Understanding of Molecular Mechanisms and Improvement of Adventitious Root Formation in Apple

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Doctoral Thesis
Swedish University of Agricultural Sciences
Alnarp 2009

Acta Universitatis agriculturae Sueciae
2010:8

Cover: From left to right: rooting test with *Arabidopsis* hypocotyls, rooting test with apple cuttings, apples, cross section of an *Arabidopsis* stem during adventitious root formation.

ISSN 1652-6880
ISBN 978-91-576-7485-2

Print: SLU Service/Repro, Alnarp 2010

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Abstract

Adventitious root formation is essential for vegetative propagation, which is the main method for propagation of many horticultural crops, especially woody species. For propagation of apple rootstocks, adventitious root formation is often a limiting step. The molecular mechanisms underlying adventitious root formation are still largely unknown and need to be extensively investigated. In this thesis approaches towards an increased understating of adventitious root formation were made using both apple and the model plant *Arabidopsis*.

For *Arabidopsis*, a protocol for efficient rooting of hypocotyls and stem segments using two pulses of auxin induction was developed. This protocol was used for studying the expression of a number of rooting related genes during adventitious root formation in both stems and hypocotyls, and for anatomical studies of the timing of the rooting process. The transcript levels of *GH3-3*, *LBD16*, *LBD29*, *LRP1* and *ARF17* were clearly affected by the auxin treatment, and some of these genes displayed considerable differences between stem segments and hypocotyls.

In apple, the function of the rooting related gene *ARRO-1* was investigated through down-regulation of the gene using RNAi. The results indicate a possible role for *ARRO-1* in promoting adventitious root initiation or regulating hormone homeostasis.

The effect of apple rootstocks transformed with the *Agrobacterium rolB* gene on non-transgenic scions was also investigated, regarding tree growth, flowering and fruit quality. The results showed that the transgenic rootstock had no clear effect on fruit quality but greatly affected vegetative growth. No translocation of transgene mRNA or DNA from rootstock to scion could be detected. Attempts were also made to produce ROLB specific antibodies to investigate if the ROLB protein is transported from the transgenic rootstock into the non-transgenic scion which could be of concern for the consumer.

Keywords: Apple, adventitious root formation, *Arabidopsis thaliana*, *ARRO-1*, *rolB*, rooting related genes

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Zwar weiß ich viel, doch möcht' ich alles wissen.

Johann Wolfgang von Goethe, Faust I, Verse 601 / Wagner

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Margareta Welander, Anders Smolka, Thomas Geier, Annelie Ahlman, Anna Holefors and Li-Hua Zhu (2009). Origin, Timing and Gene Expression of Adventitious Rooting in *Arabidopsis* Hypocotyls and Stems (manuscript).

II Anders Smolka, Margareta Welander, Peter Olsson, Anna Holefors and Li-Hua Zhu (2009). Involvement of the *ARRO-1* Gene in Adventitious Root Formation in Apple. *Plant Science* 177(6), 710-715.

III Anders Smolka, Margareta Welander, Peter Olsson and Li-Hua Zhu (2009). Production of Recombinant Protein and Antibodies for ROLB Protein Analysis (manuscript).

IV Anders Smolka, Xue-Yuan Li, Catrin Heikelt, Margareta Welander and Li-Hua Zhu (2009). Effects of Ttransgenic Rootstocks on Growth and Development of Non-Transgenic Scion Cultivars in Apple. Accepted for publication in *Transgenic Research*.

Papers II and IV are reproduced with the permission of the publishers.

The contribution of Anders Smolka to the papers included in this thesis was as follows:

I Planned and performed the molecular part of the experiments and participated in evaluation of the data and writing of the manuscript.

II Planned and performed all the experimental work, evaluated the data and wrote the paper together with co-authors.

III Planned and performed all the experimental work, evaluated the data and wrote the manuscript.

IV Took part in the field trial and experimental work, evaluated the data and took part in writing the manuscript.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ANOVA	analysis of variance
ARF17	AUXIN RESPONSE FACTOR17
ARRO-1	adventitious rooting related oxygenase
AuxRE	auxin response elements
BAP	6-benzylaminopurine
CDC27	CELL DIVISION CYCLE PROTEIN27
cDNA	complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DCL	dicer-like
DHA	dehydroascorbic acid
DNA	deoxyriboneucleic acid
DNase	deoxyribonuclease
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GA	gibberelic acid
GM	genetically modified
GMP	genetically modified plant
Gu	guanidinium
HBT	HOBBIT
HPLC	high-performance liquid chromatography
IAA	indole acetic acid
IBA	Indole-3-buturic acid
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
LBD16/29	LATERAL ORGAN BOUNDARIES-DOMAIN16/29
LRP1	LATERAL ROOT PRIMORDIUM1

miRNA	micro RNA
mRNA	messenger RNA
NAA	1-naphthaleneacetic acid
NAC1	NAM/ATAF/CUC1
nc	noncoding
nptII	neomycin phosphosphotransferase
PCR	polymerase chain reaction
PMSF	phenylmethanesulphonylfluoride
QC	quiescent centre
qRT-PCR	quantitative RT-PCR
Ri	root inducing
RISC	RNA-induced silencing complex
RNA	riboneucleic acid
RNAi	RNA interference
ROLB	rooting locus B
RT-PCR	reverse transcription PCR
SCL	SCARECROW-LIKE
SCR	SCARECROW
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR	SHORT-ROOT
TA	titratable acidity
T-DNA	transfer DNA
TDZ	Thidiazuron (1-phenyl-3-(thiadiazol-5-yl)urea)
Tris	tris(hydroxymethyl)aminomethane
TSS	Total soluble solids
UBC21	ubiquitin-conjugating enzyme 21
UTR	untranslated region

Introduction

1.1 Apple – an ancient and modern crop

The domesticated apple, *Malus domestica*, belongs to the *Malus* genus of the Rosaceae family. The genus includes 30-35 species which are native to the temperate zone in Europe, Asia and North America. *M. sieversii*, which is found growing wild in the mountains of Central Asia in southern Kazakhstan, Kyrgyzstan, Tajikistan and China, is believed to be the major maternal contributor to *M. domestica* (Harris et al., 2002). Other species which may also have contributed to the lineage of cultivated apple include *M. baccata*, *M. Prunifola*, and *M. orientalis*, as well as *M. sylvestris*, the crabapple native to Europe (Janick et al., 1996).

