Molecular Physiology of Adventitious Root Formation (ARF) in *Petunia hybrida* Cuttings

Involvement of Primary Metabolism in Root Formation

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Herr **Amirhossein Ahkami**
geb. am 21.01.1979 in Shiraz, Iran

verteidigt am
31.08.2010
begutachtet von:

Herr Prof. Dr. Ralf Bernd Klösgen (Halle University)

Herr Prof. Dr. Nicolaus von Wirén (IPK, Gatersleben)

Herr PD Dr. Philipp Franken (IGZ, Großbeeren)

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Dedicated to My Dear Parents

and My Lovely Wife

تقدیم به پدر و مادر عزیز و گرانقدرم

و به همسر مهربانم، نازنین، بخاطر از خودگذشتگی فراوانش

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Abbreviations

3PGA	3-Phosphoglyceric acid
AQC	Aminoquinolyl-N-hydroxysuccimidyl carbamate
ADP	Adenosine diphosphate
ARF	Adventitious Root Formation
ATP	Adenosine tri-phosphate
Bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CW	Cell wall
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EST	Expressed sequence tag
FBPase	Fructose-1,6-bisphosphatase
FW	Fresh weight
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GB	Gene Bank
Glc6PDH	Glucose-6-phosphate dehydrogenase
GO	Gene Ontology
H	Hour
HCl	Hydrochloric acid
hpe	Hours post excision
HPLC	High pressure liquid chromatography
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
ICMS	Ion chromatography–mass spectrometry
Inv	Invertase
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCMS	Liquid chromatography–mass spectrometry
MDH	Malate dehydrogenase
Mg	Magnesium

ml	Milliliter
mM	millimolar
mRNA	Messenger ribonucleic acid
NAA	α-Naphtalene Acetic Acid
NaCl	Sodium chloride
NAD	Nicotine amide dinucleotide
NADP	Nicotine amide dinucleotide phosphate
NaOH	Sodium hydroxide
nmol	Nanomole
NPA	Naphthylphthalamic Acid
ºC	Centigrade
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PFK	Phosphofructokinase
PK	Pyruvate kinase
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RP	Rank product
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription - polymerase chain reaction
SE	Standard Error
SGN	Solanaceae Genomics Network
STP	Sugar transport protein
SuSy	Sucrose synthase
TES	N-tris-(Hydroxymethyl)-methyl-2-amino ethansulfonic acid
UDP	Uridine di-phosphate
µg	Microgram
µl	Microlitre

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1. Introduction

1.1. Roots: structure and function

Plant roots serve as the major conduit of energy to the rhizosphere and of nutrients and water from the rhizosphere; therefore, plant roots dominate the dynamic habitat below ground. (Rost and Bloom, 2006). Roots provide **anchorage** in the soil and allow **absorption** and **transport** of water and nutrients. However, roots have adapted to fulfill a variety of other functions including storage and aeration. Lateral root, root hair, root tip and root cap, are considered as major parts of root structure (Fig.1). The development of the root system depends on the activity of the root apical meristem and the production of lateral root meristems. As shown in Figure 1, the apical region of a plant root is characterized by three zones of activity: meristematic, elongation, and maturation. The meristematic cells are located near the tip of the root. These cells generate the root cap and the upper tissues of the root. In the elongation zone, cells differentiate to produce xylem, phloem, and cortex. Root hairs, formed in epidermal cells, first appear in the maturation zone (Taiz and Zeiger, 2002).

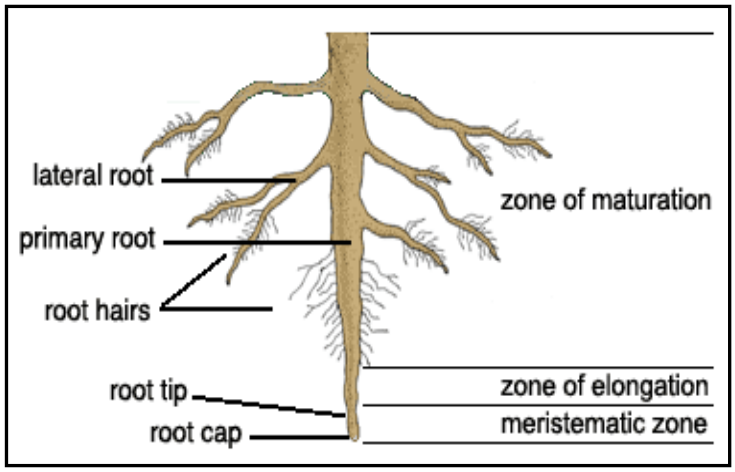


Figure 1. Schematic presentation of root structure and zones of activity in the apical region.

Generally, two different pathways are involved in determination of root system architecture: (1) intrinsic pathways which are necessary for organogenesis and growth; and (2) response pathways which are involved in

respond to external signals (Malamy, 2005). Primary roots originated from the radicle which is the first part of a seedling to emerge from the seed during the process of germination. In other words, the radicle is the embryonic root of the plant which grows downward in the soil. This primary root creates secondary roots, which in turn produce tertiary and further roots (known as lateral roots). Many plants can also give rise to other type of roots which derived from the hypocotyl called adventitious roots.

1.2. Adventitious Roots

The term “adventitious root” is used to indicate a root that arises either from a lateralized root axis or in a part of the plant that is not itself a root (e.g. on a shoot or leaf) (Esau, 1953). In one point of view, there are two types of roots: one is the “shoot-borne” type whose origin is self-defining; the other is “pole-borne” whose origin is from one of the poles of embryo. Thus, adventitious roots have been classified as shoot-borne roots (Barlow, 1994). The most basic division is between plants that will produce adventitious roots and those that will not. Of those that will root the most important sub grouping is between those that require excision and those which do not (Lovell and White, 1987). In the first case, which is also the focus of the present research, the most important feature is the vegetative propagation possibility of the individual. As defined by Casson and Lindsey (2003) roots formed de novo from differentiated cells (e.g. of stem or hypocotyl tissues) are adventitious roots. Furthermore, adventitious roots are designated as roots that are formed at unusual sites or under unusual circumstances such as wounding or hormone application (Hochholdinger et al., 2004a). In other words, adventitious roots can either form naturally on a stem cutting or develop in response to wounding of the site of the cutting (Koyuncu and Balta, 2004). Whatever view is taken, adventitious roots are referring to a root that arises out of the normal temporal sequence and/or at an unusual location. In most if not all cases, this would indicate a root which develops as a result of wounding and is therefore proof of a regenerative response. When a cutting is removed from a plant under appropriate conditions, it may produce a

new root system (Fig. 2) and finally an entire individual with a balanced root to shoot ratio (Lovell and White, 1987). These events involve various anatomical, physiological and molecular changes associated with wound responses in addition to those involved in root formation itself. The later subject is of major interest in the current work.

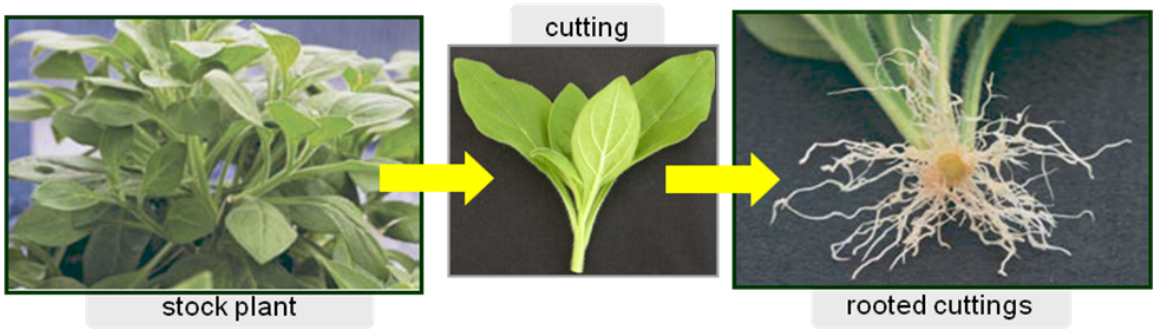


Figure 2. Adventitious root formation (ARF) in an excised leafy cutting of petunia

Research on adventitious root formation (ARF) is highly important from the practical point of view; however, at the same time it is a fascinating scientific subject matter (De Klerk, 2002). Adventitious root formation is an interesting process of postembryonic organogenesis since it describes the development of root tissues from non-root and non-meristematic tissues (Ermel et al., 2000). Besides, ARF in leafy stem cuttings is a crucial physiological process for the propagation of many ornamental plant species (Ahkami et al., 2009). This event is important in the applied sciences point of view since numerous fruit and forest tree crops, most ornamental shrubs and many commercial greenhouse crops are propagated by cuttings (Davis and Haissig, 1994). In addition, propagation by cuttings is the only practical means of preserving unique characteristics of some plants (Davis et al., 1989). Over 70% of the propagation systems used in the ornamental horticulture industry depends on successful rooting of cuttings (Davies et al., 1994). In the Netherlands, one of the major producers of starting material, six billion plants are produced per year vegetative via cuttings. This equals a value of approximately US $500 million (De Klerk et al., 1999). Furthermore, it is noted by Sorin et al. (2006) that ARF is a key step in vegetative

propagation of woody or horticultural species and despite intensive control of environmental factors in the modern propagation industry, high economic losses still occurs as a result of insufficient rooting. For example, a conservative estimation of the losses due to inadequate rooting treatments would be US $50 million per year for the Netherland (De Klerk et al., 1999). The inability to induce adventitious root formation in conventional cuttings or tissue culture is a major limiting factor when cloning plants for genetic improvement and commercial applications. Biochemical and physiological research has thus been conducted to elucidate the control of this important developmental process (Haissig and Riemenschneider, 1992).

In physiological or genetic terms, it may be questioned whether adventitious roots are similar to lateral or primary roots. As pointed out before, it is clear that the origin of these different types of roots is not the same. Primary roots are originating from the radicle, lateral roots from primary roots and adventitious roots from the hypocotyl. All of these root types are essentially identical in structure. Mutations in some root patterning genes show developmental failures in embryonic, primary and lateral roots (Malamy, 2005). Moreover, Malamy and Benfey (1997) suggested that the overall lack of mutants that are unable to produce lateral roots is due to the fact that a single developmental pathway exists for primary and lateral root formation. In fact, the *alf4* mutant in Arabidopsis (Celenza et al., 1995) and the *rtcs* mutant in maize (Hetz et al., 1996) convey defects only in lateral root formation, showing that there are genes specifically involved in this process. Similarly, the *mp* ('monopterous') mutant lacks a primary root, but is able to generate adventitious roots (Berleth and Jurgens, 1993). Consistent with this, the investigations of some mutants in maize showed that lateral, primary and adventitious root formation are genetically different (Hochholdinger et al., 2004b; Malamy, 2005).

1.2.1. Successive phases in the process of adventitious rooting

Biochemical and especially histochemical analyses revealed that adventitious root formation should be considered as a complex multi-step

process, rather than as a single phase event. The process of adventitious root formation can be divided into three stages: (1) root induction in which molecular and biochemical alterations occur before any visible cytological changes; (2) root initiation when the first anatomical modifications and cell divisions take place. In this phase, root meristems are formed and root primordia are established; and (3) protrusion, corresponding to the emergence of root primordia (Kevers et al., 1997). In apple microcuttings, developmental stages of ARF were nominated as: dedifferentiation (0-24h), induction (24h-96h) and differentiation (96h-onwards) (De Klerk, 2002). While this separation refers to the cytological analysis, physiology and molecular changes during ARF are still poorly understood.

1.2.2. Endogenous control of adventitious rooting in cuttings

The formation of adventitious roots is a very complex process which involves successive developmental phases requiring different hormonal signals and other factors (De Klerk et al., 1999). In recent decades a great progress has been made in the recognition of biochemical and molecular changes during adventitious root development in cuttings in different plant species. ARF is affected by multiple endogenous factors, including phytohormones with a central role of auxin, and environmental factors, such as wounding or light (Sorin et al., 2005). However, the influence of nitrogen supply or free amino acids (Suzuki and Kohno, 1983), mineral nutrition (Schwambach et al., 2005) or antioxidative enzymes (Metaxas et al., 2004) during root formation have been investigated in many plants. There is increasing evidence that ARF is also dependent on the action of ethylene (De Klerk et al., 1999). In this context, the first rise of ethylene production observed in cuttings is caused by wounding (Blakesley, 1994). Besides, It has been repeatedly shown that adventitious root formation of cuttings can be improved via conditioning of the donor plant e.g. by the control of nitrogen supply (Druege et al., 2000; Zerche et al., 2008) or by application of microorganisms such as arbuscular mycorrhizal fungi (Druege et al., 2006). However, the lack of knowledge of the molecular physiological basis hampers the development and establishment of reliable procedures.

1.2.2.1. Carbohydrates

It has repeatedly been shown that carbon allocation and the ability to utilize carbohydrates efficiently were the most crucial factors in adventitious root development in cuttings (Rapaka et al., 2005). Since ARF, an energy-requiring process, needs carbon skeletons, it relies on an adequate supply of carbohydrates to the region of root regeneration. However, the relationship between carbohydrates and adventitious root formation has remained controversial (Veierskov, 1988). A positive correlation between carbohydrate accumulation and adventitious root development was found by Haissig (1984) in *Pinus banksiana* seedlings. In contrast, Custodio et al. (2004) showed that except for glucose, sugars used during rooting in *Carob tree*, did not remarkably influence plantlet survival and growth at the end of the acclimatization period. Low carbohydrate levels in cuttings at the beginning of the rooting period can limit the speed or intensity of subsequent ARF (Veierskov et al., 1982a; Druege et al., 2004). In accordance to this, application of sugars to the rooting medium increased subsequent root formation (Eliasson, 1978; Li and Leung, 2000; Takahashi et al., 2003). Under sufficient light conditions, however, pre-severance carbohydrate levels are less important and ARF of leafy cuttings becomes more dependent on current photosynthesis (Eliasson, 1978; Haissig, 1989; Druege et al., 2000). In that way a storage-induced carbohydrate shortage in cuttings can be counterbalanced by higher light intensity during rooting (Rapaka et al., 2005). Under any environmental conditions, the intensity of ARF positively correlated with the sucrose level in cutting leaves during the early rooting period, supporting an important role for ARF of leaf-derived import of sugars or co-transported compounds into the stem base. Determinations of the activities of enzymes of some glycolytic and pentose phosphate pathways demonstrated that the activity of PFK and Glc6PDH increased in leafy cuttings of bean (*Phaseolus vulgaris*) and jack pine (*Pinus bansiana*) in response to applied indol acetic acid (IAA) and an endogenous root-forming stimulus (Haissig, 1982). This supports the hypothesis that the main route of glucose oxidation during ARF is through the pentose phosphate pathway and glycolysis. Moreover, application of different

sugars suggests distinct functions of glucose, sucrose and starch in the different phases of ARF (Li and Leung, 2000; Correa et al., 2005). In particular, Li and Leung (2000) could demonstrate that starch accumulation may be a biochemical marker for early critical stages of root formation in hypocotyl cuttings of radiate pine. Taken together, it is suggested that except serving a role as energy source, carbohydrates may also play other roles in the formation of adventitious roots. For instance, it is possible that carbohydrate accumulation in the rooting zone may affect osmoregulation and cellular solvent capacity. Besides, phosphorylated sugars may be considered as candidates for regulating root initiation (Veierskov, 1988). In addition, a regulatory role of sugars in plant development, their interactions with plant hormone transport, and signaling are also mentioned (Druege et al., 2000; Correa et al., 2005; Rolland et al., 2006). However, the mechanisms underlying ARF by carbohydrates remain unsolved.

The regulation of carbon partitioning between source and sink tissues in higher plants is also an important matter which should be taken into consideration. Hexoses, as well as sucrose, have been recognized as important signal molecules in source-sink regulation (Roitsch, 1999). The primary products of carbon fixation are starch and sucrose. In source leaves, starch is synthesized within the chloroplast and acts chiefly as an intermediate deposit for photoassimilates, whereas sucrose, synthesized in the cytosol, plays a central role in the distribution of photoassimilates throughout the plant. The route of sucrose unloading from the phloem in sink tissues is still poorly described, but GFP labeling indicated that symplastic unloading of sucrose from the phloem to sink tissues through plasmodesmata connections represents the principal unloading route (Imlau et al., 1999). There are three different phloem sucrose unloading routes which are illustrated in Figure 3. In the apoplastic unloading pathway, sucrose released into the apoplast can either directly enter the heterotrophic cells or be hydrolyzed to glucose and fructose by cell wall-bound invertase that are subsequently imported into the cells via the hexose transporter in the plasmamembrane (Bush, 1999) (Fig. 3). However, several studies revealed that the phloem unloading routes are changeable, and may shift in

response to sink development and function. For example, in potato tubers, during the early stages of tuberization apoplastic unloading of sucrose into swelling stolons is prevalent, followed by cleavage of sucrose into glucose and fructose with the action of cell wall invertase (Appeldoorn et al., 1997; Viola et al., 2001). After tuberization, sucrose exported from the leaves is delivered to growing tubers basically via symplastic phloem unloading (Viola et al., 2001). Moreover, a shift of phloem unloading from symplasmic to apoplasmic pathway was involved in developmental onset of ripening in grape berry (Zhang et al., 2006). Although phloem unloading is believed to play a key role in the partitioning of photoassimilates (Fisher and Oparka, 1996; Patrick, 1997), production and partitioning of photoassimilates from source (leaves) to sink organs (stem base or rooting zone) during developmental stages of ARF in excised leafy cuttings has not yet been thoroughly investigated.

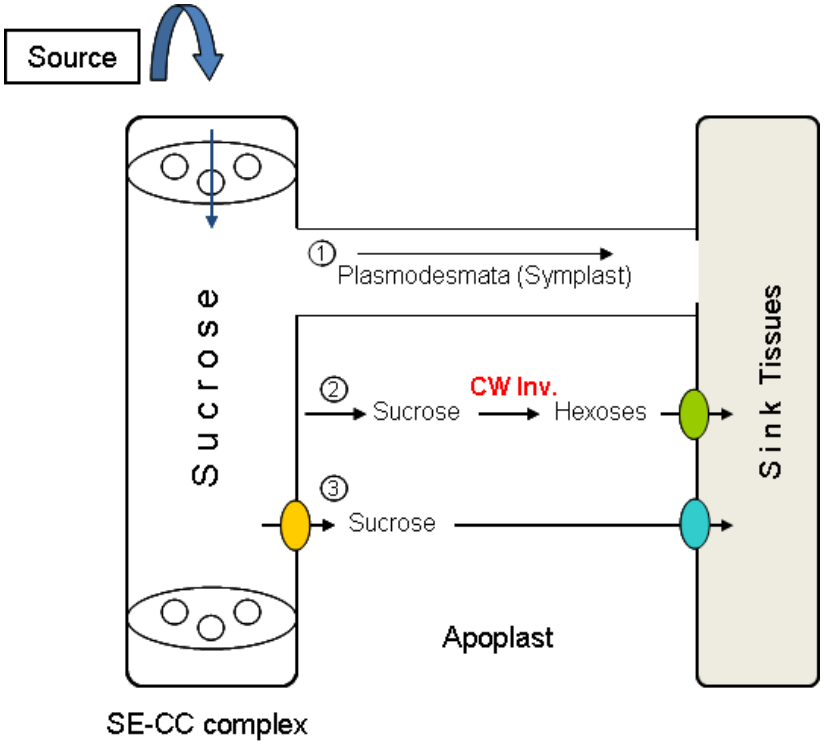


Figure 3. Three possible routes for sucrose unloading from source into sink tissues. Cw-Inv: cell-wall invertase; SE: sieve elements; CC: companion cell.

After phloem unloading in the sink tissues via one of the mentioned routes, sucrose is taken up and processed by surrounding cells through biochemical pathway of carbon metabolism including sucrose degradation, glycolysis and starch biosynthesis (Fig. 4). However, a detailed elucidation of the roles of key enzymes and intermediates involved in carbohydrate metabolism in a heterotrophic tissue of stem base of excised leafy cuttings during ARF has not been investigated to date.

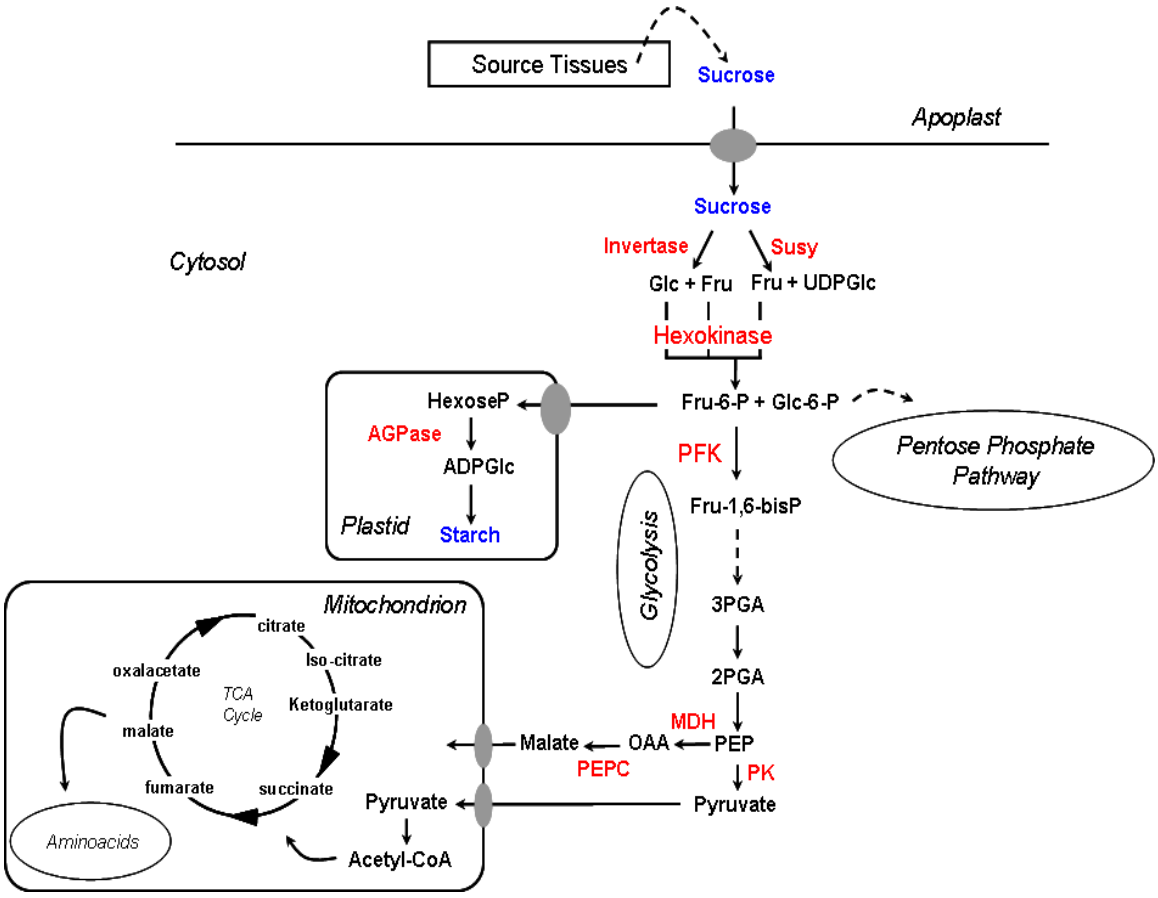


Figure 4. Schematic representation of carbohydrate metabolism in plant cells of sink tissues. AGPase: ADP-Glucose pyrophosphorylase; TP: triose-phosphate; PK: Pyruvate Kinase; MDH: Malate Dehydrogenase; PEPC; Phosphoenolpyruvate carboxylase, Susy: Sucrose Synthase; FBP: Fructose Bisphosphate; G6P: Glucose-6-Phosphate. Enzymes are illustrated in red color.

1.2.2.2. Auxin

Excision of a cutting from the donor plant has two consequences; isolation from the functional integrity of the 'whole plant' and injury. After detachment of the cutting from the shoot, basipetal polar transport of auxin from the apex of the

cuttings contributes to auxin accumulation in the stem base (Garrido et al., 2002). It is generally accepted that ARF is dependent on auxin and the rise of free auxin in the basal stem contributes to the early events of this developmental process (Blakesley, 1994; De Klerk et al., 1999; Sorin et al., 2005). Fogaca and Fett-Neto (2005) examined the roles of auxin along with the role of other factors such as irradiance, phenolic compounds and ethylene during phases of adventitious rooting in easily rooting and hardly rooting species of Eucalyptus. The results showed that the main differences in rhizogenicity between these two species were related to auxin type. In another study, Sorin et al. (2005) suggested that the Auxin Response Factor17 (ARF17) gene, a potential repressor of auxin-inducible genes, could be a major regulator of adventitious rooting in Arabidopsis. Intermediates and enzymes with central roles in auxin metabolism may also be considered as important markers during ARF. For example, peroxidases involved in the oxidative decarboxylation catabolism of auxin were suggested as predictive markers for root induction in *Betula pendula* (McDonald and Wynne, 2003). Moreover, phenolics which can have roles as regulators of peroxidases, have been regarded as important adjuvant during the first stages of the adventitious rooting process in apple (Fogaca and Fett-Neto, 2005). In addition, auxin transcriptional regulators (AUX/IAA) (Dharmasiri and Estelle, 2004), auxin-inducible genes (GH3) (Staswick et al., 2005; Sorin et al., 2006), auxin conjugators (amino acids, sugars or inositol) (Crozier et al., 2000) and auxin influx or efflux transporters (AUX1 and PIN) (Muday and DeLong, 2001) may play a regulatory role in ARF. It has been observed in different plant species that high auxin concentrations are needed during the induction phase of ARF, whereas during root formation this phytohormone acts inhibitory (De Klerk et al., 1999).

An exogenous application of auxin is also a very useful procedure to investigate its direct or indirect role during ARF. Non-woody stem cuttings are usually highly responsive to supplied auxins, although the rooting response of any cutting depends on the age of the stock material from which the cutting is taken, the type of auxin used and its concentration, the duration of the treatment

and the time interval between excision of the cuttings and the beginning of auxin treatment (Jarvis, 1987). Auxin inducers such as α-Naphtalene Acetic Acid (NAA) and Indole-3-Butyric Acid (IBA) or auxin inhibitors such as Naphthylphthalamic Acid (NPA) are widely used for exogenous applications. In this regard, Husen and Pal (2007) suggested that the concentration of exogenously applied NAA and IBA activate sugar metabolism and lead to a release of energy, proteins and peroxidase activity which are necessary for cell division and differentiation during the initiation or development of root primordia in the rooting zone of shoot cuttings. However, the complex interactions between auxin and other phytohormones, overall primary metabolism or cell division during ARF are far from being elucidated.

1.2.3. Different approaches for analysis of adventitious root formation

In addition to sugars, various intermediates involved in primary metabolism like amino acids, glycolytic intermediates like hexose-phosphates or pyruvate, multifunctional nucleotides like ATP and ADP or organic acids playing role in TCA cycle such as malate or citrate may also affect ARF in excised leafy cuttings. In this way, interactions in the structure of the metabolic and signaling network and the dynamics of transcript, protein and metabolite turnover can be investigated (Gibon et al., 2006). Previous physiological and biochemical researches have largely been focused mainly on post-translational processes of ARF on individual metabolite levels or enzyme activities. With respect to the wide range of sugars, enzymes and intermediates shown to influence ARF or to correlate with root formation in stem cuttings, omics-approaches aiming at describing changes at the metabolite level might be of particular advantage. Gas chromatography-mass spectroscopy (GC-MS) and liquid chromatography-mass spectroscopy (LC-MS) have been successfully used to detect metabolites during ARF (Kuroha et al., 2002; Anbazhagan et al., 2008). In order to achieve more knowledge on the physiological background of ARF, it is inevitable to move closer to the gene level and combine histochemical, biochemical and metabolomic data with global view on the regulation of transcripts. However, a

major obstacle in both, the molecular study and modification of rooting is the complexity of the relationship between gene/metabolite changes and rooting phenotypes (Haissig et al., 1992). Since, adventitious rooting is a complex quantitative genetic trait regulated by both environmental and endogenous factors, the molecular mechanisms by which it is regulated are still poorly understood (Sorin et al., 2005). Despite the central role of biochemical and physiological events during ARF, only a limited number of molecular studies of ARF have been performed. For example, in *Pinus contorta* hypocotyls which were treated with the auxin indole-3-butyric acid, 220 differentially expressed genes during different developmental stages of ARF have been identified. The respective gene products were involved in protein synthesis and degradation, auxin transport, photosynthesis, cell division or cell wall synthesis (Brinker et al., 2004). Furthermore, a proteomic analysis of different mutant genotypes of Arabidopsis led to the identification of eleven proteins including auxin-related and light-related proteins which positively or negatively correlated with adventitious root formation and could be suitable as molecular markers (Sorin et al., 2006). Focusing on gene expression patterns during ARF, a number of mRNAs which were up- or down-regulated or uniquely expressed during auxin-induced adventitious root formation in apple were identified using a combination of different approaches such as differential messenger RNA display (DDRT) and mRNA representational difference analysis (RDA) (Butler and Gallagher, 1998). Selected candidate genes from this type of studies can be used to generate genetically manipulated plants using heterologous overexpression or antisense-inhibition technologies to study the role of individual genes in ARF. However, regarding ARF the use of transgenic approaches is scarce till present. For a number of years, many studies have focused on *Agrobacterium rhizogenes* oncogenes, in particular, *rol* genes, to understand the mechanisms of action of these genes in inducing root formation (Altamura, 2004). For example, Dai et al. (2004) showed that the expression of the *rol*B gene enhances adventitious root formation in hardwood cuttings of aspen.

Despite intensive studies, no conclusive models concerning the relationships between the accumulation of distinct transcripts, of enzyme activities, of metabolite profiles and ARF have been reported. Moreover, most studies have compared certain conditions, rather than analyzing the temporal dynamics during a time series.

1.3. Petunia: An ornamental plant which serves as a model system

The common garden petunia, *Petunia hybrida*, is derived from *P. integrifolia* and *P. axillaris*, two of many petunia species endemic to South America (Stehmann et al., 2009). This widely-cultivated genus of flowering plants belongs to Solanaceae family. Petunia is considered to be the first cultivated bedding plant and has remained one of the favorite genera for developing new varieties (Gerats and Vandenbussche, 2005). With its trumpet-shaped flowers petunia is an ornamental plant of high economic importance in worldwide horticulture. For instance, in the United States it has been one of the top five sold bedding plants for over 100 years. Furthermore, over the past two decades petunia has served as an excellent model system for uncovering the molecular, biochemical and physiological bases of several plant processes such as gene silencing (Underwood et al., 2009). It was also a key model system in elucidating many of the features of plant transformation, including sites of insertion, and different expression levels (Conner et al., 2009). Easy growth conditions and a relatively short life cycle, cheap asexual propagation from leafy cuttings, a large and still expanding set of well characterized genes, the availability of large sets of mutants and molecular tools, such as cDNA libraries, are the most important advantages of this genus to serve as a model system (Gerats and Vandenbussche, 2005).

1.4. Aims and approaches of the current work

The main goals of the present study were to investigate the physiological events occurring during ARF in leafy stem cuttings and to characterize the suitability of *Petunia hybrida* as model system for studying this process. A focus was set on the role of primary metabolites, like sucrose and starch, in ARF. Therefore, time-course analyses of transcript levels, enzyme activities and metabolite levels were combined with a histological study of ARF in order to address questions on the role of carbohydrates, amino acids and related intermediates that change during ARF. Besides, the interaction of auxin with identified markers was analyzed in order to unravel whether auxin has any effect on the metabolic activity during ARF. Additionally, a normalized cDNA library from different developmental stages of root formation was generated for the set up of a microarray to identify candidate genes specific for ARF. It should be noted that those physiological and molecular changes specifically involved in adventitious root formation were considered as the major interests in the current investigation, rather than those associated with wound responses. Therefore, a filtration approach was chosen to eliminate primarily wound-responsive genes. These challenges were addressed in three main parts:

1. In order to describe histochemical changes and to identify molecular and physiological markers during various developmental phases of ARF in the rooting zone (stem base) of petunia cuttings, enzyme activities, metabolite levels and transcript accumulation involved in primary and specifically carbohydrate metabolism were analyzed using anatomical, biochemical and Northern blot approaches.

2. To investigate the possible impact of auxin on metabolic profiles and cell division during adventitious rooting in petunia, auxin analogs and an auxin transport inhibitor were applied to study biochemical and anatomical changes.

3. In order to identify genes specifically induced during various developmental stages of ARF and to describe the series of physiological processes during adventitious rooting, a microarray analysis in petunia cuttings was performed.

2. Materials and Methods

2.1. Plant material, growth and harvesting conditions

Leafy stem cuttings of *Petunia hybrida* cv. Mitchell were used for all experiments. Cuttings were produced on donor plants which were kept in short day condition with 10 hours light per day. All samples were collected at the same time of the day (2 h after the onset of light). Excised leafy cuttings harboring four to five leaves of similar size were placed in plastic trays with a size of 38×58 cm containing perlite ("Perligran A", particle size 0-6 mm, Knauf Perlite GmbH, Dortmund, Germany). Perlite is an aluminium silicate, which has been expanded by heating to ca. 1000 °C. The substrate is chemically inert and does not contain mineral nutrients. Trays containing cuttings were watered and covered completely with a light-permeable top to maintain a humid environment. Cuttings were put in a phytotron and cultivated under the following growth conditions: temperature 20°C (night) and 22°C (day), humidity 60% (night) and 85% (day), time regime 10 h light and 14 h dark and a light intensity of 250 µmol x m^{-2} x s^{-1}. At specific developmental stages of ARF, five mm of each cutting base (rooting zone) were harvested, immediately frozen in liquid N_2 and stored at -80°C for further analyses or fixed in a solution of formalin, ethanol and acetic acid for anatomical investigation (Gerlach, 1984). According to Brinker et al. (2004) and in order to determine early regulatory changes, material was harvested at eleven time points before any roots emerge. To simplify the description of the developmental stages, all the investigated time points are designated as hours post excision (hpe): 0, 2, 4, 6, 12, 24, 48, 72, 96, 144, 192 hpe**.**

2.2. Anatomical investigations

The anatomical examination was performed as described by Haensch (2004) and in cooperation with Dr. Klaus-T. Haensch, IGZ, Germany. Fresh basal stems of cuttings were fixed using a solution of formalin, alcohol and acetic acid (FAA), 100 ml of which consisted of 5.4 ml formalin (37%), 65.6 ml ethanol (96%), 5 ml glacial acetic acid and 24 ml distilled water (Gerlach, 1984). After

dehydration by a graded series of ethanol, stem segments were embedded in hydroxyethylmethacrylate (Histo-Technique-Set Technovit 7100; Kulzer, Wehrheim, Germany) supplemented with hardener I and II according to the manufacturer's instructions. Specimens were polymerized for 1 h at room temperature and for a further 6 h at 37 °C. Samples were cut into sections of 6 µm using a Jung CM 1800 microtome with type 818 disposable microtome blades (both from Leica Instruments, Nussloch, Germany). Sections stretched on distilled water and mounted on slides were stained with 0.05% toluidine blue O (Serva, Heidelberg, Germany), which was dissolved in 1% sodium tetraborate decahydrate buffer (Hutchinson et al., 1996). Sections were rinsed in distilled water, dried and covered with Entellan (Merck, Darmstadt, Germany) Microscopic analyses were performed using an AxioImager A1 microscope in combination with an AxioCam MRc 5 camera (Carl Zeiss, Jena, Germany).

For histochemical staining of starch which was carried out in cooperation with Dr. Melzer (IPK, Germany), transverse sections of the base part of stem from fresh petunia cuttings were used. A Leica Vibratome VT 1000S (Leica Microsystems, Bensheim, Germany) was used to prepare sections with an approximate thickness of 150 µm. To detect the presence of starch, fresh sections were incubated for 10 min in iodine and analysed at a Zeiss Axiovert 135 microscope with an attached CCD camera system (Zeiss AxioCam; Carl Zeiss, Göttigen, Germany).

