Identification and characterization of heavy metal induced genes in barley leaves (*Hordeum vulgare* L.)

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List of Abbreviations

PAR	Photosynthetic Active Radiation
µM	micromolar
mM	milimolar
µg	microgram
µl	microliter
ml	milliliter
U/µl	units per microliter
M	molarity
rpm	round per minute
min	minute(s)
h	hour(s)
°C	Grade Celsius
x g	x 9.81
mg.kg^{-1}	milligram per kilogram
m/z	Mass per Charge
MS	Mass Spectrometer
RFDD-PCR	Restriction Fragment Differential Display-Polymerase Chain Reaction
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
GFP	Green Fluorescence Protein
LC-ESI-MS	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
ICP-AES	Inductively Coupled Plasma-Atomic Emission Spectrometry
v/v	volume per volume
w/v	weight per volume
ABA	Abscisic acid
SPAD	Soil Plant Analysis Development
Fv/Fm	variable fluorescence per maximal fluorescence
PSII	Photosystem II efficiency
ATP	Adenosine Triphosphate
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
IPTG	Isopropyl-β-D-thiogalactozide
X-Gal	5-Bromo-4 chloro-3-Indolyl-B-D-Galactopyranoside
Tet	Tetracyclin
Amp	Ampicilin
LB medium	Luria-Bertani medium
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
mRNA	messenger RNA
cDNA	Complementary DNA
rRNA	Ribosomal RNA
bp	base pairs
GST	Glutathione-S-transferase
HvC2d1	Hordeum vulgare C2 domain protein 1
HvLysMR1	Hordeum vulgare lysine receptor-like kinase 1
HvLysMR2	Hordeum vulgare lysine receptor-like kinase 2

OD	Optical density
SDS	Sodium dodecyl sulfate
Dha	Dehydroalanine
MeDha	Methyldehydroalanin
Cr	Chromium
Cd	Cadmium
Cu	Copper
DNTPs	Deoxynucleotide Triphosphates
DTT	Dithiotreitol
TEA	Tris-Acetat-EDTA
MS medium	Murashige and Skoog medium
et al.	*et alii* (and others)
Da	Dalton
ORF	Open reading frame
RT	Raum temperature
EST	Expressed sequence tags
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
PAGE	Polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
DIG	Digoxigenin
KD	Kinase domain
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species

1. Introduction

1.1 Heavy metals in plants

Heavy metals such as Cu, Fe, Mn, Zn, Ni and As include elements with densities above 5g cm^{-3}, but the term was extended to a vast range of metals and metalloids (Ducic & Polle, 2005). A few metals, including Cu, Zn and Mn, are essential micronutrients required for a wide variety of physiological processes in plants (Reichman, 2002). Copper, for example, is a vital component of electron-transfer reactions mediated by proteins such as superoxide dismutase, cytochrome *c* oxidase and plastocyanin (Clemens, 2001). However, these same metals can be toxic and inhibit growth of plants when present at excessive levels (Reichman, 2002; Hall, 2002). Toxicity may result from the binding of heavy metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element for example the exchange of essential metal ions from the active centres of enzymes resulting in deficiency effects (Elstner *et al.,* 1988; Van Assche & Clijsters, 1990). In addition, an excess of heavy metals may lead to the generation of harmful reactive oxygen species (Dietz *et al.,* 1999; Clemens, 2001; Clemens *et al.,* 2002; Rüdiger & Udo, 2003; Ducic & Polle, 2005; Pittman, 2005). Reactive oxygen species destruct biological macromolecules like proteins, lipids, DNA and are involved in cell death processes (Dat *et al.,* 2000). Plants, like all other organisms, in order to maintain the concentration of essential heavy metals within the physiological limits and to minimize the detrimental effects of non-essential metals, have evolved a complex network of homeostatic mechanisms that serves to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001). However, some plants, can grow on soil contaminated with heavy metals and not only tolerate higher levels of metals but even hyperaccumulate them (Clemens, 2001). This trait can be used in the process of phytoremediation to clean contaminated soil and water (Clemens *et al.,* 2002; Baker & Whiting, 2002).

1.2 Mechanisms of metal homeostasis

Against the background that in certain regions agricultural areas are polluted by heavy metals and that on the other hand metal accumulating plants may be used in phytoremediation, in the last years there is a growing interest in understanding heavy metal homeostasis processes.

But, the mechanisms underlying uptake and transport of essential heavy metals to their cellular targets and detoxification of non-essential or excess essential heavy metals are not yet

clearly understood. The main components of plant metal homeostasis and tolerance mechanisms are uptake, transport, chelation and sequestration processes (Clemens, 2001).

1.2.1 Uptake and transport of metal ions

1.2.1.1 Mobilization of metal ions

Uptake of metals into plant roots is a complex process involving transfer of metals from the soil solution to the root surface and inside the root cells (Reichman, 2002). However, the actual bioavailability of some metals is limited because of low solubility in oxygenated water and strong binding to soil particles (Clemens *et al.,* 2002). For this, plants have developed strategies to maximise the potential uptake of metals (Welch, 1995). Some processes such as acidification of the rhizosphere and the exudation of carboxylate are considered to be potential mechanisms for enhancing metal accumulation (Clemens *et al.,* 2002). Most studies in this context deal with the uptake of iron. Iron, is mainly present as the insoluble form Fe(III). For Fe uptake, plants have developed two strategies. In dicots and nongraminaceous monocots, Fe(III) is reduced to Fe(II) by reductants in the rhizosphere which have been produced by the plant (Welch *et al.,* 1993; Marschner, 1995). In addition to this mechanism, graminaceous monocots, excrete Fe(III)-chelating phytosiderophores into the rhizosphere (Marschner, 1995; Fan *et al.,* 1997). Rhizosphere acidification is also supported by modulation of plasma membrane H^{+}-ATPase activity (Palmgren, 2001).

Another factor which has a severe impact on the availability of metal ions for plant uptake and ameliorating the effects of metal toxicity on the host plant is the mycorrhiza

(Marschner, 1995; Jentschke & Godbold, 2000). Evidence was reported by the study of Zn accumulation in *Thlaspi caerulescens* which shows that rhizosphere microbes play an important role in increasing the availability of water-soluble Zn in soil (Whiting *et al.,* 2001).

1.2.1.2 Uptake of metal ions

Metal ions uptake by plants is likely to take place through secondary transporters such as channel proteins and H^{+}-coupled carrier proteins (Clemens *et al.,* 2002). Most plants have a plasma membrane potential between -120 and -180 mV, hence a large electrical gradient exists that powers metal uptake (Welch, 1995). Most of the transporters described to play a role in the uptake of micronutrients are in the ZIP (ZRT, IRT-like protein) and the Nramp (natural resistance-associated macrophage protein) family (Guerinot, 2000; Williams *et al.,* 2000). In eukaryotes, the first transporter isolated was IRT1 from *Arabidopsis* which is

induced in roots by iron starvation (Eide *et al.*, 1996). In yeast, IRT1 was shown to mediate transport of Mn^{2+}, Zn^{2+} and possibly also Cd^{2+} (Korshunova *et al.*, 1999). ZRT1 and ZRT2 from the ZIP family were first identified in *Saccharomyces cerivisae* and shown to represent a high-affinity and a low-affinity Zn^{2+} transporter, respectively (Zhao & Eide, 1996a, b). Also, it was shown that wheat Ca^{2+} transporter LCT1 mediates uptake of Na^+ and Cd^{2+} in yeast (Schachtman *et al.*, 1997; Clemens *et al.*, 1998).

Another family involved in metal uptake in plants is the Nramp metal transporter family. The involvement of Nramp in metal transport has been reported for the SMF1 from *Saccharomyces cerivisae* (Gunshin *et al.*, 1997). In plants, two classes of Nramp metal transporters have been identified from *Arabidospsis* and rice (Curie *et al.*, 2000). AtNramp1 and OsNramp1 belonging to one class, AtNramp2-5 and OsNramp2 to the other (Curie *et al.*, 2000). The *Arabidospsis* AtNramp1 was shown to be induced under iron-limiting conditions. Therefore, it was suggested to play a role in iron homeostasis (Curie *et al.*, 2000). Thomine *et al.* (2000) showed that AtNramp3 is also involved in Cd^{2+} uptake.

1.2.1.3 Transport of metal ions

The major transport mechanisms for metals in plants are via the xylem and phloem. In xylem transport, three processes govern the movement of metals from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele and release into the xylem (Clemens *et al.*, 2002). In addition, the transport of ions into the xylem is mediated by membrane transport proteins (Gaymard *et al.*, 1998). Another factor affecting xylem transport is chelation of metal ions with certain ligands, such as histidine and nicotianamine, which direct metals to the xylem (Pich *et al.*, 1994). In *Alyssum montanum*, the supplied histidine, which is able to chelate Ni^{2+}, results in an increase in the rate of transport of Ni^{2+} into the xylem (Krämer *et al.*, 1996). In addition, the pH and redox potential of the xylem sap affects the types and amounts and therefore the movements of metal species in the xylem sap (Liao *et al.*, 2000). The same conditions will determine the loading, transport and unloading of metals in the phloem (Welch, 1995). For example, the copper can be translocated in the phloem (Schmidke & Stephan, 1995), whereas manganese mobility within the phloem is generally considered to be variable and depends on the manganese status and the plant species (Pearson *et al.*, 1996). However, during xylem transport manganese is present as a complex with organic acids or phosphate (Rauser, 1999; Luk *et al.*, 2003).

1.2.1.4 Chelation of heavy metal ions

The scarcely soluble and highly reactive heavy metal ions occur within the cell together with metal binding factors. This was demonstrated for example for copper in yeast (Rae *et al.,* 1999). The metal ions can be bound by chelators and chaperons. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations, while chaperones specifically deliver metal ions to organelles and metal-requiring proteins (Clemens, 2001). In plants, the metal chelators include phytochelatins, metallothioneins, organic acids and amino acids.

1.2.1.4.1 Phytochelatins

Phytochelatins (PCs) are a group of metal-binding peptides with the general structure (γ-glu-cys)$_n$ gly (n=2-11) (Kondo *et al.*, 1984; Grill *et al.*, 1985; Jackson *et al.*, 1987; Cobbett, 2000). Phytochelatins are synthesized from GSH by the enzyme PC synthase (Grill *et al.,* 1989). Sequestration of metal ions by phytochelatins is an important metal tolerance mechanism in a wide range of organisms including plants and certain fungi (Clemens & Simm, 2003). PCs are induced by a range of metals and metalloids like Cd, Zn, Cu, As (Grill *et al.*, 1985; De Vos *et al.*, 1992). Metals such as Cu, Ag, and As are detected in complexes with PCs (Maitani *et al.*, 1996; Schmöger *et al.*, 2000). *Arabidopsis cad1-3* mutant, which is PC-deficient, is Cd^{2+} hypersensitive (Howden *et al.*, 1995) and also highly sensitive to AsO_4^{2-} compared to wild type and slightly sensitive to Cu, Hg and Ag (Ha *et al.*, 1999). On the other hand, overexpression of *AtPCs1,* a gene encoding PC synthase in *S. cerevisiae* cells, increased Cd, Hg and As tolerance, whereas the effect on Cu sensitivity was only small (Vatamaniuk *et al.*, 1999). These data show the importance of PCs for the detoxification of a range of metals and metalloids, but also demonstrate that PCs cannot represent the only mechanism of metal tolerance in plants.

1.2.1.4.2 Metallothioneins

Metallothioneins (MTs) are a group of cysteine-rich proteins, which bind metal ions in metal-thiolate clusters (Hamer, 1986). MTs are ubiquitous among all organisms and are grouped in several classes depending on the arrangement of Cys residues. Class-I MTs from mammalian cells are known to confer Cd^{2+} tolerance (Masters *et al.,* 1994). In yeast, MTs which belong to the class II, appear to bind mainly Cu and constitute one of the cytosolic Cu stores (Clemens, 2001). In plants, the first MT identified was the wheat E_c (early Cys-labelled) protein. It was isolated from mature embryos and shown to bind Zn^{2+} (Lane *et al.*, 1987). After this, more than 50 MT-like sequences have been found in different plants (Rauser, 1999).

The classification of plant MT-like genes is based upon the arrangement of Cys residues within their predicted products which are characteristic of metal bindings motifs (Robinson *et al.*, 1993). The roles of MT-like genes in plants are not well understood. Wheat E_c was shown to form associations with metals (Lane *et al.*, 1987). Functional data have been obtained from heterologous systems. The copper-sensitivity of the *S. cerevisiae* cup1Δ strain can be suppressed by expression of Arabidopsis MT1 and MT2 (Zhou & Goldsbrough, 1994). These data suggest a role of MT-like genes and their products in plant metal homeostasis. Proposed functions include detoxification of copper, cytosolic zinc buffering, scavenging of metals during leaf senescence or involvement in metal secretion via leaf trichomes (Robinson *et al.*, 1996; Garcia-Hernandez *et al.*, 1998; Rauser, 1999). However, evidence that would explain the high ubiquitous expression of MTs and the responsiveness to various stimuli, is missing and therefore, the functions of MTs are still considered enigmatic (Palmiter, 1998; Cobbett & Goldsbrough, 2002).

1.2.1.4.3 Organic acids and amino acids

Carboxylic acids and amino acids such as citrate, malate and histidine are potential ligands for heavy metals and could play a role in tolerance and detoxification (Rauser, 1999). Citrate, malate and oxalate have been involved in transport of metal ions through the xylem and vacuolar sequestering (Rauser, 1999). Citric acid has been hypothesized to be a major Cd^{2+} ligand at low Cd^{2+} concentrations (Wagner, 1993) and has been shown to form complexes with Ni^{2+} in Ni-hyperaccumulation plants (Sagner *et al.*, 1998). Malate was suggested as a cytosolic zinc chelator in zinc-tolerant plants (Mathys, 1977). The significant and proportional change in amino acid or organic acid concentration elicited by a change in metal exposure was shown by histidine response in plants that accumulate nickel (Krämer *et al.*, 1996). In addition, heavy metal-induced proline accumulation has been described in different plants such as *Triticum aestivum* (Bassi & Sharma, 1993), and *Orysa sativa* (Chen *et al.*, 2001). The possible role of proline may be of particular importance in binding metal ions that do not form complexes with phytochelatins (Sharma & Dietz, 2006), and detoxifies the ROS under stress (Smirnoff, 1993).

1.2.1.5 Intracellular metal ion trafficking and homeostasis

Once metals enter the cell, the important task is on one hand to supply proteins with the correct metal cofactors needed for their activity and on the other hand to prevent toxic reactions of the metals. The metal ions are bound by chelators and chaperons. Chelators

contribute to metal detoxification by buffering cytosolic metal concentrations, while chaperones specifically deliver metal ions to organelles and metal-requiring proteins (Clemens, 2001).

In plant cells a Cu trafficking pathway was described which is analogous to the one found in yeast and human cells (Himelblau & Amasino, 2000). After Cu-uptake into the cell, chaperons sequester copper in a non-reactive form and interact with other transport proteins to deliver copper to where it is needed (Himelblau & Amasino, 2000). In yeast, ATX1 is involved in copper transfer to post-Golgi vesicules via interaction with CCC2, a Cu-pumping P-type ATPase residing in the membrane of post-Golgi vesicles (Pufahl *et al.*, 1997). Other cytosolic copper chaperones known from *S. cerevisiae* are COX17 which delivers Cu to cytochrome *c* oxidase complex (Glerum *et al.*, 1996), and yCCS which is essential for an active, Cu-containing form of yeast superoxide dismutase ySOD1 (Culotta *et al.,* 1997). The *Arabidopsis* ATX1 homolog CCH1, is involved in sequestering free copper ions in the cytoplasm and delivering it to RAN1 which is hypothesized to reside in post-Golgi vesicles and to be involved in delivery of copper to proteins of the secretory pathway (Himelblau & Amasino, 2000). Among those are the members of the ETR1 family of ethylene receptors, which are known to require Cu for ethylene binding (Rodriguez *et al.*, 1999). CCH1 and RAN1 are up-regulated in senescent leaves and therefore might be contributing to the scavenging of Cu ions during senescence (Himelblau & Amasino, 2000). In yeast, ISA1 and ISA2 proteins were suggested to play a role in the trafficking of iron within the mitochondria (Laran *et al.*, 2000).

Excess essential metals, as well as non-essential metals have to be removed from the cytosol. This can be achieved by compartmentation or by efflux (Clemens, 2001). The main storage compartment of metal ions in plants is the leaf cell vacuole. One well-documented example, is the accumulation of PCs-Cd complexes in the vacuole by formation of high-molecular-weight (HMW) Cd-binding complexes (Salt *et al.*, 1998). This accumulation is mediated by a Cd / H^+ antiporter and an ATP-dependent ABC transporter located at the tonoplast (Salt & Rauser, 1995; Rea *et al.*, 1998). Other heavy metals such as Zn and Mo were also found mainly in the vacuole (Brune *et al.*, 1995). Another way to prevent metal toxicity is efflux of metal ions from the cell. The manganese efflux from the cell is delivered into the Golgi apparatus and exported from the cell via secretory pathway vesicles that carry the metal to the cell surface (Ducie & Polle, 2005). This was mediated by a P-type ATPase, known as PMR1

(transporters for calcium and manganese) which pumps manganese into the secretory pathway (Durr *et al.*, 1998).

1.3 Chromium in the environment

Chromium (Cr) was first discovered in 1798 by the French chemist Vauquelin (Shanker *et al.*, 2005). It has several oxidation states ranging from Cr(-II) to Cr(+VI). The trivalent and hexavalent chromium are the most stable forms (Zayed & Terry, 2003). However, Cr(VI) is considered to be the most toxic form of chromium, which usually occurs associated with oxygen as chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) (Shanker *et al.*, 2005). It is soluble over a wide pH range and mobile in soil and groundwater (Losi *et al.*, 1994). Due to its wide industrial use (e.g. metallurgical processes, production of paints and pigments, tanning, wood preservation and paper production), chromium is considered a major contaminant of both soil and groundwater (Pandey *et al.*, 2005).

1.3.1 Chromium uptake and transport in plants

The toxic effects of Cr are primarily dependent on the oxidation state, which determines its uptake, translocation, reactivity and accumulation (Shanker *et al.*, 2005). The transport of chromate (CrO_4^{2-}) through the sulfate transport system was shown in divers microorganisms such as in *Escherichia coli* (Sirko *et al.*, 1990). In yeasts, it was shown that Cr(VI) may enter also cells via its sulfate transport systems since sulfate uptake mutants are resistant to chromate (Smith *et al.*, 1995).

Chromium is a non-essential element to plants; they do not possess specific mechanisms for its uptake. Therefore, the uptake of Cr is through carriers used for the uptake of essential metals for plant metabolism (Shanker *et al.*, 2005). The pathway of Cr(VI) transport is an active mechanism involving carriers of essential anions such as sulfate (Cervantes *et al.*, 2001). The ions Fe, S and P are known also to compete with Cr for carrier binding (Wallace *et al.*, 1976). By using metabolic inhibitors, a substantial decrease in Cr(VI) but not in Cr(III) uptake was observed in barley seedlings, indicating that Cr(VI) uptake depends on metabolic energy whereas Cr(III) uptake does not (Skeffington *et al.*, 1976). This demonstrates that the two forms do not share a common uptake mechanism. The translocation and accumulation of Cr inside the plant depends on the oxidation state of the supply (Mishra *et al.*, 1995), the concentration of Cr in the media (Kleiman & Cogliatti, 1998), as well as on the plant species. By the study of incubation of barley seedlings with $^{51}Cr^{3+}$ or $^{51}CrO_4^{2-}$, a higher content of

chromium was found in shoots when provided as CrO_4^{2-} suggesting that once Cr is in the xylem, CrO_4^{2-} moves more readily than Cr^{3+} presumably because the latter is held up by ion exchange on the vessel walls (Skeffington *et al.*, 1976).

The ability of bacteria to reduce Cr (VI) into Cr(III) has been reported in *Bacillus sp.* strain (Campos, 1995). In addition, cytochrome *c3* was reported to catalyze Cr(VI) reduction in *Desulfovibrio vulgaris* (Lovley *et al.,* 1993), suggesting that cytochrome may function as Cr(VI) reductase. Plants may also be able to reduce Cr(VI) to Cr(III), a detoxification reaction that very likely occurs in roots, and that may be catalyzed by Cr reductases similar to those found in bacteria. However, no such enzymes have been identified in plants (Lytle, 1998).

1.3.2 Chromium toxicity in plants

All chromium compounds are highly toxic to plants and are detrimental to their growth and development (Shanker *et al.*, 2005). However, Cr(III) is an essential component of balanced human and animal diet for preventing adverse effects in the metabolism of glucose and lipids (e.g., impaired glucose tolerance, elevated fasting insulin, elevated cholesterol and triglycerides and hypoglycemic symptoms) (Anderson, 1989, 1997). In animal systems, Cr(III) was identified to be the active component of the glucose tolerance factor (GTF), which acts as a cofactor to bind insulin to receptor sites in membranes and therefore improves the efficiency of insulin (Vincent, 2000). In plants, chromium inhibits photosynthesis, electron transport and enzyme activities in the Calvin cycle (Shanker *et al.*, 2005). Chlorophyll content was shown to decrease in response to various concentrations of Cr(III) and Cr(VI) in *Triticum aestivum* (Sharma & Sharma, 1996). Since Cr(VI) is a strong oxidant, it may cause severe oxidative stress in plant tissue (Pandey *et al.*, 2005). Chromium, due to its structural similarity with some essential elements, can interfere with uptake of several other ionic elements like Fe and S (Skeffington *et al.*, 1976).

1.4 Plant senescence

Senescence in plants is an internally regulated and orderly degeneration leading to the death of single cells, organs or even the whole plants during their life cycle (Nooden & Penney, 2001). It comprises a highly regulated series of cytological and biochemical events to co-ordinate the degradation of macromolecules and the remobilization of nutrients from senescing tissue into reproductive and young organs, as well as into storage tissues (Barth *et al.*, 2006). Leaf senescence progresses in an age-dependent manner, but may also be induced

by various environmental cues, such as extreme temperature, drought, nutrient deficiency, ozone, insufficient light, darkness and pathogen attack (Buchanan-Wollaston, 1997).

During senescence, cells undergo highly co-ordinated changes in cell structure, metabolism, and gene expression (Barth *et al*., 2006). The most prominent symptom of leaf senescence is the visible yellowing which correlates with physiological and biochemical changes, such as a drop in chlorophyll content and a decrease in photosynthetic activity and also degradation of RNA and proteins (Buchanan–Wollaston *et al*., 2003; Barth *et al*., 2006). Senescence is characterized by an up-regulation of senescence-associated genes (SAGs) that aid in the remobilization of biomolecules (Barth *et al*., 2006) and also specific sets of genes are down-regulated designated senescence-down-regulated genes (SDGs), including photosynthesis related genes (Miao *et al*., 2004). More than 100 SAGs have been identified from a variety of plant species (Buchanan-Wollaston, 1994). Many of these SAGs, including proteases, protease regulators, 1-aminocyclopropane-1-carboxylase (ACC) oxidase, RNAases, glutamine synthetase, lipases, and metallothioneins, have senescent-related functions (Barth *et al*., 2006). However, the biochemical function of many of these genes remains to be identified. Some senescence associated genes encode factors involved in regulation of leaf senescence. In *Arabidopsis*, for example specific WRKY proteins which are plant specific zinc-finger-type transcription factors seem to be involved in the regulation of gene expression during senescence (Miao *et al*., 2004).

1.5 Leaf senescence and heavy metals

Leaf senescence on one hand involves mobilization of nutrients released after catabolism of macromolecules, including heavy metals such as Cu and Zn (Himelblau & Amasino, 2001). On the other hand, heavy metals stress often induces senescence-like degradation processes in plants (Chen & kao, 1999; McCarthy *et al*., 2001). This indicates an overlap in the regulatory mechanisms underlying heavy metal homeostasis and leaf senescence. One common early event in both processes, heavy metal stress and leaf senescence, is the accumulation of reactive oxygen species (ROS) such as O_2^-, H_2O_2 and $^{\cdot}OH$ (Krupinska *et al*., 2003; Mithöfer *et al*., 2004). These ROS induce oxydative damages to biomolecules and have been proposed to function as signals in stress response and development (Mittler *et al*., 2004).

1.6 Aim of the work

In the past few years, responses of plants to heavy metals have received increasing attention. On one hand due to industrial activities, toxic heavy metals such as chromium have been released into the biosphere and represent a widespread environmental pollutant. Plants, like others organisms, have evolved various protective mechanisms against harmful effects of such heavy metals (Van Assche & Clijsters, 1990; De Vos *et al.,* 1991; Hall, 2002). The high potential of some plants to accumulate heavy metals has gained interest for phytoremediation technologies (Clemens *et al.,* 2002). On the other hand some heavy metals are essential micronutrients, including Cu, Mn and Zn fulfilling many crucial functions in plant metabolism. Understanding the mechanisms by which metals, both essential and non-essential, can be taken up, transported and incorporated to their target protein and also sequestered, stored and detoxified in various organisms may contribute to the optimisation of the phytoremediation processes (Clemens *et al*., 2002).

In recent years, substantial progress has been made in elucidating the mechanistic basis of the homeostasis and detoxification of metals and metalloids in plants (Krämer, 2005), but we are still far away from understanding the regulatory network underlying these processes. Furthermore, an interesting new aspect is that heavy metals can induce processes also known to act during leaf senescence. In order to study the molecular response of plants to heavy metals and the connection to leaf senescence, the model cereal plant barley (*Hordeum vulgare* L.) was used.

In the first part of this work genes which are induced in barley in response to chromium treatment were identified by Restriction Fragment Differential Display PCR technique. The second part focuses on the stress and development dependent regulation, the localization and the functional characterization of two of these newly identified heavy metal induced genes: a C2 domain protein and a lysine motif receptor-like kinase.

2. Materials and Methods

2.1 Materials

2.1.1 Plant material

Barley (*Hordeum vulgare* L. cv. Steffi) seeds from (Dr. J. Ackerman & Co Saatzucht, Germany) were used in this study.

2.1.2 Bacterial strains

Microorganism	Strain	Characteristics
Escherichia Coli	XL1-Blue MRF	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 *sup*E44 *thi*1 *rec*A1 *gyr*A96 *rel*A1 *lac*[F' *pro*AB *lac^qZΔM15Tn10* (Tet^R)]
Escherichia Coli	Rosetta™	F$^-$ *omp*T *hsd*S$_B$(r$_B^-$m$_B^-$) *gal dcm lac*Y1$_p$RARE^2 (Cm^R)

2.1.3 Plasmids

Vectors	Characteristics	Source
pET-15b	N-terminal His.Tag / T7*lac* Promoter / thrombin site / Ampicillin resistance	Novagen, Madison, USA
pGEX-2TK	GST gene region / *tac* Promoter / *lac* operator / thrombin site / Ampicillin resistance	Amersham Biosciences, Freiburg, Germany
pGEM®-T	T7 and SP6 RNA polymerase promoters / α-peptide region for β-galactoside /*lac* operator/ Ampicillin resistance	Promega, Madison, USA
pKE4xtr-G	Cauliflower mosaic virus 35S promoter	Reuter Lab, Institute of Genetic, Halle, Germany

2.1.4 Enzyms, Kits and Chemicals

The molecular biology kits, enzyms and chemicals used in this study were purchased from different companies: Promega GmbH (Mannheim, www.promega.com), Qiagen GmbH (Hilden, www.qiagen.com), Roche (Mannheim, www.roche.com), Applied Biosystems PE Germany GmbH (Weiterstadt, www.appliedbiosystems.com), Display Systems Biotech (Azign Bioscience A/S, Koppenhagen, Dänemark, www.azign.com), Biozym Diagnostics GmbH (Oldendorf, www.biozym.com), Carl Roth GmbH & Co. KG (Karlsruhe, www.carl-roth.de), MBI Fermentas GmbH (St. Leon-Rot, www.fermentas.de), Merck Euro-Lab GmbH (Darmstadt, www.merckeurolab.de), Roche-Diagnostics GmbH (Mannheim, www.roche.com/diagnostics), Sigma-Aldrich Chemie GmbH (München, www.sigma-aldrich.com). The radioactive isotopes are from Amersham Pharmacia Biotech Europe GmbH (Freiburg, www.apbiotech.com) and the Taq DNA polymerase enzym was provided by Prof. Dr. Johanningmeier (Institute of Plant Physiology, Martin Luther University, Halle, Germany).

2.1.5 Solutions, Buffers and Mediums

In this study, solutions, buffers and mediums were prepared with purified water prepared by PURELAB Plus™ – Water purifying system (USF, Ransbach Baumbach, Germany) and then autoclaved.

50 × TAE Buffer (pH 8.0)
2 M Tris
50 mM Na-EDTA, pH 8.0 adjusted with Acetic acid, autoclaved.

10 x PCR Buffer
200 mM Tris-HCl (pH 9.2)
600 mM KCl
20 mM $MgCl_2$

Lysis Buffer (RNA)
4.5 M Guanidine Thiocyanate
50 mM HEPES
2 % (w/v) N-Lauryl sarcosine
pH 7.5 with 5N KOH solution adjusted
before uses, 1% (v/v) Mercaptoethanol was added.

10 % N-Lauryl sarcosine solution

10 % N-Lauryl sarcosine

sterile filtered.

3 M Sodium Acetate Buffer

3 M Sodium Acetate

pH 4.6 adjusted with Acetic acid, autoclaved.

1 M Sodium Phosphate Buffer

1 M Sodium Phosphate

pH 7.0 adjusted with Phosphoric acid, autoclaved.

TES Buffer

10 mM Tris-Base

5 mM EDTA

1 % (w/v) SDS

pH 7.4 adjusted with HCl, autoclaved.

8 M LiCl solution

8 M Lithium chloride, autoclaved.

4 M NaCl solution

4 M Sodium chloride, autoclaved.

6 x DNA loading Buffer

60 % (v/v) Glycerol (99.8%)

60 mM Na-EDTA

0.01 % (w/v) Bromophenol Blue

autoclaved.

Ampicillin stock solution (50 mg/ml)

5 % (w/v) Sodium Ampicillin salt diluted in H_2O

sterile filtered.

Tetracyclin stock solution (5 mg/ml)

0.5 % (w/v) Tetracyclin diluted in 99.6% Ethanol, sterile filtered.

X-Gal stock solution (20 mg/ml)

2 % (w/v) 5-Brom-4-chlor-3-indolyl-ß-D-galactopyranosid (X-Gal) diluted in DMF, sterile filtered.

IPTG stock solution (0.1 M)

0.1 M Isopropyl-ß-D-thiogalactosid diluted in H_2O, sterile filtered.

Differents KOH solutions

10 M, 5 M, 1 M Potassium hydroxide (KOH), autoclaved.

LB Medium

1 % (w/v) Bacto®trypton

0.5 % (w/v) Bacto®yeast extract

1 % (w/v) NaCl

pH 7.0 adjusted with 5N NaOH, autoclaved.

LB Plates

1 % (w/v) Bacto®trypton

0.5 % (w/v) Bacto®yeast extract

1 % (w/v) NaCl

1.5 % (w/v) Agar-Agar

pH 7.0 adjusted with 5N NaOH, autoclaved, let to cool down (~50°C)

LB/Amp/Tet/X-Gal/IPTG Plattes

0.24 % (v/v) Ampicillin stock solution (50 mg/ml)

0.3 % (v/v) Tetracyclin stock solution (5 mg/ml)

0.12 % (v/v) X-Gal stock solution (20 mg/ml)

2 % (v/v) IPTG stock solution (0.1 M)

TfbI Buffer

100 mM Rubidium chloride

30 mM Potassium acetate

10 mM Calcium chloride

50 mM Mangan chloride

15 % (v/v) Glycerol

pH 5.8 adjusted with Acetic acid, sterile filtered.

<u>TfbII Buffer</u>

10 mM MOPS

10 mM Rubidium chloride

75 mM Calcium chloride

15 % (v/v) Glycerol

pH 6.5 adjusted with KOH, sterile filtered.

<u>10 × MOPS Buffer (pH 7.2)</u>

40 mM MOPS

10 mM Sodium acetate

1 mM EDTA, pH 7.2 adjusted with NaOH, autoclaved.

<u>Dye</u>

50 % (v/v) Glycerol

0.04 % (w/v) Bromophenol Blue, autoclaved.

<u>RNA Loading Buffer</u>

40 % (v/v) deionised Formamide

20 % (v/v) Dye

18 % (v/v) distillate H_2O

14 % (v/v) Formaldehyde

10 % (v/v) sterile 10x MOPS Buffer (pH 7.2)

<u>RNA Ladder Buffer</u>

50 % (v/v) deionised Formamide

20 % (v/v) Dye

3 % (v/v) distillate H_2O

14 % (v/v) Formaldehyde

10 % (v/v) sterile 10x MOPS running Buffer (pH 7.2)

<u>20 × SSC</u>

3 M NaCl,

30 mM Sodium citrate

pH 7.0 with adjusted Citric acid, autoclaved.

<u>30 × SSC</u>

4.5 M NaCl

45 mM Sodium citrate

pH 7.0 with adjusted Citric acid, autoclaved.

<u>Formamide (deionised)</u>

5 % (w/v) AG®501-X8 (D) Resin

Stirred for 1h, filtered using FILTRAK Filter paper.

<u>10 % SDS solution</u>

10 % (w/v) (SDS) autoclaved.

<u>2 M Tris-HCl Buffer (pH 9.0)</u>

2 M Tris

2.5 % (v/v) concentrated HCl

pH 9.0 adjusted with concentrated HCl, autoclaved.

<u>High-SDS-Hybridization Buffer</u>

7 % (w/v) SDS

50 % (v/v) deionised Formamid

25 % (v/v) 20 x SSC

20 % (v/v) 10 x Blocking solution (RNA)

5 % (v/v) Sodium-Phosphate Buffer (pH 7.0)

1 % (v/v) 10 % N-Lauryl sarcosine solution.

<u>Maleic acid-NaCl Buffer (pH 7.5)</u>

0.15 M NaCl

0.1 M Maleic acid

pH 7.5 adjusted with NaOH, autoclaved.

<u>10 × Blocking solution (RNA)</u>

10 % (w/v) Blocking reagent (RNA)

90 % (v/v) Maleic acid-NaCl Buffer (pH 7.5)

autoclaved, stored at -20°C

<u>1 % Blocking solution</u>

10 % (v/v) 10 × Blocking solution

90 % (v/v) Maleic acid-NaCl Buffer

<u>Wash Buffer1</u>

6.7 % (v/v) 30 × SSC (pH 7.0)

0.1 % (v/v) 10 % SDS solution

<u>Wash Buffer2</u>

1.7 % (v/v) 30 × SSC (pH 7.0)

0.1 % (v/v) 10 % SDS solution

<u>Wash Buffer3</u>

10 % (v/v) 100mM Maleic acid-NaCl Buffer (pH 7.5)

0.3 % (w/v) Tween®20

<u>Detection Buffer</u>

0.1 M Tris-Base

0.1 M Sodium chloride

pH 9.5 adjusted with 5N HCl, autoclaved.

<u>Assay Buffer</u>

50 mM Tris-HCl pH 7.6

50 mM Potassium chloride

2 mM DTT

10 % (v/v) Glycerol

<u>Running gel 15 % (SDS-PAGE)</u>

30 % Acrylamide solution + Bisacrylamide 0.8 %

3 M Tris-HCl pH 8.8

10 % SDS (w/v)

0.1 % (v/v) TEMED

10 % APS (w/v)

Sterile H_2O

Stacking gel 5 % (SDS-PAGE)

30 % Acrylamide solution + Bisacrylamide 0.8%

2 M Tris-HCl pH 6.8

10 % SDS (w/v)

0.1 % (v/v) TEMED

10 % APS (w/v)

Sterile H_2O

5 x SDS Electrophoreses Buffer

125 mM Tris-Base

959 mM Glycine

0.5 % SDS

Sterile H_2O

pH 8.3, stored Maximum 1 month at 4°C.

1 x Protein loading Buffer

0.06 M Tris-HCl, pH 6.8

0.0025 (w/v) Bromophenol Blue

2 % (w/v) SDS

10 % Glycerol

5 % 2-Mercaptoethanol were added before uses.

1 x Anode running Buffer

0.05 M Tris-Base

0.19 M Glycine

1 x Cathode running Buffer

0.05 M Tris-Base

0.19 M Glycine

10 % SDS

10 x WBB Buffer

20 mM Tris

150 mM Glycine

autoclaved

10 x TBST Buffer (1Liter)

12.1 g Tris

87.66 g NaCl

0.1 % (v/v) Tween 20

pH 8.0 adjusted with HCl, autoclaved.

Anode Transfer Buffer (Protein)

1 x WBB Buffer

30 % (v/v) Methanol

Cathode Transfer Buffer (Protein)

1 x WBB Buffer

0.1 % (w/v) SDS

Coomassie staining solution

60 ml H_2O

20 ml Methanol

stir in 20 ml 5x Roti®-Blue

PBS Lysis Buffer

150 mM NaCl

16 mM Na_2 HPO4

4 mM KH_2 PO4

pH 7.3 adjusted with HCl, autoclaved

2 % Triton X-100

<u>PBS Buffer</u>

150 mM NaCl

16 mM Na_2HPO4

4 mM KH_2PO4

pH 7.3 adjusted with HCl, autoclaved.

<u>Elution Buffer (Protein)</u>

10 mM Glutathione

50 mM Tris-HCl pH 8.0

<u>Lysis Buffer (Protein)</u>

50 mM Na_2HPO4

300 mM NaCl

10 mM Imidazole

pH 8.0 adjusted with NaOH, autoclaved.

<u>Wash Buffer (Protein)</u>

50 mM Na_2HPO4

300 mM NaCl

20 mM Imidazole

pH 8.0 adjusted with NaOH, autoclaved.

<u>Elution Buffer (Protein)</u>

50 mM Na_2HPO4

300 mM NaCl

250 mM Imidazole

pH 8.0 adjusted with NaOH, autoclaved.

<u>MS-Onion medium (250 ml)</u>

1 x MS basal Medium

30 g l^{-1} Saccharose

2.5 µg l^{-1} Amphotericin B5 µg ml^{-1} Chloramphenicol

MS-Onion Plates (250 ml)

5 g Plant-Agar (2 % (w/v))

50 ml 5x MS basal Medium

155 ml distilled H_2O

autoclaved, when cool add

50 ml 15 % Saccharose solution

20.8 µl Amphotericin B solution

36.8 µl Chloramphenicol solution

15 % Saccharose solution

15 % (w/v) Saccharose

sterile filtered

Amphotericin B solution

30 mg Amphotericin B diluted in 1ml DMSO

stored at -20°C.

Chloramphenicol solution

34 mg Chloramphenicol diluted in 1ml Ethanol

stored at -20°C.

Spermidine solution

0.1 M Spermidine diluted in sterile H_2O

Calcium Assay Buffer

10 mM MES-KOH Buffer pH 6.5

5 mM $MgCl_2$

60 mM KCl

autoclaved.

