Adventitious root formation in *Arabidopsis*: underlying mechanisms and applications

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Mehdi Massoumi

Thesis

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Chapter 1

General Introduction

Adventitious root (AR) formation: history and practical aspects

The root system of a plant consists of the primary root and lateral and adventitious roots. The primary root is initiated during embryogenesis, whereas the lateral and the adventitious roots are initiated post-embryonically from differentiated cells. Lateral roots (LR) develop from roots and adventitious roots (AR) from none root tissues *e.g.*, stem, or leaf cells.

"Adventitious" means "not expected or planned" (Cambridge Advanced Learners Dictionary) but this term seems not fully appropriate since AR formation is a normal developmental event in plants. It occurs naturally (in monocotyledonous after the primary root has died off), or is induced by stresses (wounding and flooding) (De Klerk *et al.,* 1999a). AR formation is highly important in horticultural practice when plants are propagated vegetatively as the capacity of plants to establish themselves successfully depends for the larger part on the ability to form new roots. Vegetative propagation is widely used in horticulture and forestry for multiplication of elite plants obtained in breeding programs or selected from natural populations (Hartmann *et al.*, 2011). AR formation is easy (monocotyledons), difficult or impossible (many woody crops) to achieve. If shoots are incapable of producing their own roots, grafting is being used as an 'escape' which is laborious and may suffer from problems like (postponed) incompatibility. Generally, the inability to form AR can have vast, negative economic consequences (estimated loss for the Netherlands is 25% in nursery crops and 5% in ornamental crops) (De Klerk *et al.*, 1999b). The total horticultural production in the Netherlands reached up to €8.6 billion in 2011 (Factsheet: Horticulture-Holland Alumni[1]). Bearing this in mind and considering that over 70% of the propagation systems used in horticultural industry depends on successful rooting of cuttings (Davies Jr *et al.*, 1994), a rough estimation for the economic loss would be € 0.9 billion per year in the Netherlands. Thus, research on AR formation is highly important from an economic point of view.

AR formation represents a transition of differentiated somatic cells into a new developmental pathway and the mechanisms underlying this switch are highly

[1] www.hollandalumni.nl/files/documents/career/factsheets-key-sectors/factsheet-horticulture

interesting. In rooting research, the big leap forward was made approximately 80 years ago. Briefly after the discovery of indoleacetic acid (IAA) in the 1920s, Thimann and Went (1934) reported that addition of this auxin brings about rooting of cuttings. A few years later, indolebutyric acid (IBA) and α-naphthaleneacetic acid (NAA) were synthesized chemically (Zimmerman and Wilcoxon, 1935). Nonetheless, IBA is also considered as a natural auxin as in some species *e.g., Arabidopsis*, IBA comprises up to 30% of the total free auxin pool (Ludwig-Muller *et al.*, 1993). It was found that the synthetic auxins are more effective, and that auxin can be applied via the basal cut surface of a cutting by a dip in "rooting powder" (talc powder with auxin). By the end of the 1930s Rhizopon, the first company producing rooting powder was established. The developed methods are satisfactory with 75% of the crops. Nonetheless, significant improvements are desirable to further reduce the economic losses. Researchers have attempted to develop new rooting treatments. In particular, different types of plant growth regulators have been examined (Davies *et al.*, 1994). Nonetheless, none of these efforts have resulted in development of new rooting treatments: cuttings are still rooted by dipping in rooting powder, by a short term exposure (a few seconds) to a solution with a high auxin-concentration, or by complete submergence for a short period of time in an auxin solution (Hartmann *et al.*, 2011).

In the overall process, though, significant practical improvements have been made. These concern selection and pre-treatment of the donor plants, adjustment of glasshouse and soil conditions after planting and propagation of adult plants via epicormic shoots (shoots originating from dormant axillary buds which have been formed when the plant was still juvenile and are therefore themselves juvenile) (Hartmann *et al.*, 2011).

Since the 1970s, a new propagation technology has emerged, *in vitro* propagation (or micropropagation). For many crops this has apparent advantages concerning speed of propagation and phytopathology. Shoots produced in micropropagation may be rooted *ex vitro* (like conventional cuttings) or *in vitro*. Some advantages of *in vitro* rooting as technique are (1) that the microcuttings gain considerably in weight during rooting so that they are less vulnerable and (2) that the survival rate and growth is better compared to *ex vitro* rooting. (3) It is also more cost effective for growers to

receive rooted, single shoots. The latter is highly important since individualization (excision of shoots from tufts) at nurseries is undesirable because excised shoots loose the capability to root within a few days. In addition, *ex vitro* rooting does not occur simultaneously in all cuttings. This will negatively influence the uniformity of the plants, and, therefore, rounds of sorting are needed which is laborious and causes additional costs.

Adventitious root formation: scientific aspects

Despite the major progress that has been made in understanding the physiological and molecular mechanisms controlling primary and lateral root development through studies in *Arabidopsis* (Osmont *et al.*, 2007; Petricka *et al.*, 2012; Ubeda-Tomás *et al.*, 2012), AR formation has been proven difficult to study. Fundamental research on rooting progresses very slowly because most research on the topic is usually done from a practical, applied point of view and is not targeted to an understanding of the underlying mechanisms. Moreover, because root induction occurs in only very few cells in an explant, and that processes in the thousands of surrounding cells are probably irrelevant for the rooting process, biochemical and molecular studies are complicated. Despite significant progress in studying crown root development in rice and maize (identification of the genetic determinants of root development as well as detection of quantitative trait loci for root development) (Coudert *et al.*, 2010; Hochholdinger and Zimmermann, 2008 and 2009), proper insight into the mechanisms regulating AR initiation and development in dicotyledonous species need further investigations. Nevertheless, during the past few decades some progresses have been made. Most importantly, rooting is envisaged as a multi-step developmental process (De Klerk *et al.*, 1999b). In addition, the role of plant hormones and their interaction as well as involvement of different exogenous factors for the individual steps have been studied. We summarize them in this chapter.

Rooting as a developmental process

Many researchers now recognize that rooting is a developmental process consisting of distinct steps each with its own hormonal requirements (Fig. 1, according to De Klerk, 2002a).

The rooting process consists of the following phases/steps:

Dedifferentiation

Dedifferentiation has been defined as the loss of previously developed characteristics (Wilson 1994). In the stem of some woody species like willow and poplar AR initials already exist in a dormant form (Hartmann *et al.*, 2011). However, in most of species, such initials are lacking and dedifferentiation of the cells to become AR is, therefore, an inevitable stage prior to induction. A consequence of this stage is the ability of plant cells to enter a new developmental pathway such as that required for the initiation of ARs. 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. He concluded that different factors *e.g.*, variation in lineage, age and relative position to other cells cause such differences. A support for his conclusion is the finding of Jasik and De Klerk (1997). They observed that amongst many cells dividing in response to auxin, only a small portion proceeds further to form a root meristem. In apple microcuttings it has been shown that during the initial 24 h after explant excision, they are not yet very sensitive to auxin and cytokinin. It is believed that this lag-period coincide with dedifferentiation during which cells become competent to respond to the rhizogenic stimulus, auxin (De Klerk *et al.*, 1999b). Wounding related compounds and ethylene were shown to stimulate entering this stage (De Klerk *et al.*, 1999a, b; De Klerk, 2002a). Auxin may indirectly promote this stage as it stimulates biosynthesis of ethylene (Imaseki, 1999). On the other hand, a low level of cytokinin is required (De Klerk *et al.*, 2001). In terms of root origin, it has been shown that the cells (root initials) are usually located between the vascular bundles (Ahkami *et al.*, 2013; Jasik and De Klerk, 1997; Naija *et al.*, 2008; Yoshida *et al.*, 2013; Wendling *et al.*, 2014) and accumulate starch during the initial 24 h (De Klerk *et al.*, 1999b).

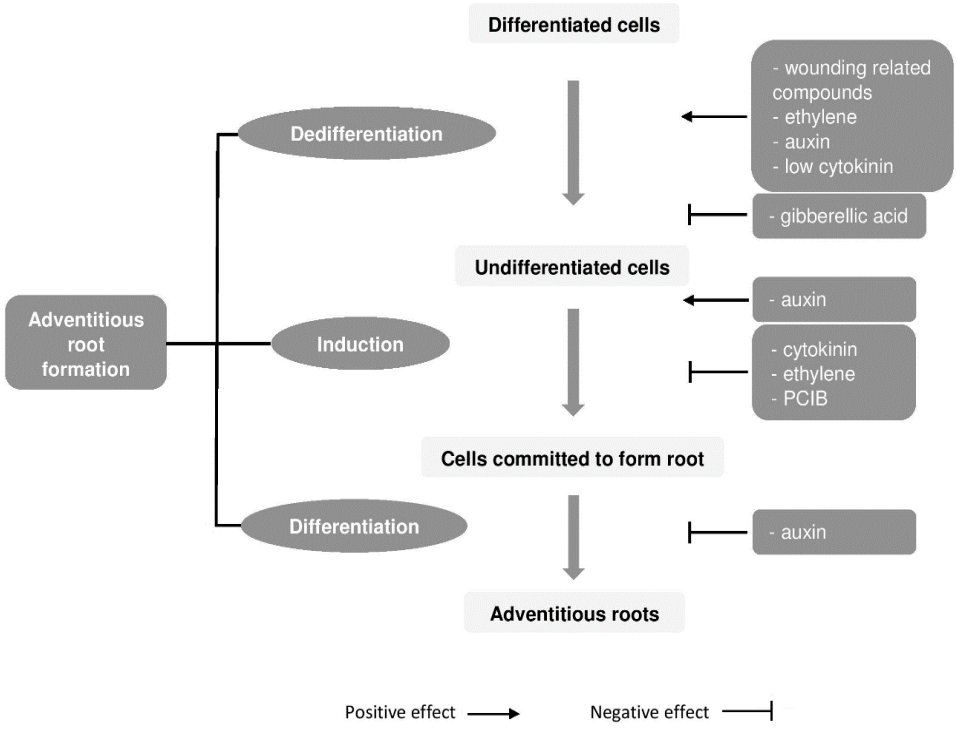

Fig. 1. Successive stages of rooting in apple microcuttings. The model is deduced based on a review by De Klerk (2002).

Induction

In this phase, competent cells respond the rhizogenic action of auxin and become committed to form root primordia. During this period, in apple up to 72 or 96 h, auxin pulses induce the highest number of roots (De Klerk *et al.*, 1999b). Auxin availability has been shown to stimulate establishment of a carbohydrate sink that serves as an additional stimulant for AR development (Agulló-Antón *et al.*, 2011). On the other hand, during this phase, a genuine anti-auxin (p-chlorophenoxyisobutyric acid, PCIB) and auxin-antagonists (cytokinins (CKs)) (De Klerk, 1995; De Klerk *et al.*, 1995) as well as ethylene are inhibitory (De Klerk *et al.*, 1999a, b). The inhibitory effect of ethylene may be related to its interference with the establishment of polarity in the meristem (De Klerk *et al.*, 1999a, b). At the histological level, changes in nuclear appearance, increased cytoplasmic density and organelle development as well as

degradation of starch grains were observed from 24-48 h. By 72 h, transverse division in the majority of cambial cells resulted in the formation of organized cell files. Finally, by 96 h, extensive division of cells in the interfascicular cambium leads to the formation of root meristemoids (De Klerk *et al.*, 1995; Jasik and De Klerk, 1997).

(Re) differentiation

During this stage, meristemoids start to differentiate into AR primordia. They further grow and penetrate the cortex before protruding from the basal surface of the stem disks (De Klerk *et al.*, 1995; Jasik and De Klerk, 1997; De Klerk *et al.*, 1999b). While auxin was favorable for the formation of meristemoids during induction, it was shown to be inhibitory during differentiation (De Klerk *et al.*, 1990). Furthermore, sensitivity to CKs has strongly decreased (De Klerk *et al.*, 1999b).

Endogenous factors influencing AR formation

AR formation is a complex heritable trait controlled by many endogenous and exogenous factors including light conditions, temperature, hormones (especially auxin), plant age, sugars, mineral salts and other molecules (Bellini *et al.*, 2014). They may function as signals and induce groups of cells to redefine their fate, resulting in and regulating AR.

Auxin

Auxin plays a key role in both AR and LR development (De Klerk *et al.*, 1999b; Lavenus *et al.*, 2013; Overvoorde *et al.*, 2010; Pop *et al.*, 2011). Its exogenous application has a consistent effect across the plant kingdom *i.e.*, *de novo* formation of roots (Pacurar *et al.*, 2014b). Cuttings in many species *e.g.*, in *Pisum sativum* and *Populus spp.*, develop ARs without the addition of auxin (Nordström and Eliasson, 1991; Rigal *et al.*, 2012). In these plants, though, endogenous auxin produced in the apex is transported basipetally to the cut surface and acts as rooting stimulus. Therefore, even in these cuttings, rooting is auxin dependent.

The most studied natural auxins are IAA and IBA. Synthetic auxins *e.g.*, NAA, 2,4-D (2,4-Dichlorophenoxyacetic acid), dicamba and picloram are available, but from

these, only NAA is effective whereas the other synthetic auxins mainly induce callus. In commercial production, IBA is the most commonly used rooting hormone (Hartmann *et al.*, 2011) probably because of its greater stability and higher root-inducing capacity compared to IAA (Zimmerman and Wilcoxon, 1935). It has been shown that various auxins cause different rooting responses (Massoumi and De Klerk, 2013; Verstraeten *et al.*, 2013). The reason might be a difference in the affinity of applied auxin for auxin receptors. For example, NAA shows a lower binding affinity to the auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1) (Kepinski and Leyser, 2005; Badescu and Napier, 2006; Spartz and Gray, 2008) as compared to IAA. However, the lower binding affinity does not correlate with its activity which suggest that the observed differences between the various auxins are most likely due to induction of a different signal transduction pathway (Verstraeten *et al.*, 2013). Moreover, different responses may also be related to the actual concentration of free auxin that reaches the target cells which is dependent on various factors, *i.e.*, uptake, transport, oxidation and conjugation.

First, the various auxins are taken up differently. For example, uptake of NAA and IBA are much faster than IAA in tobacco explants and apple shoots, respectively (Peeters *et al.*, 1991; Van der Krieken *et al.*, 1993).

Second, the metabolism of various auxins is different. In apple shoots, IAA is degraded faster than IBA (Van der Krieken *et al.*, 1993). There are two major pathways of conversion that inactivate the auxin: oxidation and conjugation. While NAA is not oxidized, IAA and to a lesser extent IBA, may be inactivated irreversibly by oxidation (Epstein and Ludwig-Muller, 1993). In contrast, conjugation is a reversible inactivation of auxin as the free auxin may be released from the conjugates (Smulders *et al.*, 1990). All auxins are conjugated.

Third, difference in transport of applied auxins can cause different rooting responses. It has been reported that IBA likely acts after its conversion to IAA in many species (Kurepin *et al.*, 2011; Schlicht *et al.*, 2013), however, the possibility of it acting as an independent auxin has also been discussed (Ludwig-Müller, 2000). Recent findings suggested that IBA uses its own specific transporters (PDR [PLEIOTROPIC DRUG RESISTANCE] family proteins, ABCG36 and ABCG37 [ATP-binding cassette

subfamily G]) when it is transported along great distances in plants (Strader and Bartel, 2009). Moreover, influx (AUX1) and efflux (PIN2, PIN7, ABCB1 and ABCB19) carriers are shown to transport IAA but not IBA (Strader and Bartel, 2009).

Depending on the propagation system, auxin may be applied for several days or weeks at a low concentration (μM range in micropropagation), or for several seconds or minutes at a high concentration (mM range in macropropagation) (Hartmann *et al.*, 2011). Auxin enters cuttings predominantly via the cut surface even in microcuttings that may have a poorly functioning epidermis (Guan and De Klerk, 2000). It is rapidly taken up in cells by pH trapping (because the pH outside the cell is relatively acidic (5.5), about 15% of IAA is in its protonated form. The protonated form is electrically neutral and can diffuse into the cell across the cell membrane) (Rubery and Sheldrake, 1973). IAA is also taken up by influx carriers (Delbarre *et al.*, 1996).

The endogenous level of auxin is largely dependent on its biosynthesis. Auxin is synthesized in most tissues (Ljung *et al.*, 2001; Marchant *et al.*, 2002) but in particular in young leaves and cotyledons (Ljung *et al.*, 2001). In *Arabidopsis*, mutations in the *SUPERROOT 1* (*SUR1*) and *SUR2* genes which are involved in the biosynthesis of indole glucosinolates (IGs), cause IAA overproduction (Mikkelsen *et al.*, 2004). It has been reported that *Arabidopsis superroot* mutants *sur1* and *sur2* (Boerjan *et al.*, 1995; Delarue *et al.*, 1998) and the dominant activation-tagged *yucca1* mutant (Zhao *et al.*, 2001), which all overproduce auxin, spontaneously generate AR on the hypocotyls of light grown seedlings. Over expression of *YUCCA1* gene in rice (*OsYUCCA1*) increases crown root formation (Yamamoto *et al.*, 2007). Reduced IAA biosynthesis negatively affects the number of ARs (Pacurar *et al.*, 2014a). These all indicate that biosynthesis of auxin is also important for AR formation.

