nmol JA $(g\ FW)^{-1}$, whereas the whole leaf tissue contained 0.1 nmol OPDA $(g\ FW)^{-1}$ and 0.06 nmol JA $(g\ FW)^{-1}$. The abundant occurrence of the AOC product OPDA in the phloem of *P. major* prompted us to inspect localization of AOC in vascular bundles of tomato in more detail by immunolabeling with an antibody raised against recombinant

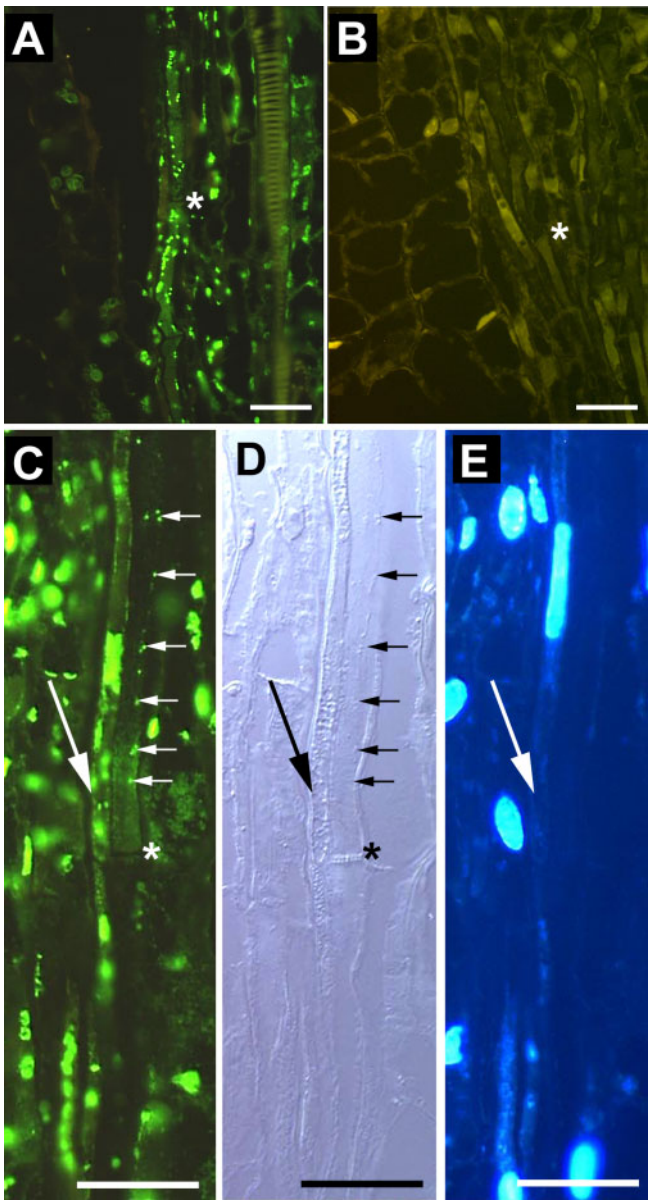

Fig. 1 Occurrence of AOC protein in companion cells and sieve elements of tomato flower stalks and petioles. Flower stalks (A, B) and petioles (C–E) of untreated, 6-week-old plants were cut for immediate embedding in PEG. Longitudinal sections (2 µm thickness) were subjected to immunohistochemical analysis as described using a 1 : 2,000 dilution of the anti-AOC-antibody (A, C) or of the corresponding preimmune serum (B) and were stained with DAPI to visualize nuclei (E). In D the differential interference contrast image of the section visualized in C is shown. In A and C, the green fluorescence, indicative for the occurrence of AOC, appears in plastids of CC (large arrows) and in plastids of SE (small arrows). The sieve plate of a SE is marked by an asterisk. Bars represent 25 µm.

AOC of tomato. The anti-AOC antibody did not show cross-reactivity with other proteins in the immunoblot analysis of total leaf extracts (Hause et al. 2000). Together with the fact that the AOC is encoded by a single copy gene in tomato (Ziegler et al. 2000), specific detection of AOC protein in the immunocytochemical analysis is highly probable. Immunocytological analysis of longitudinal sections of flower stalks and petioles revealed the occurrence of AOC in CC and SE (Fig. 1). SE were identified by the presence of sieve plates, lack of nuclei and the tube-like shape. All SE contained small but strong fluorescent dots indicating occurrence of the AOC protein. In contrast to SE of wild-type flower stalks, the AOC protein was not detectable in SE of 35S::*AOCantisense* plants (Fig. 3), thereby supporting that AOC protein was detected with the anti-AOC antibody. The neighboring CC identified by DIC-imaging and DAPI-staining of nuclei exhibited strong label in plastids (Fig. 1).

The dots within the SE appeared exclusively parietal. Since the tomato AOC carries a chloroplastic transit peptide and the AOC protein was localized immunocytologically in chloroplasts of tomato mesophyll cells (Ziegler et al. 2000), we assumed that the dots belong to SE plastids. The size of the labeled structures is about 1 µm (Fig. 1A). Electron microscopic analysis revealed that in SE only plastids have a diameter of about 1 µm (Fig. 2). Other organelles such as mitochondria or remnants of the cytoplasm are smaller (Fig. 2B). Plastids of SE identified for many different species were subdivided into S-type plastids, containing only starch inclusions, and P-type plastids, containing mainly protein inclusions (Behnke 1981). Due to the absence of starch-granules within the SE, we assume a P-type in tomato. A more detailed localization of AOC in the SE plastids by means of immunogold technique failed due to the low amount of AOC protein in SE.

Immunocytological inspection of LOX and AOS protein revealed that both proteins are also located in SE (Fig. 3). Although there was some cross-reactivity of the anti-AOS antibody and the anti-LOX-antibody presumably with callose of sieve plates, fluorescent labeling in SE was obvious. With the anti-AOS antibody one band was found in the immunoblot analysis of total leaf extracts of untreated tomato leaves (Howe et al. 2000). Thus, it is probable that this antibody recognizes both highly homologous 13-AOS forms known to occur in tomato leaves (Howe et al. 2000, Sivasankar et al. 2000). Using the anti-LOX antibody, one band was detected in the immunoblot analysis of total leaf extracts of untreated tomato leaves (Wasternack et al. 1998). It cannot be excluded that more than one LOX form is detected in the immunocytochemical analysis shown here, since at least two 13-LOX forms of the five LOX forms encoded in tomato occur in tomato leaf chloroplasts (Feussner and Wasternack 2002). The label indicative for LOX protein and AOS protein is also parietally located, but not exclusively as found with the anti-AOC antibody (Fig. 3).

In phloem sap, specific populations of mRNAs have been clearly detected (Oparka and Turgeon 1999). Among them were virus RNA and mRNA coding for the sucrose transporter SUT1. There are hints suggesting that SE-located mRNAs are translated (Kühn et al. 1997). However, most of mRNAs detected in SE were thought to be linked to long distance traffic

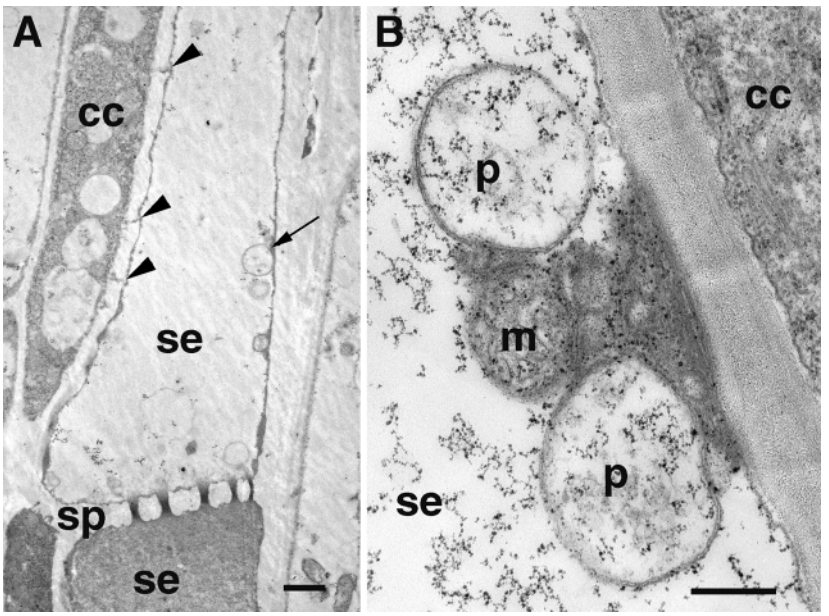

Fig. 2 Ultrastructure of SE. Two neighboring sieve elements (se) are divided by a porous sieve plate (sp) (A). Plasmodesmata (arrowheads) connect CC and SE. The electron transparent organelles in the cytoplasm of the sieve elements (arrow in A, p in B) represent plastids indicated by the double membrane. m, mitochondrion. Bars represent 1 µm (A) and 0.5 µm (B).

(Haywood et al. 2002). Here, we inspected the location of AOC-mRNA by in situ hybridization of cross-sections of flower stalks (Fig. 4). As shown by an overall view of a cross-section, AOC-mRNA was clearly detectable in the bicollateral vascular bundles, preferentially in the inner part, and in the epidermal layer. Among SE and CC inspected at higher magnifications, the AOC-mRNA occurred exclusively in CC indicated by number and position around the triangle-shaped SE which are also marked by the thick cell wall (Fig. 4D). As a control we detected, by in situ hybridization, AOS mRNA which codes for a protein larger than the exclusion limit of plasmodesmata. Among inspected SE and CC most SE revealed clearly occurrence of AOS mRNA (Fig. 4E).

The occurrence of AOC protein in CC and SE plastids accompanied with the exclusive location of AOC mRNA but not of AOS mRNA in CC suggest a traffic of AOC protein into SE via plasmodesmata. To date there is little doubt that proteins synthesized in CC are transferred into SE (Oparka and Turgeon 1999). Although the exact mechanism is unclear, green 27 kDa fluorescent protein (GFP) synthesized in CC was shown to enter the SE and to migrate within the phloem (Imlau et al. 1999). The size exclusion limit of plasmodesmata was shown to be at about 50 kDa in sink leaves and decreased by a developmental switch from non-branched to branched plasmodesmata (Oparka et al. 1999). The 26 kDa AOC protein is clearly within the range suggesting its traffic via plasmodesmata.

The occurrence of AOC in SE and CC, together with the preceding enzymes in JA biosynthesis, LOX and AOS, and the preferential generation of JA and OPDA in main veins of tomato leaves (Stenzel et al. 2003) might be of remarkable consequence for the wound response of tomato plants. Here, JA and the 18 amino acid peptide systemin were shown to be essential signals. Systemin is processed from the 200 amino acid peptide prosystemin which is also encoded in vascular bundles (Jacinto et al. 1997). Based on data on the local wound response (Ryan 2000, Ryan et al. 2002) and on transgenic tomato plants expressing *AOC* in sense and antisense orientations, an amplification in wound signaling by a systemin-dependent AOC activation and a JA-inducible prosystemin

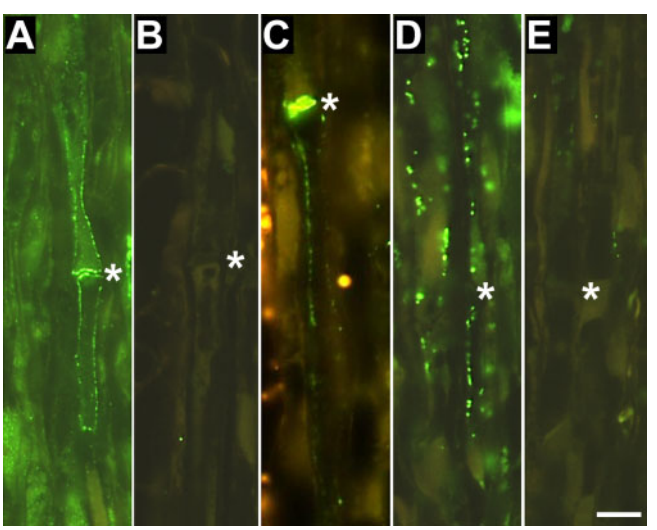

Fig. 3 Occurrence of LOX, AOS, and AOC protein in SE of flower stalks of wild-type tomato plants (cv. Lukullus, A–D) and 35S::AOC*antisense* plants (E). Flower stalks as indicated in the legend of Fig. 1A, B were used for longitudinal sections and subjected to immunocytological analyses using an 1 : 500 dilution of anti-cucumber lipid body-LOX-antibodies (A), the corresponding pre-immune serum (diluted 1 : 500, B), an 1 : 1,000 dilution of anti-AOS antibodies (C), and an 1 : 2,000 dilution of anti-AOC-antibodies (D, E). The sieve plates of SE are marked by asterisks. Bar represents 10 µm for all micrographs.