2.1.6 Oligonucleotides

Primers	Sequence 5` to 3´	Length (bp)
NewT7	GTA ATA CGA CTC ACT ATA GGG C	22
SP 6	AGC TAT TTA GGT GAC ACT ATA G	22
0-Ext	ACT GGT CTC GTA GAC TGC GTA CCC GAT	27
Eu-3	ATG AGT CCT GAC CGA AAC	18
Eu-7	ATG AGT CCT GAC CGA CAA	18
Eu-15	ATG AGT CCT GAC CGA TAC	18
Eu-6	ATG AGT CCT GAC CGA AGA	18
Eu-11	ATG AGT CCT GAC CGA ACT	18
pKEx4tr for	GGG ACT CTA GAT AAT ACG ACT C	22
pKEx4tr rev	TTT ATT GCC AAA TGT TTG AAC G	22
pUC/M13 for	CGC CAG GGT TTT CCC AGT CAC GAC	24
pUC/M13 rev	TCA CAC AGG AAA CAG CTA TGA C	22
Hexa Random	NNN NNN	6
Hv 18S rRNA for	CAG GTC CAG ACA TAG CAA GGA TTG ACA G	28
Hv 18S rRNA rev	TAA GAA GCT AGC TGC GGA GGG ATG G	25
pET15b_T7_pro	TAA TAC GAC TCA CTA TAG G	19
pET15b_T7term	GCT AGT TAT TGC TCA GCG G	19
Heat schok for (qRT-PCR)	CGG GTG CGG CTT CTC GTC AAT	21
Heat schok rev (qRT-PCR)	GTG AAG CCG GGT GGA AGA AAG	22
OMPF for (qRT-PCR)	AGG TTG AGC TTC TTG CAG TTG GT	24
OMPF rev (qRT-PCR)	GAC GGT GTT GGC GGT CTT ATC AG	23
C2 Domain for (qRT-PCR)	ACG GCG AAA ACC CCA CCT G	19
C2 Domain rev (qRT-PCR)	TAG AGC ACG GCG TCC TGG AG	20
HvKinDom-*NdeI*	GAC ATA TGA GGC GAA GAA AGG CGA AAC AGG GTG	33
HvKinDom-*XhoI*	GAC TCG AGT CAT CTC CCG GAC ATG AGG TTC ACC A	34
HvC2d1-*EcoRI*	GAG AAT TCA TGG CTC GCG GTA CGA GGT GGA GGT GAC	37
HvC2d1-*BglII*	GAA GAT CTG TAG TCG TCG TCG CCG CCG TAG TCG C	34
HvC2d1-XhoI	GAC TCG AGC TAG TAG TCG TCG TCG CCG CCG TAG TC	35
RLK-Rev 5´ Race	GGG TCT TGA GTA CAT TCA TGA GCA TAC CGT TCC AGT G	37
RLK-RevNest 5´ Race	CCG CAA ACA TCT TGA TAG ACA AAA CAC CCG TGC AAG G	37
GeneRacer 5`	CGA CTG GAG CAC GAG GAC ACT GA	23
GeneRacer 5` nest	GGA CAC TGA CAT GGA CTG AAG GAG TA	26
GeneRacer 3`	GCT GTC AAC GAT ACG CTA CGT AAC G	25
GeneRacer 3` nest	CGC TAC GTA ACG GCA TGA CAG TG	23
RLK 3´ Race	CCG AAG CTG GGA GAC GAC TAT CCT GTC GAT GC	32
RLK 3´ Nest	CTC ATG ATG ACG CAC CTG GCG AAC GCA TGC AC	32
3`Race LysMR2	CGA TGT TGG CGT CGT CAA CAT CAC CGT CAA CTG C	34
3`Nest LysMR2	CTA ATG CAA AAG GTG AAA CCA TCG ACA GCT CAA G	34

17E22F (sequencing)	ACT AGC CTA CCT ACA AGT GAT GAA TGC ATG G	31
IPK (RPK) clone- For	GAC GCT TTG CTT CCA TCT TCT GA	23
IPK (RPK) clone- Rev	CCT TTG CAC GGG TGT TCT TGT CTA TC	26
RLK-500 For (RT-PCR)	CAT TCA TGA GCA TAC CGT TCC AGT GTA CAT	30
RLK-1090 Rev (RT-PCR)	AGA CGA GGT CAT CTC CCG GAC ATT AGG TTC	30
17E22F for02 (sequencing)	GGT ACG GGA CGA CGG GCG TGG C	22
LRK-forward (RT-PCR)	TCT CCC GGT TTA TGA GTA CAT GGA AAA TGG	30
LRK-for-nested (RT-PCR)	ACA CAA GAA ACG GCC CAA CTC TAT GT	26
LRK-reverse (RT-PCR)	GCT CCC ATT CCT CAT GTA GAT ACA ACA AAC	30
LRK-rev-nested (RT-PCR)	TTG ATG CCA CAC CTT GAT GAT TGA ATA CC	30
LysMR1 for (qRT-PCR)	CAA CGT GAA CGT CTC CTA CAT CGC ATC G	28
LysMR1 rev (qRT-PCR)	GGC AGC GTG AGG CAC TTG CAT GTG A	25
LysMR2 for (qRT-PCR)	CGG CGT CCA CCT ACC TCG CCG CCT CCA T	28
LysMR2 rev (qRT-PCR)	ATG TTG ACG ACG CCA ACA TCG GGG ATG TCG T	31
5` pGEX Sequencing Primer	GGG CTG GCA AGC CAC GTT TGG TG	23
3` pGEX Sequencing Primer	CCG GGA GCT GCA TGT GTC AGA GG	23

2.2 Methods

2.2.1 Plant growth conditions

Seedlings of barley (*Hordeum vulgare* L. cv. Steffi) were grown hydroponically on Murashige and Skoog Medium (DUCHEFA BIOCHEMIE BV, The Netherlands) under controlled growth-chamber (Growth chamber KBWF 720- Wts; Binder Labor Technik GmbH; Tutlingen; Germany) conditions (16 h, 21°C and 100 µmol m^{-2} s^{-1} PAR (Photosynthetic active radiation: 400-700nm); 8 h, 16 °C and darkness).

2.2.2 Heavy metal treatment

7 days old barley seedlings grown hydroponically as described above were then treated with 50 µM or 1 mM Potassium dichromate ($K_2Cr_2O_7$), cadmium chloride ($CdCl_2$) or copper chloride ($CuCl_2$), respectively, for different time points. Untreated barley seedlings served as controls. Samples of leaves were harvested at an appropriate time-points, immediately frozen in liquid nitrogen and stored at −80°C until use.

2.2.3 Senescence experiment

For the analysis of senescence expression of newly identified genes during the chromium treatment, plants were grown for 9, 26 and 38 days at 16 h light (21°C and 100 µmol m^{-2} s^{-1}) and 8 h darkness (16°C) on soil containing 4g of fertilizer (Osmocote 5M; Urania, Hamburg, Germany) per litre of soil.

2.2.4 Calcium ionophore treatment

Primary leaves of 7 days-old barley plants grown on Muraschige and Skoog Medium were cut and then immersed in water containing 200 µM of calcium ionophore A23187 for 5, 10, 24 and 48 hours. For application of calcium ionophore A23187 (SIGMA-Aldrich, Germany) a stock solution of 1mM was preapred in dimethylsulfoxid (DMSO) (Roth, Karlsruhe, Germany) and then dissolved in distilled water by rapid mixing. Controls were treated identically except for the addition of calcium ionophore A23187. Samples of leaves were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at – 80°C until use.

2.2.5 Methylviologen treatment

In order to analyse the effects of reactive oxygen species, barley plants were grown hydroponically for 7 days as described above (see 2.2.1). Then 50 µM of methylviologen in 0.1 % (v/v) Tween 20 was sprayed onto the leaves. Control plants were treated only with 0.1 % (v/v) Tween 20. After an incubation of 1 hour in the dark, for improved uptake of the herbicide, plants were exposed to 300 µmol m^{-2}s^{-1} light to induce the accumulation of reactive oxygen species. The effect of methylviologen treatment was characterized by measuring the decrease in photosystem II efficiency. Samples of leaves were harvested at an appropriate time point, immediately frozen in liquid nitrogen and stored at –80°C until use.

2.2.6 Abscisic acid treatment

Primary leaves of 7 days old barley plants grown hydroponically were cut and treated in beakers with water containing 1 % (v/v) ethanol and 50 µM abscisic acid (ABA) for 8, 12 and 48 hours and without ABA (controls) under controlled growth conditions.

2.2.7 Drought stress

7 days old barley plants grown on Muraschige and Skoogs were exposed to drought conditions by removal of the medium.

2.2.8 Physiological characterization

2.2.8.1 Chlorophyll content

The relative chlorophyll content per unit leaf area was determined in the middle region of intact leaves by the use of a soil plant analysis development (SPAD) analyser (Minolta, by Hydro Agri, Dülem, Germany) which measures transmission of wavelengths absorbed by chlorophylls in the intact leaves. Each data point represents the mean of 10 independent measurements.

2.2.8.2 Photosystem II efficiency

Chlorophyll fluorescence measurements were performed in the middle region of intact leaves after dark adaptation as described by (Humbeck *et al.*, 1996) using a chlorophyll fluorometer (Mini PAM; Walz, Effeltrich, Germany). Mean values of the ratio variable fluorescence / maximal fluorescence (*Fv/Fm*) are based on 10 independent measurements.

2.2.9 Analyses of chromium content by ICP-AES methods

Total chromium content was analysed in 7 days old barley seedlings treated with 1mM of potassium dichromate for 24 to 144 hours and controls (no treatment). At each time point, roots, shoots and leaves samples were harvested and washed with distilled H_2O. The harvested plant tissues were dried at 80°C in an oven for 48 h. The samples were then milled and digested with a microwave using nitric acid / hydrogen peroxide. The estimation of chromium content was performed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) methods (Spectro, Germany).

2.2.10 Total RNA extraction

Total RNA was extracted from leaf tissue using the method described by Chirgwin *et al.* (1979). Approximately 3 g of primary leaf tissue was ground to a fine powder with liquid nitrogen in the mortar and transferred while frozen into 50 ml falcon glass. After incubation at room temperature for 3-4 min, 12 ml Lysis-buffer (pH 7.5) was added. The sample was homogenised using " IKA Ultra- Turrax T25 basic " from (IKA Labor technique, Staufen,

Germany) for 3 min at 24000 rpm. Then the sample was incubated for 20 min at 37 °C in Inkubator shaker " G24 environmental incubator shaker " (New Brunswick Scientific GmbH, Nürtlingen, Germany). The mixture sample was then centrifuged at 9400 x g in 50 ml falcon tube (centrifuge bottles polyallomer 50 ml) for 20 min at 4 °C in centrifuge (Beckman Coulter, Allegra™ 64R, Germany). The volume of the supernatant was estimated and transferred into a new 50 ml falcon and finally 0.02 volume of 3M sodium acetat- buffer (pH 5.0) and 0.75 volume 96 % Ethanol were added to the sample and incubated at − 20 °C overnight.

The next day, the sample was centrifuged at 4°C for 20 min, 10000 x g in an Avanti centrifuge J-25 from (BECKMAN, Germany). The pellet was resuspended with 8 ml TES-buffer (pH 7.4) and homogenised with "IKA Ultra- Turrax T25 basic" apparatus at 9000 rpm. Finally, the RNA was stored at − 80 °C until use.

2.2.11 Estimation of nucleic acid concentration

2.2.11.1 RNA concentration

RNA concentration was estimated at its absorption peak (260 nm) by 8452A Diode Array Spectrophotometer (HEWLETT PACKARD GmbH, Bad Homburg, Germany) using the formula:

RNA concentration = A_{260} x Dilution factor x 40 μg RNA / ml.

For each sample, the estimation of the concentration was performed 2 times, with the ratio of E260 / E280 representing the quality of nucleic acid isolated. The calculated ratio was between 1.8 to 2.0.

2.2.11.2 DNA concentration

DNA concentration was estimated at 260 nm by 8452A Diode Array Spectrophotometer (HEWLETT PACKARD GmbH, Bad Homburg, Germany) using the formula:

DNA concentration = A_{260} x Dilution factor x 50 μg DNA / ml.

2.2.12 Poly (A)$^+$ RNA isolation

Messenger RNA (mRNA) was isolated from the total RNA samples treated with potassium dichromate for 1 h, 5 h and 24 h or without 3 h and 24 h controls using the PolyATtract mRNA Isolation system IV (Promega, Madison, WI, USA).

1. 400 µg total RNA from 1 h chromium treated sample
2. 400 µg total RNA from 5 h chromium treated sample
3. 800 µg total RNA from 24 h chromium treated sample
4. 800 µg total RNA from 3 h control sample
5. 800 µg total RNA from 24 h control sample

The method consists of binding the Poly $(A)^+$ tail of the mRNA to oligo dTs, which are then attached to streptavidin- conjugated magnetic beads. The beads together with bound mRNA were collected using a magnet, and the rest of the RNA (tRNA and rRNA) was washed off. The elution of isolated Poly $(A)^+$ RNA was performed with RNase-free water.

2.2.13 Restriction fragment differential display PCR (RFDD-PCR)

The differential display-polymerase chain reaction was performed according to the instructions of the displayProfile Expression Profiling Kit (Qbiogene GmbH, Heidelberg, Germany). Double-stranded cDNA from each sample was synthesized, digested with *TaqI* enzyme which has a four-base recognition sequence. After ligation two adaptors to the expressed cDNA fragments and finally PCR was performed with ^{33}P- labeled primers.

The populations of cDNA fragments of each sample were loaded on a 8 % (w/v) denaturing polyacrylamide gel and autoradiographed with Kodak Biomax MR-film (Eastman Kodak, Rochester, NY, USA). The cDNA fragments differentially expressed were excised from the gel, eluted by boiling the gel pieces in 10 mM Tris-1mM EDTA (TE) for 10 min at 95°C, re-amplified by PCR with the same pair of primers as used for the first amplification, cloned by using the pGEM-T$^®$ Vector System I (Promega, Madison, USA) and sequenced.

2.2.13.1 cDNA synthesis

First strand cDNA synthesis

Poly(A)$^+$-RNA (= 400 ng mRNA)	15.0 µl
Anchored primer: 5'-T$_{25}$V (V=A,C oder G)	1.5 µl
(12.5 µM)	
10x cDNA Buffer 1 (500 mM Tris-HCl pH 8.3;	2.5 µl
800 mM KCl;100 mM MgCl$_2$; 40 mM DTT)	
dNTP Mix (5 mM each)	5.0 µl
displayTHERMO-RT (100U/µl)	<u>1.0 µl</u>
	25 µl

First strand cDNA synthesis reaction was incubated at 42°C for 2 h in thermocycler (T3-Cycler, Biometra$^®$, Göttingen, Germany).

Second strand cDNA synthesis

Sterile H$_2$O	38.0 µl
10x cDNA Buffer 2 (350 mM Tris-HCl pH 7,4;	7.5 µl
40 mM MgCl$_2$; 10 mM (NH$_4$)$_2$SO$_4$;	
30 mM DTT)	
dNTP Mix (5 mM each)	2.5 µl
DNA Polymerase I (10U/µl)	1.2 µl
RNase H (1U/µl)	<u>0.8 µl</u>
	50 µl

For each 25 µl first strand synthesis reaction, 50 µl of the above second strand master mix was added to a final volume of 75 µl. The reactions were incubated at 16°C for 2 h in Thermocycler (T3-Cycler, Biometra$^®$,Göttingen, Germany). After incubation, Phenol / Chloroform extraction was performed. For each 75 µl reaction, 125 µl of sterile water, 100 µl Phenol and 100 µl Chloroform (equilibrated to pH 8.0) were added. The reactions were mixed by vortexing and centrifuged for 5 min at room temperature at high speed (15000 x g) in the centrifuge (Biofuge fresco, Heraeus Instruments GmbH, Hanau, Germany). The aqueous phase was removed and kept in a new eppendorf tube. The cDNA was precipitated by adding

0.1Volume 3M NaAc (pH 5.2), and 2 volume 96 % Ethanol and then stored at – 20°C overnight. To the pellet cDNA, the mixture was centrifuged at maximum speed (15000 x g) for 20 min at 4°C in Heraeus centrifuge, then washed with 50 µl, 70 % (v/v) ice cold Ethanol, centrifuged for 10 min and finally, the pellet was dried for 15 min in the clean bench and resuspended in 20 µl sterile water. To confirm the efficiency of cDNA synthesis, 10 µl of the resuspended cDNA was run in 1.5 % (w/v) agarose gel containing 4 % formaldehyde, stained with ethidium bromide and then visualised under UV light.

2.2.13.2 Template preparation

Digestion reaction

For each sample 10 µl cDNA were digested with the following endonuclease digestion reaction:

Sterile H_2O	7.5 µl
10x displayPROFILE Buffer	2.0 µl
(100 mM Tris-Acetate pH 7.5; 100 mM	
Magnesium Acetat; 500 mM Calium Acetate)	
CDNA	10.0 µl
*Taq*I Restriction Enzyme (10U/µl)	0.5 µl
	20 µl

The digestion reaction was incubated at 65°C for 2 h in thermocycler.

Ligation of adaptors

To ligate the adaptors, 7.5 µl of the ligation mix was added to each sample.

Sterile H_2O	17.8 µl
10x displayPROFILE Buffer	3.0 µl
Adaptor Mix (15 µM)	3.0 µl
ATP (10 mM)	5.0 µl
T4 DNA ligase (1U/µl)	1.2 µl
	7.5 µl

The ligation reaction (27.5µl) was incubated at 37°C for 3 h in thermocycler. To confirm the efficiency of the digestion and ligation reactions, control PCR was performed with control

primer. The same PCR was performed one time with 2 µl Control Template from Kit and a second time with sterile water (6.9 µl). 10 µl of PCR products were loaded in 1.5 % (w/v) agarose gel containing 4 % formaldehyde, stained with ethidium bromide and then visualised under UV light.

Control-PCR

Sterile H$_2$O	8.7 µl
10x display TAQ FL 10x Reaction Buffer	2.0 µl
dNTP Mix (5 mM each)	0.8 µl
Control Primer (1 µM)	8.0 µl
Template	0.2 µl
display TAQ FL (5U/µl)	<u>0.3 µl</u>
	20 µl

PCR program

Temperature	Time	Cycles	Step
94°C	30 sec		Denaturation
94°C	30 sec		Denaturation
55°C	30 sec	30	Annealing
72°C	1 min		Elongation
72°C	10 min		Final Elongation

2.2.13.3 ^{33}P end-labeling for radioactive detection

The single PCR reaction was used to label the 0-extention primer. The total amount of labeled 0-extention primer was calculated by multiplication of the volume of each component with the number of PCR reactions planned. The following PCR reaction was set up,

Sterile H$_2$O	0.28 µl
10x displayPROFILE Buffer	0.10 µl
"0-extension" Primer (10 µM)	0.40 µl
[γ^{33}P]-ATP (3000 Ci / mmol)	0.20 µl
T4-Poly nucleotide kinase (5U/µl)	<u>0.02 µl</u>
	1.0 µl

Finally, the PCR reaction was incubated at 37°C for 30 min in thermocycler.

2.2.13.4 Amplification of template

The Restriction Fragment Differential Display-PCR was performed in total with 12 differents PCR with radioactive labeled "0-extention" primer, each selective primer (Eu 3, Eu 6, Eu 7, Eu 13 and Eu 15) and 4 cDNAs template from 1+5 h and 48 h chromium treated samples and 3 h and 48 h controls.

Sterile H_2O	11.7 µl
displayTAQ FL 10x Reaction Buffer	2.0 µl
dNTP Mix (5 mM each)	0.8 µl
^{33}P labeled "0 extension" Primer (4 µM)	1.0 µl
displayPROBE Primer (selective Primer Eu 3, Eu 6, Eu 7, Eu 13 and Eu 15; 1 µM)	4.0 µl
cDNA template	0.2 µl
displayTAQ FL (5U/µl)	0.3 µl
	20 µl

"Touch-down" PCR program

Temperature	Time	Cycles	Step	
94°C	1 min		Denaturation	
94°C	30 sec		Denaturation	
60°C	30 sec	10	Annealing	$\Delta T = -0.5°C$ (each cycle)
72°C	1 min		Elongation	
94°C	30 sec		Denaturation	
55°C	30 sec	25	Annealing	
72°C	1 min		Elongation	
72°C	5 min		Final Elongation	

At the end of the PCR, 15 µl of loading buffer containing (95 % (v/v) Formamide, 20 mM EDTA, 0.05 % (w/v) Bromophenol blue and 0.05 % (w/v) Xylene cyanol FF) was added, incubated at 85°C for 5 min, then placed directly on ice. Each 2 x 5 µl of PCR sample was loaded on a 8 % urea polyacrylamide sequencing gel "CastAway® Precast Sequencing Gels"

from (Stratagene, La Jolla, USA) that has been pre-run 30 min at 60 Watts with 0.6 x TBE as electrophoresis buffer. For radioactive analysis, the run was stopped when the bromophenol blue has left the gel and the xylene cyanol has passed the middle of the gel. The gel was then dried for 20 min in CastAway Gel Dryer (Stratagene, La Jolla, USA) and exposed onto a film (Kodak, BioMax MR, EASTMAN KODAK, Rochester, USA) for 20-24 h. After exposition, the autoradiograph film was developed, fixed and dried then carefully aligned with the dried gel according to the position markings. For isolation of the differentially expressed cDNA fragments, the needle was used to make a hole through a film and into the glass plate containing a gel. The cDNA fragments were cut out with the scalpel, eluted in 20 µl on TE-Buffer (pH 8.0), and then incubated at 95°C for 10 min. Finally the eluted bands were stored at – 20°C.

2.2.14 Reamplification of cDNA fragments isolated by RFDD-PCR

To amplify the gene fragment of interest isolated and eluted in TE-Buffer, the following PCR reaction was performed

Sterile H$_2$O	20.0 µl
displayTAQ FL 10 x Reaction Buffer	4.0 µl
0-extension Primer (10 µM)	0.8 µl
(Eu 3, Eu 6, Eu 7, Eu 13 and Eu 15) Primer (1 µM each)	10.0 µl
dNTP Mix (5 mM each)	1.6 µl
displayTAQ FL (5U/µl)	0.6 µl
Solution containing the gene fragment	<u>5.0 µl</u>
	40 µl

PCR program

Temperature	Time	Cycles	Step
94°C	30 sec		Denaturation
94°C	30 sec		Denaturation
55°C	30 sec	30	Annealing
72°C	1 min		Elongation
72°C	10 min		Final Elongation

Each 2 x 15 µl of PCR product was loaded in 1.5 % (w/v) agarose gel containing 4 % formaldehyde, stained with ethidium bromide and then visualised under UV light. Reamplified bands were isolated from the agarose gel using a "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany).

2.2.15 DNA agarose gel electrophoresis

The analyses of samples was performed in 1.5 % (w/v) TAE agarose gel which was prepared as follows: 1.5 g of agarose (Roth, Kahlsruhe, Germany) was melted in 100 ml 1 x TAE-Buffer (pH 8.0). The melted agarose was cast in a tome hood and allowed to set. DNA samples were diluted 6:1 with 6 x Loading Dye solution and loaded with 100 bp DNA molecular weight ladder (Fermentas, St. Leon-Rot, Germany) using 1x TAE-Buffer (pH 8.0) as gel running buffer.

2.2.16 DNA isolation from agarose gel

The "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany) was used for purification of DNA bands from agarose gel.

2.2.17 Ligation of DNA fragment

For cloning of the PCR products, a pGEM-T$^{®}$ Vector System I (Promega, Madison, USA) was used. The high copy number pGEM-T$^{®}$ Vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region with the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening (Blue-white colonies) on indicator plates.

3 µl of eluted PCR products was cloned by following the ligation reaction below and incubated overnight at 4°C.

2 × Rapid Ligation Buffer, T4 DNA Ligase	5.0 µl
pGEM$^{®}$-T Vector (50ng/µl)	1.0 µl
PCR product	3.0 µl
T4 DNA Ligase (3U/µl)	<u>1.0 µl</u>
	10 µl

2.2.18 Preparation of competent cells

The transformation of ligation fragments was performed in competent cells prepared with Rubidium chloride method using host strain *E. coli* XL1-blue MRF$^{'d}$.

10 ml LB-medium containing 30 µl Tet were inoculated with *E. coli* XL1-blue MRF$^{'d}$

host strain and incubated at 37°C, 160 rpm overnight in an INNOVA™ 4000 Thermo shaker (New Brunswick Scientific GmbH, Nürtingen, Germany). The next morning 5 ml overnight culture was used for inoculation of 100 ml LB-medium and incubated at 37°C, 160 rpm in Thermo shaker until OD_{590nm}= 0.6 (CARY 50 BIO, UV-visible spectrophotometer, VARIAN, Australia).

The liquid culture was then incubated on ice for 15 min and the bacterial cells were collected by centrifugation for 5 min at 4 000 x g and 4°C. The bacterial pellet was resuspended in 40 ml (0.4V from 100 ml overnight culture) ice cold TfbI- Buffer and incubated on ice for 10 min. At the end of incubation, the bacterial sample was centrifuged for 5 min at 4 000 x g and 4°C. The pellet was then resuspended in 40 ml (0.4 V from 100 ml overnight culture) ice cold TfbII-Buffer and incubated on ice for 20 min. Finally, the bacterial solution was eluted in 1.5 ml eppendorf tubes (150 µl each), frozen in liquid nitrogen and then stored at - 80°C until uses.

2.2.19 Bacterial cells transformation

For the transformation of PCR products ligated into pGEM-T® Vector, 5 µl of the ligation mix was added on ice into 150 µl eluted bacterial cells *E. coli* XL1-blue MRF$^{'d}$, and kept on ice for 20 min. The reaction mixture was then incubated in a water bath at 41°C to heat shock the bacteria for 60 sec and immediately returned to ice for 2 min to allow the cells to recover. 850 µl of LB medium were added to the transformed cells, which were then incubated for 1 h at 37°C and 150 rpm in an incubator shaker (New Brunswik GmbH, Nürtingen, Germany). The transformed cells were spread over the surface of the LB / Amp / Tet / X-Gal / IPTG plates and incubated at 37°C overnight.

2.2.20 Colonie-PCR

The verification of positive clones after transformation in bacterial cells of *E. coli* XL1-blue MRF$^{'d}$ was done by screening of white / blue colonies. Successful cloning of an insert in pGEM-T® Vector interrupts the coding sequence of β-galactosidase; recombinant clones can usually be identified by color screening on indicator plates. Clones that contain PCR products,

in most cases produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *LacZ* gene. On this basis, 10 colonies were picked from the plates with sterile tips and used for inoculation of new LB / Amp / Tet / X-Gal / IPTG plates and 50 µl H$_2$O. The bacterial solution was then incubated at 95°C for 5 min and used as template for the colonie PCR and vector specific primers NewT7 and SP6.

Sterile H$_2$O	16.4 µl
TAQ 10x PCR-Buffer	3.0 µl
dNTP Mix (2.5 mM each)	1.6 µl
NewT7 Primer (5µM)	3.0 µl
SP6 Primer (5µM)	3.0 µl
Template	1.0 µl
Taq DNA Polymerase	<u>2.0 µl</u>
	30 µl

PCR program

Temperature	Time	Cycles	Step
94°C	5 min		Denaturation
94°C	30 sec		Denaturation
55°C	1 min	30	Annealing
72°C	1.5 min		Elongation
72°C	10 min		Final Elongation

PCR products were run in a 1.5 % (w/v) TAE agarose gel. After resolution, the ethidium bromide stained DNA bands were visualized using the UV transilluminator (H. SAUR, Reuthingen, Germany) and then photographed for gel photography. The positive colony PCR was used for inoculation of 10 ml LB /Amp / Tet liquid medium from new inoculated LB / Amp / Tet / X-Gal / IPTG plates incubated at 37°C. The inoculated LB liquid medium was incubated overnight at 37°C and 150 rpm in an Incubator shaker (New Brunswik GmbH, Nürtingen, Germany).

2.2.21 Plasmid DNA mini-preparation

For a plasmid isolation from transformed *E. Coli*, a "QIAprep Spin Miniprep Kit" from QIAGEN (Hilden, Germany) was used.

2.2.22 Glycerol stocks of plasmid culture

Glycerol stock was prepared by mixing of each 150 µl of overnight liquid culture with 850 µl sterile glycerol, frozen in liquid nitrogen and finally stored at - 80°C.

2.2.23 Sequence analysis

Nucleotide sequences were determined by the dideoxy chain-termination method using the BigDye® Terminator v1.1 Cycler Sequencing Kit (Applied Biosystems, Forster City, CA, USA) with a sequencer ABI Prism™ 370 automatic DNA-Sequencer (Applied Biosystems). The primers NewT7 and SP6 were used for sequencing of cDNA fragments cloned into the pGEM-T® vector (Promega, Madison, USA).

BigDye ReadyReaction (RR) Mix	2.0 µl
BigDye PCR Buffer	2.0 µl
NewT7 (5 µM)	1.0 µl
Template Plasmid DNA (~400 ng)	X µl
Sterile H$_2$O to a final volume of	10.0 µl

Sequencing PCR program

Temperature	Time	Cycles	Step
94°C	5 min		Denaturation
94°C	30 sec		Denaturation
58°C	1 min	30	Annealing
72°C	1.5 min		Elongation
72°C	10 min		Final Elongation

At the end of the PCR, DNA was precipated with 1 µl 3M Na-Acetat (pH 4.6) and 40 µl 96 % Ethanol and incubated for 5 min at –20°C. The DNA was pelleted by centrifugation for 20 min, and then washed with 250 µl 70 % Ethanol. After further centrifugation for 10 min at room temperature, the pellet was air-dried and finally sent for sequencing. cDNA sequences

were analysed and compared with DNA and protein databases using the EMBL FASTA server (Pearson & Lipman, 1988), the BLAST server at NCBI (Altschul *et al.*, 1990) and the "Lasergene expert sequence analysis software" (DNA STAR Inc., Madison, USA).

2.2.24 DNA labeling for expression analyses

After sequence analyses, interesting fragments were labeled by random primer DNA-labelling using DIG-labelled dUTP "Dig-High-Prime"- Kit (Roche Diagnostics GmbH; Mannheim, Germany). The PCR labeling reaction was performed using pGEM-T® vector primers NewT7 and SP6 or the insert specific primers "0-extention" and "Eu 3, Eu 6, Eu 7, Eu 13 and Eu 15" from RFDD-PCR fragment. For the template, round 300ng of plasmid mini-preparation was used for probe labeling.

Sterile H_2O	23.0 µl
10 x PCR- Reaction Buffer	4.0 µl
NewT7 (5 µM each)	4.0 µl
SP6 (5 µM each)	4.0 µl
10 x Dig DNA Labeling Mix	2.0 µl
(1mM dATP; 1 mM dGTP; 1 mM dCTP;	
0.65 mM dTTP; 0.35 mM Dig-11-dUTP)	
Taq DNA Polymerase	2.0 µl
Plasmid mini preparation (~ 300 ng DNA)	<u>1.0 µl</u>
	40 µl

PCR program

Temperature	Time	Cycles	Step
94°C	1 min		Denaturation
94°C	30 sec		Denaturation
58°C	1 min	35	Annealing
72°C	1.5 min		Elongation
72°C	10 min		Final Elongation

After PCR labeling, each 2 x 20 µl were run on 1.5 % (w/v) TAE agarose gel and stained with ethidium bromide. DNA bands were visualized using the UV transilluminator (H. SAUR, Reuthingen, Germany). The labelled DNA was excised from agarose gel, purified and then eluted in 20-30 ml High-SDS-Hybridization buffer and stored at -20°C until uses.

2.2.25 Northern blot

2.2.25.1 Electrophoresis of RNA samples

Total RNA was electrophoretically fractionated on 1 % (w/v) agarose gels containing 4 % formaldehyde. 25 µg RNA of each sample were precipitated with 0.026V 4M NaCl solution and 2V 96 % Ethanol then stored overnight at -20°C. The next day, the samples were centrifuged for 20 min at 4°C and maximum speed, washed with 70 % ethanol and centrifuged again for 10 min at 4°C. The pellets were dried in a clean bench for 15 min, then resuspended in 18 µl RNA Probe-buffer and incubated at 68°C for 10 min in a heating block for denaturation, then chilled on ice for 2 min. The RNA samples were run with RNA molecular weight ladder and 1x MOPS as running gel buffer. The electrophoresis was stopped when the bromophenol blue dye had migrated to approximately 2/3 of the gel. The gel was washed for 15 min with destilled water, incubated in 0.1M Tris-HCl solution with ethidium bromide (1µg / ml) for 20 min at room temperatue and then washed for 2 x 30 min in 0.1M Tris-HCl solution at room temperature. The RNA bands were visualized using a UV transilluminator (H. SAUR, Reuthingen, Germany) and then photographed.

After RNA electrophoresis in agarose gel, the RNA were transferred by pressure blotting (PosiBlot® 30-30 Pressure Blotter and Pressure Control Stations, Stratagene Cloning Systems, La Jolla, USA) into positively charged Nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) using transfer buffer 10x SSC (pH 7.0) and 60mmHg pressure for 1h. At the end of the transfer, the membranes were incubated at 1200 µ joules x 100 under UV-light (UV Stratalinker® 1800, Stratagene, Germany) to allow binding of RNA to the Nylon membranes.

2.2.25.2 Hybridization

The Nylon membranes containing RNA were prehybridized in a OV3 Hybridizirung oven (Whatman-Biometra®, Göttingen, Germany) at 50°C for 1.5 h in 25 ml prehybridization solution. The solution consisted of 50 % (v/v) deionized formamide, 74.7 mM sodium citrate (pH 7.0), 747 mM sodium chloride, 50 mM sodium phosphate, 2 % (w/v) blocking reagent

(Roche, Diagnostics GmbH, Mannheim, Germany), 0.1 % (w/v) N-lauroylsarcosine, and 7 % (w/v) SDS incubated before at 68°C for 10 min in water bath. After discarding the prehybridization solution, hybridization was carried out overnight at 50°C in a solution consisting of the same chemicals as prehybridization plus a DIG-labelled probe. The hybridization solution was incubated at 68°C for 10 min and then 2 min on cold ice before uses.

2.2.25.3 Detection of mRNAs

To remove background signals, the membrane was washed twice for 15 min at room temperature in wash buffer 1 and twice for 15 min at 50°C shaking in wash buffer 2. The detection of DIG-labelled probe was performed with "DIG Luminescent Detection Kit"

(Roche, Mannheim, Germany). The membrane was equilibrated for 3 min in wash buffer 3, and incubated for 45 min at room temperature in 1x blocking solution to block unspecific binding sites on the membrane. In order to bind antibodies to the DIG-labelled probe, antibody solution consisting of dilution (anti-digoxigenin, Fab- fragments, conjugated to alkaline phosphatase 1: 10000 in 1x blocking solution) was used to incubate the membrane for 30 min at room temperature. The antibody was removed by washing the membrane twice for 15 min in wash buffer 3 at room temperature. After equilibration of membrane in 1:10 detection buffer for 2 min at room temperature, the membrane was incubated between acetate foils in CSPD$^®$- solution (Tropix) diluted 1:60 in detection buffer for 25 min at 37°C. The membrane was then wrapped in Saran plastic foil washed with 70 % ethanol and exposed on a film (Hyperfilm™ ECL™, Amersham Biosciences, UK) for 30 min to 24 h (depending on the signals). After the exposition, the film was developed first with developer solution (Kodak, GBX) then in a solution consisting of water and drops of acetic acid and finally fixed in fixer solution (Kodak, GBX).

2.2.26 Quantitative Real-time PCR (qRT-PCR)

Genes which presented low transcription levels in northern analyses were investigated by quantitative real-time PCR method. First, the total RNA was treated with DNase I and cDNA was synthesized using Omniscript Reverse Transcriptase Kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) was carried out in iCycler (BioRad, Munich, Germany) in a total volume of 25 µl including SYBR Green I fluorescence dye (QIAGEN, Hilden, Germany), 5 µM of the gene specific primers and the 18S rRNA housekeeping gene primers

with different dilutions of cDNA (1/4, 1/16 and 1/64). For checking the specificity of RT-PCR products they were separated on 1 % (w/v) agarose gel always resulting in one single product. To determine the relative expression rate based on the expression of our target gene versus the reference gene 18S ribosomal RNA, a relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR described by (Pfaffl *et al.,* 2002) was used. Each data point is based on 6 independent measurements.

2.2.26.1 RNA treatment with DNase I

Total RNA (3 µg)	x µl
10x DNase Buffer	3.0 µl
DNase I (1U/µl)	3.0 µl
Sterile H_2O to a final volume of	30.0 µl
Incubation at 37°C for 1 h in thermocycler,	
EDTA (25mM)	3.0 µl

Incubation at 65°C for 10 min to stop reaction.

2.2.26.2 cDNA synthesis

Hexa Random Primer (100 µM)	2.0 µl
RNA-DNase solution (~1µg)	11.0 µl
RNase-free H_2O (for control no R T)	1.75 µl
(for sample with template To)	2.75 µl

Incubation at 70°C for 5 min in thermocycler,

10x Reverse transcriptase buffer	2.0 µl
dNTPs (5 mM each)	2.0 µl
RNase inhibitor (40U/µl)	0.25 µl

Incubation at 25°C for 5 min,

Omniscript-Reverse Transcriptase (40U/µl)   1.0 µl

Finally, the reaction was incubated at 37°C for 2 h, then at 95°C for 5 min.

After cDNA synthesis, the samples were diluted 1/ 4, 1/ 16 and 1/ 64.

2.2.26.3 qRT-PCR reaction

2x QuantiTect SYBR Green PCR Master Mix	12.5 µl
Primer forward (5 µM)	1.5 µl
Primer reverse (5 µM)	1.5 µl
Fluorescein calibration Dye (1 µM)	0.25 µl
RNase-free H$_2$O	7.25 µl
CDNA	<u>2.0 µl</u>
	25 µl

The samples were distributed in PCR-plates (96 wells, Bio-Rad Laboratories, CA, USA) and covered with PCR-Film (Eppendorf AG**,** Hamburg, Germany). The polymerase chain reaction (PCR) was carried out in iCycler (BioRad, Munich, Germany) using the following PCR program: 1 cycle at 95°C for 15 minutes followed by 50 cycles at 58°C for 30 seconds and finally extension phase at 72°C for 30 seconds.

2.2.27 Reverse transcriptase PCR reaction (RT-PCR)

The RFDD-PCR clone identified chromi 1 (183 bp) was compared with other barley EST using the HarvEST Triticeae software version 0.99 (University of California, USA) which showed 156 / 185 bp identities to the HarvEST consensus performed with AV946685 barley EST. The cDNA was isolated by first RT-PCR using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) and the gene specific primers designed from the HarvEST consensus sequence.

5x OneStep RT-PCR Buffer	10.0µl
dNTP Mix (10 mM each)	2.0µl
Primer forward (10 µM)	2.0µl
Primer reverse (10 µM)	2.0µl
Template RNA 48 h chromium (2 µg)	x µl
OneStep RT-PCR enzyme Mix (2U / reaction)	2.0 µl
Sterile H$_2$O	x µl
	50 µl

"Touch-down" PCR program

Temperature	Time	Cycles	Step	
50°C	30 min		Reverse transcription	
95°C	15 min		HotStar Taq activation	
94°C	1 min		Denaturation	
60°C	1 min	9	Annealing	$\Delta T = -0.5°C$ (each cycle)
72°C	1 min		Elongation	
94°C	1 min		Denaturation	
55°C	1 min	25	Annealing	
72°C	1 min		Elongation	
72°C	10 min		Final Elongation	

2.2.28 <u>R</u>apid <u>A</u>mplification of <u>c</u>DNA <u>E</u>nds (RACE)

The GeneRacer™ Kit (Invitrogen, Karlsruhe, Germany) was used to obtain full-length 5` and 3`ends of cDNA clone (chromi 1, 992 bp) cloned by RT-PCR. This method is based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) methods, and results in the selective ligation of an RNA oligonucleotide to the 5`ends of decapped mRNA using T4 RNA ligase as described in The GeneRacer™ Kit (Invitrogen, Karlsruhe, Germany).

2.2.28.1 Dephosphorylating of RNA

150 ng mRNA isolated from total RNA 48 h chromium treated barley seedling using PolyATtract mRNA Isolation system IV of Promega (Madison, WI, USA) was treated with cal intestinal phosphatase (CIP) to dephosphorylate non-mRNA or truncated mRNA.

mRNA	6.0 µl
10x CIP Buffer	1.0 µl
RNaseOut TM (40U/µl)	1.0 µl
CIP (10U/µl)	1.0 µl
DEPC H$_2$O	<u>1.0 µl</u>
	10 µl

The reaction was mixed gently by pipetting, briefly vortexed and incubated at 50°C for 1 h in thermocycler. After incubation, the reaction was centrifuged briefly and placed on ice.

