# Studies of Adventitious Root Formation in Woody Species

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# Abstract

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Four different *Agrobacterium rhizogenes* wild-type strains were compared for the ability to induce transformed roots on micropropagated shoots of the apple rootstock Jork 9. The mannopine strain 8196 gave the best results in the production of chimeric plants compared to two agropine strains (A4 and 15834) and one cucumopine strain. From the transformed roots seven transgenic shoots were obtained, which rooted on a hormone-free medium. Furthermore the apple rootstock Jork 9 was transformed with *Agrobacterium tumefaciens* strain C58C1(pGV3850)(pB-B:GUS), containing the *npt*II, *rol*B and *gus* genes on the T-DNA. Transformation was confirmed by PCR and Southern blot analysis for all introduced genes. The rooting experiments showed that introduction of the *rolB* gene increased root percentage and root number, giving 13.8 roots per shoot compared to 2.3 for untransformed shoots. In addition, more than two copies of the *rol*B gene decreased the number of roots and percentage of rooted shoots. Auxin-induced expression of the *ARRO-1* (Adventitious Rooting Related Oxygenase) gene during the induction phase of adventitious root formation in both *rol*B transformed and untransformed Jork 9 stem discs and microcuttings has been studied. The higher expression of the *ARRO-1* gene in the *rol*B transformed material suggests that its expression may be rooting-specific rather than auxin-specific. We have improved synchrony of root formation from *Malus* Jork 9 stem discs through temporarily blocking DNA synthesis by application of aphidicolin (AD). The effects of different treatments with and without AD were studied at the cellular level by visualising DNA replication through the thymidin analog 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) labelling. The results show that DNA synthesis is effectively blocked by AD, and this blockage is overcome after AD withdrawal. It is concluded that AD treatment causes initial synchronization of the cell cycle and, thereby, more synchronized root initiation. BrdU labelling further revealed an increase in competence of the explants to respond to applied indole-3-butric acid (IBA) during the first day of cultivation.

*Keywords:* apple rootstock Jork 9, adventitious root formation, auxin, *Agrobacterium*, genetic transformation, *rol*B gene, *ARRO-1* gene, aphidicolin, cell synchronization, BrdU-labelling, *in vitro* culture, plant biotechnology

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# Contents

# Appendix

Papers I-IV

The present thesis is based on the following papers, which will be refered to their roman numerals

I. Pawlicki-Jullian, N., Sedira, M. & Welander, M. 2002. The use of *Agrobacterium rhizogenes* transformed roots to obtain transgenic shoots of the apple rootstock Jork 9. *Plant Cell, Tissue and Organ Culture* 70, 163-171.

II. Sedira, M., Holefors, A. & Welander, M. 2001. Protocol for transformation of the apple rootstock Jork 9 with the *rol*B gene and its influence on rooting. *Plant Cell Reports* 20, 517-524.

III. Sedira, M., Butler, E., Gallager, T. & Welander, M. 2005. Verification of auxin-induced genes during adventitious rooting in transformed and untransformed apple Jork 9. *Plant Science* 168, 1193-1198.

IV. Sedira, M., Welander, M. & Geier, T. 2006. Influence of IBA and aphidicolin on DNA synthesis and adventitious root regeneration from *Malus* Jork 9 stem discs. (Submitted to *Plant Cell Reports).*

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# Introduction

Clonal propagation is the most important method of commercial production of the majority, if not all horticultural crops throughout the world (Davies, Davis & Kester, 1994). Clearly adventitious rooting is a prerequisite for the successful production of viable plant clones, however in the case of woody plants, it is their physiological inability to produce adventitious roots which has led to the need for grafting and budding of many crop species onto rootstocks. Although such technologies have distinct advantages associated with them, there are fundamental benefits to produce plants on their own roots. These include ease of mass propagation, more economical production and avoidance of graft-union disorders (Davies, Davis & Kester, 1994). Considering that over 70 % of the propagation systems used in horticultural industry depend on successful rooting of cuttings (Davies, Davis & Kester, 1994), it becomes evident that adventitious rooting forms the backbone of the modern horticultural industry.

## Factors influencing adventitious root formation in woody plants

### *Recalcitrance*

The diversity and complexity of life requires that all organisms undergo differentiation. Plants are unique in that cells having undergone fate determination, can redifferentiate to form new organ initials (Esau, 1977). The concept of plant cell totipetency does not always translate to the reality of plant propagation. Many cells and tissues cannot acquire competence to regenerate new tissues and organs even though conditions to do so are optimal. Plants which can not regenerate with ease are said to be recalcitrant. The inability to maintain the morphological plasticity typically associated with plant cells means that recalcitrant plants cannot be propagated successfully. Although micropropagation of plants is a viable method of reproducing plants vegetatively, the application of in vitro micropropagation, as well as conventional propagation, to woody plants, is limited by the decreased potential of these plant for adventitious rooting (Mullins, 1985). Since clonal propagation is the preferred method of reproducing genetic lines of many plants it would be of considerable practical and commercial benefit if it was possible to characterise and eventually manipulate the adventitious rooting process at the genetic level. Although research has focused on the role of auxins in adventitious root formation in a wide variety of both herbaceous and woody plants (Hitchcock & Zimmerman, 1936; Zimmerman & Wilcoxon, 1935; Diaz-Sala *et al*., 1996; Blazkova *et al*., 1997), the process is still a very poorly understood phenomenon particularly in the case of woody plant species.

### *Cellular competence*

Dedifferentiation has been defined as the loss of previously developed characteristics (Wilson, 1994). A consequence of this redefinition of cellular fate in response to endogenous and exogenous signals, is the ability of plant cells to enter a new developmental pathway such as that required for the initiation of adventitious roots. Wilson (1994) suggests that there are significant differences

between cells which have the potential to initiate root formation and cells of the same type which do not have the potential to root. If a cell has the potential to initiate adventitious root formation then this cell is said to be competent for rooting. Accordingly Wilson (1994) concludes that such a difference between cells is due to variation in lineage, age and relative position to other cells. This is supported by the observation by Jasik & De Klerk (1997) that many cells divide in response to auxin but only a small number of cells proceed to form root meristems (see section anatomy and timing of adventitious root formation in stem discs of *Malus* Jork).

### *Auxin conjugation*

Within the context of auxin homeostasis, conjugated auxins have been implicated in the control of adventitious rooting. It was observed by Blakesley, Weston, & Elliot (1991) that cuttings of the woody plant *Cotinus coggygria*, examined early in the growing season, rooted easily and had high free indole-3-acetic acid (IAA) levels in contrast to the levels of conjugated IAA. The reverse was found to be true when cuttings were examinated later in the growing season when adventitious rooting was poor. This suggests that IAA conjugation can affect the rooting response by altering the free IAA levels within the plant. Nordström & Eliasson (1991) proposed that the accumulation of free IAA within the rooting zone of pea cuttings was prevented by the conjugation of excessive free IAA. Alvarez, Nissen, & Sutter (1989) examined free and conjugated levels of IAA in difficult-to-root (M.9) and easy-to-root (M.26) apple rootstocks prior to rooting. They found that a greater proportion of total IAA was present as conjugated IAA in basal sections of M.9, the difficult-to-root rootstock, than in M.26. This suggested that ease of rooting associated with the M.26 rootstock was related to a higher level of free IAA in the rooting zone. However it is unlikely that auxin conjugates play a direct role in adventitious root formation. When Nordström & Elaisson (1991) applied the principal amide conjugate of IAA, indole-3-acetyl-aspartate, to a rooting solution it did not stimulate rooting in the pea cuttings. This supports the hypothesis that once IAA has been conjugated it is not physiologically active and any potential activity is related to the amount of free IAA released by hydrolysis (Bialek, Meudt, & Cohen, 1983).