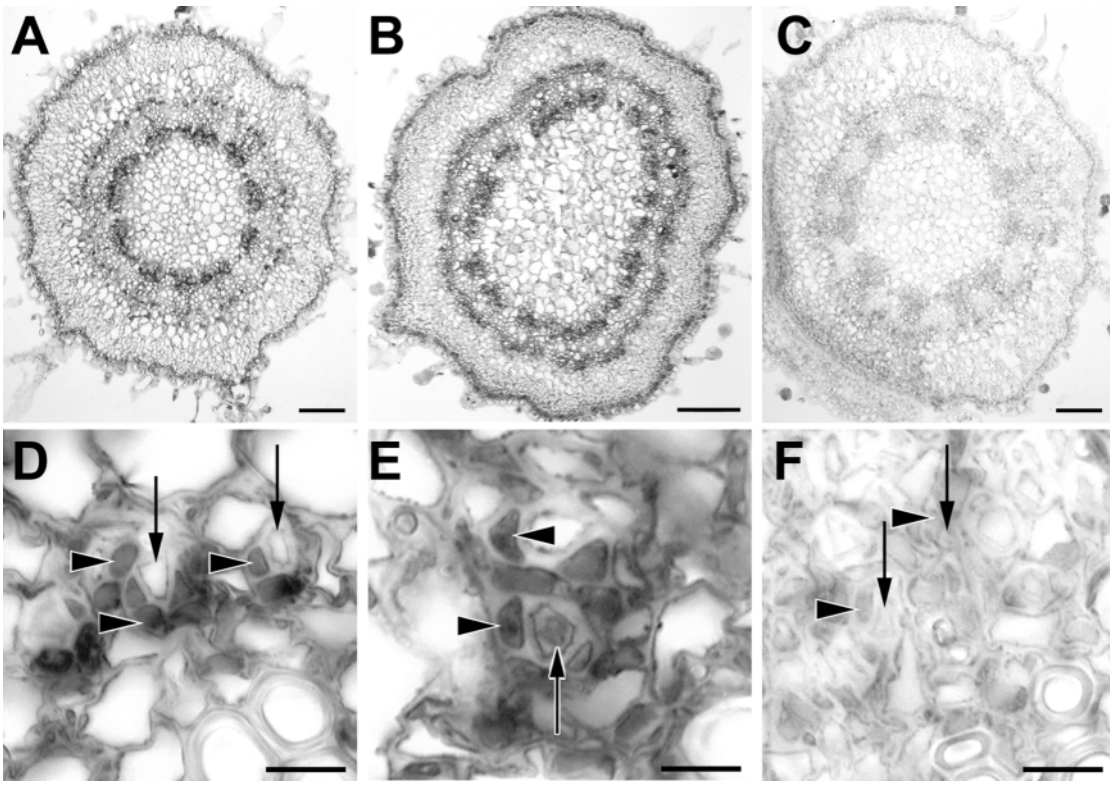

Fig. 4 Localization of AOC mRNA and AOS mRNA in flower stalks by in situ hybridization. Flower stalks as indicated in the legend of Fig. 1A, B were used for embedding in paraplast as described in Materials and Methods. Cross-sections (10 µm thickness) were subjected to in situ hybridizations with DIG-labeled *antisense* RNA probes (A, B, D, E) or DIG-labeled AOC *sense* RNA probes (C, F). In A and D an AOC *antisense* RNA probe was used. Note the AOC mRNA accumulation exclusively in the CC (arrowheads in D) adjacent to the triangle-shaped SE which are also marked by thick cell walls (arrows in D). In B and E an AOS *antisense* RNA probe was used. Note the AOS mRNA accumulation in both the CC (arrowheads in E) and SE (arrows in E). In C and F sections were probed with a DIG-labeled AOC *sense* RNA probe which is also representative for the AOS *sense* RNA probe. Bars represent 100 µm for A, B, C and 10 µm for D, E, F, respectively.

gene expression both taking place in vascular bundles was proposed (Stenzel et al. 2003). The outcome of this amplification would be a spatial and temporal coincidence of the generation of JA. Recent grafting experiments (Li et al. 2002) strengthen the hypothesis that a rapid and sufficient elevation of JA in the local leaf may trigger the systemic wound response (Ryan and Moura 2002). JA or OPDA levels (Herde et al. 1996, Landgraf et al. 2002) may elevate systemically following local wounding and pathogen attack, respectively, or might be constant (Strassner et al. 2002), but JA perception in the systemic leaf is essential (Li et al. 2002). Loading of JA into SE or its formation within the SE may support a systemic response which finally leads to expression of plant defense genes such as those coding for proteinase inhibitors (Ryan 2000). Analysis of tomato phloem sap will help to answer open questions in JA-mediated wound signaling.

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Novel plasmid vectors for homologous transformation of barley (*Hordeum vulgare* L.) with JIP23 cDNA in sense and antisense orientation

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SUMMARY

The most abundant jasmonate-induced protein (JIP) in barley leaves is a 23 kDa protein (JIP23). Its function, however, is unknown. In order to analyze its function by homologous transformation, new plasmid vectors have been constructed. They carry the cDNA coding for JIP23 in sense or antisense orientation under the control of the *Ubi-1*-promoter as well as the *pat* resistance gene under the control of the *35S* promoter. Barley mesophyll protoplasts were transiently transformed with the sense constructs. PAT activity and immunological detection of JIP23 could be achieved in transformed protoplasts but not in untransformed protoplasts indicating that the construct was active. Thus, these new vectors are suitable for stable transformation of barley. Carrying a multiple cloning site (MCS), these vectors can be used now in a wide range of transformation of barley.

Key words: *Hordeum vulgare*, jasmonate-induced protein (JIP), transformation

INTRODUCTION

Jasmonic acid (JA) and its methyl ester (JM) collectively named 'jasmonates' are ubiquitously occurring plant hormones (Sembdner and Parthier, 1993, Wasternack and Hause, 2002). Most plants analyzed so far respond to external stimuli such as wounding or other stress by an endogenous rise of jasmonates which is followed by expression of a subset of genes usually induced also upon JA treatment (Wasternack and Hause, 2002). These JA responsive genes code for various groups of proteins including proteinase inhibitors and enzymes of

phytoalexin synthesis. In barley a thionin, a ribosome-inactivating protein and enzymes of JA biosynthesis such as a lipoxygenase (LOX), and the allene oxide synthase (AOS) are among the jasmonate induced proteins (JIPs). For the most abundant JIP of barley leaves, a 23 kDa protein (JIP-23), however, no putative function could be drawn from data base searches. Due to its stress-responsive formation and tissue specific appearance, JIP23 might function as a stress-protective protein (Hause *et al.,* 1996, 1999, Kramell *et al.,* 2000). Furthermore, JIP23 may attribute to the well-known JA-induced down-regulation of photosynthetic genes as shown by heterologous overexpression in tobacco (Görschen *et al.,* 1997). Also in barley, JIP23 may act by "repressing" the amount of photosynthesis-related and nuclear-encoded proteins in cells which have yet to form functional chloroplasts. Here, the homologous overexpression or the antisense repression of JIP23 will allow to elucidate its function in different tissues and developmental stages of the barley plant.
Therefore, novel plasmid vectors have been constructed which contain the cDNA coding for JIP23 in sense or antisense orientation. Transient expression in barley mesophyll protoplast revealed that the constructs were functionally active. Additionally, the introduction of a multiple cloning site (MCS) into the vector plasmid provides easy-to-handle vectors to create further transformation vectors with other cDNAs of interest.

MATERIALS AND METHODS

Plant material and bacterium strain

Barley seedlings of the two-row spring-type cultivar 'Salome' were grown under sterile conditions on hormone-free MS medium for 7 - 10 days.
Plasmids were cloned in the *Escherichia coli* strain XL1-Blue MRF'.

Plasmids, cDNAs and oligonucleotides

pUC18 was used as cloning vector (Norrander *et. al.*, 1983). MCS was composed of the oligonucleotides ***mcs-1*** (5'-TGC CCG GGC ACT AGT ATC GAT CTA GAG CGG CCG CAT GCA-3') and ***mcs-2*** (5'-TGC GGC CGC TCT AGA TCG ATA CTA GTG CCC GGG CAT GCA-3'). ***sb-85*** (5'-AAT TTG CA-3') allowed an *Eco*RI-*Pst*I ligation.
The following plasmids and cDNAs were integrated in the novel constructs:

(i) **pAHC20** containing the *bar* gene under the control of the *Ubi-1* promoter (Christensen and Quail, 1996),

(ii) **pWD26.41** containing the *pat* gene under the control of the *35S* promoter (Dröge *et al.,* 1992), and

(iii) the cDNA coding for JIP23-3 isolated from a cDNA library obtained from barley seedlings 38 h after imbibition (Hause, unpublished).

Molecular biological methods

Basic methods of plasmid manipulation. Competent *E. coli* cells were prepared and transformed according to Inoue *et al.* (1990). Plasmid DNA was isolated by a rapid alkaline extraction and purified according to the manufacturer's instruction (QIAGEN). Restriction analysis, dephosphorylation, ligation and gel electrophoresis were performed as described by Sambrook *et al.* (1989).

Colony hybridization. Basically the method of Sambrook *et al.* (1989) was used. The radioactively labeled DNA probe was prepared from the dephosphorylated oligonucleotide mcs-2 labeled with [γ-^{32}P]ATP by a T4 polynucleotide kinase at 37 °C for 60 min. The prehybridization, hybridization and washing steps were performed according to standard protocols (Sambrook *et al.*, 1989).

Protein extraction and Western-blot analysis. Proteins were extracted from protoplasts according to Meyer *et al.* (1988). Total protein extracts were separated by PAGE in a 12% polyacrylamide gel, and transferred onto nitrocellulose membranes by semi-dry electroblotting.

For immunostaining blots were incubated for 2 h with the rabbit monospecific polyclonal antibody raised against JIP23 (Hause *et al.*, 1996) diluted 1:10000 in 1% BSA/TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% TWEEN 20). After washing, incubation with a goat anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer, Mannheim; diluted 1:2000 in 1% BSA/TBST) was performed for 1 h. Blots were stained with p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) according to the supplier's protocol.

PAT-assay. Protein extracts of transformed mesophyll protoplasts were incubated with [^{14}C]-acetyl-CoA and phosphinothricin (PPT) followed by thin-layer chromatography (de Block *et al.*, 1987, modified by Schulze, pers. comm.).

Isolation and transformation of barley mesophyll protoplasts

Primary leaves of barley seedlings were cut into pieces of about 2 mm length and were incubated in enzyme solution (4% cellulose and 0.02% pectinase in CPW medium; Frearson et al., 1973) at 25 °C for 15-16 h. Protoplasts were filtered through 56 µm nylon mesh and washed with CPW containing 0.68 M mannitol. Following centrifugation protoplasts were resuspended in 1 ml washing solution (WLW) and were layered onto a 0.6 M sucrose cushion, followed by centrifugation. The green band containing viable protoplasts was collected from the upper phase. Protoplasts were diluted with WLW, sedimented and resuspended in 1 ml WLW. Protoplast number was calculated using a haemocytometer. After sedimentation protoplasts were adjusted to 10^6 per ml with a solution containing of 0.6 mannitol, 15 mM $MgCl_2$ and 0.1% MES. Aliquots of the protoplast suspension (500 µl each) were used for transformation according to Negrutiu *et al.* (1987). Transformed protoplasts were cultured in the culture medium (T/G medium) at a density of 2 x 10^5 per ml at 25 °C in the dark. Total protein extracts were prepared from protoplasts of day 2, 3 and 6 of cultivation.

RESULTS

Construction of novel vectors containing the JIP23 cDNA

Vectors were designed which carry the barley JIP23 cDNA controlled by the *Ubi-1* promoter and the *pat* resistance gene controlled by the *35S* promoter within the same plasmid. The main steps of cloning were as follows (Fig. 1):

Step 1. The *bar* gene was removed from pAHC20 by partial digestion with *Pst*I. MCS of 35 base pairs, composed of oligonucleotides *mcs-1* and *mcs-2*, was inserted between the *Pst*I sites. As the MCS completes the restriction sites with T instead of G, *Pst*I cannot recognize the original sites any more. Following ligation and transformation, the colony-hybridization and restriction analysis revealed three positive clones. The orientation of the MCS (sense or antisense) was determined by sequencing. For this purpose, MCS-containing fragments of the three clones were inserted into pUC18 by *Eco*RI ligation.