2.2.28.2 Precipitation of RNA

To precipitate mRNA, 90 µl of DEPC water and 100 µl phenol : chlorophorm were added to the reaction. After vortexing, the reaction was centrifuged at maximum speed for 5 min at room temperature. The aquous phase was transferred to a new eppendorf tube and 2 µl mussel glycogen (10 mg/ml) plus 10 µl 3M sodium acetate (pH 5.2), were added and well mixed. 220 µl 95 % ethanol were added and then stored at - 20°C overnight. In order to pellet the mRNA, the reaction mixture was centrifuged at maximum speed for 20 min at 4°C, then washed with 70 % ethanol and resuspended in 6 µl DEPC water.

2.2.28.3 Removing the mRNA Cap Structure

After the dephosphorylation reaction of mRNA, the following reaction was set up on ice to remove the 5´cap structure from the full-length mRNA.

Dephosphorylated mRNA	6.0 µl
10x TAP Buffer	1.0 µl
RNaseOut™ (40U/µl)	1.0 µl
TAP (0.5U/µl)	<u>1.0 µl</u>
	10 µl

The reaction was mixed gently by pipetting, centrifuged briefly to collect fluid and incubated at 37°C for 1 h. After the incubation, the sample was centrifuged and placed on ice. The mRNA precipitation was precipitated as described above and the pellet resuspended in 7 µl DEPC water.

2.2.28.4 Ligating the RNA oligo to decapped mRNA

Once the mRNA was decapped, the following reaction was set up to ligate the GeneRacer RNA oligo to the 5`end of mRNA: 7 µl of dephosphorylated, decapped mRNA were added to the tube containing the pre-aliquoted, lyophilised GeneRacer™ RNA Oligo (0.25µg). The fluid was pipetted up and down, centrifuged briefly, and incubated at 65°C for 5 min to relax the RNA secondary structure. After incubation, the reaction was placed on ice to chill for 2 min and then the following reagents were added to the reaction:

10x Ligase Buffer	1.0 µl
10mM ATP	1.0 µl
RNaseOut™ (40U/µl)	1.0 µl
T4 RNA ligase (5U/µl)	<u>1.0 µl</u>
	10 µl

The reaction was incubated at 37°C for 1 h in a thermocycler. After the incubation, the mRNA was precipitated as described above (see 2.2.28.2).

2.2.28.5 Reverse transcribing mRNA

The reverse transcription of mRNA into cDNA was performed using the SuperScript™ III RT (Invitrogen, Karlsruhe, Germany). To 10 µl ligated RNA, the following components were added:

GeneRacer™ 3`primer (50 µM)	1.0 µl
dNTPs Mix	1.0 µl
Sterile H$_2$O	1.0 µl

The reaction was incubated at 65°C for 5 min to remove any RNA secondary structure. Subsequently, the following reagents were added to the reaction on ice:

5x First Strand Buffer	4.0 µl
0.1M DTT	1.0 µl
RNaseOut™ (40U/µl)	1.0 µl
SuperScript™ III (200U/µl)	<u>1.0 µl</u>
	20 µl

Finally, The reaction was incubated at 50°C for 1 h. To inactivate the reverse transcriptase the reaction was incubated at 70°C for 15 min, chilled on ice for 2 min, then 1 µl of RNase H (2U) was added to the reaction and incubated at 37°C for 20 min.

2.2.28.6 Amplifying cDNA Ends : 5´end

To amplify the 5`end of the extended clone chromi1, the following reaction was set up:

GeneRacer™ 5´ Primer (10µM)	3.0 µl
Reverse Gene Specific Primer (10µM)	1.0 µl
RT Template	1.0 µl
10x PCR Buffer	5.0 µl
dNTP (10 mM each)	1.0 µl
HotStar Taq DNA Polymerase (5U/µl)	0.5 µl
$MgSO_4$ (50 mM)	2.0 µl
Sterile H_2O	<u>36.5 µl</u>
	50µl

"Touch-down" PCR program

Temperature	Time	Cycles	Step	
94°C	15 min		HotStar Taq activation	
94°C	2 min		Denaturation	
94°C	30 sec	4	Denaturation	
72°C	1 min		Annealing	
94°C	30 sec	4	Denaturation	
70°C	1 min		Annealing	
94°C	30 sec		Denaturation	
68°C	30 sec	9	Annealing	$\Delta T = -0.5°C$ (each cycle)
70°C	1 min		Elongation	
94°C	2 min		Denaturation	
63°C	30 sec	14	Annealing	
70°C	1 min		Elongation	
70°C	10 min		Final Elongation	

The specificy of the PCR product was verified by nested PCR.

GeneRacer™ 5´ Nested (10 µM)	1.0 µl
Reverse Nested (GSP) (10 µM	1.0 µl
Initial PCR	1.0 µl
10x PCR Buffer	5.0 µl
dNTP (10 mM each)	1.0 µl
HotStar Taq DNA Polymerase (5U/µl)	0.5 µl
$MgSO_4$ (50 mM)	4.0 µl
Sterile H_2O	36.5 µl
	50 µl

PCR program

Temperature	Time	Cycles	Step
94°C	2 min		Denaturation
94°C	30 sec		Denaturation
65°C	30 sec	25	Annealing
68°C	2 min		Elongation
68°C	10 min		Final Elongation

PCR products were run on 1.5 % (w/v) TAE agarose gel, stained with ethidium bromide and then purified using a "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany).

2.2.28.7 Amplifying cDNA Ends : 3´end

The 3´end of the extended clone chromi1 was performed as descibed above in the amplification of the 5´end using the GeneRacer™ 3´ Primer and the gene specific primer for the amplification of the 3´end reaction. The GeneRacer™ 3´ Nested Primer and Nested Gene Specific Primer were used for the Nested PCR reaction.

2.2.29 Overexpression of GST-HvC2d1

2.2.29.1 Ligation of HvC2d1 into pGEX–2TK vector

To produce a recombinant GST-HvC2d1 protein in *E. coli*, the full-length coding region (ORF) of the HvC2d1 protein was amplified using the cDNA clone HV_Ceb0017E22f (BF064709) and the primers HvC2d1 For 5`-GAG AAT TCA TGG GCT CGC GGT ACG AGG TGG AGG TGA C with *Eco*RI site and HvC2d1 Rev 5`- GAC TCG AGC TAG TAG TCG TCG TCG CCG CCG TAG TC with *Xho*I site. The PCR product was first digested with *Eco*RI/*Xho*I enzymes and then ligated into the *Eco*RI / *Xho*I site of a pGEX–2TK vector (Amersham Biosciences, Freiburg, Germany) using the following reaction:

10x PCR Buffer	5.0 µl
dNTP (2.5 mM each)	1.0 µl
Primer with *Eco*RI (5 µM)	6.0 µl
Primer with *Xho*I (5 µM)	6.0 µl
Template (1:1000)	2.5 µl
Taq DNA polymerase	1.0 µl
Q-solution 5x	10.0 µl
Sterile H_2O	<u>18.5 µl</u>
	50 µl

PCR Program

Temperature	Time	Cycles	Step
94°C	3 min		Denaturation
94°C	1 min		Denaturation
57°C	1 min	30	Annealing
72°C	2 min		Elongation
72°C	5 min		Final Elongation

The PCR product was run on 1.5 % (w/v) TAE agarose gel and stained with ethidium bromide. The DNA bands were excised from the gel and purified with a "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany).

Digestion reaction

pGEX–2TK vector (~2 µg)	8.5 µl
*Eco*RI (10U/µl)	1.5 µl
Buffer (R+)	2.0 µl
Sterile H_2O	8.0 µl

After incubation at 37°C for 2 h in a thermocycler the reaction was completed with the addition of:

Buffer (R+)	1.0 µl
*Xho*I (10U/µl)	1.5 µl
Sterile H$_2$O	7.5 µl

Finally, the reaction was incubation at 37°C for 2 h and then at 80°C for 20 min in thermocycler.

The digestion reaction product was run on 1.5 % (w/v) TAE agarose gel and stained with ethidium bromide. The band corresponding to the digested vector were excised from the gel and purified with a "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany).

Ligation reaction

pGEX–2TK vector digested	
(~500ng)	14.0 µl
Insert HvC2d1 digested (~300 ng)	3.0 µl

The reaction was incubated at 45°C for 5 min, then chilled on ice for 1 min and completed with:

T4 ligase Buffer	2.0 µl
T4 ligase (5U/µl)	1.0 µl

Finally, the reaction was incubated at 4°C overnight.

The recombinant plasmid was introduced into *E. Coli* Rosetta (DE 3) pLys S (Novagen, Darmstadt, Germany). The positive colonies were used for inoculation of 10ml LB-medium supplemented with 100 µl / ml Ampicillin and 34 µl / ml Chloramphenicol and then incubated at 160 rpm in thermo shaker at 37°C overnight. The next day, the culture was used for inoculation (1:50) of 400 ml LB-medium and incubated at 37°C, 160 rpm in a thermo shaker until OD$_{590nm}$ = 0.6, where OD was determined with a UV-visible spectrophotometer (CARY 50 BIO, VARIAN, Australia). Before the protein overexpression, 2 ml LB-medium as "not induced control sample" were pelleted in eppendorf tubes, resuspended in 50 µl 2x SDS-PAGE-Buffer without bromophenol blue and incubated at - 20°C until use.

Overexpression of the protein was induced by addition of 0.1 mM IPTG (isopropyl β-D-thiogalactoside, DUCHEFA, Haarlem The Netherlands) to the culture medium. After

incubation at 30°C for 2 h, 2 ml bacterial cultures as ``induced control sample`` were pelleted in eppendorf tubes and resuspended in 100 µl 2x SDS-PAGE-buffer without bromophenol blue. The rest of the bacterial cultures was harvested and resuspended in PBS buffer (150 mM NaCl, 16 mM Na_2 HPO4, 4 mM KH_2 PO4, 2 % (v/v) Triton X-100). The lysis of bacterial cells was performed by sonification using a Model 250 Sonifier® (Branson, U.S.A) for 10 x 10 sec with 10 sec pauses at 65-70 W. The lysate was centrifuged at 10000 x g at 4°C for 25 min. The supernatant represented the soluble protein and the pellet was resuspended in the same lysis buffer (insoluble protein).

2.2.29.2 SDS-Polyacrylamide gel electrophoresis

The overexpressed protein was analysed through SDS-PAGE. Two glass plates for gel cassettes (Biometra, Göttingen, Germany) were cleaned with 70 % ethanol. For mounting the glass plates, a spacer of silicone rubber seal was placed between 2 glass plates and then the assembly was fixed with 5 clips. The running and stacking gel solutions were prepared on ice. The running gel solution was poured up to 5 cm, overlaid with distilled water and incubated at room temperature for about 45 min for polymerisation. The water was removed completely from the surface of the polymerised gel with a sheet of filter paper. After pouring the stacking gel solution, the comb was inserted between the glass plates.

The lower reservoir of the electrophoresis apparatus was filled with 50 ml of running buffer (0.05 M Tris, 0.19 M Glycin) and the upper reservoir with 50 ml of running buffer (0.05 M Tris, 0.19 M Glycin, 1 % SDS). Each 20 µl protein samples were mixed in the same volume of SDS-PAGE buffer, incubated at 95°C for 5 min and then chilled on ice for 2 min. The samples were loaded into the well of a gel with a prestained protein molecular weight marker (Fermentas, St. Leon-Rot, Germany). After the electrophoresis, the gel was stained with 20 ml Rotie R-Blue solution (Roth, Karlsruhe, Germany) mixed with 60 ml H_2O and 20 ml methanol overnight in a shaker (Heidolph, ROTAMAX 120, Germany) at room temperature. The gel was destained in 60 ml H_2O and 20 ml methanol for 2 h at room temperature.

2.2.29.3 Purifiction of GST-HvC2d1 protein

The recombinant protein GST-HvC2d1 was purified in a Glutathione- Sepharose 4B affinity column (Amersham Biosciences, Freiburg, Germany) using a pump (Minipuls 3, Abimed / Gilson®, France). The Glutathione- Sepharose 4B affinity column was washed with 20 ml PBS buffer (150 mM NaCl, 16 mM Na_2 HPO4, 4 mM KH_2 PO4, pH 7.3) and then equilibrated with 3 x 5ml PBS buffer (150 mM NaCl, 16 mM Na_2 HPO4, 4 mM KH_2 PO4, 2 % (v/v) Triton X-100). The sample containing the soluble protein was applied to the column and the protein GST-HvC2d1 bound to the gel bed was washed with 2 x 10ml PBS buffer. The recombinant protein GST-HvC2d1 was eluted with 5x 5ml elution buffer (10 mM Glutathione in 50 mM Tris-HCl pH 8.0).

2.2.30 Ca^{2+}-binding assay for HvC2d1

The ability of HvC2d1 to bind calcium was confirmed by $^{45}Ca^{2+}$ overlay assay using the method described by Maruyama *et al*. (1984). Different amounts (2 µg, 4 µg, 8 µg and 12 µg) of the purified HvC2d1 protein, of calmodulin (Sigma-Aldrich, Taufkirchen, Germany) and of bovin serum albumin (Sigma-Aldrich, Taufkirchen, Germany) were blotted to the nitrocellulose transfer membrane (PROTRAN, Dassel, Germany) using a dot-blot apparatus (MINIFOLD I, Schleicher & Schuell, Dassel, Germany). The membrane sheet was washed four times with 10 mM MES-KOH (pH 6.5), 5 mM $MgCl_2$, and 60 mM KCl. Then the membrane was incubated in the same buffer supplemented with 37 KBq / ml $^{45}Ca^{2+}$ ($^{45}CaCl_2$, Amersham Biosciences, Freiburg, Germany) at 23°C for 10 min. The membrane was washed three times in 50 % (v/v) ethanol and dried at room temperature, then exposed to an Imaging Plate for 3 days and analysed by a Fluorescent Image Analyser (FLA-3000 Series, FUJIFILM; FUJI PHOTO FILM CO; Tokyo, Japan) for autoradiogram of the $^{45}Ca^{2+}$– labelled proteins on the membrane.

2.2.31 Subcellular localisation of HvC2d1-GFP

The full-length coding region (ORF) of HvC2d1 protein was amplified using a cDNA clone HV_Ceb0017E22f (BF064709) and PCR primers HvC2d1 For 5`-GAG AAT TCA TGG GCT CGC GGT ACG AGG TGG AGG TGA C with an *Eco*RI site at the 5´end and a HvC2d1 Rev 5`-GAA GAT CTG TAG TCG TCG TCG CCG CCG TAG TCG C with *Bgl*II site at the 5´ end. The PCR product was digested with *Eco*RI and *Bgl*II enzymes and cloned into the *Eco*RI and *Bam*HI sites of smRSGFP-pKE4xtr-G construct to generate HvC2d1-smRSGFP

(smRSGFP = solubility modified red shifted-green fluorescent protein) under the control of the cauliflower mosaic virus 35S promoter. The HvC2d1-GFP and control smRSGFP constructs were used for transformation of onion epidermal cells using a self-made Vacuum-Helium-Particle-Gun as described by Barth *et al.* (2004). The epidermal layers isolated from onions were placed on MS basal medium containing 2 % (w/v) plant agar, 2.5 μgml^{-1} amphotericin B and 5 μgml^{-1} chloramphenicol (DUCHEFA, Biochemie bv). The transformed onions cells were treated either with or without 30 μM calcium ionophore and incubated in the dark at 28°C for 10-12 h. GFP fluorescence was determined by FITC-filtered visual inspection using a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.2.32 Overexpression of HvLysMR1-kinase domain

2.2.32.1 Ligation of HvLysMR1-KD into pET-15b vector

The kinase domain of HvLysMR1 was amplified by PCR from the original HvLysMR1 plasmid using the primers (forward) 5`- GAC ATA TGA GGC GAA GAA AGG CGA AAC AGG GTG  with an *Nde* I site and (reverse) 5`- GAC TCG AGT CAT CTC CCG GAC ATG AGG TTC ACC A with an *Xho* I site. The PCR product was first digested with *Nde* I / *Xho*I enzymes and then ligated into the *Nde* I / *Xho*I site of a pET-15b vector (Novagen, Darmastadt, Germany) to produce a recombinant His-HvLysMR1-KD protein.

5x PCR Buffer	10.0 μl
dNTP (2.5 mM each)	1.0 μl
Primer forward (5 μM)	6.0 μl
Primer reverse (5 μM)	6.0 μl
Plasmid Template (1:1000)	2.5 μl
Taq DNA polymerase	1.0 μl
Q-solution 5x	10.0 μl
Sterile H$_2$O	13.5 μl
	50 μl

PCR Program

Temperature	Time	Cycles	Step
94°C	3 min		Denaturation
94°C	1 min		Denaturation
57°C	1 min	30	Annealing
72°C	2 min		Elongation
72°C	5 min		Final Elongation

PCR product was run on 1.5 % (w/v) TAE agarose gel and then stained with ethidium bromide. The DNA bands were excised from agarose gel and purified using a "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany).

Digestion reaction

pET-15b vector (~2 μg)	13.5 μl
Nde I (10U/μl)	1.5 μl
Buffer (R+)	2.0 μl
Sterile H$_2$O	3.0 μl

Were incubated at 37°C for 2 h in a thermocycler, then the reaction was completed by addition of:

Buffer (R+)	1.0 μl
*Xho*I (10U/μl)	1.5 μl
Sterile H$_2$O	7.5 μl

The reaction was incubated at 37°C for 3 h and finally at 80°C for 20 min in a thermocycler. The digestion PCR product was run on 1.5 % (w/v) TAE agarose gel and then stained with ethidium bromide. The band corresponding to the size of the vector was excised from the gel and purified using a "QIAquick Gel Extraction Kit" from QIAGEN (Hilden, Germany).

Ligation reaction

pET-15b vector digested

(~500ng)   9.0 µl

Insert HvLysMR1-KD (~300 ng)   2.0 µl

Sterile H_2O 6.0 µl

The reaction was incubated at 45°C for 5 min, then chilled on ice for 1 min, and completed by adding:

T4 ligase Buffer   2.0 µl

T4 ligase (5U/µl)   1.0 µl

Finally, the reaction was incubated at 4°C overnight.

The recombinant plasmid was introduced into *E. coli* Rosetta (DE 3) pLys S (Novagen, Darmstadt, Germany). The overexpression of the protein was performed as described above (see 2.2.29) by addition of 0.2 mM isopropyl β-D-thiogalactosite (IPTG; DUCHEFA, Haarlem The Netherlands) to the medium. After the incubation at 37°C for 1 h, the bacterial cultures were harvested and resuspended in lysis buffer (pH. 8) and lysed by sonification.

2.2.32.2 Purification of His-HvLysMR1-KD

The recombinant protein His-HvLysMR1KD was purified in a Ni-NTA Superflow Column (1.5 ml) (QIAGEN, Hilden, Germany) using a pump (Minipuls 3, Abimed / Gilson$^®$, France). The Ni-NTA Superflow Column was washed with 20 ml Lysis buffer (300 mM NaCl, 50 mM Na_2 HPO4, 10 mM imidazole, pH 8) and then equilibrated with 3 x 5ml Lysis buffer (300 mM NaCl, 50 mM Na_2 HPO4, 20 mM imidazole, 2 % (v/v) Triton X-100, pH 8). The sample containing the soluble protein was applied to the column and the protein His-HvLysMR1-KD bound to the gel bed was washed with 2 x 10ml wash buffer. The recombinant protein His-HvLysMR1KD was eluted with 5 x 5ml elution buffer (50 mM Na_2 HPO4, 300 mM NaCl, 250 mM imidazole, pH 8.0).

2.2.32.3 Western blot

The overexpressed protein was separated by SDS-PAGE as described before (see 2.2.29.2) and transferred to a "WESTRAN$^®$ CLEAR SIGNAL PVDF membrane" 0.45 µm (Schleicher & Schuell, Dassel, Germany) by electroblotting. After electrophoresis, the gel was incubated

with 3 Wathman filter papers in cathode buffer. The PVDF membrane was then briefly incubated in methanol, 2 min in distillate water and finally in anode buffer for 5-10 min. At first the 3 Wathman filter papers were placed in contact with the cathode of the blotting apparatus (Biotec-Fischer, Germany), followed by the gel, the PVDF membrane and finally with 3 Wathman filter papers incubated before in anode buffer. Air bubbles were carefully removed from the space between gel and the membrane. The protein transfer was performed in the cold room (4°C) by 0.65 mA/cm^2 for 1.5 h . To prevent non-specific binding of the antibody, the membrane was incubated overnight in blocking solution (2 % milk powder in TBST buffer) at 4°C. The next day, the membrane was washed once for 15 min and twice for 5 min with 1x TBST buffer.The membrane was incubated with the first antibody (Anti-His Antibody; QIAGEN, Hilden, Germany) for 1 h at room temperature and then washed as described above. Finally, the membrane was incubated in HRP-second antibody (Anti-Rabbit; Amersham, Freiburg, Germany) for 45 min then washed once for 15 min and 4 times for 5 min with 1x TBST buffer. The detection was performed using the ECL Plus Western blotting detection reagents from Amersham Biosciences (UK) was used. For each square centimetre of membrane, 20µl of detection solution was used by mixing detection solutions A and B in a ratio of 40:1, then the membrane was incubated for 5 min at room temperature. At the end, the membrane was wrapped in Saran Wrap and exposed on a film (Hyperfilm™ ECL™, Amersham Biosciences, UK) for 1 to 15 min (depending on the signals). The film was developed first with developer solution (Kodak, GBX), then in a solution consisting of water and drops of acetic acid and finally fixed in fixer solution (Kodak, GBX).

2.2.33 In vitro phosphorylation assay

The His-tagged fusion protein HvLysMR1-KD was analyzed for its kinase activity. Phosphorylation was carried out in 25 µl volume of assay buffer (50 mM Tris-HCl, pH 7.6; 50 mM KCL, 2 mM DTT, 10 % (v/v) glycerol) each containing 0.5 µg His-tag fusion protein in the presence of 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The phosphorylation reaction was initiated by adding 12.5 µCi γ-[^{32}P]-ATP (Amersham Biosciences, Freiburg, Germany), then incubated at 22°C for 45 min. The reaction was stopped by addition of EDTA to a final concentration of 10 mM. The phosphorylated protein was analysed through 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane "WESTRAN$^®$ CLEAR SIGNAL PVDF membrane" 0.45 µm (Schleicher & Schuell, Dassel, Germany) as described above (see

2.2.32.3). The incorporated phosphate was visualized by exposition of the membrane to Imaging Plate for 2 days and analysed by Fluorescent Image Analyser (FLA-3000 Series, FUJIFILM; FUJI PHOTO FILM CO; Tokyo, Japan).

2.2.34 Peptide identification by nano LC-ESI-MS (MS^2 and neutral loss triggered MS^3)

20 µl eluate of the overexpressed His-HvLysMR1-KD which had been released from the Ni-NTA Superflow Column were digested with 20 ng trypsin (Promega, Madison, WI, USA) at 37ºC overnight following the protocol of Promega. The peptide solution was desalted with ZipTips according to Wagner *et al.* (2006) and vacuum-dried. The resulting pellet was dissolved in 5 µl of buffer A (see below) and the peptides were subjected to nano LC-ESI-MS analysis using a nanoscale C18 column (flow rate, 300 nl/min) coupled online to a linear-ion-trap mass spectrometer (Finnigan LTQ; Thermo Electron Corp., San Jose, CA). The LC system consisted of a Dionex UltiMate3000 liquid chromatography system (Dionex), including an autosampler, a flow control modul and a micropump. A gradient was used to elute peptides from the reversed-phase column (length, 15 cm; inner diameter, 75 µm; C18Pepmap100 particle size, 3 µm [Dionex P/N 160321]).

The successive steps of the applied gradient were as follows:

5 min, 96 % (v/v) *A*-4 % (v/v) *B*; 30 min, gradually shifting to 50 % (v/v) *A*–50 % (v/v) *B*; 5 min, gradually shifting to 10 % (v/v) *A*–90 % (v/v) *B*; 10 min, 10 % (v/v) *A*–90 % (v/v) *B*; and 1 min, gradually shifting to 96 % (v/v) *A*-4 % (v/v) *B*, where *A* consists of 5 % (v/v) acetonitrile 0.1 % (v/v) formic acid in water and *B* consists of 80 % (v/v) acetonitrile 0.1 % (v/v) formic acid in water.

The instrument was run in the data-dependent neutral-loss mode, cycling between one full MS scan and MS^2 scans of the four most-abundant ions. The detection of a neutral-loss fragment (196, 98, 49, or 32.66 Da) in the MS^2 scans triggered immediately an MS^3 scan of the precursor ion representing the dephosphorylated peptide. The analysis of the resulting spectra was done according to Wagner *et al.* (2006). The MS^2 and MS^3 data were used to search the database with the protein sequence of the overexpressed protein using Bioworks software (version 3.2; Thermo Electron Corp., San Jose, CA) including the SEQUEST algorithm (Link *et al.*, 1999). The software parameters were set to detect a modification of 79.96 Da in Ser, Thr, or Tyr in MS^2 and MS^3 spectra. When phosphoserine and phosphothreonine undergo gas-phase ß elimination, dehydroalanine (Dha) and 2-aminodehydrobuturic

acid(methyldehydroalanine[MeDha]), respectively, are produced. Thus, modifications of -18.00 Da in Ser and Thr residues were additionally used for database searches with MS^3 data. Searches were done for tryptic peptides, allowing two missed cleavages. Mass tolerance was set to 1.5 Da for the peptide precursor ion in MS mode. For fragment ions (MS^2 and MS^3 modes), mass tolerance was set to 1.0 Da. Scores for the Xcorr factor (Eng *et al*., 1994) were set to the following limits: Xcorr of >1.5 if the charge of the peptide was 1, Xcorr of >2 if the charge of the peptide was 2, and Xcorr of >2.5 if the charge of the peptide was 3.

3. Results

3.1 Set-up of the experimental system for heavy metal treatment of barley plants

In order to investigate responses of barley plants to heavy metals, seedlings were grown hydroponically on Murashige and Skoog medium for 7 days under controlled growth-chamber conditions (16 h at 21°C and a photosynthetic photon fluence rate (PPFR, 400-700 nm) of 100 μmol m^{-2} s^{-1}; 8 h at 16 °C in the dark), and then treated with potassium dichromate or not treated (controls). Additionally, responses to the non-essential toxic heavy metal cadmium and to the essential heavy metal copper were also investigated. In contrast to many other investigations, experiments in this work focus on the shoot, which is very important for biomass production, and not on the root system. Photosynthetic activities in the leaves specifically respond to heavy metal treatment. In preliminary tests based on the physiological stress parameters chlorophyll content and photosystem II efficiency (data not shown) an optimal concentration (1mM) for analyses of effects of heavy metals on the photosynthetic active leaves was determined. At this concentration first effects of the exposure to the heavy metals could be detected in the leaves within two days when leaves were still in their mature stage. This is important since in older leaves senescence processes start which interfere with those effects caused by the heavy metal treatment.

Uptake and translocation of chromium in the barley plants under the experimental conditions used in the following experiments, in the roots, the shoots and the leaves was analysed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) during a time period of 144 hours (Fig. 2).
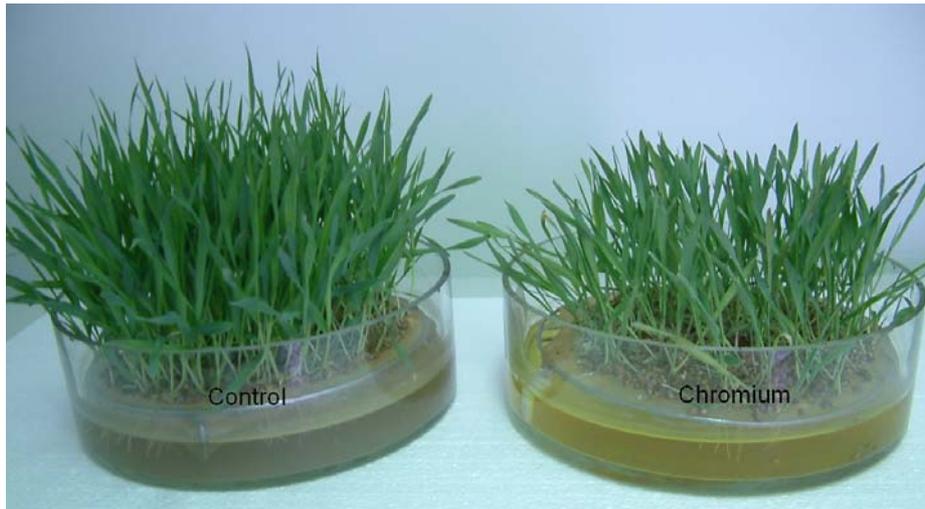
Fig. 1: Experimental system, 7 days old barley seedlings (*Hordeum vulgare* L. cv. Steffi) grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 µmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate (chromium) or not (control) for 96 hours.

3.2 Analyses of uptake and translocation of chromium by barley plants

Uptake and translocation of chromium in the barley plants under the experimental conditions used in the following experiments was analysed using inductively coupled plasma-atomic emission spectrometry (ICP-AES). This method allows measurement of total chromium content (Cr(III) and Cr(VI)) in the roots, the shoots and the leaves during a time period of 144 hours (Fig. 2). After exposure of 7 days old plants to 1mM potassium dichromate, chromium content in the roots already clearly increases within 24 hours. In contrast to the high accumulation of chromium in the roots, a much lower and delayed accumulation of chromium could be observed in the shoots and leaves. The uptake and translocation of chromium in barley seedlings increased during prolonged time of exposure (Fig. 2). After 24 h the concentration of total chromium in roots was 2486 mg per kg dry weight and only 25.21 mg per kg dry weight in the leaves. At the late stage of the treatment, the total chromium concentration in the roots and in the leaves increased up to 5068 mg per kg dry weight and 343.5 mg per kg dry weight, respectively (Fig. 2). This implies that the time dependent uptake and translocation of chromium to the upper parts of barley plants is retarded compared to the roots.
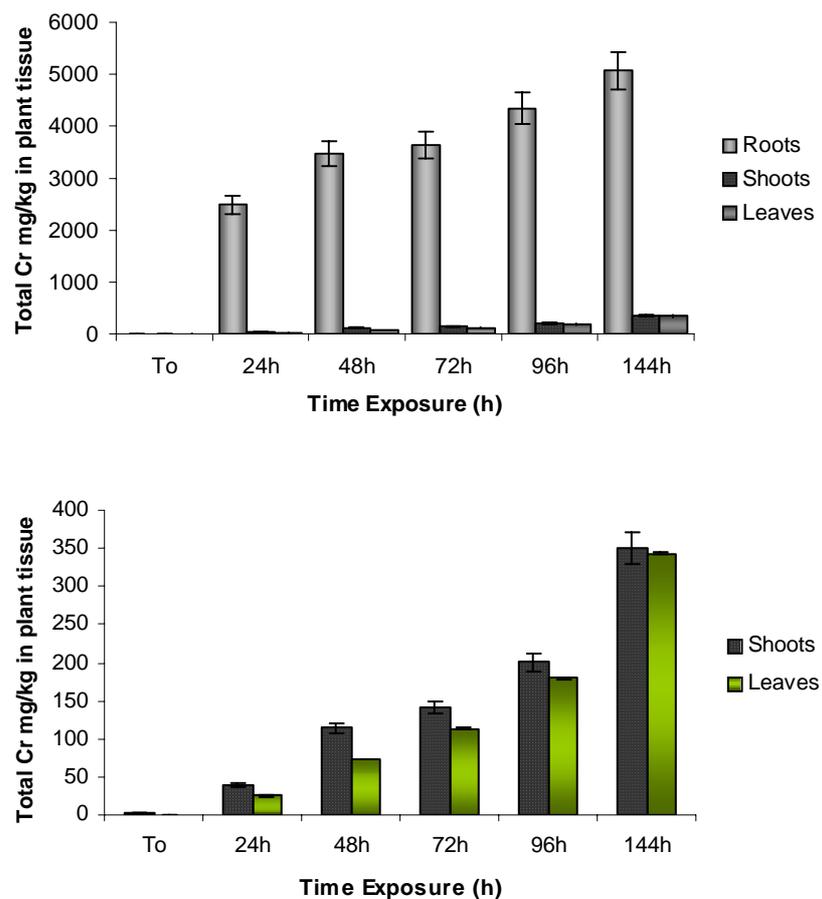
Fig. 2: Chromium concentration in roots, shoots and leaves of barley seedlings (*Hordeum vulgare* L. cv. Steffi). Seven days old barley seedlings were cultivated on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 µmol m^{-2} s^{-1}; 8 h, 16 °C and darkness) and then either treated with 1 mM potassium dichromate or not treated (controls) for different time-points. Each data point represents a mean of 3 independent measurements and bars indicate standard errors.

3.3 Physiological characterization of stress response of barley plants to the treatment with heavy metals chromium, cadmium and copper

In order to analyse the response of barley plants to chromium stress, 7 days old barley seedlings grown hydroponically as described in Materials and Methods were treated with 1mM of potassium dichromate for 24, 48, 96 and 144 hours. The stress response of the leaves was first analysed using the photosynthesis-related stress parameters chlorophyll content and photosystem II efficiency (Fig. 3). During the first 24 h to 48 h, the chlorophyll content showed a slight decrease after chromium exposure in comparison to the control, but a more significant decrease in chlorophyll was observed after extending the exposure time to

(a) Chlorophyll content
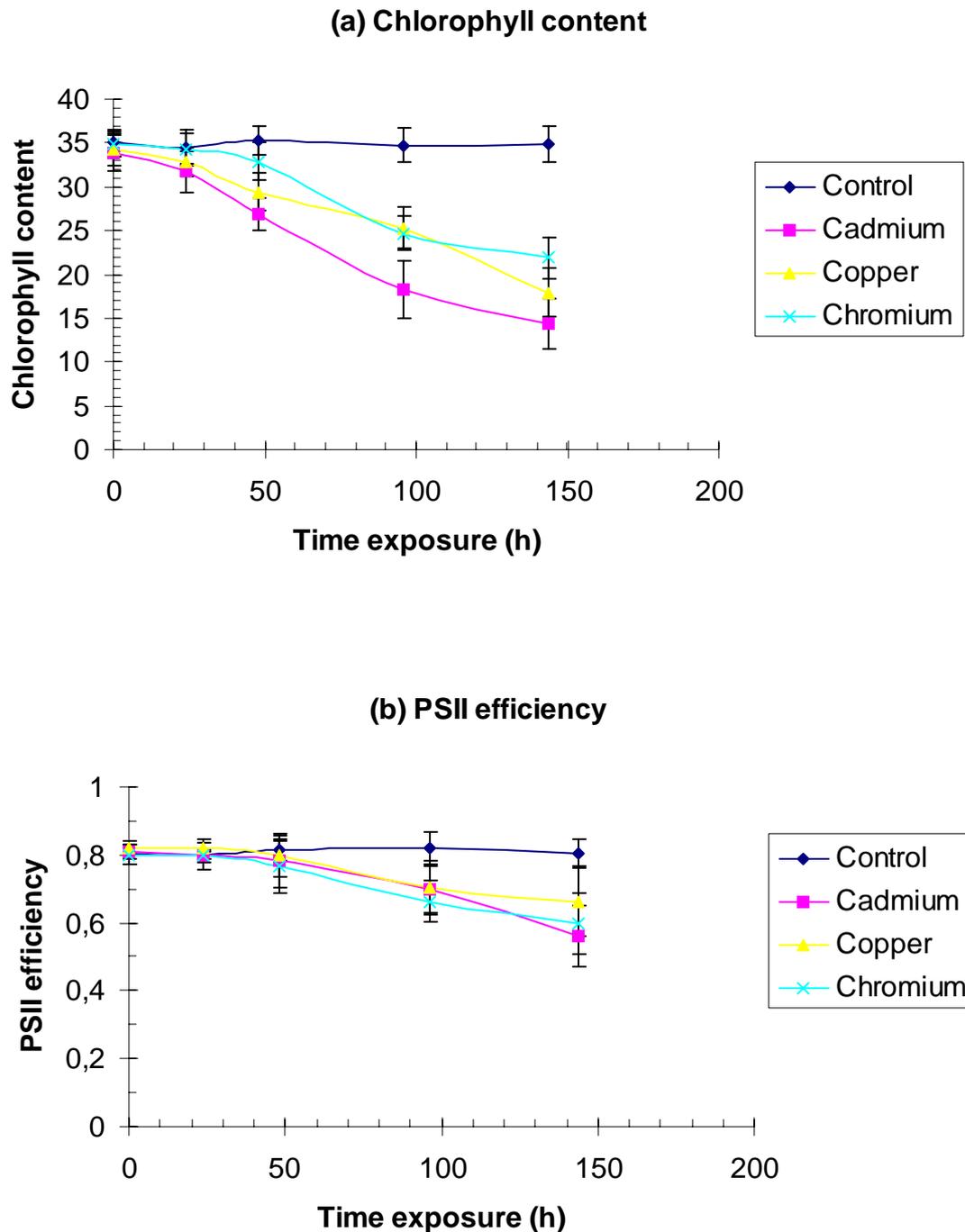
(b) PSII efficiency

Fig. 3: Effect of chromium, cadmium and copper stress on chlorophyll content (**a**) and PSII efficiency (**b**) in 7 days old barley seedlings cultivated on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate, cadmium chloride or copper chloride or not (control). Each data point represents a mean of 10 independent measurements and bars indicate standard errors.

chromium. After 144 hours, only about 65 % of chlorophyll content was left in plants treated with 1mM chromium compared to untreated controls (Fig. 3a). A second sensitive photosynthesis-related parameter investigated in this study is the PSII efficiency which was measured after dark adaptation as described by Humbeck *et al.* (1996). During the first hours of chromium treatment, almost no changes in the PSII efficiency occurred in plants in comparison to the controls (Fig. 3b). A slight decrease was observed after 48 h exposure. During prolonged time of exposure, PSII efficiency clearly decreased in comparison to the control plants. Similar effects on chlorophyll content and PSII efficiency could be observed when plants were treated with 1 mM cadmium or 1 mM copper (Fig. 3).

3.4 Analyses of changes in mRNA levels of heavy metal stress marker genes in primary barley leaves during chromium, cadmium and copper treatment

In order to characterise the response of plants to the chosen experimental conditions of chromium, cadmium and copper treatment on a molecular level and to determine the appropriate time intervals needed for the following transcriptome analysis of the chromium treated samples, expression levels of heavy metal stress marker genes *HvMT*-1a, *HvMT*-2a, *HvClpD*, *HvBsi* and *Cdi2* identified by Heise (2004) and Heise *et al.* (2007) were investigated. Total RNA was extracted from primary leaves of plants treated with 1mM potassium dichromate, cadmium chloride or copper chloride for 48, 96, and 144 h or not treated (controls).

3.4.1 Metallothioneins (MTs)

3.4.1.1 *HvMT-1a*

In order to determine whether *HvMT-1a* expression is induced during heavy metal stress conditions used in this experiment, 7 days old barley seedlings were treated with 1mM cadmium chloride, copper chloride or potassium dichromate for 48, 96 or 144 h or were not treated (controls). The changes in expression levels of *HvMT-1a* in the barley leaves during exposure to heavy metals in comparison to controls were investigated by northern analyses. Figure 4 shows that mRNA of *HvMT-1a* already accumulated to high levels after the first 48 h of cadmium, copper or chromium treatment when compared to the control. Thereafter, the mRNA level remained almost stable. This result indicates that under the conditions chosen already after 48 h of treatment the sensitive heavy metal stress marker gene *HvMT-1a* was clearly induced during the treatment with either cadmium, copper or chromium. In later stages (144h) there is also a slight increase in *HvMT-1a* mRNA level in the control which indicates the onset of senescence processes in these leaves.
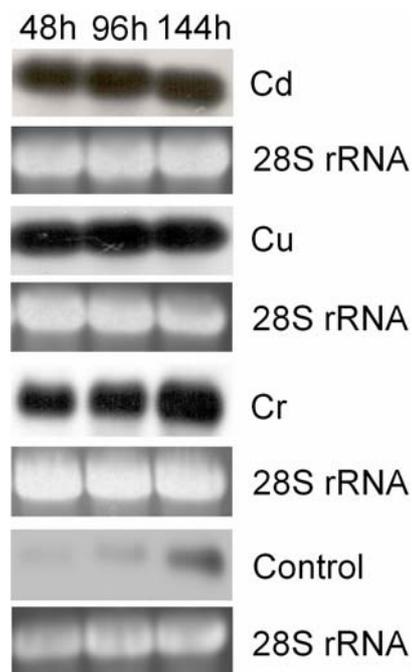
Fig. 4: Analyses of expression of *HvMT1a* gene during chromium, cadmium or copper treatment. RNA was extracted from 7 days old primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate, cadmium chloride or copper chloride or not (control). Each lane was loaded with 20 μg of total RNA. The 28S rRNA band of the ethidium bromide-stained gels are shown for loading controls.