It is not known when cultivation of apples began, but it is likely to have been cultivated in Central Asia several thousands years B.C. The apple tree might have been one of the earliest trees to be cultivated, and the fruit has been improved through selection over thousands of years. Literature on apple cultivation is available from the Greek and the Roman era, and some of the cultivars grown today were cultivated already in the thirteenth century (Morgan and Richards, 1993).

Modern breeding of apple varieties is focused mainly on improving yield, bruise resistance, shelf life and other factors that are necessary for efficient production and processing. Improved resistance towards a number of pests and diseases, such as powdery mildew, aphids and apple scab are also very important goals. Cosmetic appearance of the fruit such as colour, size and shape are also of great interest for the breeders to improve marketability.

Today, there are more than 10 000 apple cultivars, displaying a wide range of characteristics (Way et al., 1990). Apple is one of the most widely grown fruit trees and is of great economic importance to many countries,

ranking as the fourth most economically important fruit crop in the world (Janick et al., 1996). In 2005, at least 55 million tonnes of apples were produced worldwide, with a total value of about $10 billion. The annual production in Sweden is 20 000 tonnes (FAO, 2009), and for some regions it is an economically very important crop.

1.2 Apple rootstock

Most apple trees sold commercially today consist of two quite separate parts which together form a complete tree. A selected variety, referred to as the scion, is grafted onto a rootstock of choice. The art of grafting is an ancient method. The writings of followers of Hippocrates make it evident that grafting superior varieties onto rootstocks was common practice around 424 B.C., but other sources suggest that it was well known in Asia hundreds or possibly thousands years earlier (Garner, 1988). Today a large number of horticultural crops, especially fruit trees, are being grafted routinely. Apple, pear, peach, cherry, almond, apricot, plum and citrus are just a few examples of species that are commonly being produced as grafted trees (Rom and Carlson, 1987).

While the role of the scion is to produce fruits, the rootstock affects many other important traits of the tree. Each rootstock has its own distinct characteristics, regarding such aspects as winter hardiness, anchorage, insect and disease resistance, site and soil adaptability, and also controls various aspects of the scion such as degree of dwarfing, precocity and productivity (Lauri et al., 2006, Tworkoski and Miller, 2007, Ferree et al., 2001b, Ferree et al., 2001a, Hirst and Ferree, 1995, Hirst and Ferree, 1996, Tubbs, 1974, Webster et al., 1985, Drake et al., 1988, Fallahi et al., 1985). Due to graft incompatibility, not all rootstocks are compatible with all scions. Sometimes this can be circumvented by the use of an interstock, a third part that serves as a bridge between the rootstock and the scion.

Depending on the influence on the growth of the tree, rootstocks are classified as dwarf, semi-dwarf, semi-vigorous or vigorous. A dwarfing rootstock will produce trees of 15-50% the size of trees on seedling. In modern intensive fruit tree orchards, dwarfing rootstocks are commonly used to reduce tree size, enabling high-density planting and easy management, thus achieving high production efficiency. Trees on dwarfing rootstocks can also exhibit other economically important traits, such as precocious flowering, increased yield efficiency and increased disease and virus resistance (Fallahi et al., 2002, Barritt et al., 1995). Some of the most popular dwarfing rootstocks used in commercial production today come from the

Malling series, described and classified at the East Malling Research Station, Kent, England. Of these, M.9 and M.26 are the most well known (Rom and Carlson, 1987).

Breeding of apple rootstock has been carried out for almost a century, but has been performed on a larger scale at a rather limited number of locations. Not so strange perhaps, considering that rootstock breeding is a very expensive and long-lasting task. Most of these breeding projects can be considered as classic "chance" breeding. Since little or no information is available on inheritability of the desired traits, selection of parents is usually based on phenotype. Breeding has mainly been focused on improving resistance to diseases, such as fireblight (*Erwinia amylovora*) and collar or crown rot (*Phytophthora cactorum*) and to pests such as the woolly apple aphid (*Eriosoma lanigerum*). Winter hardiness and soil adaptability have also been major goals for many breeding programmes, as well as productivity and tree size control. Some programmes have also involved improved rooting, since many of the widely used rootstocks of today are either difficult to root or provide insufficient anchorage for the tree (Czynczyk, 2007, Webster, 2001), resulting in the need for support of the tree, increasing the cost of maintaining an orchard. These problems appear to be particularly pronounced for dwarfing rootstocks.

1.3 Propagation of apple rootstock

Vegetative propagation is one of the most important methods for commercial production of horticultural crops throughout the world (Davies et al., 1994). For many plants it is a fast and economic method of multiplication, and for plants with a long generation time or poor sexual reproduction it is the only practically applicable method available. In addition to this, vegetative propagation is clonal, producing new plants genetically identical to the mother plant. The formation of adventitious roots is a key step in vegetative propagation. For many woody species, including apple rootstocks, adventitious root formation is a limiting step for vegetative propagation due to poor rooting ability.

Since cuttings of many apple rootstocks do not root easily, a system of propagation using layering of mother plants in stool beds are commonly used. In this system the rootstock is pruned off a few inches above ground, and after some time several upright, vigorous vegetative shoots will develop. As these shoots grow, the lower portion is covered with moist soil or sawdust which allows rooting of the shoots while they are still attached to the mother plant. The rooted shoots are then removed for use as rootstock.

The method of propagation by layering of stool beds is both time consuming and expensive. Improvement of rooting ability is thus of great commercial interest.