Once auxin is synthesized, the plant uses a directional and active system to transport it from cell to cell using membrane-integrated carrier proteins (Takahashi, 2013). This transfer system which is referred to as polar auxin transport (PAT), is important for lateral and AR formation. Mutant analysis has helped to identify components of PAT, *e.g.,* influx and efflux carriers during different stages of LR and crown root development in *Arabidopsis* and rice (Coudert *et al.*, 2010; Lavenus *et al.*, 2013). Similarly, its importance during AR initiation in hypocotyl and stem cuttings of

several species has been shown (da Costa *et al.*, 2013; Sukumar *et al.*, 2013). Application of PAT inhibitors like naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) have been instrumental to evaluate the involvement of PAT during AR formation (Ahkami *et al.*, 2013, Ludwig-Müller *et al.*, 2005). AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) uptake permeases (influx), ATP Binding Cassette subfamily B (ABCB) transporters, and PIN-FORMED (PIN) carrier proteins (efflux) coordinate PAT (reviewed in Benjamins and Scheres 2008; Petrášek *et al.*, 2006; Zažímalová *et al.*, 2010). The direction of auxin flow is determined by the localization of these carriers (Fukaki and Tasaka, 2009).

There have been some studies to unravel the role of PAT and its components during the development of ARs (Brinker *et al.*, 2004; Xu *et al.*, 2005; Oliveros-Valenzuela *et al.*, 2008; Li *et al.*, 2012). However, they mainly focused on the expression of genes coding for the influx or efflux auxin carriers during AR formation and unlike for LRs, precise information on the molecular network controlling PAT during this process is lacking. Sukumar *et al.* (2013) have shown that ABCB19 (ATP-binding cassette B19) auxin efflux carrier plays a significant role in AR formation in *Arabidopsis* hypocotyls (Sukumar *et al.*, 2013). The involvement of auxin influx and efflux carriers during the mechanism of quiescent center (QC) cell establishment in AR tips of *Arabidopsis* has also been unraveled (Della Rovere *et al.*, 2013). Nevertheless, further investigation is still needed to fully decipher the molecular network of PAT that control AR formation.

It was mentioned earlier that metabolism and transport of auxin generate different levels of the hormones in plants parts or cells. This will consequently lead to different responses (Pacurar *et al.*, 2014b). One of the most investigated signaling pathways is the one that regulates the transcription of auxin-inducible genes. This pathway consists of different components, *i.e.*, nuclear auxin receptors TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEINs (TIR1/AFBs) and AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (AUX/IAA) family of transcriptional repressor proteins (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005). When a high level of auxin is available, auxin acts as a molecular glue between the two subunits of SCF complex (TIR/AFBs and AUX/IAAs) that adds multiple

ubiquitins to AUX/IAAs and targets them for degradation via the 26S proteasome system (reviewed in Takahashi, 2013). This consequently activates auxin response factors (ARF) resulting the expression of auxin-inducible genes (Kepinski, 2007). Whereas, in the absence or low concentrations of auxin, AUX/IAAs repressors form a heterodimer with ARFs and inhibit the expression of auxin inducible genes.

Research has unraveled some parts of auxin signaling networks during AR formation. For example, in *Arabidopsis* it has been shown that initiation of ARs is under the control of three different *ARFs* namely *AtARF6*, *AtARF8* and *AtARF17* (Gutierrez *et al.*, 2009). The authors reported that *AtARF6* and *AtARF8* are positive regulators of AR formation while *AtARF17* acts as repressor. They further showed that the balance between these repressing and activating factors is post-transcriptionally regulated by miR160 (targets *AtARF17*) and miR167 (targets *AtARF6* and *AtARF8*). Additionally, *Gretchen Hagen 3* gene, acts downstream of the *ARF*s and regulates AR initiation via the modulation of jasmonate-homeostasis (Gutierrez *et al.*, 2012). Lack of crown roots in rice mutants *crl1*/*arl1* (*crown rootless 1*/*adventitious rootless 1*) indicate a role for auxin signaling in the crown root initiation. The observed phenotype in this mutant is the result of altered expression of the auxin inducible *OsLBD3-2* gene encoding a LATERAL ORGAN BOUNDARIES domain (LBD) protein (Inukai *et al.*, 2005; Liu *et al.*, 2005). Similarly, mutation in the orthologue of the rice gene *ARL1*/*CRL1*/Os*LBD3-2* in maize, *rtcs* (*rootless concerning crown and seminal roots*), impairs the initiation of crown and seminal roots (Taramino *et al.*, 2007).

Other growth regulators

The involvement of different classes of phytohormones in the control of AR formation and their interaction has emerged from studies in different systems (intact plants, derooted seedlings or stem cuttings), and auxin likely interacts with nearly all the phytohormones (Pacurar *et al.*, 2014b and Fig. 2). Additionally, the interaction of these phytohormones during different stages of AR formation has been recently reviewed by da Costa *et al.* (2013). However, the interactions are different depending on the plant species or system utilized (Pacurar *et al.*, 2014b).

Ethylene was shown to have a positive effect on AR development and emergence in many species like mung bean, sunflower, *Rumex*, maize and rice (Drew *et al.*, 1979, 1989; Jusaitis, 1986; Liu and Reid, 1992b; Pan *et al.*, 2002). In tomato, treatment with the ethylene precursor, ACC (1-aminocyclopropane-1-carboxylic acid), and the *epi* mutation (elevated ethylene and constitutive ethylene signaling in some tissues) increase AR formation. On the other hand, in *Nr* mutants (*Never ripe*), with blocked ethylene response and delayed ripening, the number of ARs was reduced significantly (Clark *et al.*, 1999; Negi *et al.*, 2010). Application of IAA had no or little effect on AR formation in vegetative stem cuttings of *Nr* plants (Clark *et al.*, 1999). Similarly, reduced AR formation was reported in ethylene-insensitive transgenic *petunia* plants (Clark *et al.*, 1999).

Environmental conditions can influence ethylene synthesis and consequently AR formation. For example, increased AR formation upon flooding in some species is related to stimulation of ethylene production (Vidoz *et al.*, 2010; Visser *et al.*, 1996 a, b). Moreover, in deepwater rice it has been shown that ethylene facilitates AR emergence via induction of epidermal cell death (Mergermann and Sauter, 2000; Steffens *et al.*, 2006).

The effect of ethylene in inducing AR formation is often dependent on the presence of auxin. This is probably due to its interaction with auxin sensitivity and transport (Lewis *et al.*, 2011; Negi *et al.*, 2010; Riov and Yang, 1989; Růžička *et al.*, 2007; Strader *et al.*, 2010). On the other hand, ethylene biosynthesis is controlled by auxin and vice versa. In addition, ethylene also interacts with other phytohormones. The effect of ethylene in promotion of AR formation in deep water rice was shown to be co-stimulated by GA and inhibited by abscisic acid (ABA) (Steffens *et al.*, 2006).

Pacurar *et al.* (2014a) screened for suppressor mutants that produce fewer ARs than *sur2-1* (*superroot2-1*). Some of these mutants were identified as mutations in candidate genes involved in either auxin biosynthesis [*ASA1*/*WEI2*, *ASB1*/*WEI7* and *TRYPTOPHAN SYNTHASE BETA 1* (*TSB1*)] or signaling [*AUXIN RESPONSE 1* (*AXR1*), *SHORT HYPOCOTYL2*/*IAA3* (*SHY2*) and *RUB-CONJUGATING ENZYME1* (*RCE1*)]. Since *wei2* and *wei7* mutants have also been described as weak ethylene insensitive mutants (Stepanova *et al.*, 2007), this indicate an interaction between

ethylene and auxin at signaling level. In addition, mutation in *RCE1*, a gene required for a proper regulation of ethylene biosynthesis (Larsen and Cancel, 2004), causes deficiency in auxin and JA response (Dharmasiri *et al.*, 2005b) confirming the existence of a cross talk between ethylene and auxin. It seems that ethylene influences AR formation by altering auxin perception, as the suppressor mutants in the *RCE1* gene still retains the high IAA content of *sur2-1*.

Despite abundant reports on the promoting effect of ethylene on AR formation, it has also been shown that in some species ethylene has either no or even inhibitory effect (Geneve and Heuser, 1983; Mudge and Swanson, 1978). The observed contradictions may be related to the different experimental procedures *e.g.*, growth conditions, different plant tissues as well as methods of quantifying AR formation. As it was discussed in "rooting as a developmental process" section, the effect of ethylene is phase-specific; it acts as a promotor at early stage (dedifferentiation) and as an inhibitor at later stage (induction) of AR formation, respectively (De Klerk *et al.*, 1999a, b; De Klerk, 2002a).

Cytokinins (CKs) are a class of plant growth regulators involved in many plant processes, including cell division, shoot and root morphogenesis. They are known as auxin antagonists that suppress AR formation in some crops like poplar and rice (Kitomi *et al.*, 2011; Ramírez-Carvajal *et al.*, 2009). In addition, CKs modify the expression of PIN genes in such a way that auxin distribution and the formation of the required auxin gradient is hindered (Laplaze *et al.*, 2007; Růžička *et al.*, 2009). On the other hand, auxin negatively influences CK biosynthesis or transport. This has been shown for example in nodal stems of *P. sativum* and carnation cuttings (Tanaka *et al.*, 2006; Agulló-Antón *et al.*, 2014). Overexpression of cytokinin oxidase in tobacco and *Arabidopsis* resulted in an increased AR and LR formation (Werner *et al.*, 2001, 2003). In addition, increased ARs in transgenic tobacco overexpressing *ZOG1* (*O*-glucosyltransferase) gene, with reduced active CK level (Martin *et al.*, 2001), indicate an inhibitory role for CK during AR formation.

Despite the negative effect of CKs, there are some reports on use of low CK concentrations in combination with auxin in *in vitro* conditions to induce AR formation in microcuttings or thin cell layer (TCL) (De Klerk, 2002a; Leyser, 2006; Falasca *et*

al., 2004; Fattorini *et al.*, 2009) and it is possibly because of the involvement of CKs in stimulating cell divisions. Della Rovere *et al.*, (2013) also showed that CKs are important for the establishment of a functional meristem in both LRs and ARs.

Both strigolactone (SL) and GA negatively influence AR formation. In *Arabidopsis* and pea, AR formation increased in both SL-deficient and SL-response mutants (Rasmussen *et al.*, 2012b). In addition, exogenous application of synthetic SL analogues, GR24 and CISA (Cyano-Isoindole Strigolactone Analogue), strongly reduces AR formation (Rasmussen *et al.*, 2012b; Rasmussen *et al.*, 2013). SLs are shown to mitigate PAT (Bennett *et al.*, 2006; Crawford *et al.*, 2010) and, therefore, modulate the auxin level in the cells or tissues from which the AR originates.

Gibberellic acid (GA_3) inhibits AR formation in *Arabidopsis* hypocotyls (Mauriat *et al.*, 2014), poplar cuttings (Busov *et al.*, 2006) and in tomato (Lombardi-Crestana *et al.*, 2012). In addition, treatment with GA biosynthesis inhibitors *e.g.*, daminozide, paclobutrazol and triadimefon stimulate AR formation in mung bean hypocotyl cuttings and their effect was synergistic with IBA (Pan and Zhao, 1994).

Both GA biosynthesis and signaling seem to be involved in controlling AR formation. An ectopic increase of GA production in the stem of tobacco and rice reduces AR induction (Lo *et al.*, 2008; Niu *et al.*, 2013). Similarly, poplar mutants deficient in GA biosynthesis produced more ARs (Busov *et al.*, 2006). AR formation capacity is influenced in transgenic lines or mutants in which GA signaling is altered. For example, the tomato *pro* (*procera*) mutant (constitutively active GA signaling), has a very poor regeneration capacity in a root-inducing medium.

The negative effect of GA on AR formation is due to reduced PAT. This consequently reduces auxin availability required for the induction of cell division (Mauriat *et al.*, 2014; Niu *et al.*, 2013). Despite its negative effect, GA was shown to promote AR formation through interactions with ethylene in deep water rice (Steffens *et al.*, 2006). This discrepancy in the effect of GA might be due to the following: a different role of GAs during AR formation in different systems, and GAs may have different functions during various stages of the AR formation (Pacurar *et al.*, 2014b).

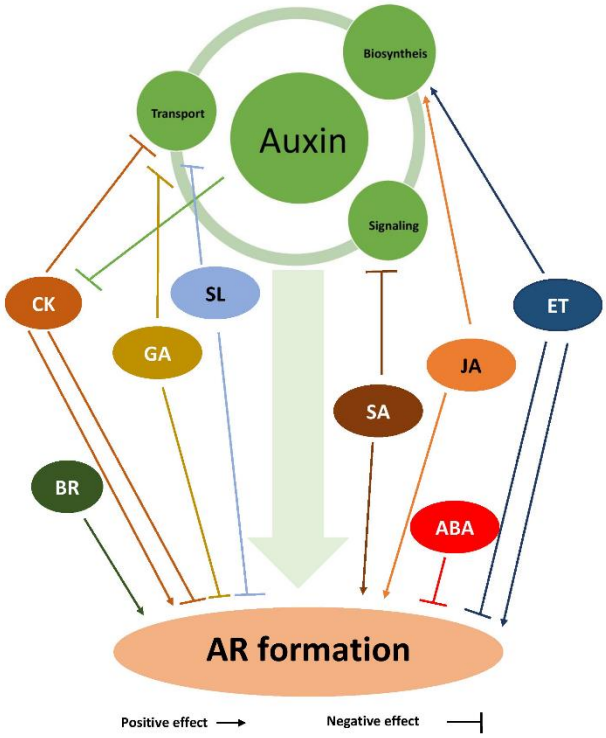

Fig. 2. Interaction of different phytohormones during the process of AR formation in cuttings. The effects of different phytohormones: abscisic acid (ABA), brassinosteroids (BR), cytokinin (CK), ethylene (ET), gibberellin (GA), jasmonic acid (JA), salicylic acid (SA), and strigolactone (SL) are shown. They affect AR formation either directly or via interaction with other phytohormones. Note that the model presented here is based on the finding from different species. Therefore, some of these interactions might be missing in some species and active in some others.

Extensive studies have described a negative role for abscisic acid (ABA) in regulating ARs, crown roots (CRs) and LRs. For example, in tomato mutants deficient in ABA (*flacca* and *notabilis*) AR formation on the stem substantially increased (Tal, 1966; Thompson *et al.*, 2004). Expression of genes involved in ABA biosynthesis reduced the number of ARs in *notabilis* mutants (Thompson *et al.*, 2004). ABA also negatively influences AR emergence in deep water rice (Steffens *et al.*, 2006). It delays ethylene-induced and GA-promoted programed cell death that facilitates root emergence (Steffens and Sauter, 2005; Thompson *et al.*, 2004). Despite a negative

effect of ABA on AR formation, in some species *e.g.*, *Vigna radiate* and *Hedera helix*, ABA promotes adventitious rooting (Chin and Beevers, 1969; Tartoura, 2001). This discrepancy might be the outcome of interaction between ABA and other phytohormones. In flooded rice plants, the altered balance between ethylene, GA and ABA upon submergence was shown to cause various adaptive responses *e.g.*, AR formation (Steffens *et al.*, 2006). This indicates an interaction network among these phytohormones in controlling AR formation.

In horticultural practices, AR formation is generally induced by stress (severance, change in the intensity or quality of light and etc.). This suggests that stress related hormones play a role during different stages of AR formation. A class of these hormones are jasmonates. They have shown opposite effects on AR formation depending on the plant organ. For example, Gutierrez *et al.* (2012) showed that in the *Arabidopsis* hypocotyls, jasmonic acid (JA) negatively regulates AR development through the *COI1* signaling pathway. In this system, auxin interacts with JA to control AR formation. This concerns regulation of JA homeostasis and negative regulation of JA signaling. On the other hand, a positive effect for JA during AR formation has also been suggested. For instance, it was reported that a transient JA accumulation at the cutting's base in *petunia* is critical for the rooting process by initiating sink establishment required for subsequent AR formation as well as increasing the level of cell wall invertases (Ahkami *et al.*, 2009). Similarly, it was shown that in JA-deficient *petunia* cuttings the number of AR decreased suggesting that jasmonates act as positive regulators of AR formation in *petunia* wild-type (Lischweski *et al.*, 2015). In addition, a positive role for methyl jasmonate (MeJA) in stimulating AR initiation in tobacco TCLs has been suggested (Fattorini *et al.*, 2009). JA interacts with auxin at different levels by modulating its biosynthesis, transport and signaling (reviewed in Wasternack and Hause, 2013). These opposite effects suggest that the interaction between auxin and JA, as for other phytohormones, is a crucial factor to be considered. Therefore, further investigation is required to fully address the role of JA during AR formation process.

Salicylic acid (SA) is another stress related hormone that has been shown to have both negative and positive effects on AR formation. De Klerk *et al.* (2011) showed that

application of SA to apple microcuttings during induction stage (24 to up to 96 h after excision) decreased AR formation. They showed that SA increases decarboxylation of IAA and as a result dose-response curve of IAA shifted to the right. In lavandin, it was shown that SA seriously impairs AR formation by a transient decrease in ethylene biosynthesis (SA impairs ACC oxidation to ethylene) (Mensuali-Sodi *et al.*, 1995). On the contrary, Wei *et al.* (2013), observed that treatment of mung bean hypocotyl with SA significantly increased AR formation in dose- and time-dependent manner. They showed that pre-treatment of mung bean explants with the scavenger of hydrogen peroxide (H_2O_2), N, N'-dimethylthiourea (DMTU), significantly reduces SA-induced AR formation indicating an interaction between SA and H_2O_2 in controlling AR formation.