### *Plant age*

Plant growth may be separated into distinct juvenile and mature phases. These phases can be distinguished from one another by a number of morphological and physiological characteristics such as leaf shape, leaf retention, phyllotaxis and pigmentation (Hackett, 1985). The juvenile phase of plant growth is characterised by an inability to initiate flowering and in *Malus* species this phase may last from 4-8 years (Clark, 1983). Adventitious root formation in woody plants is also intrinsically linked to the developmental age of the plant. The potential to initiate adventitious root formation is one of the characteristics which has been observed to change in many woody plant species with developmental age. It is a well known observation that the majority of mature woody plants do not root easily. This includes cuttings from most adult apple trees which are extremely difficult, if not impossible to root (Brown, 1975). Reasons for this are unclear but endogenous

auxin levels in the mature plants are known not to be the limiting factor (Mullins, 1987). The ability of mature plants to form adventitious roots after undergoing rejuvenation has also shown that the loss of rooting potential experienced by mature tissues is not permanent and may be reversed (Mullins, 1985). White & Lovel (1984), examining adventitious root formation in mature *Griselinia* species, suggested that either the rooting stimulus was less effective in mature plants or that target cells were less responsive than those in juvenile plants. Geneve, Mokhtari & Hacket (1991) have suggested that the rooting potential of cells in mature ivy petioles is associated with the ability of cells which have recently undergone cell division, to perceive the rooting stimulus. A consequence of these findings is that a high potential for adventitious rooting is considered a juvenile characteristic.

Other factors are known to influence rooting ability. Williams, Taji & Bolton (1984) observed that poor-rooting ability in 16 woody plant species was related to suberization of the cortex. The deposition of lignin and cellulose in the plant cell wall which occurs during the onset of maturity has also been shown to influence rooting. Favre (1970) observed that too much or too little lignification gave minimal rooting in grape cuttings, while Dalet & Cornu (1988) found lignification retarded the emergence of adventitious roots in the easy-to-root cherry clone, INRA 235. Beakbane (1961) found that an inverse relationship existed between the easiness with which some apple cultivars rooted and the continuity of a sclerenchymatous ring which blocked the phloem rays in mature apple stems. She observed, that an increased difficulty in rooting was associated with intense fibre formation in mature woody plants.

## Apple as a model system to study adventitious root formation in woody plant species

### *Origins of the cultivated apple and its genome*

Apples have been propagated by means of budding and grafting for more than 2,000 years (Brown, 1975). Today, apple is the most widely grown fruit in the world (Zimmerman, 1984). There is some disagreement on the evolution of the modern apple with *Malus pumila* generally being considered the parent of most cultivated apples (Brown, 1975). The domestic apple Malus x domestica is thought to have originated in western Asia from natural hybridisation between a number of species including *M. pumila*, *M. sylvestris* Mill., *M. sieversii* Ldb. and *M. baccata* (L.) Borkh (Roach, 1985). As a result a variety of botanical names are used to describe apple with *Malus pumila* Mill., *M. sylvestris* Mill. and *M. domestica* Borkh. being the most commonly used (Zimmerman, 1984).

The apple, in common with many of the commercially important fruits such as pears, plums, cherries, strawberries etc. belongs to the *Rosaceae* or rose family. The sub-family *Maloideae*, to which apple belongs, is considered unusual because its members have a haploid chromosome number of 17. This is in contrast to the basic chromosome number in the Rosaceae which is believed to be between 7 and 9. Consequently, it has been suggested that the apple genome may have arisen through allopolyploidy between primitive sub-families of the *Rosaceae* (Stebbins,

1958). The majority of modern, cultivated apples are diploid (2n=2x=34), although some triploid (2n=3x=51), and even tetraploid (2n=4x=68) cultivars are known (Brown, 1975).

Apple was one of the first woody plants to be successfully propagated *in vitro*. It was first micropropagated by Jones (1967), when it was found that shoot tip growth could be stimulated by the synthetic plant growth regulator, benzyl amino purine (BAP). Since then a large number of apple cultivars have been successfully micropropagated in tissue culture.

### *The use of apple rootstocks*

Apple rootstocks have been widely used for commercial production, of which dwarfing rootstocks are particularly favourable for high density planting systems. Some important effects of dwarfing rootstocks are tree size reduction, early bearing and high yield efficiency (Rom & Carlson, 1987). Currently there are many rootstocks for apple (*Malus domestica*) trees, which are available for commercial purposes, but there are different problems which are limiting the efficiency of apple production. For instance, some dwarfing rootstocks are difficult-to-root (M.9) and some are too vigorous to be used for commercial purpose (A2, M26), whereas others have problems with adaptability (Rom & Carlson, 1987). Therefore, commercially important rootstocks still need to be improved.

The apple rootstock A2 (Alnarp 2) was selected in Sweden in late 1940. A2 is winter hardy and easy-to-root. It is readily propagated by stooling and cuttings and has a high compatibility with all cultivars tested and excellent anchorage. However, it is too vigorous for commercial apple production. Introduction of dwarfing characteristics into this rootstock would have a great value (Zhu *et al*., 2001a). Another semi-dwarf rootstock, which needed the introduction of genes, causing reduced growth is M26 (Holefors, Xue & Welander, 1998). The apple rootstock M.9 is one of the most commonly used dwarfing rootstocks for apple trees in commercial production. It is used throughout the world both for one-union trees, producing 25-35% of the size of the tree, and for interstem trees (Rom & Carlson, 1987). However, it is difficult-to-root from cuttings and is mainly propagated through stool layering, an inefficient propagation method. Recently, several distinct types of M.9 have been selected by nursemen for their ease of propagation. One of them is M.9/29 and was selected in Belgium for a better rooting ability compared to the original M.9. However, the rooting ability of M.9/29 was still not optimal and the rooting ability was improved by introduction of the *rol*B gene (Zhu *et al*., 2001b).

The apple cultivar used in this project is *Malus domestica* Borkh., Jork 9. This rootstock was selected in Germany from open-pollinated seedlings of the difficult-to-root rootstock M.9 (Tiemann & Dammann, 1981). Culture of Jork 9 has been extensively investigated in terms of both shoot (Pawlicki & Welander, 1994) and root (Pawlicki & Welander, 1995) regeneration. Because Jork 9 is a woody plant and more easy-to-root compared to M.9, it provides an excellent plant material of investigating adventitious root formation both at anatomical and molecular levels.

*The Malus 'Jork 9' stem disk system*

Van der Krieken *et al.* (1993) developed a model test system to study adventitious root formation in woody plants (Fig.1). Stem disks (0.5-1 mm thick) of the apple rootstock Jork 9 are induced to form adventitious roots by incubation on a medium containing the plant growth regulator indole-3-butyric acid (IBA). This test system was later modified by Welander & Pawlicki (1993) to optimise root production and reduce the exposure time of discs to auxin. This was achieved by the use of a high concentration of IBA (15 mg/l) for a 5 hours exposure. Discs are placed with the apical side down on the rooting medium containing IBA, and after the initial induction period of 5 hours the discs were placed on an IBA-free medium until root development was completed. Cell divisions leading to the formation of root meristems occur at approximately 48 hours with dome-shaped primordia becoming visible on the cut surface of the discs by 5-6 days. Roots continue to emerge and elongate from 7-9 days and thereafter. The mean number of roots per disk is 5-6 (Welander & Pawlicki, 1993) but up to 13 adventitious roots have been observed on some discs depending on the diameter of the shoot. Among the different factors critical for *in vitro* root induction are: explant position, etiolation state and orientation as well as temperature and light intensity (Seifert *et al*.,1995).

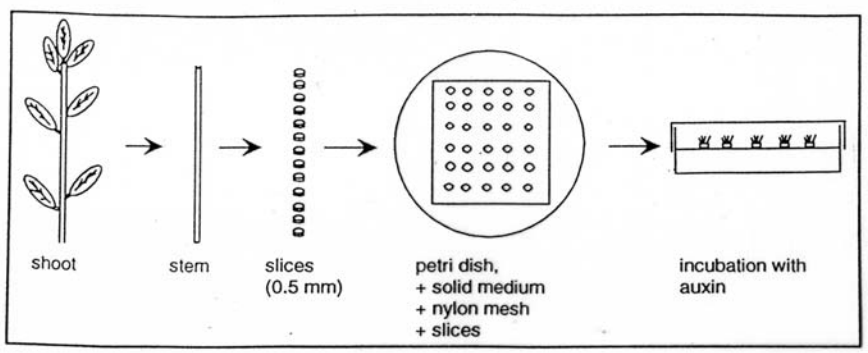



Fig.1. Diagram of the test system of root regeneration in vitro on stem discs of apple shoots (Van der Kriken *et al*., 1993).