1.4 Auxin and adventitious root formation

The plant hormone auxin is involved in a wide range of developmental processes including embryo and fruit development, organogenesis, vascular tissue differentiation, root patterning, elongation and tropical growth, apical hook formation and apical dominance (Kepinski and Leyser, 2005). Auxin plays a central role in the formation of lateral (Malamy and Benfey, 1997, Zhang and Hasenstein, 1999, Guo et al., 2005) as well as adventitious roots (Zimmerman and Wilcoxin, 1935, Sorin et al., 2005, Falasca et al., 2004).

On an anatomical level, adventitious roots are post-embryonic roots which arise from stem, leaves or any other tissue other than the roots. In difference to lateral roots, which are formed from the pericycle (for reviews see Benfey and Scheres (2000) and Hardtke (2006)), adventitious roots are formed in non-meristematic tissues after initiation of new meristems (for reviews see Han et al. (2009) and De Klerk et al. (1999)). Adventitious roots formed from hypocotyls are an exception to this since they are formed from the pericycle rather than from new meristems (Falasca and Altamura, 2003). In cuttings of apple, adventitious root primordia usually originate from cells between the vascular bundles (Naija et al., 2008, De Klerk et al., 1999, Welander and Pawlicki, 1993).

It is well known that auxin concentrations favourable for adventitious root initiation inhibit root growth (Thimann, 1936, De Klerk et al., 1990). In apple, the developmental process of adventitious root formation can be divided into three phases: dedifferentiation, induction and differentiation. Each phase has its specific auxin requirement. During the first 24 h after the microcuttings have been excised, the cells are not yet very sensitive to auxin. During this period, dedifferentiation occurs and the cells become competent to respond to the rhizogenic action of auxin. During the following induction phase, cells committed to the formation of root primordia begin to divide and form meristemoids. The first cell divisions occur 48 h after the onset of auxin induction, and by 96 h meristemoids of approximately 30 cells have been formed. After 96 h the differentiation phase starts, where the meristemoids first develop into root primordia and then into roots (De Klerk et al., 1999). During the differentiation phase, auxin is no longer required but rather inhibitory to the root growth (Kevers et al., 1997).

1.5 Rooting related genes

A number of auxin-induced and rooting-related genes have been isolated from several different species. In *Arabidopsis thaliana*, a number of genes involved in adventitious root formation in the hypcotyl have been identified, for example *AUXIN RESPONSE FACTOR 17* (*ARF17*) (Sorin et al., 2005), *LATERAL ORGAN BOUNDARIES-DOMAIN16* (*LBD16*) and *LBD29* (Okushima et al., 2007), as well as a number of GH3-like proteins (Staswick et al., 2005, , 2002). Many of the genes involved in development of the primary root, such as *HOBBIT* (*HBT*) (Willemsen et al., 1998), may also be involved in adventitous root formation. Genes that are known to be involved in lateral root formation, such as *ARF17* and *ARF19* (reviewed in Guilfoyle and Hagen, 2007), may also play an important role in the formation of adventitious roots, at least from hypocotyls. However, it is not clear if these genes are also involved in the formation of adventitious roots from stems, where the origin of adventitious roots is different from that of hypocotyls as stated above. In woody species, very few genes with a possible involvement in adventitous root formation have been isolated. The gene *5NG4* from loblolly pine (*Pinus taeda* L.) (Busov et al., 2004), the *SCARECROW-LIKE* genes from *Pinus radiata* and *Castanea sativa* (Sanchez et al., 2007) and the *SHORT-ROOT* (*PrSHR*) gene (Nakajima et al., 2001) from *P. radiata* are possible candidates, at least for involvement in adventitous root formation in hypocotyls. In apple, the *ARRO-1* gene (Butler and Gallagher, 1999) may have a role in the auxin-induced pathway of adventitious root formation.

The Adventitious Rooting Related Oxygenase (*ARRO)* gene, encoding a novel 2-oxoacid-dependent dioxygenase, has been isolated from apple (Butler and Gallagher, 1999). *ARRO-1* is constitutively expressed in the primary root and the expression is upregulated in response to IBA and IAA, but neither by 2,4-D or ACC (Butler and Gallagher, 2000). In apple stem discs, *ARRO-1* is upregulated 24-72 h after the onset of auxin treatment (Butler and Gallagher, 1999, Sedira et al., 2005), coinciding with the induction phase of adventitious root meristems as identified by De Klerk et al. (1995). This suggests the possible involvment of *ARRO-1* in the auxin-induced pathway of adventitious root formation.

The *rolB* (rooting locus B) gene, isolated from the soil bacterium *Agrobacterium rhizongenes* (Cardarelli et al., 1987, Vilaine and Cassedelbart, 1987), is a well documented rooting related gene and has been proved to stimulate rooting in different plant species when overexpressed (Spena et al., 1987, Capone et al., 1989, Rugini et al., 1991, Tzfira et al., 1996, Welander et al., 1998, Dai et al., 2004, Feyissa et al., 2007, Geier et al., 2008). Except

for its effect on adventitious rooting, the *rolB* gene can also stimulate flowering in transgenic tobacco plants (Altamura et al., 1994). The *rolB* gene has been used for improve rooting in difficult-to-root dwarfing apple and pear rootstocks both *in vitro* and *ex vitro* as well as to reduce plant size under greenhouse conditions (Zhu et al., 2001, Zhu et al., 2003). It has also been reported that, under non-limiting nutrient conditions, the relative growth rate of transgenic apple rootstocks was not altered by the *rolB* gene compared to the untransformed control (Zhu and Welander, 1999).

1.6 Application of gene technology and GM plants

Genetically modified (GM), or transgenic, crops refer to plants produced by the insertion of specific pieces of nucleic acids into the plant's DNA, for example through *Agrobacterium*-mediated transformation. This approach allows genes to be introduced into a plant genome from any source, i.e. plant, animal, bacterial or fungal, resulting in the potential transfer of a wide range of genetic material between unrelated species. Traditional plant breeding is limited to exchange of genetic material between sexually compatible close relatives of a given plant.