Other endogenous hormones/factors

In addition to above mentioned phytohormones, other endogenous factors have also been identified to play a role in AR formation, such as calcium (Ca^{2+}) (Bellamine *et al.*, 1998), sugar (Li and Jia, 2013), phenolics (Rout, 2006), polyamines (Nag *et al.*, 2001), nitric oxide (NO) (Pagnussat *et al.*, 2002, 2003, 2004), carbon monoxide (Xu *et al.*, 2006), cyclic guanosine monophosphate (cGMP), mitogen-activated protein kinase (MAPKs) (Pagnussat *et al.*, 2003, 2004), wounding related compounds (Van der Krieken *et al.*, 1997), and peroxidase (Syros *et al.*, 2004). Some of these molecules may function in signaling and mediate auxin-induced adventitious rooting and auxin-response gene expression. The influence of these factors during AR or LR formation has been extensively reviewed (Bellini *et al.*, 2014; Ling *et al.*, 2015; Pacurar *et al.*, 2014b; Verstraeten *et al.*, 2014).

Plant age

Aging is one of the crucial endogenous factors that influences AR formation. Three types of aging have been defined in plants namely chronological, physiological and ontogenetic aging (Wendling *et al*., 2014a). Among others, ontogenetic aging has been extensively studied. It indicates the transition to the next developmental stage. For rooting, the phase change from juvenile to adult is important. These phases that can be

distinguished from one another by a number of morphological and physiological characteristics (Hackett, 1985); they occur in both woody and herbaceous species (Ballester *et al.*, 1999; Diaz-Sala *et al.*, 2002; Vidal *et al.*, 2003; Rasmussen *et al.*, 2015). The length of the juvenile stage can last for a few days or even years depending on the species (Poethig, 1990). For example, in herbaceous species the length of juvenile stage is shorter and the morphological and physiological changes associated with the phase transition are less distinct.

Maturation-related loss in AR formation potential has been reported in many plant species (Ballester *et al.*, 1999; Diaz-Sala *et al.*, 2002; Rasmussen *et al.*, 2015; Vidal *et al.*, 2003). However, the ability of mature plants to form ARs after undergoing rejuvenation in crops like apple (De Klerk and Ter Brugge, 1992) indicate that the loss of rooting potential experienced by mature tissues is not permanent and may be reversed. Different techniques *e.g.,* repeated subculturing of *in vitro* grown plants, repeated *ex vitro* pruning as well as sequential grafting of adult scions onto juvenile rootstocks have been shown to rejuvenate the mature plant materials (Wendling *et al.*, 2014b).

To unravel mechanisms underlying phase change, research was first performed at the morphological and anatomical levels. For instance, Ballester *et al.*, (1999) studied the rooting process in juvenile and mature chestnut (*Castanea sativa*) shoots. They observed no difference in anatomical characteristics between these shoots. Later, researchers attempted to find biochemical and physiological features, especially with respect to distinctive phytohormones. Although auxin is the central player for the induction of roots, the phytohormone does not seem to be the limiting factor during the maturation related decline in rooting potential. It has been shown that neither auxin uptake and metabolism nor its transport correlate with the differences in the capacity of cells to form ARs in *Pinus sylvestris* or *Pinus taeda* (Diaz-Sala *et al.*, 1996).

Advent of molecular research, however, made the difference between juvenile and adult tissues more apparent, in particular differences in DNA-methylation and expression of miRNAs. Although not absolutely consistent, transition from juvenile to adult coincides with increased methylation (hypermethylation) of DNA (Valledor *et al.*, 2007). Hypermethylation has been shown to play an integral role in regulating gene

expression, as a gene that is methylated is silenced and cannot be transcribed (Grant-Downton and Dickinson 2005). This is a possible cause for the maturation-related decline of rooting observed in woody and herbaceous plant species.

More recently, small RNAs (19–24-nucleotide RNAs) have been the center of attention. The transition of juvenile vegetative phase to the mature vegetative phase has been shown to be regulated by miR156 (Wu and Poethig, 2006). The expression level of miR156 is high in juvenile phase, whereas its expression decreases dramatically during vegetative phase change (Wu and Poethig, 2006). This small RNA controls the expression of *SBP/SPL* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*) transcription factors (Wu and Poethig, 2006). External factors have been shown to influence the level of miR156 in the plants. For example, exogenous sugar application reduces whereas leaf detachment and reduced photosynthesis increase the level of miR156 (Yang *et al.*, 2013). By monitoring flowering, it was concluded that increased expression of miR156 (by genetic engineering) delays the transition to the adult phase (Wu and Poethig, 2006; Chuck *et al*., 2007). Recently, Yu *et al*. (2015a) showed that *Arabidopsis* plants overexpressing miR156 produce more LRs than plants overexpressing its target mimic, MIM156, indicating a role for miR156 in lateral root development. However, it remains a question whether the loss of competence to develop ARs associated with the phase change is also under the control of miR156. This highlights a possibility for further investigations.

Exogenous factors influencing AR formation

Exogenous or environmental factors have been shown to influence the physiological and biochemical quality of the donor plants (Osterc, 2009). Therefore, and as rooting capacity of the cuttings is highly dependent on the quality of the donor plants (Geiss *et al.*, 2009), exogenous factors seem to be important when considering rooting in practice. Here, we briefly discuss some of these factors, in particular the effect of light and nutrients.

Light

Light is one of the most important environmental factors that control plant development (Alabadi and Blazquez, 2009). It has always been considered as an important parameter in vegetative propagation practices when optimizing conditions for rooting of cuttings (Bellini *et al.*, 2014). The different aspects of light, quality, intensity and duration, have been shown to influence the rooting of cuttings (Daud *et al.*, 2013). The outcome of such studies has indicated possible synergistic or antagonistic effects of light with plant growth regulators *e.g.*, auxin and CKs (Baraldi *et al.*, 1988; Fett-Neto *et al.*, 2001; Wynne and McDonald, 2002). For example, it was shown that light induction is necessary to induce AR in intact hypocotyls of *Arabidopsis* (Sorin *et al.*, 2005, 2006). In addition, it has been reported that light has a contrasting effect on the expression of *ARF* genes. While it has a positive effect on the expression of *ARF6* and *ARF8* (both positive controllers of AR initiation), it negatively regulates the expression of *ARF17* (negative controller of AR initiation) (Gutierrez *et al.*, 2009).

On the other hand, other researchers have focused on the effect of light versus darkness (etiolation), and etiolation was shown to stimulate rooting of cuttings (Klopotek *et al.*, 2010; Shi and Brewbaker, 2006). Etiolation causes anatomical, physiological and molecular changes. It has been attempted to relate the effect of etiolation with these changes (Maynard and Bassuk, 1988; Haissig and Davis, 1994; Hartmann *et al.*, 2011; Sorin *et al.*, 2005) but the mechanism is still not understood. A complicating factor is the broad spectrum of roles that sucrose, the product of photosynthesis, plays: energy source, building block and signal molecule.

Contradicting results have been reported for the effect of etiolation on change in endogenous IAA in cuttings. In *Chrysanthemum morifolium*, a reduction in rooting and IAA content was observed only after a prolonged irradiation period of donor plants (Weigel *et al*., 1984). An increased IAA level has been reported in etiolated stem parts of eucalyptus (Fett-Neto *et al.*, 2001), carnation (Agulló-Antón *et al.*, 2011) and pea (Koukourikou-Petridou, 1998). However, Kawase and Matsui (1980) concluded that etiolation did not affect IAA content in hypocotyls of *Phaseolus vulgaris* L. Additionally, light would affect the level of endogenous auxin either by influencing its

transport or by its metabolism into conjugates or via photo-oxidation (Naqvi and Gordon, 1967; Normanly *et al.*, 2004). It was recently demonstrated that the expression and/or localization of the auxin efflux carrier proteins PIN1, -2, and -3 (PIN-FORMED 1, 2, and 3) is regulated by light (Ding *et al.*, 2011; Sassi *et al.*, 2012). Sassi *et al.* (2012) observed that differential trafficking at the shaded and illuminated hypocotyl side aligns PIN3 polarity with the light direction, and presumably redirects auxin flow towards the shaded side and consequently hypocotyls bend towards the light.

Apart from a change in auxin level, biosynthesis of CKs (Agulló-Antón *et al.*, 2011; Bollmark and Eliasson, 1990), ethylene (Cao *et al.*, 1999; Koukourikou-Petridou 1998), flavonoids (Buer and Muday, 2004), abscisic acid (Grafi *et al.*, 1994), brassinosteroids (review in Symons and Reid, 2003) and carbohydrates (Agulló-Antón *et al.*, 2011; Baque *et al.*, 2010; Takahashi *et al.*, 2003) have also been reported to be affected in response to different light intensities. The possible influence of these hormones and their interaction during AR formation was discussed in the section "Endogenous factors influencing AR formation".

It has also been proposed that increased AR formation of cuttings by lower irradiation (shading, etiolation) is either because of arresting or because of reversing the ontogenetic aging (Hartmann *et al.*, 2011; Husen, 2008). Change in rootability of the explants caused by change in the light quality and/or quantity is, therefore, the result of a complex network.

Nutrients

Mineral nutrients, classified as macro- and micronutrients, have essential and specific functions in plant metabolism (Li *et al.*, 2009). Among the various nutrients, the effect of nitrate and phosphorous on plant growth and development has been studied in greatest detail.

Nitrogen (N), which is normally obtained in the form of nitrate, is one of the most essential macronutrients for the plants' growth and development. Nitrate can also serve as signaling molecule that regulates gene expression (Krouk *et al.*, 2010). Although nitrate has been shown to affect both LR initiation and development (Robinson, 1994; Signora *et al.*, 2001; Zhang *et al.*, 1999), the relation between

nitrogen-availability and AR development still needs to be evaluated. Nitrogen affects the formation of aerenchyma in AR and thereby improves the oxygen exchange (Drew *et al.*, 1989; He *et al.*, 1992). In addition, it is clear that modified nitrogen supply strongly affects carbon assimilation, allocation and partitioning within the plants. Therefore, it seems that the balance between carbon and nitrogen is an important factor influencing AR formation. Druege *et al.* (2004) showed that donor plants supplied with high nitrogen and high-light conditions had increased endogenous nitrogen content. This has consequently improved rooting of pelargonium and poinsettia cuttings when stored under low-light conditions. However, the effect of high nitrogen is highly dependent on the availability of carbohydrate, otherwise high nitrogen might have either no or inhibitory effects on AR formation (Druege *et al.*, 2004, Zerche *et al.*, 2009).

The effect of nitrogen supply in favoring AR formation has been shown in *Eucalyptus globulus* (Bennett *et al.*, 2003; Schwambach *et al.*, 2005). Recently, the effect of various nitrogen sources *e.g.*, nitrate, urea, and glutamic acid has been studied on AR formation and root branching in microcuttings of *E. globulus* (Schwambach *et al.*, 2015). The authors reported a positive effect for both nitrate and urea on AR development and root branching. In addition, they also observed a positive effect of all nitrogen sources on *ex vitro* acclimatization of rooted microcuttings.

In *Arabidopsis*, ANR1 (ARABIDOPSIS NITRATE REGULATED 1) transcription factor is a major component of nitrate signaling that triggers LR growth (Zhang and Forde, 1998). In addition, it has been shown that in rice, four homologous genes to *ANR1* are the target of miR444 (Sunkar *et al.*, 2005; Lu *et al.*, 2008; Wu *et al.*, 2009b; Li *et al.*, 2010). Whether similar signaling pathways are involved in AR formation in response to nitrate is unclear. However, Yan *et al.* (2014) showed that overexpression of miR444a promoted rice primary and AR growth, in a nitrate-dependent manner indicating a role for nitrate signaling in growth of ARs.

Phosphorous has also been shown to influence AR formation. During phosphorous deficiency, formation of ARs and its branching seems to be an efficient adaptive mechanism to maximize phosphate absorption. Low phosphate stimulates the initiation of new AR on the hypocotyl and lower stems (Lynch and Brown, 2001; Walk

et al., 2006; Williamson *et al.*, 2001). In contrast, there are reports that phosphate deficiency negatively influences AR formation. For instance, the density and the growth of ARs in microcuttings of *E. globulus* were significantly reduced in response to phosphate deficiency in the culture medium (Schwambach *et al.*, 2005). Similarly, Dag *et al.* (2012) showed that treatment of olive tree (*Olea europea* L.) donor plants with high phosphate significantly increased the rooting rate of the cuttings.

Different factors might be involved in response to phosphate *e.g.*, interaction with auxin perception, signaling and redistribution (Al-Ghazi *et al.*, 2003; Jones and Ljung, 2012; López-Bucio *et al.*, 2002, 2005; Nacry *et al.*, 2005; Pérez-Torres *et al.*, 2008). In addition, SL biosynthesis has also been shown to be directly regulated by the presence of phosphorous (Foo *et al.*, 2013; López-Ráez *et al.*, 2008; Yoneyama *et al.*, 2012).

Although AR and mineral nutrition are intimately related, no studies have attempted to characterize the effects of specific minerals on each of the three phases of the rooting process.

The importance of AR formation from both practical and economic points of view prompted us to perform research on investigating the mechanisms via which different factors influence AR formation. Understanding the underlying mechanism is the key for further improvements in solving problems associated with low AR formation capacity in many plant species.

Outline of this thesis

The scope of this thesis is to investigate and understand the mechanisms underlying AR formation in particular the role of PAT, the effect of plant age and the influence of some donor plant's pre-treatment. There are several lines of evidence leading to a central role for auxin in controlling AR and LR development (De Klerk *et al.*, 1999b; Lavenus *et al.*, 2013; Overvoorde *et al.*, 2010; Pop *et al.*, 2011). Furthermore, the interdependent physiological stages of the rooting process are associated with changes in endogenous auxin concentrations. Several factors have been shown to affect the concentration of free auxin that is reached in the ´target´ cells including auxin biosynthesis, uptake, transport and conjugation.

Use of the model organism *Arabidopsis thaliana* has advanced the study of root development because of the simple cellular organization of its roots and application of *in vitro* techniques make the analysis much easier. The outcomes of research over the last 25 years have generated substantial knowledge of *Arabidopsis* root development, which began with classical genetic experiments and has been accelerated by the use of modern molecular biology and genomics techniques *e.g.,* reverse and forward genetic approach, tissue or cell specific transcriptomic analysis. This has led to portray the molecular state of individual cell types, at different developmental stages, and in response to various stimuli (Petricka *et al.*, 2012).

In contrast to LR formation, progress in research of AR formation has been limited as most of the researchers have focused on the practical aspects of AR formation. Molecular and genetic aspects of AR formation, are still largely unexplored. Nonetheless, in the recent decade, researchers have attempted to unravel the mechanisms underlying AR formation. However, despite some progresses new rooting treatments have not emerged and, therefore, further insights in the rooting process are highly required.

Motivated by the recent developments in research on LR formation, we started research on AR formation by studying different factors which may have effect on the rooting potential of cuttings to elucidate their underlying mechanisms. We used several tools and different approaches to clarify the role of PAT and plant age, including reverse genetics, hormonal treatments as well as anatomical observations.

In chapter 2, we established a model for AR formation in *Arabidopsis* as a prerequisite for further investigations. Adventitious rooting of various explant types in particular hypocotyl, flower stem (FS) stems, rosette leaves (RL) under the application of different auxin types (IAA, IBA and NAA) were examined. We also set up the timing of developmental phases based on auxin and cytokinin requirement during rooting of FS explants. Microscopic observations also determined the main cellular origin of ARs in FS explants.

In chapter 3, the role of PAT and one of its components, the PIN family of transporter proteins, was investigated in *Arabidopsis*. Application of PAT inhibitors and an indirect promotor of PAT, fluridone (SL biosynthesis inhibitor), provided

evidence for a positive role for PAT during AR formation. Investigating mutants affected in long-PINs together with an anatomical study identified an explant-specific role for PINs. We also investigated the timing of the phases in which PIN-proteins exert their roles during AR formation.

In chapter 4, we examined the effect of the phase transition from juvenile to adult on adventitious rooting in *Arabidopsis* cultured *in vitro*. We identified a negative correlation between AR formation and plant age. Application of the hypomethylating agent, 5-azacytidine (AzaC), promoted root formation in adult explants but not in juvenile ones indicating that increased DNA methylation status upon aging negatively affects rooting of the cuttings. It also showed that hypomethylation can be seen as an efficient method to increase rooting potential of the adult plant materials. In addition, analyzing the rooting response of juvenile and mature explants of transformants *35S::MIR156* and *35S::MIM156*, respectively overexpressing and under-expressing miR156, and wild-type *Arabidopsis* showed that phase change-associated loss of competence to develop ARs is under the control of miR156.

In chapter 5, we investigate the influence of two donor plant pre-treatments (etiolation and flooding) on AR formation of *Arabidopsis*. Gene expression assays using qRT-PCR showed that the expression of SL biosynthesis genes is different in light and darkness. On the other hand, we found that a change in the level of endogenous soluble carbohydrates (ESCs) is associated with better rooting performance of etiolated plants. In terms of flooding-treated donor plants, anatomical studies contributed to a better understanding of potential reasons for better rooting of explants excised from flooding-treated donor plants.

Finally, in chapter 6, a summary and discussion of the most important results and future perspectives of the research on AR formation are presented.