## Anatomy and timing of adventitious root formation in stem discs of *Malus* Jork 9

Adventitious root formation in woody plants has been studied comprehensively at the anatomical level. Studies have been carried out in cherry (Ranjit, Kester & Polito, 1988), hazelnut (Gonzalez, Rodriguez & Sanchez-Tames, 1991), sycamore (Wright & Northcote, 1972), pear (Baraldi *et al*., 1995) and apple (Hicks, 1987; Harbage 1993; Zhou 1992). Anatomical and ultrastructural features of the adventitious rooting process in Malus 'Jork 9' stem disks have been extensively studied by Jasik & De Klerk (1997). They observed that during the first 24 hours following induction with IBA, starch grains began to accumulate in cells of the cambium, vascular tissues and the primary rays. By 48 hours, changes in nuclear appearance, increased cytoplasmic density and organelle development coincided with the breakdown of starch grains in these cells. Cambial cells were observed to

be dividing only occasionally at this time. However, by 72 hours the majority of cambial cells which were meristematic in appearance had undergone transverse division giving rise to organised files of cells (Welander & Pawlicki, 1993; Auderset *et al*., 1994; Jasik & De Klerk, 1997). By 96 hours, those cells originating in the interfasicular cambium had continued to divide extensively and eventually formed root meristemoids (Welander & Pawlicki, 1993; Jasik & De Klerk, 1997). Meristemoids began to differentiate giving rise to adventitious root primordia which penetrated the cortex before protruding from the basal surface of the stem disks. Not all cells which had undergone division were involved in the formation of root meristems. Callus was observed to develop from those cells which had not originated from the interfasicular cambium (Jasik & De Klerk, 1997).

According to De Klerk *et al.* (1995) adventitious rooting in Malus 'Jork 9' occurs in three specific phases: dedifferentiation (0-24 hrs), induction (24-96 hrs) and differentiation (96 hrs onwards). Analysis of the kinetics of root emergence has shown that IBA acts from 24 hours onwards suggesting that dedifferentiation does not require the presence of the rooting stimulus (De Klerk *et al*., 1995). During the induction phase the rooting stimulus is essential for adventitious root initiation. Thereafter the stimulus is no longer required as root differentiation and outgrowth proceeds.

## Bacterial transgenes and adventitious root formation

*Agrobacterium* is a gram-negative soil bacteria belonging to the *Rhizobiacea* family. The two best known species are the plant pathogens *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, both can infect wounds of several dicotyledonous and some monocotyledonous species. In case of *A. tumefaciens*, undifferentiated tumours (crown galls) are produced, whereas *A. rhizogenes* infections are characterized by a massive production of adventitious roots with numerous root hairs (hairy roots). Both species transfer T-DNA , excised from large, more than 200 kb in size, plasmids from the bacteria to the plant cell. In *Agrobacterium tumefaciens* the plasmid is called the tumour-inducing (Ti) and in *A. rhizogenes* root-inducing (Ri) plasmid. The Ri plasmids encode enzymes for synthesis of opines, which are derivatives of sugars and amino acids. Different strains of *A. rhizogenes* are classified by the opine synthesis genes that are transferred: agropine, cucumopine, or mannopine (Petit *et al*., 1983).

The Ri plasmids of *A. rhizogenes* can simultaneously carry one to several different T-DNAs. Thus, the Ri plasmid in the mannopine strain 8196 contains only one T-DNA (Hansen *et al.*, 1991), which encodes nine transcribed genes. However, in the agropine pRiA4 plasmid, the T-DNA is organised in two non-contiguous subfragments, the TL (left) and TR (right) regions, both of which can be transferred to the plant cell (White *et al*., 1985). For the transfer of DNA from the bacteria to the plant cell, three blocks of gene families are necessary: the T-DNA, the virulence (*vir)* genes and the chromosomal virulence (*chv*) genes. TR-DNA contains genes for biosynthesis of auxin (*aux1* and *aux2*) and the genes encoding synthases for the opines mannopine (*mas1'* and *mas2'*) and agropine (*ags*). The TL-DNA harbours four rol genes (root loci): *rol*A, *rol*B, *rol*C and *rol*D,

which were later shown to correspond to four of the 18 open reading frames (ORFs), identified by sequence analysis of the pRiA4 plasmid (Slightom 1986). Insertion in only those four loci ORF10 (*rol*A), ORF11 (*rol*B), ORF12 (*rol*C) and ORF15 (*rol*D) affected the morphology of the hairy roots, while the others ORFs seem to have an additional role in establishing full hairy root symptoms, and in broadening the host-range of the infection.

The *rol*B gene was identified as the critical *rol* gene for the induction of roots (White *et al*., 1985; Spena *et al*., 1987; Capone *et al*., 1989), because among all the Ri genes and ORFs, is the only one capable, individually, of inducing adventitious root formation in different plant species. The *rolA* and *rolC* genes were also found separately to induce adventitious rooting in tobacco although to a lesser extent than the *rolB* gene (Spena *et al*., 1987). Maximum adventitious rooting occurred when the *rolB* gene was combined with either *rolA* and/or *rolC* suggesting that the three genes synergistically control the rooting response (Spena *et al*., 1987). Maurel *et al.* (1994) has shown that the *rolB* promoter is regulated by auxin, while Filippini *et al*. (1996) has shown that the *rolB* protein has tyrosine phosphatase activity and is localised in the plasma membrane of transformed plant cells. This strongly suggests that a kinase/phosphatase cascade plays an important role in the signal transduction of the plant growth regulator, auxin. Nilsson *et al.* (1997), using trangenic aspen, has shown that the *rolB* and *C* promoters are expressed in cells from stem and root tissues which are competent to form adventitious or lateral roots. They have suggested that the cells which are responsible for the initiation of root meristem formation are the target for *A. rhizogenes* infection.

## The key role of auxin in root formation

The early history of auxin as a root-inducing agent has been summarized by Haissig & Davis (1994). Indole-3-acetic acid (IAA) is the main auxin naturally occurring in most plants and soon after the discovery of IAA, its rhisogenic activity was reported (Thimann & Went, 1934). At first, researchers thought that auxin could only be applied in a seminatural way, namely by basipetal transport. Practical application of auxin for rooting of cuttings became feasible when it was found that auxin also acts when added to the cut surface of cuttings, i.e., passed through the base (Hitchcock & Zimmerman, 1936). In the same period of time, indole-3-butyric acid (IBA) and α-naphthalene-acetic acid (NAA) were synthesized chemically, their capability to induce roots was discovered (Zimmerman & Wilcoxon, 1935), and talc powder was introduced as a carrier for auxin (Grace, 1937).