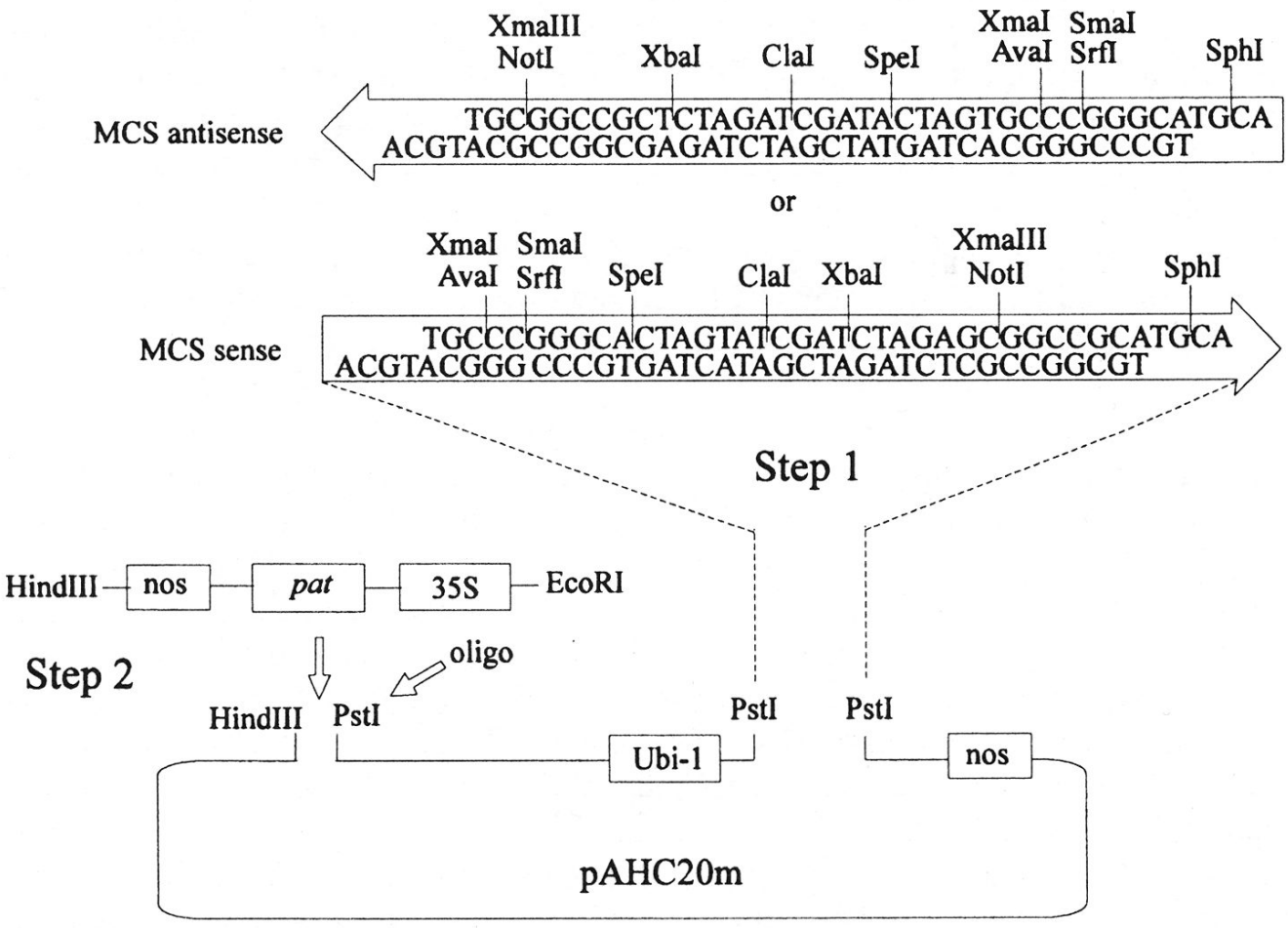

Figure 1. Scheme of construction of the novel vector (cf. text for details)

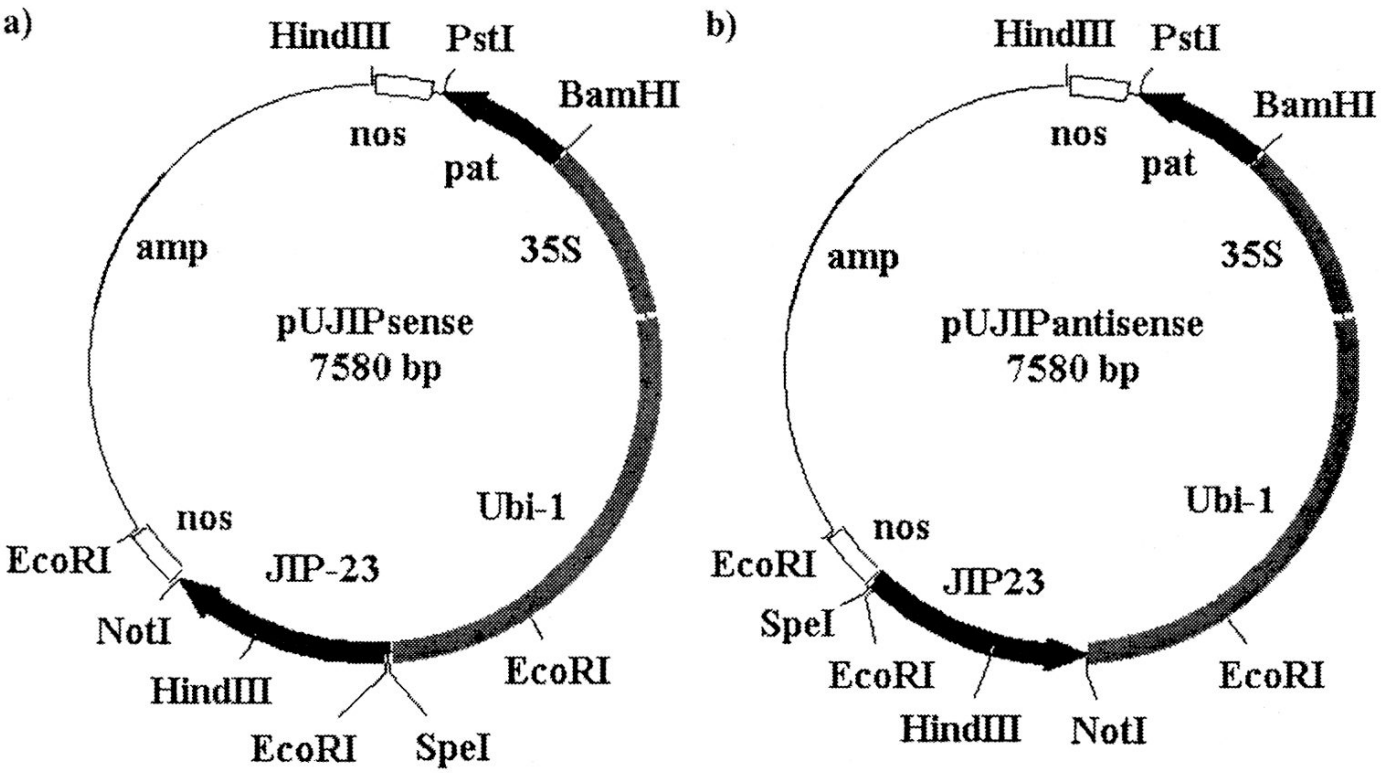

Figure 2. Vectors carrying the JIP23 cDNA in sense (a) and antisense (b) orientation.
35S: CaMV 35S promoter; Ubi-1: maize *ubiquitin* promoter and first intron; JIP23: JIP23-3 cDNA from barley; pat: *pat* gene from *Streptomyces viridochromogenes*; nos: *nopaline synthase* 3' sequence from *Agrobacterium*

Step 2. The modified pAHC20 clones (pAHC20m) containing sense or antisense MCS were cut by *Hin*dIII and *Pst*I and the 1689 bp *Eco*RI-*Hin*dIII fragment of the pWD 26.41 plasmid was inserted using a seven-base pair oligonucleotide to allow *Pst*I-*Eco*RI ligation.
Step 3. Both plasmids containing sense and antisense sequence were cut inside the MCS by *Not*I and *Spe*I. JIP23 cDNA containing terminal *Not*I and *Spe*I restriction sites was inserted into the corresponding vectors.
The vectors containing the JIP23 cDNA in sense and antisense orientation were designated as pUJIPsense and pUJIPantisense, respectively (Fig. 2).

Transient expression of the pat *gene and of the JIP23 cDNA*
In order to test the expression of both sequences, transient transformation of barley mesophyll protoplasts was performed. pUJIPsense was introduced into mesophyll protoplasts of the cultivar 'Salome' by PEG-mediated transformation. After two days of cultivation the PAT activity was determined. PAT activity was clearly detectable in barley mesophyll protoplasts transformed

with pUJIPsense and in transgenic tobacco carrying the *pat* gene (Fig. 3a) indicating *pat* gene expression. In contrast, in non-transformed protoplasts PAT activity was absent.

Also the expression of the JIP23 cDNA under the control of the *Ubi-1* promoter was checked. After transformation with the pUJIPsense vector, protoplasts were analyzed at day 2, 3 and 6 of cultivation by Western-blot analysis. JIP23 protein was clearly detectable two days after transformation of protoplasts with the pUJIPsense construct, but was absent in non-transformed protoplasts (Fig. 3b).

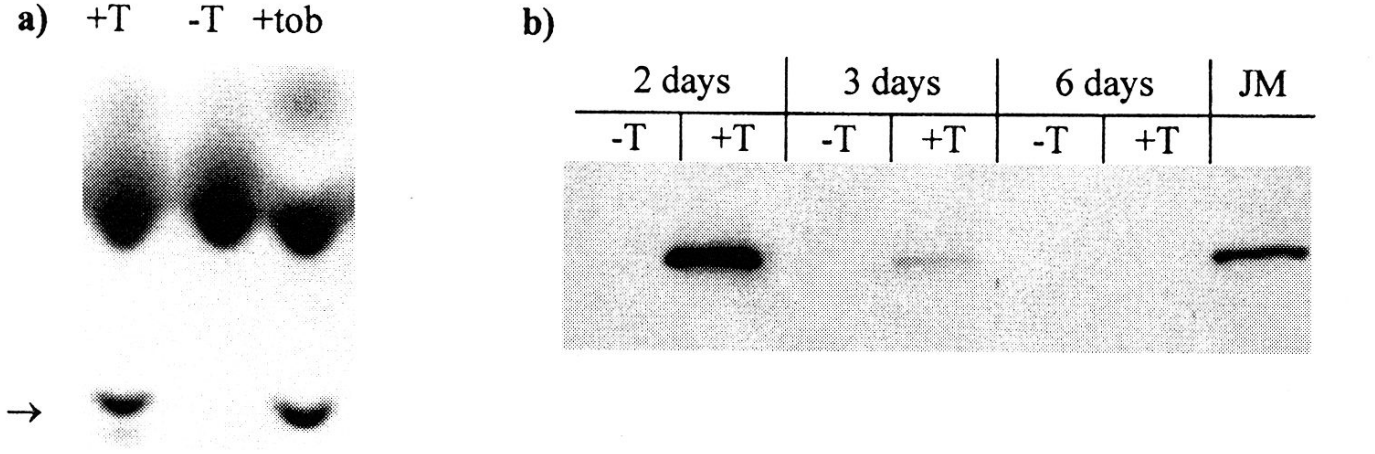

Figure 3. PAT activity (**a**) and JIP23 accumulation (**b**) in transformed barley mesophyll protoplasts.

a) +T: protoplasts transformed with pUJIPsense; -T: non-transformed protoplasts; +tob: transgenic tobacco carrying the *pat* gene; arrow: acetylated L-PPT.

b) JIP23 accumulation in non-transformed (-T) and transformed (+T) protoplasts 2, 3 and 6 days after transformation. For comparison JIP23 was detected in barley leaf segments (LS) treated with JM for 24 h.

DISCUSSION

In order to set up stable transformation of barley via particle bombardment, plasmid vectors have been prepared which contain (i) a cDNA coding for barley JIP23 in sense or antisense orientation under the control of the *Ubi-1* promoter and (ii) the *pat* resistance gene under the control of the *35S* promoter. The use of two different promoters to control a gene of interest and a resistance gene is a repeatedly performed approach to avoid or minimize transcriptional gene silencing (de Wilde *et al.*, 2000). The co-transformation of the expression cassettes (i) *35S::resistance gene* and (ii) *promoter of various origin::transgene of interest* has already been successfully used for the generation of transgenic

barley plants (Jähne et al., 1994, Ritala *et al.*, 1994, Leckband and Lörz, 1998). To date, however, the combination of *35S* and *Ubi-1* promoters controlling different genes in the same plasmid has been reported only for binary vectors used in the *Agrobacterium*-mediated transformation of barley (Horvath *et al.*, 2000, Wang *et al.*, 2001).

The present work describes the construction of a plasmid vector containing both the cDNA of interest and the resistance gene within the same plasmid, both under the control of different promoters. Expression studies in transiently transformed barley protoplasts, done by PAT activity test and immunological detection of JIP23, clearly indicate that both promoters were active and both sequences were expressed, now allowing stable transformation.

Additionally, a MCS was introduced into the basic plasmid in both sense and antisense orientation. This MCS carries restrictions sites for at least ten different restriction enzymes. Therefore, the vector is suitable for cloning other cDNAs for the stable transformation of barley, too. Such a stable transformation with the cDNA encoding JIP23 may now attribute to the functional analysis of this protein.

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Occurrence of the allene oxide cyclase in different organs and tissues of *Arabidopsis thaliana*

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Abstract

Occurrence of an essential enzyme in jasmonate (JA) biosynthesis, the allene oxide cyclase, (AOC) was analyzed in different developmental stages and various organs of *Arabidopsis thaliana* plants by immuno blot analysis and immunocytological approaches. Levels of AOC and of the two preceding enzymes in JA biosynthesis increased during seedling development accompanied by increased levels of JA and 12-oxophytodienoic acid levels after 4 and 8 weeks. Most tissues including all vascular bundles and that of flower buds contain AOC protein. Flowers shortly before opening, however, contain AOC protein preferentially in ovules, stigma cells and vascular bundles, whereas in anthers and pollen AOC could not be detected. The putative roles of AOC and JA in development are discussed.