3.4.1.2 *HvMT-2a*

Figure 5 shows that the metallothionein gene *HvMT-2a* was constitutively expressed in controls and during the chromium treatment. After 48 h of cadmium or copper treatment, mRNA levels of *HvMT-2a* were slightly reduced when compared to the control. In later stages of the treatment the *HvMT-2a* mRNA accumulated to the same high levels as in the control. This results indicates that different barley MTs exhibit quite different expression patterns.
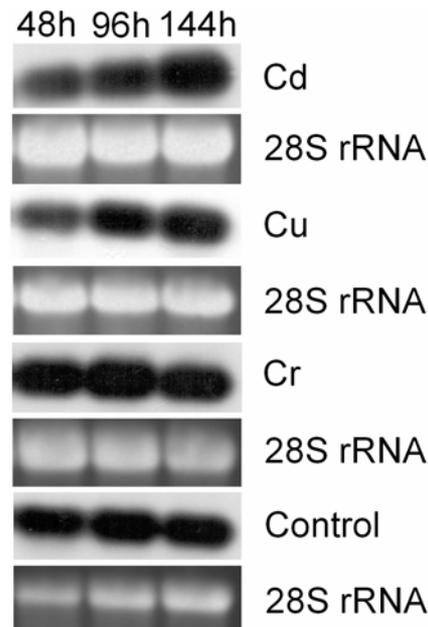
Fig. 5: Northern analyses show expression pattern of *HvMT-2a* gene in primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) during chromium, cadmium or copper treatment. RNA was extracted from 7 days old primary leaves grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate, cadmium chloride or copper chloride or not (control). Each lane was loaded with 20 μg of total RNA. The 28S rRNA band of the ethidium bromide-stained gels are shown for loading controls.

3.4.2 *HvClpD* protease

Another heavy metal stress marker gene tested in this study is *HvClpD* protease. Proteases are enzymes that require ATP for proteolytic processes (Porankiewicz *et al.,* 1999). To analyse the expression levels of *HvClpD* during heavy metal stress, 7 days old barley seedlings were treated with 1mM cadmium chloride, copper chloride or potassium dichromate for 48, 96 or 144 h or were not treated (controls). The changes in mRNA levels were investigated by northern blot analyses. The *HvClpD* mRNA had already started to accumulate during the first 48 h of chromium and cadmium treatment and after 96 h of copper exposure. During the

prolonged time of exposure, *HvClpD* mRNA level stayed almost stable during the three heavy metal treatments (Fig. 6).
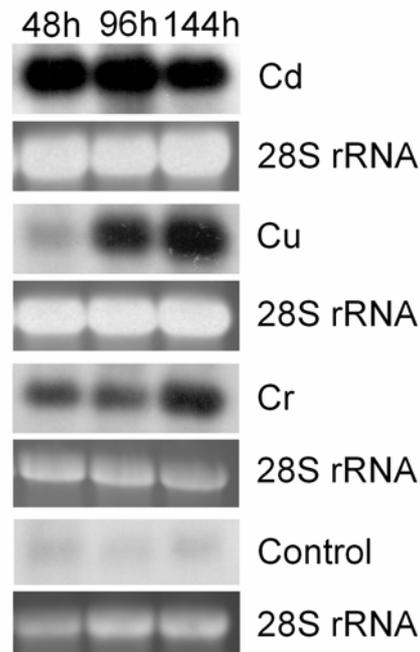
Fig. 6: Northern analyses of expression of *HvclpD* gene in primary barley leaves (*Hordeum vulgare* L. cv. Steffi) during chromium, cadmium or copper treatment. RNA was extracted from 7 days old primary leaves grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 µmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate, cadmium chloride or copper chloride or not (control). Each lane was loaded with 20 µg of total RNA. The 28S rRNA band of the ethidium bromide-stained gels are shown for loading controls.

3.4.3 *HvBsi*

Bsi is a small cysteine-rich protein containing 89 amino acids which encodes for Bowman-Birk-type proteinase inhibitors (BB-PIs) (Stevens *et al.*, 1996). Bsi presents homology to an aluminum-induced protein from wheat (Snowden & Gardener, 1993). In order to determine whether *HvBsi* expression is also induced in response to heavy metals conditions used in this study the changes in expression levels of *HvBsi* in the barley leaves exposed to 1 mM potassium dichromate, cadmium chloride or copper chloride were analysed by northern blot. Figure 7 shows that the *Bsi* transcript accumulated during the first 48 h in samples treated with chromium, cadmium or copper in comparison with control. After this time-point, the mRNA levels remained nearly stable during prolonged time of exposure to the heavy metals.
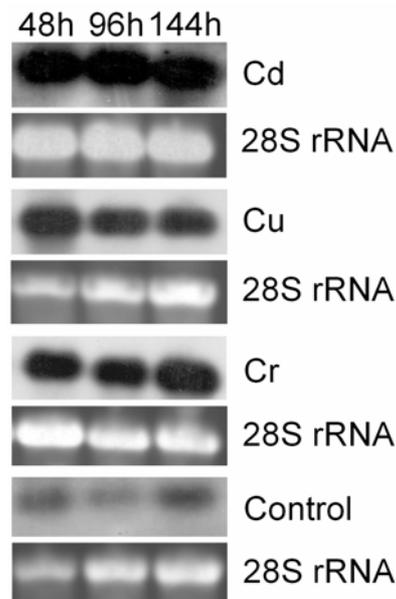
Fig. 7: Northern analyses show differential expression patterns of *HvBsi* gene in primary barley leaves (*Hordeum vulgare* L. cv. Steffi) during chromium, cadmium or copper treatment. RNA was extracted from 7 days old primary leaves grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate, cadmium chloride or copper chloride or not (control). Each lane was loaded with 20 μg of total RNA. The 28S rRNA band of the ethidium bromide-stained gels are shown for loading controls.

3.4.4 *Cdi2*

The clone Cdi2 (Aj508231) (<u>ca</u>dmium <u>i</u>nduced <u>2</u>) identified in barley showed a 83 % homology to an EST from maize with unknown function (Heise, 2004; Heise *et al.,* 2007). Northern analysis using this clone as a hybridization probe indicated that *cdi2* mRNA is constitutively expressed in the control samples, but accumulated in the leaves of plants exposed to chromium, copper and cadmium (Fig. 8).
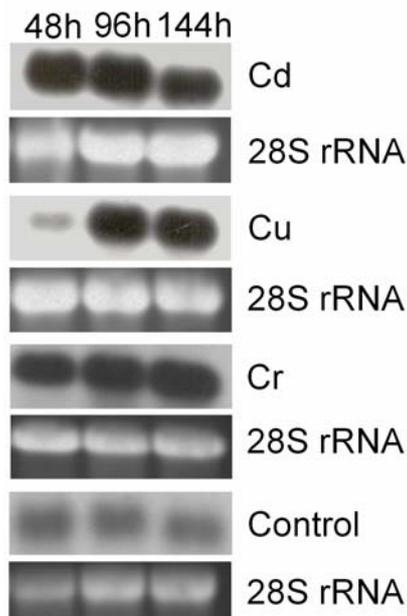
Fig. 8: Northern analyses of expression of *Cdi2* gene in primary barley leaves (*Hordeum vulgare* L. cv. Steffi) during chromium, cadmium or copper treatment. RNA was extracted from 7 days old primary leaves grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate, cadmium chloride or copper chloride or not (control). Each lane was loaded with 20 μg of total RNA. The 28S rRNA band of the ethidium bromide-stained gels are shown for loading controls.

3.5 Identification of chromium induced genes from barley leaves by Restriction Fragment Differential Display (RFDD-PCR) method

3.5.1 Continuative physiological characterization of stress response of barley leaves during early phase of chromium treatment

To identify genes that respond to chromium in barley leaves, a Restriction Fragment Differential Display (RFDD-PCR) PCR analysis was performed. Aiming at genes coding for putative regulatory factors in the heavy metal stress response in the leaves, especially those genes which are rapidly induced after the onset of the stress were of interest. Therefore in addition to the previous experiments shown in chapter 3.1, especially fast reactions of the plants to chromium stress were investigated. Hydroponically grown seedlings were treated with 1mM potassium dichromate ($K_2Cr_2O_7$) and the stress response of primary leaves in comparison to the control was analysed by the two photosynthesis-related parameters chlorophyll content and photosystem II efficiency (Fig. 9). Chlorophyll content during the

(a)

(b)
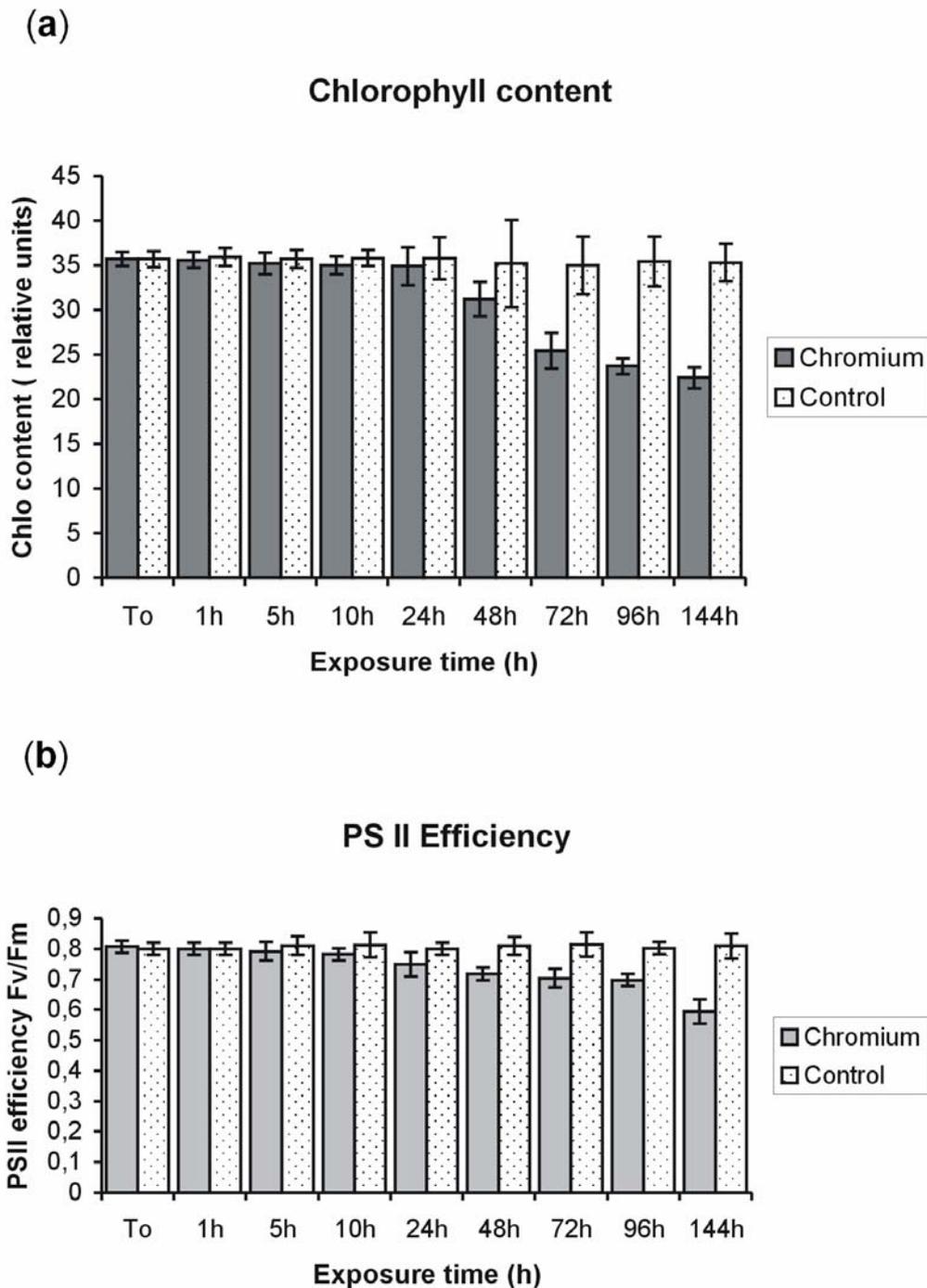
Fig. 9: Effect of chromium stress on chlorophyll content (**a**) and PSII efficiency (**b**) in 7 days old barley seedlings (*Hordeum vulgare* L. cv. Steffi) cultivated on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate (chromium) or not treated (control). Each data point represents a mean of 10 independent measurements and bars indicate standard errors.

first 24 hours of chromium stress remained almost constant. After 24 h, the chlorophyll content started to decrease. Over a long period of exposure to potassium dichromate (144 hours) the chlorophyll content was clearly reduced to *c.* 60 % of that in controls indicating stress-dependent chlorophyll degradation in the later stages of the treatment. Another sensitive parameter reflecting stress induced damage to photosynthetic activities is the PS II efficiency measured after dark adaptation with a chlorophyll fluorometer (Humbeck *et al.*, 1996). Only after 24 hours of chromium treatment, PSII efficiency started to decrease. The changes in photosynthetic parameters indicated that, under the stress conditions used in this experiment, leaves showed no response during the first 24 h, and there were first slight responses in both parameters only 48 h after the onset of chromium stress. In the later stages of chromium stress, leaves were severely damaged.

3.5.2 Isolation of cDNAs representing genes induced during chromium treatment

In order to identify genes that are either up-regulated before photosynthetic functions are severely reduced, or induced at the time of the very first effects of chromium stress on chlorophyll content and PSII efficiency, mRNAs from 1 h, 5 h and 48 h stressed leaves and of corresponding controls were extracted, cDNAs prepared, fragmented by the use of the restriction enzyme *TaqI* and after ligation of adapters amplified by PCR (restriction fragment differential display PCR, Barth *et al.,* 2004). The cDNA-fragment populations were separated on 8 % (w/v) polyacrylamide gels and compared in the differential display (Fig. 10). This method yielded 48 cDNA fragments, presumably representing genes differentially expressed during the first phase of chromium treatment.
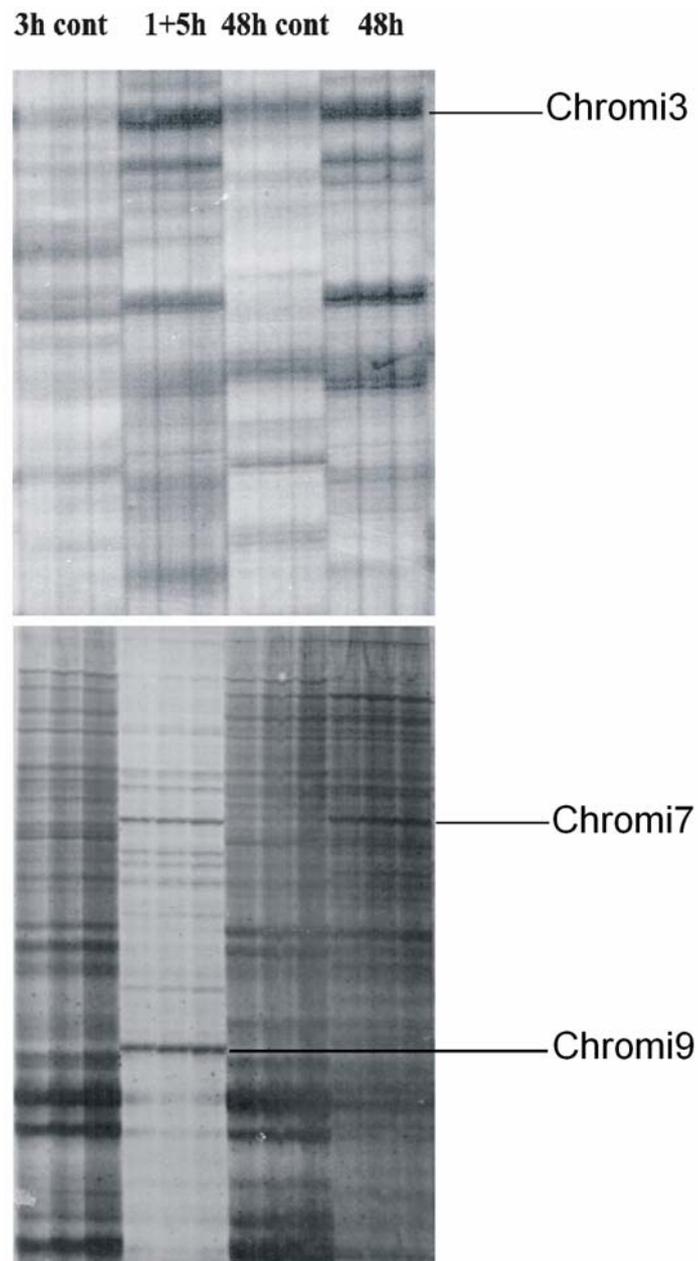
Fig. 10: RFDD-PCR polyacrylamide gels of cDNAs from barley leaves showing differential expression after chromium treatment. The first column represents 3 h control sample followed by sample 1+5 h combined mRNA populations of 1 and 5 h-treated plants. Column three represents 48 h not treated sample (control) followed by treated 48 h sample in column four.

3.5.3 Sequence analyses of novel cDNA fragments isolated by RFDD-PCR method

The isolated cDNA fragments were re-amplified by PCR, cloned and then sequenced as described in Materials and Methods. Finally, 14 clones were proved by northern analyses and quantitative real-time PCR to be up-regulated upon chromium treatment. These novel chromium-induced genes were referred as " chromi " (<u>chrom</u>ium <u>i</u>nduced gene) (Table 1). The sequenced clones were analysed by comparison with sequence protein and EST database from NCBI (National Center for Biotechnology Information) using the search programm blastn and blastx (Altschul *et al.,* 1990; 1997), the software HarvEST Triticeae version 0.99 from the University of California (Los Angeles, CA, USA) and analysed with Lasergene expert sequence analysis software (DNA STAR Inc., Madison, WI, USA).

Table 1: Comparison of identified cDNA fragment sequences with other EST and protein sequences in NCBI databases using blastx and blastn.

Clone name	Size	RFDD-PCR 1+5 h	48 h	Homology	Submitted clone	Homology with ESTs and proteins present in NCBI database
Chromi1	183bp		+	Receptor-like kinase protein	AJ630116	154/183bp homology to HarvEST consensus with *Hordeum vulgare* (CA024475) / RLK *Oryza sativa* (BAD01244)
Chromi2	287bp		+	Wheat aluminum induced protein (wali6)	/	76/84 aa homology to *Triticum eastivum* L. (AAC37417)
Chromi3	111bp	+	+	2-nitropropane dioxygenase protein	AJ630117	111/111bp homology to HarvEST consensus with *Hordeum vulgare* (BF622516) / 2-Nitropropane dioxygenase protein *Arabidopsis thaliana* (NP_568988)
Chromi4	112bp	+	+	Putative early nodulin 8 precursor protein	AJ630118	26/44 aa homology to *Oryza sativa* (BAC80099)
Chromi5	164bp		+	Ferritin protein	/	53/54 aa homology to *Triticum eastivum* (AAW68440)
Chromi6	179bp		+	Thaumatin-like protein TLP5	/	43/48 aa homology to *Hordeum vulgare* (AAW21725)

Chromi7	502bp	+	+	Outer membrane protein	AJ630119	167/167 aa identity to *Escherichia coli* (ZP00734670)
Chromi8	160bp		+	Putative heat shock protein, hsp40	/	49/53 aa homology to *Oryza sativa* (AAU10651)
Chromi9	189bp	+		ORF	/	59/62 aa homology to *Hordeum vulgare* (AAB42154)
Chromi10	261bp		+	Pathogenesis related protein	/	51/51 aa homology to *Hordeum vulgare* (CAA41446)
Chromi11	307bp		+	Glutathione S-transferase	/	100/102 aa homology to *Hordeum vulgare* (AAL73394)
Chromi12	81bp		+	Putative C2 domain-containing protein	AJ630120	81/81bp homology to HarvEST cons with *Hordeum vulgare* (BF064709) / Putative C2 protein *Oryza sativa* (BAD09616)
Chromi13	272bp	+	+	Putative chorismate mutase / prephenate dehydrogenase	AJ630121	30/43 aa homology to *Arabidopsis thaliana* (AAC73018)
Chromi14	256bp		+	unknown protein	AJ630122	97/125 aa homology to *Oryza sativa* (XP_479269)

The database search assigned putative functions to 13 genes and 1 gene with unknown function (Tab. 1). These novel chromium-induced genes could be classified into the following groups (i) genes possibly involved in signalling pathways like a receptor-like protein kinase and a C2 domain protein, pathogen responses and amino acid synthesis, and (ii) genes already known to be involved in heavy metal response, such as that encoding the wheat aluminum-induced protein (wali6) (Richards *et al.*, 1994).

3.5.4 Expression analyses of the newly identified RFDD-PCR genes during chromium treatment

To verify chromium stress-dependent expressions of the genes isolated by the differential screening to chromium response of barley seedlings, 7 days old barley seedlings were treated with 1 mM potassium dichromate for different time intervals or were not treated (controls).

The changes in mRNA levels during the early phase and prolonged time exposure to chromium were investigated via northern analyses (Fig. 11a). For those genes which present low transcript levels and therefore could not be detected by northern analyses, the changes in mRNA levels were analysed by quantitative real-time PCR by comparison of mRNA of the target gene to the reference 18S ribosomal RNA from treated samples and controls at different time points (Fig. 11b).

Northern analyses revealed that the RFDD-PCR isolated genes could be grouped concerning their induction profile. (i) transcripts of genes which start to accumulate during the first 48h chromium exposure and show maximal induction during prolonged time of exposure like chromi 4, 6 and 13), (ii) genes that start to accumulate only after 96 h chromium treatment (chromi 2, 3 and 11), and (iii) genes which present late transcript accumulation during chromium treatment like chromi 9. To summarize these results, for all genes identified by the RFDD-PCR approach as putative chromium up-regulated genes the induction in response to chromium could be proven by either northern- or quantitative real-time PCR- technique.

Based on the expression studies and the database analyses two candidates which represent putative regulatory factors involved in heavy metal stress responses were picked out for further detailed analyses. This were the clones showing homology to a C2 domain protein (chromi 12) and to a putative receptor-like kinase (chromi 1). The results are presented in chapters 3.6 (C2 domain protein) and 3.7 (receptor-like kinase).
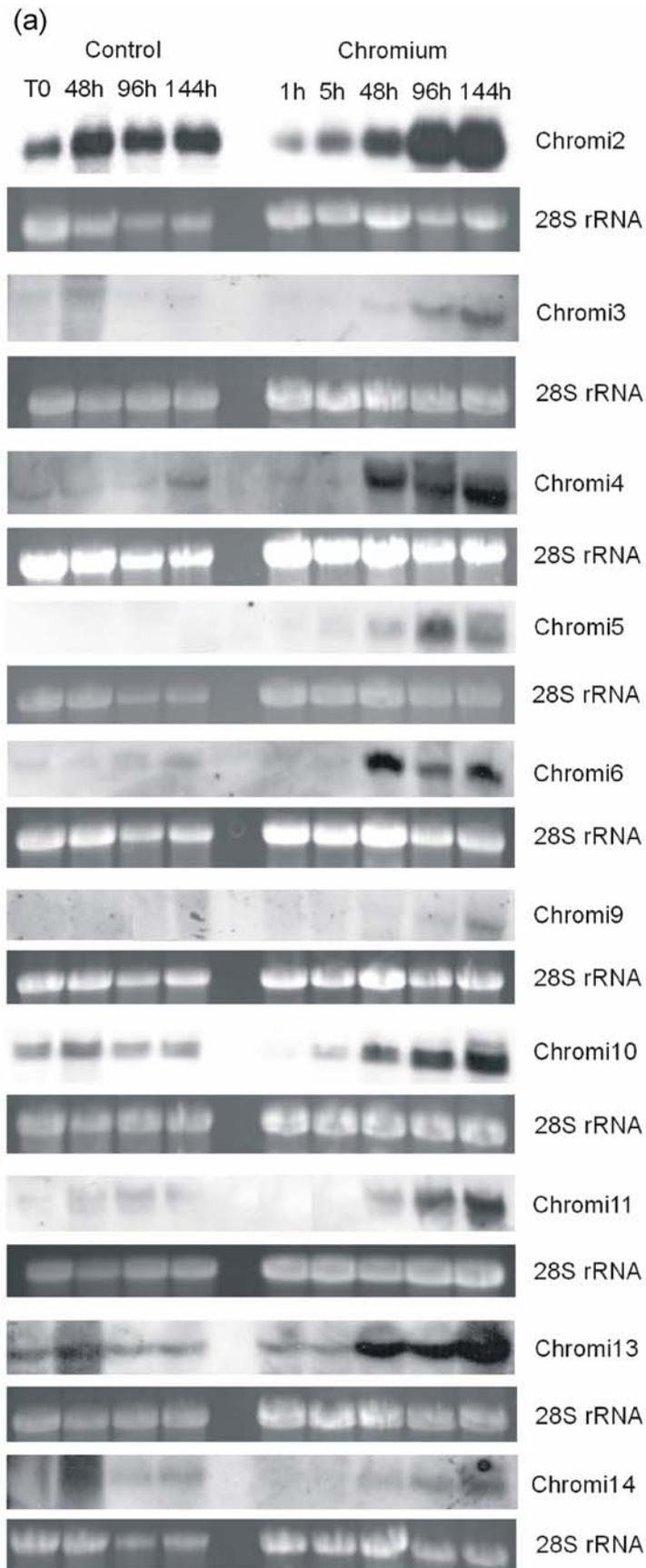
Fig. 11: (a) Northern analyses of expression of novel RFDD-PCR isolated genes in primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) during chromium treatment. RNA was extracted from 7 days old primary leaves grown on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h, 21°C and 100 μmol m^{-2} s^{-1} ; 8 h, 16 °C and darkness), then either treated with 1mM of potassium dichromate or not treated (control) for different time-points. Each lane was loaded with 20 μg of total RNA. The 28S rRNA band of the ethidium bromide-stained gels are shown for loading controls. (b) Quantitative real-time PCR analysis of Chromi 1, Chromi 7, Chromi 8, and Chromi 12 mRNA levels in primary leaves of barley (*Hordeum vulgare* L.). RNA was extracted from primary leaves of barley seedlings either treated with 1mM potassium dichromate or not treated (control). The levels of Chromi1, Chromi 7, Chromi 8 and Chromi 12 mRNA in each case are normalized to that of 18S rRNA as reference gene, and controls at each time point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).
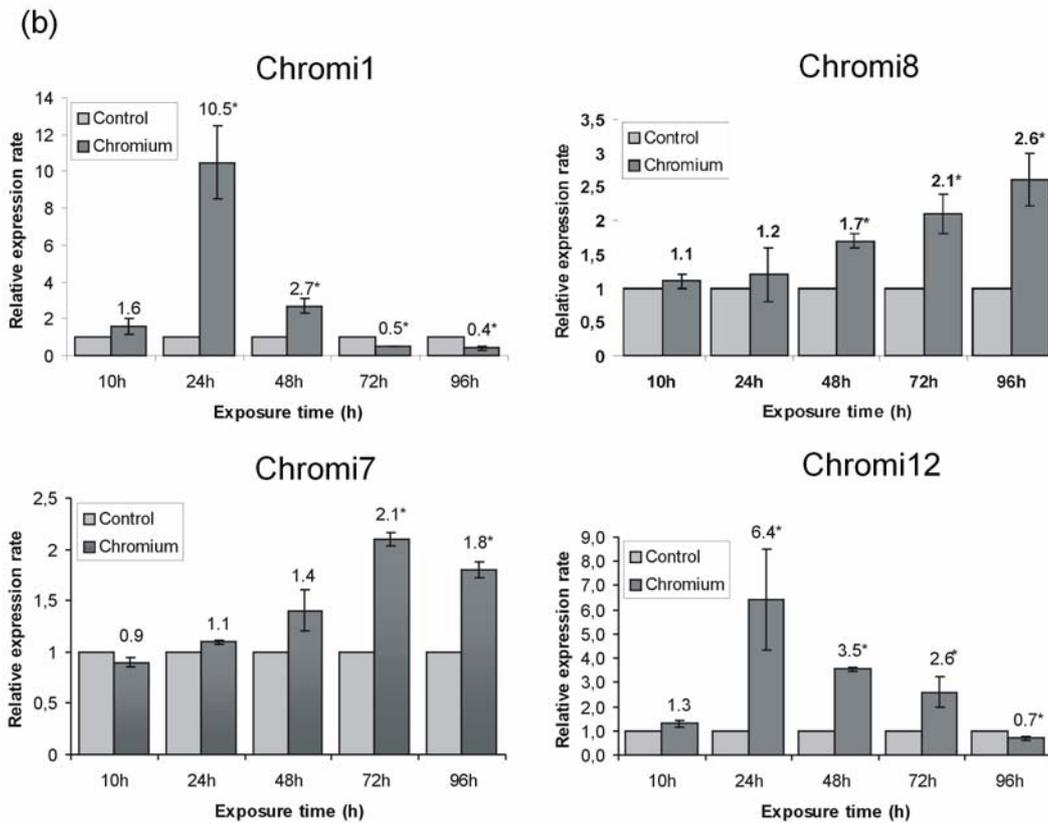
3.6 *HvC2d1*

3.6.1 Isolation of a full length cDNA encoding a putative C2 domain-like protein

Chromi 12 (AJ630120, 81bp) as a putative regulatory factor was investigated in more detail in this study. Using the software HarvEST Triticeae version 0.99 from University of California (Los Angeles, CA, USA), 100 % identity to a part of the barley cDNA clone HV_Ceb0017E22f (BF064709) was demonstrated. This clone was then sequenced. The full length sequence was 1274 bp, including an ORF of 978 bp (nucleotides 84 - 1062; CAI58613) coding for 326 amino acids. By analysis of the deduced amino acid sequence, a C2 domain-like motif comprising amino acids 7 - 95 was identified showing homology to the known C2-domain sequence (pfam00168; National Center for Biotechnology Information (NCBI) Conserved Domain Search, shown in Fig. 12a). The gene was therefore named `C2 domain 1´ (*HvC2d1*).

C2-domains are known to interact with membranes in a Ca^{2+}-dependent manner and are found in various types of protein, for example protein kinases, phospholipid-modifying enzymes such as phospholipase D, and so-called small C2-domain proteins with a single C2-domain (Kim *et al.*, 2003). On the basis of its sequence, which shows only a single C2 domain-like motif and no other conserved protein domains, the novel barley C2-domain protein HvC2d1 investigated in this study is similar to the small C2-domain protein class, which has only been found in a limited number of plants, such as *Arabidopsis AtC2-1* (AAG52148), *maize* (ZmC2-1, U64437), *pumpkin* (Cmpp 16-1 and 16-2, AF079170 and AF079171 respectively) and rice (*Oryza sativa*) (OsERG1a and OsERG1b; U95135 and U95136, respectively) (Kim *et al.*, 2003). Alignment of the sequences of these proteins with that of the novel barley C2 domain-like protein revealed clear homology in the C2-domain. In addition to the C2-domain (at the N-terminus from Val7 to Arg95), at the C-terminal end a nuclear localization signal (NLS, a K-rich region from amino acid Lys264 to Lys267) was identified (Fig. 12c).

The novel *HvC2d1* has a much higher molecular weight than the known plant small C2-domain proteins [HvC2d1(32 kDa), CmPP 16-1(16.5 kDa), CmPP 16-2 (15.6 KDa), OsERG1a (17.7 kDa), OsERG1b (17.4 kDa) and AtC2-1 (16.2kDa)], and shows the highest homology to genes with unknown functions that have only recently been added to the NCBI databases (OsBAD09616, AtC2-2, OsBAB84404 and At1g07310). As shown in the phylogenetic tree in Fig. 12b, these genes cluster in their own novel C2 domain-like group, separately from the known small C2-domain proteins. Alignment of this novel class of C2 domain-like proteins shows high homology in conserved protein sequence areas, as presented in Fig. 13. Interestingly, the deduced amino acid sequence of another protein in this novel C2 domain-like class also exhibits a NLS-like motif (Fig. 13).

(a)
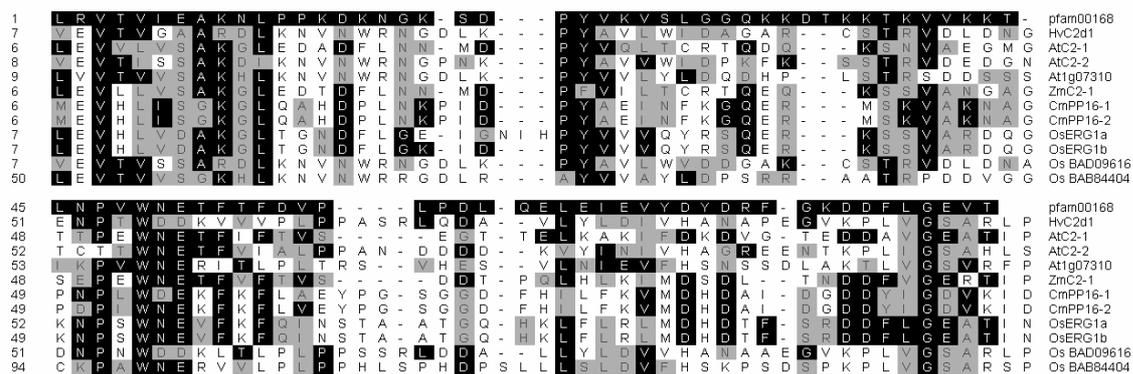
(b)
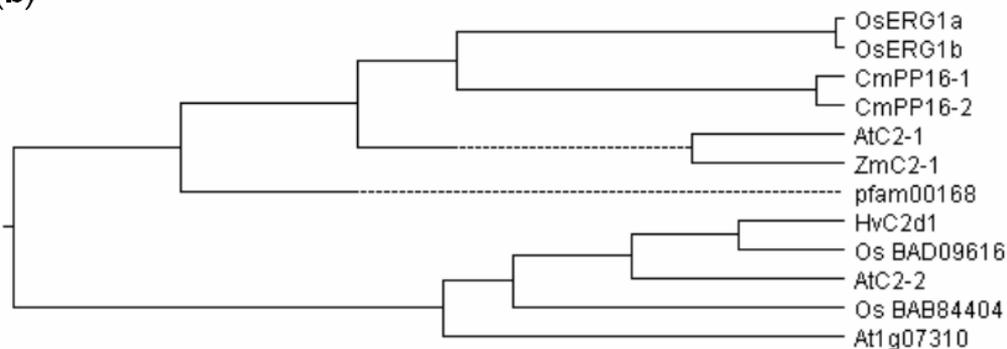
(c)

Fig. 12: (**a**) Alignment of the deduced amino acid sequence of the barley (*Hordeum vulgare* L.) protein `C2 domain 1` (*HvC2d1*; CAI58613) with the consensus C2-domain (pfam00168) and amino acid sequences from known plant small C2-domain proteins of *Arabidopsis* (AtC2-1; AAG52148), maize (*Zea mays*) (ZmC2-1; U64437), pumpkin (*Cucurbita maxima*) (CmPP16-1 and CmPP16-2; AF079170 and AF079171, respectively) and rice (*Oryza sativa*) (OsERG1a and OsERG1b; U95135 and U95136, respectively), and other plant C2-domain proteins of *Arabidopsis* [AtC2-2 (AAV85706) and At1g07310] and rice (Os BAD09616 and Os BAB84404). The Black background indicates amino acid residues that are identical and grey shading indicates amino acids that are similar to the C2-domain (pfam00168*)*. Alignment was performed using ClustalW in the Lasergene expert sequence analysis software (DNASTAR Inc., Madison, WI, USA). (**b**) Phylogenetic tree generated from alignment of HvC2d1 and C2-domain proteins shown in (**a**). (**c**) Schematic drawing of the arrangement of the C2-domain and the nuclear localization signal (NLS) motif in the HvC2d1 protein.
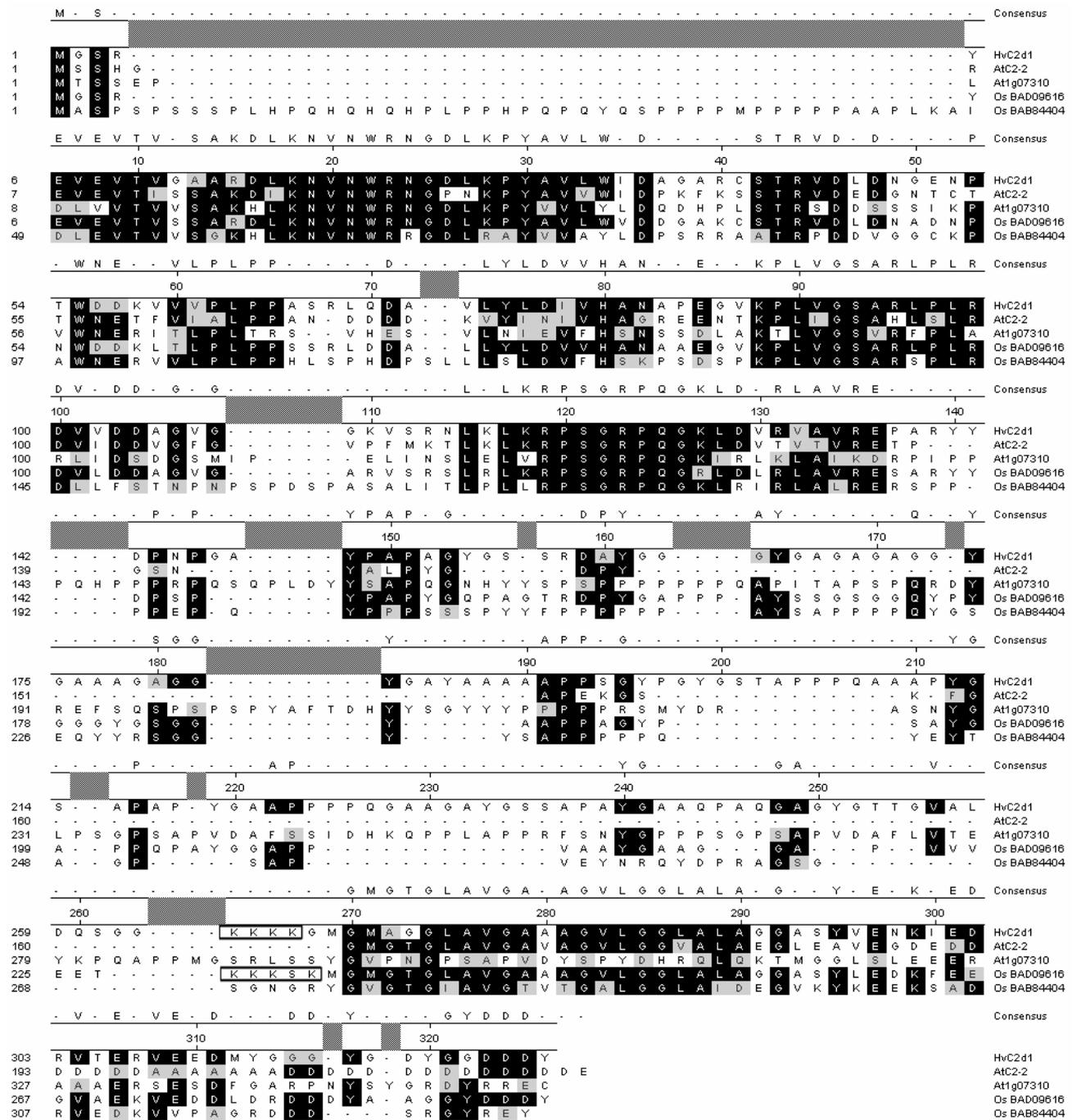
Fig. 13: Alignment of the amino acid sequence of the barley (*Hordeum vulgare* L.) protein `C2 domain 1` (HvC2d1; CAI58613) with that of the other proteins from the separate cluster in the phylogenic tree shown in Fig. 11b: *Arabidopsis* AtC2-2 (AAV85706) and (At1g07310) and rice (*Oryza sativa*) (Os BAD09616 and Os BAB84404). Amino acid residues that are identical in at least three of the five sequences aligned are shown against a black background and amino acid residues that are similar are shaded in grey. The boxes indicate the nuclear localization signal (NLS) or NLS-like positions of HvC2d1 and Os BAD09616. Alignment was performed using ClustalW in the Lasergene expert sequence analysis software (DNASTAR Inc., Madison, WI, USA).