In many species, application of exogenous auxin is required to achieve rooting (Diaz-Sala *et al*., 1996; Blazkova *et al*., 1997). Some plants regenerate roots on cuttings spontaneously, but, in these plants, endogenous auxin produced in the apex and transported basipetally to the cut surface acts as the trigger: removal of the apex reduces both the level of endogenous auxin in the basal portion of a cutting and the number of regenerated roots (Nordström & Eliasson, 1991). Moreover, in these plants, application of auxin strongly increases the number of roots (Nordström et al., 1991; Liu & Reid, 1992). IBA is most commonly used for

rooting in commercial productions. The other auxins used commercially are IAA and NAA. Many chemical analogues have been synthesized and examined for auxin-like activity (Jönsson, 1961), but none of them are being used on a large scale for rooting. The observed differences in effectivity between the various auxins may lie in the nature of the compound. For example the affinity to the auxin receptor involved in rooting (Libbenga & Mennes, 1995) or in the concentration of free auxin that is reached in the ´target´ cells. The latter depends on several factors: uptake, transport, conjugation, and, in addition, the amount of auxin synthesized by the plant itself. Auxin may be applied for several days or weeks at a low concentration (micromolar range), or for several seconds or minutes at a high concentration (milimolar range) (Hartmann, Kester & Davies, 1990). The brief exposure is practiced in macropropagation, in which cuttings are rooted by being dipped in a concentrated auxin solution or in talc-based rooting powder. Most likely, auxin is released rapidly from the talc powder but this has never been investigated. Cuttings produced in tissue culture (microcuttings) may also be treated briefly with auxin, after which they are planted ex vitro. When microcuttings are still rather small, it is preferable to root them in vitro at a low concentration of auxin and to transfer them to soil after the roots have been formed: during rooting in vitro, the microcuttings increase in size and become more robust (Hartmann, Kester & Davies, 1990).

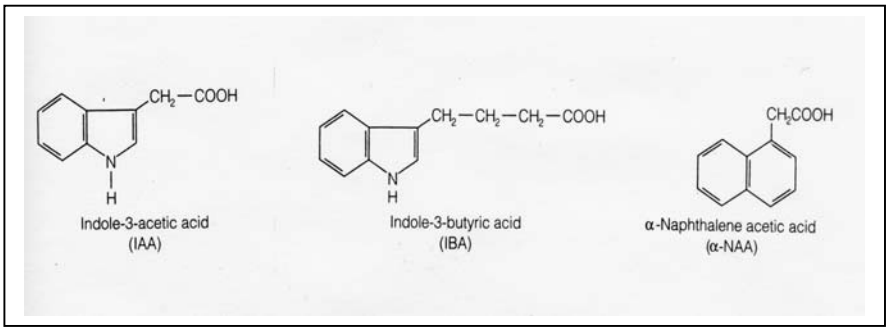



Fig.2. Chemical structures of the principle auxins. IAA and IBA consist of a benzene ring and an indole ring with a carboxyl-group side-chain. However, the indole ring is not essential for auxin activity such as in the case of NAA.

Auxin enters cuttings predominantly via the cut surface even in microcuttings that are known to have a poorly functioning epidermis (Guan & De Klerk, 2000). Auxin is rapidly taken up in cells by pH trapping (Rubery & Sheldrake, 1973) and by influx carriers (Delbarre *et al*., 1996). There are two major pathways of conversion: oxidation and conjugation. IAA, and to a lesser extent IBA, may be inactivated irreversibly by oxidation whereas NAA is not oxidized (Epstein & Ludwig-Muller, 1993). All three auxins are conjugated. Conjugation is a reversible inactivation as the free auxin may be released from the conjugates (Smulders *et al*., 1990). Because of conjugation and oxidation, only very small portion (1 % or less) of the auxin taken up by the tissue occurs in the free form (Van der Krieken, Breteler & Visser, 1992). Although auxins are primarily associated with the induction of adventitious roots, they are also intimately

involved in such processes as cell division, cell elongation and cell differentiation. Auxins have also been implicated in senescence, fruit growth and gravitropism.

## Auxin-induced gene expression

Guilfoyle (1986) categorized auxin-induced gene expression into short-term and long-term auxin responses. Long-term responses are characterized by genes which are expressed one or more hours after auxin application and which may continue to increase in expression for a number of hours or even days (Guilfoyle, 1986; Hobbie & Estelle, 1994). Abel & Theologis (1996) have since used the terms "early" and "primary response genes" to indicate those genes which are upregulated by auxin within a number of minutes. Because so called "early genes" are induced as a primary response to auxin prior to initiation of cell growth it is likely that they play a primary role in mediating the stimulatory effects of auxin (Abel & Theologis, 1996).

A number of gene superfamilies whose members exhibit early, primary response expression after auxin application have been identified and characterised. Of these, the *SAUR*, *Aux/IAA* and *GH3* genes families, involved in auxin-regulated growth and development, are the most notable. The *SAUR* (Small Auxin Up RNAs) gene family identified in soybean, consists of a cluster of five highly homologous intronless genes which are upregulated within 2 to 5 minutes of auxin application , and the response is insensitive to cycloheximide (McClure *et al.,* 1989). Members of the *Aux/IAA* gene family encode short-lived transcription factors or activators of the expression of late auxin-inducible genes. The expression of most of the *Aux/IAA* family of genes is stimulated by auxin within 5 to 60 minutes of hormone addition. All the genes encode small hydrophilic polypeptides that have putative DNA-binding motifs similar to those of bacterial repressors. They also have short half-lives (about 7 minutes), indicating that they are turning over rapidly. *GH3* early-gene family member, was first isolated by differential screening from *Glycine max* and its expression is induced within 5 min of auxin application (Hagen, Kleinschmidt & Guilfoyle, 1984). Mutations in Arabidopsis *GH3*-like genes result in dwarfism (Nakazawa *et al*., 2001) and appear to function in light-regulated auxin responses (Hsieh *et al.*, 2000). Because *GH3* expression is a good reflection of the presence of endogenous auxin, a synthetic *GH3*-based reporter gene known as *DR5* is widely used in auxin bioassays (Ulmasov *et al*., 1997). Another group of early genes is involved in adaptation to stress, such as wounding. Several genes encoding glutathione S-transferase (GSTs), a class of proteins stimulated by various stress conditions, are induced by elevated auxin concentrations (Takhashi *et al.,* 1989; Takahashi & Nagata, 1992). Likewise, ACC (1-aminocyclopropane-1-carboxylic acid) synthase, which is also induced by stress and is the rate-limiting step in ethylene biosynthesis, is induced by high level of auxin (Abel *et al.,* 1995, Zarembinski & Theologis, 1993).

Several mutants impaired in auxin response have been isolated. They give us information about the role and action of the genes and their encoded proteins in normal auxin signalling, and their significance for root initiation. For examples: AXR1 and AXR3 encode proteins that participate in the ubiquitination pathway in

protein degradation which is required for correct auxin responses, therefore the *AUX1* mutant produces fewer lateral roots than normal plants and does not exhibit a normal gravitropic response (Leyser *et al.,* 1993). *POLARIS* encodes a small polypeptide that appears to regulate root meristem cell shape and sensitivity to auxin/ethylene and root growth (Topping & Lindsey, 1997). Cell cycle genes such as cycD4, a D-type cyclin and cyclin-dependent kinase inhibitor, are required for division in the pericycle, which is the origin of lateral roots (De Veylder *et al*., 1999). Recently it has been reported that a S-adenosylmethionine synthetase (SAM) transcript cloned from *Pinus contorta* is preferentially expressed in roots and exhibits a specific pattern in the meristem at the onset of adventitious root development. This enzyme is involved at an early step in ethylene and polyamine biosynthetic pathway (Lindroth *et al.,* 2000). In spite of these achievements, there are several unknown genes involved in adventitious root formation that need to be discovered.

# Objectives of this study

The aim of this work was to identify and characterise barriers in root formation at anatomical, biochemical and molecular levels in order to improve rooting in difficult to root species. The main objectives and steps in this study can be summarised as:

- Develop and optimize a transformation protocol for the apple rootstock Jork 9 using *Agrobacterium tumefaciens*.
- Investigate the influence of the introduced *rol*B gene and its integrated copy number on rooting ability compared with the untransformed shoots.
- Study auxin-induced gene *ARRO-1* (Adventitious Rooting Related Oxygenase) expression during the induction phase of adventitious root formation in both *rol*B transformed and untransformed ´Jork 9` stem discs and microcuttings.
- Improve synchrony of adventitious root regeneration from Jork 9 stem discs through temporarily blocking DNA replication by applying aphidicolin, and visualise DNA replication through the thymidin analog 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) labelling.