Histochemical examinations in NPA-treated cuttings were performed in cooperation with Dr. Melzer (IPK, Germany). For this purpose, 1 mm thick cross-sections of stem were fixed overnight at 4°C in 50 mM cacodylate buffer, pH 7.2, containing 2% (v/v) glutaraldehyde and 2.0% (v/v) formaldehyde, followed by one wash with buffer and two washes with distilled water. For secondary fixation samples were transferred into a solution of 1% (w/v) OsO4. After 1 h, samples were washed three times with distilled water. Dehydration at 21°C was performed stepwise by increasing the concentration of ethanol as follows: 30% (v/v), 50% (v/v), 60% (v/v), 75% (v/v), 90% (v/v) and twice 100% (v/v) ethanol for 1 h each. After additional dehydration with propylene oxide for 1 h, samples were infiltrated

with Spurr resin (Plano) as follows: 33% (v/v), 50% (v/v), and 66% (v/v) resin in propylene oxide for 4 h each and then 100% (v/v) resin overnight. Samples were transferred into embedding molds, incubated there for 3 h in fresh resin, and polymerized at 70°C for 24 h. Semithin sections with a thickness of 3 µm were mounted on slides and stained for 2 min with 1% (w/v) methylene blue and 1% (w/v) Azur II in 1% (w/v) aqueous borax at 60°C before light microscopic examination.

2.3. RNA isolation and Northern blot analyses

Total RNA was extracted from petunia cutting base as described by Logemann et al. (1987). Thirty micrograms per sample were separated on a 1.5% (w : v) formaldehyde-agarose gel (Sambrook et al., 1989), transferred to a nitrocellulose membrane (GeneScreen, NEN Life Science Products, Waltham, MA, USA) and fixed by UV cross-linking. Radioactive labeling of cDNA fragments was performed using the High Prime kit (Roche Diagnostics, Mannheim, Germany) and [α-32P]-dCTP. Hybridization was carried out as described previously (Herbers et al., 1994) and signals were detected by exposure to Kodak X-ray films (Sigma, Taufkirchen, Germany). The following probes were used: the petunia *cyclin B1* (AJ250315) and the petunia *GH3* genes (CV296522) were amplified from petunia cDNA derived from the total RNA with specific primer pairs (CycB1 for. primer: 5'-AGGTACCAGCCAAGAAGAAGG- 3'; CycB1 rev primer : 5'-TGCGCTAATGCCAACTAACTG-3'; GH3 for : 5'-CACCGGCCCTTCAGTTCATC-3' ; GH3 rev: 5'-CAGCAAGGCCACCAGGAGTC-3') resulting in a fragment 449 bp for *CycB1* and 507 bp for *GH3*; fragments of the genes for potato sucrose synthase (SuSy, *c*. 900 bp, P10691), tomato cell wall invertase (800 bp, AAM28823) and potato vacuolar invertase (800 bp, ABF18956) were isolated from the appropriate vectors (Zrenner et al., 1995); for monosaccharide transporter STP4 (sugar transport protein 4), a cDNA fragment (1000 bp) was isolated using a petunia cDNA library; the potato cytosolic GAPDH gene was used according to Hajirezaei et al. (2006).

2.4. Carbohydrate measurements

Soluble sugars were determined as described by Chen et al. (2005). To extract soluble sugars two stem bases of frozen leafy cuttings were homogenized in 80% (v/v) ethanol and incubated at 80°C for 60 minutes. Subsequently, homogenized samples were centrifuged for 5 min at 4ºC and at 14,000 rpm. Supernatant was dried under vacuum at 50°C for 90 minutes and re-suspended in 0.25 ml purest water. Determination of produced glucose, fructose and sucrose was performed in a measuring buffer containing 100 mM imidazol-HCl (pH 6.9), 5 mM $MgCl_2$, 2.25 mM NAD, 1 mM ATP and 20 µl of the sugar extract in a final volume of 300 µl using corresponding auxiliary enzymes according to Hajirezaei et al. (2000). For starch measurement, the sediment resulting from sugar extraction was washed two times with one ml of 80 % (v/v) ethanol and water each. Subsequently, remaining residue was re-suspended in 0.2 ml of 0.2 M KOH and incubated at 4°C over night to degrade the starch chain. Next day, samples were homogenized in another 0.2 ml of 0.2 M KOH and incubated at 95°C for a minimum of 1 h. The pH was adjusted to 6.5-7.5 by the addition of 0.07 ml of 1 M acetic acid. Starch hydrolysis was carried out using an aliquot of samples which were incubated in the same volume of sample and a buffer containing 50 mM sodium acetate, pH 5.2 and 7 unit/mg of amyloglucosidase (Roche, Germany). Determination of produced glucose was performed according to Hajirezaei et al. (2000).

2.5. Extraction and activity measurement of enzymes

The extraction of enzymes was carried out according to the method of (Zrenner et al., 1995) with minor modifications. Frozen leafy cuttings were homogenized in liquid nitrogen and suspended in 0.5 ml of a buffer containing 50 mM Tris-HCl, pH 6.8, 5 mM $MgCl_2$, 5 mM mercaptoehanol, 15% glycerol, 1 mM EDTA, 1 mM EGTA and 0.1 mM pefabloc phosphatase inhibitor. Subsequently, homogenized samples were transferred in eppendorf tubes and centrifuged for 5 min at 4ºC and at 14,000 rpm. Aliquots of the supernatant were utilized for enzyme activity measurements using coupled spectrophotometric enzyme

assays. Protein level of enzyme extracts was determined according to (Bradford, 1976).

2.5.1. Invertases (EC 3.2.1.26)

The activities of cytosolic and vacuolar invertases were measured using a modified method of (Zrenner et al., 1995). Cytosolic invertase activity was assayed in a buffer containing 50 mM Hepes-KOH (pH 7.5), 0.5 M sucrose and 20 µl of the protein extract in a final volume of 100 µl, whereas vacuolar invertase was assayed in a buffer containing 50 mM sodium acetate (pH 5.2) with the same components. Incubations were carried out at 37ºC for 180 min and reactions were stopped at 95ºC for 5 min. For each sample a blank was prepared containing the same reaction mixture but heat-inactivated immediately for 5 minutes at 95°C. Produced glucose was measured as described in Hajirezaei et al. (2000). Cell wall-bound acid invertase was measured using the remaining pellet after enzyme extraction. Pellets were washed two times with a buffer containing 50 mM Tris-HCl (pH 6.8) and 5 mM $MgCl_2$. Incubation was carried out in a buffer containing 50 mM sodium acetate (pH 5.2), 0.5 M sucrose and complete pellets of protein extract in a final volume of 100 µl at 37°C for 180 min and neutralized by adding 10 µl of 1 M Tris-HCl (pH 8.0). The reaction was stopped at 95°C for 5 min. Blank samples were prepared without plant material. Samples were centrifuged for 1 min at 10,000 g and produced glucose was measured in the supernatant as described in Hajirezaei et al. (2000).

2.5.2. Sucrose synthase (EC 2.4.1.13)

Sucrose synthase was assayed by UDP-Glucose level determination using a modified method of Zrenner et al. (1995). The reaction mixture contained 100 mM Hepes-KOH (pH 7.0), 0.5 M sucrose, 20 mM UDP and 20 µl of the protein extract in a final volume of 100 µl. Incubation was carried out at 30ºC for 30 min and stopped at 95ºC for 5 min. Blanks had the same reaction mixture but were heat-inactivated without incubation. The determination of UDP-Glucose was performed by LCMS according to Chen et al. (2005).

2.5.3. Cytosolic glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)

Activity of cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as described by Biemelt et al. (1999). The activity of GAPDH was measured in 0.1 M Tris (pH 7.0) containing 5 mM $MgCl_2$, 1 mM sodium fluoride (NaF), 5 mM dithiothreitol (DDT), 0.5 mM NADH, 10 mM 3-phosphoglycerate (3PGA), 1 U triose phosphate isomerase (TPI) and 2 U phosphoglycerate kinase (PGK). The reaction was started by the addition of 7 mM ATP (final concentration).

2.5.4. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

Activity of glucose-6-phosphate dehydrogenase was determined in a buffer containing 100 mM glycylglycine (pH 8.0), 150 mM $MgSO_4$ and 60 mm NADP. The reaction was started by the addition of 15 mM glucose-6-phosphate and the absorption of NADP was followed at a wavelength of 340 nm.

2.5.5. Phosphofructokinase (EC 2.7.1.11)

PFK activity was assayed using a modified method of Hatzfeld et al. (1989) in a buffer containing 75 mM Hepes (pH 7.9), 15 mM $MgCl_2$, 0.1 mM NADH, 5 mM Fru-6-P, 1 U aldolase, 1 U triosephosphate isomerase, 1 U glycerol-3-P dehydrogenase, and the reaction started by adding 1 mM ATP.

2.5.6. Pyruvate kinase (EC 2.7.1.40)

The activity of pyruvate kinase was measured according to Hajirezaei et al. (1994) with minor modifications. PK activity was assayed in 0.5 M TES (pH 7.9) containing 10 mM $MgCl_2$, 40 mM KCL, 0.1 mM NADH, 2 mM dithiothreitol (DDT), 0.5 mM PEP and 4 U lactate dehydrogenase. The reaction initiated by addition of 1 mM ADP.

2.5.7. Cytosolic aldolase (EC 4.1.2.13)

Activity of cytosolic aldolase was determined as described by Haake et al. (1998) in a buffer containing 100 mM Tris (pH 8.5), 5 mM $MgCl_2$, 0.15 mM

NADH, 1 mM EDTA, 2.5 U glycerinaldehyde-3-P dehydrogenase and 6.5 U triosephosphate isomerase. The reaction was started by the addition of 2 mM fructose-1,6-bisphosphate and the absorption of NADH was followed at a wavelength of 340 nm.

2.5.8. Cytosolic fructose-1,6-bisphosphatase (EC 3.1.3.11)

The activity of cytosolic fructose-1,6-bisphosphatase (FBPase) was measured according to Kelly et al. (1982). The reaction mixture contained 100 mM imidazol (pH 7.1), 5 mM $MgCl_2$, 0.25 mM NADP, 1 U Glc-6-P-DH (Yeast) and 1 U phosphoglucoisomerase. A final concentration of 0.12 mM of fructose-1,6-bisphosphate was used as starter.

2.5.9. Phosphoenolpyruvate carboxylase (EC 4.1.1.31)

PEPC activity was determined spectrophotometrically according to Rolletschek et al. (2004).The assay was performed in 25 mM Tris (pH 8), 5 mM $MgCl_2$, 1 mM KHCO3, 0.2 mM NADH, 2 U malate dehydrogenase and 20 µl extract in a total volume of 0.3 ml and started with 5 mM PEP.

2.5.10. Malate dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase activity was determined according to Jenner et al. (2001) and assayed by monitoring the rate of change in A_{340} in the following mixture: 50 mM TES-NaOH (pH 7.2), 5 mM $MgCl_2$, 0.2 mM NADH, and 0.05% (v/v) Triton X-100. The reaction was started by the addition of 1 mM oxaloacetate (OAA).

2.6. Targeted metabolite profiling

To measure the levels of important metabolites involved in primary metabolism, two frozen excised cuttings with a fresh weight of about 100 mg were homogenized in liquid nitrogen followed by the addition of 1 ml of a 1:3:1 (v/v/v) water:methanol:chloroform solution. Samples were mixed thoroughly and incubated at 4°C for 20 minutes. The suspension was centrifuged for 5 min at

14,000 rpm. Supernatant was dried under vacuum at 35°C, re-suspended in 0.2-0.4 ml water and filtered through microtiter plate filter (microcon 10 kDa pore size; Millipore, Germany) at 4°C and 4000 rpm. The collected filtrate was used for metabolite measurements using ion chromatography coupled to mass spectrometry (IC-MS) according to Chen et al. (2005). The IC-MS instrumentation consisted of a Dionex HPLC system (Dionex, Idstein, Germany) with a MSQ mass detector (Dionex).

Soluble amino acids were determined according to Rolletschek et al. (2002). Extraction procedure was carried out as described for soluble sugars in section 2.4. Samples, including standards, were derivatized using the AccQ-Tag method (Waters Associates, Milford, Mass., USA). Derivatization was performed in a buffer containing 0.2 M boric acid (pH 8.8), 20 µl fluorescent agent AQC (aminoquinolyl-N-hydroxysuccimidyl carbamate) and 20 µl of the prepared standard mixture or sample in a final volume of 200 µl followed by Incubation at 55ºC for 10 min. Subsequently, samples were centrifuged for 1 min at 4ºC and at 8,000 rpm. Total aliquots were run on a reversed-phase HPLC system (Alliance 2795, Waters, Germany) for separation. The gradient was accomplished with buffer A containing 140 mM sodium acetate (pH 5.8) and 7 mM triethanolamine. Acetonitrile and water were used as eluents B and C. To separate the amino acids a reversed-phase column (XBridge^{TM} C18, 3.5µm, 3.0 x 150 mm) was used. Quantification of the concentration of individual amino acid was carried out by using Empower Pro software (Waters, Milford, MA).

ATP, ADP, and ADP-glucose contents were determined using a highly sensitive fluorescence method according to Haink and Deussen (2003). Adenosine nucleotides are specifically converted into fluorescent etheno-adenosine nucleotides. Prior to HPLC separation, an aliquot of the samples was derivatized with 10 % (v/v) chloracetaldehyde in 62 mM sodium citrate and 76 mM potassium dihydroxide phosphate, pH 5.2. The mixture was incubated for 40 min at 80°C, cooled immediately on ice, centrifuged at 14,000 rpm for 1 min and used for HPLC analysis. Separation was carried out using a reversed phase HPLC system (Alliance 2795, Waters). The gradient was accomplished with a

buffer containing 5.7 mM tetrabutylammonium bisulfate and 30.5 mM potassium dihydroxide phosphate, pH 5.8 and an eluent containing pure acetonitrile (Roti C Solv HPLC, Roth, Karlsruhe, Germany). A single run was set to 4.5 min following a reconditioning of 2 min with the eluent. The excitation wavelength was set at 280 nm and the emission wavelength at 410 nm. Chromatograms were integrated in all cases using the software package Empower Pro software (Waters, Milford, MA).

2.7. Determination of carbon and nitrogen concentrations

The relative content of total carbon and total nitrogen was measured according to Rolletschek et al. (2002) using dried, powdered samples by an elemental analyzer (Vario EL, Elementaranalysen systeme GmbH, Hanau, Germany). Each biological sample was measured in duplicates.

2.8. Recovery experiments (validation of the biochemical measurements)

A recovery experiment was carried out for enzyme activities and metabolites to evaluate the stability of the appropriate enzymes and metabolites during the extraction. For enzyme activity two different tissues of petunia containing cutting and leaf were either mixed together prior to extraction or used separately. Based on the results obtained for separately measured samples and the mixed tissues, the percentage of recovered enzyme activity was calculated. In the case of intermediates, a standard mix of desired metabolites was prepared. The mixture was added either to the plant material or to the extraction buffer prior to the extraction. The percentage of found metabolites was calculated after analysis by LC-MS. With that we confirmed that there were no or little losses during extraction by performing recovery experiments. The percentage of recovered enzyme activity was calculated as follows: phosphofructokinase 86±7%, cytosolic FBPase 108±2%, cytosolic GAPDH 126±16%, pyruvate kinase 85±1%, malate dehydrogenase 70±10%, cell wall invertase 75±12%, cytosolic invertase 95±4%, vacuolar invertase 108±2%, sucrose synthase 94±1%,

glucose6phosphate dehydrogenase 78±5%, cytosolic aldolase 108±8%, glucokinase 100±6% and PEP carboxylase 93±5%.

In case of metabolites following recovery percentages were determined: UDP-glucose 125±4%, citrate 87±10%, malate 72±8%, hexose-phosphate 82±7%, 3PGA 62±2%, pyruvate 70±6%, ADPGlc 106±9%, ADP: 117±2 % and ATP: 118±0.3 %.

2.9. Application of growth regulators

Combination of two different auxin analogs was used at two different concentrations. α-Naphtalene Acetic Acid (NAA) and Indole-3-Butyric Acid (IBA) (Duchefa, Haarlem, Netherlands) at 3 and 4.5 µM concentrations which were dissolved in 1 ml ethanol 96% before dilution with distilled water according to the producer's protocol. Treatment carried out in a foliar spraying manner. Growth and harvesting conditions was similar to non-treated condition as described in section 2.1. Stem bases from treated cuttings (0 hpe, no treatment) were excised at eleven time points which also described before. All biochemical analyses including measurement of metabolite levels and enzyme activities were performed using methods which explained for non-treated cuttings.

Foliar spraying of Naphthylphthalamic Acid (NPA) (Duchefa) was carried out to inhibit basal auxin transport in the cuttings. It was dissolved in 1 ml NaOH 1N according to the manufacturer's protocol, and diluted with distilled water to a final stock concentration of 100 µM. Appropriate amounts of stock solution were added to distilled water to obtain the different concentrations required. Five different concentrations of NPA including 10, 25, 50, 80 and 100 µM were tested. The concentration of 80 µM of NPA was chosen since rooting was delayed severely up to twenty days after excision without led the cuttings to be withered. Rooting zones of stem bases from treated cuttings (0 hpe, no treatment) were excised at eleven time points. Growth and harvesting conditions and all biochemical analyses including measurement of metabolite levels and enzyme activities were carried out as described before for non-treated condition.

2.10. Construction, sequencing and clustering of a normalized cDNA library of petunia cuttings

RNA was extracted from cuttings at various developmental stages grown under the conditions as described by Logemann et al. (1987) and poly(A) RNA was prepared using oligo(dT) cellulose Type7 according to manufactor's instruction (Amersham Pharmacia, Germany). Construction of the normalized cDNA library was performed as described (Lein et al., 2008), with minor modifications, using 5 µg poly(A) RNA. The cDNA library was subjected to three rounds of normalization, involving the denaturation, reassociation and removal of double-stranded cDNAs and the isolation and amplification of single-stranded cDNAs via polymerase chain reaction (PCR). After normalization, equalized cDNAs were ligated at random into the pCRblunt vector and transformed in competent E. coli cells followed by the selection of blue/white colonies. Clones were picked and sequenced by GATC Biotech AG using Capillary Sequencer systems ABI 3730 XL (Konstanz, Germany). Approximately 4,700 sequences were obtained after processing, a success rate of approximately 94%. The average reading length was 495 bp.

2.11. Microarray design, hybridization and analysis

Total RNA from different developmental stages and various tissues were extracted using QIAGEN kit (Qiagen, Hilden, Germany). To provide control tissues, the uppermost fully developed leaves of axillary shoots still attached at the stock plant were transversely pinched and used as wounded leaves. Samples were harvested two hours post wounding. In addition, petunia adventitious root system harvested 24 days post excision of cuttings (begin of lateral root formation) and used as root system. For microarray construction, the custom array service of NimbleGen was selected. The EST sequences generated in this study together with all sequences of *P. hybrida* and *P. axillaris* available (in 2007) at Genebank (15,713), TIGR (4,466), Max-Planck Institute (MPI) in Golm (17,943), University of Cologne (6), University of Fribourg (42) and the Solanaceae genomics network SGN (5,135) (Table 5), were assembled into

24,816 non-redundant unique sequences, which were used for probe design. Design of a 4-plex microarray with 72,000 features was carried out using the ArrayScribe software from NimbleGen (www.nimblegen.com) to generate three optimized independent probes per gene, with an average length of 36 base pairs per probe. Shorter sequences were represented by two probes. Array design, probe synthesis, hybridization, analysis, and data normalization was carried out by NimbleGen. A complete list of the gene IDs represented on the microarray, and the corresponding expression values in controls and the induction ratios is provided in Excel File 2 (Data-CD).

2.12. Statistical analysis of microarray data

All normalized expression values in different time points were compared with 0h. Significantly up- or down-regulated genes were recognized via Rank Product (RP) analysis (Breitling et al., 2004) using MeV (MultiExperiment Viewer, version 4.4.1.) software. For each individual time point, the expression values of each replicate were divided by expression values of all four replicates at 0h, followed by a log2-transformation for the Rank Product analysis. Then, median values of these paired log-fold changes were calculated for each gene-related sequence identifier. Finally, the median values were back-transformed by a power of two in order to obtain the real expression ratios. The same procedure was carried out for the comparison of control tissues. To extract up or down-regulated genes, median ratios with values above two (>2) were determined as up-regulated and median ratios with values below 0.5 (<0.5) were determined as down-regulated. Based on the RP statistical analysis for each gene, using 1000 permutations, P-values below (≤ 0.01) were considered as significant differentially expressed.

Plot of -Log10 of the computed P-values of the replicate effect versus the -Log10 of the P-values of the time effect was generated based on the minimum P-values derived from RP analysis according to Himanen et al. (2004) (Fig. 27). Minimum P-value within replicates of all time points was located on the abscissa

(X-axis) and minimum P-value of all ratios compared to 0h was located on the ordinate (Y-axis).

2.13. Real-Time PCR

The transcript levels of seven genes (GO_drpoolB-CL9530Contig1, cn1111, cn8317, IP_PHBS008L07u, IP_PHBS007P04u, cn3641 and cn5371) that were significantly induced during different developmental phases in the microarray experiments were confirmed by real-time PCR. RNA from five different time points (0, 2, 6, 72 and 192 hpe) was isolated from petunia cuttings according to Logemann et al. (1987). DNA was removed with RQ1 DNase (Promega, Madison), and first-strand cDNA was reverse transcribed using M-MLV RT RNase H reverse transcriptase (Promega) according to the manufacturer's protocol. This experiment was carried out using three independent biological replicates for each time point. Gene-specific primers were designed by using the online program Primer3 (http://frodo.wi.mit.edu/primer3/ ; Rozen and Skaletsky, 2000). The relative cDNA abundance was detected by the i-cycler iQ (Bio-Rad) using iQ SYBR Green SuperMix (Bio-Rad). The mRNA levels were determined by relative quantification using actin mRNA (cn1159) as a reference related to the 0h control. Primers used are listed in Table 1.

2.14. Plasmids and cloning

Standard cloning procedures such as amplification of DNA fragments with polymerase chain reaction (PCR), transformation of *Escherichia coli* (*E. coli*) cells, preparation of plasmid DNA, restriction digest and agarose gel electrophoresis were carried out as described (Sambrook et al., 1989). Cloning procedure into pCR 2.1 vector was performed using TOPO cloning reaction (Invitrogen) according to the manufacturer's protocol. DNA fragments were eluted from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden) according to the manufacturer's protocol. Plasmid DNA purification from Agrobacterium were carried out using the QIAprep Spin Miniprep Kit (Qiagen, Hilden) according to the producer's protocol.

Table 1. Gene-specific primers for Real-Time qPCR. Analysis was carried out using actin as housekeeping gene and "2^ -ΔΔCt" formula. All Real-Time qPCR reactions were repeated three times.

Seq. Id.	Function	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
GO_drpoolB-CL9530Contig1	Pectinase	CCGTCAGTATTGGGGCTTAG	ACCACACAGGGGACCTAATG
cn1111	Hexose transporter	CCCACTGCTCAGCTGTAAGA	AGAAACGTGAATTCGCAAGG
cn8317	Nitrate transporter	ACAACAAGGCCATGGTTAGC	TCCAATTCAATCCCCATTTC
IP_PHBS008L07u	F-box	ACAGCATCAGGGAGACATTG	CAAAACTGTCAGGGTTGGAC
IP_PHBS007P04u	Ubiquitin-protein ligase	TGGACAGTGCCAATCAAGTC	CCTCGCAAAGGAAAGTGAAG
cn3641	Trehalose-p-phosphatas	CGAGTGCGTGCTACTCTCTG	CTGCTCCCTAGACCATCTGC
cn5371	Zinc/Iron transporter	GGGATTGCTTAATGCATGTTC	CATGCCACCAGCACCTAGTA
cn1159	Actin	TCAGATTTGCTGGCATGAAG	ATTGTCCAAAGCAAGGATGG

2.15. Oligonucleotides and DNA sequencing

Custom sequencing and PCR oligonucleotides were purchased from Metabion (Martinsried, Germany). DNA sequencing was done at the IPK service facility.

2.16. Bacterial strains and vectors

The strain *Escherichia coli* XL1 Blue MRF was used for all cloning steps. The strain *Agrobacterium tumefaciens* C58C1 pGV2260 (Deblaere et al., 1985) was used to transform petunia plants. The vectors used in this work are listed in Table 2.

Table 2. Vectors

Vector	Application/resistance	Source/reference
pCR2.1	Cloning vector, Kan^r	Invitrogen
pBinAR	Binary vector, Kan^r	Höfgen and Willmitzer, 1990
pK2GW7	Binary vector, Kan^r	Karimi et al., 2002
p9NOS	Binary vector, Kan^r	DNA Cloning Service, Hamburg., Germany

2.17. Plant growth conditions in tissue culture

Leafy stem cuttings of *Petunia hybrida* cv. Mitchell were generated through germination of sterilized seeds and maintained in tissue culture under a 16-h-light/8-h-dark period (200 µmol m^{-2} s^{-1} light, 21°C) on Murashige and Skoog basal medium (Duchefa) containing 3% (w/v) sucrose.

2.18. Plant transformation

Transformation of petunia plants was carried out by *A. tumefaciens* mediated gene transfer using a modified method of Lutke (2006).

2.18.1. Explant preculture

After three to four weeks growth in glass cups, leaves were cut from the upper half of the plant (leaves were typically about 2-cm wide and 3-4 cm long). The explants were prepared by trimming away the edges of the leaf (about 2 mm). Horizontal slices were made through the leaf to generate explants approx. 4 x 4 mm in size. 50-60 explants were transferred to plates with sterile 7-cm filter paper containing 2 ml of liquid pre-culture media which was prepared as follows: MS-medium containing 3% (w/v) sucrose and 1 ml/l Gamborg's vitamin (Sigma, Steinheim**,** Germany) (pH 5.7). Subsequently, the plates were wrapped with parafilm and cultured at 24°C with 16:8-photoperiod and light intensity of 200 µmol m^{-2} s^{-1} for two days.

2.18.2. Agrobacterium culture preparation and infection

One day before transformation, Agrobacterium suspension was prepared according to Lutke (2006) and grown overnight on the shaker at 28°C by

agitation at 200 rpm. On the day of transformation, the 20 ml Agrobacterium suspension was transferred to a sterile 50-ml culture tube and centrifuged at 4000 rpm for 15 min at 4ºC to pellet the bacteria. The supernatant was decanted and the pellet was resuspended by vortexing in 20 ml of pre-culture media. The OD_{600} was measured and the culture was further diluted with pre-culture medium to obtain an OD_{600} 0.4 – 0.5. The inoculation procedure was carried out as described (Lutke, 2006).

2.18.3. Washing and placing on the selection media

After three days, explants were washed three times to remove residual Agrobacterium by adding 5 ml of wash solution which was prepared as follows: MS basal medium (Duchefa) (pH 5.8, filtered) and 500 µg/ml ticarcillin. Plates were gently shaken for 15 min at room temperature. After the wash period, leaf explants were dried using sterile 7-cm filter papers and placed on the MS-medium containing 3% (w/v) sucrose, 0.9% (w/v) Phyto-Agar (pH 5.7),1 ml/l Gamborg's vitamin, 1 ml/l BA (N-6-benzylaminopurine), 100 µl/l NAA (naphtaleneacetic acid), 500 µg/ml ticarcillin and 100 µg/ml kanamycin as petunia selection medium (PSM) under the following conditions: 24°C with 18:6-photoperiod and light intensity of 110 µmol m^{-2} s^{-1} for one month.

2.18.4. Selection and regeneration

Generated shoots were maintained in tissue culture on MS-medium containing 1% (w/v) sucrose, 0.7% (w/v) Phyto-Agar and 250 µg/ml ticarcillin under the following conditions: 21°C, and a 16h light , 8h dark regime (200 µmol m^{-2} s^{-1} light). Regenerants were screened for the expression of the transgene by Northern blot which is described in section 2.3. Positive transformants were vegetatively propagated in tissue culture. In the greenhouse, positive selected lines were grown on soil (Klasmann Substrat 1, Geeste, Germany) in 0.11l pots under the same light regime with illumination of 180 µmol photons m^{-2} s^{-1} at 22°C and a relative humidity of 60%.

3. Results

3.1. Anatomical, biochemical and gene expression analysis during ARF in petunia cuttings

3.1.1. Establishment of a stable rooting system for molecular and biochemical analyses in *P. hybrida*

Most of the previous studies concerning root formation were carried out with plants under defined, but artificial conditions such as experiments in petri dishes or by application of exogenous hormones. Since the results of these experiments might lead to a misinterpretation of the pathways that are modulated by endogenous regulators prior to the initiation of root formation, we decided to use *P. hybrida* as an ex-vitro model system. Cuttings were treated without any external additives to enable accurate measurement of biological parameters during ARF. To this end, a neutral substrate, Perlite, was chosen to analyze metabolic and transcriptional changes and to exclude potential complications from microbial contamination and nutritional stress. *P. hybrida* stock plants were generated from seeds of *P. hybrida* cv. *Mitchell.* To obtain homogenous and comparable leafy cuttings for the biochemical and transcriptome investigations, first and second cuttings were removed and discarded. For all experiments, shoot tip cuttings of *P. hybrida* of similar size with four to five leaves (Fig. 5) were transferred without any external additives to Perlite and watered. Roots emerged after 8 d from the first 5 mm of the cutting stem base. All analyses were therefore carried out within the first 8 d using the rooting zone (Fig. 5).

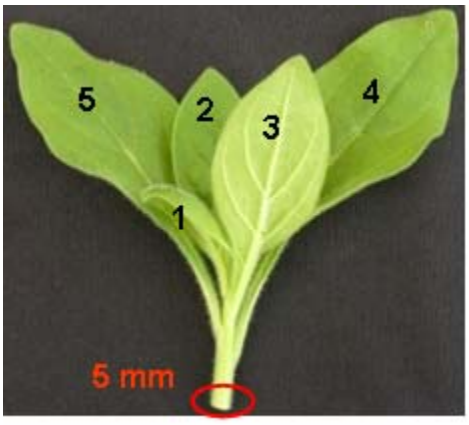


Figure 5. Rooting zone (stem base) of a petunia cutting used as starting material. Cuttings of similar size containing five leaves were harvested.

3.1.2. Anatomy of ARF in *P. hybrida* cuttings

Representative cross sections of stems showed that the main tissues of the stem base at the time of excision (0 **h**ours **p**ost **e**xcision abbreviated as hpe) are the cortex, the pith parenchyma and a ring of vessels between them (Fig. 6a) consisting of the outer phloem, the cambium, the xylem and the inner phloem (Fig. 6b). First meristematic cells of the developing root meristem, i.e. small cells with a dense cytoplasm and large nuclei were found at 72 hpe (Fig. 6c). First well developed young root meristems could be observed at 96 hpe (Fig. 6d). First root primordia with apical meristem and differentiation of the root body appeared at 144 hpe (Fig. 6e). Roots with vascular bundles surrounded by elongated cells of the elongation zone were regularly visible at 192 hpe (Fig. 6f). All basal segments of the three cuttings of the same date showed the same anatomical pattern, except 192 hpe at which only two out of the three segments contained fully developed roots. Additionally, a histochemical staining of starch granules is shown in figure 6 (g-i) which will be described in section 3.1.5 in more details.

3.1.3. Transcript accumulation of putative marker genes for ARF and of genes encoding enzymes involved in primary carbohydrate metabolism

The expression of some genes involved in cell division and auxin metabolism were analyzed by Northern blot analysis in order to test, if they could be used in further molecular analyses as markers for particular developmental stages in ARF. To check whether the first cytological signs of new meristematic cells are also detectable at the molecular level, expression of *CycB1* encoding a mitotic B1 cyclin and serving as marker for mitotic activity (Porceddu et al., 1999) was monitored. The RNA of this gene started to accumulate 48 hpe, further increased up to 144 hpe and declined at 192 hpe (Fig 7a). The second one, a GH3-auxin responsive gene, has been shown in many plants to be induced by auxin (Li et al., 1999), probably serving as a signal in ARF. The RNA accumulation of this gene exhibited a biphasic character. It showed a first maximum at 4 hpe, declined thereafter and started to increase again at 24 hpe (Fig. 7b).

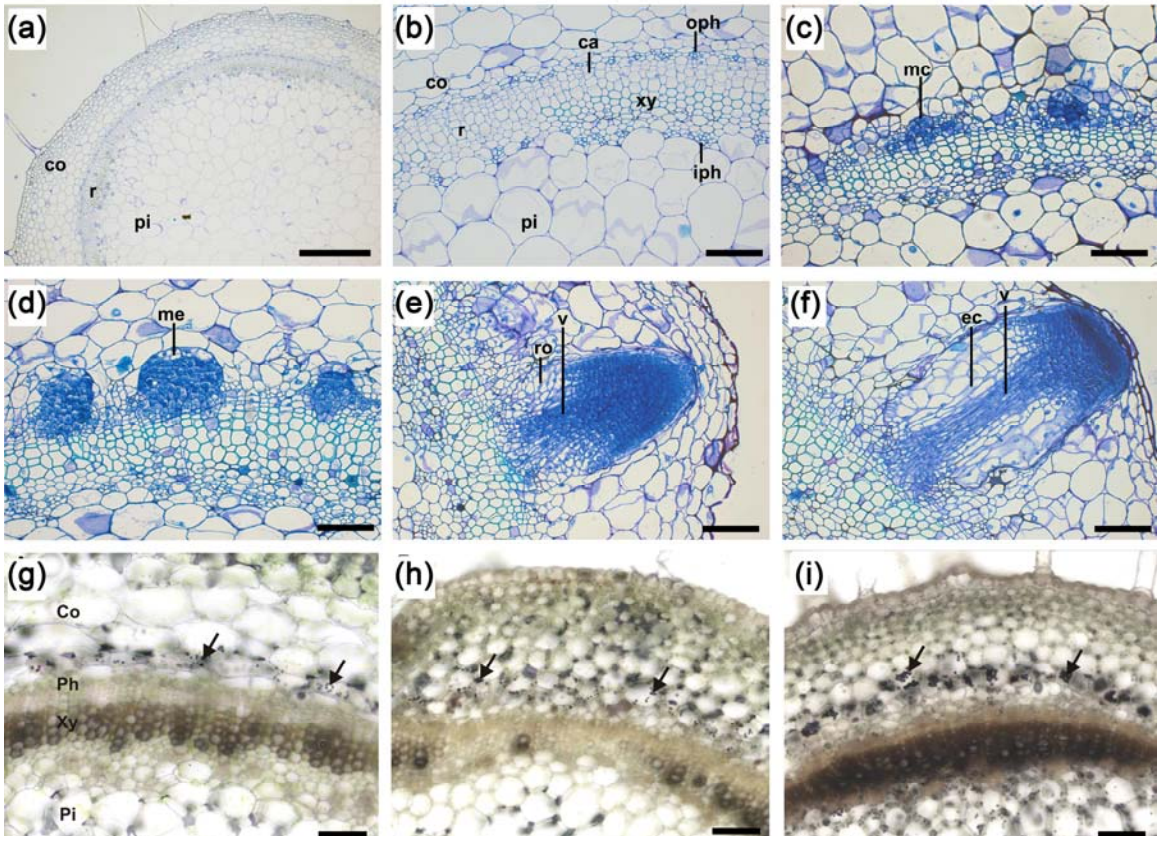


Figure 6. Anatomy of adventitious root formation (a–f) and starch accumulation (g–i) in the stem base of petunia (*Petunia hybrida*) cuttings. In all micrographs, cross-sections from *c*. 1–4 mm above the excision site are shown. (a,b) 0 hours post excision (hpe), typical stem anatomy consisting of the cortex (co), the pith parenchyma (pi) and a ring of vessels (r) with outer phloem (oph), the cambium (ca), the xylem (xy) and the inner phloem (iph); (c) 72 hpe, first meristematic cells (mc) of developing root meristems, that is, small cells with a dense cytoplasm and a large nucleus; (d) 96 hpe, first root meristems (me); (e) 144 hpe, first differentiating root primordia with an organized meristem and differentiation of cells of the root body containing root cortex (ro) and vascular bundle (v); (f) 192 hpe, first roots with vascular bundles (v) in the center surrounded by elongated cells (ec) of the elongation zone; (g–i) histochemical staining of starch granules at 0 hpe (g), 24 hpe (h) and 72 hpe (i). Arrows show the presence of starch. Bars, (a), 500 µm; (b–i), 100 µm. The images are prepared by Dr. Klaus-T. Haensch , IGZ, Erfurt.