3.6.2 Transient expression pattern of *HvC2d1* during chromium treatment

In order to analyse the heavy metal stress-dependent expression of *HvC2d1*, 7 days old barley seedlings were treated with 1mM potassium dichromate or were not treated (controls). The changes in mRNA levels were investigated via quantitative real-time PCR by comparison of mRNA levels of the *HvC2d1* gene to levels of the reference 18S ribosomal RNA from treated samples and controls at different time-points. In Fig. 14 this expression rate for *HvC2d1* is referred to that of the untreated control, which at each time-point is set as 1. The *HvC2d1* mRNA had already started to accumulate after 10 hours and reached a maximum transcript level 24 hours after onset of stress (6.4 times more than in the control, Fig. 14). In the later stages of the treatment, mRNA levels decreased again, reaching a basal transcript level after *c.* 96 hours. These data show a transient expression pattern of *HvC2d1* during the initial phase of chromium stress.
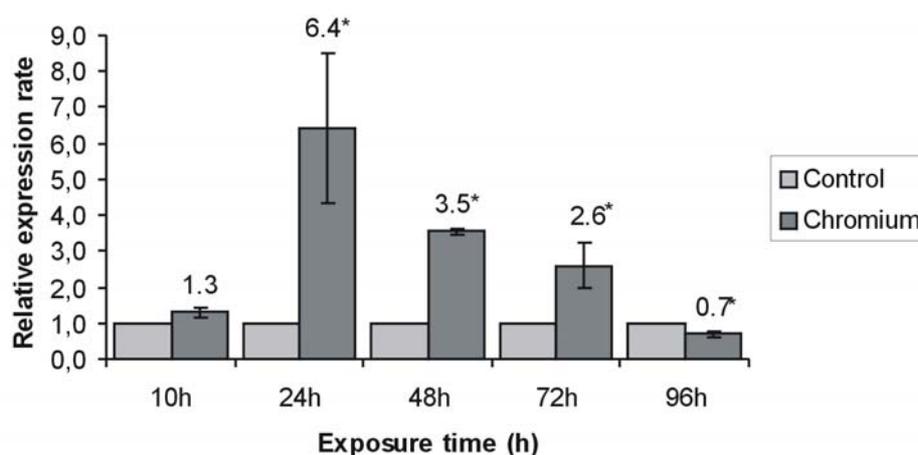
Fig. 14: Relative expression rate of the gene *HvC2d1* in primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings treated with 1mM potassium dichromate or not (control). The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and the control at each time-point is set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

3.6.3 Expression of *HvC2d1* is also induced by treatment with other heavy metals

In order to determine whether *HvC2d1* expression is also induced in response to heavy metals other than chromium, 7 days old barley seedlings were either treated with 1mM cadmium chloride or copper chloride for 48, 72 and 96 hours or were not treated (controls). In addition, the photosynthesis-related stress parameters, chlorophyll content and PSII efficiency were measured in both experiments (Figs. 15b and 15c). In cadmium-treated seedlings, chlorophyll

(a)

(b)

		0h	24h	48h	72h	96h
Control	Chlorophyll content	33.27 ± 3.43	33.69 ± 1.83	34.02 ± 2.87	33.25 ± 2.61	32.82 ± 3.56
	PSII efficiency	0.805 ± 0.06	0.805 ± 0.06	0.803 ± 0.07	0.805 ± 0.07	0.803 ± 0.10
Cadmium	Chlorophyll content	33.27 ± 3.43	29.75 ± 2.54	25.79 ± 4.76	18.75 ± 3.23	14.86 ± 3.72
	PSII efficiency	0.805 ± 0.06	0.804 ± 0.05	0.799 ± 0.13	0.774 ± 0.17	0.676 ± 0.30

(c)

		0h	24h	48h	72h	96h
Control	Chlorophyll content	34.09 ± 2.8	34.55 ± 2.34	34.02 ± 2.87	33.25 ± 2.61	32.82 ± 3.56
	PSII efficiency	0.806 ± 0.04	0.803 ± 0.05	0.806 ± 0.06	0.807 ± 0.07	0.801± 0.09
Copper	Chlorophyll content	34.09 ± 2.8	31.85 ± 2.42	29.27 ± 2.96	24.66 ± 3.19	18.58 ± 3.59
	PSII efficiency	0.806 ± 0.04	0.807 ± 0.07	0.801 ± 0.13	0.769 ± 0.22	0.701 ± 0.20

Fig. 15: (**a**) Effects of cadmium and copper treatment on mRNA levels of the barley (*Hordeum vulgare* L.) gene *HvC2d1* in primary leaves. RNA was extracted from primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings either stressed with 1mM cadmium chloride or with 1 mM copper chloride for 48 to 96 hours or not stressed (controls). The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls at each time-point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002). (**b**) Effects of cadmium treatment on chlorophyll content and photosystem II (PSII) efficiency during treatment of barley seedlings (*Hordeum Vulgare* L. cv Steffi) with 1mM copper chloride for 24 to 96 hours or not treated (control). Each data-point represents the mean of 10 independent measurements and bars indicate standard errors. (**c**) Effects of copper treatment on chlorophyll content and photosystem II (PSII) efficiency during treatment of barley seedlings (*Hordeum Vulgare* L. cv Steffi) with 1mM copper chloride for 24 to 96 hours or not treated (control). Each data-point represents the mean of 10 independent measurements and bars indicate standard errors.

content starts to decrease at 24 h after onset of cadmium treatment and accentuated during prolonged time of exposure. PSII efficiency decreased at 48 h of cadmium treatment (Fig. 15b). In seedlings treated with copper (Fig. 15c), patterns of changes in chlorophyll content and PSII efficiency similar to those during cadmium treatment were observed. The changes in expression levels of *HvC2d1* in the barley leaves exposed to cadmium chloride or copper chloride measured by quantitative real-time PCR (compared with the controls) are shown in Fig. 15. The mRNA levels of *HvC2d1* exhibited similar changes during cadmium or copper treatment. In both treatments, *HvC2d1* induction was significant after 48 hours. At this time, the *HvC2d1* transcript level was 2.8 times higher in the copper-treated seedlings and 15.2 times higher in the cadmium-treated seedlings compared to the untreated control (Fig. 15). Similar to the chromium treatment, in the later stages the relative mRNA levels of *HvC2d1* declined again, also showing a transient expression pattern after exposure to essential (copper) and non-essential (cadmium) heavy metals.

3.6.4 *HvC2d1* is also induced during leaf senescence but not by drought stress

It is known that some heavy metal-induced genes, such as methallothionein 1 (*MT1*) and blue copper-binding protein (*BCB*) are also up-regulated during leaf senescence (Himelblau & Amasino, 2000). In order to determine whether *HvC2d1* is up-regulated during leaf senesece, barley plants were grown 9, 26 and 38 days in 16 hours light (21°C with a PPFR [400-700 nm] of 100 μmol m^{-2} s^{-1}) and 8 hours darkness (16°C) on soil as described in Materials and Methods. As characterized by measurements of chlorophyll content and PSII efficiency (data not shown, Miersch *et al.,* 2000), primary leaves of 9 days old seedlings were in the mature developmental stage and those of 26- and 38-days old seedlings were in the early and late senescence stages, respectively, with decreased chlorophyll content and photosynthetic activities. The data show that the mRNA level of *HvC2d1* was significantly increased during senescence (Fig. 16a).

I further examined the effect of drought stress on transcript levels of *HvC2d1*, as the *Vr-PLC3* (phospholipid C) gene in *Vigna radiata* L. containing a C2-domain is induced under drought stress (Kim *et al.*, 2004). Barley plants were grown hydroponically on Muraschige and Skoog medium for 7 days and then removed from the medium to induce drought stress. The impact of drought stress on barley plants was characterized by measuring the decreasing water content in the leaves at different times (data not shown). The data indicated that *HvC2d1* transcripts do not accumulate but rather decrease during the drought stress (Fig. 16b).
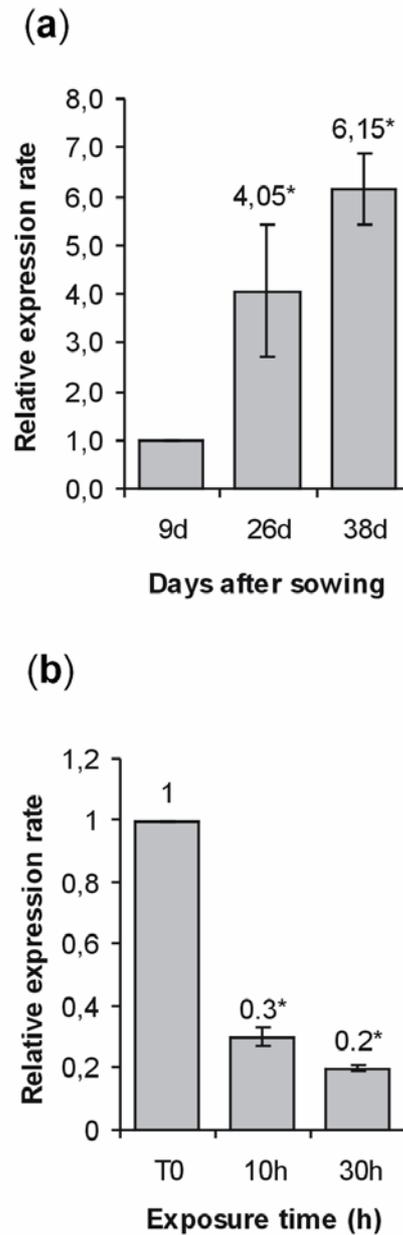
Fig. 16: (**a**) Expression analyses of the gene *HvC2d1* in primary barley (*Hordeum vulgare* L. cv Steffi) leaves during different developmental stages. At 9 days after sowing, primary leaves are in the mature stage. After 26 and 38 days these leaves are in the early and late stages of senescence, respectively. The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and the expression rate of the mature leaf is set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002). (**b**) Effect of drought stress on transcript levels of *HvC2d1*. seven-day-old barley plants grown on Muraschige and Skoog medium were removed from the medium. To represents the control (the starting point for drought stress), where the expression rate is set as 1. The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

3.6.5 Expression of *HvC2d1* responds to changes in cytosolic calcium

In plants, calcium is a possible mediator of extra cellular signals such as hormones and biotic and abiotic stressors (Takezawa, 1999). It is known that the C2-domain is a Ca^{2+}-activated membrane-targeting motif present in a wide range of Ca^{2+}-regulated proteins. In order to investigate whether the newly identified C2 domain-like *HvC2d1* also responds to changes in the Ca^{2+}-concentration, I investigated the effects of calcium ionophore A23187, which induces an increase in cytosolic calcium concentration (Takezawa, 1999; Kim *et al.*, 2003). Figure 17 shows that addition of calcium ionophore A23187 resulted in a clear increase in the level of *HvC2d1* mRNA, which started to accumulate significantly 5 hours after addition of the calcium ionophore. This result suggests that calcium signalling is involved in the regulation of *HvC2d1*.
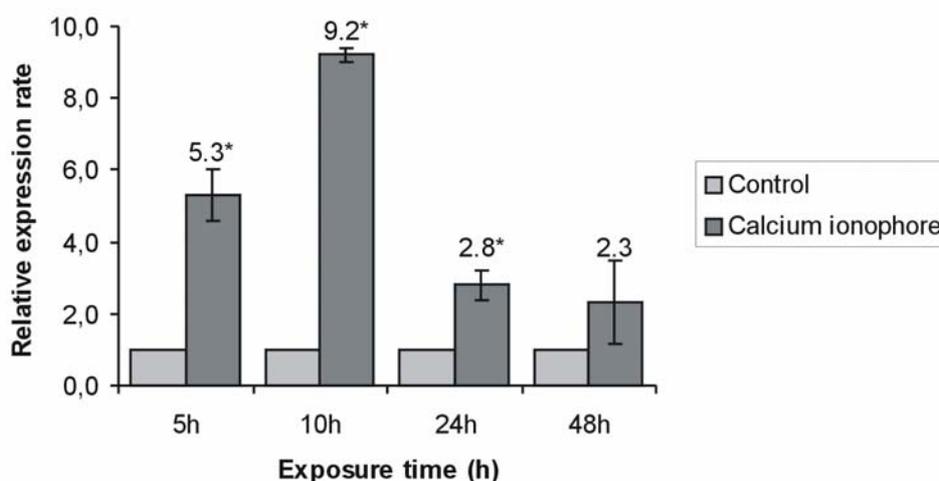
Fig. 17: Effect of calcium ionophore A23187 on mRNA levels of the barley (*Hordeum vulgare* L.) gene *HvC2d1*. Seven-day-old primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) plants grown on Muraschige and Skoog medium were treated with 200 μM of calcium ionophore for 5, 10, 24 and 48 hours or not treated (controls). The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls at each time-point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).

3.6.6 Expression of *HvC2d1* is affected by abscisic acid and methylviologen

Calcium acts as an intracellular messenger in many phytohormone signalling processes including that involving abscisic acid (ABA), which plays a major role in stress responses. In these processes, the Ca^{2+} signal is often triggered by secondary messengers such as cyclic ADP ribose (cADPR), inositol 1,4,5 trisphosphate (InsP3), myo-inositol hexakiphosphate (InsP6) or ROS such as H_2O_2 (Himmelbach *et al.*, 2003). It is also known that the small C2-

domain containing protein OsERG1 is induced in response to H_2O_2 (Kim *et al.*, 2004). In order to clarify whether ABA and /or ROS are also involved in the regulation of *HvC2d1*, two experiments were performed with ABA and Methylviologen, which generates ROS. For the first experiment, primary leaves from 7 days old barley plants were cut, then exposed in beakers to 50 µM of ABA with 1 % (v/v) ethanol for 8, 12 and 48 hours or not exposed to ABA (controls). Figure 18 shows that the expression level of *HvC2d1* was more than doubled in response to ABA treatment.
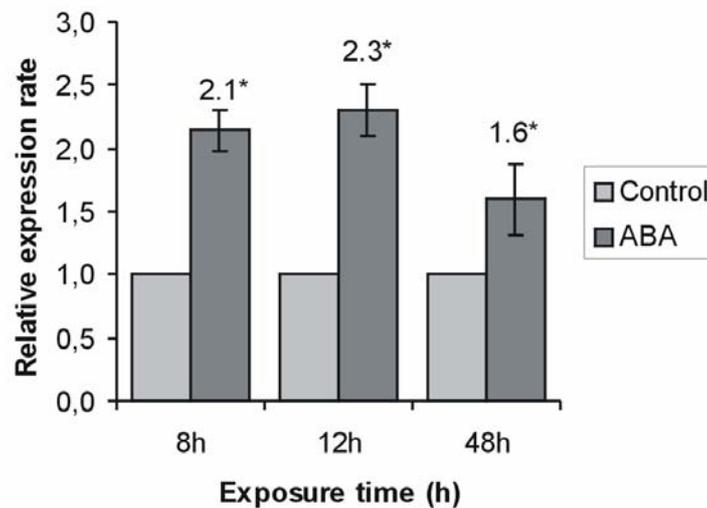
Fig. 18: Effects of abscisic acid (ABA) treatment on transcript levels of *HvC2d1*. Primary leaves of 7 days old barley plants were cut and treated in beakers with water containing 1 % (v/v) ethanol and 50 µM ABA for 8, 12 and 48 hours or not treated with ABA (controls) under controlled growth conditions. The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

For methylviologen application, 7 days old barley plants cultivated hydroponically as described in Materials and Methods were sprayed with 50 µM methylviologen in 0.1 % (v/v) Tween 20, then incubated for 1 hour in the dark for improved uptake of methylviologen. Plants were then exposed to a PPFR (400-700 nm) of 300 µmol $m^{-2}s^{-1}$ light to induce oxidative stress for 1,5, 3 and 6 hours. The control was treated only with 0.1 % (v/v) Tween 20. As shown in Fig. 19, the treatment of plants with methylviologen clearly causes the accumulation of *HvC2d1* mRNA.
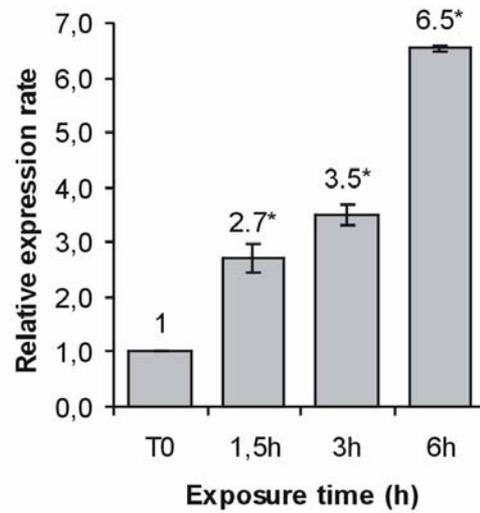
Fig. 19: Analysis of *HvC2d1* transcript levels during methylviologen application. Seven-day-old barley plants were treated with 50 µM methylviologen in 0.1% (v/v) Tween 20. After 1 hour in darkness, plants were exposed to light [with a photosynthetic photon fluence rate (PPFR , 400-700 nm) of 300 µmol m^{-2}s^{-1}]. The samples were harvested at 1, 5, 3 and 6 hours. After 1 hour in darkness, plants were exposed to light (at a PPFR, 400-700 nm of 300 µmol m^{-2}s^{-1}). The samples were harvested at 1, 5, 3 and 6 hours. T0 represents a control treated only with 0.1% (v/v) Tween 20 whose expression rate is set as 1. The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).

3.6.7 Confirmation of calcium binding of HvC2d1 by ^{45}Ca^{2+} overlay analysis

The ability of HvC2d1 to bind Ca^{2+} was tested in a ^{45}Ca^{2+} overlay analysis. Recombinant GST-HvC2d1 protein (see Fig. 20a) was overexpressed in *E. coli* and purified via glutathione-sepharose affinity chromatography (Fig. 20b). Different amounts of the purified protein, of BSA and of calmodulin were blotted to nitrocellulose membranes, exposed to ^{45}Ca^{2+} and then washed to remove non-specifically bound calcium. As shown in Fig. 20, HvC2d1 and the known calcium- binding protein calmodulin clearly bound ^{45}Ca^{2+}, whereas the negative control BSA did not. This result proves that the novel C2 domain protein HvC2d1 is able to bind calcium.

(a)

(b)
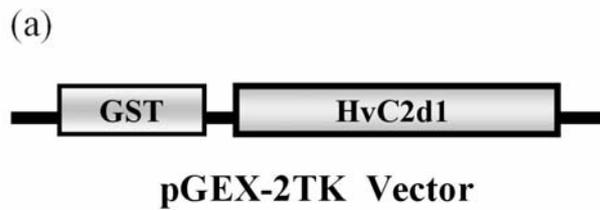
Fig. 20:(**a**)Construct of chimeric GST-HvC2d1 protein (**b**)SDS-PAGE analyses of the overexpressed GST-HvC2d1 protein in *E. Coli.* The boxes indicate the overexpressed protein. Protein Marker, not induced control (sample without IPTG), induced control
(sample with IPTG), supernatant (soluble protein after lysis), pellet (insoluble protein after lysis), washed 1-3 (GST-HvC2d1 washed 3 times), eluate 1-4 (GST-HvC2d1 eluted 4 times).
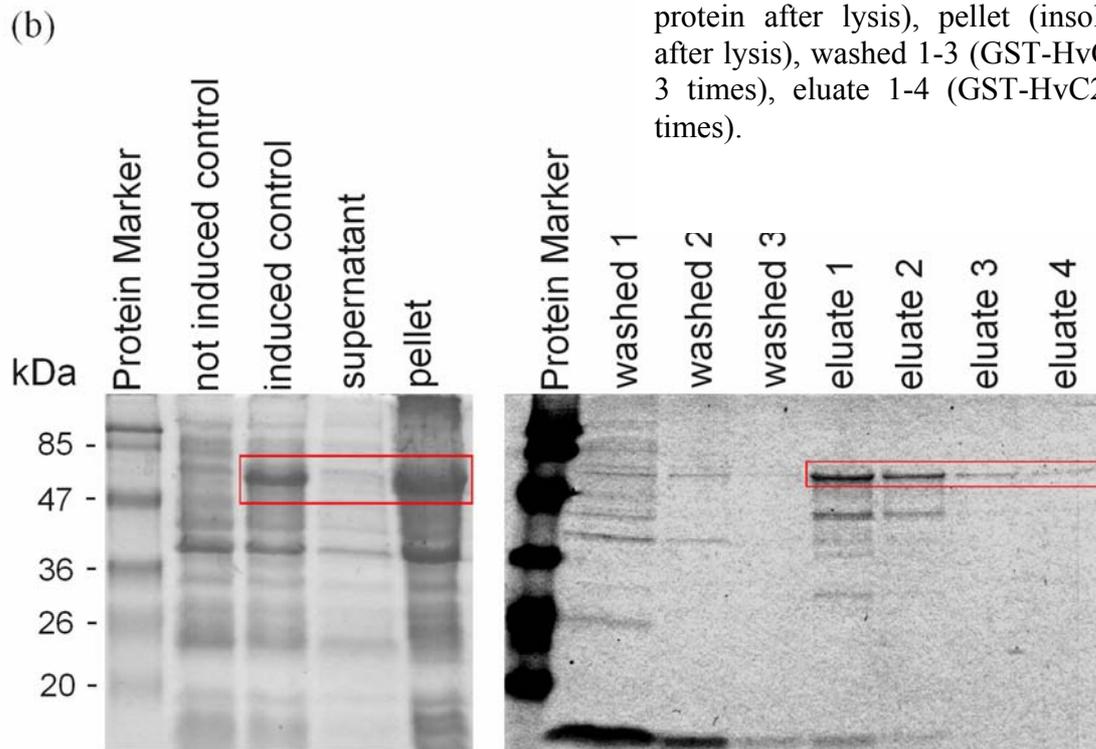
(c)
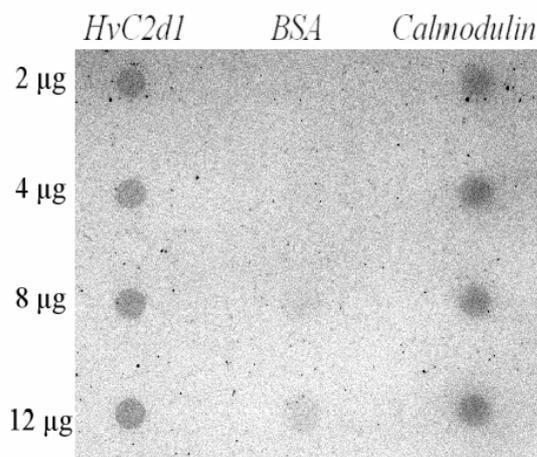
(**c**) Binding of Ca^{2+} by the barley (*Hordeum vulgare* L.) protein HvC2d1. Different amounts of overexpressed and purified HvC2d1 were blotted onto a nitrocellulose membrane. Additionally, the same amounts of the known calcium-binding protein calmodulin as a positive control and of bovine serum albumin (BSA) as a negative control were also blotted. After exposure to $^{45}Ca^{2+}$ (37 kBq / ml, 10 mM MES-KOH (pH 6.5), 5 mM $MgCl_2$, 60 mM KCl buffer) the membrane was washed with 50 % (v/v) ethanol and binding of radioactive $^{45}Ca^{2+}$ was analysed using a fluorescence image analyser.

3.6.8 Ca^{2+}-dependent subcellular localization of HvC2d1-GFP constructs

It is known that C2-domain proteins play a role in accurate Ca^{2+}-dependent spatio-temporal targeting in different regulatory signal transduction chains (Evans *et al.*, 2004). Furthermore, it has been shown that the small rice C2-domain protein OsERG1 is translocated to the plasma membrane of plant cells in a Ca^{2+}-dependent manner (Kim *et al.*, 2004). In order to determine whether the newly identified C2-domain protein HvC2d1 also exhibits calcium-dependent subcellular localization, HvC2d1-smRSGFP constructs were, after particle bombardment, overexpressed in onion epidermal cells for 12 hours. During this calcium ionophore A23187 was either added or not. As an additional control, smRSGFP alone was also overexpressed. Subcellular localization was then analysed using a confocal laser scanning microscope (Fig. 21). SmRSGFP alone was localized at the plasma membrane, the cytoplasm and the nucleus (Fig. 21a). Treatment of these cells overexpressing smRSGFP with the calcium ionophore A23178 did not change its distribution (Fig. 21d). All cells transformed with HvC2d1-smRSGFP showed a similar pattern of localization at the plasma membrane, the cytosole and the nucleus to smRSGFP alone (Fig. 21b). In contrast to this after the treatment with calcium ionophore A23178, green fluorescence was seen only in the nucleus, with higher green fluorescence intensity in the nucleoli of the examined cells (an example is shown in Fig. 21c). In all cells analysed, green fluorescence was never observed at the plasma membrane, as was always seen after transformation with either smRSGFP alone or the HvC2d1-smRSGFP construct without calcium ionophore A23178 treatment. These results indicate a calcium dependent translocation of the HvC2d1 protein to the nucleus, as expected from the presence of a NLS motif in the deduced amino acid sequence (shown in Fig. 12c).
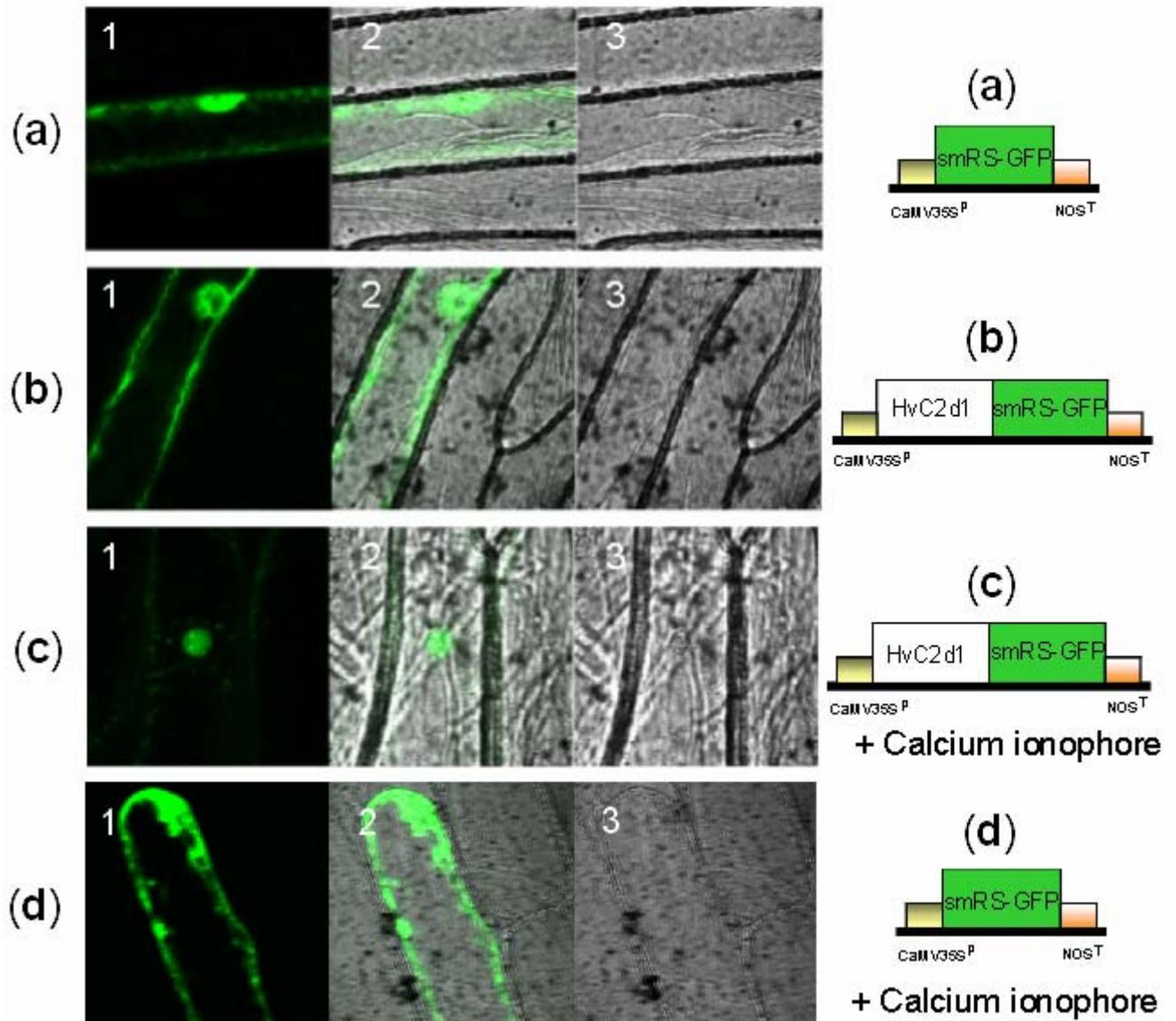
Fig. 21: Calcium-dependent subcellular localization of the barley (*Hordeum vulgare* L.) protein `C2 domain 1` (HvC2d1). Onion cells were transformed by particle bombardment using different constructs: (**a, d**) the smRGFP control, without (**a**) or with Ca^{2+} ionophore (**d**), and (**b, c**) smRS-GFP fused at the C-terminal end of HvC2d1 without (**b**) or with (**c**) Ca^{2+} ionophore treatment. The GFP fluorescence of the cells (1) was analysed 10-12 hours after transformation using a confocal laser scaning microscope. The images were captured at differential interference contrast (DIC, 3). The merged images are also shown (2).

3.7 *HvLysMR1*

3.7.1 Isolation of a full length cDNA encoding a lysine motif receptor-like kinase

The 183 bp fragment (chromi 1, AJ630116) showing homology to a receptor-like kinase was also characterized in more detail in this study. The identified clone was compared with other barley ESTs using the HarvEST Triticeae software version 0.99 (University of California, USA) showing 154/183 bp identity to the HarvEST consensus performed with AV946685barley EST. First 993 bp of this cDNA clone were amplified by RT-PCR and sequenced. The full length sequence including 5`and 3`ends of this cDNA clone was obtained by using a RACE technique (data not shown). The total length cDNA comprised 2119 bp including an ORF of 1868 bp (nucleotides 48 to 1916; CAJ14969) coding for 622 amino acids from Met1 to Arg622 (Fig. 22a).

By comparison of the deduced amino acids sequence, a LysM motif from amino acids His110 to Pro148 and Phe176 to Pro217 (underlined in Fig. 22a) could be identified showing homology to a consensus sequence LysM domain (PF01476) available at www.sanger.ac.uk/Software/Pfam/search.shtml (Fig. 22c). The LysM protein module is found among both prokaryotes and eukaryotes and was first identified in bacterial lysine and muramidase, enzymes that degrade cell wall peptidoglycan (Radutoiu *et al.*, 2003). In addition to lysine motifs (at the extra cellular region of the receptor-like kinase), the identified HvLysMR1 presents an N-terminal signal peptide from amino acids Met1 to Ala27 (square brackets), a transmembrane domain from amino acids Ala240 to Tyr262 (italicised) identified using CBS analyses (Center for Biological Sequence Analysis from Technical University of Denmark) and a kinase domain from amino acids Phe327 to Val594 with 11 characteristic subdomains (roman numerals) of the protein kinases (Radutoiu *et al.*, 2003) (Fig. 22a). Interestingly, the deduced protein contains at the extra cellular region three sets of potential metal-binding motifs, one CxxxC and two CxC motifs. Due to these characteristic domains the gene was named *HvLysMR1*(Hordeum vulgare Lysine Motif Receptor- like Kinase 1).

Alignment of the novel barley HvLysMR1 reveals homologies to others plant LysM receptors-like kinases identified in *Medicago truncatula* (AAQ73157, AAQ73154, AAQ73160, AAQ73155 and AAQ73158), *Lotus japonicus* (CAE02589 and CAE02597) and *Pisum sativum* (SyM 10) (Fig. 22c). Figure 22d shows the phylogenetic tree of these genes and of further genes with up to now unknown functions from *Arabidopsis thaliana*

(At BAB02358, At NP_56689; At AAB80675 and At NP_180916). Receptor like kinases with the lysine motif have, so far, been found only in plants and some of them are described to play a role in recognition of symbiotic bacteria in legume plants *Lotus japonicus* (Radutoiu *et al.*, 2003) and *Medicago truncatula* (Limpens *et al.*, 2003).

(a)

```
  1   [MGAPLPLLLPFLLLLLLLVASNTATAA]GDGCNMGCDLALGSYYV

 44   TPNVNVSYIASLFGFSDYRELAKYNRGFPSLDYVAAGNRLDVPFT

 89   CKCLTLPSDRASTYLAASFPHKVDTGETYVSIASKYSNLTTADWL

134   QATNTNTYPPNNIPANTILNVIVNCTCGDARISADYGLFRTFPVK

179   DWQVLASISEFSPDQKALLTIYNPAIHSGTGSGIAYIPAKDPDGS

224   YRPLKSQAGKKVPAGAIAGSVAAGLVAPVLGVLLFLFYRRRKAKQ

269   GALLPSSNESTRLASTILIQKLSPSTTEADVASLAAGITVDKSVE

314   FTYQELFNATEGFHITHKIGQGGFGAVYYAELLGEKAAIKKMDMQ
                    *               I              II
359   ATQEFLAELKVLTHVHHLNLVRLIGYCTESSLFLVYEFVENGNLS
          III                  IV              V
404   QHLHGTGYEPLSWAERVRIALDSARGLEYIHEHTVPVYIHRDIKS
                    VIa                          VIb
449   ANILIDKNTRAKVADFGLTKLTEVGGASLLTRVVGTFGYMPPEYV
                  VII                       VIII
494   RYGDVSRKVDVYAFGVVLYELISAKDAIVRSTDGSASGSRGLVYL
                  IX
539   FEEALTGLDPKEGLQKLIDPKLGDDYPVDAILMMTHLANACTEED

584   PKLRPTMRSVVVALMTLSSMTEFWDMKNPGLVNLMSGR
          XI        *
```

(b)
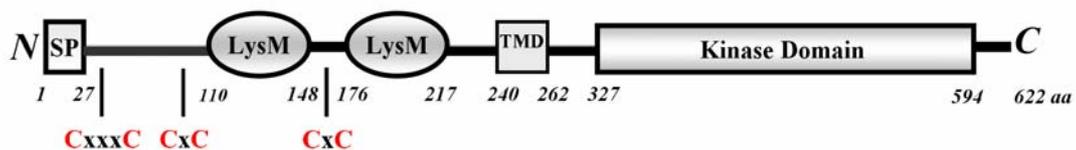
(c)
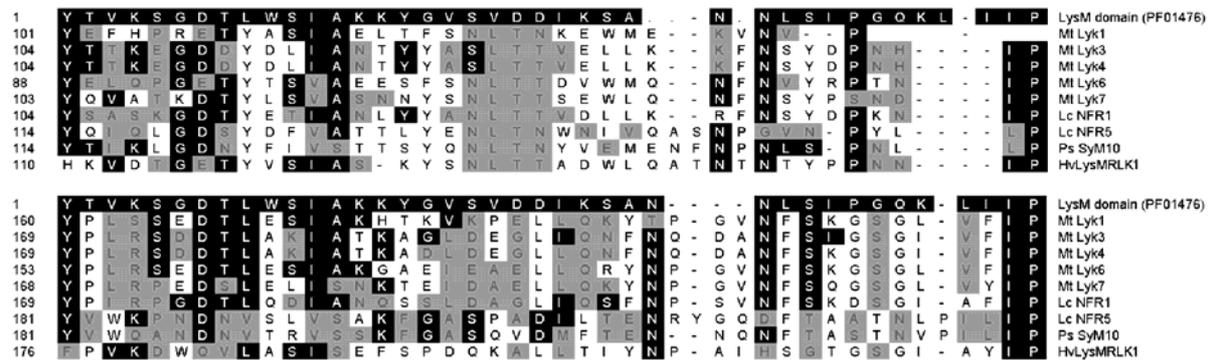
(d)
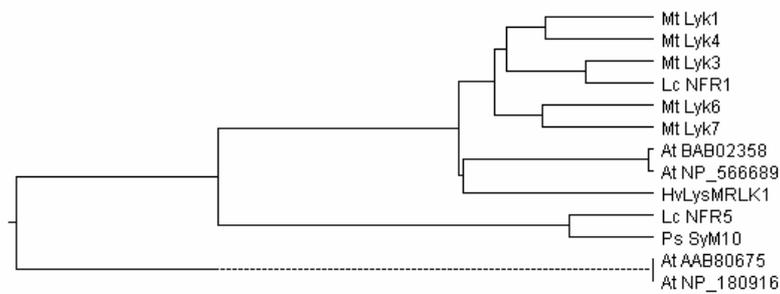
Fig. 22: **(a)** Predicted amino acid sequence of HvLysMR1. Signal peptide (SP) sequence is shown in square brackets; LysM motifs (LysM1 and LysM2) are underlined; the red boxes indicates potential metal-binding motifs; italicised amino acids indicate the transmembrane region (TMD); amino acids with asterisks indicate the beginning and the end of the kinase domain with eleven conserved subdomains (roman numerals) and highly conserved amino acids shown in bold (Radutoiu *et al.,* 2003). The black boxe indicate the phosphorylated peptide identified. **(b)** Schematic drawing of the arrangement of the Signal peptide (SP), the two LysM motifs, the transmembrane region (TMD), the kinase domain and the positions of potential metal-binding motifs of HvLysMR1. **(c)** Alignment of the deduced amino acid sequence of the two LysM motifs identified in barley (*Hordeum vulgare* L.) protein HvLysMR1 (CAJ14969) with the consensus LysM domain (PF01476) and amino acid sequences of known LysM motif receptor-like kinases of *Medicago truncatula* (Mt Lyk1: AAQ73154, Mt Lyk3: AAQ73155, Mt Lyk4: AAQ73160, Mt Lyk6:AAQ73157 and Mt Lyk7: AAQ73158), *Lotus japonicus* (NFR1: CAE02589 and NFR5: CAE02597) and *Pisum sativum* (SyM 10). The black background indicates amino acid residues that are identical and grey shading indicates amino acids that are similar to the LysM motif (PF01476). Alignment was performed using ClustalW in the Lasergene expert sequence analysis software (DNASTAR Inc., Madison, WI, USA). **(d)** Phylogenetic tree generated from the alignment of HvLysMR1 and other LysM receptor-like kinases shown in **(c)** and additionally, LysM receptor-like kinases from *Arabidopsis thaliana* (At BAB02358, At NP_56689; At AAB80675 and At NP_180916) found in the database (NCBI, National Center for Biotechnology Information). Alignment was performed using ClustalW in the Lasergene expert sequence analysis software (DNASTAR Inc., Madison, WI, USA).