# Present investigation – results and discussion

## The use of Agrobacterium rhizogenes transformed roots to obtain transgenic shoots of apple rootstock Jork 9 (paper I)

In paper I the apple rootstock Jork 9 was transformed using four different *Agrobacterium rhizogenes* wild-type virulent strains, two agropine strains: A4 and 15834, the mannopine strain 8196, and a cucumopine strain. The classification of those strains depends on the types of opines that the T-DNA of these plasmids direct the infected plant to synthesize. For inoculations, 5 weeks old micropropagated shoots were pricked at their base, dipped in the bacterial suspensions and transferred to the hormone free rooting medium. After 2 days, the shoots were rinsed in liquid MS medium containing cefotaxime, and transferred to the hormone free rooting medium. The mannopine strain 8196 gave the best results in the production of transformed roots, giving 77.8% of rooted shoots, followed by the agropine strain A4, resulting in 40.1% rooted shoots. Shoots inoculated with these two strains produced long and branched roots.

Both, untransformed and the chimeric plants obtained from shoots inoculated with the mannopine strain 8196, were used for shoot regeneration from the roots. A successful shoot regeneration from both untransformed and transformed roots was achieved, but the shoot regeneration was much higher for untransformed roots (77.8%) compared to 16.7% for transformed roots. Optimum combination and concentration of thidiazuron (TDZ) and α-naphtaleneacetic acid (NAA) was different between untransformed and transformed roots. The best results for untransformed roots were obtained when the regeneration medium was supplemented with 1 μM NAA and 1 μM TDZ. The highest regeneration of adventitious shoots from transformed roots was observed with 1 μM NAA and 0.1 μM TDZ. From the transformed roots seven shoots were obtained and propagated as individual clones. All shoots from these clones rooted on a hormone-free medium contrary to untransformed shoots that did not root under similar culture conditions. Differences in the morphology of the leaves and stems were observed between the clones. The transformed status of different clones was verified with mannopine tests, PCR and Southern blot analyses. Five clones contained the *mas1*', the ORF 13 and the *rol*B genes, whereas two clones contained only the *rol*B gene.

## Genetic transformation of apple Jork 9 with the rolB gene (paper II)

The apple rootstock Jork 9 is recalcitrant for both regeneration and transformation. An efficient system for adventitious shoot regeneration was developed by Pawlicki & Welander (1994). Different factors, like pre-treatment of the mother shoots, hormone concentrations, the carbohydrate source and the gelling agent influenced the regeneration capacity of Jork 9. Replacement of sucrose by sorbitol increased the rate of adventitious shoot regeneration and the number of shoots per explant (Pawlicki & Welander, 1994). The beneficial influence of sorbitol on callus

initiation and shoot multiplication in apple has also been shown previously by Chong & Taper (1972). The positive response of sorbitol on shoot regeneration was also enhanced when a dark and cold treatment was applied to the shoots (Pawlicki & Welander, 1994). The best hormonal combination for regeneration of adventitious shoots from untransformed Jork 9 was a low NAA concentration and a high concentration of 6-benzylaminopurine (BAP). However, in the transformation experiments the most important factor for regeneration of transformed plants was thidiazuron (TDZ), a potent cytokinin-like substance for woody plant tissue culture. TDZ stimulated callus formation and enhanced shoot production when added to both the callus promoting medium and the regeneration medium.

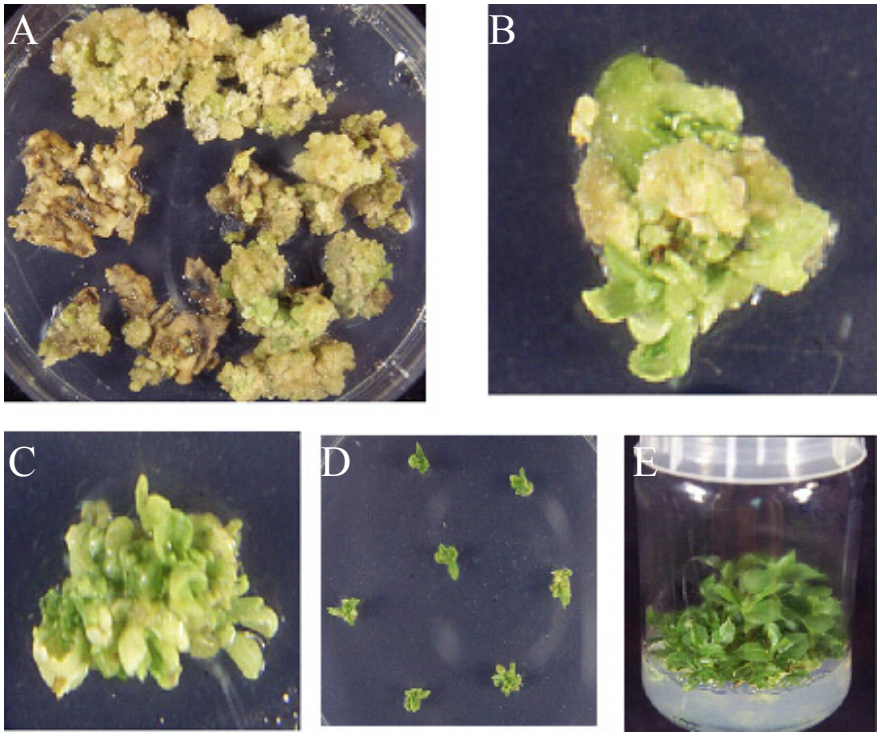



Fig.3. Genetic transformation of apple Jork 9 by *Agrobacterium tumefaciens* strain C58C1(pGV3850)(pB-B:GUS), containing the *rol*B gene. (A) Transformed callus on the leaf explants after 8 weeks of incubation on callus induction medium containing 200 mg/l cefotaxime and 50 mg/l kanamycin, (B,C) transformed shoots formed on callus explants growing on regeneration medium supplemented with 200 mg/l cefotaxime and 50 mg/l kanamycin, (D, E) transformed shoots excised from the explants and propagated on shoot multiplication medium containing 50 mg/l kanamycin.

In this study, we developed a transformation procedure for the apple rootstock Jork 9 using the *Agrobacterium tumefaciens* strain C58C1(pGV3850)(pB-B:GUS), containing on the T-DNA, the *npt*II as selectable marker, the *gus* gene as a scorable marker, and the *rol*B gene for improving rooting ability. Young expanded leaves from micropropagated shoots of Jork 9 were excised, wounded

with a scalpel and gently shaken in the bacterial suspension. Leaf explants were then co-cultivated with the bacteria in the dark for 3 days on a callus induction medium including basal nutrients with cytokinin and auxin. After co-culture the leaf explants were rinsed in a solution containing cefotaxime to kill the bacteria and transferred to a fresh callus induction medium containing both cefotaxime and kanamycin. When transformed callus appeared on the leaf explants (fig.3A) the explants were transferred to the regeneration medium. When shoots had been formed (fig.3B, 3C), the explants were transferred to light, shoots were excised from the explants and subcultured every 4 weeks (fig.3D, 3E). Only transformed cells can grow on kanamycin because incorporation of the nptII gene confers resistence to kanamycin. Figure 3D shows green transformed shoots that can grow in the presence of kanamycin, whereas untransformed shoots became pale and died eventually. From 475 infected leaves, 31 leaves regenerated one or more shoots, resulting in a transformation frequency of 6.5%. 18 of the best growing shoots, from 18 different leaf explants, were confirmed for all of the introduced genes by PCR and Southern blot analysis. Among 18 independent shoot lines obtained after transformation only ten contained at least one copy of intact T-DNA, while six lines were missing the *gus* gene and two lines were missing both the *gus* and *rol*B genes. The copy number of introduced genes varied from 1 to 7 for *npt*II, 0 to 7 for *rol*B, and 0 to 4 for the *gus* gene.