Focusing on key enzymes in carbohydrate metabolism, gene expression analyses were performed in a time course of ARF. An increased accumulation of transcripts coding for cell wall invertase was observed at 2 and 4 hpe (Fig. 7c). A strong parallel induction of STP4 mRNA encoding a plasmalemma-localized monosaccharide transporter (Truernit et al., 1996) was monitored (Fig. 7d). While the transcript level of cell wall invertase already decreased 6 hpe, there was still a high transcript level of *STP4* up to 48 hpe. Additionally, the transcript level of

vacuolar invertase was high throughout the first 5 days after excision followed by a decrease to a low level (Fig. 7e). No significant changes in the transcript levels of sucrose synthase (SuSy) and cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) could be detected during the different developmental stages of root formation (Fig. 7f,g).

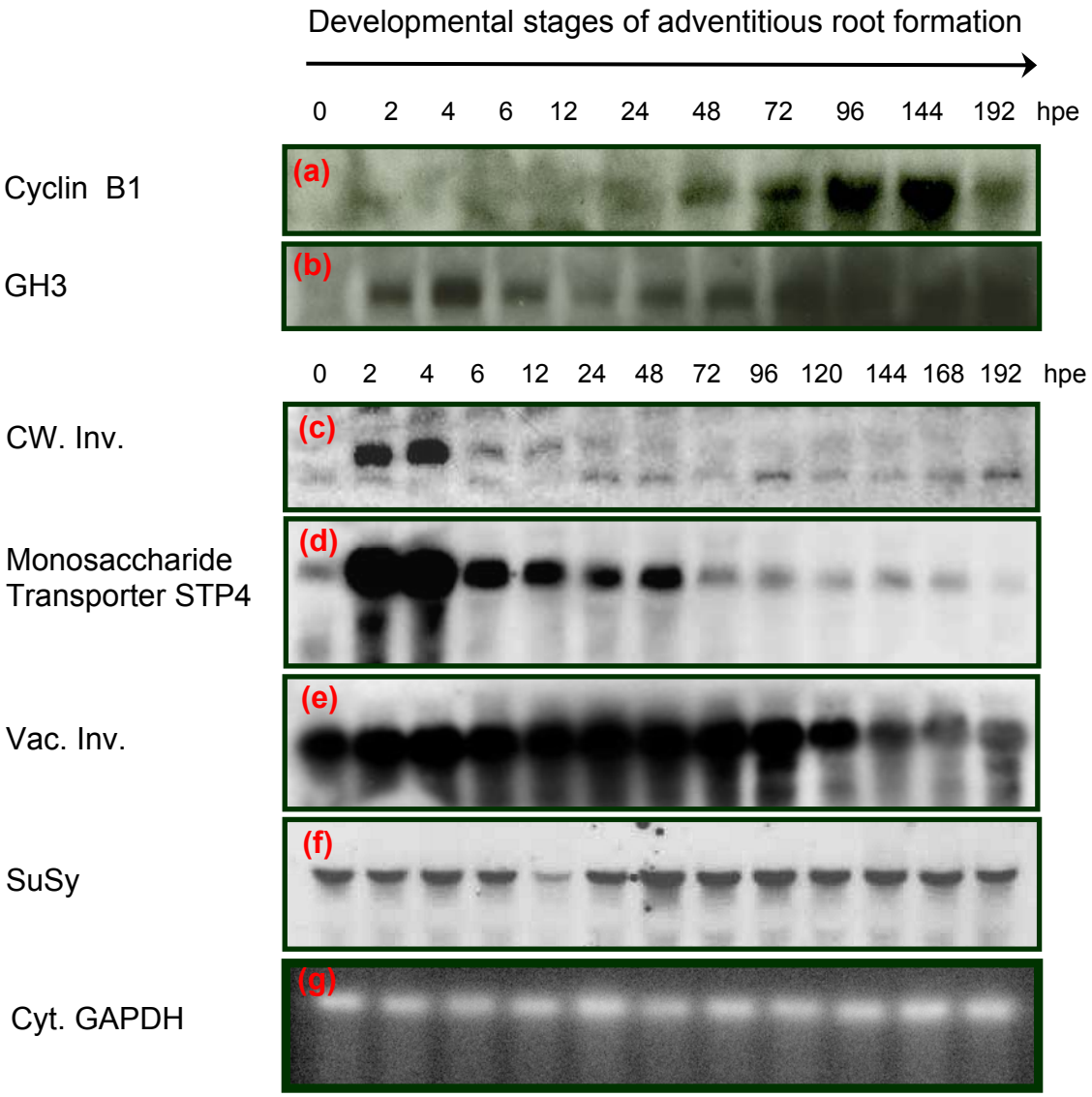


Figure 7. Transcript accumulation of putative marker genes for ARF and of genes coding for enzymes involved in carbohydrate metabolism. Northern blot analysis was performed with 20 µg RNA per sample separated on a 1.5% (w/v) formaldehyde agarose gel. After transfer of RNA to nitrocellulose membrane, it was hybridized to radioactively labeled cDNA fragments of the corresponding genes. (a) cyclin B1, (b) GH3-auxin responsive gene, (c) cell wall invertase, (d) sugar transport protein 4 (STP4), (e) Vacuolar invertase (f) sucrose synthase, (g) cytosolic glyceraldehyde-3-phosphate dehydrogenase.

3.1.4. Activities of enzymes involved in primary carbohydrate metabolism

To assess whether carbohydrate metabolism was changing during ARF, the activity of enzymes involved in various pathways was analysed (Fig. 8). To this end, several enzymes were chosen that might play an important role in the regulation of the transition of sucrose-derived carbon to energy production or protein synthesis. For all determinations of enzymatic activities it must be taken into consideration that the respective values represent their total cellular activity without distinguishing their possible functions in different subcellular compartments except where indicated therein.

A general decrease was observed for enzymes involved in sucrose metabolism, with particular differences among the enzymes. The activities of vacuolar (Fig. 8b) and cytosolic invertase (Fig. 8c) remained unchanged during the first 6 hpe and then decreased continuously until 192 hpe to 20% and 25% of the initial value, respectively. The activity of sucrose synthase (SuSy) (Fig. 8d) decreased to 40% at 4 hpe and increased again until 12 hpe to the initial value followed by a continuous decrease to 20% at 192 hpe. To find out whether the imported sucrose from source tissue is degraded in the apoplast, the activity of extracellular invertase was measured. Unlike the soluble invertases, cell wall invertase activity increased at the beginning threefold to reach a maximum at 6 hpe and decreased almost to its basal activity before roots emerged (Fig. 8a).

Among the enzymes involved in glycolysis and gluconeogenesis, the activity of phosphofructokinase (PFK), a crucial regulatory and definitive enzyme of sugar degradation via the Embden-Meyerhof pathway, increased constantly up to fourfold (Fig. 8e), whereas cytosolic fructose-1,6-bisphosphatase (FBPase) in gluconeogenesis pathway remained unchanged during the first period but was reduced to 40% in the later course of ARF (Fig. 8h). An increase of twofold was observed for the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) until 6 hpe, but it decreased later to the basal level (Fig. 8f). Cytosolic aldolase activity showed no significant change up to 144 hpe. Subsequently, a twofold increase was observed followed by a decrease at 192 hpe back to the basal level (Fig. 8g). The activity of pyruvate kinase (PK) exhibited fluctuation up to 12 hpe

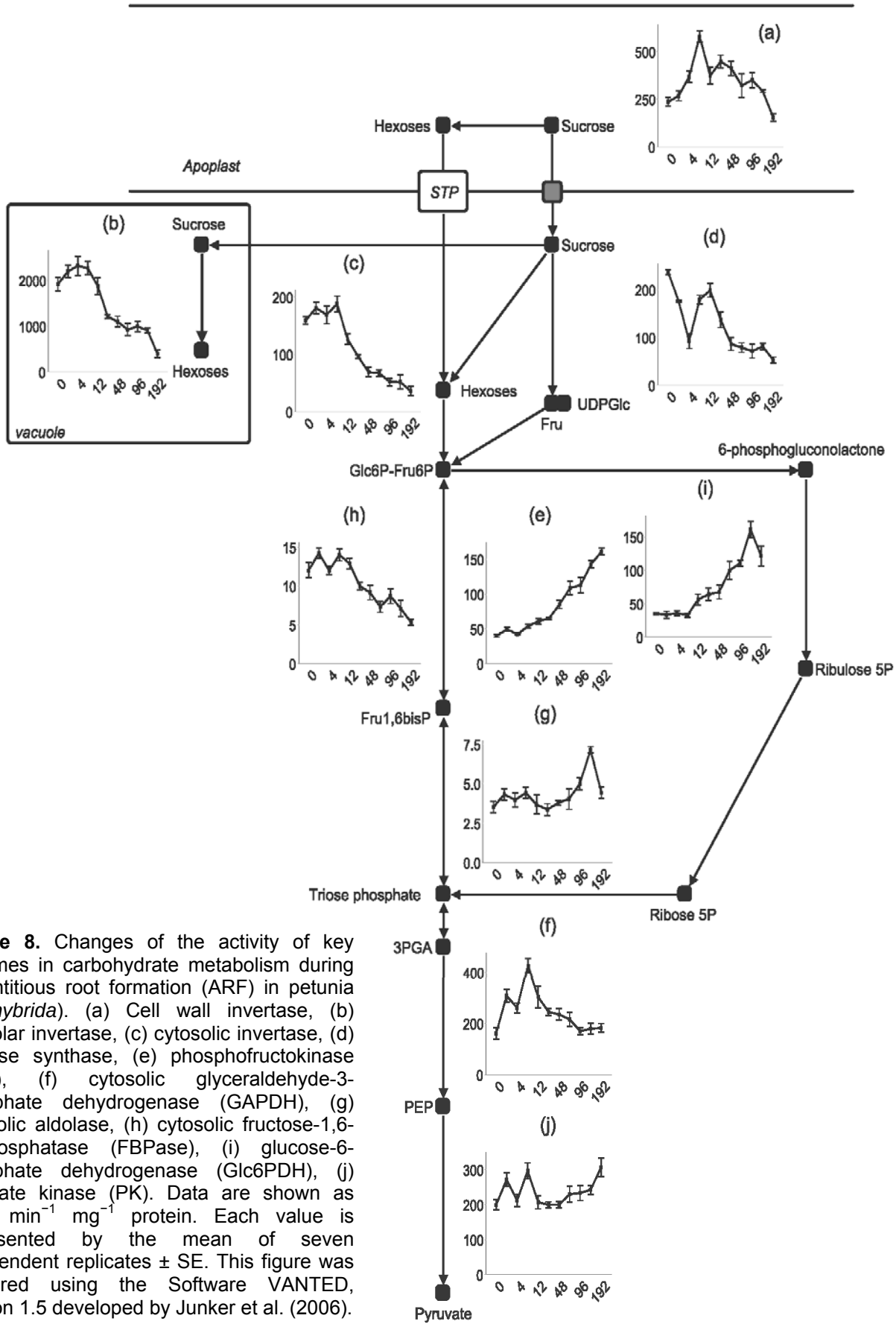


Figure 8. Changes of the activity of key enzymes in carbohydrate metabolism during adventitious root formation (ARF) in petunia (*P. hybrida*). (a) Cell wall invertase, (b) vacuolar invertase, (c) cytosolic invertase, (d) sucrose synthase, (e) phosphofructokinase (PFK), (f) cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (g) cytosolic aldolase, (h) cytosolic fructose-1,6-bisphosphatase (FBPase), (i) glucose-6-phosphate dehydrogenase (Glc6PDH), (j) pyruvate kinase (PK). Data are shown as nmol min^{-1} mg^{-1} protein. Each value is represented by the mean of seven independent replicates ± SE. This figure was prepared using the Software VANTED, version 1.5 developed by Junker et al. (2006).

and revealed a slight but continuous increase thereafter (Fig. 8j). Furthermore, the activity of glucose-6-phosphate dehydrogenase (Glc6PDH), the key enzyme in the pentose phosphate pathway, exhibited a threefold increase beginning at 12 hpe (Fig. 8i). Because the activities of cytosolic aldolase, cytosolic GAPDH and pyruvate kinase did not change significantly (Fig. 8g,f,j), the first enzymes of the corresponding pathways seem to control the metabolic activity by which sucrose is catabolized.

Finally, two enzymes involved in citric acid cycle were monitored (Fig. 9). While there was a nearly threefold increase in PEP carboxylase activity after 48 hpe (Fig. 9a), the activity of malate dehydrogenase did not change significantly up to 96 hpe, increased two times at 144 hpe and fell again to the basal value upon root emergence (Fig. 9b).

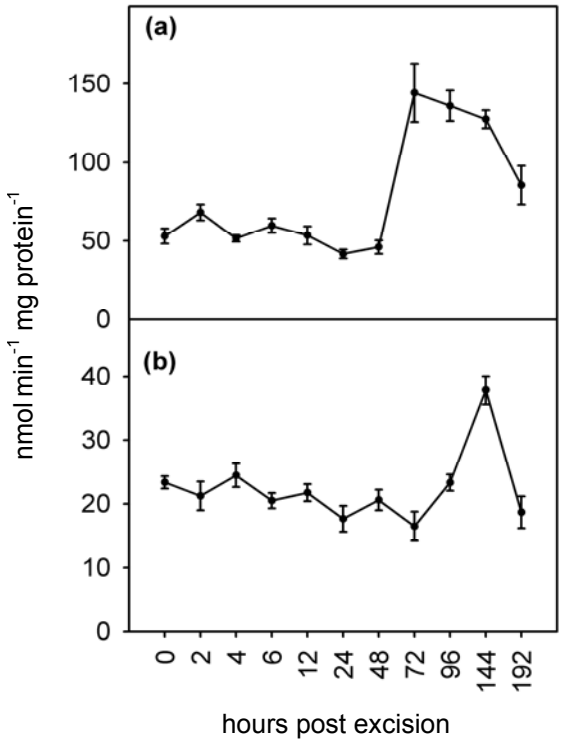


Figure 9. The activity of (a) PEP carboxylase and (b) malate dyhydrogenase during adventitious root formation in petunia cuttings. Data are shown as nmol min^{-1} mg $protein^{-1}$. Each value is represented by the mean of seven independent replicates ± SE.

3.1.5. Levels of soluble and insoluble sugars during ARF

The levels of soluble sugars including glucose, fructose and sucrose were low in the first hours post excision, but a continuous increase was observed after 24 hpe and reached the highest level before the emergence of roots (Fig. 10a,b). The level of starch began to increase after 72 hpe to a 16-fold higher level at 192 hpe (Fig. 10c).

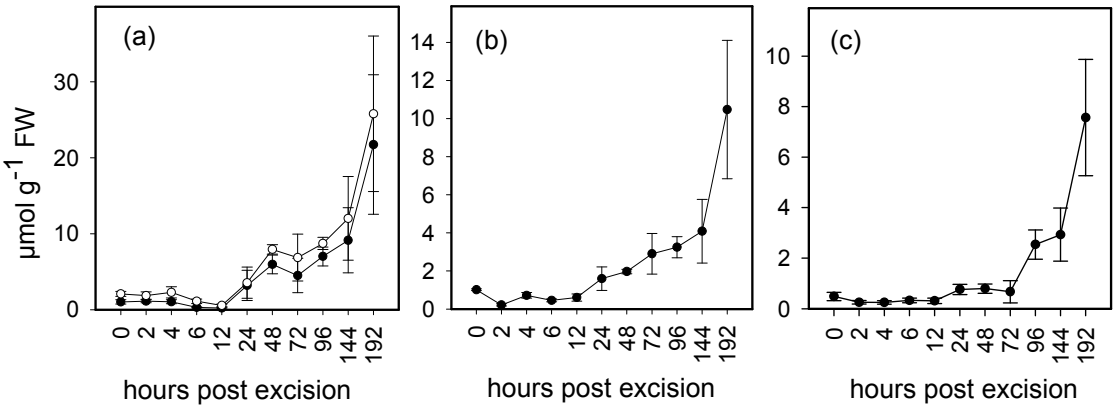


Figure 10. Alterations in the levels of soluble and insoluble sugars during adventitious root formation (ARF) in petunia (*Petunia hybrida*) cuttings. (a) glucose (closed circles) and fructose (open circles), (b) sucrose, (c) starch. All data are shown as µmol g^{-1} FW. Each value is represented by the mean of five independent replicates ± SE.

In order to find out whether starch accumulation could be monitored by an aggregation of starch granules, a detailed microscopic investigation was carried out. At early stages of ARF starch granules were only detectable in the starch parenchyma cells adjacent to the inner cortex (Fig. 6g,h). A strong accumulation of starch granules, however, was detected three days after excision. Here, starch granules were distributed over various cell types pointing to an accumulation in starch parenchyma cells and pith (Fig. 6i). Additionally, correlation analysis of soluble and insoluble sugars revealed a positive correlation between hexoses and sucrose as well as between sucrose and starch during ARF in *P. hybrida* cuttings (Appendix Fig.1).

3.1.6. Levels of intermediates involved in primary metabolism during ARF in petunia

To date, the importance of intermediates involved in primary metabolism for ARF has been poorly understood. To assess the levels of intermediates during ARF in petunia cuttings, a metabolite profiling was performed using ion chromatography–mass spectrometry (ICMS) and fluorescence HPLC. The level of UDP-glucose showed a slight increase between 4 hpe and 8 hpe (Fig. 11a), whereas the level of hexose phosphate was reduced to 50% immediately after excision, increased slightly during the next 12 h and decreased again (Fig. 11d). 3-Phosphoglycerate levels decreased slightly during ARF (Fig. 11c). Pyruvate concentrations increased fourfold at 48 hpe and decreased again at later stages (data not shown). After 12 hpe, a continuous increase in ADP-glucose was observed, reaching 16-fold higher levels until root emergence (Fig. 11b) which was associated with a similar rise in ATP levels up to fourfold (Fig. 11e).

An overall measurement of the level of intermediates involved in citric acid cycle revealed that the concentrations of all metabolites detected increased between 24 hpe and 48 hpe. For citrate, a continuous increase to 125-fold higher levels was observed, which was most pronounced between 72 hpe and 96 hpe (Fig. 11g). There was also a tenfold and an eightfold increase in α-ketoglutarate and isocitrate concentrations, respectively (Fig. 11h,f). The concentrations of malate elevated nearly fourfold at 144 hpe and decreased to the half before roots emerged (Fig. 11i).

The pool of total amino acids was depleted between 6 hpe and 48 hpe (Fig. 12a). Thereafter, a recovery was observed reaching 50% of the initial value at 192 hpe. Individual amino acids followed this general pattern with glutamine and asparagine (Fig. 12c,e) as the predominant amino acids, followed by glutamate, and aspartate (Fig. 12b,d). Total protein levels increased steadily to fourfold higher level during the whole time course of ARF (Fig. 12f). All amino acids behaved similarly during ARF being depleted to a low level between 6 and 48 hpe and increasing again to either exceed the basic levels (aspartate and glutamate), to remain the basic levels (asparagine, glutamine) or to reach the

basic levels (all other amino acids except arginine (Appendix Table 1) and proline, see section 3.2.3).

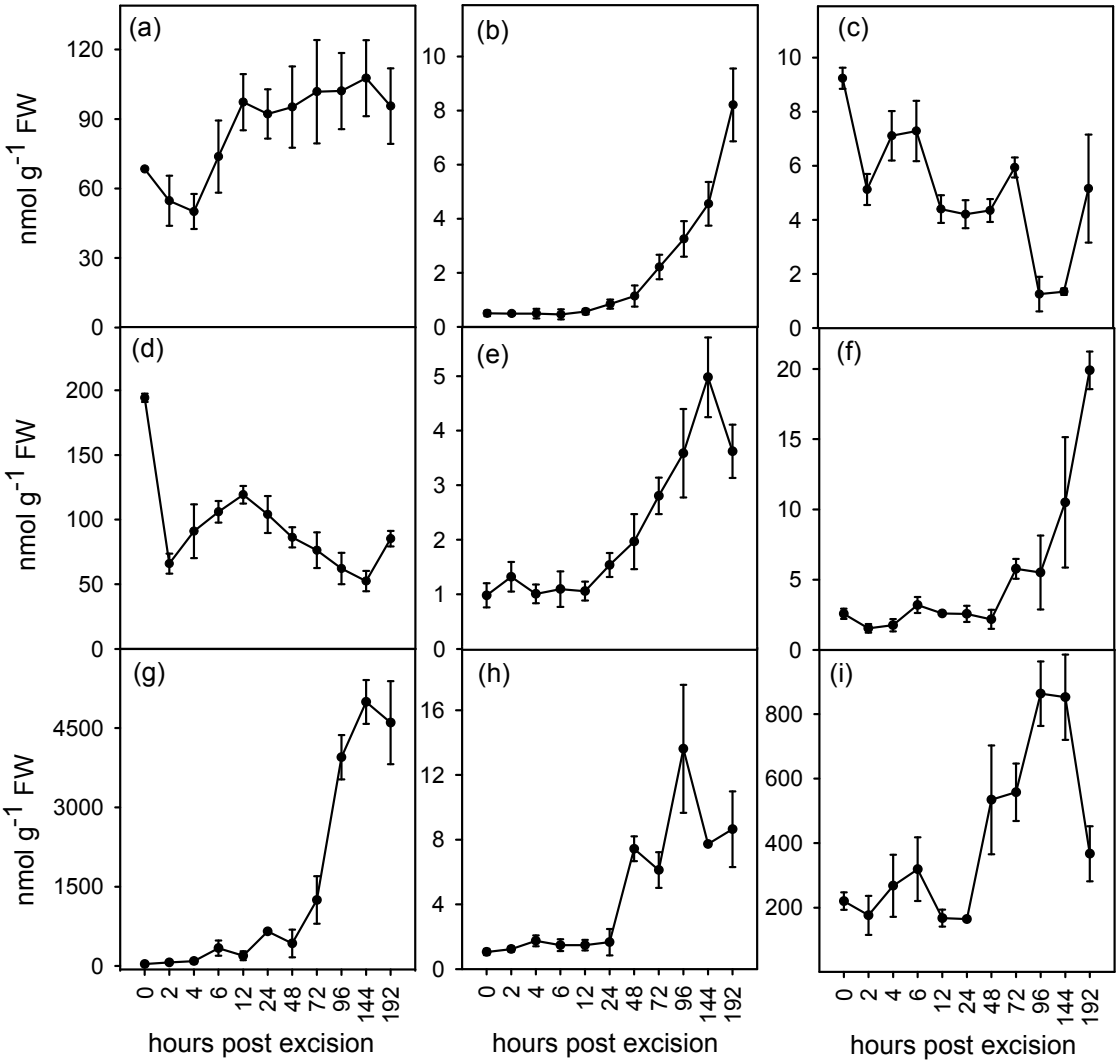


Figure 11. Alterations in the concentrations of metabolites involved in glycolysis, citric acid cycle and respiration during adventitious root formation (ARF) in petunia (*Petunia hybrida*) cuttings. (a) UDP-glucose, (b) ADP-glucose, (c) 3PGA, (d) hexose-P, (e) ATP (f) isocitrate, (g) citrate, (h) α-ketoglutarate and (i) malate. All data are shown as nmol g^{-1} FW. Each value is represented by the mean of five independent replicates ± SE.

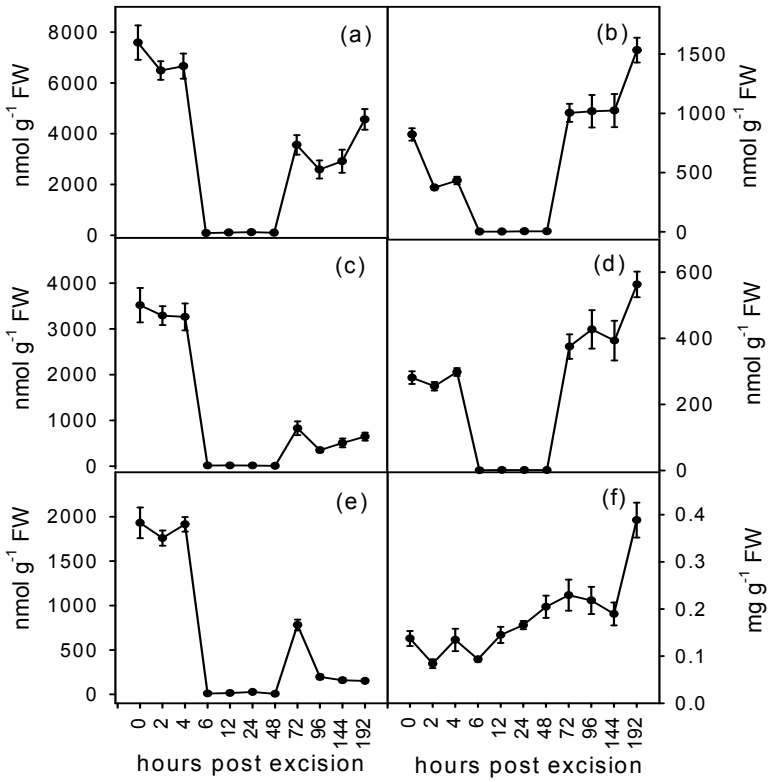


Figure 12. Levels of amino acids and total protein during adventitious root formation (ARF) in petunia (*Petunia hybrida*). (a) total amino acids, (b) glutamic acid, (c) glutamine, (d) aspartic acid, (e) asparagine and (f) total protein. All data are shown as either nmol g^{-1} FW (a-e) or mg g^{-1} FW (f). Each value is represented by the mean of five independent replicates ± SE.

3.1.7. Carbon to Nitrogen ratio during ARF

To evaluate whether the accumulation of carbon-containing metabolites was reflected by a change of carbon to nitrogen ratios during ARF, total concentrations of carbon and nitrogen were measured. The results showed that parallel to sugar accumulation, an increase in the carbon to nitrogen ratio up to 2.5-fold was monitored, beginning 48 hpe (Fig. 13).

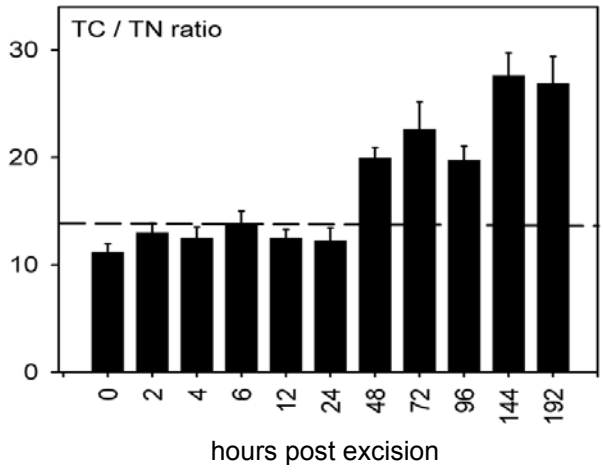


Figure 13. The relative ratio of total carbon to total nitrogen during ARF. Data are shown as percentage of dry matter. Each value is represented by the mean of six independent replicates ± SE

3.2. Effect of exogenous auxin on metabolic and anatomical changes during ARF in petunia cuttings

3.2.1. Application of exogenous auxin analogs during ARF

In order to understand the role of auxin and to unravel whether changes in enzyme activities and metabolite concentrations observed during ARF (see sections 3.1.4, 3.1.5 and 3.1.6) are due to an increased endogenous auxin concentration, two auxin analogs α-Naphtalene Acetic Acid (NAA) and Indole-3-Butyric Acid (IBA) were used. According to investigations conducted in the course of this thesis, the combination of 3 µM NAA and 4.5 µM IBA was sufficient to induce ARF in petunia cuttings (Fig. 14). The majority of the cuttings rooted 6 days after NAA and IBA application, whereas non-treated cuttings showed first visible roots between 8 and 9 days after excision. All analyses on the rooting zone were therefore carried out within the first eight days. Results showed that there were no significant differences between normal and auxin-induced conditions regarding metabolic activities except in the case of some amino acids and apoplastic invertase.

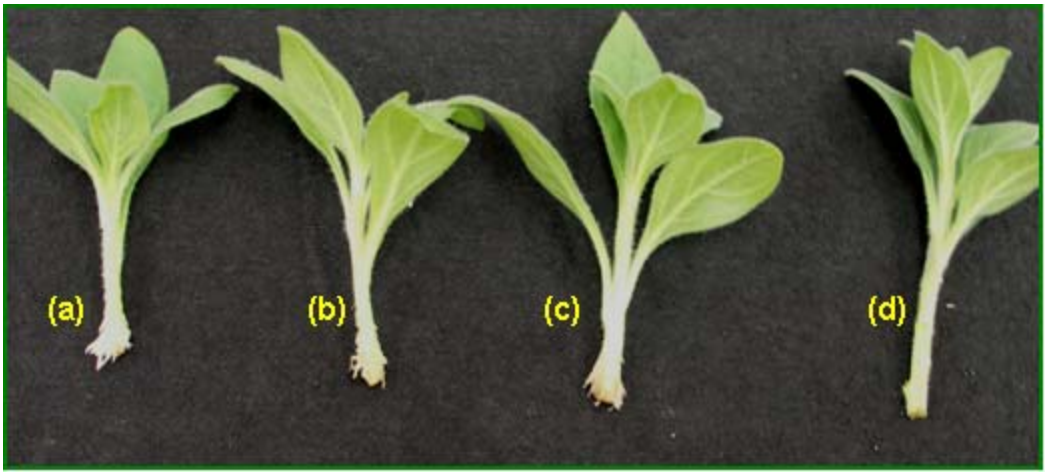


Figure 14. Comparison of rooting behavior between auxin-induced (a-c) and control condition (d), 7 days after excision. Foliar spraying of 3 µM NAA and 4.5 µM IBA was used as auxin enhancer.

Soluble sugars including glucose, fructose and sucrose accumulated during ARF and displayed a similar pattern in comparison with non-treated

cuttings except the initial values which were about two times higher (Fig. 15a,b and c).

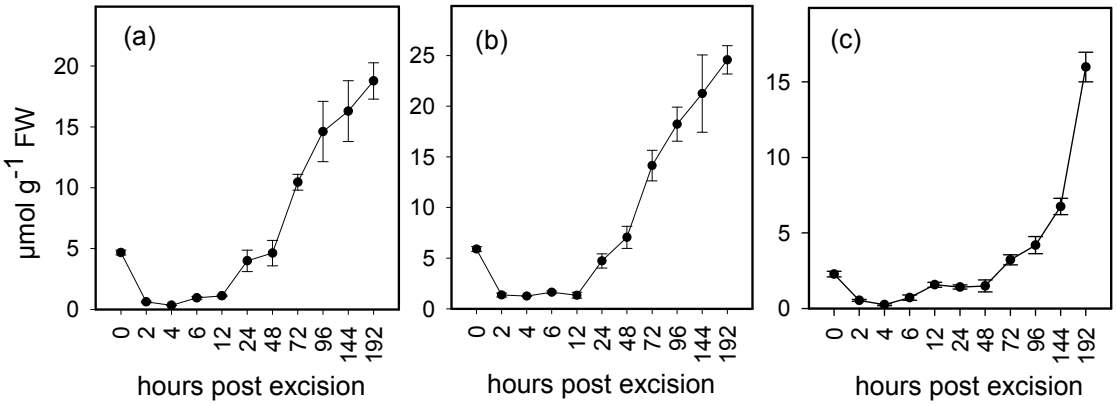


Figure 15. Alterations in the levels of soluble sugars during adventitious root formation (ARF) in petunia (*Petunia hybrida*) cuttings in auxin-induced condition. (a) glucose, (b) fructose, (c) sucrose. All data are shown as µmol g^{-1} FW. Each value is represented by the mean of five independent replicates ± SE.

A discontinuous increase in the activity of apoplastic invertase under auxin-induced condition during ARF was observed. It decreased two times at 2 hpe and 72 hpe and increased again to the initial level in both cases (Fig. 16a). Furthermore, there were no significant differences in the activity pattern of cytosolic invertase (Fig. 16b), vacuolar invertase (Fig. 16c), PFK (Fig. 16d), cytosolic FBPase (Fig. 16e), and glucose 6-phosphate dehydrogenase (Fig. 16f) in auxin-treated cuttings, relative to non-treated condition (Fig.8). Almost in every case, investigated enzymes behaved similarly independent of the presence or absence of auxin analogs.

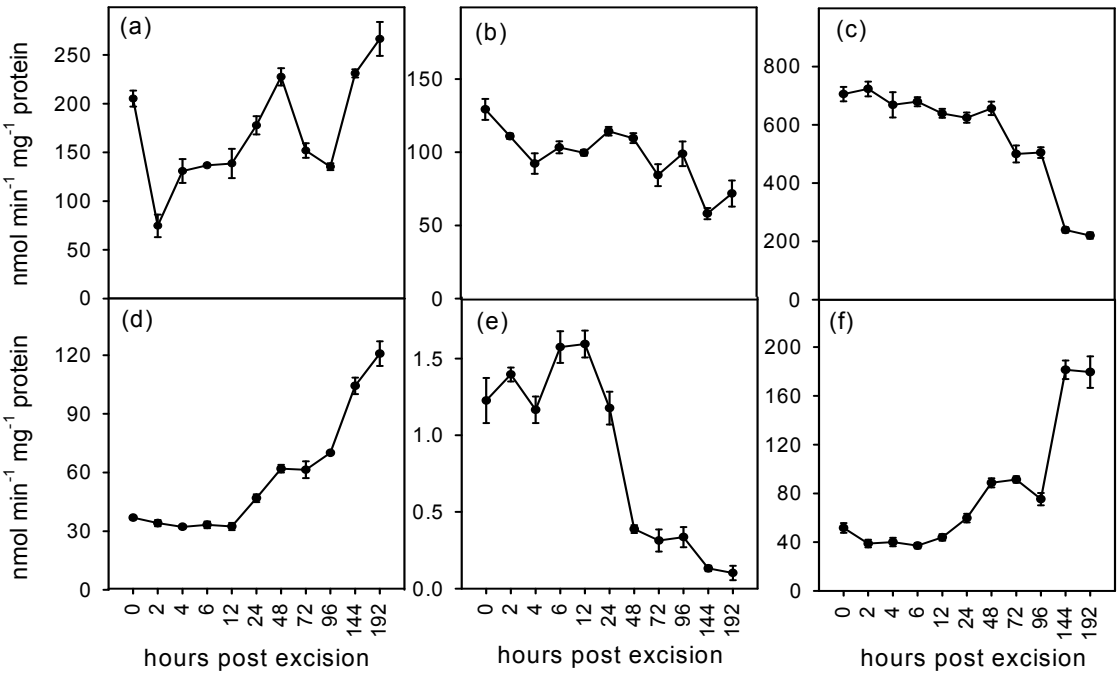


Figure 16. Alterations in the enzyme activities involved in sucrose degradation, glycolysis and pentose phosphate pathways during adventitious root formation (ARF) in petunia (*Petunia hybrida*) in auxin-induced condition. (a) Cell wall invertase, (b) Cytosolic invertase, (c) Vacuolar invertase, (d) PFK, (e) Cytosolic FBPase and (f) Glu6PDH. All data are shown as nmol $min^{-1}mg^{-1}$ protein. Each value is represented by the mean of five independent replicates ± SE.

In addition, metabolite analyses showed that there was a slight depletion of total amino acids after 24 hours. Thereafter, a fourfold increase was observed at 72 hpe (Fig. 17a). Predominant amino acids such as glutamate and aspartate behaved similarly to those under non-treated conditions (Fig. 12b,d). However, in treated cuttings a time shift in depletion and recovery events could be observed (Fig. 17b,d). The concentration of glutamine was depleted between 12 hpe and 72 hpe followed by a recovery reaching the initial level at 192 hpe (Fig. 17c). In the presence of auxin enhancers, there was an increase of asparagine at 6 hpe followed by a strong decrease which remained low during all the investigated developmental stages (Fig. 17e). Furthermore, the total protein value was constant till 48 hpe followed by elevation up to twofold of basal amount at 192 hpe (Fig. 17f). Concentrations of all amino acids during ARF in the presence of auxin analogs in petunia cuttings are listed in Appendix Table 2.