Commercial cultivation of genetically modified plants has been steadily increasing worldwide in the past ten years. By 2008, the cultivation of GM crops had reached over 100 million hectares in 25 countries (James, 2008). So far, commercial crop plants have been genetically modified mainly to achieve resistance to herbicides or pathogens, but resistance to abiotic stresses is also being studied. More recently, genetically modified plants with improved nutritional qualities have been developed, such as plants containing higher proportions of unsaturated fatty acids or containing β-carotene. The only GM tree species that are commercially cultivated are poplar with insect resistance and papaya with virus resistance (James, 2008).

Compared with conventional breeding, genetic modification could be a more efficient method for improving currently available rootstocks where only one or a few traits need to be improved. Using genetic engineering, only a very small portion of foreign DNA will be transferred to the host plant, as compared to the case with traditional breeding using crossing. Another advantage is the relatively short time span needed for genetic engineering, omitting the need for multiple back-crossings. Genetic engineering could therefore be a very valuable tool for improving rooting in apple rootstocks.

1.7 Aim and scope of this study

The overall aim of my PhD project was to study and investigate the molecular mechanisms of adventitious rooting. A better understanding of this process would give a platform for improving this important trait in apple as well as in other crops.

Even though the central role of auxin in adventitious root initiation is well characterised, the signalling pathways and gene expression during the whole rooting process are still poorly understood, especially in woody species. To better understand and possibly manipulate this process, the rooting-related genes must be identified and their functions elucidated. As part of this project, the expression of a number of rooting related genes during adventitious root formation in *Arabidopsis* was investigated through qRT-PCR. To achieve this, efficient rooting methods for both hypocotyls and stems were developed and the differences in timing of adventitious root formation in these two explants were examined. The use of the model plant *Arabidopsis* instead of apple in this part of the project had several advantages. The complete genome of *Arabidopsis* has been sequenced, facilitating the design of primers for qRT-PCR. Much work has been done to assign functions to its 27,000 genes and the 35,000 proteins they encode, and a considerable number of rooting related genes have been identified. Information of this kind is not available for apple, but hopefully the knowledge gained from working with *Arabidopsis* will be applicable for apple.

A possible involvment of the *ARRO-1* gene in the auxin-induced pathway of adventitious root formation has been suggested, but the exact role of this gene is still unclear. The function of *ARRO-1* during adventitious root formation in apple was studied through downregulation of this gene using RNAi technique. Attempts were also made to study the effects of overexpression of this gene.

rolB has shown some very promising results regarding improvement of rooting, but it is unknown how the *rolB* transgenic rootstocks will affect non-transgenic scion cultivars under field conditions. The answer to this question would provide the information about the potential use of the *rolB* gene in breeding of fruit tree rootstocks. In this study we evaluated the effects of *rolB* transgenic apple rootstocks on growth, flowering and fruit quality of non-transgenic scion cultivars grafted onto these rootstocks, as well as the stability of the *rolB* gene expression and the possibility of translocation of transgene or its mRNA from rootstock to scion. Another aspect, which could be of major interest for the consumer, is that the use of transgenic rootstock could result in the presence of transgenic proteins in the

fruit. As part of this project, antibodies against the ROLB protein were raised and used to test for the presence of ROLB in protein extracts of plant material from the field trial.

2 Materials and methods

2.1 Investigation of adventitious root formation in *Arabidopsis*

In vitro grown *Arabidopsis* Col-0 was used as plant material for expression studies of rooting related genes, using qRT-PCR. Protocols for induction of adventitious roots in hypocotyls and stem segments were developed by optimising factors such as IBA concentration, medium composition and induction time. For both hypocotyls and stem segments the use of two pulses of IBA induction, separated by a period without induction, proved to be most effective. Using this method, high rooting frequency was achieved, with little or no callus being formed.

For gene expression studies, hypocotyls were sampled at 10 time points, ranging from 0 to 72 h incubation on rooting medium. Due to their relative recalcitrance towards root induction, stem segments were sampled during a longer period of time, covering 15 time points ranging from 0 to 156 h. At each time point, plant material was flash frozen in liquid nitrogen and used for RNA extraction. Using qRT-PCR, the expression of the rooting related genes *ARF17*, *GH3-3*, *HBT*, *LBD16*, *LBD29*, *LRP1*, *NAC1* and *SCR* was studied at each time point during the auxin induced adventitious root formation. For anatomical studies of the rooting process in hypocotyls, samples were taken after 10 h to 2 days on the rooting medium, while for stem segments samples were taken after 2 to 6 days.

2.2 Functional studies of the apple *ARRO-1* gene

The *ARRO-1* gene was cloned from apple, using the published sequence information, and used for preparation of constructs for overexpression as well as for downregulation of the *ARRO-1* gene. For overexpression, the

gene was placed under the control of the constitutive 35s promoter. This will give a constant expression of the gene in any tissue of the plant, and during any developmental stage, displaying a pronounced phenotype as determined by the overexpressed gene. An RNAi construct was used for downregulating the expression of the *ARRO-1* gene. This technique takes advantage of an endogenous control system in the plant, which can inactivate the expression of a specific gene. A vector expressing a short sequence of a target gene can induce down-regulation of this gene, either on a transcriptional or on a translational level. The altered phenotype of the plant may help reveal the function of the gene. These two constructs were used for transformation of apple, using leaves from micropropagated shoots of the M26 rootstock as explants. The leaves were wounded and infected with the soil bacterium *Agrobacterium tumefaciens*, which is capable of transferring its T-DNA to a target plant cell. Transformed calli were induced from the leaves, and from these calli shoots of transformed plants were induced (Figure 1, left).

Both transformed and non-transformed shoots were used for rooting tests with stem discs and microcuttings (Figure 1, centre and right). Rooting percentage, root number and maximum root length were recorded. Transcript levels of the *ARRO-1* mRNA were analysed with qRT-PCR.