Chapter 2

Development of a model system for adventitious root formation in *Arabidopsis thaliana*

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Abstract

Adventitious root (AR) formation is an essential step when crops are propagated via *in vitro* or conventional vegetative propagation techniques. Considerable progress has been made in the understanding of rooting from a physiological point of view. The availability of numerous *Arabidopsis* mutants has facilitated the understanding of developmental processes in plants. However, an efficient adventitious root model system in *Arabidopsis* is lacking and establishment of such a system is, therefore, needed prior to mutant analysis. In the present study rooting response of different explant types, in particular, hypocotyls, flower stems (FS) and rosette leaves (RL) were examined. The best performing explant, in terms of number of roots and root growth, was the hypocotyl followed by FS and RL explants, respectively. For the *in vitro* rooting of *Arabidopsis* hypocotyls, IBA and IAA proved the auxins of choice. IAA was the best performing auxin for *in vitro* rooting of both RL and FS explants.

We also investigated the timing of developmental phases in AR formation of FS explants based on their sensitivity to auxin and cytokinin. The results showed that induction occurs at 24 h after explant excision and the presence of auxin for 72 h is critical for AR formation in FS explants. Hardly any roots developed when the auxin was applied too late after excision, *i.e.*, beyond 72 h. In addition, microscopic observations in FS explants showed that the starch sheath cells adjacent to phloem are the main origin of ARs.

Key words: Adventitious root formation, *Arabidopsis thaliana*, explant type, anatomy, and developmental phases.

Abbreviations: adventitious root (AR), flower stem (FS), fluorescence activated cell sorting (FASC), laser capture microdissection (LCM), lateral root (LR), polar auxin transport (PAT), rosette leaves (RL), thin cell layer (TCL), wounding related compounds (WRCs).

Introduction

Adventitious root (AR) formation is indispensable for vegetative propagation of elite genotypes and difficult, if not impossible, to accomplish in many crops. Over 70% of the propagation systems used in the horticultural industry depends on successful rooting of cuttings (Davis and Haissig, 1994). However, the lack of proper progress in understanding the mechanisms underlying AR formation makes this research an important topic from both scientific and economic point of view. Rooting represents a transition of differentiated somatic cells into a new developmental pathway and the mechanisms underlying this switch are highly interesting. Although a major breakthrough in research of AR formation was made more than 80 years ago by the discovery of the effect of auxin (Thimann and Went, 1934), progress in research has been limited. Nevertheless, during the past few decades some progress has been made; rooting is envisaged as a developmental process consisting of different stages each with its own hormonal requirement (De Klerk *et al.*, 1999b). The role of plant hormones and their interaction as well as involvement of different exogenous factors *e.g.*, light, mineral nutrients and biotic factors have been studied in more detail (Bellini *et al.*, 2014; Geiss *et al.*, 2009; Pacurar *et al.*, 2014b).

Arabidopsis thaliana is an herbaceous plant with sporadic formation of ARs *in planta* (Worley *et al.*, 2000). Acquired knowledge on the overall genetics of *Arabidopsis* (The *Arabidopsis* Genome Initiative 2000) as well as availability of numerous mutants (King *et al.*, 1998; Konishi *et al.*, 2003; Sorin *et al.*, 2006) plus its short life cycle make it an attractive model plant for studies on adventitious rooting.

Various AR formation systems have been reported so far in *Arabidopsis* using different explant types. Ozawa *et al.* (1998) obtained ARs from hypocotyl segments using a two-phase method: pre-culturing on callus-inducing medium followed by transferring onto root-inducing medium. However, hypocotyls have a root like structure, having pericycle cells that provide the founder cells for lateral root (LR) development (Busse and Evert, 1999; Goldfarb *et al.*, 1998). So, it could be argued that the use of these explants in research of AR formation is not justified because of the high similarity to LR formation. Nonetheless, according to the definition of Esau (1960) which characterizes ARs as roots arising from above ground organs, root

formation from hypocotyl explants can be perceived as AR formation. This discrepancy has led to confusion in the *Arabidopsis* literature. Some authors have defined roots formed from hypocotyl explants as "adventitious" (King and Stimart, 1998; Ullah *et al.*, 2003; Worley *et al.*, 2000), whereas others *e.g.*, Malamy and Ryan (2001) refer to them as LR.

Apart from hypocotyls, other *Arabidopsis* explant types have been implemented in research of AR formation, such as thin cell layers (TCL) taken from flower stem (FS) (Falasca *et al.*, 2004), leaves and FS segments (Ludwig-Müller *et al.*, 2005) and de-rooted seedlings (da Rocha Correa *et al.*, 2012). However, in some cases the efficiencies are low and the methods are rather complex. For example, Ludwig-Muller *et al.* (2005) proposed a method for rooting of FS including several transient applications of IBA as exogenous auxin, followed by transferring the explants to hormone free medium at different time points. They determined rooting as percentage of rooted explants without counting the number of roots per explants. Therefore, *in vitro* rooting in *Arabidopsis* still needs further investigation.

The timing of phases in which auxin has its highest efficiency to bring about rooting is important for many reasons. For instance, molecular studies aimed at studying the changes in expression of genes involved in induction and initiation stage can benefit much from knowledge about this timing. De Klerk *et al.* (1995) established such a time frame in apple microcuttings. Knowledge on such timing is still lacking in *Arabidopsis* and needs to be established. In the present research, we first established a rooting system in *Arabidopsis* by testing the rooting response of different explant types under the influence of different auxin types in a broad range of concentrations. In addition, the timing of individual phases based on hormonal requirements was determined by application of auxin/cytokinin pulses.

In order to unravel cell cycle regulation mechanisms that result in the initiation of new ARs, detailed molecular studies are required. However, the small numbers of cells involved in the early events during AR initiation provide a challenge to such studies, however, successes have been reported (Taylor and Scheuring, 1994) and recent new technical developments open up a myriad of possibilities (Birnbaum *et al.*, 2003; Jiao *et al.*, 2009; Tang *et al.*, 2011). New approaches like tissue or organ-specific

transcriptional profiling has been used to identify genes that are transcriptionally regulated in that tissue or organ. However, the major setback with these approaches is that different organs consist of different cell types with totally different gene expression pattern. Therefore, when different cells are pooled, the RNA of cells of interest might be masked by the RNA of thousands of surrounding cells and the detection of the most interesting transcriptional changes would be nearly impossible (Dinneny and Benfey, 2009). Cell-specific transcriptional profiling has been improved to overcome this obstacle through laser capture microdissection (LCM) and fluorescence activated cell sorting (FACS) (Birnbaum *et al.*, 2003; Jiao *et al.*, 2009; Tang *et al.*, 2011). Microscopic analysis to detect the founder cells of AR is, therefore, a prerequisite of such approaches. In the current research, we performed a microscopic analysis in *Arabidopsis* FS to determine the cells where ARs originate from. FS compared to other explant types have a histological structure similar to cuttings or *in vitro* micropropagated shoots (Verstraeten *et al.*, 2013) and, therefore, would provide a better model system to study the adventitious rooting process in plants than hypocotyls that are structurally similar to root (Busse and Evert, 1999; Goldfarb *et al.*, 1998).

Materials and methods

Plant materials

Arabidopsis thaliana (Col-0) seeds (Lehle Seeds, Round Rock, USA) were surface-sterilized with 70% (v/v) ethanol for one minute followed by sodium hypochlorite 2% (w/v) for 10 min. Then the seeds were washed three times, each time 10 min, with sterilized distilled water. They were germinated in Petri dishes or containers (depending on the explant type) using half-strength MS basal salt mixture including vitamins (Murashige and Skoog, 1962), 3% (w/v) sucrose and 0.7% (w/v) Micro-agar (Duchefa, Netherlands). To synchronize germination, the seeds were first stratified in the dark for 3 days at 4^{o}C. Then they were transferred to 20^{o}C under long day (16 h light/8 h dark) conditions (30 µmol m^{-2} s^{-1}, Philips TL33).

Explant type

We examined the rooting response of four different explant types, *viz.*, 10 mm-long hypocotyl, 10 mm-long root segments, 5-7 mm-long and node-free FS segments and finally excised rosette leaves (RL). Hypocotyl explants were taken from etiolated seedlings that were prepared in the following way. We first incubated the Petri dishes containing seeds vertically in the growth chamber in the dark for 12 days. In this way seedlings grow up alongside the medium surface. Then after 12 days the seedlings were de-rooted and decapitated. Ten millimeter hypocotyl segments were placed horizontally on the surface of the rooting medium. Similarly, 10 mm-long root segments were taken from root system of 12 day-old seedlings.

RL and FS segments were taken from 5 week-old plants according to Ludwig-Müller *et al.* (2005). Five to seven millimeter node free FS segments were used as explants. They were placed in rooting medium like hypocotyl explants, but the only difference was that they were pushed slightly into the medium. We found that if they are fully submerged the rooting response drops dramatically. RL explants were taken from the vegetative adult part of the rosette according to morphological markers described by Wu *et al.* (2009a). Based on these markers, the adult leaves, mainly located at apical part of the rosette, are elongated, serrated, and produce trichomes on their abaxial sides.

Rooting treatment

Different auxin types, *i.e.*, Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) in a range of 0-100 μM were applied. Considering that most of the auxins are sensitive to photo-oxidation and auxins are only required during the first few days after explant excision (De Klerk *et al.*, 1990), we kept the cultures in darkness for the first week of the rooting treatment and after that the explants were transferred onto new MS medium without auxin and into the light. Rooting was determined at the indicated times as percentage of rooted explants and as mean number of roots per explant.

Timing of phases

To establish the timing of the auxin sensitive phase, FS segments were transferred at the indicated time into the medium containing auxin and after 72 h back to auxin-free medium. In our preliminary experiments IAA (30 µM) was first used as 24-h pulses. We selected IAA as it was the best performing auxin for *in vitro* rooting of FS explants (see Fig. 2A). However, rooting was scarce and am increased concentration (up to 100 µM) or an increased pulse duration (up to 72 h) did not improve the rooting of FS explants. Therefore, for this experiment IBA, a more stable auxin compared to IAA, was applied. The concentration of IBA and its duration were 100 µM and 72 h, respectively.

The timing of the cytokinin (CK) sensitive phase was also established. FS segments were transferred at the indicated time onto medium containing both IBA (100 µM) and 6-Benzylaminopurine (BAP) (30 µM). After 24 h, the cultures were transferred back onto medium containing only IBA (100 µM). The selected BAP concentration is based on our preliminary experiments in which a wide range of BAP concentrations was applied. The results showed that when BAP was applied at 10 µM and higher rooting was inhibited. We chose 30 µM for further experiments (data not shown).

Histological analysis

At different time points (1, 3, 5 and 7 days) after auxin treatment (IAA 30 µM), FS segments of *Arabidopsis* were fixed in 5% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. Plant materials were then rinsed four times (15 min each) in 0.1 M phosphate buffer (pH 7.2) followed by four times rinsing in demi-water (15 min each). Then the materials were dehydrated in a gradient series of ethanol (v/v: 10, 30 and 50% each for 15 min, 70, 90% and absolute ethanol for 2 h each step) before processing further with glycol-methacrylate-based resin (Technovit 7100, Heraeus-Kulzer Technik, Germany). Infiltration in Technovit was performed according to the manufacturer's instruction. Sections (5 µm thick) were cut with a rotary microtome, mounted onto glass slides, dried on a heater (60 °C) and stained with 0.25% (w/v) toluidine blue in distilled water.

Statistical tests

For all rooting experiments, three repeats each with 10 explants were used in every treatment. The means ± SE are given in the graphs. The significance of difference between root numbers was determined with a Student *t*-test and between the percentages with a χ^2- test. All the experiments were carried out at least twice.

Results

Development of a model system

Rooting of rosette leaves (RL)

Fig. 1 shows the response of RL to different concentrations of IAA, IBA and NAA.

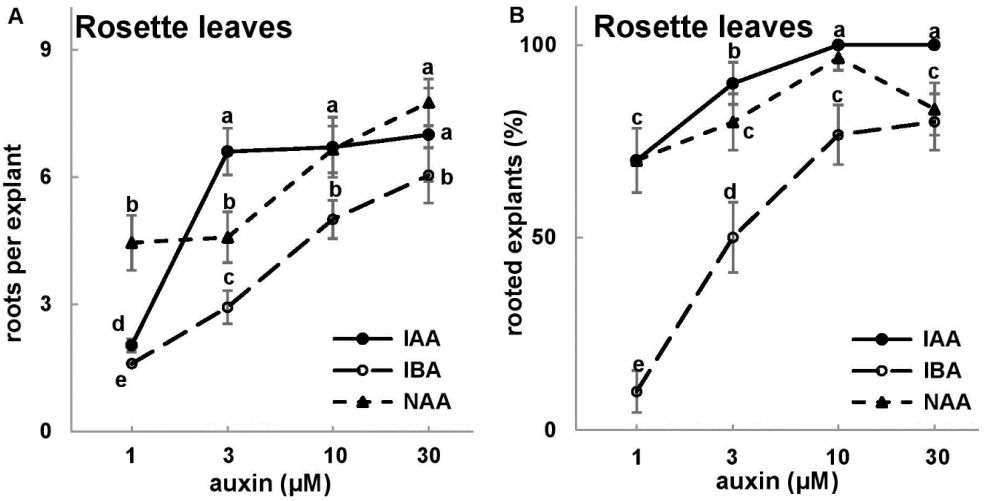


Fig. 1. Rooting of *Arabidopsis* RL after 7 days' exposure to a range of IAA, IBA and NAA concentrations. A) Number of roots per explant B) rooting percentage. Error bars (SE) represent error range of three biological replicates. Different letters represent means that are significantly different at $P < 0.05$.

The highest rooting response, with respect to the number of roots (~ 8 ± 0.55, Fig. 1A) and the rooting percentage (100%, Fig. 1B), was observed with NAA and IAA, respectively. Except for IAA, NAA and IBA showed similar dose-response

curves. In general, the effect of NAA and IAA on root induction was higher than IBA. ARs regenerated from the cut surface of the RL in contact with the medium (Supplementary Fig. S1). The appearance of the RL was also different: growth of RL was poor in NAA-treated explants and they turned to yellow/brown at the end of the experiment. Callus formation was stimulated by NAA and not by IBA or IAA. Furthermore, the maximum number of roots was observed over a wide range of IAA concentrations (3, 10 or 30 μM) indicating that the cells in RL are more sensitive to IAA compared to the other tested auxins. IAA can, therefore, be seen as the auxin of choice for rooting of RL in *Arabidopsis*.

Rooting of flower stem (FS) segments

The response of *Arabidopsis* FS segments to *in vitro* rooting treatments is shown in Fig. 2. In terms of number of roots per explant, IBA was the best auxin, followed by IAA and NAA respectively (Fig. 2A). All three auxins showed a bell shaped response curve with optimum concentration of 30 μM for IAA and IBA, while 3 μM was recorded optimal for NAA. The percentage of rooting was close to 100% or 100% with the exception of 3 and 10 μM IBA (Fig. 2B). Roots emerged directly from the cut surface of the explants.

Phenotypic observations showed that the type and concentration of applied auxins strongly affected the quality of the root system at the end of the rooting period. For example, high concentrations of NAA strongly inhibited the growth of roots; root length decreased and the roots were covered with many root hairs in comparison to lower concentrations. Less inhibition of growth occurred on IBA and IAA-containing medium (Fig. 3). The length of the longest roots at the optimal concentrations for rooting was 20 mm (NAA), 32 mm (IBA) and 28 mm (IAA).

Because the maximal number of roots in FS explants was reached over a wide range of IAA concentrations but was restricted to only higher concentrations of IBA (30 and100 μM) (Figs. 2 and 3), IAA is proposed as the best performing auxin for this type of explant.

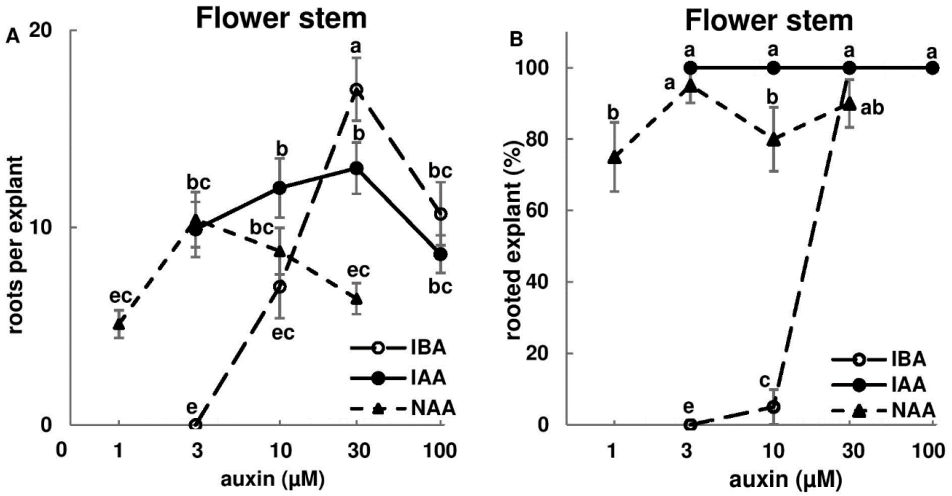


Fig. 2. Rooting of *Arabidopsis* FS segments after 7 days' exposure to a range of IAA, IBA and NAA concentrations. A) Number of roots per explant B) rooting percentage. Error bars (SE) represent error range of three biological replicates. Different letters represent means that are significantly different at $P < 0.05$.