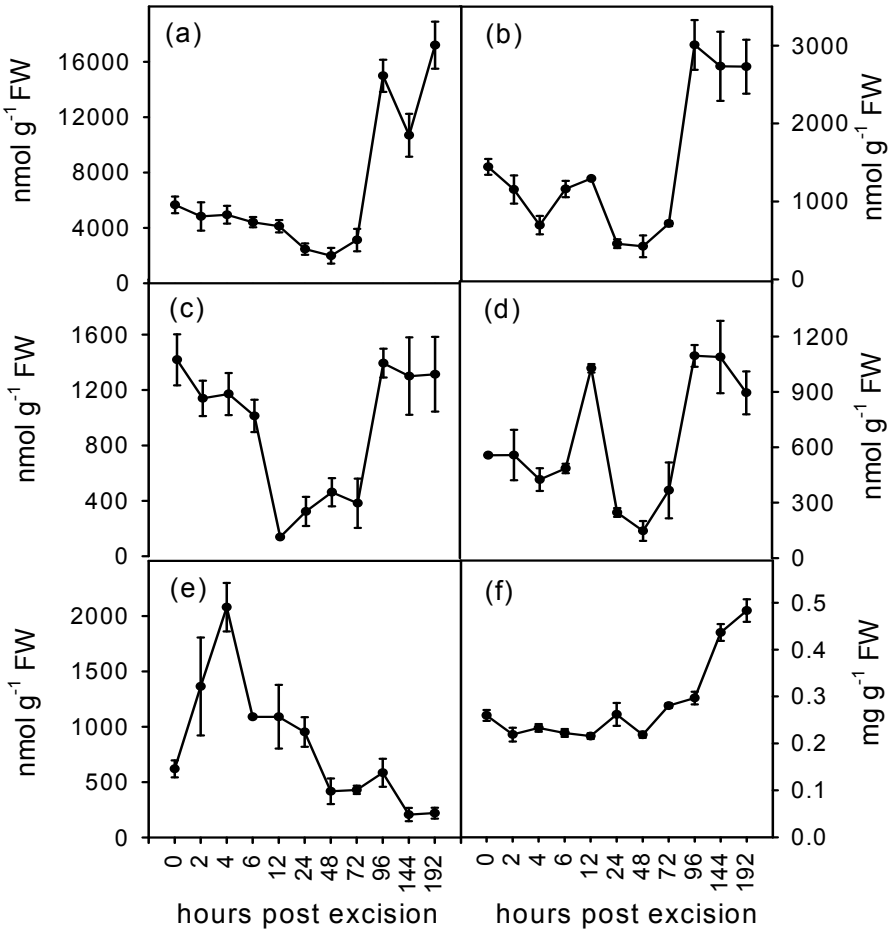


Figure 17. Concentrations of amino acids and total protein during adventitious root formation (ARF) in petunia (*Petunia hybrida*) in auxin-induced condition. (a) total amino acids, (b) glutamic acid, (c) glutamine, (d) aspartic acid, (e) asparagine and (f) total protein. All data are shown as either nmol g^{-1} FW (a-e) or mg g^{-1} FW (f). Each value is represented by the mean of five independent replicates ± SE.

3.2.2. Application of exogenous auxin inhibitor during ARF

The auxin inhibitor Naphthylphthalamic Acid (NPA) was employed to inhibit basal auxin transport and to find out whether ARF depends on local auxin synthesis or basipetal transport from leaves and whether inhibition of auxin transport influences the metabolic activity during ARF as demonstrated in previous experiments in the current work. Using 80 µM of NPA, rooting was delayed up to twenty days after excision (Fig.18).

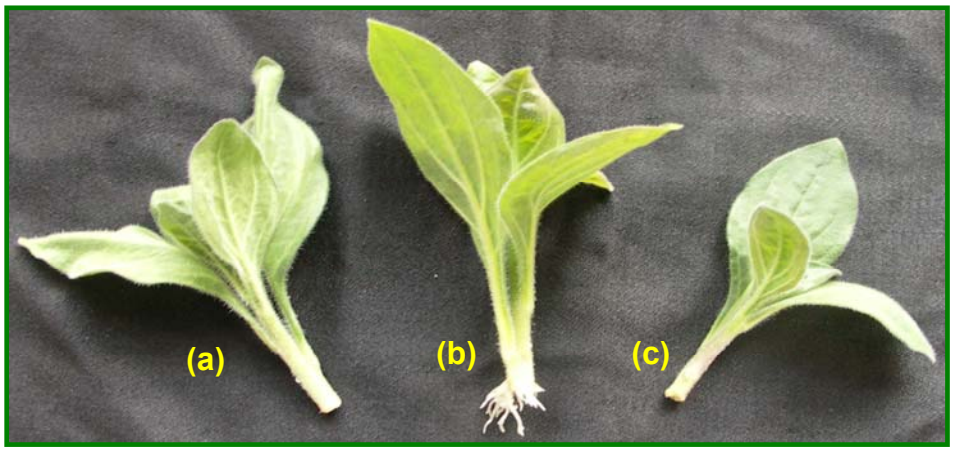


Figure 18. Comparison of rooting behavior between auxin-inhibited (a and c) and control condition (b), 16 days after excision. Foliar spraying of 80 µM NPA was used as auxin inhibitor.

Then, a histochemical analysis of ARF in NPA-treated cuttings was performed. Formation of meristematic cells was inhibited at 144 hpe in the NPA-treated cuttings (Fig. 19a,d), whereas at the same developmental stages, visible meristematic cells, root primordia and first roots were observed under non-treated condition (Fig 6e,f). However, first meristematic cells and first differentiating root primordia were generally visible at 192 hpe (Fig. 19b,e) and 336 hpe (14 days after excision) (Fig. 19c,f) in NPA-treated cuttings, respectively.

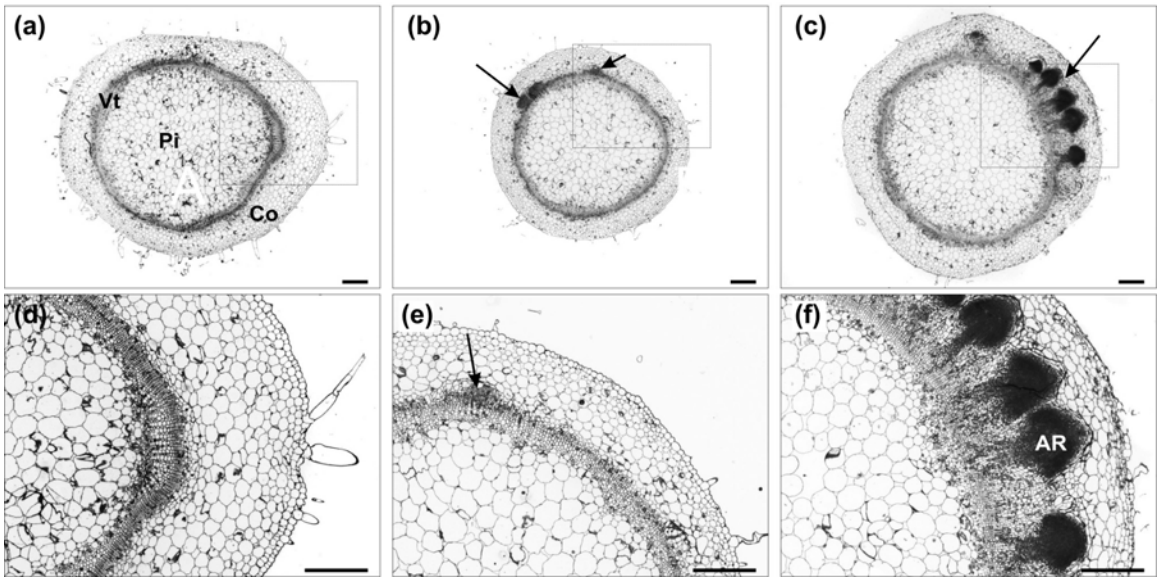


Figure 19. Anatomy of adventitious root formation in auxin-inhibited condition in the stem base of petunia (*Petunia hybrida*) cuttings. In all micrographs, cross-sections from *c*. 1–4 mm above the excision site are shown. (a,d) 144 hpe, (b,e) 192 hpe and (c,f) 336 hpe. Vt: vascular tissues, Co: cortex, Pi: pith, AR: Adventitious Root primordia. Bars, (a-c), 500 µm; (d–f), 100 µm.

Biochemical analyses were performed within the first eight days using the rooting zone of NPA-treated cuttings during ARF. As indicated in figure 20, a continuous increase of glucose and fructose were observed after 24 hpe reaching 10-fold higher levels at 192 hpe in the presence of auxin inhibitor (Fig. 20a, b). Likewise, sucrose levels (Fig. 20c) followed a similar pattern as in the absence of NPA starting to increase at 24 hpe and to reach a similar level as shown for non-treated cuttings at 192 (hpe) (Fig.10c).

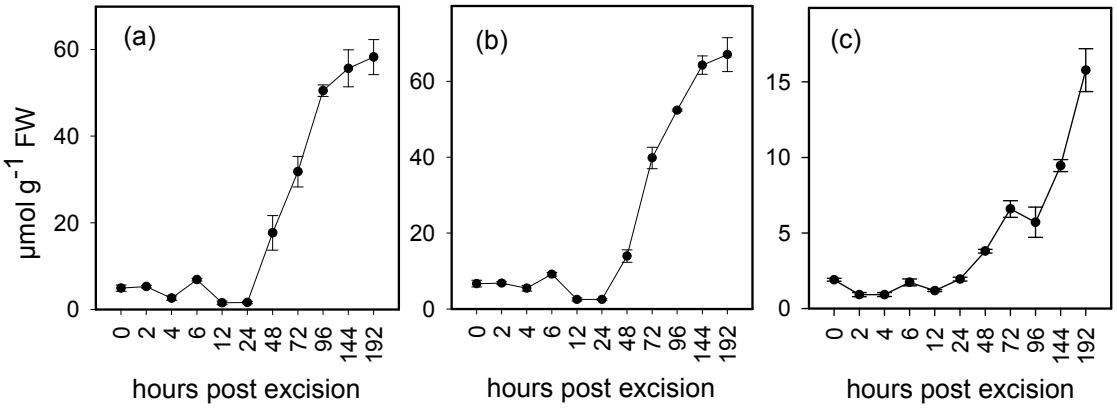


Figure 20. Alterations in the concentrations of soluble sugars during adventitious root formation (ARF) in petunia (*Petunia hybrida*) cuttings in auxin-inhibited condition. (a) glucose, (b) fructose, (c) sucrose. All data are shown as µmol g^{-1} FW. Each value is represented by the mean of five independent replicates ± SE.

In addition, the activity of some important enzymes involved in primary metabolism was analyzed. In almost every case, investigated enzymes showed a similar activity irrespective of the presence of the NPA. The activity of apoplastic invertase increased after 6 hours and reached a threefold higher level at 144 hpe before decreasing to approximately its initial activity at 192 hpe (Fig. 21a). Cytosolic invertase activity exhibited fluctuations up to 72 hpe and decreased thereafter to 20% of the basal level at 192 hpe (Fig. 21b). The activity of vacuolar invertase remained unchanged during 72 hours after excision and declined strongly until 192 hpe to reach approximately 30% of the initial value (Fig. 21c). The activity of cytosolic FBPase showed a slight decrease during ARF in auxin-inhibited condition (Fig. 21e), while PFK activity (Fig. 21d) exhibited a twofold increase beginning at 24 hpe. Furthermore, the activity of glucose-6-phosphate dehydrogenase declined during the first period but elevated in the later course of ARF (Fig. 21f).

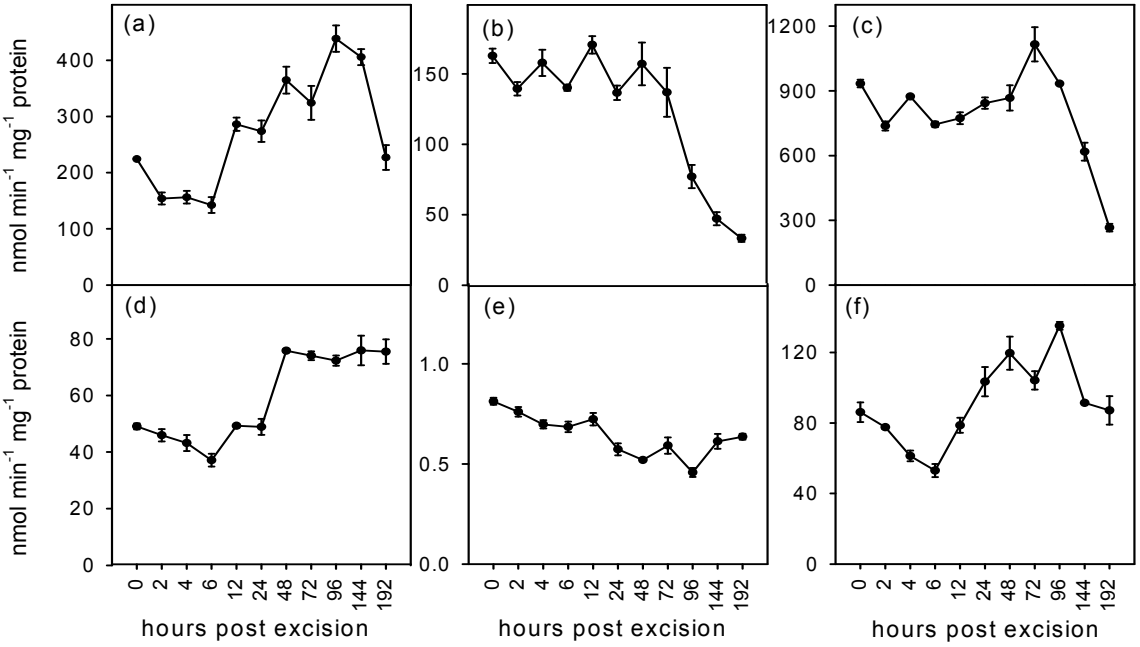


Figure 21. Alterations in the enzyme activities involved in sucrose degradation, glycolysis and pentose phosphate pathways during adventitious root formation (ARF) in petunia (*Petunia hybrida*) in auxin-inhibited condition. (a) Cell wall invertase, (b) Cytosolic invertase, (c) Vacuolar invertase, (d) PFK, (e) Cytosolic FBPase and (f) Glu6PDH. All data are shown as nmol $min^{-1}mg^{-1}$ protein. Each value is represented by the mean of five independent replicates ± SE.

The concentrations of total amino acids under NPA-treated conditions decreased slightly during the first two days followed by strong increase beginning at 72 hpe reaching a twofold higher level at 192 hpe (Fig. 22a). The level of glutamate and aspartate were reduced to 50% immediately after excision, increased slightly during the next stages reaching more or less the initial value (Fig. 22b,d). After 4 hpe, a continuous decrease in glutamine concentration was observed until 48 hpe, increased sixfold at 72 hpe and leveled off (Fig. 22c). The level of asparagine exhibited a eightfold elevation at 96 hpe. Then, asparagine concentration declined to lower levels than the initial value (Fig. 22e). Total protein remained unchanged during ARF in NPA-treated petunia cuttings (Fig. 22f). Concentrations of all amino acids under auxin-inhibited condition are provided in Appendix Table 3.

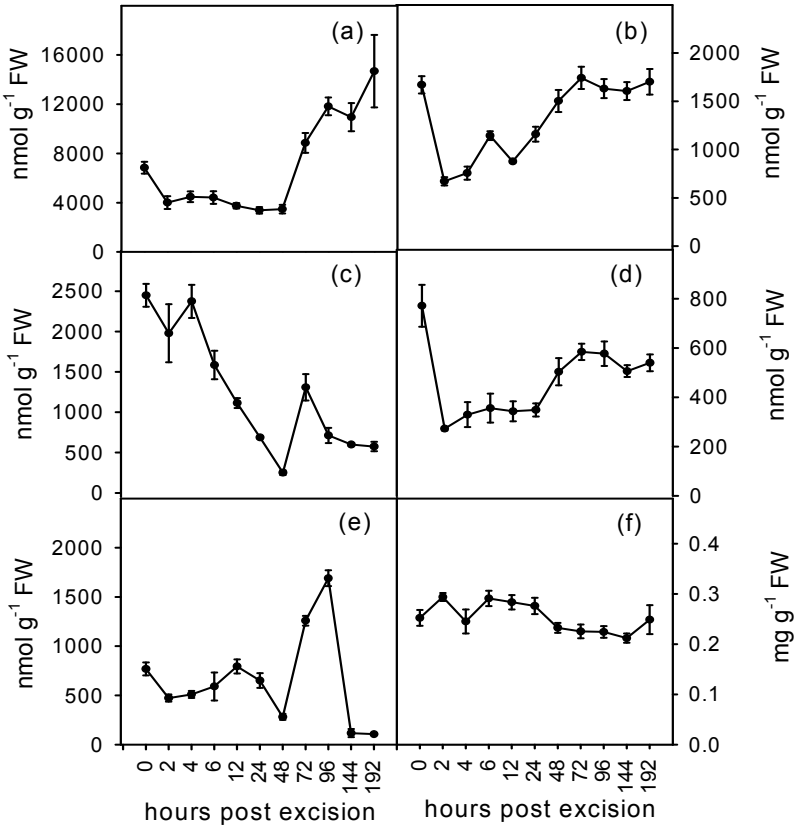


Figure 22. Concentrations of amino acids and total protein during adventitious root formation (ARF) in petunia (*Petunia hybrida*) in auxin-inhibited condition. (a) total amino acids, (b) glutamic acid, (c) glutamine, (d) aspartic acid, (e) asparagine and (f) total protein. All data are shown as either nmol g^{-1} FW (a-e) or mg g^{-1} FW (f). Each value is represented by the mean of five independent replicates ± SE.

3.2.3. Comparison of proline accumulation during ARF

The proline levels increased after 96 hours, reached 3-fold and 10-fold higher values at 144 hpe and 192 hpe, respectively, in non-treated cuttings of petunia (Fig. 23a). Nevertheless, in the presence of NAA and IBA, a rapid and strong increase of proline (55-fold) was observed at 72 hpe. This increase continued to reach a maximum of 70-fold at 192 hpe (Fig. 23b). In the presence of NPA, however, proline started to accumulate at 48 hpe continuously and reached a maximum at 192 hpe (Fig. 23c).

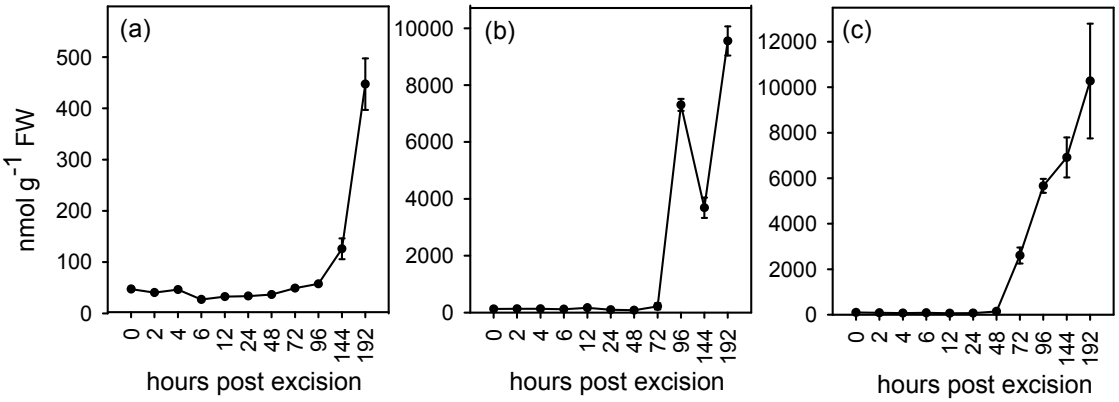


Figure 23. Proline concentrations in three different conditions. (a) non-trated condition, (b) auxin-induced condition and (c) auxin-inhibited condition during adventitious root formation (ARF) in petunia (*Petunia hybrida*). All data are shown as nmol g^{-1} FW. Each value is represented by the mean of five independent replicates ± SE.

3.3. Identification of specific genes crucial for adventitious root formation in leafy cuttings of petunia using microarrays

In order to find out whether physiological changes are accompanied by corresponding changes in gene expression, transcriptome analysis during adventitious root formation in the stem base of the cuttings of *Petunia hybrida* plants (line W115) were performed (in cooperation with Dr. Philipp Franken, IGZ, Germany and Dr. Uwe Scholz, IPK, Germany).

3.3.1. Generation of a normalized cDNA library at different developmental stages of adventitious root formation in petunia

In order to target specific genes crucial for adventitious root formation a normalized cDNA library was generated. Cutting ends were harvested at the same time points as used in the biochemical analyses and complete RNA from petunia cuttings was isolated and pooled. Since the quality of total RNA and mRNA is crucial for construction of a high-quality cDNA library, the RNA was verified to be non-degraded, high-quality, and without genomic DNA (Fig. 24). Table 3 indicates the RNA purity in different time points during ARF which is between 1.64 (at 6 hpe) and 2.1 (at 2 hpe).

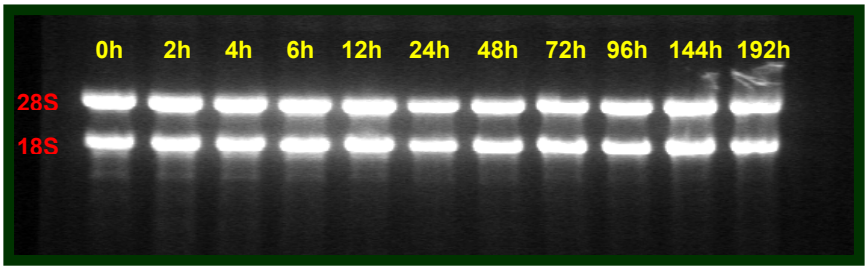


Figure 24. Extracted RNA from petunia cuttings in different developmental stages. The ratio of intensity of 28S RNA to 18S RNA (for total RNA) is 1:1.

Table 3. Ratio of DNA and RNA to other contaminants that absorb strongly at or near 280 nm like proteins or phenols in extracted RNA from petunia cuttings in various developmental. Stages of ARF.

hours post excision (hpe)	0	2	4	6	12	24	48	72	96	144	192
Ratio (260/280nm)	1.9	2.1	1.7	1.6	1.9	1.8	1.9	1.7	1.7	1.8	1.7

After picking and sequencing desired clones, a normalized cDNA library containing 4,700 EST's was generated. From these EST's, 1,964 (42%) were singletons and 2,736 (58%) had a consensus sequence with an average sequence length of 561 bp (Table 4).

Table 4. Characsteristics of the generated normalized cDNA library during ARF in Petunia cuttings

Accumulated ESTs (Project-New ESTs)	4,700
Singletones (percentage of accumulated ESTs)	1,964 (42%)
ESTs member in consensus sequence (Percentage of accum. ESTs)	2,736 (58 %)
Consensus sequences	1,013
Consensus with 3'-EST members only (Percentage of all consensus sequences)	1,013 (100 %)
Average consensus length	561

Annotation of sequences showed that 607 out of 4,700 ESTs belonged to genes putatively encoding particular proteins. These putative proteins could be clustered into different categories using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database (Fig. 25). The largest categories were amino acid metabolism (19%), energy metabolism (17%), carbohydrate metabolism (16%) and biosynthesis of secondary metabolites (12%). More information about the generated cDNA library is also available at CR-EST: The IPK Crop EST Database, (Kunne et al., 2005) ; (http://pgrc.ipk-gatersleben.de/cr-est/index.php).

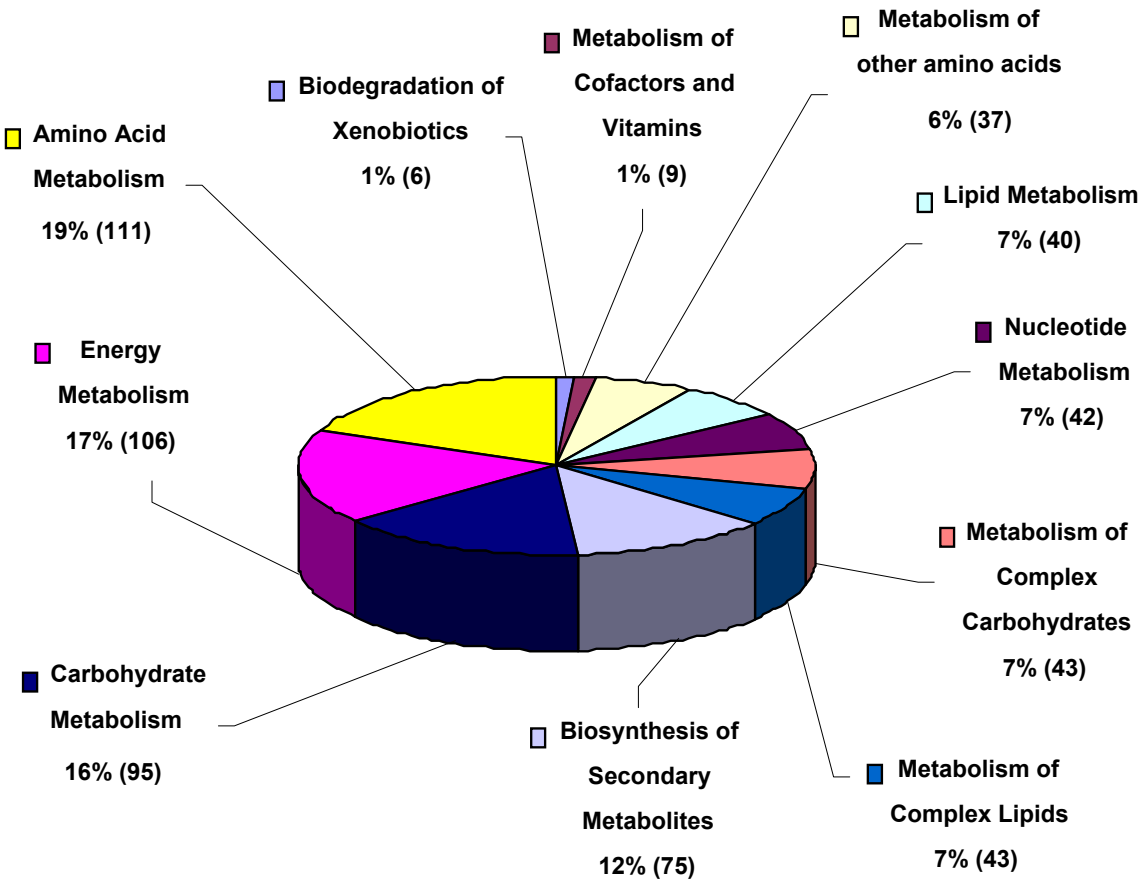


Figure 25. Classification of putative proteins into different categories of metabolism during various developmental stages of adventitious root formation in petunia cuttings. Numbers of putative proteins in different categories of the KEGG superpathway are shown in parentheses. Total protein number was 607 (out of ca. 4,700 EST's).

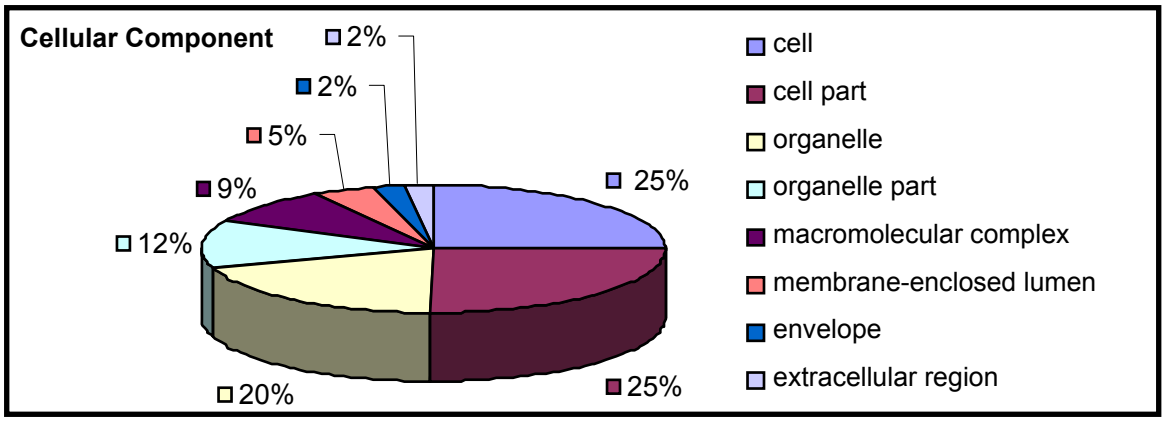
3.3.2. Assembly of a petunia microarray

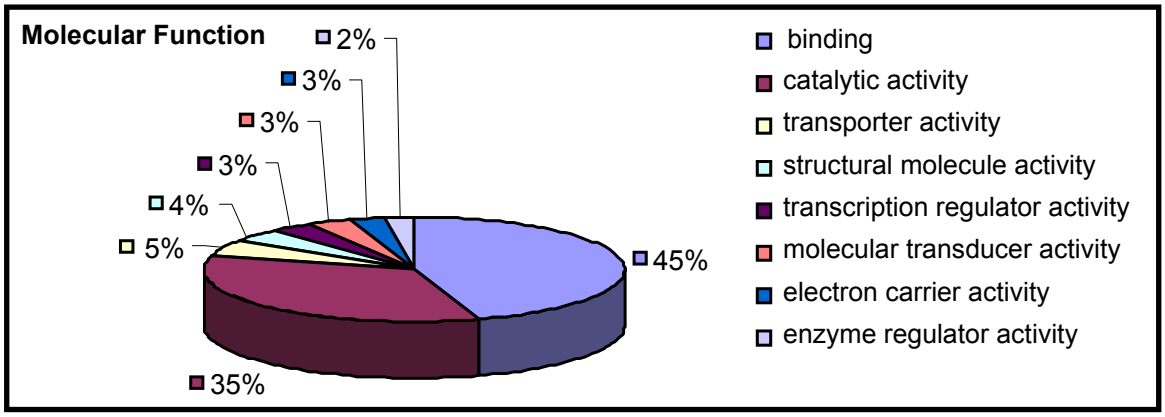
The cDNA library containing 4,700 ESTs of different developmental stages of adventitious root formation in petunia along with all published and unpublished petunia sequences available in Genebank of computational Biology and Functional Genomics Laboratory (GB), Max-Planck-Institute in Golm (MPI), TIGR, and the Solanaceae Genomics Network (SGN) were used to generate a petunia sequence collection (Table 5). A total of 48,005 sequences passed the quality check were clustered and assembled using StackPACK software (Christoffels et al., 2001) by Dr. Uwe Scholz (IPK, Germany). Subsequently, 25,726 unigenes (10,534 consensus sequences and 15,192 singletons) were

submitted to NimbleGen Company. The final clustering verification performed by NimbleGen resulted in 24,816 unique non-redundant sequences (10,450 consensus sequences and 14,366 singletons) were used for the design of a custom microarray. Distribution of the sequences to functional groups based on GO (Gene Ontology) annotation (http://www.geneontology.org/) is shown in Figure 26.

Table 5. Published (underlined) and unpublished sequences used for assembling petunia microarray. Unigenes are sequences which are produced using *in silico* clustering tools and can be singletons or contigs (consensus sequences).

Database	Prefix in Seq. Id.	Number of Sequences	Type/Comment	File
IPK	IP	4,700	ESTs	IPK_petunia_ests_4700.fasta
TIGR	GI	4,466	Unigenes (Singl + Contigs)	PHGI.fasta
SGN	SG	5,135	Unigenes (Singl + Contigs)	SGN_petunia_hybrida.fasta
Add on Venail	VE	30	_____	Add_on_Venail.fasta
Add on DR	DR	12	_____	Add_on_DR.fasta
Download from Golm	GO	7,793	Singletons	GO_drpoolB_singl.fasta
Download from Golm	GO	10,150	Contigs	GO_drpoolB_contigs.fasta
Genbank	GB	15,713	ESTs	GB_petunia_ESTs.fasta
University of Cologne	KO	6	_____	KO_PhPT1-6.fasta
	Total:	**48,005**		





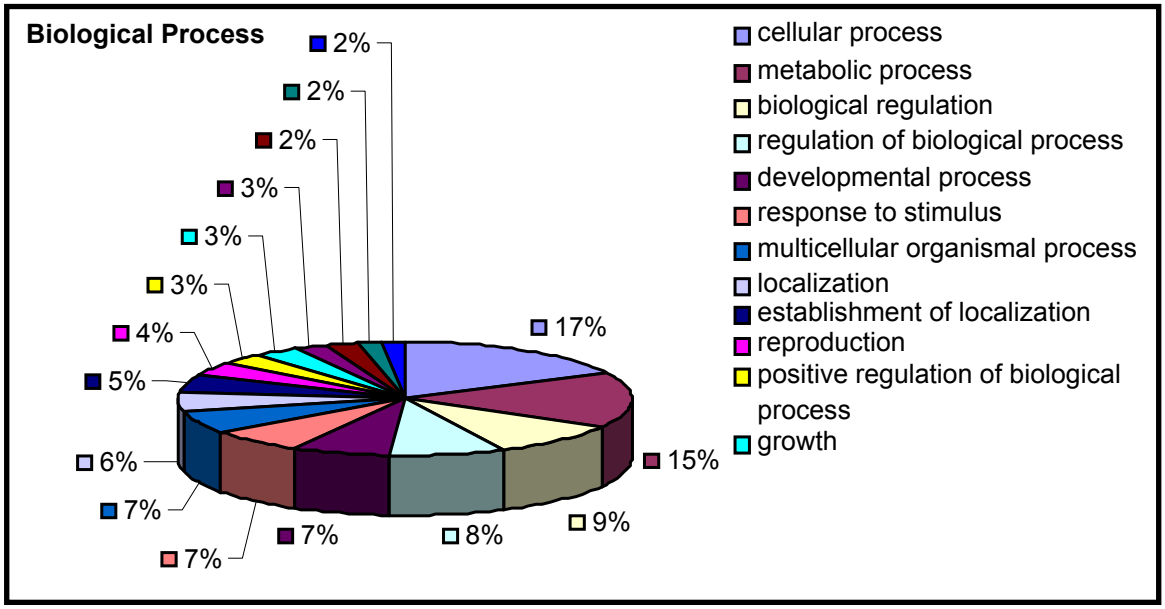


Figure 26. Gene Ontology (GO) classification (version March 2009) of 24,816 non-redundant petunia sequences used for microarray assembling. BlastX of petunia unigenes against representative proteins, extracted broad level terms (shown here) as well as lower level terms (not shown here) of three main categories of GO: Cellular Component, Molecular Function and Biological Process.

Hence, the set of non-redundant unigenes comprising the entire known petunia transcriptome were grouped to three main categories of GO: **biological process** which refers to a biological objective to which the gene or gene product contributes, **molecular function** which is defined as the biochemical activity (including specific binding to ligands or structures) of a gene product and **cellular component** which refers to the place in the cell where a gene product is active (Ashburner et al., 2000). The GO vocabularies have a hierarchical structure that permits a range of detail from high-level, broadly descriptive terms to very low-level, highly specific terms. The description of all GO annotation hits of this investigation including more specific levels is available in Excel File 1 (Data-CD).

3.3.3. Experimental setup and data analysis

As mentioned in the introduction (see section 1.2.1), the process of adventitious root formation can be separated into three stages: (1) induction; (2) initiation; and (3) emergence. It was shown before (sections 3.1.1 and 3.1.2) that petunia cuttings produce roots eight days after excision from the stock plant. Based on these physiological events (see section 3.1 or Ahkami et al. 2009*)* and in order to determine early regulatory changes, the rooting zone of leafy cuttings from following time points during ARF (before emergence of roots) were selected for the array experiment: 2 and 6hpe; representatives for very early physiological events after excision, 72 hpe; which is anatomically characterized by the first new meristematic cells (dense cytoplasm and large nucleus) (Fig. 6c) and 196hpe; representative for the last day before root emergence which covers the time point from the appearance of first roots with vascular bundles in the center surrounded by elongated cells of the elongation zone (Fig. 6f). For comparison, the rooting zone of leafy cuttings at 0hpe, fresh leaves, wounded leaves and root tissues were used (Table 6).