The rooting experiments showed that introduction of the *rolB* gene increased root percentage and root number, giving 13.8 roots per shoot compared to 2.3 for untransformed shoots. However, the number of roots per shoot varied between shoots containing different copy numbers of the *rol*B gene. Optimum rooting ability was obtained for shoots containing two copies of intact T-DNA, whereas more than two copies of the *rol*B gene decreased the number of roots and percentage of rooted shoots (Fig.4).


Fig.4. Rooting of untransformed shoots (NT) and transformed shoots containing different copy number of the *rol*B gene (clone C14 contains 1 copy of rolB gene, clone C6 - 2 copies, clone C1 - 4 copies and clone C3 - 7 copies of the *rolB* gene). The shoots were grown on rooting medium without auxins.

## The ploidy of transgenic plants of apple Jork 9 (unpublished results)

Somaclonal variation is a typical side effect in several plant species after short or long term tissue culture. Especially when plants regenerate through a stage of unorganized cell proliferation (callus), phenotypic and genetic changes are not uncommon. Chromosome doubling, among others things, depends on the donor plant genotype, type and age of explant, composition of the culture medium as well as the duration of the callus stage and frequency of transfer (Geier, 1991).
The most reliable conventional method of ploidy level analysis is the counting of chromosomes of metaphase plates. However, the preparation and microscopical analysis is time consuming, and in apple species rather difficult due to the high number (2n=34) of small chromosomes. Furthermore, chromosome counting requires the availability of appropriate developmental stages as young root tips. In cell suspensions or calli the number of dividing cells is rather low, and the detection of such cells is mostly difficult.

Therefore the flow cytometry (FCM) is a very useful method for the rapid detection of ploidy level variation in plants . FCM is based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence intensity of stained nuclei. The most simple preparation for FCM includes disintegration of plant tissue (preferably small pieces of leaves) by chopping with a razor blade releasing the nuclei into a buffer solution and the following staining with a fluorescent dye, e.g. 4´,6-diamidino-2-phenylindole (DAPI). Using the hydrodynamic focusing effect, the stained nuclei pass a flow chamber one after another and traverse an UV-light beam. The resulting fluorescence emission can be quantified by an optical system, and directly be related to the nuclear DNA content. FCM is widely used in plant science, especially for analysis of nuclear DNA amounts (Bennet & Leitch 1995) or for ploidy level detection (Carle, Jung-Heiliger & Schröder, 1993).

In our experiments we analysed 19 plants by flow cytometry. The plant material consisted of *in vitro* cultivated shoots of apple rootstock Jork 9, of which 18 were independent clones transformed with the *rol*B gene from *Agrobacterium rhizogenes* and one untransformed clone. Small pieces of leaves from each clone were chopped with a razor blade in order to release the nuclei into a buffer solution (DAPI HR KIT Typ P, Solution A, PARTEC, Germany). The samples were stirred gently for 10 minutes and then filtrated through 50 µm nylon gauze filter. The filtrate containing the nuclei was stained with the fluorescent dye DAPI (PARTEC-DAPI HR KIT Typ P, Solution B) for 15 min in the dark. The stained nuclei were analysed using a PARTEC CA II flow cytometer; the resulting data were evaluated using the DPAC software package (PARTEC, Germany) or Microsoft Excel. The measurements were repeated and other parts of the plants (as stem, callus, big leaves and small (young) leaves) were also investigated, in order to check the variation within the plant.

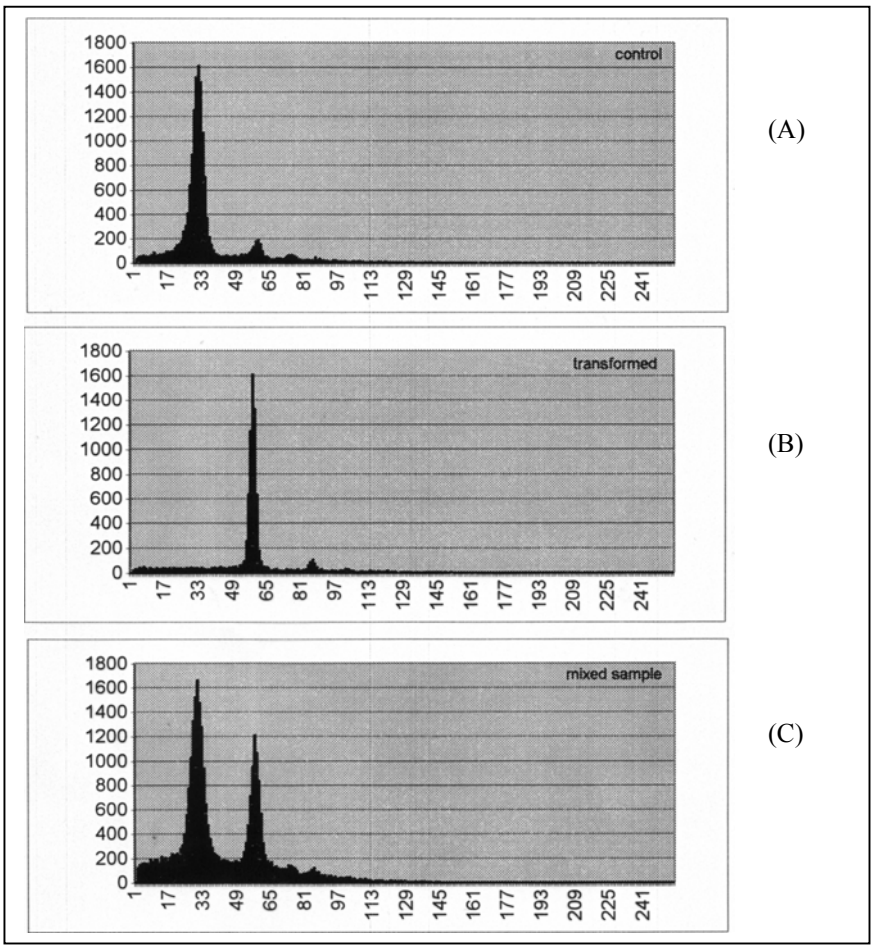



Fig.5. Flow cytometry detection of (A) diploid untransformed plant, (B) tetraploid transformed clone C14 and (C) mixed sample from both diploid and tetraploid plants of apple Jork 9.

The analysis resulted in the detection of 2 tetraploid clones among the 18 transformed plants, and the remaining 16 of the transgenic apple clones retained the diploid level (Fig. 5). This gives the rate of tetraploid transgenic plants of 11%. This result suggests that it is necessary to take into account this kind of analysis in order to adequately perform a selection from the initial population of primary transformants.

## Induction of adventitious roots on Apple Jork 9 stem discs and expression of the ARRO-1 gene (paper III)

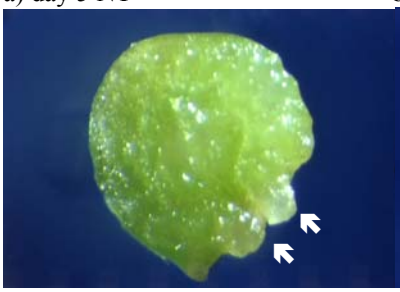
Cell divisions leading to adventitious root meristem formation, occur approximately 48 hours after the application of auxin to stem discs of *Malus* Jork 9. Changes in gene expressions which precede these cell divisions are thought to control the process of root initiation. *ARRO-1* (Adventitious Rooting Related

Oxygenase) gene is one of the rooting related genes, isolated from auxin-induced thin-stem-discs culture of the apple rootstock Jork 9 (Butler 2000) and is one of the first genes whose expression is known to be associated with the adventitious root formation process in woody plants (Butler & Gallagher, 1999). *ARRO-1* is 1,282 base pairs long, and consists of a 921 base pair open reading frame (ORF) which encodes a polypeptide of 307 amino acids. Sequence analysis of the *ARRO-1* cDNA has relevated it to be a member of the 2-oxoacid-dependent dioxygenase (2-ODD) superfamily. Dioxygenases are soluble enzymes which catalyse the addition of two oxygen atoms from $O_2$ into an organic substrate or substrates (Prescott & John 1996). In this paper we investigated auxin sensitivity in stem discs from untransformed and *rol*B-transformed shoots, and expression of *ARRO-1* gene at 0, 24, 48 and 72 hour time-points in both *rol*B-transformed and untransformed Jork 9 stem discs and microcuttings.