Keywords: Jasmonates; Allene oxide cyclase; JA biosynthesis; Development; Immunocytochemical localization; *Arabidopsis*

1. Introduction

Different lipid-derived signals are formed when plants adjust to biotic and abiotic stresses. Jasmonates are most prominent among these signals. The term "jasmonates" is commonly used for **j**asmonic **a**cid (JA), its **m**ethyl **e**ster (JAME) and derivatives such as JA amino acid conjugates, whereas the precursor 12-**o**xo-**p**hyto**d**ienoic **a**cid (OPDA) and its derivatives are designated as "octadecanoids". All oxidation products of **p**oly**u**nsaturated **f**atty **a**cids (PUFAs) are designated as "oxylipins".

Jasmonates are synthesized by one of seven different branches of the **l**ip**ox**ygenase (LOX) pathway which is initiated by the LOX-catalyzed oxygenation of α-**l**inol**e**nic **a**cid (α-LeA) (Feussner and Wasternack, 2002). Insertion of oxygen can take place at position 13 by a 13-LOX or at position 9 by a 9-LOX. The 13-LOX product with α-LeA as a substrate is a hydroperoxide (13*S*-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid, 13-HPOT) (Fig. 1). 13-HPOT can be metabolized by an allene oxide synthase (AOS) into an unstable allene oxide which is cyclized by an allene oxide cyclase (AOC) to *cis*(+)-OPDA (9*S*,13*S*) carrying the enantiomeric structure of the naturally occurring JA. Subsequent reduction of the cyclopentenone ring and three steps of β-oxidation lead to (+)-7-*iso*-JA which equilibrates to the more stable (-)-JA (Fig. 1).

Except for β-oxidation, all genes encoding enzymes of JA biosynthesis have been cloned from several plants including *Arabidopsis* (Creelman and Rao, 2002; Feussner and Wasternack, 2002). Among the six cDNAs coding for LOXs of *A. thaliana* (Bell and Mullet, 1993; Melan et al., 1993; Feussner and Wasternack, 2002), several might be involved in JA biosynthesis. AOS is encoded by a single copy gene (Laudert et al., 1996), whereas for AOC, four different genes were identified (Stenzel et al., 2003b). Among the three OPDA reductases (OPRs) of *A. thaliana*, only the OPR3 is converting specifically *cis*-(+)-OPDA (Müssig et al., 2000; Sanders et al., 2000; Schaller et al., 2000).

Abbreviations: α-LeA, α-linolenic acid, AOC, allene oxide cyclase, AOS, allene oxide synthase, JA, jasmonic acid, LOX, lipoxygenase, OPDA, 12-oxophytodienoic acid, OPR, OPDA reductase, PUFA, polyunsaturated fatty acid

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13-LOXs, AOS and AOCs of *A. thaliana* carry a putative target sequence for chloroplast import, and chloroplast location for all three enzymes were shown immunocytochemically and by import studies, respectively (Bell et al., 1995; Laudert et al., 1996; Stenzel et al., 2003b). OPR3 carries a peroxisomal target sequence (Stintzi and Browse, 2000) and was detected immunocytochemically in peroxisomes (Strassner et al., 2002).

Transcripts coding for LOXs, AOS, AOCs and OPRs of *A. thaliana* accumulate upon wounding, pathogenic attack or other stresses (Reymond et al., 2000; Strassner et al., 2002; Stenzel et al., 2003b), and most plants analyzed so far accumulate JA transiently within the first hour upon wounding or other external stimuli (Stintzi et al., 2001; Stenzel et al., 2003b). The JA biosynthesis seems to be regulated by substrate availability, since leaves of transgenic plants overexpressing *AOS* or *AOC* did not show constitutively elevated JA levels, but form more JA than the wild type upon wounding (Wang et al. 1999; Laudert et al., 2000; Stenzel et al., 2003a). Since expression of all corresponding genes are JA-inducible, a feed forward regulation of JA biosynthesis is also supposed. As shown by comparison to the wild type, the JA-deficient mutant *opr3* contains less AOC but unchanged level of AOS and LOX, respectively. Therefore, the AOC seems to be the preferential target in regulation of JA biosynthetic capacity (Stenzel et al., 2003b).

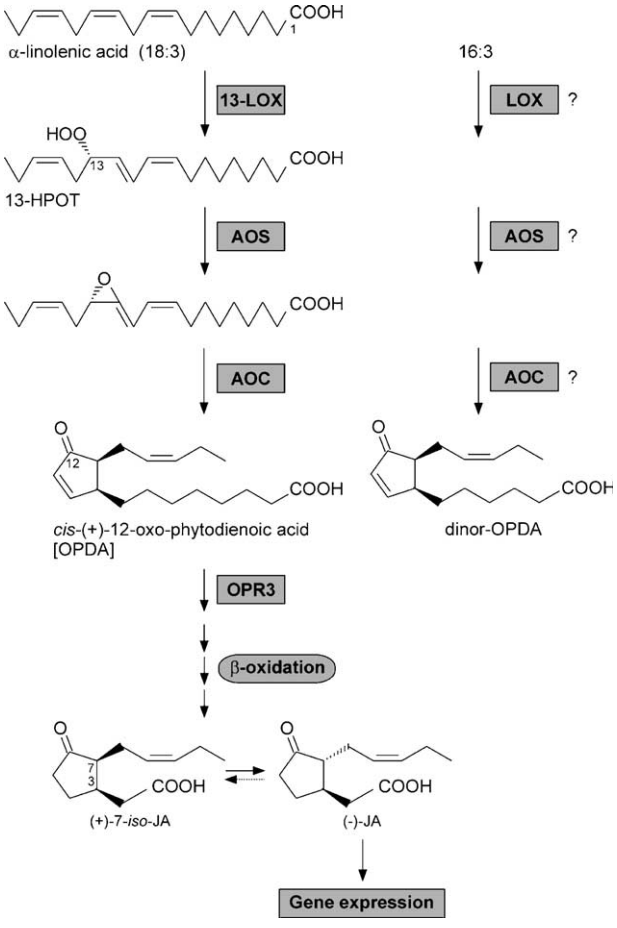

Fig. 1. Scheme of JA biosynthesis.

The formation of JA and OPDA and their role in plant responses to biotic and abiotic stresses is well-documented (reviewed in Wasternack and Hause, 2002). Less is known about biosynthesis of JA during development (Creelman and Mullet, 1997). Several mutants of *A. thaliana* affected in JA biosynthesis indicate a role of JA in flower development: (i) The mutant *dad1*, defective in a chloroplast located phospholipase A1, is JA-deficient and male sterile (Ishiguro et al., 2001). (ii) The mutants *opr3* and *dd1*, both of them defective in *OPR3*, are male sterile and affected in proper pollen development (Sanders et al., 2000; Stintzi and Browse, 2000). (iii) Expression of *AOS* (Kubigsteltig et al., 1999) and *OPR3* (Sanders et al., 2000) in distinct stages of flower development suggest an altered JA biosynthetic capacity during flower formation. Furthermore, tissue-specific *AOS* expression and stage-specific alteration of JA levels has been observed for soybean and barley (Creelman and Mullet, 1995; Maucher et al., 2000).

Recently, LOX, AOS and AOC protein was shown to occur abundantly in fully developed rosette leaves of *A. thaliana* but much less in young developing leaves (Stenzel et al., 2003b). Here, we show that during development the JA biosynthetic capacity increased in terms of LOX, AOS and AOC protein content and altered levels of JA/OPDA/dinor-OPDA. Immunocytological analysis revealed an occurrence of AOC in all flower tissues except anthers and pollen.

2. Results and discussion

2.1. LOX, AOS and AOC protein accumulate steadily during development

The occurrence of LOX, AOS and AOC protein was analyzed by immunoblot analysis in different stages of seedling development and of rosette leaves. Already one week after imbibition, all three enzymes were detectable (Fig. 2A). LOX protein was maximal in 4-weeks-old seedlings, whereas the AOS protein increased steadily even in leaves of 9–14-weeks-old plants. A steady increase appeared also in the amount of AOC protein. The corresponding mRNAs for AOS and AOC were detectable after three weeks and decreased after 11 weeks (Fig. 2B). Among the six LOX sequences identified in *A. thaliana* (Melan et al., 1993; Bell et al., 1995; Feussner and Wasternack, 2002), at least LOX2 (Bell et al., 1995), LOX3 and LOX4 (Feussner, unpublished) are candidates to function in JA biosynthesis. With the polyclonal anti-LOX-antibodies used here only the bulk of LOX proteins was detected. Due to the preferential

occurrence of LOX2 (Bell et al., 1995), the immuno blot analysis shown here is at least partially indicative for JA-related LOX protein during development. In case of AOS, a single copy gene product, the anti-AOS-antibodies should reflect the actual AOS protein level. For AOC, most presumably the detected protein shown in Fig. 2A is preferentially indicative for AOC2. Among all four recombinant AOCs, AOC2 was preferentially recognized by the anti-AOC antibodies, and the *AOC2* gene is most actively expressed (Stenzel et al., 2003b).

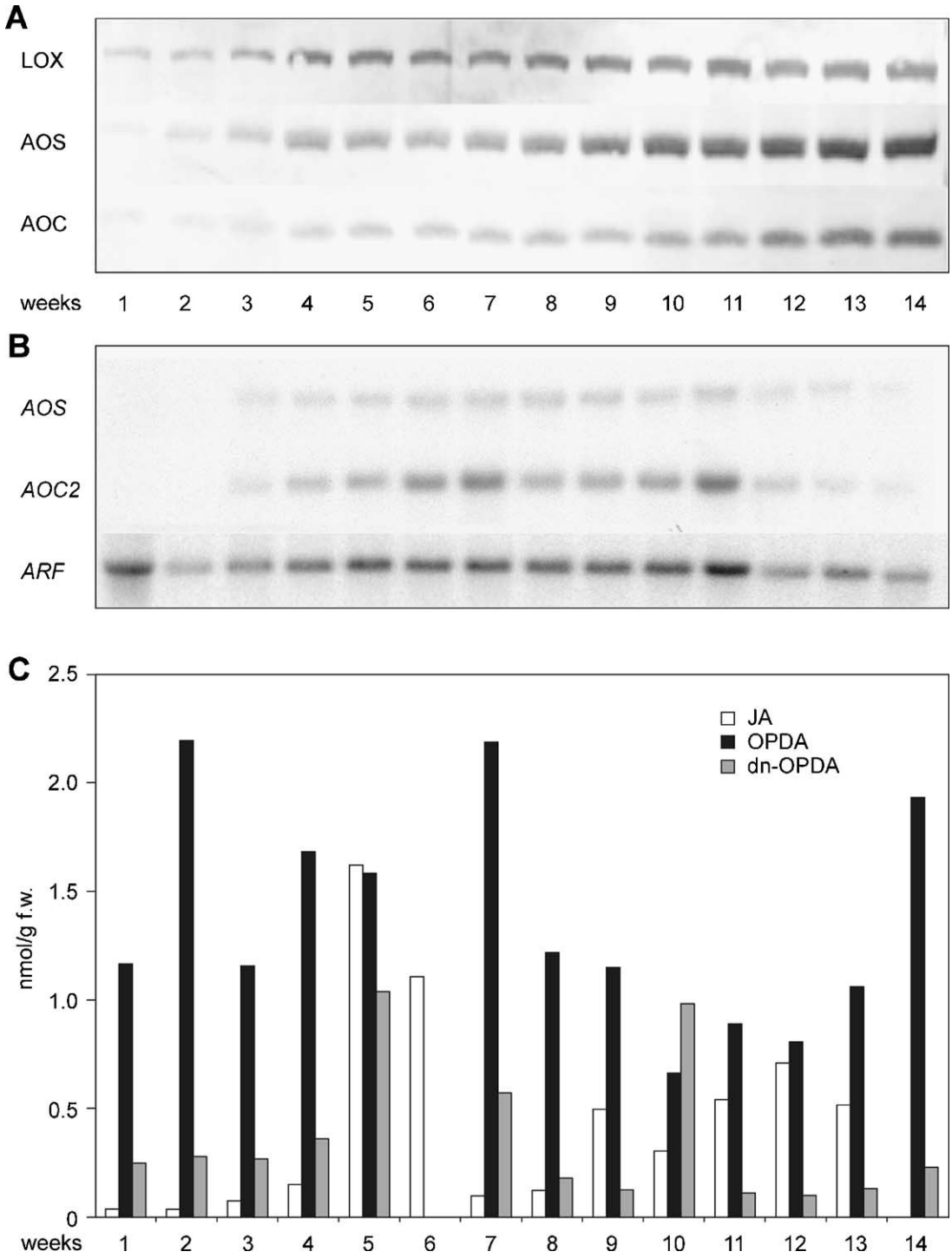

Fig. 2. Immunoblot analysis (A), Northern blot analysis (B) and content of JA, OPDA and dinor-OPDA (C) during development of *A. thaliana* ecotype Columbia. Aliquots of identical leaf material pooled from 70 to 100 plants were subjected to extraction of proteins, total RNA and the oxylipins. Per lane 20 μg total RNA and 5 μg total protein, respectively, were loaded. RNA loading was checked by probing with an ARF probe. Blots were hybridized with a ^{32}P-labeled full-length cDNA of AOC2 and AOS, respectively. Immuno blots were probed with anti-LOX antibodies at a dilution of 1:1000, with anti-AOS antibodies at a dilution of 1:2500, and with anti-AOC2 antibodies at a dilution of 1:5000.