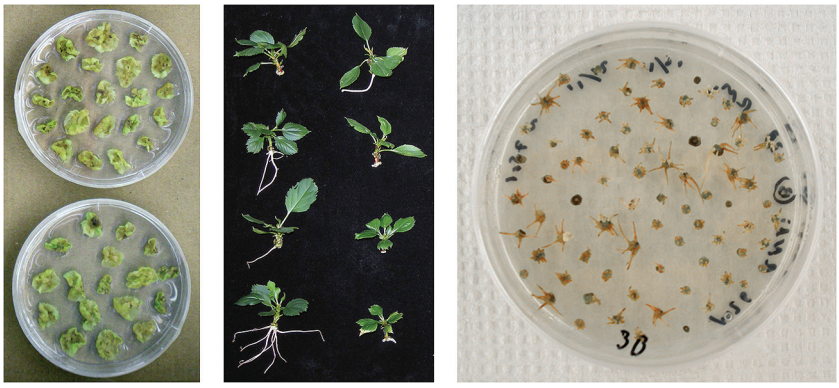

Figure 1. Left: two Petri dishes with transformed apple leaves on shoot induction medium. Middle: shoots from rooting test with microcuttings. Right: rooted stem discs.

2.3 Production of ROLB antibodies

The coding sequence of *rolB* was cloned into an expression vector, which was used for expression of His-Tag ROLB fusion protein in *Esherischia coli*. Inclusion bodies were purified and used for purification of recombinant

ROLB using Ni spin columns. The purified protein was used for antibody production in rabbits. The protein was further purified and used in affinity purification of ROLB specific antibodies from the serum. These antibodies were used in Western blot for detection of recombinant ROLB and for detection of ROLB in plant protein extracts. We also evaluated ROLB antibodies which previously had been raised against synthetic peptides and were available in our lab at the beginning of this PhD project. A new attempt was also made to raise antibodies against a new synthetic peptide, and these antibodies were used for detection of recombinant ROLB and for detection of ROLB in plant protein extracts.

2.4 Evaluation of transgenic rootstocks under field conditions

A field trial with five non-transgenic cultivars budded onto three different transgenic rootstocks and their control counterparts, was initiated in Alnarp, Sweden in 2001 (Figure 2). The cultivars Aroma, Discovery, Elise, Elstar and Jonagold were budded on the transgenic rootstocks M26-rolB M9-rolB1 and M9-rolB2 (Welander et al., 1998, Zhu et al., 2001), as well as non-transgenic control rootstocks. Non-grafted rootstocks of both transgenic and non-transgenic control rootstocks were also planted in the field for investigation of the stability of the *rolB* gene expression under field conditions.

Figure 2. Overview of the field trial in Alnarp, Sweden, established in 2001. The trees consist of transgenic rootstocks grafted with non-transgenic cultivars.

Tree height, trunk diameter and annual shoot growth was registered, as well as the number of flower buds, flowers and fruits. The fruit quality parameters fresh weight,

diameter, firmness, colour, total soluble solids, titratable acidity, total phenols and vitamin C were analysed. Cutting experiment using non-grafted rootstocks was carried to confirm that the *rolB* gene can still stimulate adventitious rooting under field conditions. Semi quantitative RT-PCR analysis was performed to investigate the stability of the *rolB* gene expression under field conditions, and PCR analysis was performed to investigate the possible presence of the transgene in the scion. Semi quantitative RT-PCR analysis was performed to investigate the possible presence of *rolB* mRNA in the scion.

2.5 Statistical analysis

Differences in the rooting frequency from the rooting tests were analysed using the Chi-Square test. Differences in root number and root length were analysed using the Mann-Whitney test. These statistical analyses were performed with the Minitab Statistical Software (Minitab, State Collage, PA, US). Data from growth registration and fruit quality analysis was subjected to analysis of variance (ANOVA) with Duncan's multiple range test using the Statgraphics program (StatPoint Technologies, Warrenton, VA, US).

3 Results and discussion

3.1 Adventitious root formation in *Arabidopsis*

We developed an efficient rooting method, using two pulses of IBA induction, resulting in a high rooting percentage, high root number and no or very little callus. Presumably, the first IBA pulse affects the cell competence, making them competent to respond to the second IBA pulse. The second pulse probably induces root initiation, thereafter auxin stimulus is no longer required as the roots differentiate and the outgrowth proceeds. We found that the concentration of nutrients influenced rooting more than the IBA concentration. Rooting medium with Lepoivre nutrients of half strength and 15 mg/l IBA gave the best results for both stem segments and hypocotyls. Timing of the rooting process showed that adventitious root formation is much faster in hypocotyls than in stems. In hypocotyls all explants had formed roots at day 4, while in most cases roots were not visible with the naked eye until day 7 in stems. Under a microscope, the first evidence of root primordia in the hypocotyls could be observed already after 12 h on the rooting medium. In stem segments, root primordia were not visible until day 5 (Figure 3). The first basal internode of the stem formed more roots than internodes 2 and 3.

Our results from gene expression analysis showed that *GH3-3*, *LBD16*, *LBD29* and *LRP1* were clearly affected by auxin induction, while *HBT*, *SCR* and *NAC1* displayed much smaller changes in transcript levels in response to auxin. *GH3-3* transcript levels were increased in stems as well as in hypocotyls, with a much more rapid increase in the hypocotyls (Figure 4). These results indicate a possible involvement of this gene in adventitious root initiation in both tissues, also reflecting the more recalcitrant nature of the stems towards root initiation. In hypocotyls, the transcript levels of

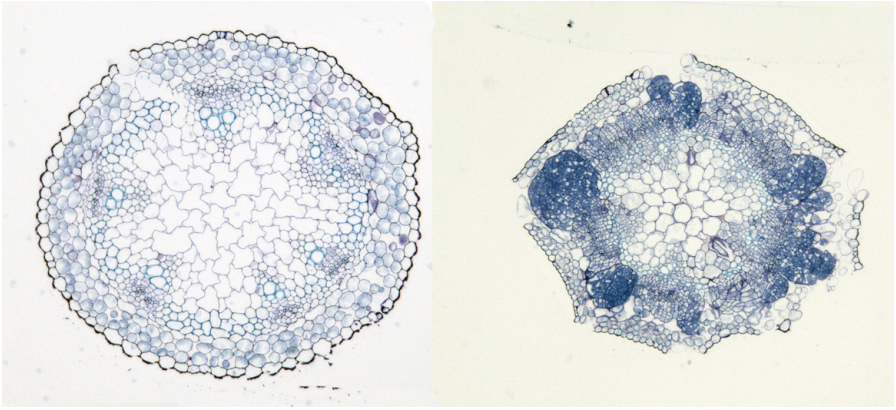

Figure 3. Cross section of an un-induced *Arabidopsis* stem (left, 20 x magnification) and after 5 days of root induction (right, 10 x magnification), where a number of root primordia (dark blue) are clearly visible.