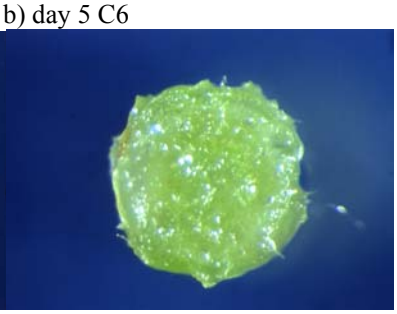
For induction of adventitious roots on stem discs, shoots of the apple rootstock Jork 9, both untransformed and *rol*B-transformed, were grown for a maximum of six weeks to allow sufficient nodal elongation. Stem discs approximately 1mm thick were cut using a scalpel blade and placed with the proximal end on rooting medium (Pawlicki & Welander, 1995) containing indole-3-butric acid (IBA) at the concentration of 73.8 μM. Only the basal 10 mm of the shoot stem was used for cutting of stem discs since this region exhibits greater competence for rooting. Stem discs were incubated for 5 hours in the dark and subsequently transferred to fresh rooting medium without IBA and placed in the light. Stem discs were harvested at 0, 24, 48 and 72 hours time-points for extraction of total RNA. Approximately 10 % of all stem discs were continued to grow in order to estimate rooting efficiency. Discs were examined under a stereo microscope on a daily basis for external evidence of root formation from the beginning of auxin application until roots had elongated and the same disc was photographed from untransformed shoot (NT) and *rol*B transformed clone C6, respectively (Fig.6). By day four, there was no visible external evidence of root formation on the basal surface of the stem discs. However, by day five, dome-shaped adventitious root promordia were observed protruding from the basal surface of stem discs from the untransformed shoots (see arrows fig.6a). On the stem discs from *rol*B-transformed shoots the root primordia were difficult to distinguish from the callus until day ten, due to thinner roots on transformed discs and large amount of callus. Roots began to elongate and at day 15 we could observe fully formed roots (the pink colour, fig.6). There were more roots on discs from *rol*B-transformed shoots than from untransformed shoots, however the former roots were thinner and some intervening callus between roots was observed (perhaps why we do not see them at an early stage).

Study of the effects of IBA concentration and duration of exposure on the Jork 9 stem discs showed that the optimum time for root induction in the transformed plants was 7 hours with 24.6 μM IBA, compared to 7 hours with 49.2 μM IBA for untransformed stem discs. These results showed that insertion of the *rol*B gene increased the sensitivity to IBA.

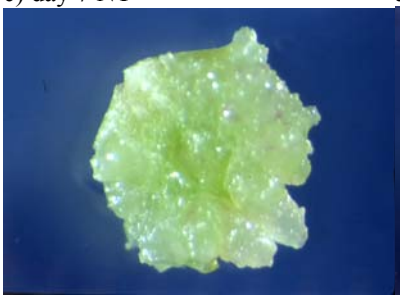
a) day 5 NT



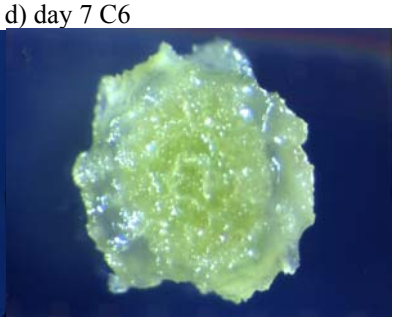
b) day 5 C6



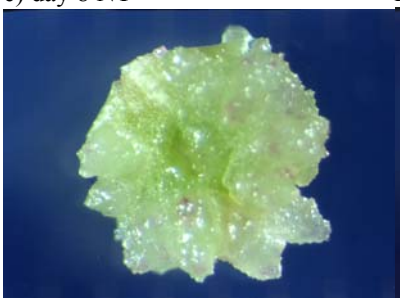
c) day 7 NT



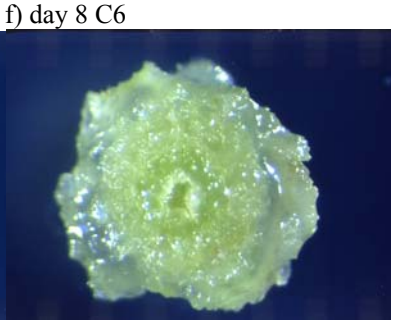
d) day 7 C6



e) day 8 NT

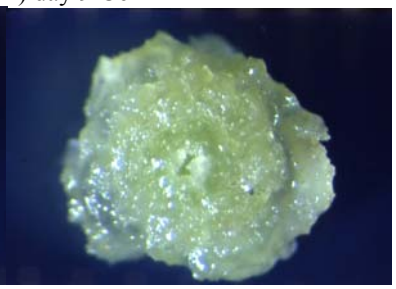


f) day 8 C6



g) day 9 NT

h) day 9 C6



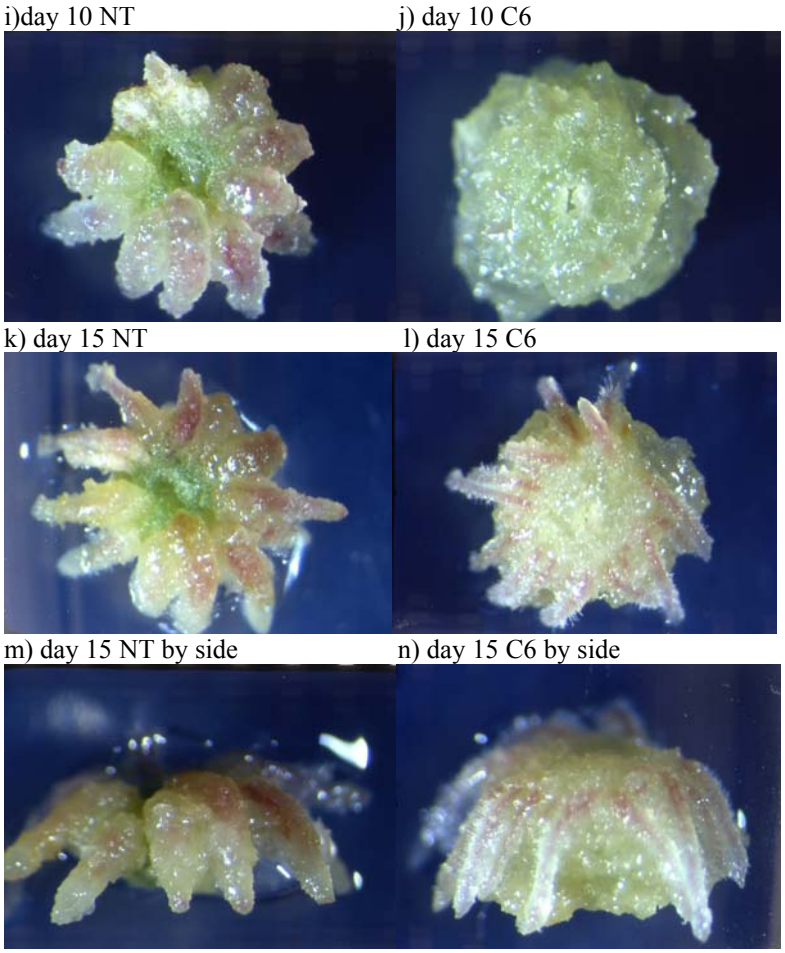



Fig.6. Adventitious root formation in *Malus* Jork 9 stem discs. The stem discs were incubated for 5 hours in the dark on the rooting medium containing 73.8 μM IBA and then transferred to hormone free rooting medium . The same stem disc from untransformed (NT) and the same stem disc from *rol*B-transformed clone 6 (C6) were pictured starting from day 5, when roots (the pink colour) first began to protrude from its basal surface, until day 15. Day 5 (magnification x 40), days 7, 8 and 9 (magnification x 32), days 10 and 15 (magnification x 25).