3.7.2 *HvLysMR1* is transiently induced during chromium treatment

In order to determine expression patterns of the newly identified *HvLysMR1* during chromium exposure, barley plants were grown 7 days on murashige and skoog medium and then treated with 1mM of potassium dichromate or not treated (controls) as described in Materials and Methods. Changes in mRNA levels of *HvLysMR1* during exposure to 1mM chromium were investigated using quantitative real-time PCR. Transcript levels of *HvLysMR1* in the leaves were compared to those of the reference gene (18S ribosomal RNA). mRNA levels in controls were always set as 1 at the different time-points (Fig. 23a). *HvLysMR1* expression in the leaves was already slightly induced during the first 10 h of chromium treatment and maximum transcript levels were detected after 24 h of treatment (10.5 times higher than in the control; Fig. 23a). After this time, mRNA levels decreased again during prolonged time of stress. This result indicates a transient expression pattern of *HvLysMR1* during chromium stress.
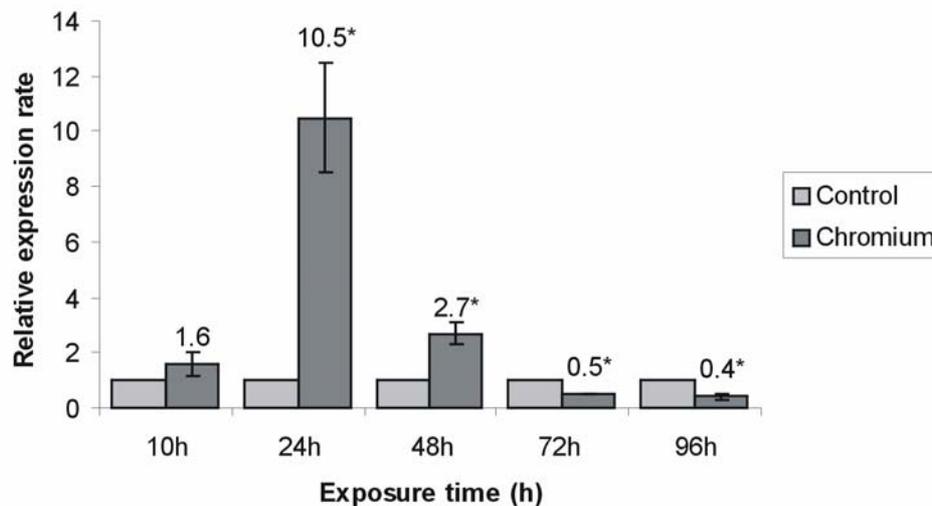
Fig. 23: Relative expression rate of *HvLysMR1* in primary leaves of barley seedlings (*Hordeum vulgare* L. cv. Steffi) treated with (chromium) or not treated (control) 1mM potassium dichromate. The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as reference gene and the control at each time point is set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).

3.7.3 *HvLysMR1* is also induced during cadmium and copper treatment

In order to investigate whether the expression of *HvLysMR1* is also affected by other heavy metals than chromium, 7days old barley seedlings were treated either with 1 mM of cadmium chloride or 1mM of copper chloride for 24, 48, 72 and 96 hours or not treated (controls). In addition, changes in the two photosynthesis related stress parameters chlorophyll content and PSII efficiency were measured in the two experiments (Fig. 24b and Fig. 25b). In cadmium treated seedlings, chlorophyll content started to decrease 24 h after the onset of the cadmium treatment. This decrease was accentuated during prolonged time of exposure. PSII efficiency decreased at 48 h of cadmium treatment. In seedlings treated with copper (Fig. 25b), similar patterns of changes in chlorophyll content and PSII efficiency were observed as during cadmium treatment (Fig. 24b).

The expression levels of *HvLysMR1* under cadmium and copper treatment were again investigated via quantitative real-time PCR in comparison to the controls. During cadmium treatment, after 24 h of exposure, the mRNA level of *HvLysMR1* increased significantly and high levels of this transcript could be identified 48 h after onset of treatment (147.4 times more then in control, Fig. 24a). 1 mM copper treatment also resulted in increased mRNA levels of *HvLysMR1* already 24 h after the onset of the treatment (3.4 times more then in controls). After this time point, the relative mRNA levels of *HvLysMR1* declined to reach basal levels at 48 h. These data indicate a fast and transient expression pattern after exposure of the plant to high concentrations of the essential heavy metal copper and the non-essential heavy metal cadmium.
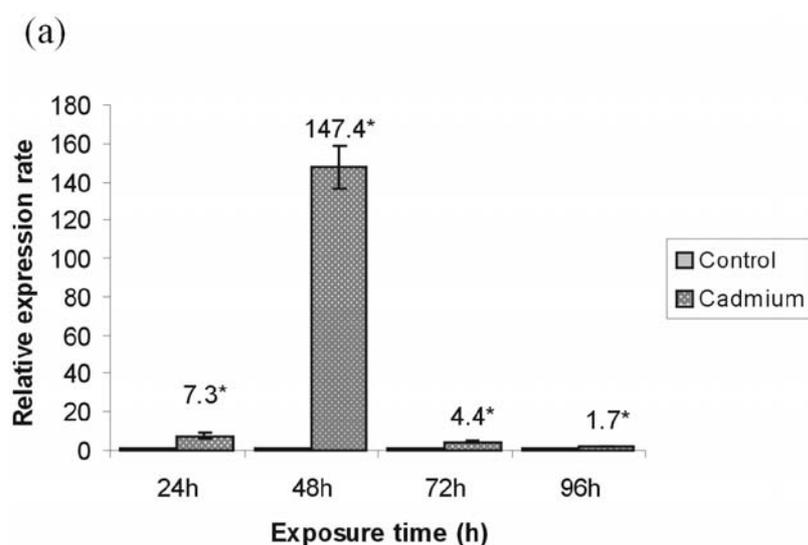
(a)

(b)

		0h	24h	48h	72h	96h
Control	Chlorophyll content	33.27 ± 3.43	33.69 ± 1.83	34.02 ± 2.87	33.25 ± 2.61	32.82 ± 3.56
	PSII efficiency	0.805 ± 0.06	0.805 ± 0.06	0.803 ± 0.07	0.805 ± 0.07	0.803 ± 0.10
Cadmium	Chlorophyll content	33.27 ± 3.43	29.75 ± 2.54	25.79 ± 4.76	18.75 ± 3.23	14.86 ± 3.72
	PSII efficiency	0.805 ± 0.06	0.804 ± 0.05	0.799 ± 0.13	0.774 ± 0.17	0.676 ± 0.30

Fig. 24: (**a**) Effects of cadmium treatment on *HvLysMR1* mRNA levels in primary leaves (*Hordeum vulgare* L. cv Steffi). RNA was extracted from primary leaves of barley seedlings either stressed with 1mM cadmium chloride during a period from 24 to 96 hours or not stressed (controls). The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as reference gene and controls at each time point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002). (**b**) Effects of cadmium treatment on chlorophyll content and photosystem II (PSII) efficiency during treatment of barley seedlings (*Hordeum Vulgare* L. cv Steffi) with 1mM cadmium chloride for 24, 48, 72 and 96 hours or not treated (controls). Each data-point represents the mean of 10 independent measurements.

(a)

(b)
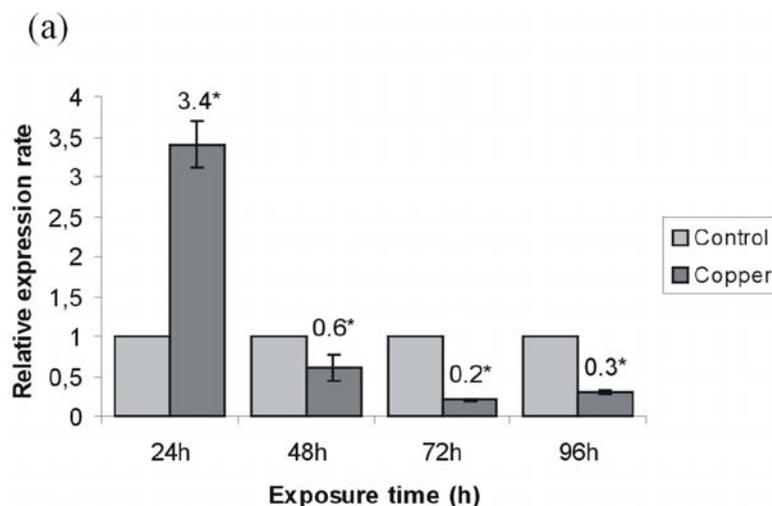
		0h	24h	48h	72h	96h
Control	Chlorophyll content	34.09 ± 2.8	34.55 ± 2.34	34.02 ± 2.87	33.25 ± 2.61	32.82 ± 3.56
	PSII efficiency	0.806 ± 0.04	0.803 ± 0.05	0.806 ± 0.06	0.807 ± 0.07	0.801± 0.09
Copper	Chlorophyll content	34.09 ± 2.8	31.85 ± 2.42	29.27 ± 2.96	24.66 ± 3.19	18.58 ± 3.59
	PSII efficiency	0.806 ± 0.04	0.807 ± 0.07	0.801 ± 0.13	0.769 ± 0.22	0.701 ± 0.20

Fig. 25: (**a**)*HvLysMR1* gene expression during copper treatment of barley seedlings (*Hordeum vulgare* L. cv Steffi). RNA was extracted from primary leaves of barley seedlings either stressed with 1mM copper chloride during a period from 24 to 96 hours or not stressed (controls). The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as reference gene and controls at each time point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002). (**b**) Effects of copper treatment on chlorophyll content and photosystem II (PSII) efficiency during treatment of barley seedlings (*Hordeum vulgare* L. cv Steffi) with 1mM copper chloride for 24, 48, 72 and 96 hours or not treated (control). Each data-point represents the mean of 10 independent measurements.

3.7.4 *HvLysMR1* mRNA accumulates during leaf senescence

As outlined before, some heavy metal induced genes are also up-regulated during leaf senescence (Himelblau *et al.*, 1998; Himelblau & Amasino, 2000; Guo *et al.*, 2003) indicating an overlap in the response of plants to these two conditions. Analogous to this, the induction of the newly identified receptor-like kinase was tested during leaf senescence. Barley plants were grown for 9, 26 and 38 days at 16 h light (21°C and 100 μmol m^{-2} s^{-1}) and 8 h darkness (16°C) on soil. The characterization of the different developmental stages of the primary

leaves was performed by measurement of chlorophyll content and PSII efficiency which decrease with onset of senescence (data not shown). At 9 days after sowing, primary leaves were in their mature stage. After 26 and 38 days the leaves were in the early and late stages of senescence. Figure 26 shows that the *HvLysMR1* mRNA significantly accumulated during leaf senescence showing a 15 – 19 times higher level during senescence compared to the mature leaf.
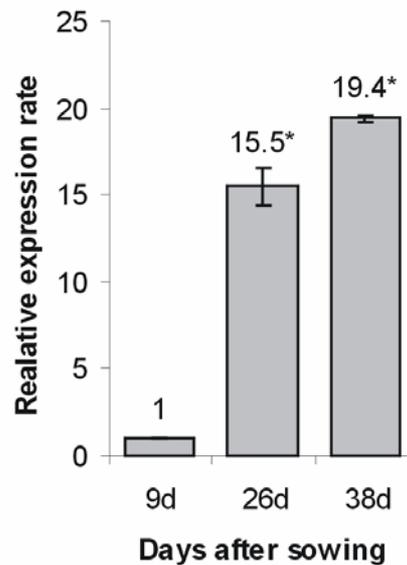
Fig. 26: Expression of *HvLysMR1* gene in primary barley (*Hordeum vulgare* L. cv Steffi) leaves during different developmental stages. 9 d after sowing, primary leaves are in the mature stage. At 26 and 38 d these leaves are in the early and late stages of senescence, respectively. The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as reference gene and the expression of the mature leaf is set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).

3.7.5 *HvLysMR1* mRNA level responds to changes in cytosolic calcium

The two other known LysM receptor-like kinases *Lj* NFR5 and *Lj* NFR1 have been shown to be involved in Nod-factor signal transduction which implies Ca^{2+}-signalling (Oldroyd & Downie, 2004). Such changes in cytosolic calcium play a central role during the transduction of a wide variety of abiotic and biotic signals and in growth and developmental processes (Reddy, 2001; Sanders *et al.*, 2002) and it is known that many stress related genes are regulated in response to intracellular calcium levels (Albrecht *et al.*, 2003). In order to investigate whether changes in cytosolic calcium are also involved in the regulation of the newly identified LysM motif receptor-like kinase HvLysMR1, an experiment was performed with calcium ionophore A23187 as described in Materials and Methods, which induces an

increase in cytosolic calcium concentration (Kim *et al.,* 2003). A fast and significant accumulation of *HvLysMR1* mRNA was observed already 5 h after addition of calcium ionophore A23187 (Fig. 27). In later stages of the treatment the mRNA levels decrease again.
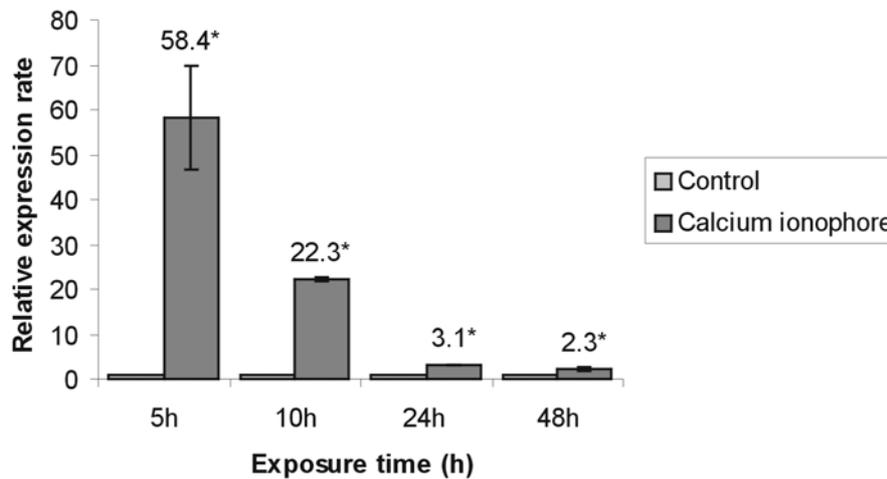
Fig. 27: Effect of calcium ionophore A23187 on mRNA levels of the barley (*Hordeum vulgare* L) gene *HvLysMR1*. Primary leaves of 7 days old barley (*Hordeum vulgare* L cv. Steffi) plants grown on Muraschige and Skoog medium were treated with 200μM of calcium ionophore for 5, 10, 24 and 48 hours or not treated (controls). The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as reference gene and controls at each time point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

3.7.6 Methylviologen treatment

Another common signal in response to different stress conditions including heavy metal treatment and also during onset of senescence is the accumulation of reactive oxygen species (ROS) (Krupinska *et al.,* 2003; Mithöfer *et al.*, 2004). Changes in amounts of reactive oxygen species (ROS) were described to also play a role in Ca^{2+}-signalling (Himmelbach *et al.,* 2003). In order to verify whether ROS are involved in the regulation of *HvLysMR1*, methylviologen was used as source which generates superoxide anion radicals by uptake of an electron from photosystem I (Donahue *et al.*, 1997). Figure 28b shows that methylviologen caused a very slight but significant accumulation of *HvLysMR1* mRNA.
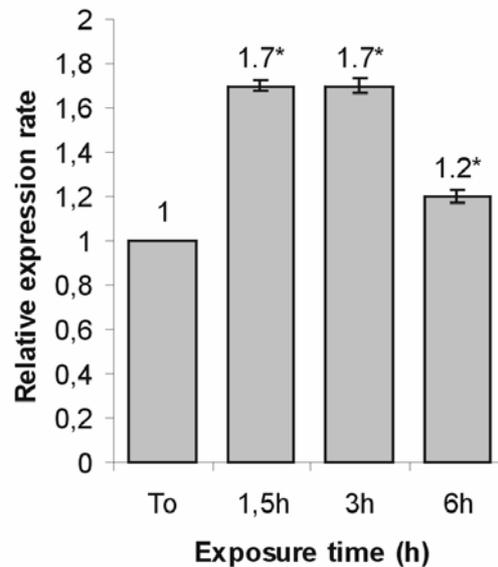
Fig. 28: Analysis of *HvLysMR1* transcript levels during methylviologen application. Seven days old barley seedlings were treated with 50 μM methylviologen in 0.1% (v/v) Tween 20. After 1 h in darkness, plants were exposed to light (300 μmol m^{-2}s^{-1}). The samples were harvested at 1,5, 3 and 6 hours. T0 represents a control treated only with 0.1% (v/v) Tween 20. whose expression rate is set as 1. The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as reference gene. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

3.7.7 The HvLysMR1 intracellular domain encodes a functional kinase

It is known that the intracellular part of receptor-like kinases undergoes autophosphorylation which plays a role in signal transduction (Yoshida & Parniske, 2005). In order to investigate whether the newly identified HvLysMR1 protein possesses such an active kinase domain, the His-tagged HvLysMR1-kinase domain (Fig. 29a) was overexpressed in *E. coli*, purified using a Ni-NTA Superflow Column and immunologically analysed with an Anti-His tag Antibody (QIAGEN, Hilden, Germany). Figure 29c shows a band of about 42 KDa corresponding to the molecular mass of the overexpressed protein. Autophosphorylation was tested by incubation of the purified protein with [γ-^{32}P]ATP. The phosphorylated protein was analysed after SDS-PAGE, transfered onto a PVDF membrane, and visualized by autoradiography (Fig. 29d). The results prove a weak phosphorylation of a protein showing a similar molecular mass to the immunologically identified chimeric protein (Fig. 29c). The weak phosphorylation indicates that the overexpressed protein was already phosphorylated to a great extent in *E. coli*. In order to prove that the intracellular domain of HvLysMR1 can indeed be phosphorylated as
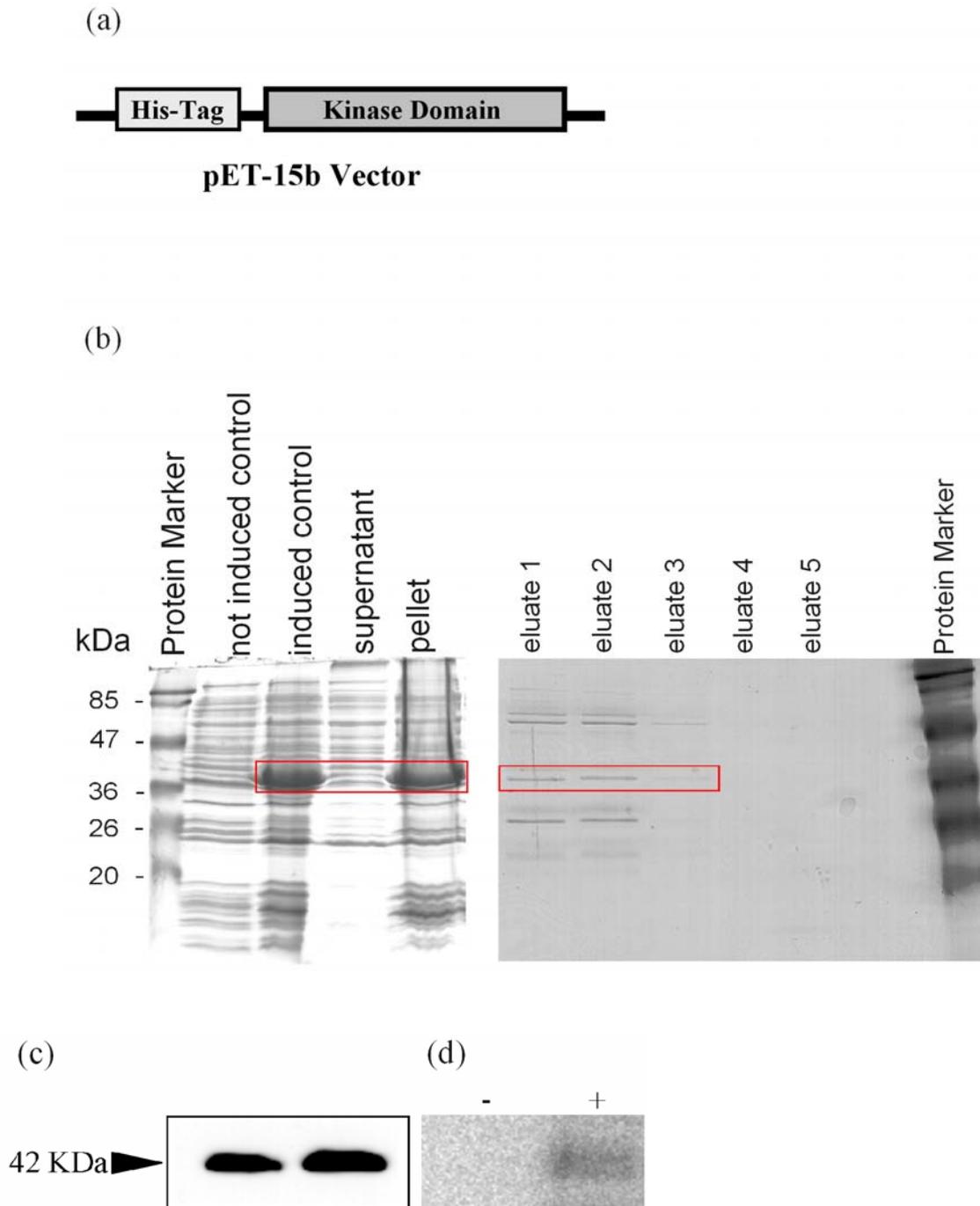
Fig. 29: **(a)** Construct of chimeric His-HvLysMR1-kinase domain **(b)** SDS-PAGE analyses of the overexpressed His-HvLysMR1-kinase domain in *E. coli*. The boxes indicate the overexpressed protein. Protein Marker, not induced control (sample without IPTG), induced control (sample with IPTG), supernatant (soluble protein after lysis), pellet (insoluble protein after lysis), eluate 1-5 (His-HvLysMR1-kinase domain eluted 5 times). **(c)** Western blot analyses of the overexpressed and purified His-HvLysMR1-KD protein. Purified protein was subjected to SDS-PAGE, transferred onto PVDF membrane, and then incubated with an Anti-His tag Antibody. **(d)** (+) indicates that the overexpressed His-tagged protein was incubated with [γ-^{32}P]ATP for 45 min and (-) the control not incubated with radioactive ATP. After separation by SDS-PAGE, it was transferred onto PVDF membrane. Autoradiography of the membrane revealed a signal at 42 kDa corresponding to the His-tagged HvLysMR1-KD protein (42 kDa) shown in **(b)**.

known for active receptor-like kinases, the overexpressed protein was additionally analysed using nano LC-ESI-MS by MS^2 and MS^3 spectra using the data dependent neutral loss mode (Wagner *et al.*, 2006). Thus, neutral loss events (loss of phosphoric acid from a phosphorylated serine or threonine resulting in a dehydroalanine (Dha) or methyldehydroalanine (MeDha)) that occurred during MS/MS, are used to trigger an MS^3 automatically. MS analysis was carried out in two experiments with independent eluates from the His-HvLysMR1-KD overexpression. It revealed 13 different peptides with significant Xcorr (Tab. 2) giving an amino acid coverage of 47.8%. From the detected peptides only one (LASTILIQK) could be identified as phosphopeptide. However, it was also present in its non-phosphorylated form, indicating that only a portion of the protein is phosphorylated. The phosphopeptide LASpTILIQK was found with Dha instead of 284-Serin indicating a neutral loss on the phosphorylated side chain of Ser. We were able to positively assign the complete y-ion series from this peptide, and all b-ions with a mass >300 *m/z* resulting in a clear identification of this phosphopeptide (Fig. 30, Tab. 2). In addition, we found the same peptide with phosphorylation at the Thr residue (LASTpILIQK), however only with a relatively low abundant y-ion at the relevant position. The experiments showed that either Ser- 284 or to a lower extent Thr- 285 within peptide LASTILIQK that is located at the juxtamembrane region is phosphorylated. Earlier studies of phosphorylation sites of plant receptor-like kinases revealed that phosphorylation at this juxtamembrane region is a common feature (Nühse *et al.*, 2004; Wang *et al.*, 2005).

Table 2: Identification of peptides from HvLysMR1 by LC-ESI-MS with MS^2 and neutral-loss-triggered MS^3 spectra.

Peptide	MH+	z	Xcorr
LSPSTTEADVASLAAGITVDK	2047.2	2	7.62
IGQGGFGAVYYAELLGEK	1873.1	2	5.25
VVGTFGYMPPEYVR	1615.9	2	4.48
GLEYIHEHTVPVYIHR	1964.2	2	4.18
MDMQATQEFLAELK	1655.9	2	4.01
LTEVGGASLLTR	1217.4	2	3.94
LASTILIQK	987.3	2	3.53
NPGLVNL(Mox)SGR [a]	1174.4	2	3.43
GLVYLFEEALTGLDPK	1766.0	2	3.36
VLTHVHHLNLVR	1438.7	2	3.22
VADFGLTK	851.0	2	2.79
QGALLPSSNESTR	1360.5	2	2.46
SANILIDK	874.0	2	2.16
LASpTILIQK [b]	1067.3	1	2.84
LASTpILIQK [b]	1067.3	1	2.69
LASTILIQK	987.3	1	2.18

[a] Methionine in its oxidized form.
[b] Phosphopeptide; p indicates a phosphorylation at the prefixed amino acid.

(a) Full MS [1000-1200 m/z]

(b) MS/MS of 1067.34, 1+

Insufficient fragment information

(c) Neutral loss triggered MS3 scan of 969.3, 1+

LA (Dha)TILIQK
Xcorr: 2.84
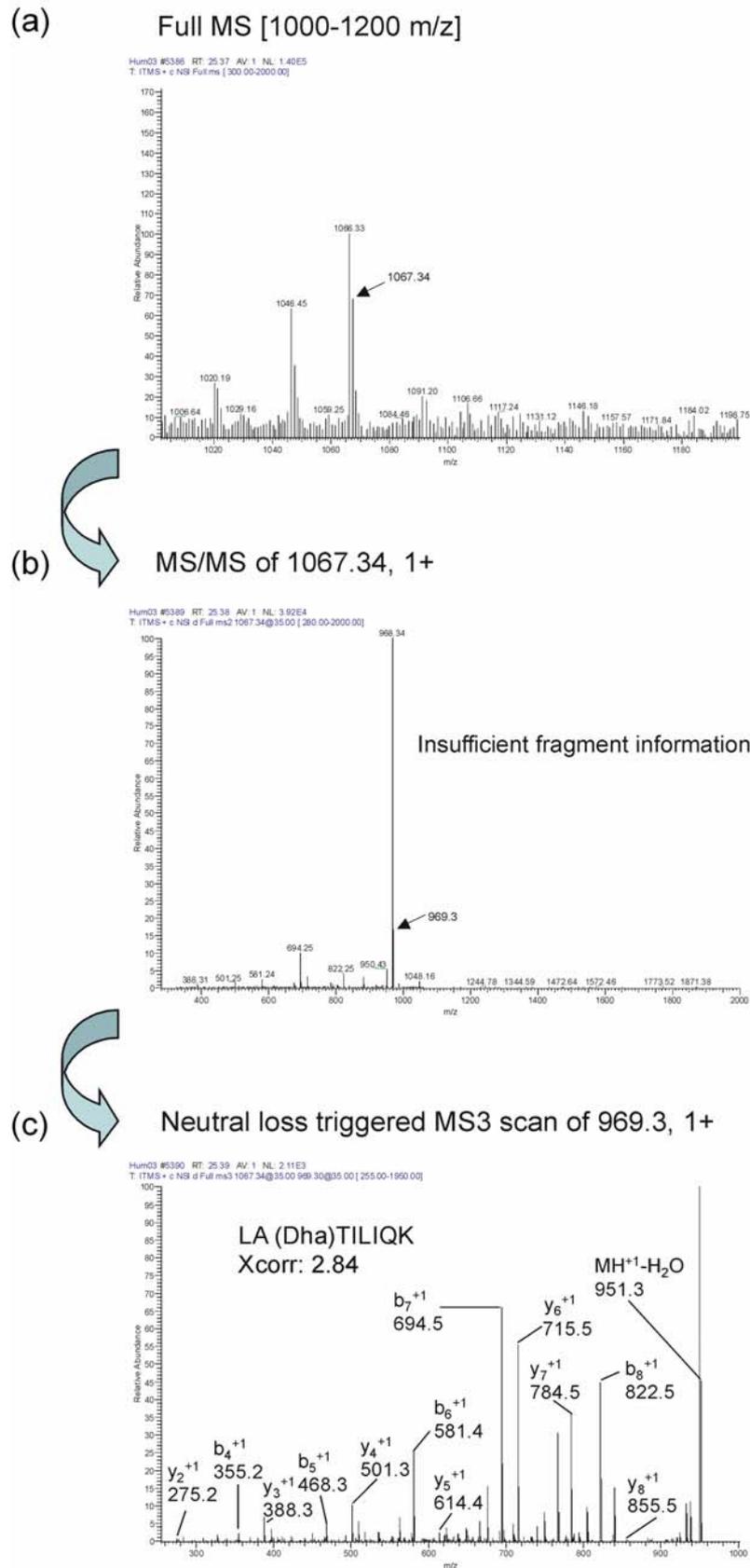
Fig. 30: Identification of the phosphopeptide LASpTILIQK by neutral-loss-triggered nano-LC-ESI-MS[3]. (a) Full MS scan in the m/z range of 1000-1200 detects the prominent peptide ion 1067.34. (b) The MS[2] fragmentation spectrum of peptide ion 1067.34 reveals the neutral loss (fragment ion 969.30) but no fragment information sufficient for peptide identification. (c) Identification of the peptide LA(Dha)TILIQK by neutral-loss-triggered MS[3] of peptide ion 969.30. Dha indicates the site of the neutral loss of phosphoric acid from phosphoserine. Only prominent y- and b- fragment ions have been labeled.

3.8 *HvLysMR2*

3.8.1 Identification of second LysM receptor-like kinase

By performing a RACE technique to get the 5´end of extended cDNA clone chromi 1 (993 bp), a second cDNA clone could be identified. The full length nucleotide sequence of this cDNA comprised 1971 bp including an ORF of 1706 bp (nucleotides 65 to 1771; AM400870) coding for 568 amino acids from Met1 to Arg568 (Fig. 31). By comparison of the deduced amino acid sequence, a LysM motif from amino acids Tyr49 to Pro94 and Lys117 to Gly162 (underlined in Fig. 31a) could be identified showing homology respectively to LysM domain (smart00257 and Pfam01476). The deduced protein presents a N-terminal mitochondrial targeting peptide from amino acids Met1 to Gly36 (italicised), identified using CBS analyses (Center for Biological Sequence Analysis from Technical University of Denmark) and a kinase domain from amino acids Phe273 to Val541 with 11 characteristic subdomains (roman numerals) for the protein kinases (Radutoiu *et al*., 2003) (Fig. 31a). In addition, the deduced protein shows the presence of a potential metal-binding motifs CxxC and two CxC motifs. Due to these characteristic domains the gene was named *HvLysMR2* (Hordeum vulgare Lysine Motif Receptor-like kinase 2). The second HvLysMR2 identified lacks the presence of the transmembrane domain. By performing an alignment with blastx from NCBI, the novel barley HvLysMR2 revealed homologies to other plant receptor-like kinases identified in *Medicago truncatula* (AAQ73157, AAQ73154, AAQ73156 and AAQ73159) and genes with unknown functions from *Arabidopsis thaliana* (AT BAB02358, At NP_56689).

(a)

```
1    MVPGFLPWGRRVMQAPLPLVLPLFLLVASKTATVAGDGCRGCSLA
45   FGSYYVTKDTNLTFISQLFGLSDYRDLAKYNRGLPNLDNAAAGDR
90   VDVPFPCECLTRPSHPASTYLAASIPYKVATGETYVSIASNYNNL
136  TTADWLQATNTYYPPNDIPDVGVVNITVNCSCGDARISTDYGLLRT
181  FPLRDWETLDSVAATRDLSSPKRMDQLRRYNPGMEGATGSGIVYI
226  PAQASTILMQKVKPSTAQADVASLAADITVDKSVEFTYQELFNAT
271  EGFXITHKIGQGGFGAVYYAELXGEKAAXKKMDMQATQEFLAELK
          *             I              II            III
316  VLTHVHHLNLVRLIGYCTDSSLFLVYEFVENGNLSQHLRGTGYEP
                    IV              V
361  LSWPERVRIALDSARGLEYIHEHTVPVYIHRDIKSANILIDKNTR
              VIa                  VIb
406  AKVADFGLTKLTEVGGASLQTRVVGTFGYMPPEYVRYGDVSRKVD
            VII                  VIII
451  VYAFGVVLYELISAKDAIVRSTDGSASGSRGTGLSVSRGSHGLDP
        IX
496  KEGLQKLIDPKLGDDYPVDAILMMHLANACTEEDPKLRPTMRSVV
                                      XI          *
541  VALMTLSSMTELWDMKNPGLVNLMSGR
```

(b)
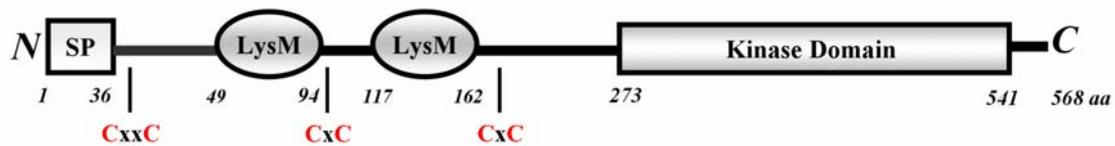
Fig. 31: **(a)** Predicted amino acid sequence of *HvLysMR2*. Italicised amino acids indicate the signal peptide (SP); LysM motifs (LysM1 and LysM2) are underlined; the boxes indicates the potential metal-binding motifs; amino acids with asterisks indicate the beginning and the end of the kinase domain with eleven conserved subdomains (roman numerals) and highly conserved amino acids shown in bold (Radutoiu *et al.,* 2003). **(b)** Schematic drawing of the arrangement of the signal peptide (SP), the two LysM motifs, the kinase domain and the positions of potential metal-binding motifs of HvLysMR2.

3.8.2 *HvLysMR2* is transiently induced during chromium treatment

In order to determine the expression of *HvLysMR2* gene during the chromium treatment, 7 days old barley seedlings were treated with 1mM potassium dichromate or not treated (control) for different time-points. The changes in mRNA levels were analysed by quantitative real-time PCR by comparison of mRNA levels of the *HvLysMR2* to levels of the reference 18S ribosomal RNA from treated samples and controls at different time-points. The expression rate of *HvLysMR2* is referred to that of the untreated control, which at each time-point is set as 1. The *HvLysMR2* mRNA had already started to accumulate 24 h after chromium treatment (6.9 times higher than in the control; Fig. 32). The transcript level of *HvLysMR1* remained almost stable between 24 h to 48 h. After this time-point, mRNA levels decrease again, reaching a basal transcript level after *c.* 96 h. These result indicates a transient expression pattern of *HvLysMR2* during chromium treatment.
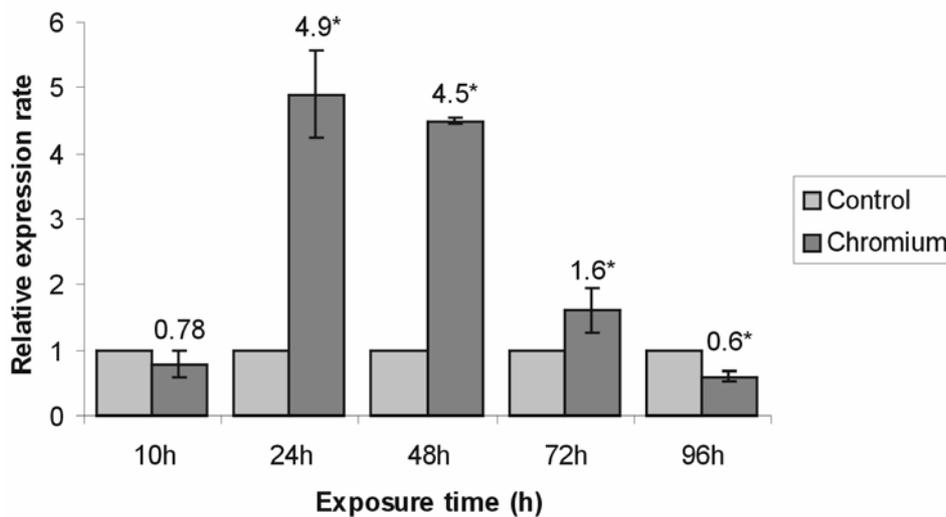
Fig. 32: Relative expression rate of the gene *HvLysMR2* in primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings treated with (chromium) or not treated with (control) 1mM potassium dichromate. The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and the control at each time-point is set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

3.8.3 Changes in mRNA levels of *HvLysMR2* in response to cadmium and copper treatment

In order to analyse whether *HvLysMR2* is also induced by other heavy metals such as cadmium and copper as shown above for *HvLysMR1*, 7 days old barley seedlings were grown hydroponically as described in Materials and Methods, and then treated either with 1 mM of cadmium chloride or 1 mM of copper chloride for 24, 48, 72 and 96 hours or not treated (controls). During cadmium treatment, the *HvLysMR2* mRNA had already started to accumulate 24 h and reached a maximum transcript level 48 h after onset treatment (26.3 times higher than in the control; Fig. 33). After this time-point, the relative mRNA levels of *HvLysMR2* decreased again. In the copper treatment, a fast and transient expression pattern was observed with maximum accumulation of *HvLysMR2* mRNA at 24 h after onset of treatment (7.1 times higher than in control; Fig. 34).
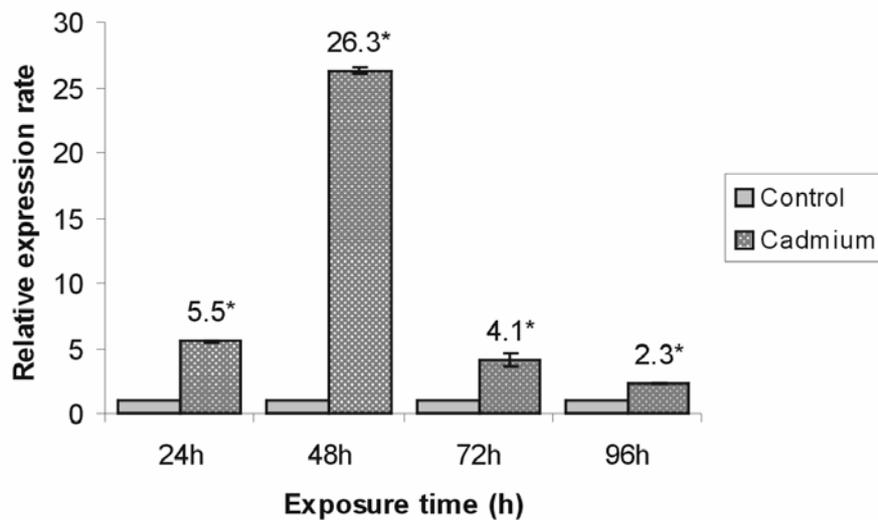
Fig. 33: Changes of mRNA levels of the barley (*Hordeum vulgare* L.) gene *HvLysMR2* in primary leaves during cadmium treatment. RNA was extracted from primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings treated with 1 mM cadmium chloride for 24 to 96 h or not treated (controls). The level of *HvLysMR2* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls at each time-point are set as 1. Error bars indicate the standart deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).
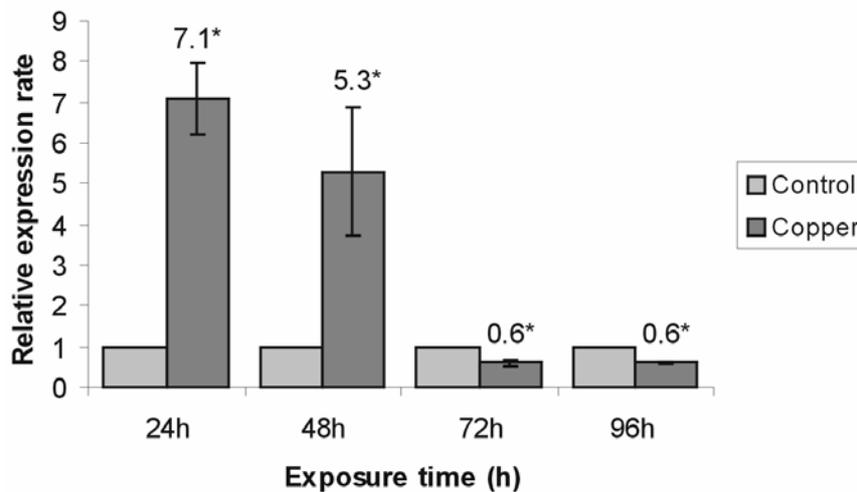
Fig. 34: Expression of barley (*Hordeum vulgare* L.) gene *HvLysMR2* in primary leaves during copper treatment. 7 days old barley (*Hordeum vulgare* L. cv. Steffi) seedlings were treated with 1mM copper chloride for 24 to 96 h or not treated (controls). The level of *HvLysMR2* mRNA in each case is normalized to that of 18S rRNA as reference gene and controls at each time-point are set as 1. Error bars indicate the standart deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).