Table 6. Sample descriptions and number of replicates for hybridization in array experiment

Tissue	Number of replicates	Description
Stem base of cutting (0hpe)	4	Control
Stem base of cutting (2hpe)	3	Very early events in root induction phase
Stem base of cutting (6hpe)	3	
Stem base of cutting (72hpe)	4	Formation of first meristematic cells in root initiation phase
Stem base of cutting (192hpe)	4	Formation of first roots with vascular bundles (still invisible) in root emergence phase
Leaves	4	Controls
Wounded leaves	4	
Roots	4	

Rank Product (RP) analysis (Breitling et al., 2004) was performed to identify significantly up- and down-regulated genes (Excel File 2, Data-CD) using MeV (MultiExperiment Viewer) software. Only a small fraction of the genes varied in expression across the biological replications; consequently, the reproducibility of the experiment was high. The significances of the time and replicate effects, expressed as the negative 10-base logarithm of the *P* values assigned to each effect based on the Rank Product (RP) analysis, were plotted against each other (Fig. 27) according to Himanen et al. (2004). The accumulation of the dots (each dot represents one sequence) on the left side of the plot, illustrating the negative correlation between the significance of both effects, indicates a high reproducibility of the replicated time courses.

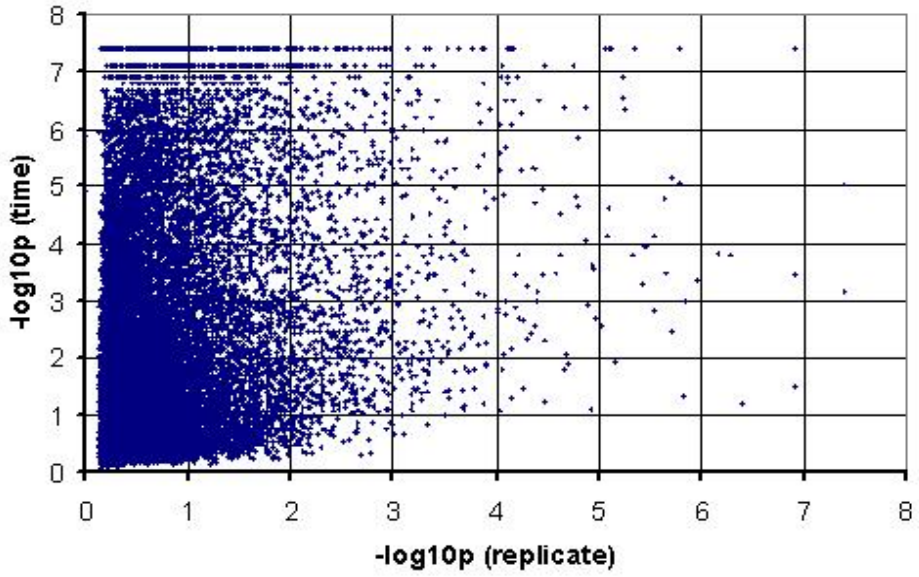


Figure 27. Plot of -Log10 of the significance of the replicate effect (Y-axis) versus the -Log10 of the significance of the time effect (X-axis), indicating a much stronger time effect than undesired variation between replicates.

For reaching the goal of finding those genes which were specifically induced during ARF, a filtration process was carried out to remove wound responsive and root related genes (Fig. 28). Accordingly, genes only induced significantly at 2 or 6 hpe and induced significantly in control ratio wounded leaf/fresh leaf were removed in order to eliminate those genes which were wound-induced by the cutting process (Fig. 28a). In addition, genes only induced significantly at 192 hpe and induced significantly in control ratio root system/fresh leaf were removed in order to discard root system-related genes (Fig. 28b). Applying such a stringent filter identified 3,561 sequences out of 24,816 sequences (Excel File 3, Data-CD).

	2h/0h	6h/0h	72h/0h	192h/0h	Wounded leaf / Fresh leaf	Root system / Fresh leaf
(a)	Induced	Induced	Not induced	Not induced	Induced	
(b)	Not Induced	Not Induced	Not Induced	Induced		Induced

Figure 28. Approaches to remove (a) wound responsive and (b) root system-related genes from differentially expressed genes in order to select genes specifically induced during ARF. Applying this filter, 3,561 sequences out of 24,817 sequences were isolated. In other words, obtained genes are: 2 hpe or 6hpe-induced genes (minus wound induced) + 72 hpe-induced genes + 192 hpe-induced genes (minus root specific genes).

Data analysis revealed that out of 24,816 tested sequences, 10,611 were differentially expressed, while 3,561 were induced specifically during ARF. Since one of the main goals of this work was to identify genes crucial for adventitious root formation, functional classification was accomplished for 3,561 genes specifically induced during ARF (see below).

The analysis of up- and down-regulated genes at different stages showed that most up-regulated genes, namely 2,375 were induced at 72 hpe while most down-regulated genes namely, 176 were repressed at 6 hpe. The lowest number of up- and down-regulated genes was observed at 2 hpe and 192 hpe with 1,551 and 122 genes, respectively (Fig 29a). With regard to the different developmental stages of ARF, the majority (524 genes) was up-regulated only at 6 hpe while the minority (121 genes) was up-regulated only at 72 hpe (Fig. 28b). In contrast, 107 genes were down-regulated only at 6 hpe and 16 genes were down-regulated only at 192 hpe (Fig. 29b). Likewise, 824 genes were up-regulated at all time points.

3.3.4. Functional classification of genes specifically induced during ARF

All 3,561 genes specifically induced during different phases of ARF were blasted against National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov) using nrpep-blastx program and The Arabidopsis Information Resource (TAIR8) (http://www.arabidopsis.org/) using pep-blastx program (In cooperation with Dr. Uwe Scholz, IPK). Ten different hits (five from NCBI and five from TAIR8) were assigned for each sequence and subsequently the best one was selected manually according to associated e-values and scores. Afterwards, they were grouped into 17 functional categories (In cooperation with Dr. Philipp Franken, IGZ) based on the classification reported by Covitz et al. (1998) (modified).

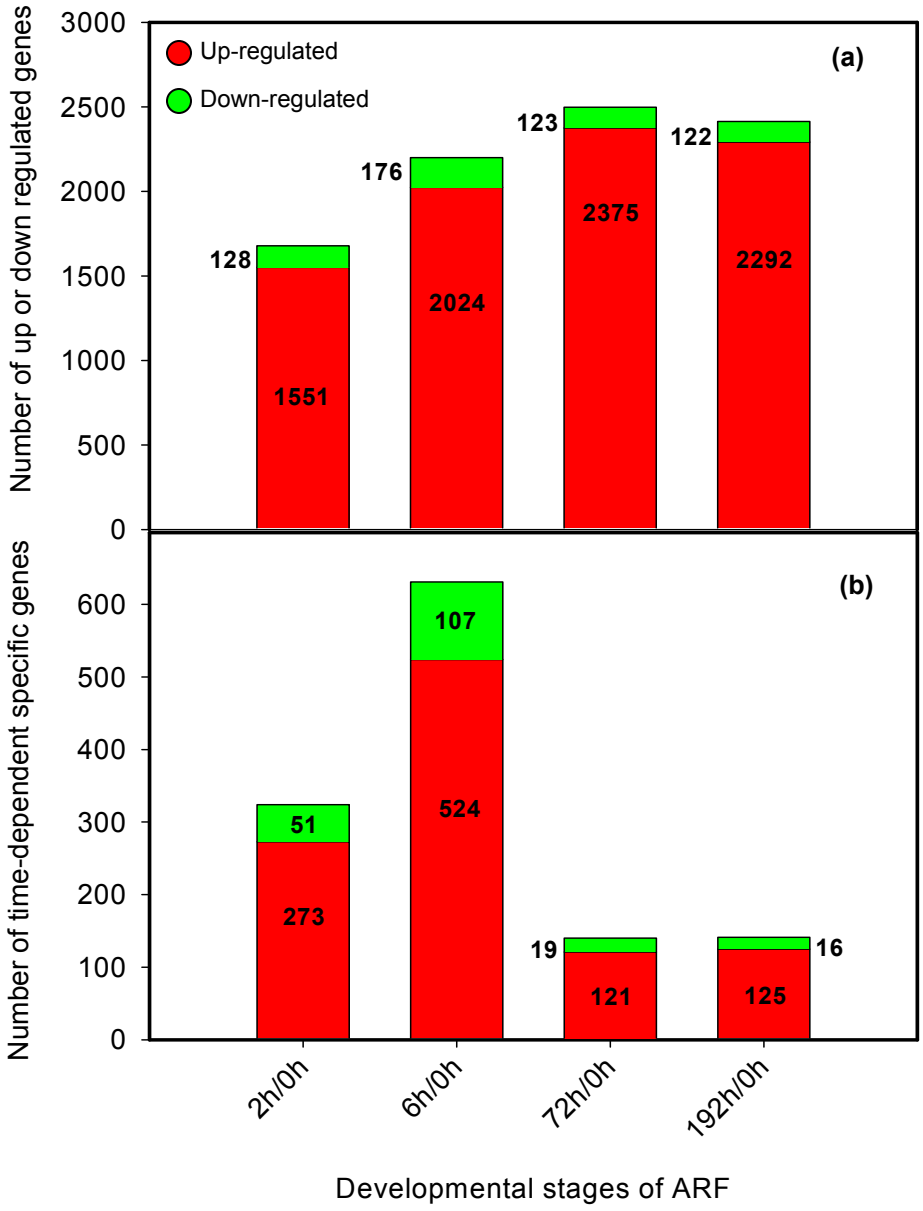


Figure 29. Data analysis among the 3,561 ARF-specifically-induced genes in petunia cuttings. (a) Number of up- or down-regulated genes at different dates (b) Number of time-dependent specific up- or down-regulated genes.

A total of 2,328 out of 3,561 genes specifically induced during ARF were grouped according to the physiological process with which they are associated (Table 7). The majority of the genes were belonging to the functional categories primary metabolism (14.6%), signaling (13%), miscellaneous (10.3%), gene

expression and RNA metabolism (10.2%), secondary metabolism (8%), protein synthesis, processing and degradation (7.2%) and membrane transport (6.5%).

Table 7. Functional classification and number of down- or up-regulated genes specifically induced during different phases of ARF in petunia cuttings in each individual category.

Function	2 or 6 hpe		72 hpe		192 hpe	
	Up	Down	Up	Down	Up	Down
Primary metabolism	238	20	208	23	204	23
Signaling	229	23	186	8	174	10
Gene expression and RNA metabolism	178	6	139	2	129	0
Secondary metabolism	124	14	149	5	149	4
Protein synthesis, processing and degradation	127	6	95	8	86	6
Membrane transport	102	5	111	2	114	1
Hormone metabolism and perception	89	7	88	5	84	6
Biotic stress response	72	7	88	3	96	3
Cell wall and storage	62	11	81	5	82	6
Antioxidative metabolism and Redox state	55	2	84	0	83	1
Chromatin and DNA metabolism	25	17	57	0	52	0
Mineral nutrient acquisition	46	4	58	2	54	2
Abiotic stress response and development	33	2	29	1	29	0
Cytoskeleton	14	10	27	0	27	0
Vesicular trafficking, secretion, protein sorting	26	2	13	2	14	2
Cell cycle	11	4	17	1	19	1
Miscellaneous	151	18	175	4	173	5
Total	**1535**	**153**	**1554**	**66**	**1517**	**64**

Out of the 3,561 genes, 1,233 genes were not included because these sequences displayed no similarity to known proteins or were homologs to hypothetical proteins. When hierarchical trees and their associated expression graphs were prepared, it turned out (Fig. 30) that genes highly up-regulated at 2 and 6hpe were placed in cluster I, whereas genes down-regulated in these stages were grouped in cluster II. Additionally, genes up-regulated at 72 and 192hpe were profiled in cluster III and genes highly down-regulated in these stages of ARF in petunia cuttings were classified as cluster IV. Major functional

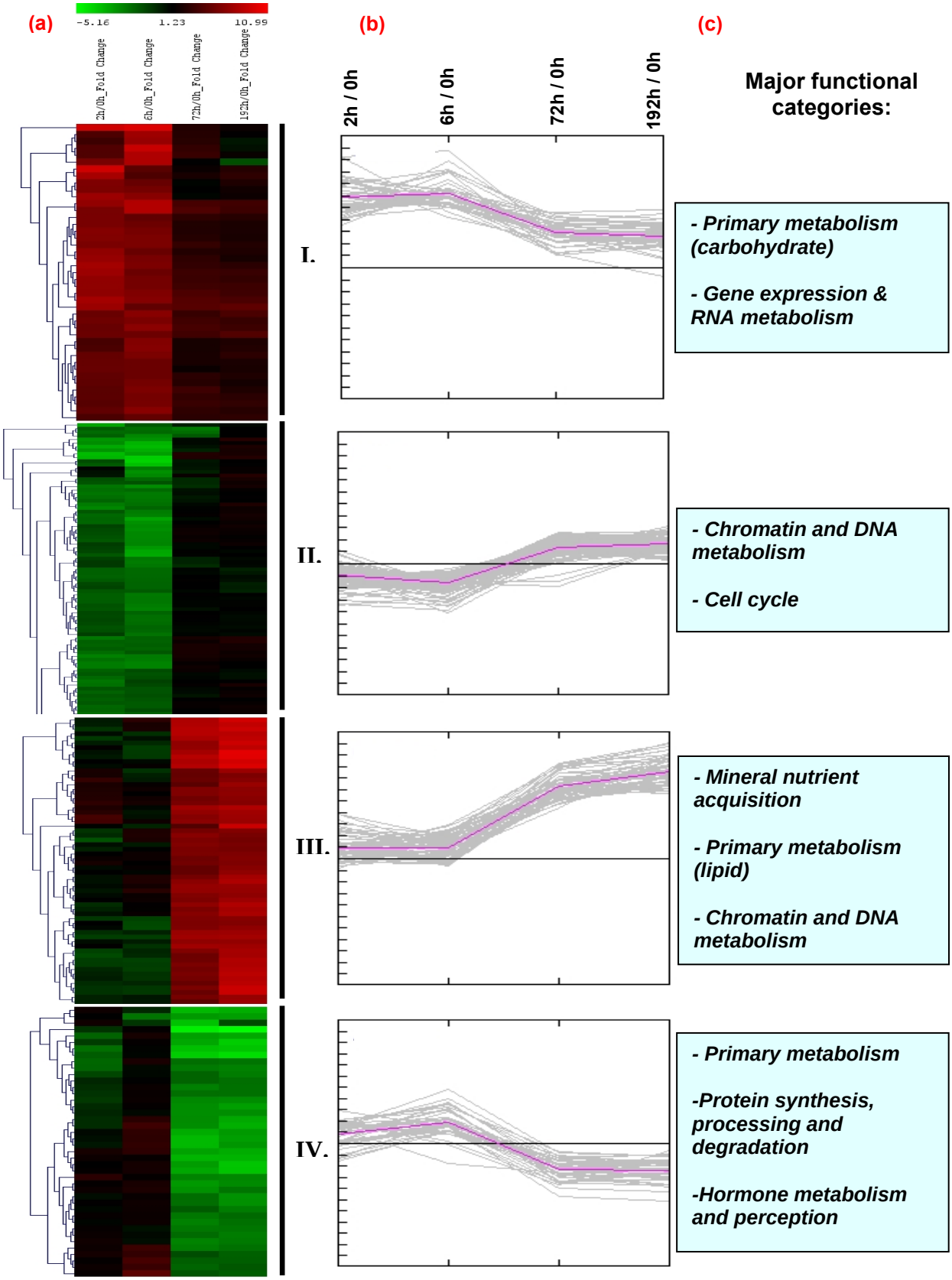


Figure 30. Four major clusters of genes specifically induced during different phases of ARF created via K-means method by Mev (MultiExperiment Viewer) software. (a) hierarchical trees (b) their associated expression graphs and (c) main functional categories of each cluster. (I) Up- regulated genes at 2 and 6hpe (II) Down-regulated genes at 2 and 6hpe (III) Up-regulated genes at 72 and 192 hpe (IV) Down-regulated genes at 72 and 192 hpe.

categories associated to each profile are indicated in Figure 30c which represents the most dominant categories on the basis of alterations in fold changes.

3.3.5. Distribution of identified genes among metabolomic pathways during ARF

Based on the functional classification, different genes were shown to be up- or down-regulated in various phases of ARF in different metabolomic pathways. Here we focus mainly on genes involved in primary metabolism, cell replication, membrane transporters and hormone metabolism. The fold change amount for all genes in each individual date is presented in Excel File 3 (Data-CD).

(A). First 6 hours after excision

Among those genes specifically induced during different phases of ARF, 1,535 genes were up-regulated and 153 genes down-regulated during the first 6 hours after excision, but not by wounding. Most of the induced genes were belonging to the functional categories primary metabolism (15.4 %), signaling (14.9%) or gene expression and RNA metabolism (11.6%). Transcripts encoding key enzymes and transporters in primary carbohydrate metabolism, such as phosphofructokinase (PFK), cell wall invertase, cytosolic invertases, trehalose-6-phosphate phosphatase, malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase, mannose-6-phosphate isomerase, acetyl-CoA synthetase, sucrose phosphate synthase (SPS) and hexose transportes (especially of the STP gene family) were highly induced during this time. Furthermore, proline dehydrogenase (oxidase) which catalyzes the first step in proline catabolism in mitochondria was highly up-regulated only in this phase, whereas transcripts encoding zinc/iron transporters and phosphate transportes were repressed or remained unchanged during the first six hours after excision. Moreover, a transcript encoding ribonucleotide reductase was repressed, while a 3-hydroxyacyl-CoA dehyrogenase-encoding gene involved in ß-oxidation was up-

regulated in this phase. Focusing on hormone metabolism, seven different isoforms of auxin responsive genes of the GH3 family (indole-3-acetic acid amido synthetase) were induced at all dates of ARF, and GH3.3 was induced even up to 100-fold at 6hpe. Likewise, transcripts encoding an auxin influx transport protein, a PIN1-like auxin transport protein, the auxin-responsive transcription factor IAA4, an ABA-inducible protein, a cytokinin binding protein, S-adenosylmethionine synthetase (SAMS) and a jasmonate ZIM-domain protein were induced in the first phase of ARF. Several genes related to ethylene and gibberellin metabolism were also induced at early stages. As components of signaling pathways, transcript abundances of two isoforms of sucrose non-fermenting 1- related protein kinase (SnRK1) and two types of glutamate receptors were up-regulated in this phase.

(B). 72 hours after excision

Among the genes specifically induced during ARF, 1,554 genes were up-regulated and 66 genes down-regulated three days post excision. The majority of the significantly induced genes were belonging to the functional categories primary metabolism (13.4 %), signaling (12%) and secondary metabolism (9.5%). In addition, phosphoenolpyruvate (PEP) carboxylase, glutamine synthetase and cytosolic pyruvate kinase were the most strikingly up-regulated transcripts in primary metabolism encoded at 72 hpe. However, a transcript encoding trehalose-6-phosphate phosphatase was repressed as well as cytoplasmic and apoplastic invertases. Furthermore, genes involved in chromatin and DNA metabolism, cytoskeleton, cell cycle (CycB1, CycA and ribonucleotide reductase), mineral nutrient transport (phosphate and nitrate transporters) and primary lipid metabolism (enoyl-CoA hydratase, acyl-CoA synthetase and GDSL-motif lipase) started to be highly up-regulated in this developmental stage. In addition, transcripts of several types of peroxidases, GA2 ent-kaurene synthase, GA7-oxidase, 1-pyrroline-5-carboxylate dehydrogenase (P5CDH), and uridine 5'-monophosphate synthase (UMP synthase) were up-regulated in this phase.

(C). 192 hours after excision

1,517 genes were up-regulated and 64 genes down-regulated eight days after excision of the cuttings from the stock plant, but were not higher expressed in roots than in leaves. The majority of significantly induced genes were belonging to the functional categories primary metabolism (13.4%), signaling (11.5 %), and secondary metabolism (9.8%). In general, most genes showed a similar expression pattern at 72 and 192hpe. Several genes involved in lipid metabolism, such as fatty acid dehydrogenase, fatty acid omega-hydroxylase, omega-6 fatty acid desaturase, lipid transfer and lipid binding proteins were overpresented in last date of ARF. Likewise, transcripts encoding important mineral nutrient acquisition genes, such as transporters for metals, zinc/iron, phosphate, nitrate, or boron were highly up-regulated at 192 hpe. Furthermore, different types of peroxidases, GA2 ent-kaurene synthase, GA7-oxidase, P5CDH, UMP synthase, alpha-L-fucosidase and cyclin family of cell cycle regulators accumulated at 72hpe and continued to remain at an elevated level until 192 hpe. In addition, transcripts encoding CycD and a proton-pumping ATPase (H^+-ATPas) were up-regulated in this developmental stage.

3.4. Verification and validation of differentially regulated genes during ARF

3.4.1. Comparison of gene expression data between microarray and Northern blot analysis

Several genes involved in carbohydrate metabolism and cell division showed a similar time-dependant expression pattern based on microarray and Northern blot analysis. For example, the expression pattern of cell wall invertase showed an elevation early after excision followed by a decrease at later stages. This was in agreement with the Northern blot analysis of this gene (Fig. 31a). Moreover, transcript levels of sucrose synthase (SuSy) exhibited no significant changes during various phases of ARF in both, the array experiment and Northern blot analysis (Fig. 31b). Additionally, a similar expression pattern was observed for apoplastic localized monosaccharide transporter (STP) in the

microarray experiment and Northern blotting (Fig. 31c). It should be noted that in Northern blot analysis the transcript level of *STP4* was analyzed, however another member of this transporter family (STP9 or STP13) was specifically induced during first phase of ARF in the microarray. Moreover, expression of *CycB1* encoding a mitotic B1 cyclin, increased at 72hpe in microarray experiment which was in coincident with the scheme indicated in Northern blot analysis (Fig. 31d). Furthermore, glyceraldehyde-3-phosphate dehydogenase (GAPDH) gene expression pattern based on microarray exhibited no significant alteration during ARF which confirmed its associated Northern blot analysis (Fig. 31e).

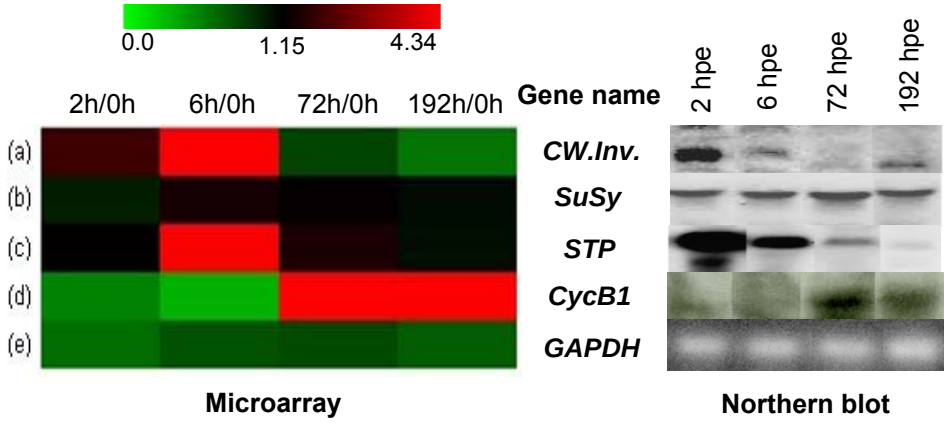


Figure 31. Comparison of the expression pattern of five genes by microarray and Northern blotting. (a) Cell wall invertase (cn490), (b) Sucrose synthase (GO_dr001P0012G11_F_ab1), (c) Sugar transport protein STP9 or STP13 (SG_SGN-U208608), (d) Cyclin B1 (GO_dr004P0003J17_F_ab1) and (e) glyceraldehyde-3-phosphate dehydogenase (cn100). Sequence Id. of each gene is presented in parenthesis in front of their names.

3.4.2. Real-Time PCR data

To validate gene expression patterns obtained by petunia microarray hybridizations, we analyzed the RNA accumulation of seven genes specifically induced during different phases of ARF by qPCR. The selection criteria for the investigated genes was based on their differential expression pattern over time: (a) repressed genes in first stages followed by induction in the last stages (pectinase) (b) genes being highly induced (F-box and hexose transporter), (c) constantly induced genes (ubiquitin-protein ligase), (d) genes induced at the first

stage of ARF and being involved in carbohydrate metabolism (trehalose-p-phosphatase), (e) genes induced at later stages involved in mineral nutrient transport (nitrate transporter, zinc/iron transporter). Results obtained by Real-Time PCR coincided with the expression data observed in the microarray experiment (Table 8). Although, the extent of regulation was in some cases larger than deduced from microarray analysis (nitrate and zinc/iron transporters). This was in consistent with the larger dynamic range of Real-Time qPCR analysis compared to microarray analysis.

Table 8. Comparison of fold change differences of seven genes specifically induced during different phases of ARF based on microarray and Real-Time qPCR. Each value in qPCR data is represented by the mean of three independent replicates ± SE. Sequence ID for each gene is: pectinase (GO_drpoolB-CL9530Contig1), hexose transporter (cn1111), nitrate transporter (cn8317), F-box family protein (IP_PHBS008L07u), ubiquitin-protein ligase (IP_PHBS007P04u), trehalose-p-phosphatase (cn3641) and zinc/iron transporter (cn5371).

Putative function	Fold change Microarray				Fold change qPCR			
	2h	6h	72h	192h	2h	6h	72h	192h
pectinase	0.21	0.10	17.30	23.62	0.13 ± 0.02	1.12 ± 0.53	8.38 ± 0	36 ± 7.92
hexose transporter	51.1	94.90	33.77	26.63	193.7 ± 27	84.18 ± 11.9	92.7 ± 22	29.4 ± 2.8
nitrate transporter	3.7	12.0	141.0	184.7	1 ± 0.19	5.0 ± 2.0	1206 ± 265	2645.1 ± 250
F-box	75.8	64.15	6.58	4.71	95.8 ± 9.6	32.8 ± 0.7	15 ± 4.3	13.8 ± 1.5
ubiquitin-protein ligase	11.6	2.56	10.19	11.37	2.16 ± 0.52	3.22 ± 0.72	7.79 ± 3.03	6.70 ± 2.5
trehalose-p-phosphatase	3.33	3.86	0.77	0.80	5.33 ± 0.54	1.76 ± 0.33	1.49 ± 0.10	0.71 ± 0.04
zinc/iron transporter	0.79	0.79	181	187	0.3 ± 0.16	0.2 ± 0.01	166.0 ± 47	3426 ± 273

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3.5. Generation of transgenic plants using candidate genes to improve ARF in petunia

3.5.1. Generation of transgenic lines

By positioning the coding regions of selected genes under the transcriptional control of the cauliflower mosaic virus 35S promoter a number of fourteen genes was chosen for overexpression on the basis of their biochemical analysis or gene regulation, as well as on the basis of previous reports from the literature. A complete list of selected genes, as well as other information such as: related vector, source organism, associated accession number and current status of generated transgenic lines for each individual gene are presented in Table 9. Due to time restrictions for the course of this thesis, transformation of candidate genes started before the results of the microarrays study could be obtained. Functional analysis of the ARF in these transgenic lines will be performed in future.

3.5.1.1. Phosphoribulokinase (PRK)

Ribulose-5-phosphate kinase or Phosphoribulokinase is responsible for the conversion of ribulose-5-phosphate to ribulose 1, 5-bisphosphate in the Calvin cycle. Next to Rubisco, it is one of the key enzymes of the reductive pentose phosphate pathway as it catalyzes the only reaction by which Calvin cycle intermediates can be provided for further CO_2 fixation (Michels et al., 2005). PRK was introduced as a marker associated with adventitious root formation in Arabidopsis (Sorin et al., 2006). So, overexpression of Arabidopsis *PRK* in petunia was performed followed by examination of transgenic plants for the presence of PRK RNA by Northern blot analysis which revealed four positive transgenic lines.

3.5.1.2. Superroot 1 (SUR1)

The superroot1 (*sur1*) and superroot2 (*sur2*) mutants are auxin overproducers which spontaneously generate adventitious roots on the hypocotyl (Boerjan et al., 1995; Delarue et al., 1998). The superroot genes have been

described to encode enzymes of the indole-glucosinolate pathway. *SUR1* encodes the carbon-sulfur lyase catalyzing the conversion of S-alkylthiohydroximate to thiohydroximate (Bak et al., 2001; Mikkelsen et al., 2004). The Arabidopsis *SUR1* gene was used for the production of transgenic petunia plants and five independent overexpressing lines were generated.

3.5.1.3. Genes involved in hormone metabolism

The GH3 family is one of three auxin-responsive gene families (besides Aux/IAA and SAUR) which have not been extensively studied in other species than rice or Arabidopsis (Khan and Stone, 2007). Moreover, three auxin-inducible GH3-like proteins were shown to positively correlate with the number of mature adventitious roots in Arabidopsis (Sorin et al., 2006). In this study the Arabidopsis *GH3.5* gene was overexpressed in petunia and three transgenic lines were generated.

As several genes encoding Gibberellic Acid (GA) biosynthetic enzymes are now available, genetic engineering of GA contents has become practicable (Hedden and Phillips, 2000). Up to now, several reports described that different types of GAs inhibit or promote adventitious or lateral root formation in various plants under different environmental conditions (Hansen, 1976; Jarvis, 1987; Radi et al., 2006). In this study, two different types of tobacco GAs, GA2-oxidase which converts the active form of GA to the inactive form and GA20-oxidase which plays a role in the production of the active form of GA, were selected and overexpressed in petunia. Subsequently, seven and four transgenic lines were generated for GA2-oxidase and GA20-oxidase, respectively.

3.5.1.4. Genes involved in the TCA cycle

Malate dehydrogenase (MDH) catalyzes the reversible conversion of malate to oxaloacetate (OAA) in the TCA cycle. A proteomic analysis of different mutant genotypes of Arabidopsis resulted in identification of this enzyme to act as one of the markers for adventitious root development (Sorin et al., 2006). The

Arabidopsis *MDH* gene was used to overexpress this enzyme in petunia which finally resulted in generation of four transgenic lines.

Citrate synthase converts oxaloacetate (OAA) to citrate using acetyl-CoA in TCA cycle. As citrate accumulated strongly in the last stages of ARF in petunia cuttings (Fig. 11h) and regarding the fact that it was proposed as a biochemical marker for the late stage of ARF in the current investigation (Ahkami et al., 2009 or see discussion section 4.5.), the overexpression of Arabidopsis citrate synthase in petunia was carried out and four transgenic lines were produced.

3.5.1.5. Genes involved in carbohydrate metabolism

ADP-glucose pyrophosphorylase (AGPase) is one of the key enzymes in starch biosynthesis which converts glucose-1-phosphate to ADP-glucose by ATP consumption. Due to the strong accumulation of starch in the last stage of ARF (Fig. 6g-i), a potato *AGPase* gene was overexpressed in petunia. The selection of transgenic homozygous lines is currently running.

One of the key regulatory steps of sucrose biosynthesis is supposed to be the interconversion of fructose 1,6-bisphosphate (FBP) and fructose 6-phosphate (F6P) (Tamoi et al., 2007). So, cytosolic fructose-1,6-bisphosphatase (FBPase) that catalyses the formation of F6P plays a role in the sucrose biosynthesis pathway. Additionally, it was shown in the current investigation that the activity of this enzyme remained unchanged during the first hours after excision but decreased in the later course of ARF in petunia (Fig. 8h). This result along with the activity patterns of other enzymes (PFK and Glu6PDH Fig.8e,i) propose that the glucose is catabolized in parallel in the pentose phosphate pathway and during glycolysis in petunia during ARF (Ahkami et al., 2009 and see discussion). In order to verify this hypothesis, as well as evaluating the role of this enzyme in sucrose biosynthesis during ARF, a potato fragment of *cyt.FBPase* was introduced in petunia. The production of transgenic homozygous lines is currently running.

Plastidic Aldolase converts triose phosphate to fructose 1,6-bisphosphate (FBP) in the plastid. In the current work, a potato plastidic aldolase gene was overexpressed in petunia but so far only one transgenic line was generated.

Plastidic FBPase converts fructose 1,6-bisphosphate (FBP) to fructose 6-phosphate (F6P) which may convert to Glu-1-P and can be utilized in the starch biosynthesis pathway in the chloroplast. In this investigation, a potato plastidic *FBPase* gene was overexpressed in petunia for the generation of transgenic homozygous lines that is currently running.

3.5.1.6. Genes involved in carbohydrate transport

The role of STP4 is to import hexoses generated from sucrose after phloem unloading and hydrolysis by cell wall-bound invertases (Sturm and Chrispeels, 1990; Truernit et al., 1996). The RNA of this monosaccharide transporter was specifically induced during the first phase of ARF (Fig. 7d). Overexpression of the Arabidopsis *STP4* gene in petunia led to the generation of nine transgenic lines.

The Tonoplast Monosaccharide Transporter (TMT) family comprises three isoforms in *Arabidopsis thaliana. AtTMT1* and *AtTMT2* expression is induced by stresses like drought, salt and cold and by sugars indicating that *AtTMTs* play a role in vacuolar hexose transport, mainly under stress conditions. Furthermore, TMT1 is required for cytosolic glucose homeostasis (Wormit et al., 2006; Buttner, 2007). The over expression of *AtTMT1* and *AtTMT2* in petunia, resulted in the generation of five and four transgenic lines, respectively.

Possible effects of over expression of described selected genes on various phases of adventitious root formation will be analyzed by a current or future student using rooting behavior experiments, as well as biochemical and molecular approaches in different generated transgenic lines of petunia.

Table 9. Selected genes used for the generation of transgenic lines in *Petunia hybrida*. PRK: Phosphoribulokinase, FBPase: cytosolic fructose-1,6-bisphosphatase, AGPase: ADP-glucose pyrophosphorylase, GA: gibberellin, SUR1: Superroot1, TMT: Tonoplast Monosaccharide Transporter, STP4: Sugar Transport Protein 4, MDH: Malate Dehydrogenase, CS: Citrate Synthase.

Gene	Function	Source	Accession Number (NCBI)	Vector	Fragment Size	Number of Transgenic Lines
PRK	Photosynthesis	Arabidopsis	BT000019	pk2GW7	1200bp	4
Cytosolic.FBPase	Gluconeogenesis	Potato	X76946	pk2GW7	1000bp	-
Plastidic.Aldolase	Glycolysis	Potato	EU344848	pk2GW7	1000bp	1
Plastidic.FBPase	Glycolysis	Potato	AF134051	p9 NOS	1400bp	-
AGPase	Starch synthesis	Potato	X55155	p9 NOS	1200bp	-
GA2-oxidase	Gibberelin catabolism	Tobacco	AB125232	pK2GW7	1000bp	7
GA20-oxidase	Gibberelin anabolism	Tobacco	AB012856	pk2GW7	1000bp	4
GH3.5-Auxin	Auxin responsive gene	Arabidopsis	NM_118860	p9 NOS	1600bp	3
SUR1	indole-glucosinolate pathway	Arabidopsis	NM_201760	pK2GW7	1300bp	5
TMT1	Sugar transporter	Arabidopsis	NM_101937	pBin AR	2200bp	5
TMT2	Sugar transporter	Arabidopsis	NM_179234	pBin AR	2200bp	4
STP4	Sugar transporter	Arabidopsis	X66857	pK2GW7	1600bp	9
Mitochondrial MDH	TCA cycle	Arabidopsis	NM_001084687	p9 NOS	900bp	4
CS	TCA cycle	Arabidopsis	AF387018	pk2GW7	1300bp	4

4. Discussion

A cutting removed from the donor plant undergoes various anatomical changes accompanied by changes in metabolic activity and gene expression during the wound response and subsequent rhizogenesis. To investigate the occurrence and temporal sequence of specific anatomical, molecular and biochemical changes during adventitious root formation, different developmental stages were investigated using the model *P. hybrida*. For this purpose, the earliest time point of the occurrence of typical structures in ARF was registered and considered as being a mark for the transition from one developmental phase to the next.