Expression of the auxin-induced adventitious rooting related oxygenase (*ARRO-1*) gene was examinated at 0, 24, 48 and 72 hours time-points in both *rol*B-transformed and untransformed Jork 9 stem discs and microcuttings. The untransformed material showed no expression in the non-IBA treated discs and high expression in IBA treated discs. In the rolB-transformed discs without IBA treatment, there was a low, but detectable expression of *ARRO-1* after 48 and 72 hours. The expression pattern of *ARRO-1* in microcuttings showed a high expression in IBA treated shoots from untransformed material and very low

expression in the untreated shoots. The *rol*B-transformed shoots showed very strong levels of *ARRO-1* expression in IBA treated material and also strong *ARRO-1* expression in non-IBA treated shoots. This suggests that *rol*B transformation has enhanced the constitutive expression of *ARRO-1*. As rooting occurred in this instance without the addition of exogenous auxin, it appears that *ARRO-1* expression may be rooting-specific rather than auxin-specific.

## Influence of IBA and aphidicolin on DNA synthesis and adventitious root regeneration from Malus Jork 9 stem discs (paper IV)

The sequence of cell division is thought of as a circuit, strictly controlled in a cell-specific manner. The cell cycle tends to be controlled by the entry into the S-phase. Hence, the G1-phase could be of various lengths depending on cell position (Barlow, 1973). There is evidence that cell size is important in determining when an arrested cell leaves G1 and enters the S-phase (John, Zhang & Dong, 1993). Although a higher degree of synchrony of rooting can be achieved, by appropriate choice of carbohydrate source, auxin concentration and time of exposure (Pawlicki & Welander, 1995), there is still a problem with synchronized rooting. For this reason in paper IV we have attempted to improve synchrony of root regeneration through temporarily blocking DNA replication that occurs during dedifferention prior to the first mitoses by applying aphidicolin (AD), an inhibitor of DNA polymerases-α and -δ (Wang, 1996). This inhibitor has been used for the synchronization of cell cultures by temporarily blocking entry of cells into the S-phase (Villemont *et al.*, 1997). The root induction treatment by auxin was interrupted for 24 h by AD application . Besides scoring the numbers of roots per disc, the effects of different treatments with and without AD were studied at the cellular level, by visualising DNA replication through supplying the thymidin analog 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) and subsequent immunolocalisation of BrdU-labelled DNA.

Three different concentrations of AD were added to the rooting medium: 5 μM, 15 μM and 30μM and compared for their effects on root formation. Concentration of 30 μM AD in the medium had a slightly inhibiting effect on rooting process compared to control as well as 5 and 15 μM AD. Among those last two concentrations, 15μM AD resulted in more roots per rooted disc and at the same time more roots appeared during the first 10 days.

BrdU labelling experiments confirmed that 24 hours application of 15 μM AD effectively inhibits DNA synthesis in apple stem discs. It was also shown that this inhibition is reversible, and more than 4 hours after withdrawal of AD cells eventually enter S-phase. Histological investigations have revealed that adventitious roots originate from the vascular zone of apple stem discs and microcuttings. In the case of stem dics, roots are initiated in the vascular zone close to the basal cut surface (Jasik & De Klerk, 1997). This is exactly the region in which the maximum proportion of BrdU-labelled nuclei was detected in the present study. Altogether, the results strongly suggest that AD application causes

an initial synchronization of those cells, thereby leading to more synchronized initiation and outgrowth of root primordia.

Apart from confirming the inhibitory effect of AD on DNA synthesis, BrdU labelling also revealed temporal changes in the competence of explants to respond to exogenously applied auxin. Thus, it is shown that the proportion of replicating nuclei present during 28-32 hours is significantly increased in the split IBA treatment (0-4 hours IBA, 24 hours BM (the basal rooting medium) and 28-32 hours IBA + BrdU; treatment C3), compared with a single IBA application during 0-8h (0-8 hours IBA, 20 hours BM and 28-32 hours BrdU; treatment C3.1). This clearly shows that during the first hours of culture, apple stem discs have lower competence to respond to exogenously applied auxin, as has been reported previously for microcuttings (DeKlerk *et al.,* 1995).

# Conclusions

From an economic point of view, rooting is an important phenomenon for horticulture, agriculture, and forestry. Adventitious root formation is a key step in vegetative propagation, which is widely used to multiply elite plants obtained in breeding programs or selected from natural populations (Hartmann, Kester & Davies, 1990). Losses occur because often cuttings do not form roots. From a scientific point of view, rooting is a highly interesting developmental pathway. It is one of the three main routs of organ regeneration beside adventitious shoot formation and somatic embryogenesis. In all three, cells undergo dedifferentiation and again become meristematic.

In spite of the considerable progress in the understanding of rooting, our knowledge is still fragmentary and circumstantial. More research in the area of molecular biology is required to further clarify the mechanisms of rooting and to develop new rooting treatments. Just as in biochemical studies, molecular examinations suffer from the complication that only a very few cells in an explant are directly involved in regeneration (De Klerk, 1996). On the molecular level, various approaches may be used to study adventitious root formation and the present work is a small contribution to this research.

Up to now the apple rootstock Jork 9 has been very recalcitrant to transformation. Several initial experiments have been performed using different types of *Agrobacterium* strains and vectors without any success. Successful transformation was obtained using the *Agrobacterium tumefaciens* strain C58C1(pGV3850)(pB-BGus). The high transformation frequency of 6.5% was the result of optimization of the regeneration and transformation protocol. The transformation with the *rol*B gene makes now Jork 9 even more attractive as a model plant, especially in studying gene expression during root induction. This is because of a higher sensitivity to auxin followed by increased rooting and higher number of produced roots. Most woody cuttings require the treatment of exogenous auxin to form adventitious roots. The use of genetically modified *rol*B plants abolishes the need for additional hormones in culture. This is a great advantage in studies of root inducing genes since gene expression during the induction phase of rooting is of most interest as genes controlling root initiation are most likely expressed during this phase. However, auxin, which is required for the induction of root meristems also induces the expression of many other genes, a large number of which are unrelated to the rooting process. Consequently, the task of isolating the genes that are specifically responsible for adventitious root initiation has proven difficult. Despite this, a number of rooting related genes have been isolated in recent years, one of which is the *ARRO-1* gene. The *ARRO-1* gene was isolated from auxin-treated stem discs cultures of the apple rootstock Jork 9, and is one of the first genes whose expression is known to be associated with the adventitious root formation process in woody plants. High levels of *ARRO-1* expression in non-IBA treated *rol*B transformed microcuttings suggests that *rolB* transformation has enhanced the constitutive expression of *ARRO-1*. As rooting occurred in this instance without the addition of exogenous auxin, it appears that *ARRO-1* expression may be rooting specific rather than auxin-specific. In response

to these findings, more direct analysis might involve the localisation of *ARRO-1* expression using in-situ hybridization. Verification of *ARRO-1* expression within root-initial cells would conclusively establish the relationship between *ARRO-1* expression and the formation of root meristem initials.

BrdU labelling experiments confirmed that 24 hours application of 15 µM AD effectively inhibits DNA synthesis in apple stem discs. It was also shown that this inhibition is reversible, and more than 4 hours after withdrawal of AD, cells eventually enter the S-phase. In the case of stem discs, roots are initiated in the vascular zone close to the basal cut surface. This is exactly the region in which the maximum proportion of BrdU-labelled nuclei was detected in our study. Altogether, the results strongly suggest that AD application causes an initial synchronization of cells, thereby leading to more synchronized initiation and outgrowth of root primordia. Apart from confirming the inhibitory effect of AD on DNA synthesis, BrdU labelling also revealed temporal changes in the competence of explants to respond to exogenously applied auxin. Our investigations clearly show that during the first hours of culture, apple stem discs have lower competence to respond to exogenously applied auxin.

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