3.8.4 *HvLysMR2* mRNA accumulates during leaf senescence

Since *HvLysMR1* mRNA was up-regulated during leaf senescence and some heavy metal induced genes were also induced during senescence as described above (see chapter 3.6.3) the changes of *HvLysMR2* mRNA during leaf senescence was investigated via quantitative real-time PCR. Total RNA was isolated from barley primary leaves at different stages of development. Three stages of senescence were identified by measuring chlorophyll content and PSII efficiency which decrease with onset of senescence (data not shown). At 9 days after sowing, primary leaves were in their mature stage. After 26 and 38 days the leaves were in the early and late stages of senescence. Figure 35 shows that the mRNA level of *HvLysMR2* significantly accumulated during leaf senescence.
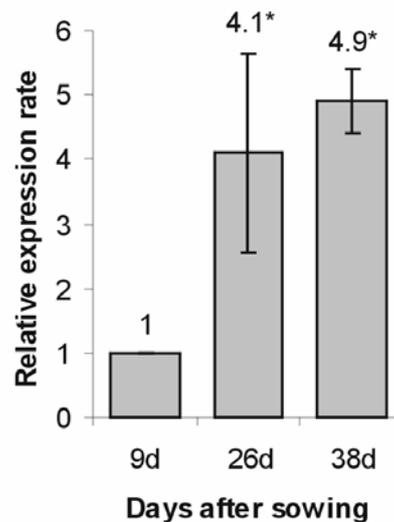
Fig. 35: Expression analyses for the gene *HvLysMR2* in primary barley (*Hordeum vulgare* L. cv. Steffi) leaves during different developmental stages. At 9 d after sowing, primary leaves are in the mature stage. At 26 and 38 d these leaves are in the early and late stages of senescence, respectively. The level of *HvLysMR2* in each case is normalized to that of 18S rRNA as a reference gene and the expression rate of the mature leaf is set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.*, 2002).

3.8.5 *HvLysMR2* mRNA accumulates in response to changes in cytosolic calcium

From the literature, it is known that changes in cytosolic calcium play a role during the transduction of a wide variety of abiotic and biotic signals and in growth and developmental processes (Reddy, 2001; Sanders *et al.,* 2002). Since *HvLysMR1* mRNA was up-regulated during calcium ionophore A23187 treatment it was tested whether changes in cytosolic calcium are involved in the regulation of the second identified LysM motif receptor-like kinase. In Figure 36 this expression rate of *HvLysMR1* is compared to that of the untreated control, which at each time-point is set as 1. A fast and significant accumulation of *HvLysMR2* mRNA at 5 h after the addition of calcium ionophore A23187 (117.1 times higher than in the control; Fig. 36) was detected. After this time-point, the relative mRNA levels of *HvLysMR2* declined again, showing a transient expression pattern.
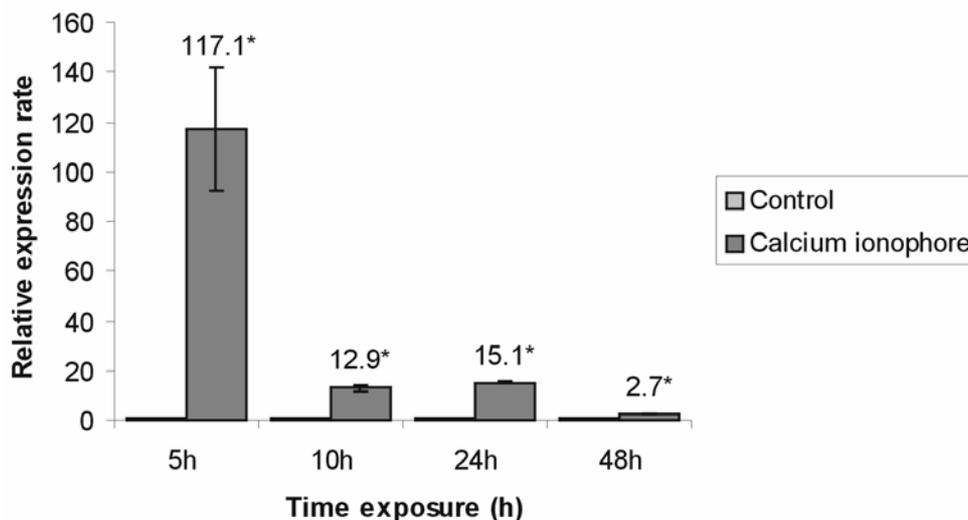
Fig. 36: Effects of calcium ionophore A23187 treatment on mRNA levels of the barley (*Hordeum vulgare* L.) gene *HvLysMR2* in primary leaves. Seven-day-old primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) plants grown on Muraschige and Skoog medium were treated with 200µM of calcium ionophore for 5, 10, 24 and 48 hours or not treated (controls). The level of *HvLysMR2* mRNA in each case is normalized to that of 18S rRNA as reference gene and controls at each time-point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al.,* 2002).

3.9 *HvC2d1*, *HvLysMR1* and *HvLysMR2* are induced during exposure to low concentrations of chromium, copper and cadmium

In order to investigate the fast reactions of the shoots to exposure to heavy metals, relative high concentrations of chromium, cadmium and copper have been chosen. As shown in Figure 2, in barley plants uptake and transport of such heavy metals to the leaves is rather slow (see also: Skeffington *et al*., 1976; Shenker *et al.,* 2001). Therefore barley plants have to be treated with relative high concentrations of heavy metals to analyse quick responses in the shoots. This is also proven by the physiological measurements (see chapter 3.3) showing the first stress reactions under the high concentrations only after 24 hours and by analyses of chromium content in the leaves (Fig. 2).

To further analyse expression of the genes *HvC2d1*, *HvLysMR1* and *HvLysMR2* in the leaves under very low concentrations of heavy metals, plants were treated with a 20 times lower concentration of the heavy metals (50 µM) and then expression of the receptor-like kinases and C2 domain protein were examined. Barley plants were grown 7 days on Murashige and Skoog medium and then treated with 50 µM of the same metals described above for 24, 48 and 72 h or not treated (controls). After exposure to low concentrations (50 µM) of chromium and cadmium, the stress parameters chlorophyll content and PSII efficiency did not change

(a) Chlorophyll content
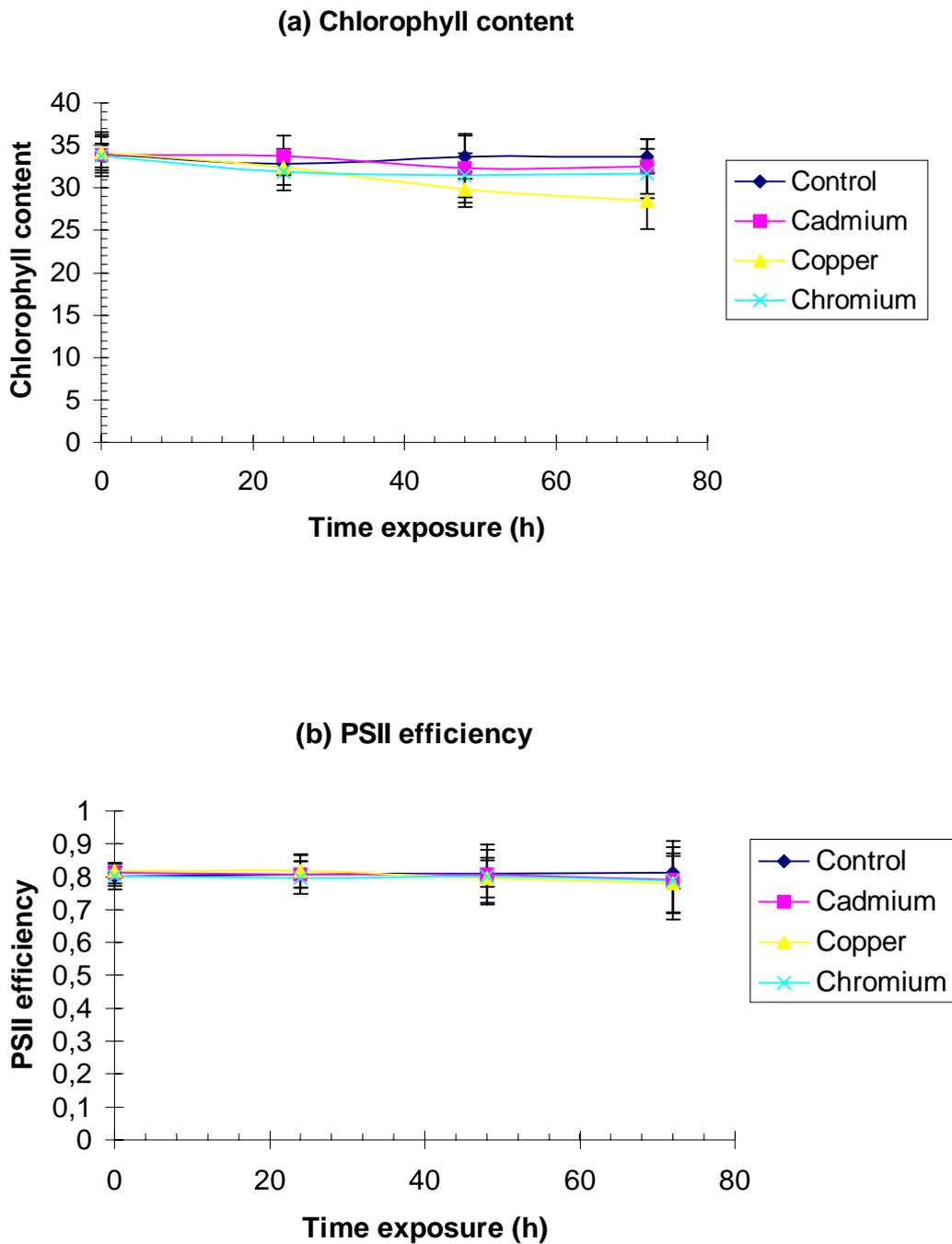
(b) PSII efficiency

Fig. 37: Effects of chromium, copper and cadmium treatment on chlorophyll content (**a**) and photosystem II (PSII) efficiency (**b**) in 7-d-old barley (*Hordeum vulgare* L. cv Steffi) seedlings cultivated on Muraschige and Skoog medium under controlled growth-chamber conditions (16 h at 21°C and a photosynthetic photon fluence rate (400-700 nm) of μmol m^{-2} s^{-1}; 8 h at 16 °C and in darkness), then either treated with 50 μM potassium dichromate or with 50 μM copper chloride or with 50 μM cadmium chloride for 24 to 72 h or not treated (controls). Each data-point represents the mean of 10 independent measurements and bars indicate standard errors.

significantly in the leaves during 72 h (Fig. 37a and 37b). The treatment with 50 μM copper resulted in a slight decrease in both parameters after 48 h (Fig. 37a and 37b). The changes in mRNA levels were investigated via quantitative real-time PCR by comparison of mRNA levels of the *HvLysMR1*, *HvLysMR2* and *HvC2d1* genes to levels of the reference 18S ribosomal RNA from treated samples and controls at different time-points.

3.9.1 *HvLysMR1*

The results of qRT-PCR showed that in barley seedlings treated with 50 μM copper chloride, *HvLysMR1* mRNA had already started to accumulate after 24 h and reached a maximum transcript level 48 h after onset of stress (6.4 times higher than in the control; Fig. 38). After this time-point, mRNA levels decreased again. These data indicate a transient expression pattern of *HvLysMR1*during the low concentration copper treatment. Low concentrations of chromium and cadmium (50 μM) caused a slight but significant increase in *HvLysMR1* mRNA levels after 72 h (2.1 times higher in the chromium-treated seedlings and 1.8 times higher in the cadmium-treated seedlings than in the control; Fig. 38).

3.9.2 *HvLysMR2*

The expression levels of *HvLysMR2* under low concentration (50 μM) of the heavy metals chromium, cadmium and copper was again investigated via quantitative real-time PCR in comparison to the controls. During copper treatment, the mRNA level of *HvLysMR2* started to accumulate after 24 h after treatment and reached a maximum level of this transcript after 48 h after onset of treatment (2.7 times more than in the control, Fig. 39). After this time-point, the relative mRNA levels of *HvLysMR2* decreased. These data indicate a fast and transient expression pattern after the exposure of the plant to the essential heavy metal copper. Cadmium and chromium treatments also resulted in increased mRNA levels of *HvLysMR2* 48 h after onset of treatments. At 72 h, the mRNA levels of *HvLysMR2* was 2 times higher in the chromium-treated seedlings and 1.4 times higher in the cadmium-treated seedlings compared to the control (Fig. 39).

Fig. 38: Effects of low concentration of chromium, cadmium and copper treatment on mRNA levels of the barley (*Hordeum vulgare* L.) gene *HvLysMR1* in primary leaves. RNA was extracted from primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings either treated with 50 μM potassium dichromate or with 50 μM copper chloride or with 50 μM cadmium chloride for 24 to 72 h or not treated (controls). The level of *HvLysMR1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls at each time-point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al*., 2002).

Fig. 39: Effects of low concentration of chromium, cadmium and copper treatment on mRNA levels of the barley gene *HvLysMR2* in primary leaves. RNA was extracted from primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings either treated with 50μM potassium dichromate or with 50 μM copper chloride or with 50 μM cadmium chloride for 24 to 72 h or not treated (controls). The level of *HvLysMR2* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls at each time-point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al*., 2002).

3.9.3 *HvC2d1*

During the treatment with low concentrations (50 μM) of copper or cadmium, the mRNA levels of *HvC2d1* exhibited similar expression pattern during both treatments. A maximum transcript level of *HvC2d1* was obtained after 48 h (15.6 times higher in the copper-treated seedlings and 12.7 times higher in the cadmium treated-seedlings compared with the untreated control, Fig. 40). In the later stages the relative mRNA levels of *HvC2d1* declined again. These results indicate a transient expression pattern of *HvC2d1* during exposure to essential (copper) and non-essential (cadmium) heavy metals. During chromium treatment, the mRNA level of *HvC2d1* increased significantly (Fig. 40).
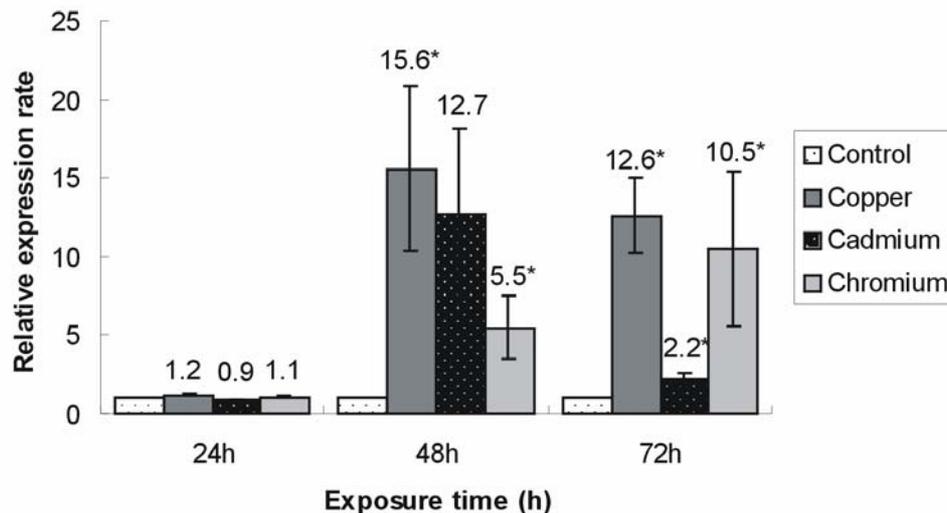
Fig. 40: Effects of low concentration of chromium, cadmium and copper treatment on mRNA levels of the barley gene *HvC2d1* in primary leaves. RNA was extracted from primary leaves of barley (*Hordeum vulgare* L. cv. Steffi) seedlings either treated with 50 μM potassium dichromate or with 50 μM copper chloride or with 50 μM cadmium chloride for 24 to 72 h or not treated (controls). The level of *HvC2d1* mRNA in each case is normalized to that of 18S rRNA as a reference gene and controls at each time-point are set as 1. Error bars indicate the standard deviation (n=6) and an asterisk indicates significant differences (calculated using the formula of Pfaffl *et al*., 2002).

4. Discussion

4.1 Heavy metal stress in plants

During their entire life cycle, plants are constantly faced with diverse environmental stresses. Some of these stress factors may change slowly and gradually affect plant growth conditions (Schützendübel & Polle, 2002). The adverse environmental conditions, known as `environmental` or `abiotic stresses´, are major limiting factors of crop productivity. It has been estimated that more than 70 % of the maximum genetic potential yield of major crops may be lost by various abiotic stresses (Boyer, 1982). The term abiotic stress comprises physical stressors like high or low temperatures or radiation and also chemical stressors like heavy metals. Among the approximately 90 elements present in the earth`s crust, about 80 % are metals and 60 % are heavy metals with specific weights higher than 5 g cm^{-3} (Sharma & Dietz, 2006). Other elements with only partial metal propreties such as As and with a specific weight lower than 5 g cm^{-3} such as aluminum also need to be considered due to their toxicity to plants (Sharma & Dietz, 2006). Human activities, especially over the past 200 years, have resulted in the massive release of heavy metals into the environment (Nriagu & Pacyna, 1988). Uptake of the metal ions by crop plants is the main entry pathway into human and animal food (Roth *et al.,* 2006).

In plants as in other organisms, heavy metals can severely impair central metabolic processes. One primary target in plants is the photosynthetic apparatus. Heavy metals can inhibit photosynthesis at several structural and metabolic levels. One obvious result of heavy metal stress is often a decrease in chlorophyll content (Krupka *et al*., 1993; Chugh & Sawhney, 1999). This can be explained on one hand by the inhibition of chlorophyll biosynthesis at the level of the enzyme ALA-dehydratase and on the other hand by the destruction of active chlorophylls via the substitution of the central Mg^{2+} ion of the chlorophyll (Küpper *et al*., 1996, 2003), and is proposed as an important damage mechanism leading to inhibition of photosynthesis (Küpper *et al*., 1996, 2003). Chlorophyll content is also known to be reduced by heavy metals because of oxidative stress related to the action of heavy metals (Gallego *et al*., 1999). Furthermore, heavy metals such as chromium induce disorganisation of the chloroplast ultrastructure and inhibition of electron transport processes (Shanker *et al.,* 2005). In addition, a diversion of electrons from the electron-donating side of PSI to Cr(VI) is a possible explanation for Cr-induced decrease in photosynthesis rate (Shanker *et al*., 2005). It is possible that electrons produced by the photochemical process were not necessarily used for

carbon fixation as evidenced by a low photosynthetic rate in Cr-stressed plants (Shanker *et al.*, 2005). Furthermore, cadmium was shown to also inhibit calvin cycle activities mainly by the reduction in rubisco activity (Cagno *et al.*, 2001). Another potential cause of toxicity is the high affinity of copper and cadmium ions to functional groups of biological molecules, in particular SH groups, O- and N-containing groups, which render molecules inactive (Weber *et al.*, 2006). Cd^{2+} ions are hypothesized to replace metal cofactors such as Zn^{2+} ions from proteins or compete with Ca^{2+} for binding to Ca^{2+}-binding proteins (Stohs & Bagchi, 1995). In addition, heavy metals such as Cu can elicit the formation of hydroxyl radicals through the Fenton and Haber-Weiss reactions (Halliwell & Gutteridge, 1990). This rather incomplete listing shows that heavy metals cause severe damages in plants and that there are multiple molecular targets of heavy metal damage.

But, heavy metals do not have only toxic effects. Some metals are actually essential micronutrients fulfilling many crucial functions in plant metabolism. For example, copper acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses and hormone signalling (Marschner, 1995; Raven *et al.*, 1999). In order to cope with the dilemma that on one hand to a certain amount some metals have to be taken up, transported and finally assembled with their specific target apoproteins and that on the other hand surplus and toxic metal species have to be detoxified, plants evoked a complex regulatory network (Clemens *et al.*, 2002). We are still far from understanding the mechanisms underlying this network in plants which involves signal perception and signal transduction and changes in gene expression. Up to now our knowledge about how plants perceive the specific signals involved in heavy metal stress and heavy metal homeostasis and how they subsequently trigger signals to activate physiological responses is rather incomplete (Hung *et al.*, 2005). In the next chapter (4.2) some factors known to be related to these processes are discussed.

4.2 Factors involved in heavy metal stress response and heavy metal homeostasis in plants and other organisms

In general, membrane-bound receptors-like kinases play fundamental roles in signal transduction chains in all organisms by recognizing signals from the environment and from others cells, and consequently activating downstream signalling cascades (Santiago & Frans, 2006). Further components of abiotic stress signalling cascades are mitogen-activated protein kinases (MAPKs) (Nakagami *et al.*, 2005), calcium and phosphatidic acid (Testerink &

Munnik, 2005). Compared to several other stresses (drought, cold, high salt), the mechanisms by which plants perceive and transmit heavy metal signals to cellular systems to initiate physiological responses are poorly understood. To date only a few of the key players in the signalling processes connected to heavy metals have been identified in plants. Mitogen activated protein kinases (MAPKs), known as mediators of stress responses, were found to be activated under Cu and Cd excess in *Medicago sativa* (Jonak *et al.*, 2004). The identification of different MAPK kinases and their different expression kinetics lead the authors to suggest that distinct metal-specific signalling pathways exist in plants (Jonak *et al.*, 2004). Furthermore, specific up-regulated responses to Cd exposure have been identified in the unicellular organisms *S. cerevisiae* and *Schizosaccharomyces pombe* (Vido *et al.*, 2001; Chen *et al.*, 2003). In *S. cerevisiae*, the sulphur amino acid biosynthesis pathway is activated by Cd, but not by other abiotic stress treatments (Chen *et al.,* 2003). Recently, Weber *et al.* (2006) showed a specific up-regulation of genes in response to Cd exposure and not by Cu in *Arabidopsis thaliana* roots. Among them you find zinc finger (C_2H_2) proteins, metal transporters (ZIP9), calmodulin-binding protein and ethylene receptor-related protein. Interestingly, a specific Cd metabolic regulation mediated by ubiquitin-ligase pathways could be shown recently in both *S. cerevisiae* and *S. pombe* (Harrison *et al.*, 2005; Barbey *et al.*, 2005).

Another signalling molecule, the phytohormone jasmonate was proposed to be involved in the transcriptional control of GSH biosynthesis genes under metal excess (Xiang & Oliver, 1998). It was shown that jasmonate treatment, but not application of other stress signals such as salicylic acid or H_2O_2, leads to an up-regulation of *gsh1* and *gsh2* in *Arabidopsis* (Xiang & Oliver, 1998). In addition, copper and cadmium treatment induced accumulation of jasmonates in mature leaves of *Arabidopsis thaliana* and in *Phaseolus coccineus* plants (Maksymiec *et al.*, 2005). Furthermore, bZIP Nrf2 protein has been shown to be an important transcription factor for cadmium response and its overexpression leads to up-regulation of genes involved in glutathione production in certain cell types (Hayes *et al.*, 2000). In bacteria, the ArsR-SmtB family of repressors is one group of DNA-binding proteins that detect metals to maintain supplies for metalloproteins while avoiding toxic excesses (Harvie *et al.*, 2006). One example is Cmtr from *Mycobacterium tuberculosis* which represses the expression of Cd^{2+}-efflux pumps (Cavet *et al.*, 2003).

In *Saccharomyces cerevisiae*, metal-sensing transcriptional regulators described specifically bind Fe (Atf 1), Cu (Mac1, Ace1) or Zn ions (Zap1) (Winge *et al.*, 1998). They modulate expression of genes that encode proteins involved in cellular uptake of copper, iron and zinc ions (Winge *et al.*, 1998). In other fungi, copper homeostasis is controlled in a similar manner by related transcription factors, Amt1, Cuf1, and GRISEA (Rutherford & Bird, 2004).

Copper homeostasis in plants has been studied in the *Chlamydomonas* model (Merchant, 1998). Among several responses to copper deficiency in *Chlamydomonas*, the *Cyc6* gene is activated to provide a copper-independent substitute for plastocyanin in the photosynthesis electron transfer chain (Quinn & Merchant, 1995). The *Cyc6* gene is activated in copper-deficient cells via a signal transduction pathway that requires copper response elements (CuREs) that contain a critical GTAC core sequence and the copper response regulator (*CRR1*) (Quinn *et al.*, 2003). This sequence is different from the fungal CuREs bound by the Ace1/Mac1 family of transcription factors and from the metal response elements bound by MTFs, suggesting a different DNA-binding domain in the *Chlamydomonas* copper signalling pathway (Kropat *et al.*, 2005).

The transcriptional activator MSN1 was found to promote Cr accumulation in yeast (Chang *et al.*, 2003). Furthermore, Kim *et al.* (2006) showed that overexpression of transcriptional activator MSN1 enhances Cr and sulphur accumulation in transgenic tobacco. In addition, the mRNA levels of *NtST1*(*Nicotiana tabacum* sulfate transporter 1) was elevated in MSN1-expressing transgenic tobacco, suggesting that chromate and sulfate are taken up via the sulfate transporter in plants (Kim *et al.*, 2006). This was also suggested by Smith *et al.* (1995), since the sulfate uptake mutants are resistant to chromate.

In mammals and other metazoans, the zinc finger transcription factor MTF-1 (metal transcription factor-1) plays a central role in the metal-inducible transcriptional activation of metallothionein and other genes involved in metal homeostasis and cellular stress response (Saydam *et al.*, 2001, 2002). In addition, it has been reported that metal-activated metallothionein transcription via MTF-1/MRE interactions is controlled by signal transduction pathways that involves protein kinase C, casein kinase II, tyrosine kinase, calcium and the MAP-kinase signalling pathway (Saydam *et al.*, 2002). Recently, Qi *et al.* (2006) showed that the bean PvSR2 gene (*Phaseolus vulgaris* stress-related gene 2) responds to heavy metals but not to other forms of environment stresses. The promoter region of PvSR2 gene contains two motifs (MRE-like sequence). The first motif is similar to the

consensus metal-regulatory element (MRE) of the animal metallothionein gene and the second had no similarity to known *cis*-acting elements involved in heavy metal induction, suggesting the presence of a novel heavy metal regulatory element (HMRE). In plants no metal-sensor protein has been identified yet.

In order to further clarify the mechanisms underlying heavy metal stress response and/or regulation of metal homeostasis in plants in this work I tried to identify novel factors involved in these processes by transcriptome analysis of rapid chromium response in barley leaves. The aim was to identify candidate genes coding for putative regulatory factors involved in heavy metal stress response.

4.3 The RFDD-PCR approach yielded novel heavy metal induced genes

Because of its increasing importance as pollutant (Krassoi & Julli, 1994) potassium dichromate was chosen for the treatment. The first step on the way to identify novel chromium induced genes was to set up an experimental system which allows analysis of fast responses of chromium stress in the leaves which are important for biomass production. Based on the physiological data which measure the impact of chromium on the sensitive photosynthesis process using a hydroponic growth system an experimental set-up was established which allows to analyse early stress responses (see 3.1). Prior to the transcriptome analysis with this system, the expression of several already known heavy metal induced genes was investigated. Since these studies yielded new results concerning the chromium dependent expression of these known genes, in the following chapter I will first discuss the expression patterns of these genes, before in the thereon following chapters I will deal with the novel genes investigated in this thesis.

4.3.1 Chromium dependent expression of known heavy metal regulated genes

To characterise the response of plants to the chosen experimental conditions of chromium, cadmium and copper on a molecular level and to determine the time point needed for the fast transcriptome analysis of the chromium treated barley leaves, the expression of the known heavy metal stress marker genes was analysed.

Among the heavy metal marker genes tested in this study, the *HvMT-1a* and *HvMT-2a* genes encoded for metallothioneins proteins. Metallothioneins are small cysteine-rich proteins (50-70 amino acids) and are known to play a role in detoxifying heavy metals by sequestration using cysteine residues (Bertini *et al.,* 2000). In plants, MTs are thought to play an important

role in metal tolerance and homeostasis (Cobbett & Goldsbrough, 2002). They have been divided into two classes based on amino acid sequence. Class I includes primarily mammalian MTs that contain 20 highly conserved Cys residues (Klaassen *et al.,* 1999). Metallothioneins from plants and fungi, as well as invertebrate animals, are grouped in Class II (Robinson *et al.*, 1993). MTs are regulated by many factors, including wounding (Choi *et al.*, 1996), pathogen infection (Choi *et al.*, 1996; Butt *et al.*, 1996), heavy metal stress (Zhou & Goldsbrough, 1994; Guo *et al.*, 2003; Kohler *et al.*, 2004) and during leaf senescence (Bhalerao *et al.*, 2003). This suggests that MTs may be expressed as part of a general stress response (Cobbett & Goldsbrough, 2002). It has been proposed that MTs may be involved in chaperoning released metal ions to protect cells from metal toxicity or metal induced oxidative stress during the complex senescence program (Buchanan-Wollaston, 1994; Butt *et al.*, 1998). However, their exact function is still not completely understood. In mammals, MTs maintain zinc and copper homeostasis and protect cells against cadmium toxicity and oxidative stress (Palmier, 1998; Klaassen *et al.*, 1999; Coyle *et al.*, 2002). The induction of *HvMT-1a* during chromium, cadmium and copper treatments (Fig. 4) confirmed that the transcription of MT genes is regulated by different heavy metals (Thiele, 1992). In addition, this results indicates that different barley MTs exhibit quite different expression patterns. Finally, the increase in *HvMT-1a* mRNA in later stages (144h) in the control which indicates the fast onset of senescence process in barley leaves (Fig. 4).

A second heavy metal marker gene analysed is homologous to a ClpD protein. Most knowledge about the Clp protease is derived from the studies of the model enzyme of *Escherichia coli*. The Clp holoenzyme consists of two adjacent heptameric rings of the proteolytic subunit known as ClpP, which are flanked by a hexameric ring of a regulatory subunit from the Clp/Hsp100 chaperone family at one or both ends (Porankiewicz *et al.,* 1999). Clp proteins are found to play a role in different processes such as sporulation, DNA replication, protein turnover, stress tolerance, and gene regulation in many different bacteria and eukaryotes (Porankiewicz *et al.,* 1999). In plants, ClpD proteins are induced during different stress situations like wounding, salt, cold and heat stress (Zheng *et al.*, 2002). In the previous work of Heise (2004) and Heise *et al.* (2007), it could be shown that barley ClpD was induced during leaf senescence and heavy metal stress. Furthermore, senescence induced Clp protease in barley plants was reported by Humbeck and Krupinska (1996). In *Arabidopsis,* distinct ClpP isomers with different localisations were reported (Zheng *et al.*, 2002). The function of Clp proteins in *Arabidopsis* remains unknown, especially those

localised in chloroplasts like ClpC, -D, -P1, P6 and ClpP3-5 (Zheng *et al.*, 2002). During chromium treatment, the *HvClpD* mRNA had already started to accumulate during the first 48 h and stayed almost stable during the prolonged time of treatment (Fig. 6).

In addition, the *HvBsi* gene encoded for a small cysteine-rich protein was reported to be induced by pathogens, heavy metals and during leaf senescence (Stevens *et al*., 1996; Heise, 2004).

To summarize the results of this experiment, northern blot analysis showed that heavy metal marker genes (*HvMT-1a*, *HvClpD*, *HvBsi* and *Cdi2*) are induced during cadmium and copper treatments. In addition, these marker genes are also chromium dependent expression. They are modulated during the first 48 h after onset of chromium treatment (Figs. 2, 4, 5 and 6). Therefore the 48 h chromium treated probe was selected for the transcriptome analysis. Furthermore, this result indicates that the response of barley plants to chromium stress is modulated by changes in gene expression.

4.3.2 Transcriptome analysis of chromium response in barley leaves

The RFDD-PCR polyacrylamide gels showed differential transcription of cDNAs in barley leaves after chromium treatment compared with controls (Fig. 10). This approach yielded 48 cDNA fragments, presumabely representing genes up-regulated during chromium exposure (see Table 1). The sequence analyses revealed that the identified genes belong to different functional groups, which indicates that Cr affected different physiological and biochemical pathways. Among the identified genes, two appeared to encode proteins involved in signalling pathways, such as a receptor-like kinase and a C2 domain protein. Chromi1 with 183 bp length presented homology to a receptor-like kinase from *Oryza sativa* (BAD01244). Receptor-like kinases play a role in the perception of the extra cellular signal and its transmission via phosphorylation cascades which lead to changes in gene expression patterns (Robatzek & Somssich, 2002). Chromi 12 showed homology to a C2 domain-containing protein from *Oryza sativa* (BAD09616). The C2 domain is a Ca^{2+}-dependent membrane targeting module found in many cellular proteins involved in signal transduction or membrane traffiking, like protein kinases and phospholypid-modifying enzymes such as phospholipase D (Nafelski & Falke, 1996; Cho, 2001). The results concerning these two genes are discussed below in more detail.

Another class of genes identified are genes involved in pathogen responses (Chromi 6 and Chromi 10). Chromi 6 showed (43/48 aa) a homology to barley thaumatin-like protein TLP5 (AAW21725). Thaumatin-like proteins (TLPs) are polypeptides of about 200 residues synthesized by plants in response to fungal infection (Brandazza *et al.,* 2004). Heavy metals induction of thaumatin-like protein were reported by Rakwal *et al.* (1999) and Sarowar *et al.* (2005). In addition, the overexpression of a pepper basic pathogenesis-related protein 1 gene in tobacco plants enhances resistance to heavy metal stress (Sarowar *et al.,* 2005). In Arabidopsis, so-called pathogenesis-related protein of the PR-10 family was shown to be induced during Cd^{2+} treatment (Roth *et al.,* 2006). The identifiction of pathogen response genes indicated that Cr induced defence reactions. The biological reason for this might be that during the stress plants are more susceptible to pathogen attack and therefore up-regulation of at least part of the pathogen response program as a protection mechanism is reasonable.

One gene (Chromi 8) that responded to Cr shows homologies to a heat shock protein. Heat shock proteins (HSPs) are found in all groups of livings organisms and are classified according to their molecular size. They are known to be expressed in response to a variety of stress conditions including heavy metals such as copper (Lewis *et al.*, 2000). They may also act as molecular chaperones in normal protein folding and assembly, but can also function in the protection and repair of proteins under stress conditions (Hall, 2002). There have been several reports of an increase in HSP expression in plants in response to heavy metal stress. Neumann *et al.* (1994), have shown that HSP70 also responds to cadmium in cell culture of *L. Peruvianum*. In addition, localisation studies revealed that HSP70 was present in the nucleus and cytoplasm, but also at the plasma membrane. This suggests that HSP70 could be involved in the protection of membranes against cadmium damage. Furthermore, the identification of HSP in this study, indicated that protein denaturation is one of the effects of Cr toxicity.

Among the Cr-regulated genes detected, Chromi 3 showed a homology to 2-nitropropane dioxygenase protein from *Arabidopsis thaliana* (NP_568988) found in the database (data not published). It is known from others organisms, that the enzyme catalyses the oxidative denitrification of primary or secondary nitroalkanes to the corresponding aldehydes or ketones with the production of hydrogen peroxide and nitrite (Gadda *et al.,* 2000). Nitroalkanes are widely used as industrial solvents and are toxic. Thus, an enzymatic activity that converts nitroalkanes into less harmful species has significant potential for bioremediation (Gadda *et*

al., 2000). Interestingly, a 2-nitropropane dioxygenase like protein has recently been found to be activated under Cd treatment in *Arabidopsis thaliana* (Roth *et al.*, 2006).

In addition, two genes identified in this study, Chromi 2 and Chromi 5, belong to the family of genes already known as heavy metal induced genes. Chromi 2 showed 90 % homology to the wali 6 protein already known to be induced during aluminum treatment in wheat roots (*Triticum eastivum* L.) (Richards *et al.*, 1994). Wali 6 encodes a small protein of 9.4 kDa including an hydrophobic leader and shows a homology to Bowman-Birk proteinase inhibitors (Ikenaka & Norioka, 1996) and to wound-inducible genes from maize (Rohrmeier & Lehle, 1993). The second gene chromi 5 encodes for a ferritin protein. The iron storage ferritin protein has a structure highly conserved among plants, animals, and bacteria that can store 4500 atoms of iron (Masuda *et al.,* 2001; Zancani *et al.*, 2004). However, specific features characterise plant ferritins, among which are plastid cellular localisation and transcriptional regulation by iron (Briat *et al.,* 1999; Wei & Theil, 2000). Furthermore, Zancani *et al.* (2004) showed the presence of ferritin protein in plant mitochondria. *Arabidopsis* contains four genes that encode ferritin (*AtFer1- AtFer4*) which are differentially expressed during high iron treatment in roots and leaves (Connolly & Guerinot, 2002). Another gene chromi 7 showed a homology to an outer membrane protein from *Escherichia coli* known to be involved in iron transport in bacteria (Waschi *et al.,* 1996). This suggests that the response is not specific to the chromium in plants. Also, some effector proteins are responsive to more than one heavy metal.

Sequencing analysis revealed that one gene identified (Chromi 11) is homologous to a barley glutathione S-transferase (AAL73394). Glutathione S-transferases are encoded by a large and diverse gene family in plants, which can be divided on the basis of sequence identity into the phi, tau, theta, zeta and lambada classes (Dixon *et al.*, 2002). Glutathione S-transferases (GSTs) catalyse the conjugation of the tripeptide glutathione (GSH, γ-glutamyl-cysteinyl-glycine) to a wide range of xenobiotic or natural, often cytotoxic compounds via the nucleophilic thiol residues (Moons, 2003; Grzam *et al.*, 2006). Plants GSTs have adapted to fulfill a diverse range of functions such as oxidative stress tolerance, cell signalling like the induction of chalcone synthase and transport of flavonoid pigments to the vacuole (Dixon, 2002; Moons, 2003). Tau class glutathione S-transferases, first identified as being induced by auxins, have been shown to be involved in the response to a variety of endogenous and

exogenous stresses including pathogen attack, wounding, heavy metal toxicity, oxidative and temperature stress (Marrs & Walbot, 1997; Suzuki *et al.*, 2001; Frova, 2003).

The gene chromi 4 showed sequence similarity to nodulin 8-precursor protein from *Oryza sativa*. The genes coding for nodulin proteins are induced during the formation and function of a root nodule (Asif *et al.*, 2004). It was shown that by overexpression of a gene encoding nodulin protein from *Phaseolus vulgaris* L in *Escherichia coli,* cells confer protection against oxidative stress suggesting its possible role in plant host protection from oxidative toxicity during the *Rhizobium*-legume symbiosis (Asif *et al.*, 2004). The newly identified gene might therefore encode a protein also involved in the response to oxidative stress caused by the presence of the heavy metal. The isolation of a chorismate mutase known to be involved in amino acid synthesis (Mobley *et al.*, 1999) could also indicate an increase in amino acid synthesis in Cr-treated plants.

Finally, the heavy metal stress-dependent expression of the 14 genes isolated during chromium treatment could be confirmed by northern analyses and qRT-PCR (Fig. 11). In addition, the RFDD-PCR technique allowed the identification of genes whose expression was modulated by Cr. Furthermore, this study revealed that a multitude of processes are implicated in determining response to metal in plants which leads to the activation of differents sets of genes.

As discussed above, the mechanisms by which plants perceive and transmit heavy metal signals to cellular systems to initiate these physiological responses are not yet understood. Therefore, it was of interest to further characterize the genes showing homology to proteins involved in signalling pathways. In this thesis two genes were further characterized: chromi 12 encoding a putative C2-domain protein and chromi 1 encoding a novel LysM receptor-like kinase.