These data indicate a steadily increased capacity for JA biosynthesis during development of *A. thaliana* plants. Quantitative measurements of OPDA, dinor-OPDA and JA revealed a constitutive high level of OPDA. JA levels peaked in 5- to 6-weeks-old plants and increased again in plants older than 8 weeks (Fig. 2C). Dinor-OPDA exhibited an average level of about 200–300 pmol per g f.w., but reached also levels of 1 nmol per g f.w. in 5- and 10-weeks old plants. These data are in accordance with the previously described positive feed back loop in JA biosynthesis (Sasaki et al., 2001; Stenzel et al., 2003b). Whereas the wild type is able to accumulate increasing amounts of JA biosynthetic enzymes and JA during development (Figs. 2A and C), the JA-deficient mutant *opr3* accumulates much less AOC protein in fully developed leaves (Stenzel et al., 2003b). The remarkable high level of free OPDA throughout the development of *A. thaliana* corresponds to previously described levels of 1–2 nmol per g f.w. in untreated fully developed leaves (Laudert and Weiler, 1998; Müller et al., 2002; Stintzi et al., 2001). Beside this amount of free OPDA, *A. thaliana* leaves carry an up to 10-fold higher amount of OPDA esterified to the α-LeA moiety of lipids (Stelmach et al., 2001). This esterified OPDA unknown in function to date is, however, released upon wounding.

LOX-, AOS- and AOC-encoding genes of *A. thaliana* are inducible by OPDA as analyzed by treatment with

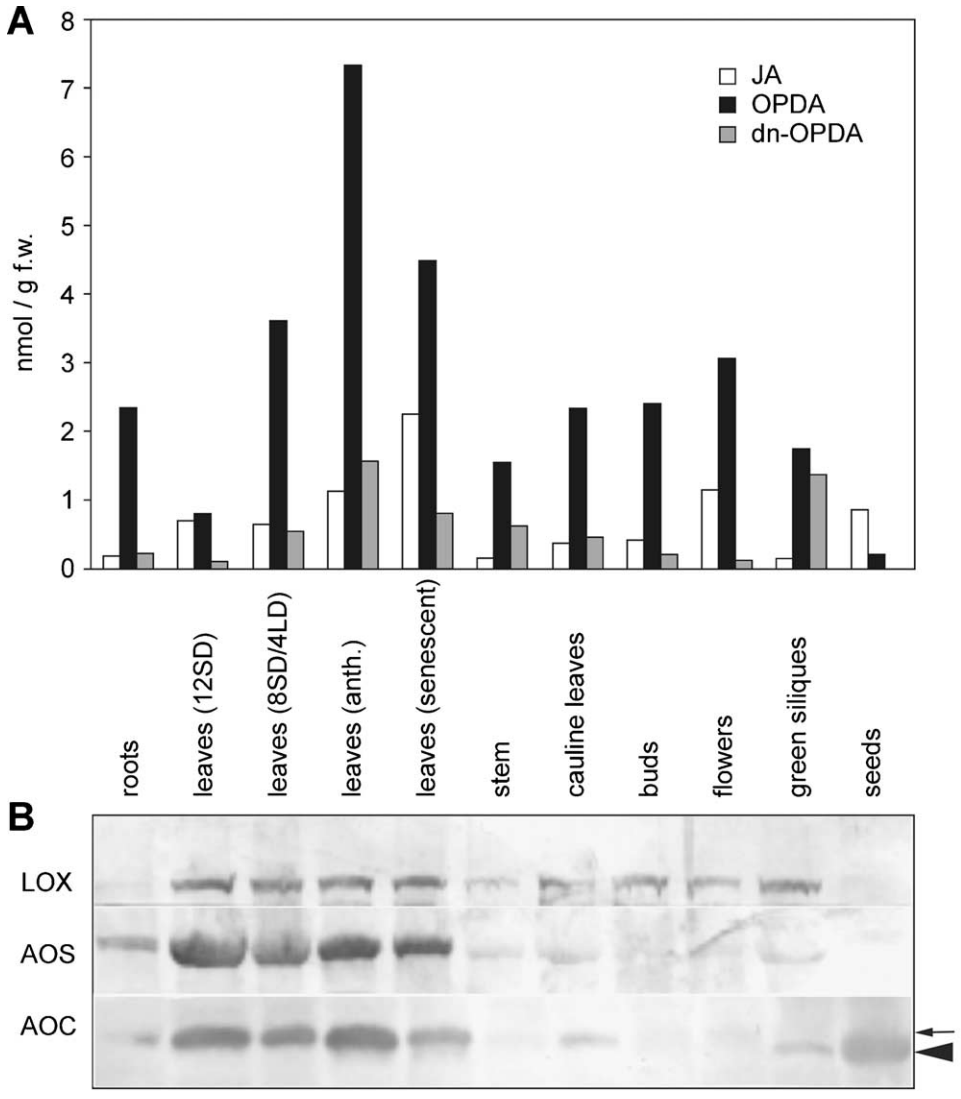

Fig. 3. Content of JA, OPDA and dinor-OPDA (A) and immunoblot analysis (B) in different organs and developmental stages of *A. thaliana* ecotype Columbia. Aliquots of identical tissues pooled from 70 to 100 plants were subjected to extraction of oxylipins and proteins. Per lane 5 μg total protein were loaded, and blots were probed as described in the legend of Fig. 2. In protein extracts of dry seeds and less in that of green siliques, AOC protein could not be detected properly, since the anti-AOC-antibodies cross-react with protein(s) lower in kDa than the AOC. This cross-reactivity appeared already with the pre-immune serum.

OPDA (Laudert and Weiler, 1998; Stintzi et al., 2001; Stenzel et al., 2003b). The endogenous OPDA, however, might be unable to express genes due to its confinement to the plastids, but is coming active in gene expression upon wounding (Stintzi et al., 2001).

2.2. LOX, AOS and AOC accumulate preferentially in leaves

Immunoblot analysis of total proteins, extracted from various organs revealed abundant appearance of LOX, AOS and AOC in rosette leaves of different age (Fig. 3B). The high levels are kept under long day conditions. Also anthocyan-accumulating rosette leaves and senescent leaves carry high level of LOX, AOS and AOC protein. Corresponding high levels were found in these leaves for OPDA and JA (Fig. 3A). These OPDA and JA levels are in accordance with data detected by the recently described multiplex GC-MS/MS technique for acidic phytohormones (Müller et al., 2002), where similar levels were found in fully developed leaves, stems and cauline leaves. The role of JA in senescence was suggested for *A. thaliana* leaves by transcriptional up-regulation of genes encoding LOX, AOS, AOC and OPR as well as by elevated JA levels in senescent leaves (He et al., 2002). The high JA levels shown here for fully senescent leaves is in line with that. The accumulation of other oxylipins is also senescence-associated in *A. thaliana* (Berger et al., 2001). These oxylipins, however, were formed autoxidatively, as indicated by a decline in their *S*/*R* ratio between early and late stages of leaf development. A link between anthocyanin synthesis and JA, first described for soybean (Staswick et al., 1992), is indicated by the high endogenous levels of JA and OPDA in anthocyanin-containing rosette leaves (Fig. 3A). In long term processes such as leaf development the amount of LOX, AOS and AOC protein may allow the plants overtime to accumulate jasmonates leading to accumulation of metabolites formed JA-dependently such as anthocyanins.

Roots, stems, cauline leaves, flower buds, flowers and green siliques exhibited low or undetectable amounts of LOX, AOS and AOC protein in the immuno blot analysis (Fig. 3B). The immunocytological inspection, however, for AOC (see below) clearly indicates occurrence of AOC in all vascular bundles, thus being in accordance with the levels of OPDA and JA in these organs (Fig. 3A). In case of roots, the JA-biosynthetic capacity is also indicated by a raise in JA observed upon infection with the root-pathogen *Fusarium oxysporum* ssp. *matthiolae* (Bohlmann et al., 1998). Dry seeds contrast from other organs by a residual amount of OPDA and higher amount of JA, which may attribute to the effect of JA on seed germination (Koda, 1997), and together with other factors such as abscisic acid, JA may sustain seed germination inhibition.

2.3. AOC protein accumulates in all vascular bundles

The increase in AOC protein levels during *A. thaliana* development prompted us to inspect different vegetative organs immunocytochemically (Fig. 4). In fully developed rosette leaves the vascular bundles and the surrounding mesophyll cells contained AOC protein (Figs. 4A and B). Due to the location of AOC within chloroplasts (Stenzel et al., 2003b), the label indicative for AOC is not randomly distributed but highly localized within the cells. A residual label appeared with the preimmune serum (Fig. 4A′). In stem tissues (Figs. 4C and D), the phloem and the surrounding green tissues contained AOC, whereas the preimmune serum revealed only some cross-reactivity (Fig. 4C′). In primary roots, the central cylinders and the cortex cells accumulated significant amount of AOC (Figs. 4E and F), whereas the preimmune serum gave cross-reactivity only with some epidermal cells of the root (Fig. 4E′). At higher magnification label was detectable even in phloem cells and xylem parenchyma cells (Fig. 4F). These data indicate occurrence of AOC protein in most if not all cell types of vegetative tissues.

2.4. Flower buds contain AOC protein randomly, but in flowers shortly before opening AOC appears preferentially in ovules, stigma cells and vascular bundles, whereas anthers and pollen lack AOC

The low or even undetectable amount of AOC protein found by immunoblot analysis of flowers contrasts from the remarkable OPDA and JA levels detected in flowers (Fig. 3A versus B). For a detailed inspection of the occurrence of AOC protein in flowers, we analyzed immunocytochemically longitudinal and transverse sections of flowers at different developmental stages (Figs. 5A–H′). The overall view of an inflorescence showed label in vascular bundles and parenchyma cells, as well as in the petals, filaments and anthers of very young flower buds (Fig. 5A). The corresponding longitudinal sections probed with the preimmune serum revealed minute cross-reactivity in anthers of the buds (Fig. 5A′). A more detailed inspection of this very early stage of flower development showed label in cells of the ovule primordia including the microgametophyte mother cells and the parietal layer surrounding them (Figs. 5B and C), but negligible cross-reactivity with the preimmune serum (Fig. 5B′). In flowers shortly before opening, however, label appeared more specifically. The anthers including the pollen were free of label in longitudinal (Fig. 5D) and cross-sections (Fig. 5F), whereas the filaments both longitudinally and transversely sectioned showed label (Figs. 5D and F). Again, in vascular bundles and the surrounding parenchyma cells of petals and sepals occurrence of AOC was indicated (Figs. 5D and E). A strong label indicating AOC was

detected in the ovaries, within the pistil (Figs. 5D, E and G) and in stigma cells as well as the enlarged papillar cells (Figs. 5D and H). All corresponding tests with the preimmune serum revealed residual or no label (Figs. 5D′, E′ and H′). Summarizing, the AOC protein occurs in all cell types and tissues of flower primordial. In flowers shortly before opening, however, AOC was undetectable in anthers and pollen and appeared preferentially in ovules, stigma cells and vascular bundles.