Fig. 3. Rooting of *Arabidopsis* FS at various concentrations of, IBA (A-E), IAA (F-J) and NAA (K-O). Concentrations for top two rows from left to right are 0, 3, 10, 30 and 100 µM, respectively. For the bottom row concentrations from left to right are 0, 1, 3, 10, and 30 µM. Scale bar applies to all pictures: 5 mm.

Rooting of hypocotyls

Fig. 4A shows the response of *Arabidopsis* hypocotyls to *in vitro* rooting treatments. The highest number of roots was observed in IBA, followed by NAA and IAA, respectively. These were achieved at 100 µM for both IBA and IAA, but at 10 µM for NAA, indicating a higher effectiveness of NAA compared to other applied auxins. Except for NAA (especially at high concentration), the induction of ARs was not accompanied by callus formation. Regardless of the type of applied auxin, the percentage of rooting was always very close to 100% or 100% indicating a very high sensitivity of hypocotyl cells to respond to the stimulating action of auxin.

At higher concentrations of auxins, all three auxins were similarly effective. However, since the rooting process was accompanied by callus formation and the roots were stunted or covered with more root hairs when NAA was used, we suggest IBA and IAA as the best hormone for rooting of hypocotyl explants.

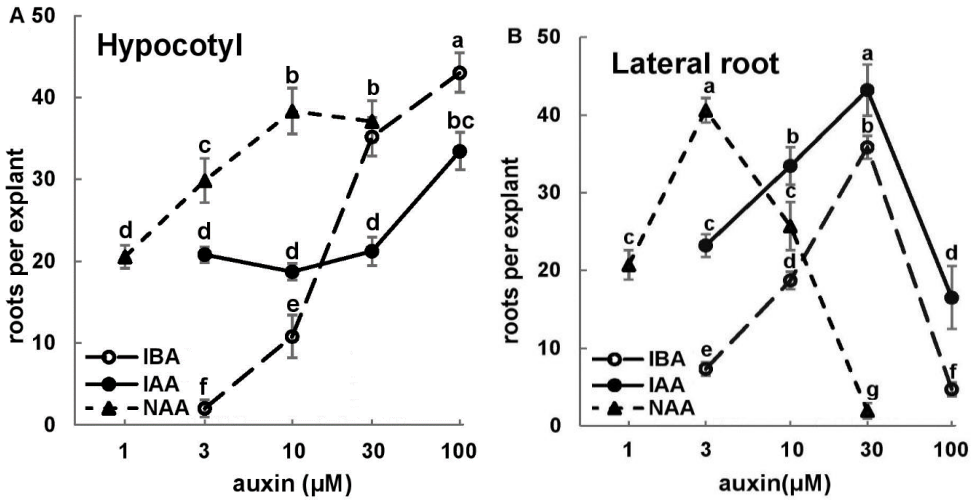


Fig. 4. Rooting of *Arabidopsis* A) hypocotyl and B) root segments after 7 days' exposure to a range of IAA, IBA and NAA concentrations. Error bars represent the standard error of three biological replicates. Means are presented with SE. Different letters represent means that are significantly different at $P < 0.05$.

According to Busse and Evert (1999), the larger part of the hypocotyl is root-like in structure. In order to check the similarities in number of produced roots, we studied the formation of LR from root segments. The results are presented in Fig. 4B. Both root and hypocotyl explants produced nearly the same amount of roots. However, for root explants, rooting response dropped profoundly at highest concentration of applied auxins. Moreover, the concentration at which the highest number of roots was observed for all three auxins was clearly lower in root explants compared to hypocotyl explants.

The position of roots on the explant

In order to determine the position in an explant where ARs originate from, we marked two ends separately (basal vs. apical end) in horizontally cultured FS explants. In this way the explants were able to absorb nutrients and growth regulators from both ends. The explants were classified into three different groups namely those rooted only at basal ends, those rooted at both ends and those rooted alongside the explants. The root formation was determined as rooting percentage (Fig. 5) and number of roots per explant (Supplementary Fig. S3) for each group. Comparison of number of roots generated at different ends (Supplementary Fig. S3) indicated that in all tested concentrations of the three auxin types, basal ends showed a higher rooting competence than apical ends and that this difference is more obvious at lower concentrations of applied auxins.

At low concentrations of IBA, root induction in FS explants took place only at basal ends. Gradually, with increasing concentrations of IBA, the percentage of explants which showed rooting at both ends increased up to 90% (Fig. 5B). At the lowest concentration of the other two auxins (NAA and IAA) nearly 50 % of the explants produced roots at basal end and 50 % at both ends. At higher concentrations almost the same trend as IBA was observed and the percentage of explants rooted at both ends reached its highest amount (90% for IAA and 65% for NAA, Fig. 5A and C).

Regarding hypocotyl explants, at low concentrations of auxin root induction occurred at both ends (Supplementary Fig. S2B) in about 75% of the explants. Increase in auxin concentration promoted root formation not only in both ends but also in other sites and alongside the explants (Supplementary Fig. S2A).

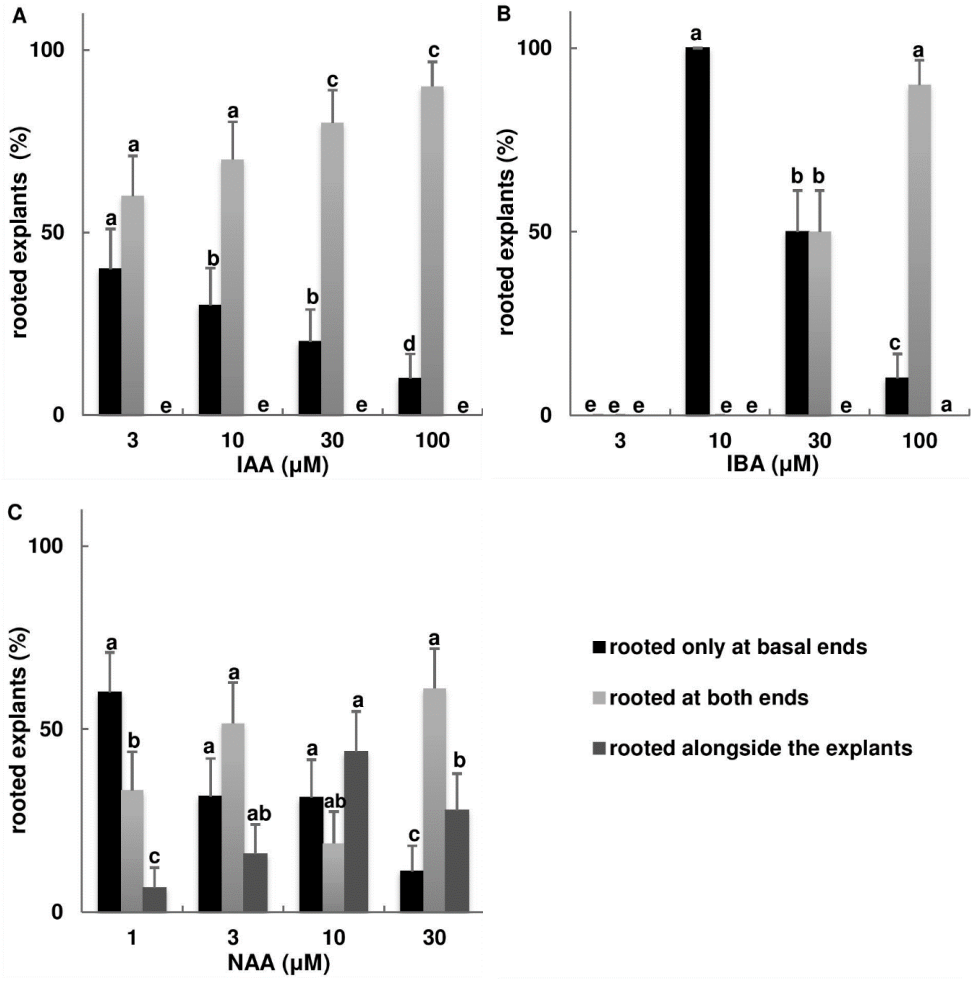


Fig. 5. Root formation at different positions in FS explants. Percentage of rooted explants at different positions at various concentrations of A) IAA, B) IBA and C) NAA. The explants were categorized in three groups; namely explants with roots at basal ends, at both ends, and lastly at alongside the explants (including basal and apical ends). Error bars represent the standard error of three biological replicates. Different letters shown for different categories in each concentration represent means that are significantly different at $P < 0.05$.

Timing of phases in rooting of *Arabidopsis* FS explants

In order to determine the timing of auxin/cytokinin sensitive phases in rooting of *Arabidopsis* FS segments, the explants were exposed to auxin (IBA, 72 h) or cytokinin (BAP, 24 h) pulses at different times. The results are presented in Fig. 6.

We first applied 24 h IBA pulses but the rooting response was negligible (data not shown). Hence, we increased the auxin exposure time to 72 h (Fig. 6A). The best rooting response was observed at the time interval of 0-72 h and 24-96 h. However, when 72 h pulses were applied from 48 h onward, rooting dropped significantly ($P < 0.001$) and reached its lowest value ($\sim 2.6 \pm 0.45$) at 96-168 h time interval. It can be, therefore, concluded that auxin is critical during the early stage of AR formation from 24-48 h.

As a negative control, we applied 24 h BAP pulses (Fig. 6B). The first decline (not significant) in rooting response of FS explant was observed at 24-48 h pulse and it reached to its lowest value at 48-72 h pulse. Then the rooting response showed increase and was highest at 96-120 h.

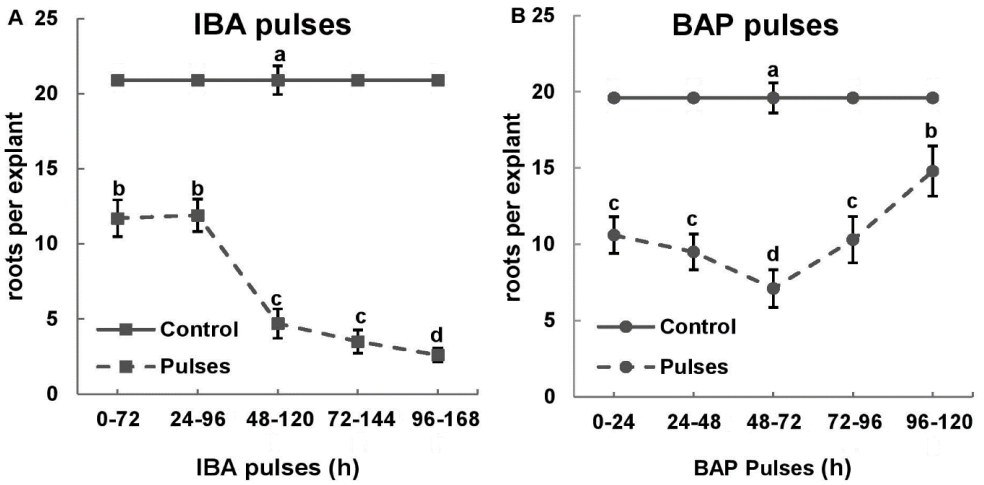


Fig. 6. Rooting of *Arabidopsis* FS explants after IBA or BAP pulses at the indicated times. A) FS explants were cultured on IBA free medium and received a 72 h IBA (100 µM) pulse at the indicated times. In control, FS segments were continuously (168 h) treated with IBA. B) FS explants were cultured on medium with 100 µM IBA and received a 24 h BAP (30µM) pulse at the indicated times. Control represents the FS explants that were treated continuously (120 h) with IBA (100 µM).

Origin of AR in FS explants

The first visible morphological changes were observed at 72 h after auxin treatment (Fig. 7) when both ends were swollen. The anatomical structure of the *Arabidopsis* FS has been addressed previously and consists of one row of epidermis cells as outermost layer and few rows of cortex cells. The innermost cortical cell layer has been reported as a starch sheath. Remaining cell layers toward the center of FS are interfascicular tissues, phloem and xylem cells which are separated by procambial cells. Protoxylem with parenchyma constitutes the innermost part of the vascular bundle. Different cells are indicated in Fig. 7B.

No significant changes were observed at the anatomical level after one day exposure to auxin compared to day 0. At day 3, however, we observed cell expansion and enlargement (mainly cortical cells) (Fig. 7E), with mitotic activity at cells adjacent to the phloem.

At 5 d after auxin treatment, the population of dividing cells in areas close to the phloem cells can be easily seen (Fig. 8G & H). In longitudinal sections root primordia are clearly visible (Fig. 7I).

Finally, after 7 days of auxin treatment, root primordia are formed and commenced outgrowth with a massive division of cells (Fig. 7J-L). Intriguingly, formation of root primordia from epidermis cells (Fig. 7L) was observed in some explants indicating that these cells can also act as AR initials.

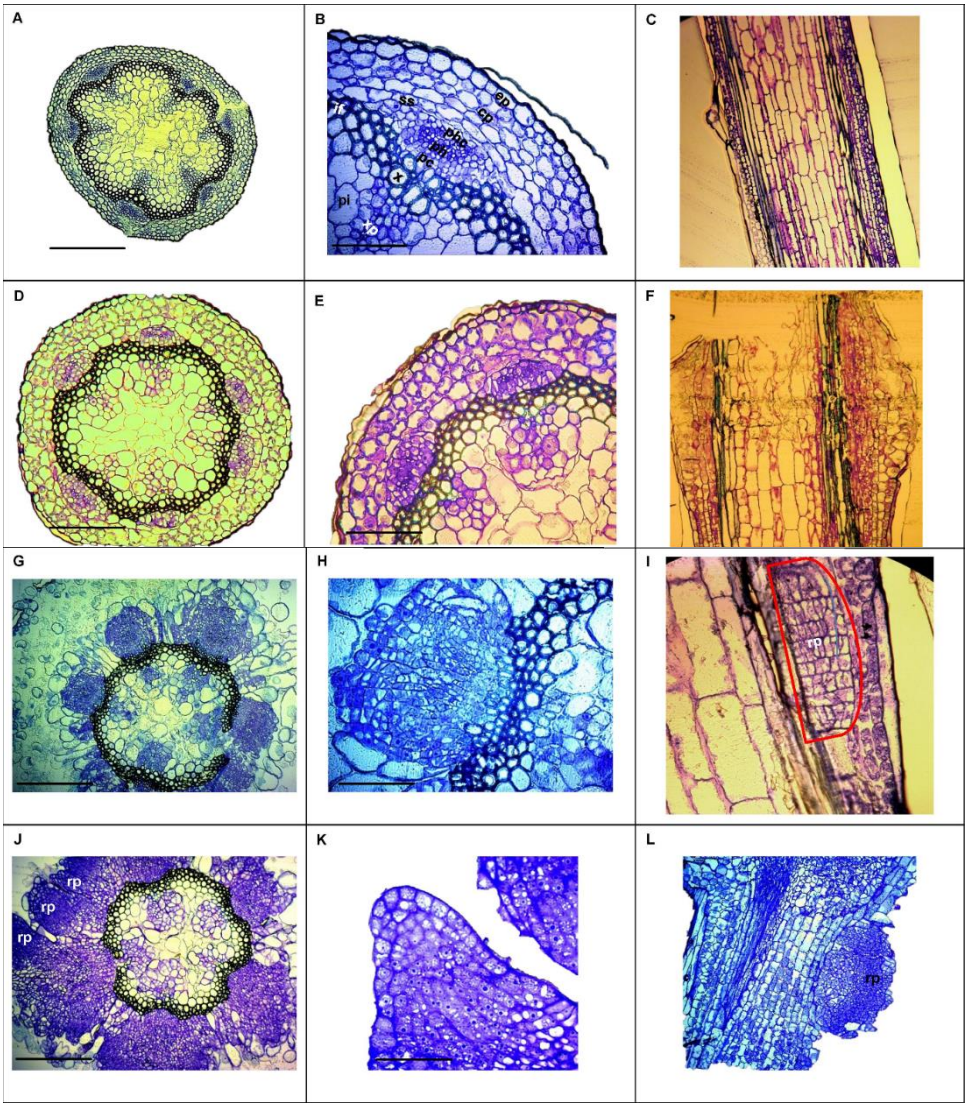


Fig. 7. Microscopic analysis of *Arabidopsis* FS explants at different time points after exposure to IAA (30 µM). Each row of images represents one time point from top to bottom 1, 3, 5 and 7 d after auxin treatment. From left to right, cross section with 10X, 40X magnifications and longitudinal section, respectively. Scale bars: (50 µm). cortical parenchyma (cp), epidermis (ep), interfascicular fibers (if), procambium (pc), phloem (ph), phloem cap (phc), pith (pi), protoxylem (px), root primordia (rp), starch sheath (ss), xylem (x), xylem parenchyma (xp).

Discussion

Establishment of a rooting system

Explant type

In general, our results indicated that different plant organs and tissues possess different rooting potential. This may be related to different factors including age (Ballester *et al.*, 1999; Vidal *et al.*, 2003), endogenous levels of phytohormones (most importantly auxin) (Malamy 2005; Osmont *et al.*, 2007), vascular patterning (as in most of the cases cells adjacent to vascular cells are the origins of ARs) (Davis and Haissig, 1994; Naija *et al.*, 2008; Bellini *et al.*, 2014) and distance from the root system (Dick and Leakey, 2006; Leakey 2004).