ARF17 were not influenced by auxin, while transcript levels in stems were clearly down-regulated. Possibly, this gene has a regulating role in adventitious root formation in stems but not in hypocotyls. *LBD16* displayed very similar transcript levels in stems and in hypocotyls, with a distinct peak at 5-10 h (Figure 4). *LBD29* was also up-regulated in both tissues, with a quicker up-regulation in hypocotyls (Figure 4). These genes are previously known to be involved in lateral root formation, and our results show their possible involvement in adventitious root formation in hypocotyls as well as in stems. It has previously been shown that *LRP1* is likely to be involved in both adventitious root formation in hypocotyls and lateral root formation. In our experiment, *LRP1* was up-regulated both in hypocotyls and in stems, but the increase was slow, indicating that this gene is more likely to be involved in root development than in root initiation (Figure 4). *HBT*, *SCR* and *NAC1* were not clearly affected by auxin induction either in hypocotyls or in stems, indicating that these genes are probably not involved in adventitious root induction.

3.2 Functional studies of the apple *ARRO-1* gene

Transformation of apple with the *ARRO-1* overexpression construct failed to produce any transformants, due to extreme difficulties in organogenesis. When transforming with the RNAi construct, a number of transformants were obtained. These transformants displayed an altered phenotype including longer internodes and a higher degree of branching, as compared

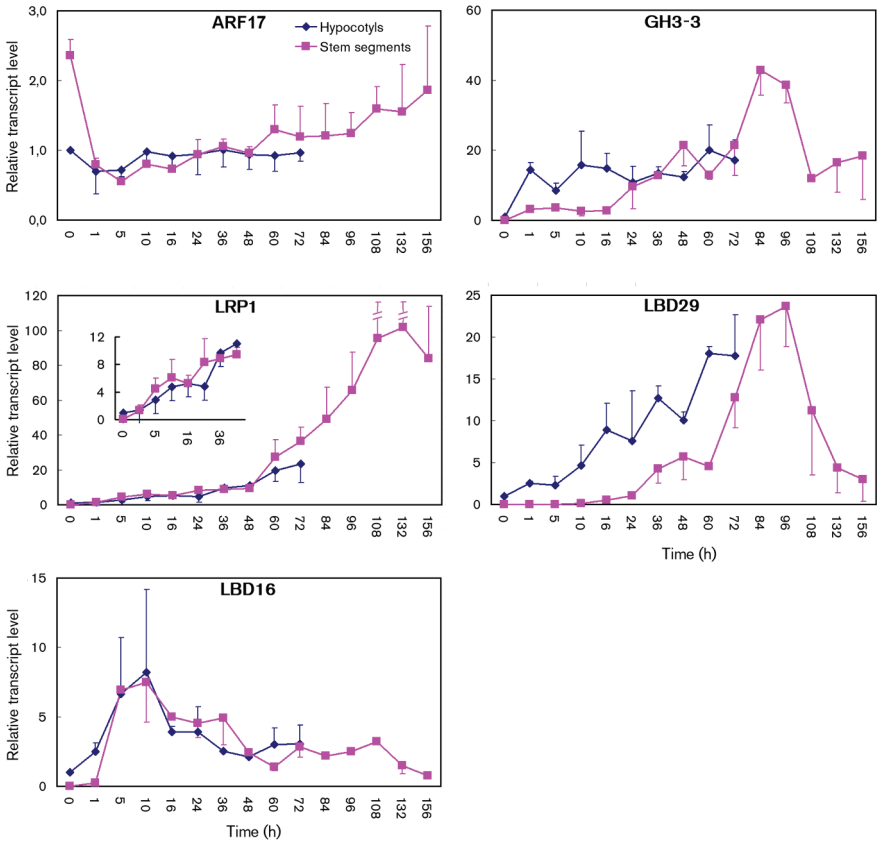

Figure 4. Relative transcript levels of *ARF17*, *GH3-3*, *LBD29*, *LRP1* and *LBD16* in hypocotyls and stem segments of *Arabidopsis* ecotype Col-0. Error bars represent error range of two biological replicates. Samples were taken at different time intervals after auxin induction. Notice that different scales are used in different figures.

to untransformed control plants. When maintained on normal shoot multiplication medium, the leaves of the transformants developed brown spots and many shoots displayed a high degree of hyperhydricity. This suggests that the transformed shoots were more sensitive to exogenous IBA or BAP than the untransformed control, indicating that the endogenous hormone balance might have been changed.

In the rooting tests with stem discs, both the rooting frequencies and the number of roots per rooted stem disc were significantly lower for all six transgenic clones than for untransformed control plants (Figure 5 and 6). In rooting tests with microcuttings the rooting frequency was also considerably lower for all the transgenic clones as compared to the untransformed control

(Figure 7). These results indicate that the rooting process has been affected negatively in the transformed clones. The fact that the root length of the transformants was similar to that of the control suggests that only root initiation had been affected, not root elongation.

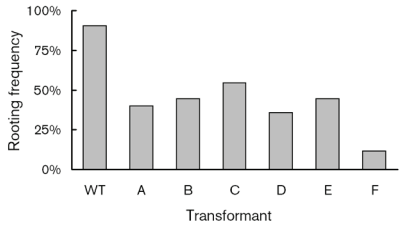

Figure 5. Rooting frequency of stem discs from the transformed clones A to F (n=70) and untransformed control plants (WT) of apple rootstock M26 (n= 42), recorded after 24 days of root induction.