4.1. Combined anatomical, biochemical and gene expression analyses in petunia cuttings allow separating ARF in three distinct physiological phases

4.1.1. Sink Establishment Phase

During the first hours after excision molecular and biochemical alterations were occurred before any visible cytological changes (Fig. 6). In agreement with the low sucrose level directly after excision, cell wall invertase activity was increased in the first hours after excising and decreased again to the basal level before root formation (Fig. 8a). By contrast, activities of vacuolar and cytosolic invertases decreased continuously to a low level (Fig. 8b,c). When activity measurements were confirmed by the transcriptome analysis, transcript levels of cell wall invertase were higher at early stages after cuttings had been excised from the donor plants. This was in agreement with previous studies showing that increased sucrose levels coincided with enhanced mRNA levels of extracellular invertase (Sinha et al., 2002). Cell wall invertase is not only a key enzyme of the apoplastic phloem unloading of transported sucrose but also links phytohormone action with primary metabolism (Roitsch and Gonzalez, 2004) (See also section 4.2.2.). Being present in the apoplast, it can establish a sink function of a certain

tissue and thus provide a mechanism for flexible and appropriate adjustment to a wide range of internal and external stimuli (Roitsch et al., 2003). Interestingly, a parallel accumulation of transcripts coding for the sugar transporter STP4 (Fig. 7d), which imports hexoses generated from sucrose after hydrolysis by apopalstic invertase (Truernit et al., 1996), has also been detected. Although enhanced transcript accumulation does not always result in enhanced protein levels, this observation suggests that at early stages of ARF basipetally translocated sucrose is degraded via cell wall invertase and the hexoses produced are transported into the cytosol via STP4 for further metabolism. As wounding causes not only an induction of cell wall invertase, but also a rapid increase of STP4-driven GUS activity (Truernit et al., 1996), it is no surprise that both genes were induced at the early stages of ARF to meet the increased carbohydrate demand in the cells. Since STP4 is a member of a large transporter family (Williams et al., 2000), It can not be excluded that also other sugar transporters are involved in the transport of sugars from the apoplastic space to cytosol in the sink tissues.

In most plant systems analysed, the most abundant amino acids in vascular tissues are glutamate, glutamine and in some cases asparagine (Urquhart and Joy, 1982; Schobert and Komor, 1989; Lohaus and Moellers, 2000). Analysis of petunia stems prior to excision indicated that mainly glutamine and asparagine were synthesized in source tissues and transported downwards to sink tissues (Fig. 12c,e). Since glutamine biosynthesis is a central step in nitrogen assimilation in plants (Joy, 1988), a basipetal translocation of glutamine is an important process to meet the N demand of the cells during root formation.

Taken together, the enhanced unloading of sucrose and transport of low molecular weight carbohydrates as well as of amino acids into the stem base indicated that this part of the tissue establishes as a sink.

4.1.2. Recovery Phase

After severance of the cuttings from the stock plant, it took 72 h to initiate the earliest anatomical event that was unambiguously related to ARF (Fig. 6c).

This was characterised by the occurrence of small cells with a large nucleus and a dense cytoplasm – both typical signs for meristematic cells. The appearance of such meristemoids marks the transition from the root initiation phase to the root primordium formation phase. This first cytological sign of ARF at 72 hpe was preceded by the RNA accumulation of the *cyclin B1* gene at 48 hpe (Fig. 7a). The expression of cyclin genes is typical for cells shortly before they divide (Ferreira et al., 1994). Additionally, since *CycB1* is not expressed in roots (Porceddu et al., 1999), it might serve as a marker gene for the root initiation phase of ARF.

Cell division and cell enlargement during ARF require high input of energy. In the stem base of petunia cuttings, the levels of soluble and insoluble sugars started to accumulate at 24 hpe despite a high metabolic activity from 12 to 24 hpe onwards. The transport form of carbon in plants is sucrose, which is formed in photosynthetically active tissues and translocated towards the sink organs of the plant. After cleavage into hexoses sucrose can be used directly as a carbon source or is converted to storage compounds such as starch. Since sugars are present in the bases of cuttings at higher levels than any other energy source such as lipids, they are supposed to play a crucial role during ARF (Haissig, 1982). Moreover, there is growing evidence that sugars have a regulatory role in ARF (Takahashi et al., 2003; Correa et al., 2005; Gibson, 2005).

The predominant amino acids in the cutting base were glutamine, glutamate, asparagine and aspartate. Almost all amino acids depleted strongly between 6hpe and 48hpe during ARF in petunia cuttings (Fig. 12). This depletion was observed for total amino acid as well, however, the pools of amino acids returned back to 50% of the initial levels three days after excision. This was interpreted in terms of a recovery of the physiological status of the stem base.

4.1.3. Maintenance Phase

Anatomically, at 96 hpe, the first well-developed young root meristems became visible during the root primordium formation phase (Fig. 6d). At 144 hpe these, at first globular structures, developed into root primordia with the typical dome shape (Fig. 6e) (De Klerk et al., 1999), which included the meristem and

behind it the first cells of the root body. After eight days (at 192 hpe) the first roots with elongated cells of the elongation zone appeared (Fig. 6f). These structures were still inside the stem, but revealed typical properties of a root organ. They mark the transition to the root elongation phase, which resulted in the emergence of the earliest roots visible after one additional day. Therefore, phases of root formation in petunia were designated anatomically as root initiation phase, followed by the root primordium formation phase and the root elongation and/or emergence phase which is in agreement with previous investigations regarding ARF (Kevers et al., 1997). These anatomical observations could be considered as powerful guidelines for the temporal sequence of root formation events.

The most pronounced increase in sugar levels was found during the later stages of ARF (Fig. 10a–c). In source leaves a continuous increase in sucrose was observed from 144 hpe onwards, probably owing to a high photosynthetic capacity (data not shown). Consequently, sucrose can be translocated towards the stem base for further metabolism. Even though a regulatory action of sugars cannot yet be excluded, the data support the suggestion that carbohydrates in petunia cuttings may play an important role in root elongation rather than in root initiation. This is supported by the fact that no direct correlation between carbohydrate levels and root initiation has been observed in leafy cuttings of *Pisum sativum* (Veierskov et al., 1982b) and in seedling cuttings of jack pine during propagation (Haissig, 1984).

The basipetally translocated sucrose is, however, not only used to deliver energy for differentiation (cell division and cell enlargement). A considerable portion of sucrose is converted into starch, which probably acts as the major carbon source when the adventitious roots grow. Starch accumulation is thought to be independent of the presence of regulatory hormones such as auxin. In leafy cuttings, auxins enhance the transport of carbohydrates to the basal part of the stem from where the roots regenerate (Haissig, 1984). As shown for *Pinus radiata*, sucrose applied to the growing medium leads to higher levels of sugars and starch in rooting regions of IBA-treated hypocotyl cuttings and enhances root

formation (Jasik and DeKlerk, 1997; Li and Leung, 2000). A further confirmation of starch accumulation was provided in the current study by histological staining of starch granules (Fig. 6g-i). Considering the only marginal increase of starch at early stages, starch seems not to be involved in root initiation. Interestingly, the present analyses indicate that starch can be synthesized and stored in different cell types to meet their C demand during enhanced metabolic activity when the adventitious roots emerge.

The successive increase of both, PFK and Glc6P DH activities (Fig. 8e,i), and the decrease of cytosolic FBPase activity (Fig. 8h) strongly suggest that catabolism of glucose occurs in parallel in the pentose phosphate pathway and in glycolysis, to produce ATP as an energy source and amino acids for protein synthesis. This was also shown for the activity of PFK and Glc6PDH, which increased in leafy cuttings of bean (*Phaseolus vulgaris*) and jack pine (*Pinus bansiana*) in response to applied indole acetic acid and an endogenous root-forming stimulus (Haissig, 1982).

Metabolomic data indicated that tricarboxylic acid cycle and energy metabolism contributed to the maintenance of ARF. Among the organic acids, citrate increased most dramatically during the last stages of ARF and increases of other organic acids were not comparable to that of citrate. Accumulation and exudation of citrate from roots have been shown to be induced by several stress factors, such as the presence of aluminum (Yang et al., 2006) or phosphorous deficiency (Zhu et al., 2005). During ARF, however, citrate accumulated in the stem tissue and was probably stored in vacuoles. The arising question is why citrate levels increased up to 125-fold (Fig. 10i), whereas that of other intermediates of citric acid cycle increased at the most fourfold. A possible explanation might be provided by a model for developing *Brassica napus* embryos: In the presence of inorganic nitrogen and glucose the refill of α-ketoglutarate is reduced to supply glutamine, glutamate, proline and arginine for protein synthesis (Junker et al., 2007). This adjustment is regulated by a balance of the activity of mitochondrial malic enzyme and PEP carboxylase. The strong accumulation of citrate might be explained by an accelerated activity of PEP

carboxylase (Fig. 9a) resulting in an increased production of oxaloacetate (OAA), which is further converted to citrate by citrate synthase in the mitochondria. The surplus of citrate is transported into the cytosol where it may either be converted back to oxaloacetate by citrate lyase or transported into the vacuole where it is stored. The obtained data strongly suggested that accumulated citrate allows a flexible adaptation of flux in response to ARF. Considering the dramatic increase of citrate during the formation of meristems, citrate level may be used as a biochemical marker for later stages of ARF in petunia.

Levels of glutamine and asparagine remained low at the later stages (Fig. 12c,e). This may indicate a considerable turnover of translocated amino acids which appears essential for the accelerated protein synthesis during ARF.

Consequently, the last phase reflected a new metabolic balance that coincided with adventitious root elongation. Therefore, it was named maintenance phase.

4.1.4. ARF in petunia cuttings is characterized by the transition of apoplastic to symplastic unloading of sucrose

Based on the mentioned results a three-phase mechanism is postulated for the metabolic response involved in ARF in petunia (Fig. 32). In the first phase designated as sink establishment phase**,** wounding leads to an induction of genes coding for enzymes that degrade sucrose to hexoses in the apoplast. The hexoses are taken up by monosaccharide transporters such as STP4, and are then used for the production of energy required for wound healing and cell division. Therefore, wounding initiates the establishment of a sink tissue in which all the resources are depleted. The second phase called recovery phase is characterized by the replenishment of resources and lasts up to 72 hpe ending with the formation of meristemoids. The last phase designated as maintenance phase is most likely characterized by symplastic transport (or by direct apoplastic transport independent of cell wall invertase) of sucrose translocated from source leaves to the stem base. Then, sucrose converted either into carbon metabolites or amino acids for root development or into starch as a transient storage

compound for reduced C. In addition, TCA metabolism, energy production and protein synthesis are increased during this phase.

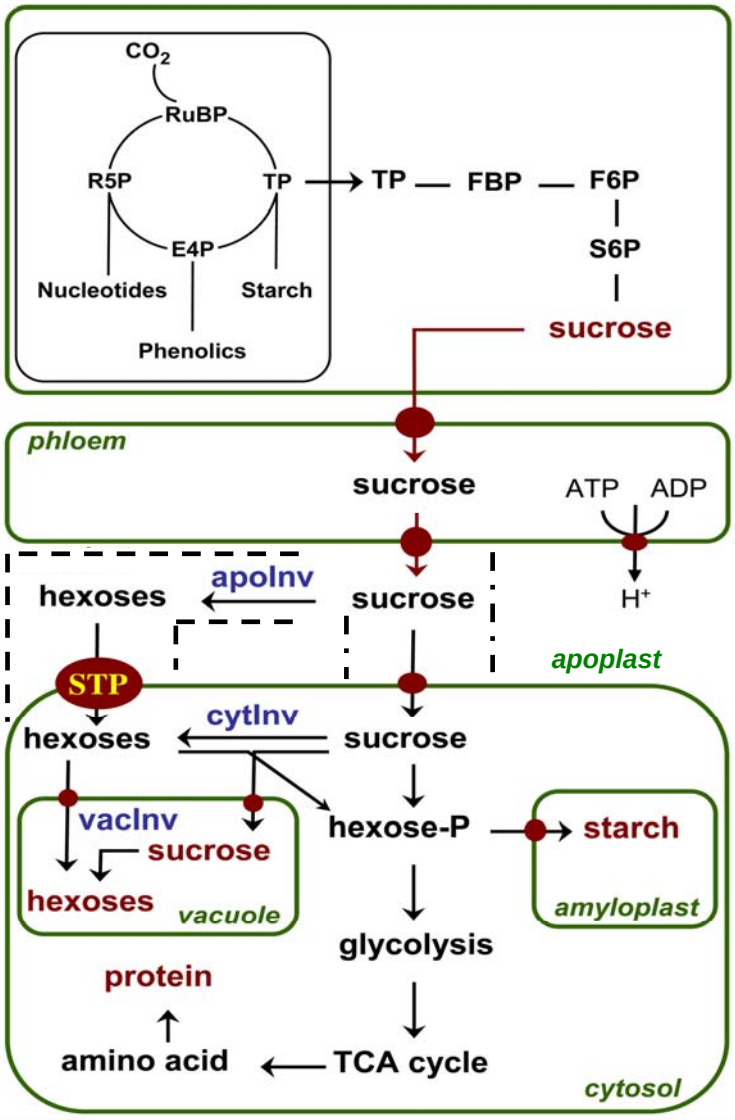


Figure 32. Schematic presentation of the metabolic mechanisms involved in adventitious root formation (ARF) in petunia (*Petunia hybrida*). Assimilates are produced in source tissues and translocated towards the cutting base to establish a sink organ. Wounding induces among others the accumulations of transcripts coding for cell wall invertase leading to cleavage of sucrose and transport of the hexoses into the cell, e.g. by STP-like monosaccharide transporters. Two days after excision the recovery phase starts followed by the maintenance phase characterized by symplastic transport of sugars translocated from source leaves into the stem base and used either for energy production or accumulated in the vacuole. Discontinuous line indicates the sink establishment phase and dash-dotted line represents the recovery and maintenance phase of ARF regarding different sucrose unloading routes.

4.2. The role of auxin during ARF in petunia cuttings

The role of auxin during ARF was investigated through two different methods. Firstly, the transcript accumulation of a GH3-responsive gene was monitored during a time series of adventitious rooting in petunia cuttings. Secondly, exogenous application of two auxin analogs and an auxin transport inhibitor was carried out to investigate metabolic, enzymatic and anatomical changes.

4.2.1. Auxin action precedes first signs of cell division

The petunia *GH3* gene expression was used as an early auxin marker (Hagen and Guilfoyle, 1985) to obtain information on the involvement of auxin action at certain developmental stages of ARF. The time course of *GH3* gene expression reflected a strong increase of auxin activity peaking at 4 hpe, followed by a transient decrease and a subsequent rise at 24 hpe (Fig. 7b) before *Cyclin B1* expression indicated the beginning of cell division. Considering the dose-dependent induction of the *GH3* promoter by active auxins as shown in transgenic tobacco (Hagen et al., 1991; Li et al., 1999), the first rise in *GH3* expression stays in accordance with observations in other plant species that a rise in the level of free indole-3-acetic acid (IAA) precedes initial events of cell division in root meristem formation (Blakesley 1994; Kevers et al., 1997). These observations support the view that an early rise of auxin levels is causally involved in initiating the primary events of ARF in petunia and suggest the petunia *GH3* gene as a suitable candidate for monitoring these early processes. Increased expression of a *GH3* promoter-reporter has also been observed in the basal cut ends of tobacco shoots or in tips of developing roots (Li et al., 1999). Proteomic analysis of different mutant genotypes of Arabidopsis revealed that three auxin-inducible GH3-like proteins were positively correlated with the number of mature adventitious roots (Sorin et al., 2006). However, since auxin levels were not correlated with the expression of *GH3* in any case, the authors concluded that *GH3* expression may also be dependent on other regulatory mechanisms, which may be mediated by action of repressors of auxin-inducible

genes (Sorin et al., 2005). Indeed, later processes of ARF can be inhibited by high auxin levels (De Klerk et al., 1999). Considering this and that several *GH3* genes in Arabidopsis encode IAA-amido synthetases, which are important to maintain auxin homeostasis by conjugating excess IAA to amino acids (Staswick et al., 2005), the second rise in *GH3* expression during ARF in petunia may indicate enhanced auxin conjugation to reduce the level of active auxin and thus to avoid inhibitory processes in ARF.

4.2.2. Enzymatic and metabolic activity during ARF in petunia cuttings after application of growth regulation

It is generally accepted that endogenous auxin has a critical function in the initiation and development of adventitious roots. Apical meristems, young leaves and active buds which are sources of auxin increase rooting in some cuttings. These promotive affects may be completely, or partially, replaced by exogenous auxin supply (Camper, 1996). There are several reports in this area, in which cuttings were treated with growth regulators, in order to investigate the relationship between auxin and biological compounds, sugar or metabolic changes (Altman and Wareing, 1975; Fogaca and Fett-Neto, 2005; Husen and Pal, 2007) during ARF. Auxin might affect the root formation by acting directly in these cells which initiate root primordia or indirectly through its engagement in the overall metabolism (Altman and Wareing, 1975). However, the state of auxin effects on primary metabolism or cell division during ARF is still matter of debate. Therefore, to answer this question whether auxin promoted root formation in cuttings might be mediated enzymatic and metabolic changes at the site of root formation, cuttings were treated with auxin analogs or inhibitors followed by a quantification of key enzymes and metabolites in the stem base of the cuttings.

Although root formation was initiated earlier after supply of IBA and NAA (Fig. 14) and was delayed using NPA (Fig. 18), there were no differences between non-treated and treated cuttings regarding sugar levels during ARF. This suggested that auxin was not involved in the accumulation of soluble sugars

during the final stages of ARF in petunia cuttings (Fig. 10, 15 and 20). By contrast, Altman and Wareing (1975) reported that treatment of bean cuttings with Indole-3-acetic acid (IAA) resulted in a considerable accumulation of soluble sugars at their base. Moreover, application of NAA and IBA to shoot cuttings resulted in an increase in the level of total soluble sugar, starch, protein, and peroxidase-activity in the rooting zone in *Tectona grandis* (Husen and pal 2007). Stimulatory effects of auxin applications on the rooting of stem cuttings have been documented in numbers of plant species (Nanda and Anand, 1970; Davis and Haissig, 1994; Hartmann et al., 1997). However, the mechanism of this physiological response yet remains disputed. Any discrepancies among those and the present study could be related to different growth conditions of the cuttings or hormone applications or to different responses in different plant species.

The present results showed that enzyme activities during ARF in the stem base of petunia cuttings were not influenced significantly by enhancing or inhibiting basipetal auxin transport, except in the case of cell wall invertase which exhibited a discontinous increase over time during ARF (Fig. 8a, 16a and 21a). This might reflect a specific relationship between extracellular invertase and basipetal auxin transport during ARF in petunia cuttings. Importantly, one of the proposed functions of cell wall invertase is to act as a linker between hormonal responses and primary metabolism (Roitsch and Gonzalez, 2004). An increase in acid invertase activity in response to hormones such as auxins (Morris and Arthur, 1984) was observed in several plant species. However, it is not clear whether these effects are due to direct regulation of invertase genes by plant hormones or via stimulated cell proliferation creating new sinks for sucrose (Sturm, 1999). Moreover, it has been mentioned that the increase in acid invertase activity induced by auxin redistribution in maize pulvinal cells has a key role in producing an asymmetrical accumulation of hexoses leading to the differential longitudinal growth of cells in gravitropic responses (Long et al., 2002). This might be provided an additional evidence for a link between invertase action and cell proliferation (Roitsch and Gonzalez, 2004). The question arising

here is why the activity of cell wall invertase increased over also in NPA-treated cuttings time during ARF, even more than under auxin-induced conditions. This should be answered by further experiments in future.

Asparagine concentrations showed completely different behavior in non-treated (Fig.12e), IBA- and NAA-treated (Fig.17e) and NPA-treated (Fig. 22e) cuttings. This amino acid is suggested to be the main nitrogen transport compound in the initiating roots of cuttings (Suzuki and Kohno, 1983). In the presence of auxin analogs, cell division initiated earlier by accelerating auxin levels in rooting zone which may lead to increase for demand of nitrogenous compounds in the stem base of the cuttings early after excision. The opposite trend may be suggested for NPA-treated cuttings regarding elevated levels of asparagine at later stages of ARF.

A strong accumulation of proline was monitored at later stages of ARF under both conditions of applying auxin analogs and auxin transport inhibitor (Fig. 23b, c). These results showed a relationship between auxin treatment and proline level in stem base of petunia cuttings during ARF. A significant increase in the rate of proline levels in auxin-induced condition in mung bean hypocotyls suggested the possibility that auxin may stimulate directly or indirectly the synthesis of a special protein with a high ratio of proline and/or hydroxyproline to other amino acids (Kuraishi et al., 1967). In agreement with this, genes for some proline-rich proteins (PRPs) involved in lateral root formation or root hair production showed a positive regulation of their mRNA levels by auxin supply (Ebener et al., 1993; Neuteboom et al., 1999; Bernhardt and Tierney, 2000). Besides, auxin is suggested to act as a regulator of PRP genes in a particular time-dependent or a concentration dependent manner (Thomas et al., 2003). However, it is very likely that the high proline levels observed in this study are a consequence of a stress response, especially in NPA-treated cuttings since high concentrations of NPA (80 µM) were applied to delay rooting. High concentration of NPA induces multiple stress responses. Moreover, NAA and IBA can induce stress responses for different reasons; NAA binds to targets other than auxin response proteins and transporters, while IBA at higher concentrations can

interfere with beta oxidation in peroxisomes. In particular, NAA can induce a high degree of oxidative stress (personal communication with Prof. Murphy, Purdue University, USA). However, whether the accumulating proline is transported from the leaves to the stem base or directly synthesized in the stem base remains unclear (See also 4.3.1).

Moreover, high concentrations of NPA delayed the onset of root primordia formation in petunia cuttings up to eight days (Fig. 19c,f vs. Fig.6e). Similarly, cell division in the tobacco cell line VBI-0 was slowed down by high concentrations of NPA (Campanoni et al., 2003). Regarding the role of auxin in cell division and enlargement, a complex interaction between auxin, cytokinin and cyclin-dependent kinases (CDK) was supposed (Meijer and Murray, 2000). Consistently, it has been suggested that CDKs are induced by auxin (Miao et al., 1993; Murray et al., 1998; Mironov et al., 1999). NPA and similar compounds can disrupt auxin efflux from the cell even though their mode of action is still a matter of debate. It has been further reported that the inhibitors of polar auxin transport prevent the traffic of PIN1 (a putative auxin efflux catalyst) and other rapidly cycled proteins to and from the plasma membrane in Arabidopsis root cells (Geldner et al., 2001). In addition, NPA has also been shown recently to interact with members of the P glycoprotein/ABCB/multidrug resistance family in Arabidopsis that are also identified as auxin transporters (Noh et al., 2001; Murphy et al., 2002; Bailly et al., 2008; Nagashima et al., 2008). In particular, TWD1 (TWISTED DWARF1) was shown recently to mediate modulation of PGP1 (P-glycoprotein) efflux activity by auxin efflux inhibitors, such as NPA and flavonoids (Bailly et al., 2008).

Taken together, auxin experiments in petunia cuttings suggested that auxin may influence ARF by stimulating the process of primordia formation rather than acting on the overall metabolism.

4.3. Transcriptome analysis reveals the involvement of genes from different metabolic pathways in ARF

To identify candidate genes specifically induced during adventitious root formation of petunia cuttings a microarray analysis was performed. For the design of a custom-made microarray a total number of 24,816 unique, non-redundant sequences were used. The RNA of stem bases of petunia cuttings in four different time points namely: 2hpe, 6hpe (representative for sink establishment phase), 72hpe (representative for recovery phase) and 192hpe (representative for maintenance phase) as well as 0hpe (without incubation in growth chamber) as control were used for hybridization. In order to monitor intact physiological events during ARF, no exogenous factor was used in the substrate. Likewise, fresh leaf, wounded leaf and root tissues were used to eliminate those genes that are not specifically related to ARF (see section 3.3.3. and Fig.28). Out of 24,816 examined sequences, 3,561 were finally considered as genes specifically induced during ARF and a total of 2,328 showed homology to known sequences in public data bases. Thereafter, the identified genes were clustered according to the physiological process to which they are associated (Table 6). Although the goal was to discarding purely wound-induced genes, some genes associated with wounding such as jasmonic acid (JA) or wound-induced proteins remained in the final list of ARF specific genes. One explanation for this is that the analyzed tissues were different from each other and might express different genes after wounding. So, these genes could be wound-inducible in particular tissues (e.g. the stem), but not in others (e.g. the leaves). Another explanation is that wounding is a more or less essential factor to induce ARF, as Hochholdinger et al. (2004a) defined adventitious roots as roots which are formed at unusual sites or under unusual circumstances such as wounding or hormone application. Consequently, the final number of 3,561 filtered genes assigned to ARF still included some genes that are related to wounding.

With regard to the large number of the genes specifically induced during ARF, a focus was set on key genes involved in primary metabolism, cell

replication and auxin metabolism. Out of the 3,561 genes, more than 1000 genes were not matched to known sequences in data bases based on annotation results. This provides a potential informative source to identify new candidates for adventitious rooting by means of reverse genetics. The complete list of all 3,561 genes specifically induced during ARF and their corresponding fold changes in each individual phase is presented in Excel File 3 (Data-CD). A short list of selected genes which are more emphasized in the current work are provided in Appendix Table 4.

4.3.1. Primary metabolism

Approximately 15% of the known and functionally classified genes specifically induced during ARF belong to the functional category primary metabolism. The number of up-regulated genes involved in primary metabolism decreased during different phases of ARF, whereas the number of down-regulated genes remained almost unchanged (Table 7). In the following, genes that are considered to play a major role in carbohydrate metabolism during different phases of ARF in petunia cuttings are discussed in more detail.

Sucrose unloading: Transcript levels of apoplastic invertase and some sugar transporters accumulated at early stages followed by a reduction during the recovery and maintenance phases, which is indicative for a switch from apoplastic to symplastic phloem unloading of sucrose during ARF. This interpretation is in agreement with previous biochemical and gene expression analysis and postulated hypothesis (section 4.1.4, Fig. 32). Carbohydrates may have to be imported into the affected tissues to expel the risk of energy limitation, and this could be accompanied by a rapid and simultaneous induction of genes coding for extracellular invertases (Sturm and Chrispeels, 1990) or putatively plasmalemma-localized monosaccharide transporters (Truernit et al., 1996) like those of the STP family in this study. In other words, the possible symplasmic carbohydrate import into the affected cells through plasmodesmata would depend on simple diffusion, which might not be fast enough to allow a rapid

wound or defense response (Truernit et al., 1996). A similar event could be observed during tuberization in potato where sucrose utilization changes from hydrolytic to sucrolytic breakdown (Appeldoorn et al., 1997; Hajirezaei et al., 2000). This process is associated with a switch from apoplastic to symplastic phloem unloading (Viola et al., 2001). Likewise, the developmental and organ specific expression of sucrose-cleaving enzymes suggested a transition between apoplastic and symplastic phloem unloading in the tap roots of sugar beet (Godt and Roitsch, 2006). As the supply of the main transport sugar sucrose is a limiting step for the growth and metabolism of sink tissues (Farrar, 1996), the insight into the underlying mechanism of sucrose transport will contribute to better understand the growth and development of higher plants as well as to successfully manipulate carbohydrate partitioning by transgenic approaches (Godt and Roitsch, 2006).

Trehalose metabolism: Trehalose metabolism, a short side-branch of primary carbon metabolism which is controlled by a large gene family, is emerging as an important new regulatory pathway in plants (Ramon and Rolland, 2007). In the sink establishment phase transcripts encoding three key enzymes involved in trehalose metabolism including trehalose-6-phosphate synthase (TPS), trehalose-6-phosphate phosphatase (TPP) and trehalase were induced. Trehalose-6- phosphate (T6P), essential for the coordination of metabolism with plant growth adaptation and development (Paul, 2007), is formed from glucose-6-phosphate and uridine-5-diphosphoglucose (UDPG) by TPS and is then dephosphorylated to trehalose by TPP (Eastmond et al., 2003). Subsequently, trehalose may be converted to glucose by trehalase. Besides the role of TPS for embryo development beyond the torpedo stage (Eastmond et al., 2002) TPS1 is expressed at low levels in all tissues, peaking in metabolic sinks such as embryos, flower buds, young rosette leaves (van Dijken et al., 2004) or in the stem base of petunia cuttings in current study. However, Schluepmann et al. (2003) presented evidence that the active component of the trehalose pathway that regulates metabolism is T6P. In the same direction, Paul et al. (2001)

suggested that T6P either directly or indirectly controls carbon assimilation. It has also been supposed that T6P is a metabolic regulator that has an impact like that of a hormone (Paul, 2007). In some yeasts, including *S. cerevisiae*, TPS1 plays a critical role in the regulation of glycolysis (Thevelein and Hohmann, 1995). The mechanism by which TPS1 controls glycolysis in yeasts is not fully understood, but the predominant site of action may be related to hexokinase (HXK) (Thevelein and Hohmann, 1995). Nevertheless, in Arabidopsis T6P is not an inhibitor of AtHXK1 or AtHXK2 activity in vitro (Eastmond et al., 2002). Similarly, T6P has no effect on HXK activity in spinach leaf extracts (Wiese et al., 1999). Alternatively, T6P may directly activate ADP glucose pyrophosphorylase (AGPase), the key enzyme in starch synthesis (Kolbe et al., 2005). Sucrose leads to the redox activation of AGPase in a Sucrose non-fermenting 1- Related protein Kinase (SnRK1)-dependent manner when fed to intact leaves (Tiessen et al., 2003). SnRK1 is a protein kinase involved in the regulation of carbohydrate metabolism. In the current investigation, a SnRK1 homolog for petunia was induced in the sink establishment and recovery phases of ARF. When micromolar T6P was fed to isolated chloroplasts, it activated AGPase, providing the possibility that T6P is the conduit for sucrose and possibly trehalose-mediated activation of starch synthesis (Paul, 2007). In this way, T6P can be seen as a sugar signal that communicates the sucrose status of the cytosol to the chloroplast (Paul, 2007). It has further been suggested by Lunn et al. (2006) that T6P acts as a signaling metabolite of the sugar status in plants, and that it mediates sucrose-dependent changes in the rate of starch synthesis. Besides, T6P was reported before to be associated with the expression of SnRK1 (Schluepmann et al., 2004). Taken together, it may be concluded that T6P, the product of TPS, along with SnRK1 may have a signaling effect on starch biosynthesis and accumulation in the later stages (Fig. 6g-i and 10c) of ARF. Additionally, induction of a plastidic aldolase gene in the recovery phase is also indicative of starch biosynthesis in the chloroplast. However, the mentioned hypothesis about trehalose metabolism should be confirmed by measuring the

level of T6P in stem base of petunia cuttings during ARF and by generating anti-sense lines for functional analysis.

Glycolysis: The transcript levels of five out of total ten enzymes involved in glycolysis were specifically induced during ARF in petunia cuttings. These included hexokinase (HXK, two isoformes), phosphofructokinase (PFK, three isoformes), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGM, two isoformes) and pyruvate kinase (PK). Induction of HXK, PFK (catalyzing a rate-limiting step) and PK as regulatory enzymes in glycolysis, all catalyzing irreversible reactions in C catabolism, indicates that C flux through this pathway is important during ARF. Except fundamental roles of glycolysis (Plaxton, 1996), in recent years, additional non-glycolytic functions such as regulation of transcription have also been attributed to glycolytic enzymes (Kim and Dang, 2005). It should be mentioned that the previous biochemical results, which were discussed before (section 4.1), are in agreement with the microarray data in this area.

Lipid metabolism: The present transcriptome study suggested an active lipid metabolism in petunia cuttings especially at later stages of ARF. Transcript levels of acetyl-coenzyme-A (acetyl-CoA) synthetase (ACS) and ATP-citrate lyase (ACL) were induced in the sink establishment phase. The generated acetyl-CoA, one of the products of these enzymes, may be used for *de novo* fatty acid synthesis in plastids (Ke et al., 2000; Rawsthorne, 2002) which is required for the assembly of membrane lipids.

Transcript levels of two enzymes involved in plant peroxisomal fatty acid β-oxidation including 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase were induced in early and later stages of ARF, respectively. Beyond its role in the breakdown of storage lipids, which seems to be not relevant for the current investigation, β-oxidation in plants has been shown to be active in a variety of other developmental processes, including the emergence of the radicle from the seed coat, embryo and flower development, production of jasmonic acid

(JA) in the wounding response or generation of the phytohormone indole-3-acetic acid (Poirier et al., 2006). The fact that ARF is a developmental process which utilizes carbohydrates as a main carbon source for energy production and requires auxin for its initiation step, suggests that β-oxidation during ARF may play a role other than in the degradation of reserve lipids. Indole-3-butyric acid (IBA), which has been shown to occur in several plant species (Li et al., 2009), is a natural auxin that can be converted to indole-3-acetic acid (IAA) via one round of β-oxidation. Several mutants deficient in enzymes of the β-oxidation cycle were shown to be resistant to the supply of IBA (Zolman et al., 2000). On the other hand, JA synthesis requires the β-oxidation of its precursor molecule OPC:8 (Castillo et al., 2004; Pinfield-Wells et al., 2005), and Rylott et al. (2003) suggested an important role for the β-oxidation of short-chain acyl-CoA esters in the development of embryo.

Transcripts encoding five different Acyl-CoA synthetase (ACyS) were specifically up-regulated during ARF in petunia cuttings. The induction of some of those exhibited a 10 to 50-fold enhancement in the recovery or maintenance phases. ACyS catalyzes the conversion of free fatty acids to acyl-CoAs, which are key intermediates in both fatty acid degradation and cellular lipid synthesis (Groot et al., 1976). It may also be involved in the regulation of cell signaling, enzyme activity, and protein modification (Johnson et al., 1994; Faergeman and Knudsen, 1997). A recent study revealed that a new group of ACySs is involved in jasmonate biosynthesis in Arabidopsis (Kienow et al., 2008; Wang and Li, 2009).

GDSL esterases/lipases might play an important role in the regulation of plant development and morphogenesis (Akoh et al., 2004). Transcript levels of several GDSL-motif lipases were mainly up-regulated in the recovery and maintenance phases with an elevation of up to 200 or 1000-fold in the maintenance phase. Various functions have been considered for these enzymes such as a fatty acyl-ester hydrolase function in germinated sunflower seeds (Beisson et al., 1997), or a role in yield production (Akoh et al., 2004). Interestingly, three esterases involved in the degradation of polysaccharides

were found to belong to the GDSL-family (Dalrymple et al., 1997). However, the biochemical function of many of these enzymes is still unknown

Likewise, an up-regulation of fatty acid dehydrogenase, Cytochrome P450-dependent fatty acid omega-hydroxylase, omega-6 and omega-3 fatty acid desaturase, lipid transfer and lipid binding proteins, 72 hours after excision of the cuttings further indicated a prominent role of lipid metabolism in the recovery and maintenance phases of ARF.

At this point it can not be concluded what is the precise role of lipid metabolism in ARF. Since many genes from different processes in lipid metabolism were altered, but metabolite analysis could not cover corresponding changes, further studies are required to define the role of lipid metabolism during ARF.

Other induced genes involved in primary metabolism: Besides its cardinal roles in the initial fixation of atmospheric CO_2 during C_4 photosynthesis and Crassulacean Acid Metabolism (CAM), PEP carboxylase (PEPC) functions anaplerotically in a variety of nonphotosynthetic systems such as for C/N partitioning in C_3 leaves, seed formation and germination, or ripening (Chollet et al., 1996). Induction of PEPC transcripts during the recovery phase of ARF in petunia cuttings, which was accompanied by an increased PEPC activity (Fig. 9a), confirmed that PEPC has a role in refilling TCA cycle during ARF. Likewise, in consistency with a proteome analysis of adventitious rooting in Arabidopsis (Sorin et al., 2006), carbonic anhydrase (CA)-RNA levels were induced at 6hpe. The corresponding enzyme functions in the production of bicarbonate, which serves as a substrate for PEPC (Melzer and Oleary, 1987). The product of PEPC activity, oxalacetate, can be converted to aspartate via aspartate aminotransferase (AAT) (Melzer and Oleary, 1987), which was also induced at the transcript level at the same time point in the current study. This observation is supported by a continuous increase of aspartate levels three days after excision (Fig. 12d). Thus, CA, PEPC and AAT may represent regulated steps in the pathway for the synthesis of aspartate.