In our system, poorest performance was observed with RL whereas hypocotyls performed best. FS explants showed an intermediate response. The high performance of hypocotyls may be related to their ontogenetic (juvenile vs. adult) and physiological age as aging determines the organogenic capability of the cells (Diaz-Sala *et al.*, 1996, 2002; Rasmussen *et al.*, 2010). It can also be related to the etiolation pre-treatment that is normally applied to elongate the hypocotyls. Etiolation has been reported to increase AR formation in some species (Fett-Netto *et al.*, 2001; Koukourikou-Petridou 1998; Klopotek *et al.*, 2010;). In a pilot experiment, dark-grown hypocotyls produced more roots compared to the light grown counterparts (data not shown). On the other hand, hypocotyls have a root like structure with pericycle cells that provide the founder cells for LR development (Busse and Evert, 1999; Goldfarb *et al.*, 1998). Xylem pole pericycle cells in these explants are the origin of roots (Boerjan *et al.*, 1995; Sukumar *et al.*, 2013) indicating a high similarity to the process of LR formation. In our assay, both hypocotyl and root segments produced nearly the same number of roots possibly as a result of such similarity. However, the question is how similar are the processes of root formation in hypocotyl and root explants. Previous findings have highlighted the similar response of hypocotyl and root explants to different hormones including strigolactone (Koltai *et al.*, 2010; Rasmussen *et al.*, 2012b, 2013), CK (Della Rovere *et al.*, 2013) and gibberellic acid (Lo *et al.*, 2008; Niu *et al.*, 2013). Additionally, similar molecular factors have been revealed in both processes (Verstraeten *et al.*, 2014 and

references therein). In contrast, hypocotyl and root explants respond differently to jasmonates (Gutierrez *et al.*, 2012; Raya-Gonzalez *et al.*, 2012) and ethylene (Clark *et al.*, 1999; Negi *et al.*, 2008). Moreover, while auxins and auxin signaling are essential for all stages of LR development (Peret *et al.*, 2009, 2012; Lavenus *et al.*, 2013), exogenous auxin is only stimulating during the first stages of AR development and inhibits later developmental stages (De Klerk *et al.*, 1995, 1999b). In our current assay, although both explants produced similar number of roots, the optimum concentration of applied auxins for root formation from root segments was less compared to hypocotyls indicating the higher sensitivity of founder cells in root segments to the applied auxins. In addition, at higher concentrations of auxin, a significant drop was observed in rooting of root segments, whereas such decline was not observed in hypocotyl explants. This indicates that both root-types have a different sensitivity to exogenous auxin. Several genetic factors, specific to hypocotyl adventitious rooting in *A. thaliana*, have been unraveled which display a dedicated signaling network that drives AR formation in the *Arabidopsis* hypocotyl (Sorin *et al.*, 2005; Gutierrez *et al.*, 2009; Verstraeten *et al.*, 2014). Therefore, despite a degree of similarity between the process of root formation in hypocotyl and root, there are still differences that distinguish them and with the definition of Esau (1960) in mind we will refer to the roots formed on hypocotyl as ARs.

In conclusion, because of structural similarity of *Arabidopsis'* FS to cuttings or *in vitro* micropropagated shoots (Verstraeten *et al.*, 2013) these explants seem to be the best model system to study the AR formation process in plants. However, other explant types (*e.g.*, hypocotyl) have been beneficial in understanding the mechanisms underlying AR formation and should not be overlooked.

Type of auxin

Various auxins caused different rooting responses in the four tested explants. Subsequently, we determined the best suited auxin for each explant type. It should be noted that the reason of the differences in effectiveness amongst the various auxins is unknown. The actual concentration of free auxin in the cells, from which the roots develop, does not reflect the medium concentrations and is, therefore, dependent on

other factors such as transport, oxidation and conjugation, and, in addition, the amount of auxin synthesized by the plant itself.

Firstly, the uptake of the three auxins is different. For example, in tobacco explants, NAA is taken up six times faster than IAA (Peeters *et al.*, 1991), and in apple shoots, IBA four times faster than IAA (Van der Krieken *et al.*, 1993).

Secondly, the three auxins are metabolized differently. In apple shoots, IAA is shown to be degraded faster than IBA (Van der Krieken *et al.*, 1993). There are two major pathways of inactivation: oxidation and conjugation. IAA, and to a lesser extent IBA, may be inactivated irreversibly by oxidation whereas NAA is not oxidized (Epstein and Ludwig-Muller, 1993). In contrast to oxidation, conjugation is a reversible inactivation of auxin as the free auxin may be released from the conjugates (Smulders *et al*., 1990). All three auxins are conjugated. 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 *et al.*, 1992).

Thirdly, the differences in effectiveness observed among the three auxins may also reflect different affinities for auxin receptors. For example, NAA compared to IAA shows a lower binding affinity to the auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1) (Kepinski and Leyser, 2005; Badescu and Napier, 2006; Spartz and Gray, 2008). However, the lower binding affinity does not correlate with its activity which suggest that the observed differences between the various auxins are most likely due to induction of a different signal transduction (Verstraeten *et al.*, 2013).

Lastly, difference in transport of applied auxins can cause different rooting responses. It has been reported that IBA likely acts after its conversion to IAA in many species (Kurepin *et al.*, 2011; Schlicht *et al.*, 2013), however, the possibility of it acting as an independent auxin has also been discussed (Ludwig-Müller, 2000). Recent findings suggested that IBA uses its own specific transporters (PDR [PLEIOTROPIC DRUG RESISTANCE] family proteins, ABCG36 and ABCG37 [ATP-binding cassette subfamily G]) when it is transported along great distances in plants (Strader and Bartel, 2009). On the other hand, the influx (AUX1) and efflux (PIN2, PIN7, ABCB1 and ABCB19) carriers transport IAA but not IBA (Strader and Bartel, 2009).

Therefore, as indicated above, different effectiveness of applied auxin in promoting AR formation relies on different factors which determine the concentration of active auxin reaching the target cells as well as the responsiveness of cells within a tissue to the applied auxin.

Rooting position

The better rooting competence of basal ends versus the apical ends especially when lower concentrations of auxin were applied could be because of polar auxin transport (PAT). It seems that at low auxin concentrations, auxin absorbed via the apical end is transported to the basal end by the basipetal auxin transport system. This together with the auxin taken up via the basal ends increases the free endogenous level of auxin in that area. Consequently, this increase acts as a trigger to activate founder cells and root primordia formation. Therefore, lower concentrations of auxin can only increase the endogenous auxin to the threshold level required for root formation at basal ends and not at other regions. Gradually, with increase in the exogenous concentration of auxin, this threshold level is also reached in entire explant and can bring about rooting at the apical ends as well.

Intriguingly, rooting was mainly limited to the ends (basal and apical) and not alongside the FS explants. This might be because cutting (wounding) stimulates the production of wounding related compounds (WRCs) and ethylene biosynthesis at the cut surface (De Klerk, 2002a; De Klerk *et al.*, 1999a and b). It has been suggested that WRCs and related compounds play an important role in rooting (Van der Krieken *et al.*, 1997). De Klerk *et al.*, (1999b) studied the mode of action of WRCs. They showed that WRCs influence neither the uptake and metabolism of auxin nor the endogenous levels of IAA. Instead, they showed that the WRCs play a main role in the dedifferentiation phase by enhancing the competence of the tissue to respond to plant hormones. In addition to WRCs, wounding also increase the endogenous synthesis of ethylene (Meyer *et al.*, 1984). Stimulation of rooting by ethylene was reported for the first time in the 1930s (Zimmerman *et al.*, 1933). Although ethylene inhibits induction stage, it has been shown to be important during the first stage of AR formation when certain cells in the stem become competent to respond to the rhizogenic signal (De

Klerk *et al.*, 1999a and b; Jasik and De Klerk, 1997; De Klerk, 2002a). The positive effect of ethylene in this stage may be related to the regulation of auxin transport (Lewis *et al.*, 2011) or to an increased responsiveness of cells to auxin (Liu and Reid, 1992a; Visser *et al.*, 1996b; De Klerk and Hanecakova, 2008). Therefore, cut surfaces compared to other areas seem more appropriate for the formation of ARs.

In hypocotyl explants, even at low auxin concentrations rooting occurred at both ends indicating that hypocotyl cells are more rooting competent. The potential reasons for better performance of hypocotyl versus FS explants have been discussed earlier (see earlier in discussion "explant type").

Timing of phases in rooting of *Arabidopsis* FS explants

Considering that AR formation consists of different stages each with its own hormonal requirements, here we determined the timing of successive phases with respect to auxin and cytokinin. To this end, IBA and BAP pulses were given to achieve transient increase in the level of hormones. It has been previously shown that the level of free hormone shows a sharp rise during the pulse (after few hours) (De Klerk *et al.*, 1995).

The best rooting response of FS explants with IBA pulses at 0-72 h and 24-96 h (similar level) indicated that induction occurs between 24-72 h (overlap between two different pulses). However, a significant drop in rooting response when the IBA pulse was applied at 48-120 h may suggest that induction occurred in a narrower window at 24-48 h. Unfortunately, application of 24 h and 48 h IBA pulses, generated very few roots (data not shown). Otherwise a more precise conclusion could have been drawn. However, based on the available data, it can be concluded that induction approximately occurs at 24 h after explant excision and presence of auxin for 72 h is vital for AR formation in FS explants.

De Klerk *et al.*, (1995) established the timing of rooting phases in apple microcuttings. They observed a lag period (24h after excision) for the action of auxin which is necessary for the dedifferentiation process. This is similar to what we can conclude from our results. Moreover, the observed significant drop in rooting response of FS explant when auxin was applied at later time points, is also in accordance with the previous findings (De Klerk *et al.*, 1995).

The observed decline in the rooting response to BAP pulses at 24 and 48 h indicate that this time frame coincides with induction. Outside the 24-72 h timeframe, BAP promotes formation. Considering that BAP acts as an auxin antagonist (Kuroha and Satoh, 2007; Della Rovere *et al.,* 2013), this result suggested that auxin inhibits the later stages of rooting. The inhibitory effect of auxin on root primordia outgrowth and emergence has been discussed in other plant species (De Klerk *et al.,* 1995, 1999; Bellamine *et al.*, 1998).

Determination of AR origins in FS explants of *Arabidopsis*

The formation of ARs from cells adjoining phloem is in accordance with previous findings in other crops. For example, in maize and rice, it has been reported that crown root primordia develop from cells adjacent to the vascular cylinder of the stem (Hochholdinger *et al.*, 2004; Inukai *et al.*, 2005). Cells located at the junction of phloem/cambium in poplar stem cuttings (Rigal *et al.*, 2012) as well as interfascicular cambium cells adjacent to phloem cells in apple cuttings have been shown to be the AR origins (Jasik and De Klerk, 1997; Naija *et al.*, 2008).

We also observed that in some cases root primordia form from epidermis cells indicating that these cells can also act as AR initials. This is in accordance with the findings of Falasca *et al.* (2004) where they reported adventitious rooting from *Arabidopsis* thin cell layers (epidermis and cortex).

These findings are of major importance for cell-specific transcriptional profiling, when studying early events that happen in root initials and cause their developmental fate change are of interest. Application of specific techniques (*e.g.*, laser capture micro-dissection (LCM) and fluorescence activated cell sorting (FACS)) might help to perform cell specific transcriptomic analysis in order to unravel the mechanisms via which the identity of founder cells can be determined.

Conclusions

Poor rooting of cuttings is the major obstacle in clonal propagation. The lack of an efficient model system for adventitious rooting of *Arabidopsis* prompted us to first establish such model. In our model system, rooting performance was best in hypocotyl

explants followed by FS and RL, respectively. For the *in vitro* rooting of *Arabidopsis* hypocotyls, IBA and IAA proved the auxins of choice. IAA was the best performing auxin for *in vitro* rooting of both RL and FS explants. This model can be applied for further studies concerning analysis of mutants or transgenic lines in order to decipher the role of specific pathways in controlling AR formation.

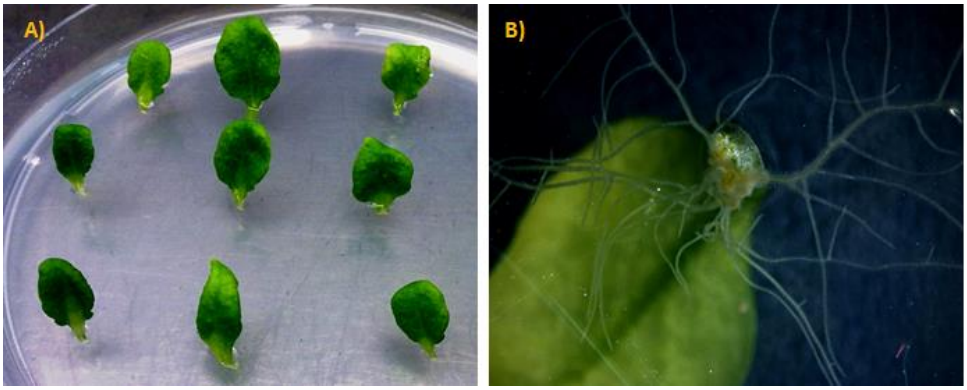
We also determined the timing of phases during adventitious rooting in FS explants based on sensitivity to auxin and cytokinin. The results showed that induction occurred at 24 h after explant excision and the presence of auxin for 72 h is vital for AR formation. Apart from that, our anatomical studies marked starch sheath cells adjacent to the phloem part as the main origins of ARs. These results together with the results of timing of phases are beneficial for cell specific transcriptional profiling. This may lead to unravel the early events that happen in some cells and cause their developmental fate change into the formation of ARs.

Supplementary documents

Supplementary Fig. S1. Rooting of *Arabidopsis's* RL.

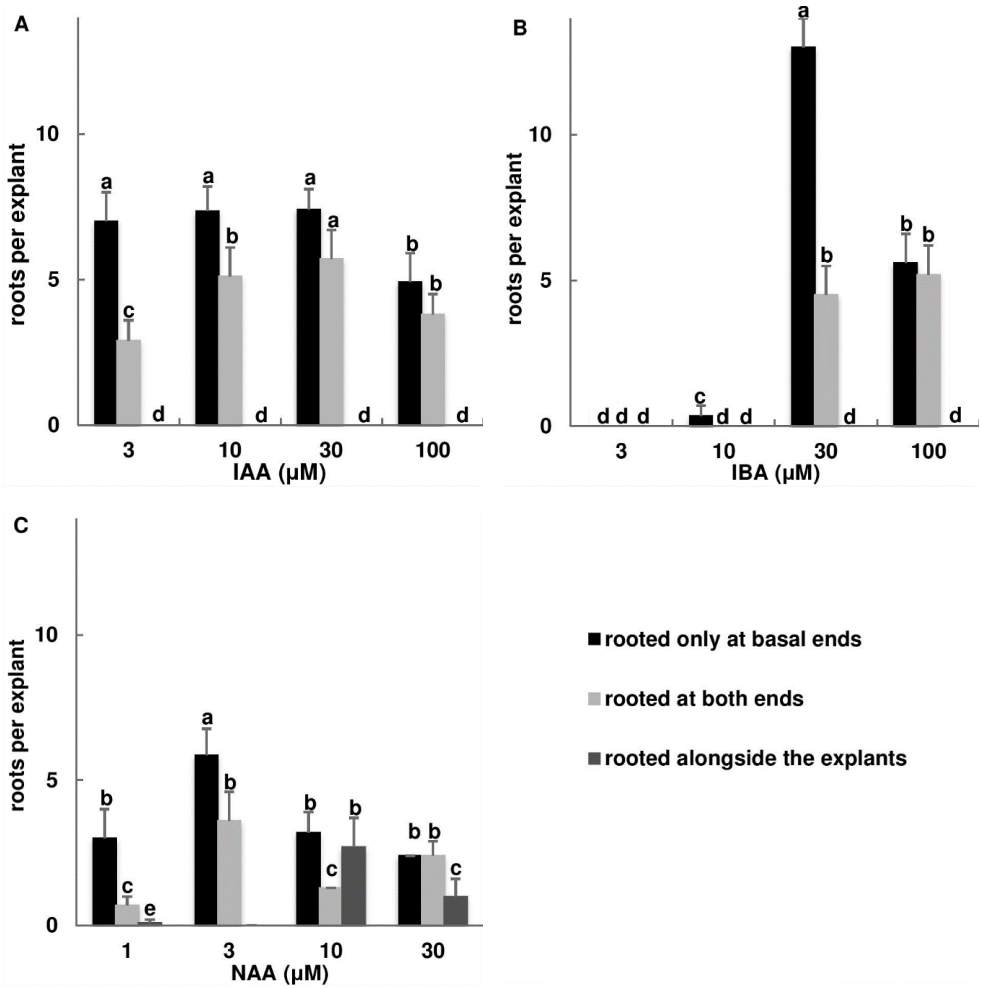
Supplementary Fig. S2. Rooting of *Arabidopsis's* hypocotyls with high and low auxin concentrations.

Supplementary Fig. S3. Root formation at different positions in flower stem explants.



Supplementary Fig. S1. Rooting of RL. A) The appearance of leaves in rooting media. B) Rooting and the position of roots after 3 weeks.

Supplementary Fig. 2. Rooting of hypocotyl with high A) and low auxin B) concentrations after 12 days.



Supplementary Fig. S3. Root formation at different positions in FS explants. Number of roots per explant at different positions when various concentrations of A) IAA, B) IBA and C) NAA were applied. The explants were categorized in three groups; namely explants with roots at basal ends, at both ends, and lastly at alongside the explants (including basal and apical ends). Error bars (SE) represent error range of three biological replicates. Different letters shown for different categories in each concentration represent means that are significantly different at $P < 0.05$.