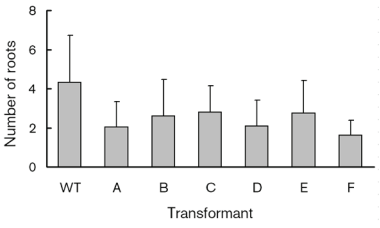

Figure 6. Number of roots per rooted disc for transformants A to F and untransformed control plants (WT) of the apple rootstock M26, recorded after 24 days of root induction. Error bars representing SD.

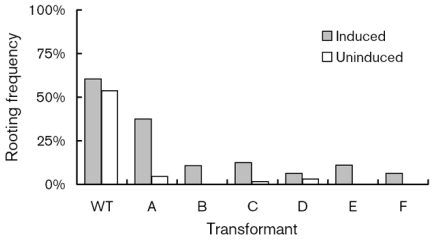

Figure 7. Rooting frequencies of microcuttings of transformants A to F and untransformed control plants (WT) of the apple rootstock M26 on IBA containing rooting medium (n>64) and on IBA-free rooting medium (n>59), respectively. Rooting frequencies were recorded after 25 days of root induction.

The qRT-PCR results did however not show any obvious change in *ARRO-1* mRNA level in the transformed clones compared to the untransformed control. It is possible that the downregulation of *ARRO-1* by the RNAi-construct was exerted on a translational level rather than on a transcriptional level. RNAi-downregulation on a translational level has been reported in plants (Aukerman and Sakai, 2003, Chen, 2004) and is now believed to be more common than previously acknowledged (Brodersen et al., 2008). In such a case, a downregulation of mRNA would not necessarily occur.

3.3 Production of ROLB antibodies

The sensitivity of all the antibodies tested for detection of ROLB was much lower than expected, which made detection of ROLB protein at nanogram

levels from plant material impossible. We encountered great difficulties in purifying the recombinant ROLB protein and ROLB antibodies, probably because the ROLB protein is likely to be a membrane protein (Filippini et al., 1996). The low sensitivity of the antibodies could possibly be explained by the low solubility of the protein. If this low solubility also applies to the synthetic peptides used for raising antibodies, this could greatly limit the number of epitopes available for antigen-antibody interaction in the rabbit. If this epitope was not properly exposed during the *in vitro* conditions of immunodetection, this could limit the binding of the antibodies to the ROLB protein. The specificity of the antibodies was generally rather good, producing very few unspecific bands and very little background. It has been reported that the phloem provides a pathway for the transport of small and macromolecules including soluble proteins (Bortolotti et al., 2005). As mentioned above, the ROLB protein is not water soluble and may not be possible or easy to transport from rootstock to scion, but this remains to be proven

3.4 Evaluation of transgenic rootstocks under field conditions

In this study, all growth parameters of the scions grafted onto the transgenic rootstocks were generally lower than those grafted onto the non-transgenic rootstocks, indicting that the *rolB* gene could reduce the tree growth of scion cultivars. This result is in accordance with previous reports concerning the *rolB* gene (Zhu et al., 2001, Zhu et al., 2003). For the cultivar Discovery grown on the transgenic rootstocks, flowering and fruiting were significantly reduced as compared to the control. This is likely due to the significantly reduced tree growth (Figure 8). It has previously been reported that yields are lower for scions grown on dwarfing rootstocks compared with those on vigorous rootstocks (Di Vaio et al., 2009). Fruit quality was generally not affected by the transgenic rootstocks, indicating that the *rolB* transgenic rootstocks do not clearly affect the fruit quality of non-transgenic scion cultivars. The results on cutting and RT-PCR showed that the *rolB* gene is stably expressed in the transgenic rootstocks under field conditions over a period of several years. PCR and RT-PCR could not detect the *rolB* gene or its mRNA in scion cultivar, suggesting no translocation of the *rolB* gene or its mRNA from rootstock to scion.

Figure 8. The author inspecting the field trial in Alnarp, Sweden. The use of transgenic rootstock has strongly influenced the tree size.

4 Conclusions and future prospects

4.1 Adventitious root formation in *Arabidopsis*

In conclusion, we have developed an efficient rooting method resulting in a high rooting percentage, high root number and no or little callus. Timing of the rooting process in stems and hypocotyls has been determined. This work offers a valuable platform for further studies of adventitious root formation in *Arabidopsis*. Since our analyses included only 8 genes, 3 of which are not obviously affected by auxin treatment, it is still difficult to make any conclusions about possible differences in molecular mechanisms underlying adventitious root formation in hypocotyls compared to stems. The expression profiles of many more genes should be studied in the future. To determine the exact origin of adventitious roots it would be desirable to use a reporter gene driven by a promoter of a rooting related gene. This work is currently being performed in our laboratory.

4.2 Functional studies of the apple *ARRO-1* gene

The altered phenotype of the RNAi-transformants suggests that the transformed shoots were more sensitive to exogenous IBA or BAP, indicating that the endogenous hormone balance might have been changed. It would be interesting to investigate if downregulation of *ARRO-1* affects the levels of hormones such as auxin, cytokinin and gibberellin. This would further show if *ARRO-1* is indeed involved in regulating hormone sensitivity or hormone homeostasis.

The lowered rooting frequency of microcuttings and stem discs of the transformants further strengthens the theory that *ARRO-1* is involved in adventitious root initiation, possibly through regulating hormone

homeostasis. To further characterise the role of this gene, localization of the ARRO-1 protein would be desirable. For this purpose, production of ARRO-1 specific antibodies has been initiated and the antibodies will be delivered within the nearest months. In future projects, these antibodies will be used for immunolocalization of the ARRO-1 protein in plant tissue.

Due to the inconclusive results from the qRT-PCR, further research is necessary to definitely prove the involvement of *ARRO-1* in adventitious root initiation. Comparison of levels of the ARRO-1 protein would help to prove that expression of the protein actually has been downregulated.