These findings correspond only partially with AOS promoter activity data analyzed with *AOS::uidA* plants (Kubigsteltig et al., 1999). Since GUS activity data are not directly comparable with immunocytological data, we inspected for comparison the occurrence of AOS protein in flower tissues immunocytologically. An interesting difference between AOS and AOC became obvious for the petal abscission zone, the anthers and pollen. The AOS appeared abundantly in the petal abscission zone and the mature pollen (Figs. 5I and K), whereas a residual amount was found with the corresponding preimmune serum (Figs. 5I′ and K′). These results correspond to the GUS activity data detected with the *AOS::uidA* plants (Kubigsteltig et al., 1999), but contrasts from the occurrence of AOC. We can not rule out that AOC protein which is clearly detectable in microgametophyte mother cells (Figs. 5B and C) becomes below the detection limit in mature pollen and anthers. The detection of AOC protein in microgametophyte mother cells and AOS promoter activity in anther development (Kubigsteltig et al., 1999) suggest that these tissues have the capacity to form OPDA or even JA. An essential role of JA in anther and pollen

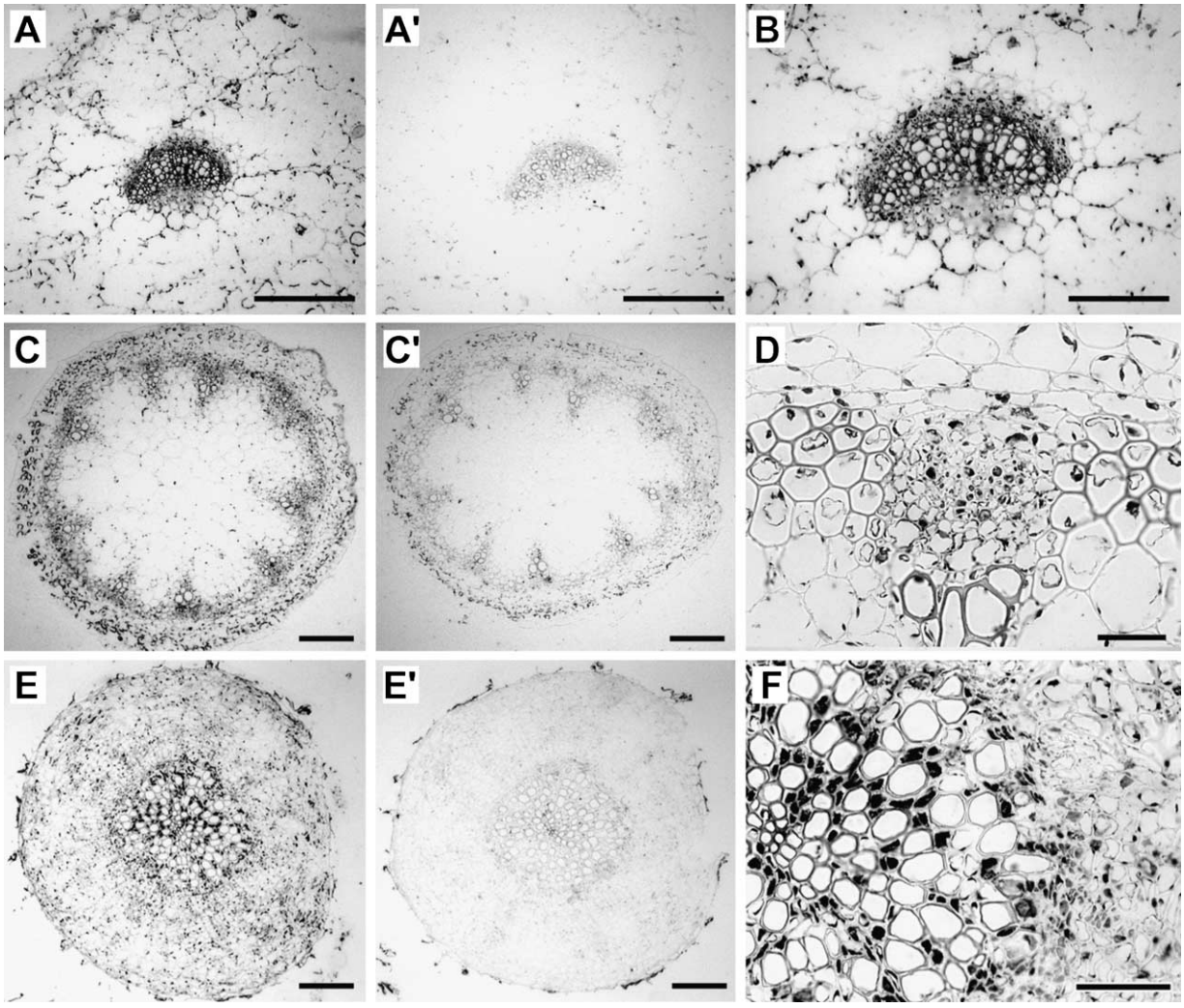

Fig. 4. Distribution of AOC protein in vegetative organs of *A. thaliana*. Roots from 3-weeks-old plants, leaves and stems from 8-weeks-old plants were used for immunolabeling as described in methods. A-F immuno stain, A′, C′, E′ controls performed with pre-immune serum. A, A′ main vein of a rosette leaf, bar = 200 μm. B higher magnification of A showing the vascular bundle, bar = 100 μm. C, C′ cross section of stem, bar = 100 μm. D higher magnification of C showing a vascular bundle, bar = 25 μm. E, E′ cross section of the primary root, bar = 100 μm. F higher magnification showing the central cylinder, bar = 50 μm.

development is clearly indicated by several male sterile mutants. The JA-deficient *fad3-2fad7-2fad8* and JA-deficient mutants *opr3* and *dd1* are affected in a proper release of pollen from the tapetum (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000). Male-sterility occurs also in the JA insensitive mutant *coi1* indicating JA-dependent processes in pollen development (Feys et al., 1994). Furthermore, a mutation of the single copy gene AOS is male sterile (Park et al., 2001).

The JA-deficient mutant *dad1* has an interesting flower-phenotype (Ishiguro et al., 2001). In *dad1* flowers the elongation of stomium filaments is delayed before flower opening. Consequently, *dad1* is male sterile, since the self-pollination, characteristic for *Arabidopsis* flowers cannot occur. These data led to the hypothesis that JA-regulated water uptake into stamen filaments might be responsible for synchronization of pollen maturation, anther dehiscence and flower opening (Ishiguro et al., 2001). The occurrence of AOC protein in stamen filaments (Figs. 5D and F) supports that these tissues may generate JA.

The occurrence of AOC in ovules (Figs. 5E and G) is in accordance with its abundant and specific occurrence in corresponding tissues of tomato (Hause et al., 2000). JA is not a limiting factor for ovule development, since all mutants mentioned above are maternally fertile. JA-inducible genes, however, coding for defense proteins

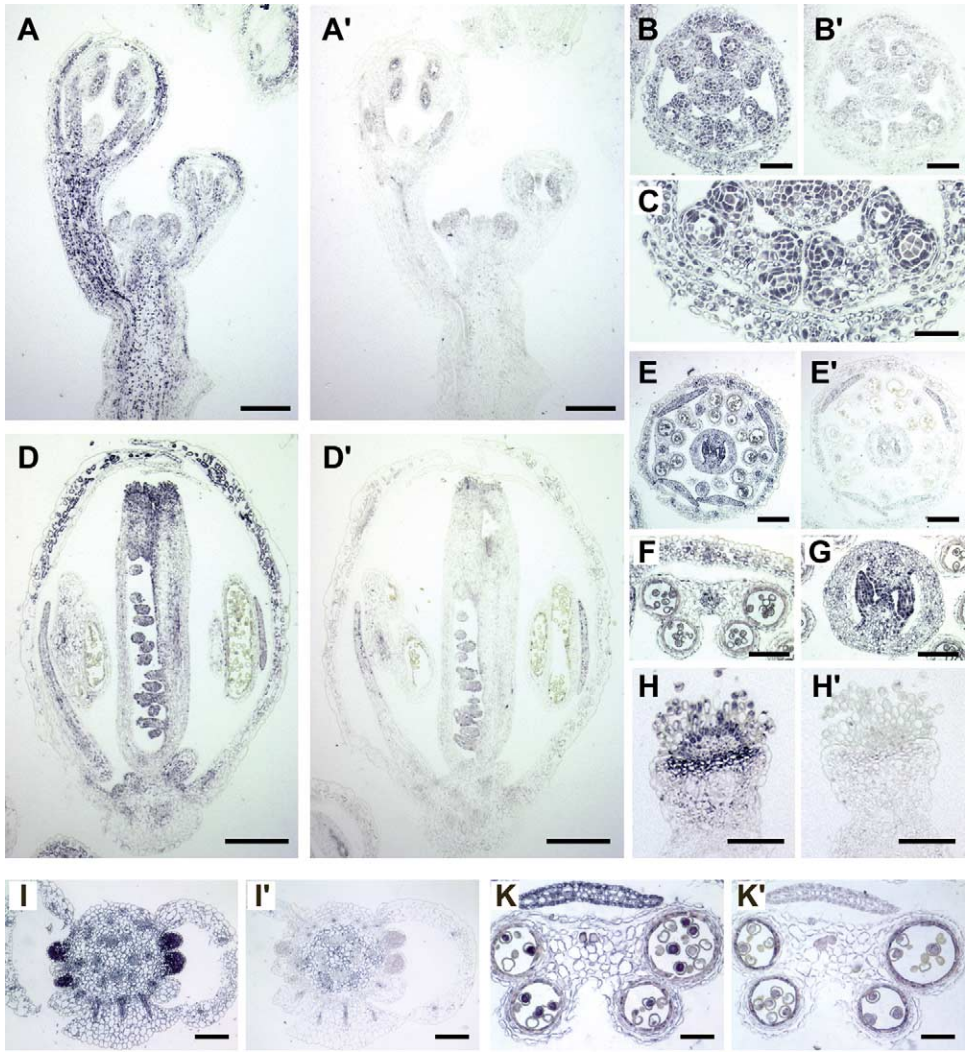

Fig. 5. Distribution of AOC protein (A–H′) and of AOS protein (I–K′) in the organs and different developmental stages of 8-weeks-old flower buds of *A. thaliana*. A–K immuno stain, A′, B′, D′, E′, H′, I′, K′ controls performed with pre-immune serum. A, A′ flower meristem and young flower buds, longitudinal section, bar = 100 μm. B, B′ young flower bud, cross section, bar = 50 μm. C higher magnification of B, bar = 25 μm. D, D′ flower bud with developing ovules, longitudinal section, bar = 100 μm. E, E′ flower bud as in D, cross section, bar = 100 μm. F higher magnification of E showing an anther, bar = 50 μm. G higher magnification of E showing the ovary, bar = 50 μm. H, H′ stigma of a fully developed flower bud shortly before anthesis, bar = 100 μm. I, I′ cross section near the bottom of a flower bud with petal bases, bar = 100 μm. K, K′ cross section of anthers with mature pollen, bar = 25 μm.

such as PINs, γ-thionins, defensins, and enzymes of phytoalexin synthesis were found to be expressed preferentially in ovules and epidermal cell layer of flower organs (Lantin et al., 1999; Lay et al., 2003; Peña-Cortés et al., 1991). It will be interesting to see, whether the antifungal activity detected recently for a flower-specific defensin (Lay et al., 2003) increases the defense status of flowers.

The strong occurrence of AOC protein in stigma cells and the enlarged papillar cells (Figs. 5D and H) points to some specific JA formation in these tissues. Due to remarkable amount of jasmonates in mature pollen (Miersch et al., 1998; Yamane et al., 1982), JA may function in pollen germination, pollen tube growth and/or synthesis of phytoalexins known to be formed in the stigma upon pollination (Vogt et al., 1994).

Among the accumulating data of microarray analyses, there are several examples of JA dependent expression of JA/ethylene-responsive transcription factors such as ERF1 and ERF2 (Lorenzo et al., 2003; Schenk et al., 2000; Spoel et al., 2003). Interestingly, *Myb*-like transcription factors with function in flowers and expression of flower identity genes such as *AGAMOUS* were among the JA dependently activated genes (Mahalingam et al., 2003). This points to a putative role of JA in early stages of flower development. The various putative functions of JA already suggested for flower tissues raise the question, how a spatially and temporally different formation of JA can be regulated. One possibility would be a non-redundant function of the four AOCs of *A. thaliana* which could not be detected in the approach used here. Analysis of various AOC knockout lines and of transgenic lines of *A. thaliana* carrying promoter::*uidA* constructs will answer the question on non-redundancy of AOCs.

3. Experimental

3.1. Materials

The internal standards for GC-MS analysis, [$^2H_6$]-JA and [$^2H_5$]-OPDA were prepared as described (Zimmerman and Feng, 1978; Miersch, 1991). Rabbit polyclonal antibodies raised against the lipid body LOX of cucumber (Feussner et al., 1996), the recombinant AOS of *A. thaliana* (Laudert and Weiler, 1998) and the recombinant AOC2 of *A. thaliana* (Stenzel et al., 2003b) were used.

3.2. Plant growth

A. thaliana ecotype Columbia was cultivated in controlled chambers (Percival, CLF) at 70% relative humidity. If not otherwise indicated short day conditions were used of 8 h light, 210 μE m^{-2} s^{-1} for 6 weeks.

3.3. RNA and immunoblot analysis

Total RNA was extracted and subjected to Northern blot analysis as described (Stenzel et al., 2003b). Proteins were isolated from the phenolic phase of RNA extraction and used for separation and immunoblot analysis as described (Hause et al., 2000). Antibodies were used in the following dilution: anti-LOX, 1 : 1000; anti-AOS, 1:2500; and anti-AOC2, 1:5000.

3.4. Quantitative measurement of jasmonic acid, 12-oxophytodienoic acid and dinor-phytodienoic acid

Plant tissue (0.5–1 g fresh weight) was frozen with liquid nitrogen, homogenized in a mortar and extracted with 10 ml of methanol, containing 100 μg of [$^2H_6$]-JA and 100 ng of [$^2H_5$]-OPDA as internal standards. Purification and separation by HPLC and quantification by GC-MS were performed as described (Stenzel et al., 2003a). Plant material pooled from 70–100 plants was used. Up to three independent extractions and measurements were done deviating by 15%.