4.4 Characterization of *Hordeum vulgare* C2-domain protein

After full-length sequencing it was shown that the chromi 12 gene encodes a protein with a conserved calcium binding C2 domain-like motif at the N-terminal end and a nuclear localization signal (NLS) (Fig. 12c). C2 domains are known to interact with membranes in a Ca^{2+}-dependent manner and are found in various types of protein, such as protein kinases,

phospholipid-modifying enzymes, and so-called small C2-domain proteins with a single C2 domain (Kim *et al.*, 2003).

The expression studies (Fig. 14) showed that the mRNA of this gene is transiently induced in the early phase of chromium treatment (high concentration) and accumulated also during low chromium concentration exposure (Fig. 40). Sequence alignment revealed that the novel chromium induced gene is similar to a subgroup of C2-domain genes coding for the so-called small C2-domain proteins which, in contrast to other known C2-domain proteins, contain only a single C2 domain and do not have further conserved domain motifs such as, for example, protein kinase domains (Kim *et al.*, 2003). Only a few small C2-domain proteins are known in plants and none have been found in animals (Kim *et al.*, 2003). The functions of the already described small C2-domain proteins are not yet clear. In pumpkin a small C2-domain protein has been reported to increase the size of mesophyll plasmodesmata to transport cellular materials, including RNA molecules, from cell to cell (Xoconostle-Cazares *et al.*, 1999). In rice, the isolation and characterization of two small C2-domain proteins, named OsERG1a and OsERG1b, which are induced by a fungal elicitor have been reported (Kim *et al.*, 2003). Kim *et al.* (2003) proposed that small C2-domain proteins play a role in the defense signalling systems in plant cells. There are no other reports on the function of small C2-domain proteins in plants.The novel protein HvC2d1 has a much higher molecular weight than the known small C2-domain proteins. Four other sequences recently added to the NCBI database show these characteristic features of HvC2d1 (AtC2-2, At1g07310, OsBAD09616 and OsBAB84404 in Fig. 13), and cluster in their own group (Fig. 12b). Interestingly, one of these sequences also has a NLS-like motif (OsBAD09616).

Many but not all known C2-domain proteins have conserved aspartate residues implicated in Ca^{2+} coordination (amino acids 15, 21, 71, 73, 78 and 79 in the consensus C2 domain pfam00168). As shown in Fig. 12a, the novel protein HvC2d1, the Arabidopsis proteins AtC2-2 (AAV85706) and At1g07310, and also the rice proteins OsBAD09616 and OsBAB84404 do not exactly follow this pattern but have either aspartates nearby or other amino acids which might also be capable of Ca^{2+} coordination (threonine, glutamine, serine and asparagine). In spite of this variability, HvC2d1 is clearly able to bind calcium, as shown by the $^{45}Ca^{2+}$ overlay analysis (Fig. 20c), indicating the presence of a functional C2-domain. Such variability in the Ca^{2+} binding sites of different C2 domain proteins is also found in other C2-domain proteins and seems to be important for the specialization of these different

C2-domains, presumably to provide optimized Ca^{2+} binding parameters, to allow specific changes in conformation upon Ca^{2+} binding or docking interactions for different biological functions (Nalefski & Falke, 1996). From work with animal systems it is known that, in general, the C2-domain is a Ca^{2+}-dependent membrane-targeting module found in many cellular proteins involved in signal transduction or membrane trafficking (Nalefski & Falke, 1996; Cho, 2001). This membrane targeting domain shows a wide range of lipid selectivity for the major components of cell membranes (Stahelin *et al.*, 2003). The majority of C2-domains bind the membrane in a Ca^{2+}-dependent manner and thereby play an important role in Ca^{2+}-dependent membrane targeting of peripheral proteins (Stahlen & Cho, 2001). Recent investigations of Ca^{2+}-activated membrane docking of the C2-domain protein phospholipase A2 have revealed an ordered binding of two Ca^{2+} ions with positive cooperativity (Malmberg *et al.,* 2004).

The novel barley *HvC2d1* has revealed a stress- and development-dependent expression pattern. I have shown for the first time the induction of a gene encoding a C2 domain protein during heavy metal stress and leaf senescence. Senescence is the last stage of leaf development and one type of programmed cell death that occurs in plants (Quirino *et al.*, 2000). During senescence, nutrients released after catabolism of macromolecules, including heavy metals such as Cu and Zn, are remobilized to younger leaves (Himelblau & Amasino, 2001). It is known from the literature that heavy metal stress results in degradation processes which partly resemble those observed during leaf senescence (Chen & Kao 1999; McCarthy *et al.*, 2001). This is also shown by the decrease in chlorophyll content and in photosystem II efficiency which occurs in the heavy metal stress experiments (Figs. 24 and 25) shown in this study as well as during leaf senescence (Miersch *et al.*, 2000). Also, it could be shown that several heavy metal induced genes are also up-regulated during leaf senescence (Himelblau *et al.*, 1998; Himelblau & Amasino, 2000; Guo *et al.*, 2003). This may indicate an overlap in the regulatory mechanisms underlying heavy metal homeostasis and leaf senescence. In addition, senescence processes can be either induced by internal signals like age or phytohormones or by external stressors (Gan & Amasino, 1997; Bleecker & Patterson, 1997; Kleber-Janke & Krupinska, 1997; Quirino *et al.*, 2000; Humbeck & Krupinska, 2003; Jing *et al.*, 2003).

Not much is known about the signalling pathways underlying the induction of heavy metal-induced genes or the onset of leaf senescence. One common signal could be reactive oxygen species (ROS), which accumulate in response to abiotic and biotic stressors (Neill *et al.*, 2002;

Mithöfer *et al.,* 2004), and have also been suggested to play a role in the induction of leaf senescence (Ye *et al.,* 2000; Krupinska *et al.,* 2003). In addition, the abscisic acid hormone has been shown to have a wide range of important roles in plant growth and development including embryo development, seed maturation, root and shoot growth, leaf transpiration and stress tolerance (Koornneef *et al.,* 1998; Leung & Giraudat, 1998; McCourt, 1999). Furthermore, H_2O_2 was demonstrated in *Arabidospsis* to play a prominent role in the transduction of ABA signals by regulating the activity of phosphatase (Meinhard & Grill, 2001; Meinhard *et al.,* 2002). In order to determine whether the accumulation of ROS also induces the novel *HvC2d1* gene, plants were treated with methylviologen to generate superoxid anion radicals by uptake of an electron from photosystem I (Donahue *et al.,* 1997). The data show a clear induction of *HvC2d1* during the methylviologen treatment. Interestingly, *HvC2d1* is induced by ABA but not by drought, indicating a signalling pathway different from that of many other genes regulated by ABA. A similar expression pattern was shown for a pathogen-related gene that was induced by high salinity, ABA and wounding, but not by drought and cold stress (Jung *et al.,* 2004). These results indicate that ROS and ABA are involved in the signalling pathway.

Another secondary signal involved in growth, developmental regulation and in plant responses to biotic and abiotic stressors is Ca^{2+} (Knight, 2000; Reddy, 2001). It has been shown that calcium-dependent kinases play a role in different stress reactions in plants (Harmon *et al.,* 2000; Romeis *et al.,* 2001; Cheng *et al.,* 2002). A second class of calcium sensor proteins a calcineurin B-like protein (CBL) was identified from *Arabidopsis* and has been implicated as an important relays in Ca^{2+} signalling in abiotic stress response (Kudla *et al.,* 1999). These proteins harbor EF-hand motifs for calcium binding and interact specifically with a group of serine-threonine protein kinases (CBL-interacting protein kinases) (Shi *et al.,* 1999). Furthermore, there are several reports indicating that signalling via Ca^{2+} and via ROS is connected (Himmelbach *et al.,* 2003; Olmos *et al.,* 2003). It is also known that the small C2-domain containing protein OsERG1 is induced in response to H_2O_2 (Kim *et al.,* 2003). The present data concerning the novel heavy metal and senescence-induced gene *HvC2d1,* which has a calcium related C2-domain and is induced during the accumulation of ROS, also indicate that calcium and ROS might be involved in the underlying signalling pathways. Such C2-domain proteins are known to be expressed in a Ca^{2+}-dependent manner (Kim *et al.,* 2003). I also clearly showed that the novel gene *HvC2d1* is induced in response to calcium ionophore A23187, which is known to enhance the calcium influx (Torrecilla *et al.,* 2001).

Known C2-domain proteins are involved in Ca^{2+} signalling or membrane trafficking processes mediated by Ca^{2+}-dependent binding of C2-domains to membrane phospholipids (Kim *et al.*, 2003). By using GFP constructs, Kim *et al.* (2003) showed that the small C2-domain protein OsERG1 is translocated to the plasma membrane of plant cells by treatment with a Ca^{2+} ionophore and also by a fungal elicitor. Immunocytochemical analyses with the other known small C2-domain protein from pumpkin (CmPP16-1) also suggested an association with the plasma membrane (Xoconostle-Cazares *et al.*, 1999). Analyses of mammalian C2 proteins, for example phospholipases, synaptogamin I and protein kinase C, also showed that these proteins migrate after binding of Ca^{2+} from the cytosol to the plasma membrane and thus are able to transduce foreign signals into the cell (Pepio & Sossin, 2001; Teruel & Meyer, 2002; Ananthanarayanan *et al.*, 2002). In contrast to these other C2-domain proteins, the novel HvC2d1 protein contains a NLS, indicating a nuclear localization. Consistent with the presence of this motif, my localization studies using HvC2d1-GFP constructs showed a calcium-dependent nuclear localization. This is the first time that a calcium-dependent translocation of a C2-domain protein to the nucleus has been shown. It is well known that cytosolic Ca^{2+} fluctuations act as intracellular secondary messengers in the responses of plants to a number of stimuli, including ABA, osmotic stress, ionic stress and oxidative stress (Knight, 2000; Sanders *et al.*, 2002). In these signalling processes the kinetics, amplitude and duration of Ca^{2+} transients are important for the transmission of specific informations (Allen *et al.*, 2000; Rudd & Franklin-Tong, 2001; Sanders *et al.*, 2002). Recent data have shown that Ca^{2+} concentrations in response to different external stimuli vary not only in the cytosol but also in other cell compartments such as chloroplasts (Johnson *et al.*, 1995) and the nucleus (Pauly *et al.*, 2000; Xiong *et al.*, 2004), indicating the complex spatio-temporal characteristics of Ca^{2+} signatures. In the Ca^{2+}-mediated signalling networks in plants, the calcium signatures are decoded by calcium sensors such as calmodulin and other calcium-binding proteins (Yang & Poovaiah, 2003). Interestingly, there are several reports showing that such calcium-binding proteins are not only localized in the cytoplasm but also in the nucleus. Among these proteins with nuclear localization are calcium-dependent protein kinases (Dammann *et al.*, 2003; Chehab *et al.*, 2004), a novel calmodulin-binding protein (Perruc *et al.*, 2004) and an activator of a H^+/Ca^{2+} antiporter (Cheng *et al.*, 2004). In addition, the calcium-dependent protein kinase McCPK1 from ice plant (*Mesembryanthemum crystallinum*) was shown to undergoe a reversible change in subcellular localization from the plasma membrane to the nucleus, endoplasmatic reticulum and actin filaments of the cytoskeleton in response to environmental stimuli (Chehab *et al.*, 2004). The novel HvC2d1

protein identified with a calcium-binding C2 domain-like motif which also shows, in a calcium-dependent manner, localization in the nucleus. From my results, I can not definitely conclude that the nuclear localization is a direct response of the binding of calcium to the C2 domain. Other calcium-dependent factors could also be involved in this process. However, despite this uncertainty, the results strongly indicate that HvC2d1, in the context of other calcium-dependent factors, plays a role in nuclear localized calcium signalling processes both in response to external stressors such as heavy metals and also in the specific developmental phase of senescence. Further studies are needed to elucidate the function of this novel C2-domain protein in the calcium signalling network.

4.5 Characterization of *Hordeum vulgare* LysM receptor-like kinases

Chromi 1 was the second gene selected for futher detailed analysis in this study. The derived amino acid sequence exhibits at the N-terminal end a hydrophobic stretch of 27 amino acids which is predicted to act as a signal peptide for the secretory pathway by the TargetP 1.1 Server from CBS analysis (Center for Biological Sequence Analysis from Technical University of Denmark). Furthermore, the sequence shows two LysM motifs which are typical for the lysine motif receptor-like kinases (Limpens, 2003; Madsen *et al.,* 2003), a hydrophobic membrane-spanning segment and a conserved serine / threonine kinase domain with eleven characteristic subdomains of proteins kinases (Radutoiu *et al.*, 2003). The LysM protein module is found among both prokaryotes and eukaryotes (Pontig *et al.*, 1999), and was first identified in bacterial lysin and muramidase enzymes that degrade cell wall peptidoglycans (Joris *et al.*, 1992; Pontig *et al.*, 1999). The extracellular domain of the novel protein contains two LysM motifs showing homologies to the conserved LysM domain (PF01476) of plant receptor-like kinases and amino acid sequences of LysM motifs of known LysM receptor-like kinases of *Medicago truncatula* (*MtLyk1*: AAQ73154; *MtLyk3*: AAQ73155; *MtLyk4*: AAQ73160; *MtLyk6*: AAQ73157 and *MtLyk7*: AAQ73158), *Lotus japonicus* (*LjNFR1*: CAE02589 and *LjNFR5*: CAE02597) and *Pisum sativum* (SyM 10) (Fig. 22c). This derived structure strongly suggests that the encoded protein is a lysine motif receptor-like kinase with an extracellular LysM part responsible for perception of incoming extracellular signals, which is by its hydrophobic membrane spanning segment fixed at the plasma membrane and transduces the signal to intracellular signalling pathways via its intracellular kinase domain (Fig. 22a). In agreement with this, the identified receptor-like kinase was classified to be a member of the LysM receptor-like kinase subfamily and named *HvLysMR1*.

In addition, a second LysM receptor-like kinase of 568 amino acids could be identified. As shown in Figure 31, the deduced amino acid sequence displays characteristics of a receptor-like kinase, including a signal peptide of 36 amino acids at the N-terminal end. This sequence is likely to act as a mitochondrial targeting peptide identified by the TargetP 1.1 Server from CBS analysis (Center for Biological Sequence Analysis from Technical University of Dernmark). Furthermore, the sequence shows two LysM motifs at the extracellular domain and a conserved serine / threonine kinase domain at the C-terminal with eleven characteristic subdomains of protein kinases (Radutoiu *et al.,* 2003). The presence of these three prominent domains indicates that the encoded protein is a lysine motif receptor-like kinase. The name of the gene *HvLysMR2*, refers to this. The second *HvLysMR2* identified lacks a transmembrane segment. Such a structure was already reported for ethylene receptor-like kinase CTR1 in *Arabidopsis*. Despite the lack of a transmembrane region in CTR1, it was found to be localized at the endoplasmic reticulum and was shown to participate in ethylene receptor signalling complexes (Gao *et al.,* 2003). It will be interesting to investigate in further experiment the subcellular localization of HvLysMR2.

Alignment of the deduced amino acid sequence of HvLysMR1 with the amino acid sequence from HvLysMR2 shows that the two novel receptor-like kinases present a high sequence similarity, especially at the kinase domain (data not shown). The two novel HvLysMR1 and HvLysMR2 exhibit several motifs that are highly conserved in the protein kinase superfamily. The known Asp-Phe-Gly (DFG) motif in the subdomain VII (see Fig. 22a) is suggested to chelate Mg $^{2+}$ ions required for autophosphorylation activity (Nishiguchi *et al.,* 2002). Another conserved subdomain VIII, which is assumed to be involved in the recognition of the substrates, consists of an Ala-Pro-Glu (APE) motif (Nishiguchi *et al.,* 2002). In both receptors HvLysMR1and HvLysMR2, the alanine of this motif is replaced by proline. Such a divergence in the conserved activation loop (subdomain VIII) or even the complete absence of this activation loop in the kinase domain of the lysine motif receptor-like kinase NFR5 from *Lotus japonicus* was already discussed by Madsen *et al.* (2003). Alterations in the conserved subdomains of the kinase domain were also reported for other serine / threonine receptor-like kinases from pea, *Medicago* and rice. They lack the aspartic acid residue in domain VII, and the activation loop in domain VIII is highly diverged or absent (Madsen *et al.,* 2003). This divergence in the conserved activation loop indicates that these two receptors are activated by

mechanism different from that predicted in many plant receptor-like kinases based on conservation of phosphorylated residues in the activations loop (Wang *et al.,* 2005).

Interestingly, the two novel lysine receptor-like kinases present a potential metal-binding motifs CxxxC, CxxC and CxC with a characteristic spacing of cysteine residus at there extracellular domains (Fig.22a; Fig. 31a). Such motifs have been identified in metalloproteins. The CxxxC motif has been shown to constitute a copper binding site in yeast Cox2p (Coruzzi & Tzagoloff, 1979), Scop1 (Rentzsch *et al.,* 1999), and an iron-binding site in bacterial ferredoxins (Bruschi & Guerlesquin, 1988). Scop1 protein is involved in the transfer of copper from the carrier Cox17p to the mitochondrial cytochrome *c* oxidase subunits 1 and 2 (Rentzsch *et al.,* 1999). The CxxC motif is present in the MxCxxC sequences found in number of metalloproteins MerP, ATX1, CCC2, CCS, Wilson`s and Menkes disease copper transporter ATPases and metallochaperone CdI19 protein (Yuan *et al.,* 1995; Culotta *et al*., 1997; Pufahl *et al*., 1997; Himelblau *et al*., 1998; Polowski & Sahlman, 1999; Huffman & O`Halloran, 2001; Suzuki *et al*., 2002). Bacterial MerP has been shown to transport Hg across the plasma membrane (Powlowski & Sahlman, 1999). Yeast ATX1, CCC2 and CCS are reported to bind Cu and to deliver it to other metalloproteins (Yuan *et al*., 1995; Culotta *et al*., 1997; Pufahl *et al.,* 1997). The CxC motif is copper-binding motif found in copper chaperones proteins yCCs and AtCCs (Chu *et al*., 2005). The CxC motif of these two proteins was found to play a role in copper transfer to metalloenzymes CuZnSOD involved in defense system against ROS (Schmid *et al.,* 1999). In addition, the CxC motif was always found between the LysM domains of *Medicago truncatula* proteins and is present in the corresponding regions of Arabidopsis and rice proteins (Arrighi *et al*., 2006). The authors suggested that disulfide bridges might participate in the spatial distribution of LysM domains separated by CxC motifs. The two novels barley LysM receptor like kinases present CxC motif between and near the LysM domains. Such a distribution could suggest that CxC motif might have others functions rather than their involvement in spatial distribution of LysM domains.

The identification of such potential metal-binding motifs at the extracellular domains in HvLysMR1 and HvLysMR2 raises important questions. Are metals bound to these motifs ? What is the function of these different heavy metal-binding motifs ? And do metal-coordinating ligands interact with HvLysMR1 and HvLysMR2 ?

From the literature it is known that in animals, binding different ligands can cause heterooligomerization between different receptor combinations and stimulation of different responses. In plants, heterooligomerization of receptor-like kinases is a recurring theme and

there is preliminary evidence for combinatorial heteromeric pairing in response to different ligands (Johnson & Ingram, 2005). In addition, it was shown that the ethylene receptor ETR1 forms a disulfide-linked dimer in the membrane, with dimerization mediated by two cysteines located near the N terminus (Schaller *et al.*, 1995).

Plant receptor-like kinases (RLKs) belong to a large gene family with at least 610 members that present nearly 2.5 % of *Arabidopsis* protein coding genes and most of them described in plants, so far, encode for serine / threonine kinases (Shiu & Bleecker, 2001). They fulfil fundamental functions in the perception and processing of various extracellular signals via cell surface receptors and according to their divergent extracellular receptor domains can be grouped into 15 different subfamilies (Shiu & Bleecker 2001; Shiu & Bleecker, 2003). This divergence allows them to respond to a wide range of external signals. Receptor-like kinases with LysM motifs have, so far, been found only in plants (Radutoiu *et al.*, 2003). Recently, they could be shown to be involved in legume perception of rhizobial signals (Limpens *et al.*, 2003; Madson *et al.*, 2003; Radutoiu *et al.*, 2003). In RNA interference studies investigating the function of the LysM receptor-like protein kinase *LYK3* from *Medicago truncatula* a role in the rhizobial-plant symbiotic process could be proven (Limpens *et al.*, 2003). In addition, it was shown that the specific signal molecule lipochitin oligosaccharide between rhizobial and leguminous plants is detected by the plant LysM receptor-like kinases LYK3, NFR1 and NFR2 (Radutoiu *et al.*, 2003; Limpens *et al.*, 2003; Madson *et al.*, 2003; Spaink, 2004). The backbone of this N-acetyl glucosamine Nod-factor is similar to peptidoglycans known to interact with prokaryotic LysM domain proteins (Riely *et al.*, 2004). The involvement in the signalling process in the legume-rhizobia symbiosis is up to now, as far as I know, the only clear functional assignment of plant LysM receptor-like kinases. So far, there are no others reports about other biological functions of plant LysM receptor-like kinases.

The two novel genes *HvLysMR1* and *HvLysMR2* are induced during high and low concentrations of heavy metals. The fast response of *HvLysMR1* and *HvLysMR2* to low concentrations of the essential heavy metal copper could be explained by the fact that copper is efficiently taken up by specific transporters (Aller *et al.*, 2004), while the non-essential heavy metal cadmium was shown to be transiently retained in the root system and only slowly transported to the shoot (Page & Feller, 2005). Cadmium is taken up into plant cells, most likely via Ca^{2+}, Fe^{2+} and Zn^{2+} uptake systems such as *LCT1* protein that mediates both Ca^{2+} and Cd^{2+} transport into the cytosol of cells (Clemens *et al.*, 1998). Since plants lack a specific

transport system for chromium, it is taken up by carriers of essential ions such as sulfate or iron (Shanker *et al.*, 2005) and predominantly accumulates in the roots while only low concentrations are transported to the shoots (Han *et al.*, 2003).

As discussed in chapter 4.4, its is known that some heavy metal induced genes are also induced during leaf senescence (Himelblau *et al.*, 1998; Himelblau & Amasino, 2000). The reason for these overlapping expression patterns might be the degradation of proteins including those containing metals during leaf senescence. The liberated metals have to be sequestered and a part of it is transported to the growing tissues of the plant (Himelblau & Amasino, 2001). Therefore the same regulatory factors might be involved in both processes, the plant`s response to heavy metals and leaf senescence. Consequently, the induction of the novel heavy metal induced *HvLysMR1*and *HvLysMR2* was tested during leaf senescence. Interestingly, the results obtained show that the *HvLysMR1*and *HvLysMR2* genes were also accumulated during senescence. From the literature it is shown that two other receptor-like kinases which belong to the Leucine-rich repeat receptor kinases subfamily are already known to be induced during senescence: the *Phaseolus vulgaris* (*SARK*) senescence-associated receptor-like kinase (Hajouj *et al.*, 2000) and the *Arabidopsis thaliana* (*At SIRK*) senescence-induced receptor-like kinase (Robatzek & Somssich, 2002). But there are still open questions about their exact functional integration in the complex signalling pathways underlying regulation of leaf senescence. To my knowledge, up to now no receptor-like kinase is reported to be involved in heavy metal stress response in plants. In this study, for the first time, I show the induction of a LysM receptor-like kinases during heavy metal stress and leaf senescence.

Reactive oxygen species (ROS) commonly accumulate during the response to biotic and abiotic stressors (Neill *et al.*, 2002; Mithöfer *et al.*, 2004; Rentel & Knight, 2004). They are extremely reactive molecules that have high affinities to membranes, DNA, or proteins in plant cells (Hung *et al.*, 2005). However, ROS also function as signalling molecules that mediate responses to various stimuli (Desikan *et al.,* 2004). In addition, they have been suggested to play a role in the onset of leaf senescence (Ye *et al.*, 2000; Krupinska *et al.*, 2003). H_2O_2 also is involved in mediating biological processes, including PCD (programmed cell death) (Desikan *et al.,* 1998). A model for an H_2O_2 signalling pathway was proposed by Hung *et al.* (2005). According to them, the H_2O_2 signal may be perceived by a receptor and then result in elevated Ca^{2+} concentration in the cytoplasma. The increase in Ca^{2+} may activate a signalling protein such as a protein kinase or phosphatase to trigger a cascade, which in turn alters the activity of a transcription factor by phosphorylation or

dephosphorylation. In addition, H_2O_2 may activate the transcription by direct oxidation of H_2O_2-responsive transcription factors via oxidation of thiols of cysteine residues in proteins. In either case, the activated transcription factor interacts with its corresponding *cis*-acting element on target promoters to regulate gene expression in the nucleus. The isolated heavy metal and senescence induced gene *HvLysMR1* is slightly induced during the methylviologen treatment (Fig. 28), indicating, if any, only a minor role of ROS in induction of this novel LysM receptor-like kinase.

From the literature it is known that heavy metals such as aluminum induce changes in the cytosolic Ca^{2+} concentration (Lindberg & Strid, 1997; Plieth *et al.,* 1999). It is also known that Ca^{2+} signals are involved in nodulation which also involves the action of LysM receptor-like kinases (as discussed above). Recently it has been shown that in root hairs of legumes nanomolar amounts of Nod factors results in the onset of Ca^{2+} cytoplasmic spiking (Bothwell & Y.NG, 2005). In addition, pharmacological studies show that calcium spiking is essential for Nod factor induced gene expression (Geurts *et al.,* 2005). The data obtained in this study show that *HvLysMR1* and *HvLysMR2* mRNAs respond to increases of cytosolic Ca^{2+} concentrations induced by calcium ionophore A23187 treatment. This indicates that calcium could be involved in the signalling pathways leading to the expression of *HvLysMR1* and *HvLysMR2*.

An essential feature of the function of receptor-like kinases is the autophosphorylation of the intracellular part which is required for the interaction with downstream regulatory factors in the connected signalling pathways (Robatzek & Somssich, 2002; Yoshida & Parniske, 2005). In addition, the interaction between plant receptor like kinases and extracellular ligands such as a cysteine-rich protein (SCR) and a secreted protein (CLV3) have been shown to induce receptor oligomerization and autophosphorylation, two critical steps in receptor activation (Cock *et al.,* 2002). In order to test whether the novel LysM receptor-like kinase HvLysMR1 is functional in this aspect, autophosphorylation of this protein was analyzed using two approaches: first, it was demonstrated that the overexpressed kinase domain of HvLysMR1 is able to incorporate ^{32}P-labeled phosphate from ATP (Fig. 29d). Second, either Ser-284 or to a lower extend Thr-285 that are situated in the juxtamembrane region were identified as phosphorylations sites by LC-ESI-MS with neutral loss triggered MS^3 spectra. Therefore it can not be excluded that HvLysMR1 has additional phosphorylation sites which are functionally active. Earlier studies of phosphorylation sites of plant receptor-like kinases

revealed that phosphorylation at the juxtamembrane region is a common feature and that there are multiple phosphorylation sites responsible for the interaction with downstream signalling factors (Nühse *et al.*, 2004; Wang *et al.*, 2005). Most of the juxtamembrane residues are not highly conserved among RLKs, suggesting that phosphorylation of HvLysMR1 at these sites might be involved in conferring specific signalling propreties (e.g. generation of docking sites for specific downstream substrate recognition).

To date, only a few receptor-like kinases have been linked to certain plant processes. These include CLV1 in meristem organization, ERECTA in organ shape, BRI1 in brassinolide signalling, FLS2 in flagellin signalling, HAESA in floral organ abscission, and BrSRK1 in self-incompatibility (Clark *et al.*, 1993; Torii *et al.*, 1996; Stein *et al.*, 1996; Li & Chory, 1997; Gomez-Gomez & Boller, 2000; Shiu & Bleecker, 2001). The identification in this study of the two novel receptor-like kinases HvLysMR1 and HvLysMR2, is of particular interest, since up to now the knowledge about regulatory components underlying the leaf senescence and heavy metal processes is very limited. As outlined in Weber *et al.* (2006), it is not clear whether responses of plants to heavy metals are primarily induced by a direct interaction between the heavy metal and specific receptor or whether they are induced by signals originating from harmful effects of heavy metals within the cell. The data presented here show the involvement of a lysine motif receptor-like kinases in both processes, leaf senescence and heavy metal stress (low and high concentrations), can also not finally answer this question. HvLysMR1 and HvLysMR2 could either sense a senescence signal or the accumulation of heavy metals. Alternatively it could only interact with a senescence signal which is also elicited by the harmful effects of the heavy metal. Since this is the first study about a receptor-like kinase induced during heavy metal stress, further functional studies have to provide insight into the molecular mechanisms to understand the role of LysM receptor-like kinases during the plant response to heavy metal stress and senescence process.

4.6 Model of the novel C2-domain protein and the LysM receptor like kinase in heavy metal stress response and leaf senescence

Taken together these data, a model of the novel C2 domain protein and the LysM receptor like kinase 1 in heavy metal stress response and leaf senescence is proposed in figure 41. Two possible pathways are discussed here (Fig. 41). In the first pathway, heavy metal or senescence signals may be perceived by the membrane bound LysM receptor like kinase 1 by interaction with specific factors (ligands) and consequently activating dowstream signalling

cascades. In further step, the phosphorylation of kinase domain of the LysMR1 may activate a signalling protein such as a mitogen activated protein kinases known to be induced during heavy metal stress (Yeh *et al*., 2003; Jonak *et al*., 2004), or phosphatase to trigger a cascade to the nucleus and finally activate a target gene. In the second pathway, once heavy metals are in the cytoplasma, a redox signal may also be generated leading to the production of reactive oxygen species (ROS) via Fenton and Haber-weiss reactions. From the literature it is known that reactive oxygen species such as H_2O_2, activate the membrane Ca^{2+} channels and mediate the influx or release of Ca^{2+} from internal stores. This generates an increase in Ca^{2+}_{cyt} concentration. In addition, ROS activate directly the mitogen activated protein kinases cascade. Furthermore, the Ca^{2+} signal may activate a C2 domain protein and its Ca^{2+}-binding triggers interaction with the transcription factor to form a protein complex and their translocation to the nucleus. Finally, the transcription factor interacts with its corresponding *cis*-acting element on target of the promoter to regulate gene expression.
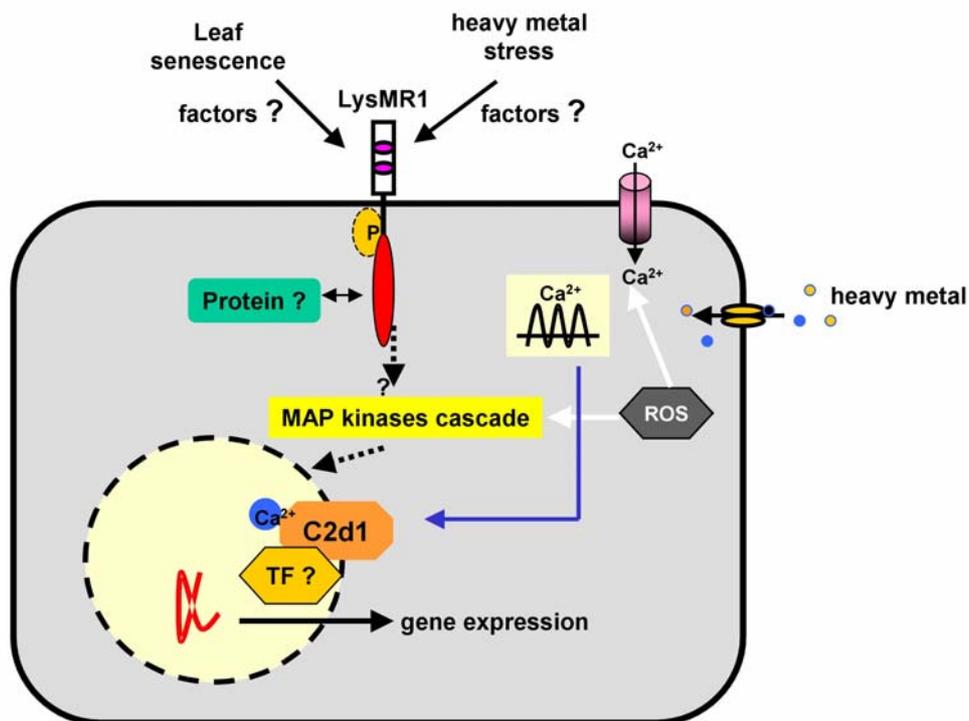
Fig. 41: Model for the functional role of LysMR1 and C2 domain protein in plant perception of heavy metal and senescence signals.

5. Outlook

In the present study novel heavy metal induced genes have been identified from barley. This is the first time where a C2 domain protein and LysM receptor-like kinases are described in response to heavy metal stress and leaf senescence. In order to improof our understanding of the signalling pathways underlying the induction of heavy metal response genes or the onset of leaf senescence further studies are necessary to understand the roles of the C2-domain protein and LysM receptor-like kinases in more detail. These could be performed by continuative experiments including the model plant *Arabidopsis* to study the orthologous genes. Of special interest in this context are the following points:

- Identification of the interaction partners of LysM receptor-like kinases and the C2 domain protein and to clarify how they interact during the signal transduction process using yeast two hybrid screens.

- Characterization of *Arabidopsis* wild-types and *LysMR1, LysMR2* and *C2d1* mutants under heavy metal stress and senescence or generation of transgenic plants by overexpressing barley LysM receptor-like kinases and C2-domain protein in *Arabidospsis* plants. With RNA interference studies the individual functions of each receptor-like kinase HvLysMR1 and HvLysMR2 could be elucidated.

a) Physiological parameters (e.g. PSII efficiency, chlorophyll content).
b) Transcriptome analysis using cDNA Microarray in wild-types and mutants after stress.

- To study the ability of HvLysMR1 and HvLysMR2 to bind heavy metal ions using site- directed mutagenesis, directed against the cysteine residues localised at the potential metal-binding motifs.

6. Summary

Contamination of soil and water by toxic heavy metals such as chromium represents a major environmental problem. Plants growing on such soil can tolerate heavy metals to very different extents. Some plants species are severely damaged by low concentrations of heavy metals while others are not affected even by high concentrations because they have evolved adaptative mechanisms to cope with this stress. However, heavy metals do not only have toxic effects. Some metals are actually essential micronutrients and act as cofactors for proteins involved in many vital cellular processes in plants. Consequently, plants have evolved a complex regulatory network for detoxification of surplus toxic metal species and for maintenance of homeostasis of essential heavy metals (Clemens *et al.,* 2002).

We are still far from understanding the mechanisms underlying this network in plants, and to date only a few of the key players in the signalling processes, such as mitogen-activated protein kinases, have been identified (Yeh *et al.*, 2003; Jonak *et al.,* 2004).

In the present study, by comparing cDNA populations derived from chromium-stressed primary leaves of barley (*Hordeum vulgare* L.) with controls using restriction fragment differential display-PCR, 48 differentially expressed cDNA fragments could be identified. Because of sequence analyses and expression studies three of them, representing novel heavy metal induced genes from barley with putative regulatory functions, were investigated in more detail in this study.

The deduced amino acid sequence of one of these cDNAs [named `C2 domain 1` (HvC2d1)] exhibits a motif that is similar to the known C2 domain and a nuclear localization signal (NLS). Expression of this member of a novel class of plant C2 domain-like proteins was studied using quantitative real-time PCR. The results obtained in this study show that, *HvC2d1* is transiently induced after exposure to high concentrations (1mM, fast response) and also induced during treatment with low concentrations (50 μM, slow response) of different heavy metals (Cr, Cu and Cd). Its mRNA accumulates also during the phase of leaf senescence. In addition, *HvC2d1* responds to changes in calcium levels caused by the calcium ionophore A23187 and also to treatment with methylviologen resulting in the production of reactive oxygen species (ROS), indicating the involvement of these factors in the pathway regulating stress response and leaf senescence.

In further experiments, using overexpressed and purified HvC2d1 protein, the binding of calcium to the C2 domain protein could be confirmed biochemically. Using chimeric HvC2d1-GFP, protein localization at the plasma membrane, cytoplasm and the nucleus could be shown in onion epidermal cells. Interestingly, after addition of calcium ionophore A23187 the green fluorescence was only visible in the nucleus. These data suggest a calcium dependent translocation of HvC2d1 to the nucleus and for the first time assign a C2 domain protein to heavy metal stress and leaf senescence and also for the first time prove a calcium dependent nuclear localization of such a C2 domain protein.

A second *Hordeum vulgare* cDNA clone, *HvLysMR1* that encodes a putative receptor-like protein kinase, was also identified in this study. The full length sequence codes for a protein with 622 amino acids which includes characteristic domains of lysine motif receptor like kinases: an N-terminal signal peptide, two lysine motifs, a transmembrane region and serine / threonine kinase domain at the C-terminal end.

The expression of *HvLysMR1* is transiently induced during exposure to high concentration and is also induced during exposure to low concentrations of different heavy metals (Cr, Cu and Cd). During senescence, *HvLysMR1* transcript accumulates. Changes in cytoplasmic calcium concentration by addition of the calcium ionophore A23187 induce the *HvLysMR1* expression again indicating the involvement of Ca^{2+} in the regulation of *HvLysMR1*.

In vitro phosphorylation of HvLysMR1 could be proven with radioactive ^{32}P-ATP. Using overexpressed and purified HvLysMR1-kinase domain. The phosphorylation of HvLysMR1 could also be confirmed by nano-liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) with neutral-loss-triggered MS-MS-MS spectra at amino acids localised at the juxtamembrane region.

In addition, a second receptor like-kinase protein HvLysMR2 with 568 amino acids could be identified. Characteristic domains of HvLysMR2 protein includes: an N-terminal signal peptide, two lysine motifs and serine / threonine kinase domain at the C-terminal end. Expression studies reveal that *HvLysMR2* is transiently induced during heavy metal treatment with high concentrations and its mRNA is also affected during exposure to low concentrations of different heavy metals (Cr, Cu and Cd). *HvLysMR2* mRNA accumulates during leaf senescence. Calcium ionophore A23187 also induce the *HvLysMR2* expression.

For the first time, the data obtained in this study suggest a possible role of HvC2d1, HvLysMR1 and HvLysMR2 in heavy metal stress- and development-dependent signalling indicating overlapping regulatory pathways during heavy metal stress response and leaf senescence. Further experiments are needed to elucidate the functions of each protein in the signalling processes.

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Publication of the results

Reviewed Manuscripts

Akli Ouelhadj, Peter Kuschk, Klaus Humbeck. 2006. Heavy metal stress and leaf senescence induce the barley gene *HvC2d1* encoding a calcium dependent novel C2 domain-like protein. *New Phytologist* 170: 261-273.

Akli Ouelhadj, Marc Kaminski, Maria Mittag, Klaus Humbeck. 2007. Receptor-like protein kinase HvLysMR1 of barley (*Hordeum vulgare* L.) is induced during leaf senescence and heavy metal stress. *Journal of Experimental Botany* (Advance access published February 23, 2007. doi:10.1093/jxb/erl304).

Scientific Meeting Attendance

Akli Ouelhadj, Klaus Humbeck. 2004. Identification and characterization of chromium induced genes in barley leaves (*Hordeum vulgare* L.). Poster presented at the 14th congres of Federation of European Societies of Plant Biology (FESPB), 23-27 August 2004, Cracow, Poland. Abstract (AS-091) published in *Acta Physiologiae Plantarum* Vol. **26**, No 3: 1-317.

Akli Ouelhadj. 2004. Identification and characterization of heavy metal induced genes in barley leaves (*Hordeum vulgare* L.). Oral presentation at Annual Report Meeting of DFG Graduate School "416" Martin-Luther-University, Halle, Wendgräben, 26-27 November 2004, Germany.

Wiebke Zschiesche, **Akli Ouelhadj**, Olaf Barth, Klaus Humbeck. 2005. Identification and characterization of factors possibly involved in heavy metal homeostasis in plants. Poster presented at the Annual Meeting of the American Society of Plant Biologists, 16-20 July 2005, Seattle, Washington, USA. Abstract (N[°]83) published in *Plant Biology 2005 Final Program*: 1-365.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter vermendung der angegeben Literatur und Hilfsmittel angefertigt habe.

Diese Arbeit wurde in keiner anderen Einrichtung zur Begutachtung eingereicht.

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