4.3 Production of ROLB antibodies

It remains to be investigated if the ROLB protein could be translocated from rootstock to scion. Different approaches to concentrating the ROLB protein, such as microsomal fractioning, might aid detection of the protein. Possibly, detection of minute amounts of ROLB could be achieved through the use of monoclonal antibodies.

4.4 Evaluation of transgenic rootstocks under field conditions

Our study showed that the use of *rolB* transgenic rootstock in most cases reduced growth and flowering as well as fruiting of non-transgenic scion cultivars, especially for cultivars with weak growth vigour. This reduction could be positive for vigorous cultivars in commercial production where thinning is often required to reduce excessive flowering. It could therefore be recommended using *rolB* transgenic rootstocks in combination with vigorous scion cultivars, using the *rolB* gene for dwarfing vigorous rootstocks. Another potential use for the *rolB* gene may be for making bonsai plants of fruit trees and ornamentals where extremely slow growth rates are desirable.

4.5 Final remarks

Despite of the huge economic values depending on adventitious rooting, very little research has been performed to improve this important trait. It seems that most efforts to improve adventitious rooting are done on a trial and error basis, rather than by a scientific approach. For many species that are vegetatively propagated, even a limited improvement in adventitious rooting would result in large economic profits. For species which today are

impossible to propagate vegetatively, an improved rooting could result in altogether new methods for propagation.

In *Arabidopsis*, studies of the molecular mechanisms of adventitious rooting is well under way, but results are still fragmentary, especially regarding genes involved in the very early stages of the process. Since most current research regarding adventitious rooting in *Arabidopsis* is based on hypocotyls, more effort should be put on exploring adventitious rooting in stems. This is the focus of our research group. Insight gained from this model plant could easily be applied to commercial crops.

ARRO-1 is likely to play a role in the mechanisms underlying adventitious rooting, but the possible use of this gene for improving rooting needs to be further evaluated. To clarify the role of this gene might help to further understand the mechanisms of adventitious rooting.

Transformation with the *rolB* gene is likely to be an efficient way to improving rooting for difficult-to-root woody species. The commercial application of *rolB* transgenic plants should be preceded by thorough evaluation of the plants under field conditions. From our field trial results, some rolB transgenic clones showed a promising commercial value.

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6 Acknowledgements

First of all, I would like to thank my main supervisor Li Hua Zhu for her scientific supervision during my whole PhD project, always being around and always having time for questions and discussions, and for being around when I was not around, taking samples at every hour of the day and every day of the week. I am very thankful to my co-supervisors who each have contributed with his or her valuable expertise: Margareta Welander for helping with setting the general outlines of the project, always planning with great enthusiasm, Peter Olsson for his invaluable advice and patience and Zhongtian Xue for some advice on molecular biology. I am also very thankful to Anna Holefors for teaching me about qRT-PCR and for being a great moral support. I also thank Annelie Ahlman for all her excellent technical support. In addition to these persons, a great number of other colleagues have contributed with advice and support, for which I am very grateful. I would like to thank all the people in the H-house for creating a friendly workplace atmosphere, and I would like to thank the helpful staff at ScanBi Diagnostics for making my qPCR analysis run so smoothly.

The work for this thesis was made possible by the economic support from the LTJ Faculty and the Department of Plant Breeding and Biotechnology, SLU. FORMAS, The Royal Physiographic Society in Lund, C. F. Lundströms foundation, Gösta and Anna-Birgit Henrikssons foundation and Nilssons foundation are also recognised for financial support.

I also thank my parents who once awoke my interest in biology, and who allowed a wide range of animals, plants, rocks and other pieces of nature to enter our home.

Finally, I would like to thank the ones who I am most grateful to for being able to go through this task; all my wonderful, beautiful friends. Without you, my work with this thesis would not only have been impossible, but also meaningless. So thank you for all the fantastic

adventures we have experienced together, and for the even more amazing adventures that lie ahead of us!

I am chaos.
I am the substance from which your artists and scientists build rhythms.
I am the spirit with which your children and clowns laugh in happy anarchy.
I am chaos.
I am alive, and I tell you that you are free.
/Eris

7 Popularised summary in Swedish

De flesta personer med det minsta intresse för krukväxter eller trädgårdsodling har nog någon gång försökt ta sticklingar. Ibland lyckas det, ibland inte. Med vissa växter lyckas det alltid, med andra aldrig. Ofta beror utgången på huruvida sticklingen lyckas bilda rötter eller inte. Denna typ av rötter, som bildas från blad eller stam, kallas adventivrötter och är alltså nödvändiga för sticklingsförökning. Det är inte bara för hobbyodlaren som det är intressant att ta sticklingar, den här typen av förökning används i stor skala av kommersiella odlare för många olika grödor, bl.a. för grundstammar av äpple (*Malus domestic*a). De flesta äppelträd som säljs i handeln idag består av en fruktbärande sort ympad på en grundstam som utgör den underjordiska delen av trädet. Trots att grundstammen aldrig producerar några frukter eller blad så är den av stor betydelse för trädets tillväxt och utveckling. Idag använder kommersiella äppelodlare ofta svagväxande grundstammar som reducerar trädets storlek, vilket underlättar skörd och skötsel av trädet. Många grundstammar av den här typen är dock mycket svåra att föröka med sticklingar, och man tvingas använda kostsamma och tidsödande metoder.

Forskningsprojektet som presenteras i den här avhandlingen hade som mål att kartlägga de genetiska mekanismerna bakom adventivrotsbildning för att på så sätt ge insikt i hur rotbildningen skulle kunna förbättras. Bland annat undersöktes en gen benämnd *ARRO-1* och dess roll vid adventivrotsbildning. Vi utvärderade även ett fältförsök med genmodifierade grundstammar för att se hur dessa påverkade trädets tillväxt och en fruktkvalité. Mycket arbete gjordes även på modellväxten backtrav (*Arabidopsis thaliana*) där adventivrotsbildning studerades med både genetiska och fysiologisk metoder.