3.5. Immunocytochemistry

Immunocytochemical analysis was performed according to (Hause et al., 2000). Cross-sections or longitudinal sections (2 μm thickness) of the corresponding tissues were immuno-labelled with anti-AOC2 antibodies (diluted 1:2000) or anti-AOS antibodies (diluted 1:1000). Controls were done by using the respective pre-immune serum at the same dilutions. As a secondary antibody a goat anti-rabbit IgG conjugated with alkaline phosphatase (Roche Diagnostics) was used according to the suppliers' instructions. The microscope Axisokop (Carl Zeiss, Jena, Germany) combined with a video camera HZ300 (Fuji) was used to obtain micrographs of each immunocytochemical analysis.

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Applied jasmonates accumulate extracellularly in tomato, but intracellularly in barley

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Abstract Jasmonic acid (JA) and its derivatives are well-characterized signaling molecules in plant defense and development, but the site of their localization within plant tissue is entirely unknown. To address the question whether applied JA accumulates extracellularly or intracellularly, leaves of tomato and barley were fed with ^{14}C-labeled JA and the label was localized in cryofixed and lyophilized leaf tissues by microautoradiography. In tomato the radioactivity was detectable within the apoplast, but no label was found within the mesophyll cells. By contrast, in barley leaf tissues, radioactivity was detected within the mesophyll cells suggesting a cellular uptake of exogenously applied JA. JA, applied to leaves of both plants as in the labeling experiments, led in all leaf cells to the expression of JA-inducible genes indicating that the perception is completed by JA signal transduction.

Key words: ^{14}C-labeled jasmonic acid; Barley; In situ hybridization; Microautoradiography; Tomato

1. Introduction

Jasmonic acid (JA) and its derivatives, commonly known as jasmonates, are lipid-derived cyclopentanones which are signals in plant defense and in distinct developmental stages [1]. Jasmonates are synthesized from α-linolenic acid via 12-oxo-phytodienoic acid (OPDA). Initial steps in JA biosynthesis catalyzed by a 13-lipoxygenase, an allene oxide synthase (AOS), and an allene oxide cyclase (AOC), are localized within the chloroplast, whereas the final steps including reduction of OPDA and β-oxidation of the carboxylic acid side chain occur in peroxisomes [2,3]. JA, exogenously applied or generated intracellularly following stresses such as wounding or osmotic stress, induces the expression of numerous genes. Among them are those encoding proteinase inhibitors (PINs) of tomato (*Lycopersicon esculentum*) [4], defensins or thionins of *Arabidopsis* [5,6], and a 23 kDa protein (jasmonate-induced protein, JIP-23) of barley (*Hordeum vulgare*) whose function is so far unknown [7,8].

In the last decade several jasmonate signaling pathways were described. Among them the wound-response pathway is studied in detail, particularly in tomato. Here, JA as well as the 18 amino acid peptide systemin were shown to be essential signals at least of a local wound response. Systemin and its precursor protein are upstream-located components of a wound-induced, intercellular signaling pathway that requires both the biosynthesis and action of JA. Systemin is processed from the 200 amino acid peptide prosystemin which is constitutively expressed in vascular bundles [9]. The binding of systemin to its membrane-located receptor [10] is followed by rapid signaling events including altered levels of cytosolic Ca^{2+} or the activation of mitogen-activated protein kinases (for review, see [1,11]), and leads to the expression of 'early genes'. Concomitantly, a systemin-dependent activation of AOC occurring upon substrate generation may result in a rapid increase in JA, which is known to induce the expression of prosystemin [4,12]. Consequently, an amplification in wound signaling by a systemin-dependent AOC activation and by JA-inducible prosystemin gene expression may occur in vascular bundles, where both processes are located [9,13,14]. However, the expression of PIN genes takes place in spongy and palisade parenchyma [15] suggesting that an active transfer or a diffusion of a signal compound from phloem to the neighboring mesophyll cells should occur. Despite these indications for a role of JA in local wound signaling, it is unknown how and where JA accumulation and perception takes place [16]. Although the existence of a JA receptor is reasonable, no such receptor has so far been identified and it is even unknown whether the JA perception and/or the accumulation of jasmonates occur extracellularly or intracellularly.

Here, the site of JA accumulation is addressed in tomato and barley leaf tissues. The monocotyledonous plant barley shows obvious differences to dicotyledonous plants with respect to JA biosynthesis [17,44] and the function of JA during development of seedlings [7]. In particular, wounding of barley plants does not result in JA biosynthesis and a concomitant gene expression (Wasternack, personal communication). Moreover, for barley different JA-signaling pathways have been proposed, since exogenously applied JA and levels of endogenously formed JA led to the expression of different genes possibly due to independent perception sites [18,19]. The data presented here suggest that following application of JA jasmonates accumulate extracellularly in tomato, but intracellularly in barley, accompanied in both plants by a complete JA signaling as indicated by the expression of respective marker genes.

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Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; DIG, digoxigenin; JA, jasmonic acid; JIP, jasmonate-induced protein; OPDA, 12-oxo-phytodienoic acid; PBS, phosphate-buffered saline; PIN, proteinase inhibitor

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2. Materials and methods

2.1. Plant materials, treatments and determination of radioactivity

L. esculentum Mill. cv. Lukullus or plants of the transgenic line *35S::AOCantisense* were grown as described [20]. *H. vulgare* cv. Salome was grown in soil for 7 days as described previously [7]. (±)-[2-^{14}C]JA was synthesized according to [21]. For treatments, tomato leaves cut at the petiole and 5 cm long segments of primary barley leaves were floated on 50 μM JA, or 50 μM (±)-[2-^{14}C]JA (specific radioactivity 1 mCi/mmol), respectively, and incubated at 25°C under continuous white light (120 μmol/m^2/s, provided by fluorescent lamps, Narva, Berlin, Germany, NC 250/01) for 4 or 24 h, respectively. Incubated leaves were used either for cryofixation and embedding or were extracted by methanol. JA metabolites were separated according to [22]. After adding scintillant to the high performance liquid chromatography (HPLC) eluents, radioactivity was directly measured in a Tri-Carb Liquid Scintillation Analyzer (Beckman, Germany).

2.2. Sample preparation for microautoradiography

Small pieces of leaves were cryofixed by plunging in supercooled propane and freeze-dried under low temperature and high vacuum conditions (CFD, Leica, Bensheim, Germany). To prevent a re-crystallization of water in the plant tissues, the freeze-drying was started at −100°C for 7 days and continued for 2 days each at −90°C, −80°C and −60°C. The samples were then slowly warmed up to room temperature, followed by a pressure infiltration according to the method described by [23] directly in 100% epoxy resin [24]. After polymerization dry sections of 1 μm thickness were made with glass knives and mounted on poly-L-lysine-coated slides.

2.3. Microautoradiography

The dry sections were overlaid with a thin layer of the nuclear research emulsion L4 (Ilford, Dreieich, Germany) with a crystal size of 0.11 μm. The thickness of the dried layer was approximately 5 μm. After exposure at 4°C for at least 6 months, the film was developed with the fine grain developer D19 A/S [25], rinsed in water and then fixed with a commercial b/w fixer (Tetenal, Norderstedt, Germany). As controls, slides without sections and with unlabeled leaf sections were processed in the same way as described for the labeled samples. On these slides almost no silver grains were visible. Sections were counterstained with toluidine blue and examined with a microscope Axioskop (Zeiss, Jena, Germany). Micrographs were taken with a CCD camera (Sony, Tokyo, Japan) and processed by Photoshop (Adobe, Seattle, WA, USA).

2.4. In situ hybridization

Small pieces of tomato leaves (freshly harvested or floated on 45 μM JA for 24 h) were fixed with 3% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, 135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.2), dehydrated in a graded ethanol series and embedded in Paraplast (Sigma, Taufkirchen, Germany). Cross-sections of 8 μm were mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated. In situ hybridization was carried out as described recently [17] using digoxigenin (DIG)-labeled antisense and sense RNA probes obtained from PIN2 cDNA by in vitro transcription.

The leaf segments of barley were fixed in 3% (w/v) paraformaldehyde in PBS, dehydrated, embedded in polyethylene glycol, and cut into 5 μm sections according to [7]. To visualize JIP23 transcript accumulation in barley epidermal cells, the leaves were fixed and the abaxial epidermal strips were isolated and treated with cell wall-digesting enzymes to facilitate penetration of the cDNA probe into the cells [26]. Both sections and epidermal strips were processed for in situ hybridization with the DIG-labeled cDNA of JIP23 as described in [7].

2.5. Extraction of RNA and Northern blot analysis

Total RNA was purified from frozen tissues by treatments with buffered phenol:chloroform:isoamyl alcohol 25:24:1 (v/v/v) and 20 μg per lane was subjected to RNA gel blot analysis according to [27]. Blots were hybridized at 60°C for 16 h with ^{32}P-labeled cDNAs of tomato *PIN2* (800 bp fragment) according to [12]. The loading control was performed by ethidium bromide staining of rRNA.

3. Results

3.1. After application of radioactive JA, the radioactivity is restricted to the apoplast in a tomato leaf, but occurs intracellularly in a barley leaf

To analyze where JA is located in tomato and in barley leaf tissues following treatments with JA, leaves of both species were floated on [^{14}C]JA and processed for microautoradiography. By commonly used preparation techniques of light and electron microscopy any diffusible compounds such as phytohormones cannot be localized within tissues, since aqueous chemical specimen preparation does not preserve the in vivo distribution of these compounds. Therefore, we used a combination of cryofixation, freeze-drying and pressure infiltration in an epoxy resin to prevent losses and dislocations of water-soluble compounds during specimen preparation. This 'water-free' method does not significantly affect the distribution of highly diffusible compounds or ions [28]. Moreover, the structural preservation of the tissue was sufficient, and only mild shrinking artifacts were detectable in the palisade parenchyma of tomato (Fig. 1A).

The distribution of the radioactively labeled JA is indicated in the micrographs by the occurrence of black silver grains in the film layer covering the sections. In tomato leaves treated for 24 h with [^{14}C]JA, the label was restricted to the apoplastic compartment (Fig. 1A–C). After 4 h of incubation the pattern of labeling was identical, but the labeling was weaker (not shown). The enlargements of the spongy (Fig. 1B) and the palisade parenchyma (Fig. 1C) show clearly that most of the radioactivity has accumulated in the intercellular space and in the cell wall area. Only a few silver grains can be seen within the cells. This might be due to some naturally occurring radioactivity during the exposure time of about 6 months.

By contrast, in barley leaf segments the radioactivity was homogeneously distributed throughout the tissue (Fig. 1D,E). Outside the leaf tissue almost no silver grains were found (not shown). The label occurred evenly distributed over the complete cell area mostly occupied by the central vacuole. An accumulation of radioactivity in cytoplasm or organelles like nuclei and chloroplasts could not be detected. This points to a localization of radioactivity mainly in the vacuole of leaf cells. Whether there is a specific transport of jasmonates into the vacuole and how such a transport could be facilitated is completely unknown to date.

In the experimental set-up used for microautoradiography a metabolic conversion of JA could not be excluded. Therefore, parallel samples were used to extract neutral and acidic metabolites followed by separation by radio-HPLC. In both plant tissues, a main part of the radioactivity was due to free [^{14}C]JA, whereas the rest consisted of a mixture of acidic JA metabolites.

3.2. Accumulation of mRNA of JA-inducible genes occurs in all cells of leaves after JA treatment

To test whether the accumulation of jasmonates in tomato and barley was accompanied by a JA response in all cells which accumulate jasmonates at their outside and inside, respectively, the expression of corresponding JA-inducible genes such as *PIN2* for tomato and *JIP23* for barley was examined by in situ hybridization (Figs. 2 and 3). Hybridizations with the DIG-labeled PIN2 antisense probe revealed an occurrence of PIN2 mRNA in all mesophyll cells of tomato after treat-