Chapter 3

Polar auxin transport and PIN-proteins play a determining role in adventitious root formation in *Arabidopsis thaliana*

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Abstract

Adventitious root (AR) formation is a critical step in the vegetative propagation of plants. It is conceived as a developmental process consisting of distinct physiological and morphological phases controlled by hormonal signaling. Despite high importance of AR formation in the horticultural industry, the underlying molecular and genetic aspects are still largely unexplored. We have investigated the role of polar auxin transport (PAT) and its components (PIN-proteins), during AR formation in *Arabidopsis'* hypocotyl and flower stem (FS) explants. *PIN1* and *PIN2* play a major role during AR formation in hypocotyls. For *PIN1* and *PIN2* we propose a role during AR primordium formation and during outgrowth. In FS explants, however, *PIN2*, *PIN3*, *PIN4* and *PIN7* are all shown to be involved in regulating AR formation.

Keywords: adventitious root formation, polar auxin transport, *Arabidopsis thaliana***,** PIN-proteins

Abbreviations: adventitious root (AR), adventitious root primordia (ARP), ATP-binding cassette protein subfamily B/P-glycoprotein (ABCB/PGP), auxin transporter protein 1 (AUX1), auxin influx carrier LIKE AUX (LAX), flower stem (FS), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), lateral root (LR), 1-Naphthaleneacetic acid (NAA), N-1-Naphthylphthalamic Acid (NPA), 2,3,5-triiodobenzoic acid (TIBA), polar auxin transport (PAT), PIN-FORMED auxin efflux carrier protein (PIN-protein).

Introduction

Vegetative propagation is a commonly used method for propagation of cultivars of ornamentals, forestry crops and food crops like potato, cassava, oil palm and banana (Hartmann, 2011). Vegetative propagation depends for the larger part on the ability of cuttings to form new roots (Pacurar *et al.*, 2014a). Thus, adventitious root (AR) formation is a key step in vegetative propagation. The capacity to induce AR depends on the genetic, physiological and developmental status of the 'donor plant' from which the cuttings are taken. It is known since the 1930s that auxin plays a key role in rooting and that applied auxin is a *sine qua non* in commercial vegetative propagation (de Klerk *et al.*, 1999). Unfortunately, though, many horticulturally interesting species are still incapable of adequate rooting and the underlying characteristics responsible for this rooting recalcitrance are largely unknown.

The plant growth regulator auxin is synthesized in most plant tissues but young leaves and cotyledons display the highest synthesis (Ljung *et al.*, 2001). Plants use an active system consisting of transport proteins, influx (AUX1 and LAX) and efflux (PIN-proteins and ABCB transporters) carriers, to transport auxin over long distances (reviewed in Benjamins and Scheres, 2008; Zazımalova *et al.*, 2010). The polarity of this transport, referred to as polar auxin transport (PAT), is determined by the localization of specific auxin PIN-protein efflux carriers (Muday and Murphy, 2002; Friml, 2003). By PAT, auxin gradients are formed throughout the entire length of the plant and these gradients are involved in *de novo* organogenesis, such as leaves and roots. Moreover, gravitropism and phototropism, two directional growth responses that shape the plant, are regulated by auxin gradients.

Application of auxin in hypocotyls and in stems in *Arabidopsis* (Massoumi and De Klerk, 2013; Verstraeten *et al.*, 2013), results in stimulation of AR formation. Moreover, auxin overproducing mutants (*sur1* and *sur2*) form more ARs (Delarue *et al.*, 1998; Pacurar *et al.*, 2014b), which confirms the importance of auxin as the root-inducing hormone in different tissue explants from *Arabidopsis thaliana* (Ludwig-Müller *et al.*, 2005; Massoumi and De Klerk, 2013; Verstraeten *et al.*, 2013).

By monitoring root formation at different positions on an explant, Massoumi and de Klerk (2013) showed that in both hypocotyl and FS explants the basal ends generate

more ARs compared to the apical ends indicating an auxin gradient toward the explant base. In addition, in many studies, application of auxin at the base of the cuttings resulted in the formation of roots at this side (reviewed in Oinam *et al.*, 2011). In general, it can be concluded that local auxin application at the base of a cutting, results in the accumulation of auxins at this side where it is involved in AR formation. Moreover, application of PAT inhibitors, such as N-1-Naphthylphthalamic Acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) have been shown to negatively influence AR formation (Diaz-Sala *et al.*, 1996; Tyburski and Tretyn, 2004; Ahkami *et al.*, 2013). This demonstrates the importance of PAT for AR initiation and development.

Mutant analysis has been instrumental in identifying components of PAT during different stages of lateral root (LR) development in *Arabidopsis* and crown root formation in rice (Coudert *et al.*, 2010; Lavenus *et al.*, 2013). The role of PIN-proteins in mediating various developmental processes such as vascular tissue and flower development (PIN1; Galweiler *et al.*, 1998; Benková *et al.*, 2003), tropisms (PIN2, PIN3; Muller *et al.*, 1998; Friml *et al.*, 2002b), root meristem activity (PIN4; Friml *et al.*, 2002a), quiescent center (QC) cell positioning in the primary roots (PIN1; Friml *et al.*, 2003), as well as early embryo development (PIN7; Friml *et al.*, 2003) has been studied. With the exception of studies on the expression of genes encoding influx or efflux auxin carriers during the development of ARs in de-rooted pine seedlings (Brinker *et al.*, 2004), intact rice plants (Xu *et al.*, 2005), and mango cotyledons (Li *et al.*, 2012), there is little information about the molecular mechanisms controlling PAT during AR formation. Recently, Sukumar *et al.* (2013) have shown that ABCB19 (ATP-binding cassette protein subfamily B19), an auxin efflux transporter, plays a significant role during AR formation in *Arabidopsis* hypocotyls. Furthermore, the involvement of auxin influx (LAX3) and efflux (PIN1) during the establishment of QC cells in the meristem of AR of *Arabidopsis* has been unraveled (Della Rovere *et al.*, 2013).

In the current study, we aimed to identify the role of PIN-proteins in regulating AR formation in *Arabidopsis* hypocotyls and flower stem (FS) explants. Compared to other studies, we did specify the role of individual PIN-proteins during different stages

of AR formation. In addition, our results showed that PIN-proteins play an important and explant-specific role during AR formation.

Materials and methods

Plant materials

Arabidopsis thaliana (Col-0) seeds (obtained from Lehle Seeds, Round Rock, USA) were surface-sterilized with 70% (v/v) ethanol for one minute followed by sodium hypochlorite 2% (w/v) for 10 min. Then the seeds were washed three times for 10 min with sterilized distilled water. They were germinated in Petri dishes using half-strength MS basal salt mixture including vitamins (Murashige and Skoog, 1962) Duchefa), 3% (w/v) sucrose and 0.7% (w/v) Micro-agar (Duchefa, Netherlands). To synchronize germination, the seeds were first stratified in the dark for 3 days at 4^{o}C. Then they were transferred to 20^{o}C under long day conditions (16 h light/8 h dark, 30 µmol m^{-2} s^{-1}, Philips TL33).

Single *PIN* mutants; *pin1-1*, *pin2*, *pin3-5*, *pin4-3*, and *pin7-1* were generously donated by Dr. R. Offringa, (Department of Molecular and Developmental Genetics, Leiden University, The Netherlands). Hereafter, we refer to them as *pin1*, *pin2*, *pin3* *pin4* and *pin7*. These lines have been described previously (Benkova *et al.*, 2003).

Rooting treatments of Arabidopsis hypocotyl and FS explants

Rooting responses of hypocotyl and FS explants were examined according to the previously established method (Massoumi and De Klerk, 2013). IAA was used at a range of concentrations (from 0 to 100 µM) to determine if increased rooting ability was because of changes in responsiveness of cells toward the applied auxin or because of other reasons. Growth conditions were similar to those described in the plant materials section. Rooting was determined at the indicated times (12 and 21 days after culture establishment on rooting media for hypocotyl and FS explants, respectively) as mean number of roots per explant and rooting percentage. For each determination, 30 explants were used.

Histological analysis

We used chloral hydrate solution in 30% (v/v) glycerol (2.5 grams of chloral hydrate to 1 ml of 30% (v/v) glycerol) according to Berleth and Jurgens (1993) to optically clear hypocotyl explants for examination under the light microscope and to be able to detect ARs at different developmental stages. Observations were performed either with Axiophot light microscope (Zeiss, Obberkochen, Germany) equipped with AxioCam ERc5S digital camera (Zeiss) or SteREO Discovery.V8 stereo microscope (Zeiss) equipped with AxioCam MRc5 digital camera (Zeiss).

Quantitative Real-Time PCR

One hundred *Arabidopsis* FS were harvested at different time points after auxin treatment, pooled and ground to fine powder in liquid nitrogen. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and subjected to a treatment with RNase-free DNase I (Qiagen) following the manufacturer's instructions. The extracted RNA served as template for the synthesis of single-stranded cDNA templates with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Quantitative Real-Time PCR (qRT-PCR) was performed using the SYBR Green Supermix with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). All qRT-PCR assays were performed as follows: 95 °C for 3min, 40 cycles of 95 °C for 10s, 55 °C for 30s. At the end of the PCR, the temperature was increased from 55 °C to 95 °C to generate the melting curve. The expression of *PIN2 (At5g57090), PIN3 (At1g70940), PIN4 (At2g01420)* and *PIN7 (At1g23080)* was measured to determine the expression pattern of these four auxin efflux carriers during AR development in the explants. The primer pairs used for qRT-PCR are shown in Supplementary Table 1S. The relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001); the expression levels of genes of interest were normalized to the expression level of *actin-2* (*ACT2*; *At3g18780*), a constitutively expressed gene.

Results

Rooting of tissues treated with PAT inhibitors

To determine the importance of PAT during AR formation, two of the most frequently used PAT inhibitors, 2,3,5-triiodobenzoic acid (TIBA) and the phytotropin 1-N-naphthylphthalamic acid (NPA) were applied in the presence of exogenous auxin. Application of TIBA in both FS and hypocotyl explants reduced the number of roots per explant (Fig. 1A).

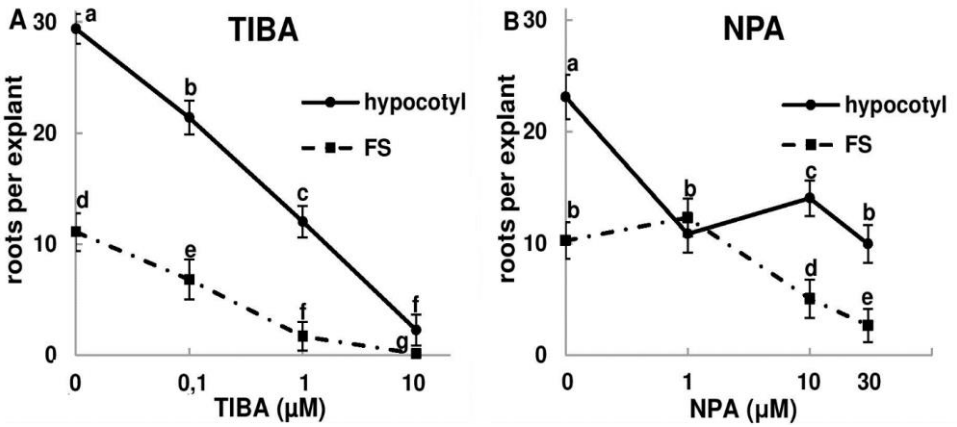


Fig. 1. Rooting of *Arabidopsis* FS and hypocotyl explants in the presence of IAA (10 µM) plus varying concentrations of TIBA (A) and NPA (B). Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$ (Student's t test).

An increases of the TIBA-concentration resulted in a significant decrease in the number of emerged roots and rooting reached the lowest value at 10 µM TIBA. In the absence of TIBA rooting was observed at both apical and basal ends for FS explants (Fig. 2A) and in hypocotyl explants, rooting occurred both at basal and apical sides, but also along the axis of the explants (Fig. 2C). TIBA application reduced root formation significantly and if any root was formed it was restricted to the basal ends in both explants (Fig. 2B and D).

The results of NPA treatment on AR formation in hypocotyl and FS explants are shown in Fig. 1B. In both explants, increased concentration of NPA caused a significant drop in rooting response.

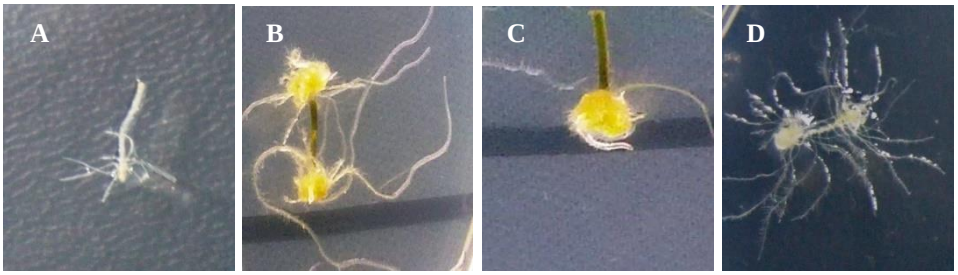


Fig. 2. *Arabidopsis* FS explants treated with A) IAA (10 µM) and B) IAA (10 µM) + TIBA (10 µM). *Arabidopsis* hypocotyl explants treated with C) IAA (10 µM) and D) IAA (10 µM) + TIBA (10 µM).

Rooting of tissues treated with fluridone

Strigolactones (SLs), a new class of plant hormones have been shown to dampen PAT by influencing the expression of PIN-proteins (Bennett et al., 2006; Crawford *et al.*, 2010). In addition to their role in shoot branching, SLs have been shown to inhibit AR formation (Rasmussen *et al.*, 2012b). In our study, we applied the terpenoid biosynthesis blocker, fluridone to block SLs biosynthesis (and to indirectly increase PAT) and see if it can affect AR formation. Under these conditions, elevated numbers of ARs and AR primordia (ARP) formation were anticipated.

The expected stimulatory effect of fluridone on FS explants was not observed when IAA was used as AR inducer (Supplementary Fig. S1). However, when IBA (10 µM) was applied, the rooting response was significantly increased by fluridone ($P < 0.01$) (Fig. 3B). Intriguingly, application of fluridone did not improve AR formation in hypocotyl explants (Fig. 3A).

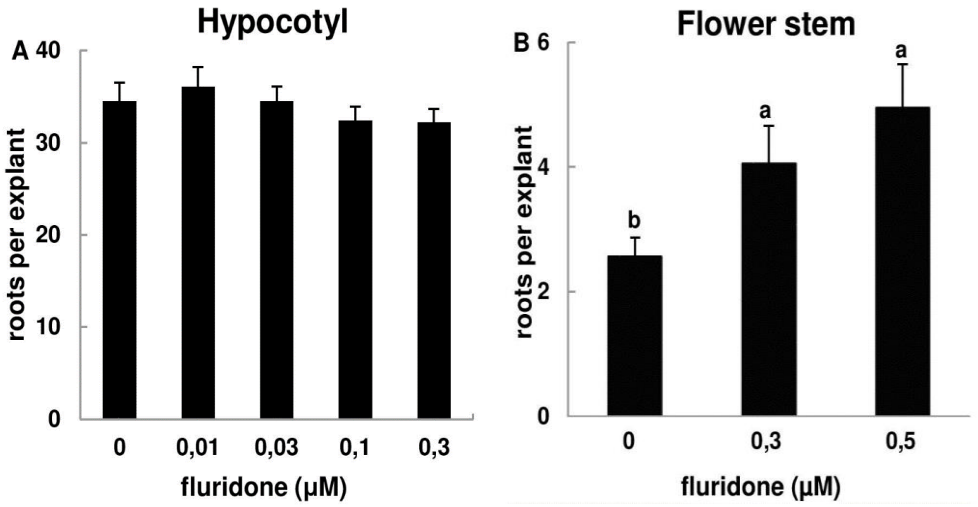


Fig. 3. Rooting of *Arabidopsis* hypocotyl and FS explants in the presence of IBA or IAA (10 µM) and at various concentrations of fluridone. A) Number of roots per hypocotyl explants, B) Number of roots per FS explants. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.01$ (Student's t test). Note that different scales are used in A and B.

Rooting of tissues in pin mutants and wild-type plants

Application of PAT inhibitors (TIBA and NPA) showed that PAT is important for AR formation (Fig. 1 and 3B). The directional transport of auxin is coordinated by different transporters, among which the PIN auxin export carrier proteins. We focused on the role of PIN-proteins by analyzing the rooting response of hypocotyl/FS explants in single *pin* mutants (*pin1*, *pin2*, *pin3*, *pin4* and *pin7*) and in wild-type (*WT*) plants (Fig. 4A and Supplementary Fig. S2). Amongst the various *pin* mutants, hypocotyl explants taken from *pin1* and *pin2* mutants formed significantly ($P < 0.001$ and $P < 0.01$, respectively) less ARs compared to the *WT* plants. The percentage of rooting, however, was not affected in these two mutants and all explants formed at least one root.

In FS explants, *pin2* ($P < 0.05$), *pin3* ($P < 0.01$), *pin4* ($P < 0.01$) and *pin7* ($P < 0.001$) produced significantly less roots compared to the *WT* (Fig. 4B). The number of

roots per explant in *pin1* mutants and *WT* was not statistically different. In contrast to hypocotyl explants, the rooting percentage of FS explants in various *pin* mutants was affected and *pin4* and *pin7* mutants showed the lowest rooting percentage (data not shown).