Regarding ribonucleotide synthesis, a transcript encoding ribonucleotide reductase (RNR) was down-regulated in the sink establishment phase followed by an up-regulation in the recovery and maintenance phases. RNR catalyses the rate-limiting step in the production of deoxyribonucleotides needed for DNA synthesis (Elledge et al., 1992). A critical role of RNR in cell cycle progression, DNA damage repair, and plant development was recently reported (Wang and Liu, 2006). In coincidence with the formation of the first new meristematic cells at 72 hpe (Fig. 6c), up-regulation of RNR in the recovery phase of ARF may be expected. In the same direction, a transcript encoding Uridine 5'-monophosphate synthase (UMP synthase), which is the rate-limiting step in pyrimidine biosynthesis (Santoso and Thornburg, 1998), was up-regulated in the recovery and maintenance phases. Therefore, the recovery phase is characterized by the induction of genes involved in ribonucleotide synthesis, such as RNR and UMP synthase.

A significant portion of nitrogen is continuously released as NH_3 and reassimilated via glutamine synthetase (GS) (Miflin and Habash, 2002). It has been reported that two different isoforms of GS including GS1 and GS2 function as the major enzyme for the assimilation of ammonia produced by N_2 fixation or derived from nitrate or ammonium uptake (Miflin and Habash, 2002). Transcript levels of GS (the isoform could not be recognized) were overpresented in the recovery and maintenance phases, indicating a requirement for ammonium re-/assimilation during the later phases of ARF in petunia cuttings. One possible source of ammonium may derive from protein degradation, because asparaginase-RNA levels were induced. Asparaginase deaminates asparagine and generates ammonium.

The degradation of proline is catalyzed by the sequential action of two mitochondrial enzymes, Pro-dehydrogenase (ProDH) and P5C-dehydrogenase (P5CDH). Transcript levels of ProDH, also known as proline oxidase (POX), which is catalyzing the rate-limiting oxidation/dehydrogenation of proline to pyrroline-5-carboxylate (P5C) (Hu et al., 2007) was highly up-regulated in the sink establishment phase followed by a reduction at later time points. In contrast,

lower transcript levels of P5CDH, catalyzing the conversion of P5C to glutamate, in the sink establishment phase was followed by an acceleration in the recovery and maintenance phases. These results suggested that the generated P5C in the sink establishment phase may have been further converted to glutamate in the later phases. Consistently, the amount of glutamate increased at 72 hpe (Fig. 12b). Nevertheless, these data are not in agreement with proline accumulation in maintenance phase (Fig. 23a). The apparent discrepancy between high proline concentrations and high ProDH/P5CDH (proline degradation enzymes) transcripts levels may be explained by post-transcriptional regulation of the corresponding proteins or by proline export via the xylem to the stem base in the maintenance phase. Induction of a transcript encoding a proline transporter during ARF in the current study may confirm the later hypothesis. Considering the role of proline transport, Raymond and Smirnoff (2002) implied that changes in biosynthesis and oxidation rate which led to proline accumulation are at least partly controlled by changes in gene expression and enzyme activity. A previous conflict between low proline concentrations and high P5CS/P5CR (proline synthesizing enzymes) transcripts levels in roots was explained by proline export via the xylem to the shoot (Verbruggen et al., 1996). Proline has been suggested to act as a compatible osmolyte and to be a way to store carbon and nitrogen (Hare and Cress, 1997). In this regard, Schwacke et al. (1999) proposed that the increase of compatible solutes is achieved either by altering metabolism (increasing biosynthesis and/or decreasing degradation) or by transport (increased uptake and/or decreased export). Moreover, proline transport to flowers is also active, as it can be a compatible solute to transfer nitrogen, carbon and energy to developing flowers and seeds (Verbruggen and Hermans, 2008). Likewise, proline has also been suggested to function as a molecular chaperone stabilizing the structure of proteins, and its accumulation can provide a way to buffer cytosolic pH and to balance the cellular redox status (Verbruggen and Hermans, 2008). Finally, proline accumulation may be part of a stress signal influencing adaptive responses (Maggio et al., 2002). The hypothesis of proline transport to the stem base of petunia cuttings at the later stages of ARF and its

possible role as a compatible osmolyte should be further investigated in the future.

A transcript encoding S-adenosylmethionine synthetase (SAMS) was also induced in the sink establishment phase, which is in agreement with earlier investigation showing that two SAMS-encoding genes were induced during adventitious root development in *Pinus contorta* (Lindroth et al., 2001a). In addition, several sequences showing homology to SAMS were also up-regulated in *Pinus contorta* during adventitious root formation using microarray (Brinker et al., 2004). SAMS catalyzes the production of S-adenosylmethionine (AdoMet) which has a central position in several physiological processes (Lindroth et al., 2001a). In plants, AdoMet serves as a methyl group donor in the transmethylation of lignin, DNA and alkaloids, or as a donor of aminopropyl moieties in ethylene and polyamine synthesis (Galston and Kaur-Sawhney, 1995; McKeon et al., 1995; Chiang et al., 1996). Regarding the fact that the expression of this enzyme is not generally up-regulated under stress conditions (Mayne et al., 1996) and considering the consistent results in previous adventitious rooting investigation, SAMS could be nominated as a significant candidate gene which may play important role specifically during ARF.

4.3.2. Chromatin and DNA metabolism, Cell cycle and Cytoskeleton

In total, 73 genes specifically induced during ARF in petunia cuttings were involved in chromatin and DNA metabolism. The number of up-regulated genes in this category were two-fold higher in the recovery and maintenance phases of ARF in comparison with the first six hours after cutting, while no genes were down-regulated (Table 7). Precisely, the same expression pattern was observed for a total of 33 genes involved in the class of cytoskeleton-related functions. (Table 7). In addition, the number of up-regulated genes involved in cell cycle processes increased during ARF, while the opposite trend was seen for down-regulated genes (Table 7). These results indicate an increase in the induction of DNA metabolism, cell cycle and cytoskeleton-related genes starting 72 hours

after excision which coincided with the formation of new meristematic cells at 72 hpe (Fig. 6c).

A total number of 15 histone-encoding genes including H_2A, H_2B, H_3 and H_4 were up-regulated in the recovery and maintenance phases indicating the proliferation of cell replication during the last stages of ARF. Similar observations were reported for adventitious root development in *Pinus contorta* (Lindroth et al., 2001b; Brinker et al., 2004) and rice (Lorbiecke and Sauter, 1999).

Cyclin-dependent protein kinases (CDKs) have emerged as important components of a conserved regulatory complex that functions in cell division in eukaryotes (Nasmyth, 1996). Transcripts encoding B1- and B2-types of CDK were repressed in the sink establishment phase and overpresented during later phases of ARF in petunia cuttings. Moreover, transcripts encoding cyclin (CDK's regulatory proteins) B1, B2 and A were induced three days after excision. Northern blot analysis of the *cyclin B1* gene showed a similar time-dependent pattern during ARF (Fig. 7a or Fig. 31d). Interestingly, transcripts encoding cyclin D were only induced in the maintenance phase. D-type cyclins regulate the progression of cells through the G_1 phase of the cell division cycle in response to extracellular signals such as auxin, cytokinin or sucrose (Meijer and Murray, 2000).

Transcript levels of annexin showed a 60-fold and 200-fold increase in the recovery and maintenance phases, respectively. In a previous study, annexin has been reported to be differentially expressed during adventitious root development in *P. contorta* (Brinker et al., 2004). It has been hypothesized that annexins play a role in the Golgi-mediated secretion of newly synthesized plasma membrane and cell wall material in plant cells (Clark et al., 2001). Annexins have also been documented in plant nuclei where they may participate in DNA replication (Clark et al., 1998; Kovacs et al., 1998). There are two tobacco annexins that are cell cycle regulated and expressed mainly in dividing tissues (Proust et al., 1999). Thus, annexin could be considered as a marker for adventitious rooting three days after excision in dividing cells of the stem base of the petunia cuttings.

In addition, transcript abundance of an alpha-L-fucosidase was highly up-regulated in the recovery and maintenance phases. It has been reported that alpha-L-fucosidase plays a role in the control of cell elongation by regulating the levels of bioactive fucosylated xyloglucan oligosaccharides generated within the growing cell wall (Augur et al., 1995).

4.3.3. Mineral nutrient acquisition

Nutrition is a key factor determining root morphogenesis (Assis, 2001) through effects on primary root length or lateral root formation (Schwambach et al., 2005). Although adventitious rooting and mineral nutrition are intimately related, only a few studies have attempted to characterize the action of certain mineral elements on the rooting process. However, recently Santos et al. (2009) reported that nutrient availability at the stem base of petunia cuttings at root emergence phase improves adventitious root development. In the current transcriptome analysis of ARF in petunia cuttings, a total of 64 genes were specifically induced during root development, in particular at later stages of ARF.

Transcript levels of several nitrate transporters (NRT) were induced during ARF. There were induced in the sink establishment phase followed by a high up-regulation in the recovery and maintenance phases up to 200 to 300-fold in some cases. The induction of NRTs at 6hpe most likely indicated nitrogen deficiency of the cuttings early after excision. Besides being a source of nitrogen, nitrate serves as a metabolite to buffer acidification from ammonium assimilation and as a signal for growth (Crawford, 1995; Stitt, 1999; Zhang and Forde, 2000). A high induction of NRT1.1 (CHL1), a dual-affinity NO_3^- transporter, at later stages of ARF is in consistent with the well-established role of this nitrate transporter in regions containing rapidly dividing cells (Guo et al., 2001). Besides its metabolic role of transporting nitrate in nascent organs, NRT1.1 activity may contribute to nitrate signaling or pH homeostasis during lateral root formation (Guo et al., 2001).

In consistency with the fact that ammonium is a major N form for plant nutrition and may show a heterogenic distribution in the rooting zone (Loque and

von Wiren, 2004), five different ammonium transporter encoding genes were induced in different phases of ARF. This suggests a consistent need for N uptake and retrieval during ARF.

Plants have evolved sophisticated metabolic and developmental strategies to maximize inorganic phosphate (P_i) acquisition when P_i is limited. One of the main strategies is to increase the expression of high-affinity P_i transporters (Ticconi et al., 2004). In the current study, transcripts encoding seven phosphate transporters were induced at different time points of ARF with the trend of a preferential up-regulation at later stages. Phosphate is necessary for various developmental and physiological processes such as root formation, cell division and nucleotide synthesis (Marschner, 1995). It is a precursor for starch biosynthesis which accumulated at later stages as well (Fig. 6g-i).

Transcript levels of iron (Fe), zinc (Zn) and zinc/iron transporters were also overpresented in the recovery and maintenance phases. These micronutrients may function as cofactors for enzymes required during ARF in petunia cuttings, such as ribonucleotide reductase or fatty acid desaturase which are iron-dependent or carbonic anhydrase which uses zinc as its prosthetic group. Moreover, Zn is required for the biosynthesis of tryptophan which is an auxin precursor (Dell and Wilson, 1985; Blazich, 1988). In agreement with this, high zinc concentrations in the induction phase of adventitious root formation in microcuttings of *Eucalyptus globulus* influenced auxin concentrations, thereby favoring the rooting response (Schwambach et al., 2005). Since iron participates in the biosynthesis of peroxidases (Campa, 1991), increase of iron transporters, which are at least one of the requirements to accelerate Fe levels, during the later phases of ARF could cause an enhancement of peroxidase activation involved in auxin catabolism (see below). A similar explanation was suggested by Fang and Kao (2000) as they implied iron reduction in rice leaves could cause a decrease in the activities of peroxidases. Besides, decreasing Fe concentrations in the induction phase of adventitious rooting in microcuttings of *Eucalyptus globulus* showed only a trend towards higher root number and longer root length (Schwambach et al., 2005). However, Fe may also act on other root

morphological traits via micronutrient signaling effects that also interfere with hormonal regulation (Giehl et al., 2009).

Boron (B) deficiency resulted in poor rooting of *E. globulus* microcuttings (Schwambach et al., 2005). Boron deprivation inhibits cell division and cell expansion (Lukaszewski and Blevins, 1996), and therefore root growth in intact plants (Josten and Kutschera, 1999). A high B demand could explain the present finding of a continuous increase of a boron transporter gene during different phases of ARF in petunia cuttings.

Moreover, in the maintenance phase, a high transcript abundance was observed of a gene encoding a plasma membrane proton ATPase, which is most likely contributing to cell elongation (Boot et al., 1993) or nutrient up-take (Michelet et al., 1994).

4.3.4. Antioxidative metabolism and Redox state

52 out of 94 genes on the microarray that are involved in antioxidative metabolism encoded peroxidases, indicating their possible important functions in root development. They were highly up-regulated in the recovery and maintenance phases, in some cases up to 400-fold. A certain peroxidase isoform has been reported as a predictive marker of ARF in *Betula pendula* (McDonald and Wynne, 2003). Besides, changes in peroxidase activity and peroxidase isoform patterns have been proposed as biochemical markers of successive rooting phases (Metaxas et al., 2004; Syros et al., 2004; Rout, 2006). However, they have also been implicated in several physiological processes including the regulation of growth, cell expansion (Goldberg et al., 1986), auxin metabolism (Grambow and Langenbeckschwich, 1983), and lignification (Grisebach, 1981). In addition, peroxidases may play a key role in several stress-related processes such as wound healing (Espelie et al., 1986). Although these are important biological processes, it is difficult to prove or disprove a role of individual isoform during ARF based on the microarray investigation in the current study. Thus, It might be worthwhile to utilize these genes for overexpression or suppression approaches in transgenic plants to elucidate the functions of individual isoforms

during ARF in petunia cuttings. For the present study a main role of peroxidases in auxin catabolism is proposed, since peroxidases exhibited a high induction levels at later stages when auxin becomes inhibitory in ARF (De Klerk et al., 1999).

4.3.5. Hormone metabolism and perception

A total of 113 genes involved in the metabolism of seven different hormones were specifically induced during various phases of ARF in petunia cuttings. Among them, genes involved in ethylene, auxin and gibberellin synthesis, transport or metabolism turned out to be more activated than those of other hormones. Indeed, fluctuations in transcript levels during various phases of ARF and induction of different types of hormones and their isoformes make it difficult to work out a clear pathway of hormonal activity required during root development in petunia cuttings, solely based on the array experiment. Maybe, this indicates a hormonal cross-talk between different hormones during ARF in petunia cuttings, as very recently Negi et al. (2010) reported a positive role for ethylene in adventitious root formation in tomato with the modulation of auxin transport as a central point of ethylene–auxin interactions.

As one could expect in the case of a developmental process regulated by auxin, genes involved in the control of auxin homeostasis were identified. Several auxin-responsive genes from the *GH3* family encoding IAA (Indole-3-acetic acid)-amido synthetase, were up-regulated at all time points of ARF. In four cases, associated transcript levels exhibited a first maximum at 2 hpe, declining at 6hpe and started to increase again at 72 hpe, which was the same in Northern blot analyses using the *GH3* gene (Fig. 7b, discussed in section 4.2.1). By contrast, two genes (one of them had a very high homology with *GH3.3*), showed an approximately 100-fold increase at 6hpe. Interestingly, proteomic studies of Arabidopsis mutants identified three auxin-inducible GH3-like proteins which positively correlated with the number of mature adventitious roots (Sorin et al., 2006). Up-regulation of *GH3* genes in the sink establishment phase of ARF in petunia suggested that auxin-regulated gene expression is under rapid

transcriptional control (Hagen and Guilfoyle, 1985). In addition, transcriptional activation of *GH3* genes in the recovery and maintenance phases of ARF, when excess auxin might be inhibitory (De Klerk et al., 1999), would lead to more IAA-amido synthetase, which might convert excess auxin to amino acid conjugates that are either inactive or degraded (Staswick et al., 2005). This conjugation of IAA to amino acids or other small molecules is essential for auxin homeostasis (Normanly and Bartel, 1999; Woodward and Bartel, 2005).

Regarding other auxin responsive genes, five transcripts encoding SAUR (small auxin-up RNA) genes were induced at different time points of ARF. Although their function is largely unknown, auxin-mediated cell elongation (Kant et al., 2009), apical hook development (Park et al., 2007) and negative regulation of auxin synthesis and transport (Kant et al., 2009), have been proposed as possible functions of SAURs.

Transcript levels of 5NG4, an auxin-induced protein, were up-regulated at all time points with a maximum induction by 50-fold in the maintenance phase. Busov et al. (2004) characterized a 5NG4 gene from juvenile loblolly pine shoots, which was highly and specifically induced by auxin prior to adventitious root formation. It has been proposed that the corresponding gene product is a transmembrane protein with a possible transport function.

Furthermore, an elevated mRNA expression level of auxin efflux (PIN1) and influx proteins during ARF may be indicative for enhanced basipetal auxin transport from leaves to the stem base of petunia cuttings at all time points. The reduced polar auxin transport in *pin1* mutants and its effects on plant development indicate a role of PIN1 in polar auxin transport, most likely by mediating auxin efflux out of the cell (Galweiler et al., 1998).

Finally, transcript levels of several isoformes of ACC (1-aminocyclopropane 1-carboxylate) synthase, ACC oxidase (ethylene synthesizing enzymes), a DELLA protein acting as a GA response repressor and two isoformes of SAM carboxymethyl transferase were induced during various phases of ARF. Although it is difficult at this point to discuss the potential role of these genes in adventitious root development, our results confirm at the

transcript level the observations made at the protein level by Sorin et al. (2006) for ACC oxidase and SAM carboxymethyl transferase.

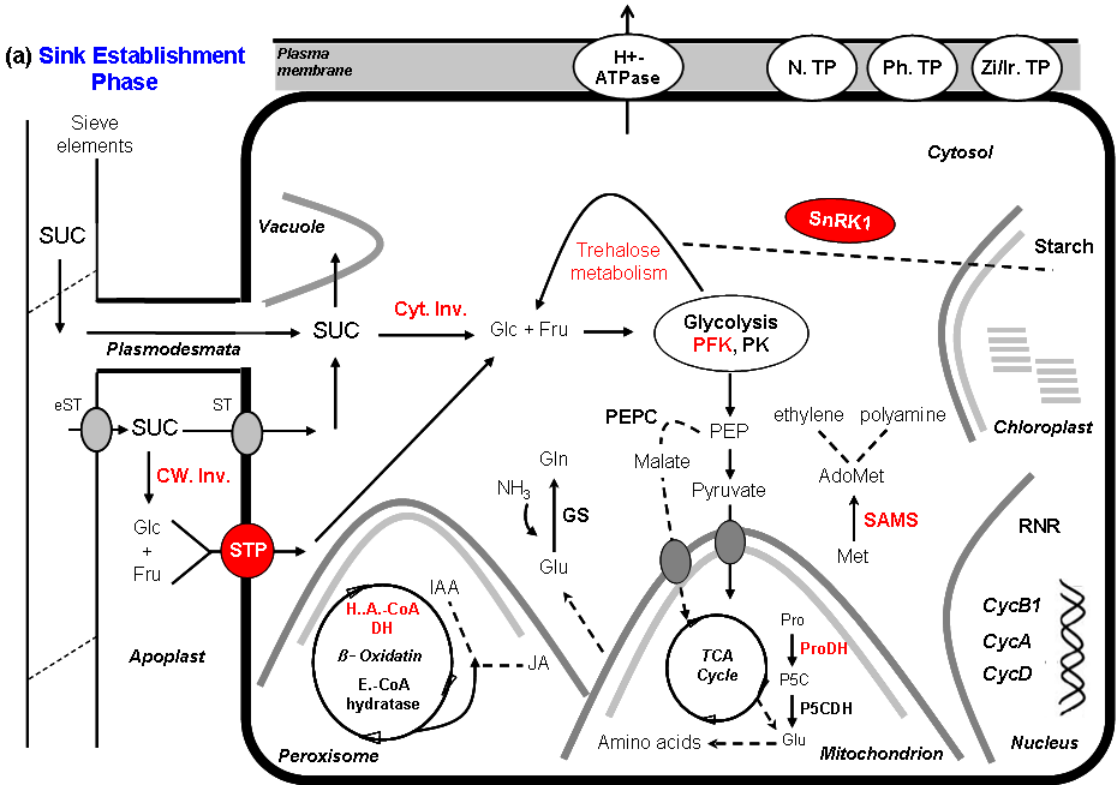
4.3.6. Working Model

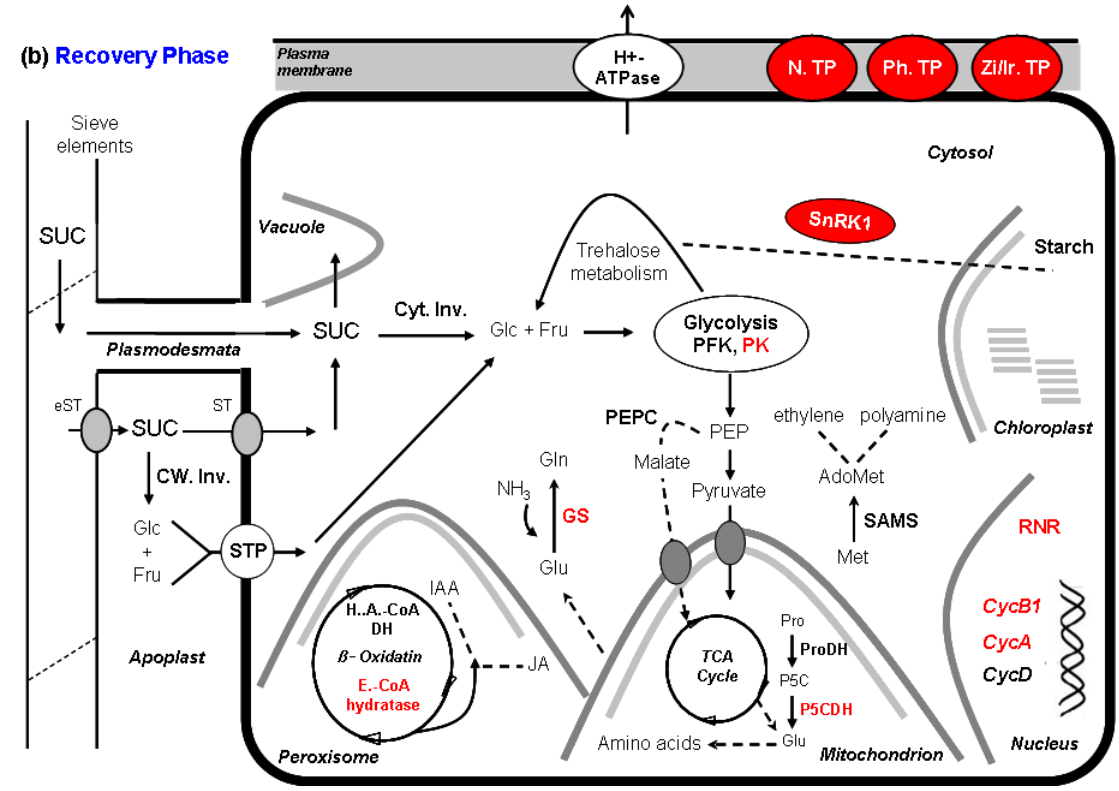
By examining changes in the global gene expression in petunia stem cuttings, a temporal sequence of molecular events taking place during adventitious rooting was established. Of course, changes in mRNA levels do not necessarily lead to alteration in protein levels or to changes in developmental processes, but the importance of transcription as an important regulatory level in developmental processes has been well established (Alberts et al., 2002). Based on the present data and focusing on changes in transcript abundances of genes involved in primary metabolism, membrane transporters, cell replication or auxin metabolism following working model (Fig. 33) has been set up.

During the first six hours after excision of the cuttings, carbohydrate metabolism in the stem base is initiated by an increase of cell wall invertase and of hexose transporters of the STP family to import the products of sucrose degradation from the apoplast into the cytosol. Simultaneously, several genes involved in trehalose metabolism are up-regulated which may regulate carbohydrate metabolism in cooperation with SnRK1 in order to convey the sucrose status of the cytosol to the chloroplast. A transcript encoding PFK is increased showing active glycolysis during the sink establishment phase. Key genes involved in nucleotide biosynthesis and in the cell cycle are repressed indicating that cell division is not a cellular process required during the sink establishment phase. Transcript levels of GH3-auxin responsive genes increase two hours after excision and decrease at 6hpe when auxin exporters (PIN1) are up-regulated indicating active auxin transport at this time. In addition, a SAMS-encoding gene suggested to function as an intermediate in ethylene or polyamine biosynthesis is active. Peroxisomal beta oxidation seems to be active at least by induction of one associated enzyme, indicating its possible role in JA or IAA production (Fig. 33a).

Three days after excision, the cell replication machinery is up-regulated coinciding with the formation of new meristematic cells. Transcript levels of extracellular invertase and hexose transporters are decreased indicating a switch from apoplastic to symplastic sucrose unloading. An activation of genes necessary for mineral nutrient acquisition takes place and glutamine synthetase is up-regulated in order to stimulate ammonia assimilation. PEPC is induced to refill the TCA cycle or to support aspartate biosynthesis. Glycolysis and peroxisomal beta oxidation seem to be still active as indicated by the induction of PK and enoyl-CoA hydratase, respectively. The transcript level of a plastidic isoform of aldolase is up-regulated suggesting active glycolysis in the chloroplast which may support starch biosynthesis. The generated P5C in the sink establishment phase is now converted to glutamate through P5CDH activation in mitochondria. Auxin inactivation is triggered by increasing transcript abundances of GH3-auxin responsive or by induction of peroxidises (Fig. 33b).

The development of non-emerged root buds, eight days after excision, is accompanied by an activation of G_1 phase-specific genes of the cell cycle as indicated by the up-regulation of *CycD*. The plasma membrane H^+-ATPase may be up-regulated to support cell elongation or nutrient uptake. The induction of transcripts encoding genes involved in lipid metabolism, cell replication, mineral nutrient acquisition and GH3-dependent auxin responses is continued. In comparison to the recovery phase, a significant enhancement of transcript levels for annexin, alpha-L-fucosidase and several isoforms of peroxidases was monitored supporting the idea that the cell replication machinery and auxin catabolism are more active shortly before root emergence. In general, most of the observed expression patterns in the recovery phase were continued in the maintenance phase, suggesting 72 hpe as a central time point at which developmental processes switch or initiate (Fig. 33c).


(a) Sink Establishment Phase
Plasma membrane
H+-ATPase
N. TP
Ph. TP
Zi/Ir. TP
Sieve elements
Cytosol
SUC
Vacuole
SnRK1
Starch
Trehalose metabolism
Cyt. Inv.
Glc + Fru
Glycolysis PFK, PK
Plasmodesmata
SUC
PEPC
PEP
ethylene
polyamine
Chloroplast
eST
ST
SUC
Gln
Malate
AdoMet
CW. Inv.
NH3
GS
Pyruvate
SAMS
Glc + Fru
STP
Glu
Met
RNR
IAA
H..A.-CoA DH
ß- Oxidatin
E.-CoA hydratase
JA
TCA Cycle
Pro
ProDH
P5C
P5CDH
CycB1
CycA
CycD
Apoplast
Amino acids
Glu
Peroxisome
Mitochondrion
Nucleus


(b) Recovery Phase
Plasma membrane
H+-ATPase
N. TP
Ph. TP
Zi/Ir. TP
Sieve elements
Cytosol
SUC
Vacuole
SnRK1
Starch
Trehalose metabolism
Cyt. Inv.
Glc + Fru
Glycolysis PFK, PK
Plasmodesmata
SUC
PEPC
PEP
ethylene
polyamine
Chloroplast
eST
ST
SUC
Gln
Malate
AdoMet
CW. Inv.
NH3
GS
Pyruvate
SAMS
Glc + Fru
STP
Glu
Met
RNR
IAA
H..A.-CoA DH
ß- Oxidatin
E.-CoA hydratase
JA
TCA Cycle
Pro
ProDH
P5C
P5CDH
CycB1
CycA
CycD
Apoplast
Amino acids
Glu
Peroxisome
Mitochondrion
Nucleus

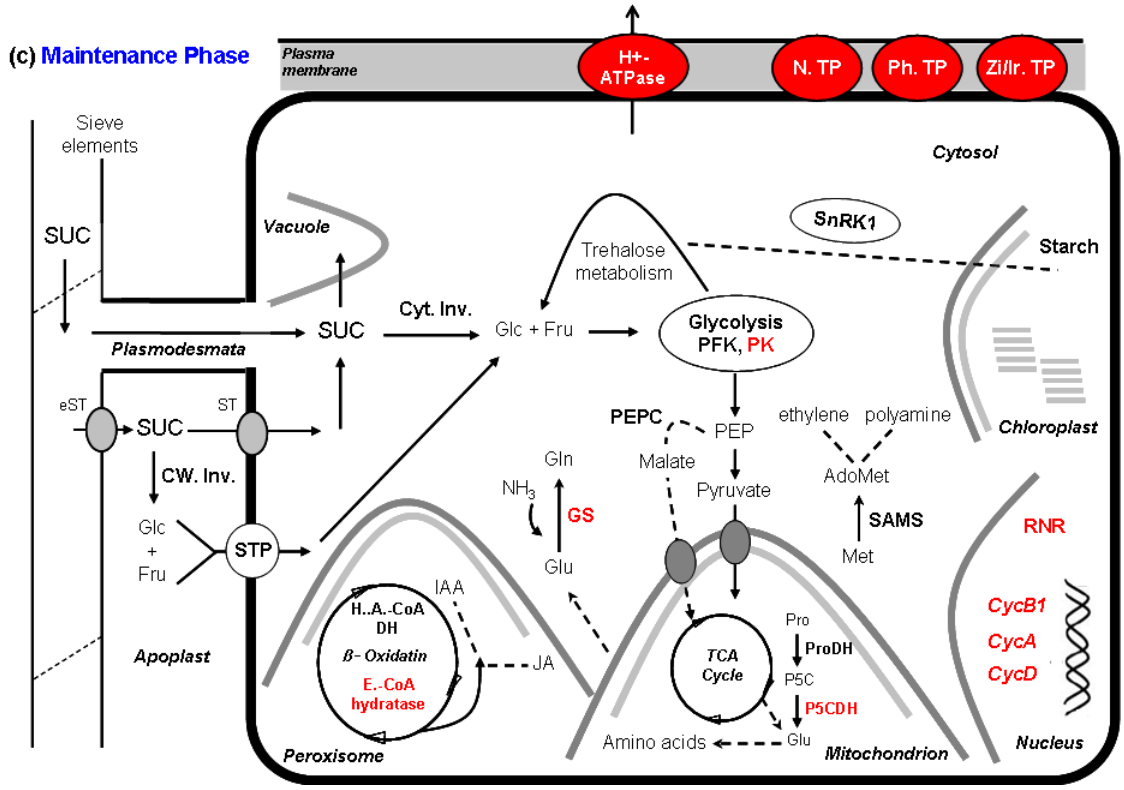


Figure 33. Schematic illustration of processes occurring during ARF in petunia cuttings based on the microarray analysis. In the figure, only induced key genes in primary metabolism, cell replication machinery and membrane transport are included. (a) sink establishment phase, (b) recovery phase, (c) maintenance phase. NTP: nitrate transporter, PhTP: phosphate transporter, Zi/IrTP: zinc/iron transporter, Cyt. Inv: cytosolic invertase; CW Inv: cell wall invertase; GS: glutamine synthetase; Glu: glutamate; Gln: glutamine; STP: sugar transport protein; RNR: ribonucleotide reductase; PEPC: phosphoenolpyruvate carboxylase; H.A.CoA DH: 3-hydroxyacyl-CoA dehyrogenase; E.CoA hydratase: enoyl-CoA hydratase; Pro: proline; ProDH: proline dehydrogenase; P5CDH: pyrroline-5-carboxylate dehydrogenase; Met: methionine; SAMS: S-adenosylmethionine synthetase; AdoMet: S-adenosylmethionine. Induced genes are shown in red color.

Several candidate genes have been identified in the current microarray analysis that require further investigations by generating transgenic lines through sense or anti-sense approaches. These include genes involved in trehalose metabolism (TPP and TPS), beta oxidation pathway, mineral nutrient acquisition, sucrose unloading process as well as SnRK1 or SAMS. In this way, the microarray approach would have served as a tool to uncover genes that play a critical role in ARF.

5. Summary

The excision of stem cuttings from the mother plant is a common practice in ornamental horticulture. The successful establishment of the cutting as an individual plant requires adventitious root formation. Regulatory processes underlying metabolic changes during ARF remained unclear. Therefore, the present thesis aimed at elucidating developmental and physiological processes during ARF by combining histochemical, biochemical and gene expression approaches.

After severance of the cuttings from the stock plant, it took 72 h until the first anatomical change, which was formation of the meristematic cells, appeared. The appearance of meristemoids marked the transition from the root initiation phase to the root primordium formation phase. Before adventitious roots emergence, the first roots with vascular bundles in the center surrounded by elongated cells appeared at 192 hpe marking the transition to the root elongation phase.

The activity of important enzymes and the level of key intermediates involved in primary metabolism during ARF were measured using photometric assay, HPLC and LC-MS. In addition, gene expression analysis using the most striking genes involved in carbohydrate metabolism, cell replication and auxin metabolism was carried out. Results revealed that soluble and insoluble sugars increased during ARF probably to serve as substrates for the production of cell components and energy in petunia cuttings. Moreover, enhanced expression levels of apoplastic invertase and hexose transporters early after excision indicated the establishment of a sink. Continuous increase of key enzymes involved in glycolysis and pentose phosphate pathways suggested an important role of these pathways for ARF. The metabolite citrate and expression levels of *CycB1* and *GH3* are suggested as markers for the different phases of ARF. Amino acids that showed predominant changes in the cutting base were recognized as glutamine, glutamate, asparagine and aspartate. Based on these

observations, the process of ARF in petunia cuttings was separated into three successive but interdependent phases which were nominated as: sink establishment phase, recovery phase and maintenance phase.

To unravel the possible effect of auxin on ARF and metabolic changes in the cuttings, auxin analoges (NAA and IBA) or an auxin transport inhibitor (NPA) were externally supplied. However, the profiles of enzyme activities and metabolites during ARF in petunia cuttings were not significantly altered by exogenous auxin except for extracellular invertase, asparagine and proline. This suggests that auxin may affect the root formation by acting directly in the cells which initiate the primordia and not through its engagement in the overall metabolism.

Microarray analyses of gene expression during ARF in petunia cuttings led to identification of 3,561 genes induced significantly and specifically during various phases of this developmental process. Based on the observed alterations in global gene expression patterns in petunia stem cuttings during different phases of ARF and following induced genes in different functional groups a working model was set up. The majority of genes involved in chromatin and DNA metabolism, cell cycle, nutrient transport and lipid degradation were up-regulated in the final stages of ARF, while key genes involved in primary carbohydrate metabolism such as those mediating apoplastic sucrose unloading were induced in early stages. The microarray data confirmed the physiological model obtained from biochemical and Northern blot analyses regarding sucrose unloading during ARF. Trancriptome changes also pointed to a possible role of trehalose metabolism and SnRK1 in sugar sensing during sink establishment phase. Symplastic sucrose unloading (or apoplastic sucrose unloading independent of cell wall invertase), cell division, nucleotide biosynthesis and auxin catabolism were the major processes induced during the recovery and maintenance phases. Moreover, peroxisomal beta oxidation was activated, maybe to stimulate functions other than lipid catabolism such as JA or IAA production during ARF. In

addition, the activation of G_1 phase-specific genes in the cell cycle was specifically induced in the maintenance phase. With regard to time-dependent developmental changes during ARF, 72 hpe was assigned as a central and key time point at which many physiological events were altered or started.

6. Zusammenfassung

Die Vermehrung der Stecklinge von Mutterpflanzen ist eine gängige Praxis im Gartenbau. Eine erfolgreiche Stecklingsvermehrung geht mit der Bildung von Adventivwurzeln (ARF) einher. Die regulatorischen Prozesse und damit zusammenhängende metabolische Änderungen sind derzeit wenig erforscht. Aus diesem Grund wurden in dieser Arbeit verschiedene Methoden inklusive histochemische, biochemische und Genexpressionsanalysen kombiniert, um die entwicklungsabhängigen und physiologischen Prozesse während der ARF aufzuklären.