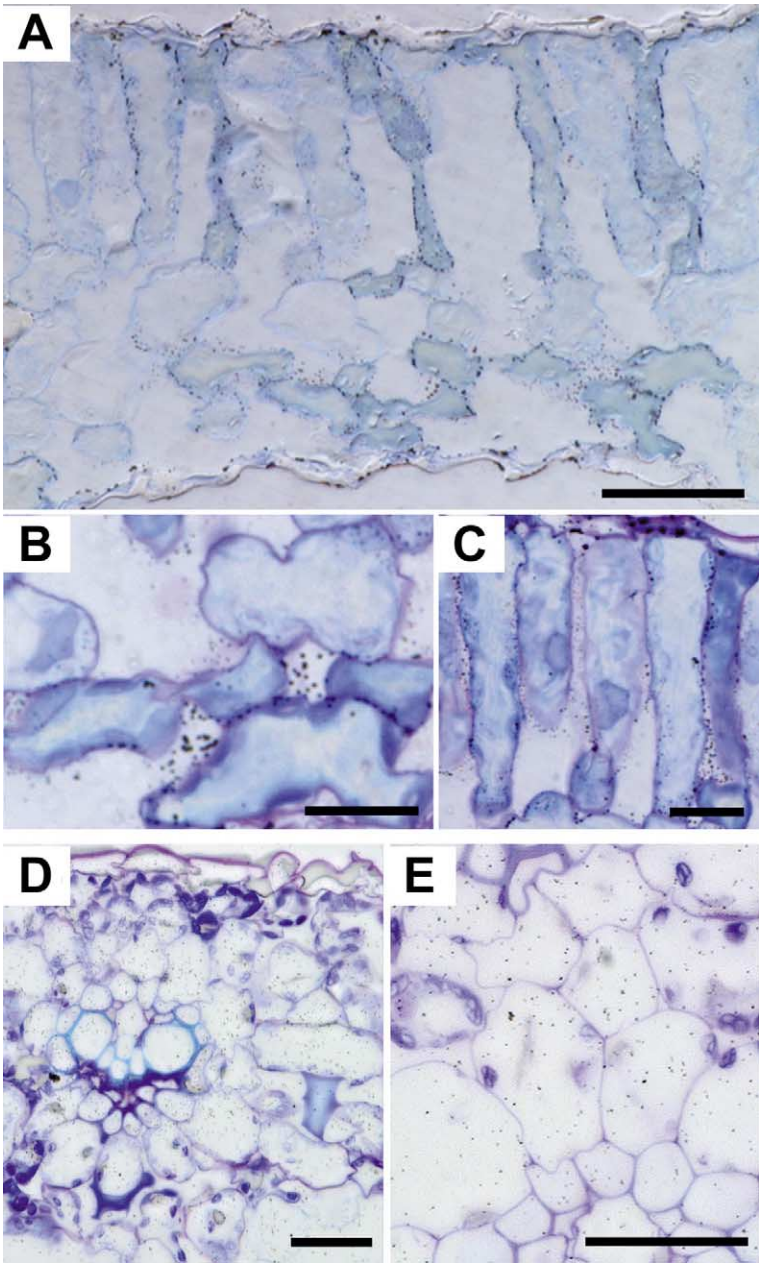

Fig. 1. Subcellular localization of radioactivity in leaves of tomato (A–C) and barley (D,E) after application of [^{14}C]JA. Tomato leaflets (A–C) and barley leaf segments (D,E) were floated on a 50 μM solution of (±)-[2-^{14}C]JA for 24 h and processed for microautoradiography followed by staining with toluidine blue. The presence of ^{14}C can be seen as black dots. Bars represent 25 μm in A,D,E and 10 μm in B,C.

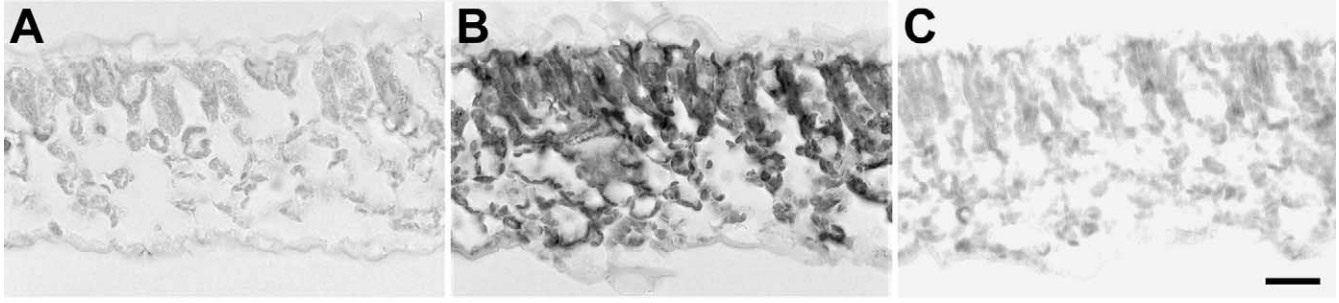

Fig. 2. Accumulation of PIN2 mRNA in excised leaves of 6 week old tomato plants cv. Lukullus after JA treatment. Excised leaves were floated for 24 h on water (A) or on 50 μM JA (B,C) and embedded in paraplast. Cross-sections were subjected to in situ hybridizations with a DIG-labeled antisense RNA probe (A,B) or a DIG-labeled sense RNA probe (C). Note the positive staining with the antisense RNA probe in all mesophyll cells of a JA-treated leaf (B). Bar represents 25 μm for all micrographs.

ment with JA (Fig. 2B), whereas in untreated leaf tissues no label could be detected (Fig. 2A). The control performed by hybridizations of treated leaves with the DIG-labeled PIN2 sense probe did not show any label (Fig. 2C).

In barley leaf segments, untreated or treated with JA, in situ hybridizations were performed by using DIG-labeled JIP23 cDNA. The untreated leaf showed a clear staining of the vascular bundles (Fig. 3A,B, untreated). This corresponds to the constitutive expression of *JIP23* during development of barley seedlings, where JIP23 mRNA is detectable in the scutellum and in the companion cells of the scutellar node and the primary leaf [7]. After JA treatment, JIP23 mRNA accumulated in all mesophyll cells of the leaf (Fig. 3A,B, treated). A clear signal within the epidermis was not detectable in leaf cross-sections, presumable due to the thin cytoplasmic seam of these cells. However, whole-mount in situ hybridizations of isolated epidermal strips showed that JIP23 mRNA is also clearly detectable in epidermal cells of leaves treated with JA for 24 h (Fig. 3C). These JA responses indicate a complete JA signal transduction in both plant species irrespective of whether radioactivity accumulated extracellularly or intracellularly.

3.3. JA application to tomato leaves leads to PIN2 mRNA accumulation in the absence of a JA biosynthesis

Tomato leaves treated with JA accumulate mRNAs encoding enzymes of JA biosynthesis such as AOC [12]. To test whether the extracellularly accumulating jasmonates (Fig. 1A–C) are sufficient for an intracellular response (Fig. 2B), the expression of *PIN2* was examined in transgenic tomato plants expressing *AOC* in an antisense direction. These plants are strongly reduced in their capacity to synthesize JA upon wounding [12]. In leaves of the *AOCantisense* plant PIN2 mRNA could be detected at a similar level as in a wild type plant (Fig. 4). These data suggest that *PIN2* expression does not need endogenous JA biosynthesis if JA is applied exogenously.

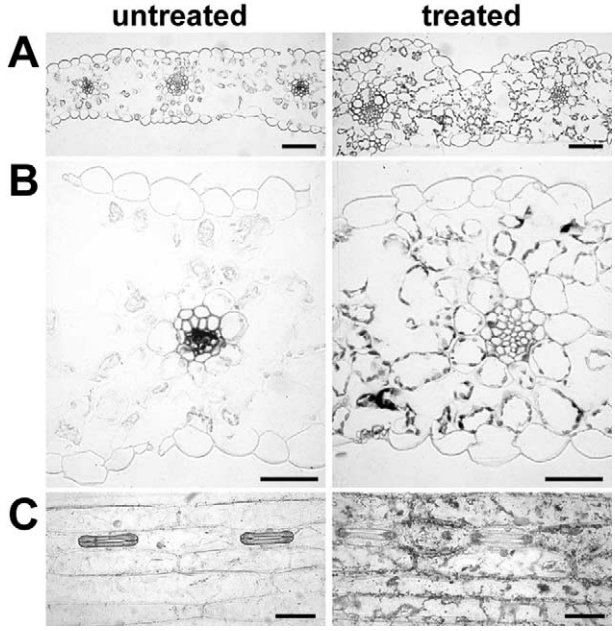

Fig. 3. Accumulation of JIP23 mRNA within barley leaf segments. Segments of primary leaves of 6 day old barley seedlings were processed for in situ hybridization directly or after a treatment with 50 μM JA for 24 h. Labeling was performed with a DIG-labeled cDNA encoding JIP23 using cross-sections (A,B) or isolated epidermal strips (C). The label is visible within all mesophyll and epidermal cells after treatment of leaf segments with JA, whereas in untreated leaves only the phloem exhibits staining. Bars represent 50 μm in A and 25 μm in B,C.

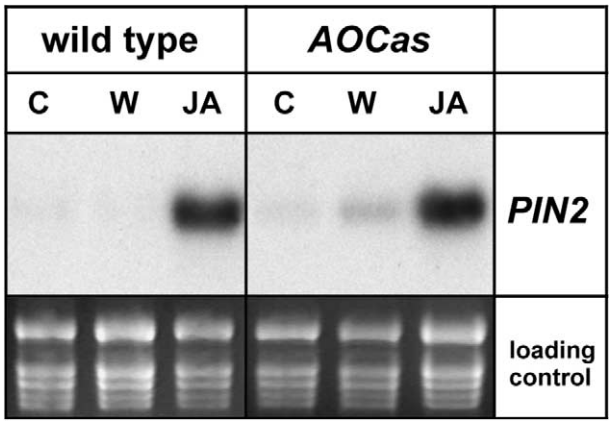

Fig. 4. PIN2 mRNA accumulation in response to JA treatment of tomato cv. Lukullus (wild type) and *35S*::*AOCantisense* (*AOCas*) plants. Leaves of 6 week old plants were freshly excised (C) or were floated on distilled water (W) or on 50 μM of JA (JA) for 24 h. Total RNA was extracted and subjected to Northern blot analysis. Loading of total RNA (20 μg per lane) was controlled by ethidium bromide staining.

4. Discussion

Plants adapt to changes in the environment by altered gene expression mediated by JA in an intra- and intercellular as well as in an interorganismic manner [29]. In particular, upon wounding of tomato leaves, JA was shown to be a signal in local as well as in systemic responses (for review, see [1]). Upon wounding of tomato, JA is synthesized preferentially within vascular tissues [12], whereas the expression of JA-responsive genes is induced in mesophyll cells [4]. Based on these data, a signal transfer from the phloem to the mesophyll cells was proposed [30]. Jasmonates generated in the phloem and released in response to wounding might be readily transported through the apoplast and diffuse to cells surrounding the veins. Here, wound-induced JA may be perceived and cause the expression of *PINs*.

However, nothing is known on the mobility and perception of JA. It is even unknown whether jasmonates are localized extracellularly or intracellularly following application. To get first answers to this question, we localized radioactivity which was applied as ^{14}C-labeled JA to tomato leaves. Using microautoradiography the radioactivity was detectable exclusively within the apoplast, whereas mesophyll cells were free of label (Fig. 1). However, in situ hybridization of PIN2 mRNA, which occurs JA-responsively, indicated a complete perception and transduction in JA signaling. The expression of *PIN2* was detected in all mesophyll cells of a JA-treated tomato leaf (Fig. 2), and was unchanged in leaves of transgenic plants impaired in the biosynthesis of JA by antisense expression of *AOC* (Fig. 4). These data suggest that exogenously applied JA is sufficient to induce the expression of a JA-responsive

gene without additional endogenous biosynthesis. This conclusion is strengthened by the fact that the positive feedback in JA biosynthesis, observed in several plants such as *Arabidopsis* and *Nicotiana tabacum* [31–33], does not lead to measurable quantities of endogenous JA in tomato during the first 24 h of treatment [34].

In the used experimental set-up a metabolic conversion of JA could not be hindered. Up to now, different metabolic routes have been detected, resulting in methylation, amino acid conjugation or adenylation at the carboxylic acid group [35–37], in decarboxylation [38] or in hydroxylation of the pentenyl side chain [39]. It is not known whether the metabolic conversion of JA takes place extracellularly or intracellularly, but most of the metabolic products exhibit biological activity [40]. From the data presented here, it is not possible to shed light on whether or not the radioactively labeled JA entered the cell and where a putative metabolism takes place. Nevertheless, because of the exclusive location of radioactivity within the apoplast in the case of tomato, one can speculate that JA does not enter the cells and that the exogenously applied JA might be perceived at receptors on the plasma membrane. This speculation is supported by the fact that in potato the elevation of the amount of endogenous JA by constitutive overexpression of AOS failed to induce the JA-responsive gene *PIN2*, probably because of jasmonate sequestration [41].

In contrast to the data shown for tomato, the radioactivity could be detected within the mesophyll cells when [^{14}C]JA was applied to leaves of barley. This result highlights significant differences with respect to the role of JA in both species. Contrary to tomato, barley leaves do not exhibit an increase in endogenous levels of JA upon wounding (C. Wasternack, personal communication). Several JA-responsive genes, including JIP23, respond to both exogenous JA and elevation of the amount of endogenous JA, whereas others respond exclusively to exogenous JA [18,19]. The authors supposed that exogenous JA localized in the apoplast might be recognized by plasma membrane receptor(s), and endogenous JA by cytoplasmic receptor(s). Our results support this model: the radioactivity was detected *within* the mesophyll cells when [^{14}C]JA was applied to leaves of barley. Furthermore, it could be confirmed that the expression of *JIP23* was induced in all leaf cells after application of JA (Fig. 3). These data suggest that exogenously applied JA can penetrate the mesophyll cells to be recognized both by plasma membrane receptors and by cytoplasmic receptors in barley leaves.

Binding proteins or receptors for JA have not yet been identified so far, either by biochemical approaches or by screens for mutants [42,43]. This might be due to the existence of several redundant JA receptors, which would hinder their identification by screening for JA insensitivity. Nevertheless, the different sites of accumulation of applied jasmonates shown here for a dicotyledonous and a monocotyledonous plant represent another facet of the complexity of the action of JA.

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Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst habe. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen wurden als solche kenntlich gemacht.
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