Nach der Trennung der Petuniensteckling von der Mutterpflanze dauerte es 72 h bis erste sichtbare anatomische Änderungen wie die Bildung meristematischer Zellen beobachtet wurden. Das Erscheinen der Meristemoide deutete auf den Übergang von der Wurzel-Initierungsphase zu der Wurzel-Primordiumsphase hin. Bevor Adventivwurzeln gebildet wurden, wurden erste Ansätze mit bereits ausgebildetem vaskulären Gewebe nach 192 h im Meristem beobachtet. Dieses vaskuläre Gewebe war von Streckungszellen umgeben und war charakteristisch für den Übergang zu der Elongationsphase.

Die Aktivität wichtiger Enzyme und der Gehalt verschiedener Intermediate, die am Primärstoffwechsel beteiligt sind, wurden mittels photometrischer Methoden, HPLC und LCMS während der Adventivwurzelbildung bestimmt. Weiterhin wurde eine Genexpressionsanalyse für die wichtigsten Gene, die in Kohlenhydratstoffwechsel, Zellreplikation und Auxinmetabolismus vorkommen, durchgeführt. Die Ergebnisse zeigten, dass die löslichen und unlöslichen Zucker während der ARF akkumulierten, um vermutlich als Substrate für die Produktion der Zellkomponenten und Energie zu dienen. Darüber hinaus konnte eine Stimulierung der Genexpression für apoplastische Invertase und den Hexosetransporter STP4 in der frühen Phase nach dem Schneiden der Stecklinge beobachtet werden, die auf die Etablierung eines Sinks hindeutete. Die kontinuierliche Aktivitätszunahme der Schlüsselenzyme, die in der Glykolyse

und im Pentosephosphatstoffwechsel vorkommen, führte zu der Schlussfolgerung, dass diese Stoffwechselwege eine wichtige Funktion während der ARF ausüben. Ausserdem wurden Zitrat und *CycB1* und *GH3* als mögliche Marker für verschiedene Entwicklungsstadien der ARF festgelegt. Bei den gemessenen Aminosäuren wurden Glutamin, Glutamat, Asparagin und Aspartat als wichtige Aminosäuren bestimmt. Auf der Grundlage der erzielten Ergebnisse konnte der Prozess der ARF in Petuniensteckingen in drei aufeinanderfolgenden aber voneinander unabhängigen Phasen definiert werden, die als Sink-, Wiederherstellungs- und Aufrechterhaltungsphasen bezeichnet wurden.

Um die möglichen Auswirkungen der Auxine auf ARF und die metabolische Änderungen der Stecklinge aufzuklären, wurden Auxinanaloga (NAA und IBA) und/oder ein Auxintransport-Blocker (NPA) extern appliziert. Die Messung der Enzymaktivitäten und der Metabolitengehalte zeigte keine signifikanten Änderungen nach Auxinapplikation ausser für apoplastische Invertase, Asparagin und Prolin. Die erzielten Ergebnisse führten zur Schlussfolgerung, dass Auxin einen Einfluss auf die Bildung der Wurzeln durch die direkte Einwirkung in den Zellen ausübt und nicht massgebend an metabolischen Änderungen beteiligt ist.

Eine detaillierte Analyse der Genexpression durch Microarray führte zur Identifizierung von 3561 Genen, die spezifisch während der verschiedenen Entwicklungsstadien der Adventivwurzelbildung induziert wurden. Basierend auf der Veränderung der Genexpression in Petuniensteckingen und der Klassifizierung der Gengruppen wurde eine Arbeitshypothese aufgestellt. Die meisten identifizierten Gene waren am Chromatin- und DNA-Stoffwechsel, Zellzyklus, Nährstofftransport und Lipidabbau beteiligt und wurden im letzten Entwicklungsstadium der ARF hochreguliert. Die Schlüsselgene des Primärstoffwechsels und diejenigen, die an der Entladung der Saccharose beteiligt sind, wurden in der ersten Entwicklungsphase induziert. Die Expressionsergebnisse bestätigten die Hypothese für die Entladung der

Assimilate in dem physiologischen Modell, das basierend auf der biochemischen und Northernblot-Analysen, aufgestellt wurde. Weiterhin erbrachte die Transkriptomanalyse neue Erkenntnisse welche auf eine wichtige Funktion des Trehalose-Stoffwechsels und SnRK1 als mögliche Sensoren während der Sink-Phase hindeuteten. Außerdem konnte festgestellt werden, dass symplastische Saccharose-Entladung (oder die von der Zellwand-Invertase unabhängige apoplastische Saccharose-Entladung), Zellteilung, Nukleotidbiosynthese und Auxinabbau die wichtigsten Prozesse während der Wiederherstellungs- und der Aufrechterhaltungsphasen waren. Darüber hinaus wurde die Betaoxidation in den Peroxisomen aktiviert, um vermutlich weitere Stoffwechselwege außer dem Lipidabbau wie Jasmonat- und/oder Indolessigsäure-Biosynthese zu stimulieren. Gleichzeitig wurde eine Aktivierung der Gene, die in der G_1-Phase im Zellzyklus vorkommen, in der Aufrechterhaltungsphase beobachtet. Aufgrund der erzielten Ergebnisse wurde die 72. Stunde nach der Entfernung der Stecklinge von der Mutterpflanze als der zentrale Zeitpunkt definiert, bei dem viele wichtige physiologische Ereignisse stattfinden und/oder beginnen.

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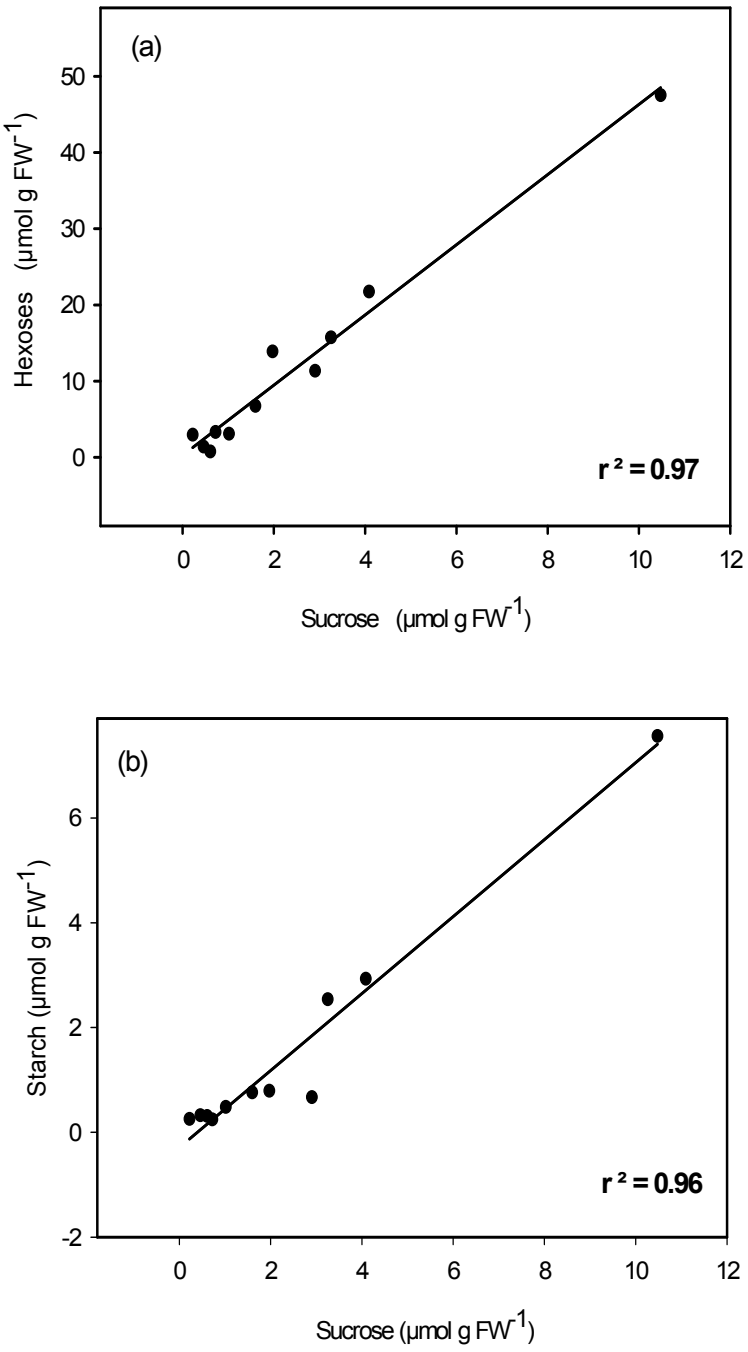
8. Appendix



Appendix Figure 1. Correlation analysis of soluble and insoluble sugars during ARF in *P. hybrida* cuttings. (a) between hexoses (Glc + Fru) and sucrose (b) between sucrose and starch.

Appendix Table 1. Amino acid concentrations during adventitious root formation in *Petunia hybrida* cuttings. Each value is represented by the mean of five independent replicates in nmol g^{-1} FW.

Hours Post Excision (hpe)	Asp	Glu	Ser	Asn	Gly	Gln	His	Thr	Arg	Ala	GABA	ACC	Pro	Tyr	Val	Met	Leu	Phe
0	281.1	822	225	1929	45.6	3521	22.4	149.3	18.4	327.1	8.5	19.6	47.3	16.2	72.1	3.0	31.8	18.3
2	255.3	373	170	1759	47.9	3292	11.5	97.3	1.4	278.4	7.2	13.9	40.4	10.7	60.7	5.5	27.7	12.0
4	298.7	433	159	1914	50.0	3264	9.6	89.0	1.6	255.6	7.7	11.9	46.2	10.0	53.3	5.7	20.9	12.7
6	0.90	2.37	1.75	10.39	23.31	15.77	0.12	0.90	0.41	2.01	0.15	0.23	26.92	0.18	0.27	0.21	0.33	0.20
12	1.51	2.07	1.49	15.34	27.56	18.75	0.15	0.92	0.63	1.57	0.15	0.40	32.64	0.17	0.28	0.26	0.32	0.22
24	1.23	4.75	1.36	27.28	28.60	15.18	0.17	0.97	1.20	1.74	0.31	1.16	33.62	0.54	0.21	0.29	0.33	0.25
48	1.18	4.52	1.23	6.77	26.32	6.68	0.27	0.28	6.86	1.45	0.31	0.84	36.72	0.98	0.39	0.18	0.30	0.24
72	375	1004	99	781	46.6	829	8.7	77.5	65.1	85.6	7.2	15.9	49.2	13.7	39.3	3.0	26.6	12.3
96	427	1017	116	196	26.0	351.3	12.6	75.4	77.2	84.8	7.7	9.4	57.5	12.6	49.1	2.9	36.8	10.7
144	393	1023	163	160	30.3	509.1	17.3	85.6	143.8	115.5	8.9	12.4	125.8	13.1	52.4	2.3	25.8	13.3
192	563	1533	311	151.6	35.8	648	24.5	113.6	336.7	186.0	13.8	28.9	447	18.4	64.9	5.3	39.7	20.6

Appendix Table 2. Amino acid concentrations during adventitious root formation in *Petunia hybrida* cuttings in presence of auxin analogs (NAA + IBA). Each value is represented by the mean of five independent replicates in nmol g^{-1} FW.

Hours Post Excision (hpe)	Asp	Glu	Ser	Asn	Gly	Gln	His	Thr	Arg	Ala	GABA	ACC	Pro	Tyr	Val	Met	Leu	Phe
0	557.0	1443.1	306.4	620.1	39.6	1418.7	7.6	142.1	21.6	596.1	186.5	3.4	127.0	7.1	69.5	3.4	29.5	16.2
2	558.0	1153.4	123.9	1363.8	27.9	1139.4	1.5	47.9	3.6	162.2	31.6	1.8	129.8	7.0	2.5	1.3	22.5	14.8
4	425.1	696.1	91.6	2079.6	27.6	1170.6	0.7	48.5	14.8	113.8	20.9	2.5	129.8	3.4	43.0	0.3	18.5	14.2
6	485.8	1160.0	76.0	1089.0	26.3	1012.6	0.6	48.8	18.0	172.3	58.1	37.0	118.3	4.5	31.0	0.3	14.5	16.4
12	1028.3	1294.1	74.4	1090.1	14.9	137.6	0.2	18.3	24.6	75.1	27.6	81.5	169.1	5.9	16.6	0.3	11.4	15.3
24	247.1	458.4	35.7	952.6	31.3	322.8	1.8	37.6	21.1	79.4	49.5	32.8	99.3	5.6	25.6	0.3	10.7	17.7
48	147.0	424.2	28.9	417.3	26.7	461.1	1.3	25.4	22.4	48.7	25.2	206.1	81.1	8.4	19.2	0.2	6.8	11.9
72	367.1	716.4	110.3	430.0	25.9	381.7	3.8	64.6	35.2	156.7	115.5	290.7	217.3	9.2	74.0	1.3	31.3	28.9
96	1095.7	3007.9	347.0	584.0	49.1	1393.9	17.0	111.9	59.6	327.3	183.6	145.4	7304.7	12.9	140.4	3.8	69.5	21.8
144	1089.1	2734.2	294.1	206.9	43.6	1300.5	4.1	139.7	407.8	305.2	98.5	4.2	3687.9	21.1	132.6	4.2	74.9	34.0
192	895.5	2730.0	569.0	220.1	66.6	1313.3	5.2	151.0	730.4	462.8	132.6	4.9	9553.3	23.4	152.0	4.4	61.2	31.1

Appendix Table 3. Amino acid concentrations during adventitious root formation in *Petunia hybrida* cuttings in presence of auxin transport inhibitor (NPA). Each value is represented by the mean of five independent replicates in nmol g^{-1} FW.

Hours Post Excision (hpe)	Asp	Glu	Ser	Asn	Gly	Gln	His	Thr	Arg	Ala	GABA	ACC	Pro	Tyr	Val	Met	Leu	Phe
0	771.3	1670.9	229.0	768.9	52.8	2447.8	7.3	149.0	21.5	349.3	104.4	21.3	96.8	9.6	55.3	5.4	28.8	15.6
2	272.6	671.6	60.8	472.6	31.4	1978.4	0.6	43.6	18.0	235.5	44.1	17.2	79.5	5.4	27.3	2.7	14.1	9.2
4	329.6	756.5	52.0	509.6	32.1	2373.5	3.7	61.4	22.2	120.9	27.0	15.9	66.9	7.3	37.0	2.3	20.2	11.9
6	356.1	1144.6	135.6	590.2	35.4	1584.4	3.5	64.7	21.6	214.6	53.0	8.6	76.6	8.8	40.9	2.1	22.4	14.0
12	343.3	878.1	116.0	794.1	38.8	1113.4	1.5	59.0	16.8	176.3	30.6	22.5	59.1	7.9	26.9	1.6	14.0	11.7
24	348.6	1159.4	69.6	651.0	29.0	688.3	1.0	35.6	33.5	117.3	56.8	18.7	67.0	10.6	24.2	1.7	18.5	14.0
48	503.7	1503.7	160.4	281.4	31.9	251.5	3.7	52.1	49.9	152.2	140.0	25.3	138.2	13.8	50.0	2.6	39.0	15.6
72	584.7	1742.8	360.5	1258.3	51.6	1309.1	27.5	166.8	64.6	195.9	136.5	42.7	2601.3	21.0	107.3	6.1	60.3	25.8
96	576.9	1631.6	468.1	1689.2	59.9	712.5	38.7	180.3	78.4	170.4	136.6	40.6	5663.3	21.7	148.3	7.7	57.0	27.6
144	506.2	1606.3	334.5	118.4	45.9	599.1	16.8	116.6	141.1	166.5	130.2	33.1	6913.7	15.4	82.9	3.5	34.9	21.6
192	540.1	1701.7	329.1	84.9	40.5	574.8	10.6	77.3	462.9	141.0	208.1	37.9	10273.3	13.9	61.4	2.3	27.9	20.9

Appendix Table 4. Selected genes specifically induced during adventitious root formation in *P. hybrida* cuttings. To extract up or down-regulated genes, median ratios with values above two (>2) were determined as up-regulated and median ratios with values below 0.5 (<0.5) were determined as down-regulated. See Excel File 3 (Data-CD) for a complete list of all 3,561 ARF specific genes.

Seq. Id.	Putative Function	Developmental Stages			
		2h/0h	6h/0h	72h/0h	192h/0h
Primary Metabolism					
cn5583	cell-wall invertase	3.73	7.12	1.27	1.17
cn8044	cell wall invertase	205.29	493.48	81.76	86.78
GO_drpoolB-CL9125Contig1	trehalose synthase	3.23	1.66	0.98	0.99
GO_drpoolB-CL6907Contig1	trehalose-6-phosphate phosphatase	3.15	1.25	0.41	0.59
cn3641	trehalose-phosphate phosphatase	3.33	3.85	0.76	0.80
GO_drpoolB-CL3077Contig1	trehalose-phosphate phosphatase	2.32	2.64	0.61	0.73
GO_drpoolB-CL1679Contig1	trehalase	3.88	36.62	35.06	23.33
cn1946	plastidic aldolase NPALDP1	0.89	1.02	2.18	1.25
cn9155	carbonic anhydrase	1.78	2.81	1.04	0.75
GO_drpoolB-CL8994Contig1	hexokinase	1.35	2.61	1.30	1.50
GO_drpoolB-CL7928Contig1	hexokinase	0.51	0.26	2.29	3.51
GO_drpoolB-CL1857Contig2	neutral invertae (cytosolic)	13.61	14.27	4.37	5.42
cn7412	neutral invertase (cytosolic)	4.58	2.63	0.56	0.48
cn3388	Phosphoenolpyruvate carboxylase	0.76	1.55	2.42	1.71
cn3389	phosphoenolpyruvate carboxylase	0.60	1.25	2.04	1.60
GO_dr001P0013L06_F_ab1	phosphofructokinase	1.26	2.43	1.84	1.62
GO_dr004P0014F13_F_ab1	phosphofructokinase	0.80	0.95	2.19	2.18
GO_drpoolB-CL2084Contig1	phosphofructokinase	0.82	2.25	1.49	1.71
cn8946	ribonucleotide reductase	0.42	0.38	2.32	1.93
GO_drpoolB-CL5407Contig1	ribonucleotide reductase	0.67	0.41	2.37	2.13
cn1458	cytosolic pyruvate kinase	1.19	1.74	2.22	2.32

Appendix Table 4. (Continued from previous page.)

Seq. Id.	Putative Function	2h/0h	6h/0h	72h/0h	192h/0h
cn1452	glyceraldehyde 3-phosphate dehydrogenase	1.13	1.46	2.09	2.08
DY395678_1	phosphoglycerate mutase	2.44	1.28	4.88	6.33
GO_drpoolB-CL2702Contig1	acetyl-CoA synthetase	0.89	2.91	0.98	0.92
GO_drs13P0026B05_R_ab1	acetyl-CoA synthetase	1.00	2.02	1.64	1.10
GO_drpoolB-CL9147Contig1	ATP citrate lyase	1.00	3.57	1.03	1.11
DC240356_1	ATP:citrate lyase	1.15	2.93	1.09	1.13
cn8627	3-hydroxyacyl-CoA dehyrogenase	1.92	2.81	1.18	1.08
GO_drpoolB-CL2994Contig1	3-hydroxyacyl-CoA dehyrogenase	2.07	0.93	1.65	1.57
GO_drpoolB-CL5661Contig1	enoyl-CoA hydratase	1.08	1.00	3.08	2.95
cn1013	acyl-CoA synthetase	2.50	4.60	2.29	2.46
cn1014	acyl-CoA synthetase	7.66	5.02	2.98	2.40
GO_drpoolB-CL7334Contig1	acyl-CoA synthetase	1.39	0.73	9.48	51.28
GO_drpoolB-CL9321Contig1	acyl-CoA synthetase	1.65	0.88	10.01	36.80
GO_drpoolB-CL6305Contig1	GDSL-motif lipase	1.19	1.02	5.27	23.12
GO_drpoolB-CL9708Contig1	GDSL-motif lipase	1.06	0.45	7.03	10.59
GO_drpoolB-CL5325Contig1	GDSL-motif lipase 3	0.84	0.93	40.43	52.39
GO_dr001P0003K17_F_ab1	GDSL-motif lipase carboxylesterase	1.23	0.53	25.15	34.01
cn7243	GDSL-motif lipase	0.64	1.16	42.41	200.67
cn7244	GDSL-motif lipase	1.56	0.87	324.04	1011.41
GO_drpoolB-CL2580Contig1	fatty acid dehydrogenase	0.87	0.86	9.71	28.36
cn10511	cytochrome P450-dependent FA hydroxylase	183.81	122.26	28.98	28.52
cn6162	cytochrome P450-dependent FA hydroxylase	201.56	84.39	22.83	17.41
cn6510	omega-3 fatty acid desaturase	1.12	0.42	2.43	2.67
cn6525	omega-6 fatty acid desaturase (chloroplastic)	2.04	0.55	0.42	0.40
GO_drs12P0005E10_F_ab1	omega-6 fatty acid desaturase	2.70	0.81	121.96	382.27
cn4804	Aspartate aminotransferase	0.80	1.51	2.39	2.51
GO_dr004P0012B09_F_ab1	aspartate aminotransferase	1.37	2.94	4.10	3.80
cn1185	Uridine 5'-monophosphate synthase	1.01	1.54	2.34	2.20

Appendix Table 4. (Continued from previous page.)

Seq. Id.	Putative Function	2h/0h	6h/0h	72h/0h	192h/0h
cn1768	glutamine synthetase	0.67	1.36	2.01	1.79
cn2200	glutamine synthetase	0.86	1.38	2.08	2.23
GO_drpoolB-CL6449Contig1	proline oxidase/dehydrogenase	18.51	29.90	4.15	6.87
DY395394_1	proline oxidase/dehydrogenase 1	3.45	5.12	0.19	0.31
cn2413	proline oxidase/dehydrogenase 2	4.18	13.25	1.13	2.18
cn4338	proline oxidase/dehydrogenase 2	2.71	4.65	0.16	0.21
cn6459	proline oxidase/dehydrogenase 2	2.97	3.23	0.29	0.37
cn6731	proline oxidase/dehydrogenase 2	13.49	17.74	3.35	5.61
GO_drpoolB-CL1012Contig1	1-pyrroline-5-carboxylate dehydrogenase	0.74	1.03	2.28	2.08
cn8851	S-adenosylmethionine synthetase 2	1.41	2.77	1.10	1.15
Signalling					
GO_dr001P0008K02_F_ab1	SNF1-related kinase	3.12	3.25	0.81	0.77
cn9516	SnRK1-interacting protein	1.73	3.49	2.07	1.50
GO_drpoolB-CL7621Contig1	Alpha-L-fucosidase 2	8.17	9.28	80.23	2029.56
Membrane transport					
SG_SGN-U208608	sugar transporter (STP family)	1.2	15	1.5	1.1
GO_drpoolB-CL1483Contig1	proline transporter	95.86	179.47	69.78	115.68
cn1203	plasma membrane proton ATPase 5	1.30	1.18	1.68	2.75
Chromatin and DNA					
cn5095	histone h2a	1.01	0.52	5.22	9.19
cn63	histone h2a	0.66	0.65	1.87	2.17
cn9045	histone H2A	0.69	0.43	2.87	2.98
cn4627	histone H2B	1.25	0.87	2.92	3.44
cn4604	histone H3	0.62	0.33	2.62	3.57
GO_dr004P0029F02_F_ab1	histone H3	1.17	0.93	5.73	5.42
cn5315	histone H3	0.76	0.57	2.33	2.77
cn1359	Histone H4	0.98	0.77	2.10	2.90

Appendix Table 4. (Continued from previous page.)

Seq. Id.	Putative Function	2h/0h	6h/0h	72h/0h	192h/0h
cn5413	histone H4	1.02	0.58	2.01	2.35
cn5414	histone H4	0.82	0.30	3.90	5.70
Cell cycle					
GO_drpoolB-CL3774Contig1	B2-type cyclin dependent kinase	0.68	0.46	4.02	2.88
GO_drpoolB-CL8300Contig1	B2-type cyclin dependent kinase	0.81	0.39	3.02	2.89
GO_dr004P0003J17_F_ab1	CYCB1	0.56	0.35	5.39	4.34
cn3361	CYCB1-1 protein	0.65	0.28	2.94	2.78
GO_drpoolB-CL6689Contig1	cyclin B2 cyclin-dependent protein kinase	0.67	0.40	5.94	4.75
cn7126	Cyclin d	1.86	1.13	1.49	2.03
GO_dr001P0002N15_F_ab1	CYCLIN D	1.09	0.44	1.88	2.70
GO_dr004P0019F04_F_ab1	cyclin d2	1.37	1.32	1.64	2.31
GO_drpoolB-CL7924Contig1	cyclin-dependent kinase B1	0.60	0.32	3.11	2.68
Cytoskeleton					
cn8351	annexin	1.32	2.66	63.00	202.09
Mineral nutrient aquisition					
GO_drpoolB-CL4971Contig1	ammonium transporter	1.01	2.75	1.43	2.60
GO_drpoolB-CL5358Contig1	ammonium transporter	0.84	3.08	1.32	2.54
GO_drpoolB-CL8140Contig1	ammonium transporter	6.31	5.87	4.85	5.79
GO_drs12P0011A24_F_ab1	Ammonium transporter	6.33	6.91	6.98	6.50
cn3402	ammonium transporter AMT2	12.98	12.82	5.42	5.46
SG_SGN-U208324	Boron transporter	2.68	3.72	14.22	17.04
cn7095	nitrate transporter	0.90	0.53	2.75	1.72
cn7729	nitrate transporter (NRT1.1)	2.40	1.23	4.92	7.07
cn8317	nitrate transporter (NRT1.1)	3.71	11.98	140.99	184.74
cn8348	nitrate transporter	1.40	62.52	242.47	331.07
cn8506	nitrate transporter (NRT1.1)	1.33	2.25	62.27	52.73
GO_drpoolB-CL2981Contig1	nitrate transmembrane transporter	1.71	112.75	314.68	293.54

Appendix Table 4. (Continued from previous page.)

Seq. Id.	Putative Function	2h/0h	6h/0h	72h/0h	192h/0h
cn8619	high affinity nitrate transporter	2.23	76.42	227.14	326.89
GO_drpoolB-CL2736Contig1	high affinity nitrate transporter	1.29	86.25	4.51	3.62
GO_drs31P0006M14_R_ab1	high affinity nitrate transporter	3.46	156.65	11.09	10.76
cn8665	high affinity nitrate transporter	1.28	0.94	4.56	4.48
GO_drpoolB-CL5640Contig1	iron transporter protein	1.42	1.17	115.79	577.92
GO_drpoolB-CL5141Contig1	iron transporter protein IRT1	0.91	1.49	23.19	117.87
cn8235	phosphate transporter	1.04	1.43	36.55	14.64
cn8239	phosphate transporter	1.04	1.30	143.47	158.12
GO_dr001P0002B23_F_ab1	phosphate transporter	1.88	1.03	315.38	332.15
GO_drpoolB-CL9490Contig1	phosphate transporter	0.55	0.98	33.46	34.69
GO_drpoolB-CL5016Contig1	zinc transporter	0.92	1.31	26.39	69.48
cn5371	zinc/iron transporter	0.79	0.79	181.09	187.02
Antioxidative metabolism					
cn8269	peroxidase	1.99	3.95	126.97	132.98
cn8388	peroxidase	0.69	0.50	3.79	13.61
cn8454	peroxidase	0.52	0.47	6.58	20.99
cn8498	peroxidase	1.78	5.25	54.86	197.70
cn8688	peroxidase	1.99	1.58	13.18	8.10
cn8689	peroxidase	1.07	0.85	6.84	7.43
cn872	peroxidase	1.84	31.16	27.42	10.87
cn9069	peroxidase	3.60	14.25	86.81	197.61
GO_drpoolB-CL6825Contig1	peroxidase	1.06	0.96	10.56	109.55
GO_drpoolB-CL7003Contig1	peroxidase	0.87	1.81	227.39	387.50
cn8269	peroxidase	1.99	3.95	126.97	132.98
Hormone metabolism					
GO_drpoolB-CL1376Contig1	GH3 Indole-3-acetic acid-amido synthetase	8.21	3.54	7.88	8.93
GO_drpoolB-CL7856Contig1	GH3 Indole-3-acetic acid-amido synthetase	8.75	3.96	9.23	9.80

Appendix Table 4. (Continued from previous page.)

Seq. Id.	Putative Function	2h/0h	6h/0h	72h/0h	192h/0h
GO_drpoolB-CL985Contig1	GH3 Indole-3-acetic acid-amido synthetase	10.35	3.92	10.23	10.89
cn5291	GH3 Indole-3-acetic acid-amido synthetase	5.55	119.86	20.09	15.31
GO_drpoolB-CL42Contig2	GH3.3; indole-3-acetic acid amido synthetase	7.87	99.43	21.08	19.52
GO_dr004P0031O04_F_ab1	auxin efflux carrier	0.85	1.57	2.28	1.97
GO_drs31P0009M18_F_ab1	auxin influx carrier	0.83	0.23	2.04	3.48
GO_drpoolB-CL7262Contig1	auxin influx transport protein	0.59	2.11	0.73	0.61
GO_drpoolB-CL4639Contig1	PIN1-like auxin transport protein	0.40	2.71	0.77	0.57
GO_drpoolB-CL7812Contig1	Auxin-induced protein 5NG4	7.14	12.27	7.19	55.68
cn7956	auxin-responsive protein-related SAUR	36.84	41.54	4.67	4.84
cn6747	SAUR auxin-responsive protein	1.22	0.51	3.15	16.98
IP_PHBS010D15u	SAUR family protein	2.81	5.15	3.77	6.33
IP_PHBS002A17u	auxin-induced SAUR-like protein	2.26	1.04	0.46	0.35
cn7580	Auxin responsive SAUR protein	3.08	21.58	35.33	19.51
Miscellaneous					
cn4640	lipid binding protein	1.68	0.22	3.26	6.36
GO_drpoolB-CL1246Contig1	lipid binding protein	3.07	4.02	3.42	3.10
cn7168	lipid binding protein,	1.34	0.97	62.41	357.01
cn2952	lipid body membrane protein	2.55	0.84	3.09	5.22
cn4557	lipid transfer protein	2.22	1.25	21.82	532.32
cn4515	lipid transfer protein	0.25	0.28	4.54	3.84
GO_drpoolB-CL1714Contig1	Lipid transfer protein	0.71	0.85	15.28	61.70
GO_drpoolB-CL4902Contig1	lipid transfer protein LTP family protein	1.68	0.22	3.26	6.36
GO_dr001P0002O21_F_ab1	lipid-transfer protein	1.40	0.52	7.16	215.81
GO_drpoolB-CL3775Contig1	lipid-transfer protein	1.51	1.38	2.86	2.69

9. Publications and Proceedings related to the submitted thesis

Franken P., **Ahkami A.H**., Olivi M., Lischewski S., Zerche S., Steuernagel B., Scholz U., Lohse M., Hänsch K.T., Hause B., Bucher M., Drüge U. and Hajirezaei M.R., 2010, Coordinated regulation of adventitious root development by phytohormones and mineral nutrients (In preparation).

Ahkami, A.H., Lischewski S., Haensch K.T., Porfirova S., Hofmann J., Rolletschek H., Melzer M., Franken P., Hause B., Druege U., and Hajirezaei, M.R., 2009. Molecular physiology of adventitious root formation in Petunia hybrida cuttings: involvement of wound response and primary metabolism. New Phytologist, 181: 613-625.

Druege U., Haensch K.T., Klopotek Y., Franken P., Lischewski S., Hause B., **Ahkami, A.H.**, Hajirezaei, M.R., 2008. The root formation on the track, an integrated research beginning for the support of the young plant production (Der Wurzelbildung auf der Spur, Ein integrierter Forschungsansatz zur Förderung der Jungpflanzenproduktion). ForschungsReport, 1: 21-25.

Ahkami, A.H., Melzer M., Franken P., Hause B., Druege U., and Hajirezaei, M.R., 2010. Influence of auxin on primary metabolism at various developmental stages of root formation in Petunia. The 20th IPGSA (The International Plant Growth Substances Association) June 28 – July 2, 2010, Tarragona, Spain.

Franken, P., Drüge, Scholz U., **Ahkami A.H.**, Hajirezaei, M.R. 2010. Petunia as a model for molecular analysis of adventitious root development. Abstract Symposium Regulation of Plant Growth, Potsdam, Germany, P. 40.

Ahkami, A.H.; Melzer, M.; Haensch, K.T.; Franken, P.; Hause, B.; Druege, U.; Hajirezaei 2008. Possible involvement of carbohydrate metabolism in adventitious root formation in Petunia hybrida cuttings. Abstracts Book, 5th International Symposium on Adventitious Root Formation, Alcala-Madrid, Spain, p 81.

Ahkami, A.H.; Druege, U.; Bucher, M.; Franken, P.; Gianinazzi, S.; Hause, B.; Porfirova, S.; Hajirezaei, M.-R. 2007. Elucidation of molecular and biochemical processes involved in adventitious root development in petunia. 9th World Petunia days, Amsterdam, The Netherlands, 28.-31. October 2007, Book of Abstracts, 29.

Porfirova, S.; Haensch, K.-T.; Hajirezaei, M.R.; **Ahkami, A.**; Druege, U.; Bucher, M.; Gianinazzi, S.; Hause, B.; Franken, P. 2007. Genetic and molecular approaches towards understanding adventitious root development. 9th World Petunia days, Amsterdam, The Netherlands, 28.-31. October 2007, Book of Abstracts, 31.

Ahkami, A.H.; Druege, U.; Bucher, M.; Franken, P.; Gianinazzi, S.; Hause, B.; Haensch, K.-T.; Hajirezaei, M.R. 2007. The role of invertases in phloem unloading of sucrose in petunia during adventitious root development. International Conference on Plant Vascular Biology, Taipei, Taiwan, May 7-11 2007, Book of Abstracts, 1p.

Herr Amirhossein Ahkami
Correnssstraße 3
06466 Gatersleben

10. Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Naturwissenschaftliche Fakultät I - Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.
Ich erkläre ferner, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe.

Gatersleben, den .....

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Amirhossein Ahkami

11. Curriculum Vitae

Family name: Ahkami
Given name: Amirhossein
Address: Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Physiology and Cell Biology Dept. Molecular Plant Nutrition Group, Correnssstr. 3, 06466, Gatersleben, Germany.
Phone number: +49-39482-5514
E-mail: ahkami@ipk-gatersleben.de
E-mail: a_ahkami@yahoo.com
Nationality: Iranian
Date of Birth: 21/01/1979
Place of Birth: Shiraz / Iran
Gender: male
Marital status: married

Academic Education:

- **Ph.D.**; Plant Molecular Physiology, Martin Luther University, Halle, Germany (Sep. 2006 - August 2010), Thesis prepared at: Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany
- **M.Sc.**; Plant Biotechnology, Agricultural College, Tehran University, Karaj, Iran (Sep.2003 - Sep.2005.), Thesis prepared at: Agricultural Biotechnology Research Institute of Iran, Karaj (ABRII).
- **B.Sc.**; Agronomy and Plant Breeding, Agricultural College, Shiraz University, Shiraz, Iran (Sep.1998 - June 2002).
- **Diploma**; Life Sciences, Shiraz, Iran (1993-1997).

Researches:

- Molecular and physiological analysis of adventitious root formation (ARF) in petunia which built on biochemical, transcriptome and transgenic analyses (Ph.D. thesis).
- Gene mapping of resistance to Shiraz strain of wheat streak mosaic virus in two bread wheat cultivars, using SSR markers (Co-researcher).
- Study of genetic diversity in durum wheat (*Triticum durum*) germplasm using AFLP markers (M.Sc thesis).

Job Experiences:

- Working on Ph.D. thesis as an employee at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (Sep.2006-March 2010).
- Working on research projects as junior scientist in Plant Virology Research Center (PVRC) of Shiraz University, Shiraz, Iran (Dec.2005- June 2006).
- Teaching English for Specific Purpose for the students majoring in plant biotechnology at Tehran University, Karaj, Iran (Oct.2004-Dec.2004).

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Amirhossein Ahkami