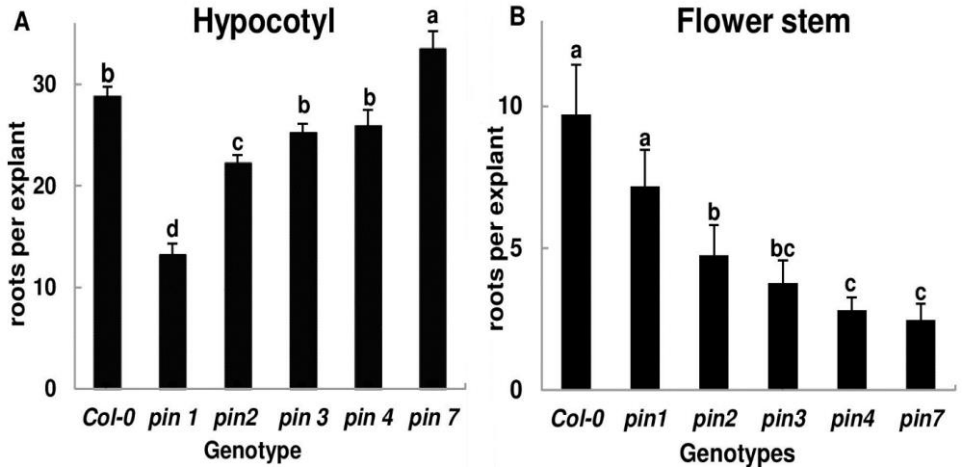


Fig. 4. Rooting of *Arabidopsis* explants in *WT* and mutants treated with IAA. Number of roots per A) hypocotyl and B) FS explants. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$ (Student's t test). Note that different scales are used in A and B.

Next, auxin response curves of explants (hypocotyl and FS) taken from *WT* and AR-involved *pin* mutants were compared. In hypocotyl explants, *pin1*, compared to *pin2* and *WT* plants, produced the lowest number of roots at all auxin concentrations evaluated (Fig. 5A). At lower IAA concentrations (3 and 10 µM) no significant difference was observed between rooting of hypocotyl in *pin2* and *WT*, whereas at 30 and 100 µM rooting responses differed. Higher concentrations of auxin did not restore the *WT* phenotype in these mutants.

Auxin response of FS explants in selected *pin* mutants and *WT* plant is shown in Fig. 5B. The lowest rooting response was observed in FS explants of the *pin7* mutant and it was statistically different ($P < 0.001$) from *WT* plants. The rooting response of

FS explants in the *pin4* mutant was better than in the *pin7* mutant but still significantly different from *WT* plants. An increase in auxin concentration slightly improved, albeit not significantly, rooting of FS explants in *pin4* mutants.

In *pin3* mutants, rooting response was not significantly different from *WT* plants with auxin concentrations up to 10 μM. However, at higher concentrations a strong decrease in root numbers was observed ($P < 0.001$). Similar to other *pin* mutants, increased auxin concentrations were not effective in restoration of the *WT* phenotype in FS of *pin3* mutant.

At lower auxin concentrations, no differences were observed between rooting of FS explants in *pin2* and *WT* plants. However, at higher auxin concentrations, the rooting responses were significantly different between these genotypes. An increase in auxin concentration increased the rooting response of FS in *pin2* mutants, but its response was still significantly different from *WT* plants when higher concentrations were applied.

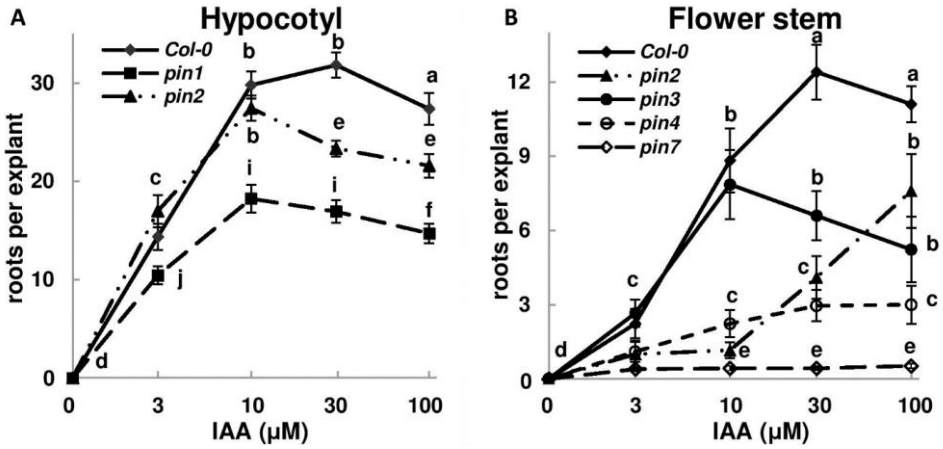


Fig. 5. Rooting of *Arabidopsis* explants in *WT* plants and mutants. Number of root per A) hypocotyl and B) FS explants to various concentrations of IAA. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.01$ (Student's t test). Note that different scales are used in A and B.

Developmental stages in which PIN1 and PIN2 are expressed during AR formation in hypocotyl explants

AR formation is a developmental process consisting of different stages including induction, initiation, and root growth and emergence. To further address the role of different PINs in controlling AR formation, histological analysis was performed. In chloral hydrate-cleared hypocotyls of *pin1*, *pin2* and *WT* plants, the roots were classified based on their developmental stages (emerged or arrested root primordia) and their number were compared after auxin treatment (Fig. 6).

In *WT* plants, no arrested root primordia were recorded and all initiated root primordia had emerged. In *pin1* mutants, less emerged roots were recorded in comparison to *WT* and *pin2* plants ($P < 0.001$). On the other hand, a portion of hypocotyls in *pin1* plants (~ 35%) had arrested root primordia. Even when the number of arrested root primordia (0.75) was taken into account, the total root number in *pin1* was still significantly different ($P < 0.001$) from *WT* and *pin2* plants.

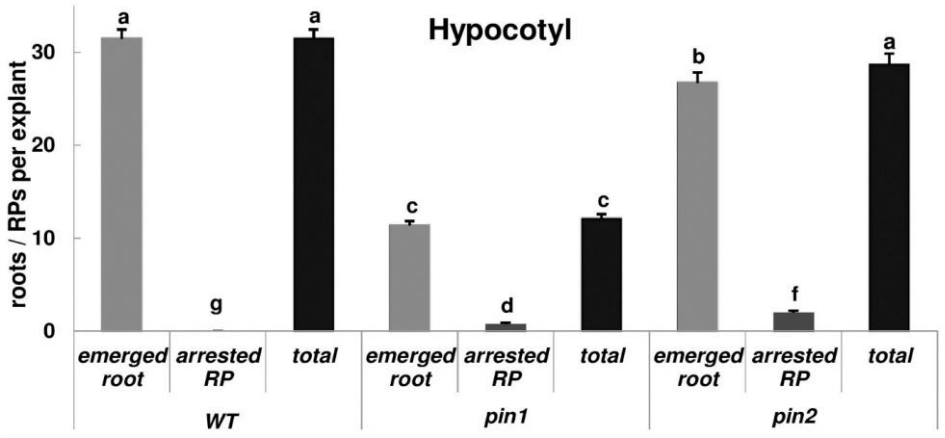


Fig. 6. Number of roots at different developmental stages (emerged roots, arrested root primordia (RP)) and their aggregate value in hypocotyl of *pin1*, *pin2* and *WT* plants. Means are presented with SE. Different letters represent means that are significantly different at least at $P < 0.01$ (Student's t test).

In hypocotyls of *pin2* plants, the number of emerged roots (~ 28) is significantly less than that of *WT* plants (~31, $P < 0.01$). However, arrested root primordia were observed in a substantial portion of the hypocotyls (70%) which is considerably higher than those in *pin1* and *WT* plants, respectively (35% and 0). In *pin2* plants the number of arrested root primordia is significantly higher (1.95, $P < 0.001$) than in *pin1* and *WT* plants (Fig. 6). When the number of arrested root primordia was taken into account, the aggregate value was not significantly different from *WT* plants.

Temporal expression patterns of selected PIN genes in FS explants

Because of the more complex structure of FS explants and problems associated with histological analysis in these explants, we utilized qRT-PCR to evaluate the expression of the selected genes (*PIN2*, *PIN3*, *PIN4* and *PIN7*) during AR formation (Fig. 7A-D).

The transcript level for *PIN2* remained low until 48 h after auxin treatment. It clearly increased from 48 h after auxin treatment onwards (Fig. 7A). For *PIN3*, however, the opposite trend was observed (Fig. 7B). Its expression was high even at 6 h after auxin treatment reaching a peak at 12 h. Afterwards, the transcript level declined significantly and reached its lowest value at 120 h.

For *PIN4*, the transcript level was highest at 6 and 12 h and decreased to almost one third at 24 h (Fig. 7C). After some fluctuation at 48 and 72 h, another peak was observed at 96 h and it remained unchanged afterwards.

The increase in transcript levels of *PIN7* (20 fold) was clearly evident at 6 h after auxin treatment and showed an ascent with a peak at 12 h (Fig. 7D). Then it dropped significantly at 24 and 72 h after auxin treatment. The expression pattern of *PIN7* is more or less similar to that of *PIN3*.

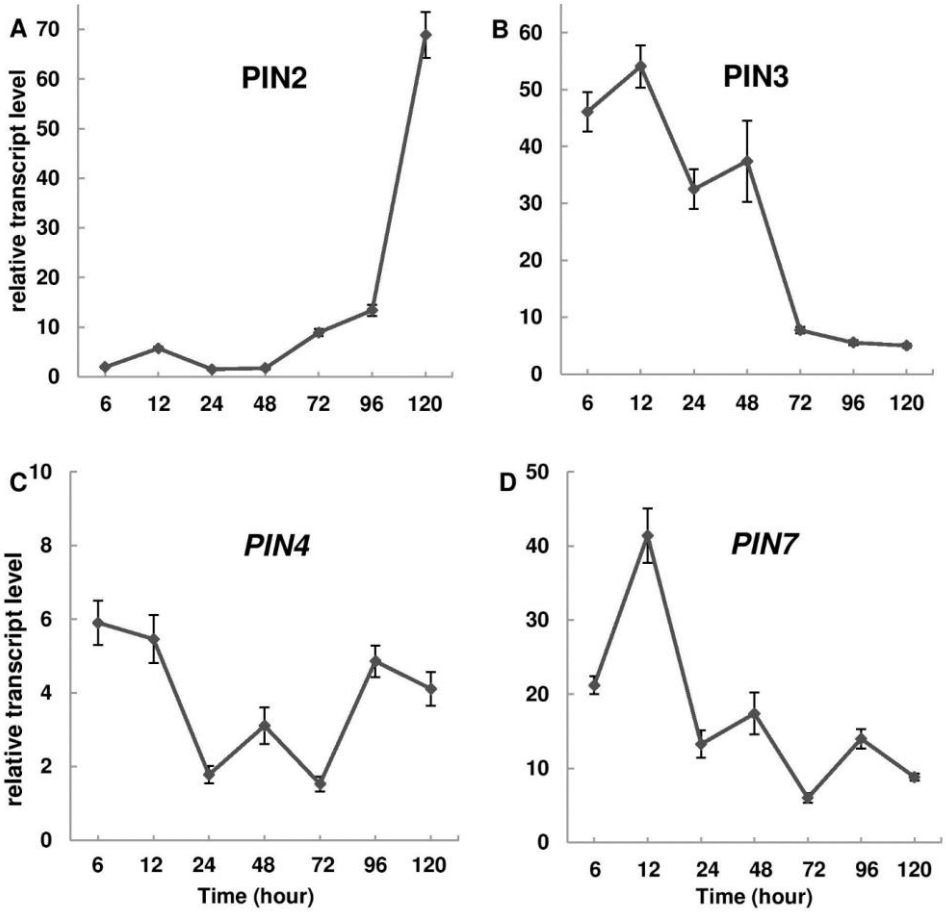


Fig. 7. Relative transcript levels of *PIN2*, *PIN3*, *PIN3*, *PIN4* and *PIN7* in FS explants of *Arabidopsis* ecotype *Col-0*. Error bars (SD) represent error range of different biological replicates. Samples were taken at different time intervals after auxin exposure. Note that different scales are used in different figures.

Discussion

Rooting of tissues treated with PAT inhibitors

The significant decline in rooting response of both FS and hypocotyl explants upon TIBA treatment is in line with previous reports on the effect of TIBA on AR formation of *Arabidopsis* stem segments (Ludwig-Müller *et al.*, 2005). However, these authors

observed a small promoting effect after longer incubation times which might be because of a different experimental set up.

In terms of rooting position, we observed that after TIBA treatment rooting was limited to the basal ends whereas in previous findings on tobacco and lily a pronounced increase in the number of scattered buds was reported (Smulders *et al.*, 1988; Van Aartrijk *et al.*, 1985). However, in our assay, the explants were placed horizontally on the auxin-containing medium and auxin uptake occurred at both ends. Despite the inhibitory effect of TIBA on basal auxin transport, the occurrence of roots at the basal site could be a consequence of the auxin uptake at this end. Moreover, it is possible that some of the absorbed IAA is transported via non-specific auxin transport through phloem with the sap flow (Morris and Kadir, 1972; Tromas and Perrot-Rechenmann, 2010) and hence the delivery at the rooting zone does not entirely depend on TIBA-inhibited PAT. This IAA transportation through membrane-less phloem channels is rapid and has been shown to reach up to 7 $cm \cdot h^{-1}$ in the roots of *Populus tremula* and *Vicia faba* (Eliasson, 1972; Tsurumi and Wada, 1980).

We also studied the influence of NPA, another PAT inhibitor. The results of NPA treatment on the capacity of hypocotyl and FS explants to form ARs supported our findings on TIBA application, indicating that PAT is important for AR formation.

The role of auxin transport in AR formation has been described in a diversity of plant species (Garrido *et al.*, 2002; Nicolás *et al.*, 2004; Li *et al.*, 2008). Impaired AR formation caused by PAT inhibitors has been reported in other studies (Diaz-Sala *et al.*, 1996; Tyburski and Tretyn, 2004; Ahkami *et al.*, 2013) and together with our results point to an important role for PAT during AR formation.

Rooting of tissues treated with fluridone

Increased capacity of fluridone-treated FS explants to form AR is in accordance with the findings of Rasmussen *et al.* (2012a and b) who showed a negative influence of SL on AR formation in *Arabidopsis* and pea (Rasmussen *et al.*, 2012b) as well as the stimulatory effect of fluridone on AR formation in some other plant species (Rasmussen *et al.*, 2012a). However, the stimulatory effect of fluridone on FS explants

seems to be dependent on the type of auxin as its effect was not observed when IAA was used as AR inducer. This might be related to the different effectiveness of IAA compared to IBA to induce AR. For example, in FS explants, it has been shown that IAA is more effective than IBA (Massoumi and De Klerk, 2013). Therefore, the concentrations of IAA applied in this experiment might already have caused the highest rooting potential in such a way that fluridone cannot cause an additional induction of ARs. Based on these observations, application of fluridone alone or in combination with lower IAA-concentrations could better show the additive effect of fluridone in rooting of FS explants. Another explanation may be related to the differences between IBA and IAA concerning their affinities for auxin receptors and or transport across cells and tissue. It has been reported that IBA is a precursor for IAA that may promote accumulation of IAA by local enzymatic conversion (Strader and Bartel, 2011; Kurepin *et al.*, 2011; Schlicht *et al.*, 2013), but other reports suggest that IBA is a biologically active entity (Ludwig-Müller, 2000). Moreover, other findings have suggested that IBA uses specific transporters for long distance transport (PDR; PLEIOTROPIC DRUG RESISTANCE family proteins; ABCG36 and ABCG37) (Strader and Bartel, 2009) and IBA is not converted to IAA during the long-distance transport (Strader and Bartel, 2011). The influx (AUX1) and efflux carriers (PIN2, PIN7, ABCB1 and ABCB19) are shown to transport IAA but not IBA. Fluridone may, therefore, influence the long distant transport of IBA and not that of IAA. This, however, needs further investigation.

In contrast to FS explants, fluridone was not effective in improving AR formation in hypocotyl explants. That could be because of different structure of stem versus hypocotyl tissues. Stem tissues consist of more differentiated tissues. Hence, stem cuttings can be considered more rooting recalcitrant and the rooting potential of hypocotyls is higher. Hypocotyls might, therefore, be more sensitive to auxins, especially because of the presence of a pericycle meristematic cell layer and a root-like structure (Busse and Evert, 1999; Goldfarb *et al.*, 1998). At the concentration of auxin applied (10 µM), the highest root induction is already obtained for the hypocotyl explants, so that eventual positive effects of fluridone are masked.

Rooting of tissues in pin mutants and wild-type plants

Comparing the rooting response of hypocotyl explants in single *pin* mutants and *WT* plants showed that PIN1 and PIN2 proteins play a role in controlling AR formation. In FS explants, AR formation is highly dependent on PIN2, PIN3, PIN4 and PIN7-mediated auxin transport as both number of roots and number of rooted explants are affected in their respective mutants. These results are in line with previous studies that reported an important role for influx and efflux-mediated PAT during AR formation (Brinker *et al.*, 2004; Del Rocío *et al.*, 2008; Li *et al.*, 2012; Xu *et al.*, 2005). Moreover, our results in hypocotyls and FS explants showed that the effect of PIN-proteins on AR formation is explant-specific, but that PIN2 is a main regulator of auxin transport in both explants.

Higher concentrations of auxin did not restore the *WT* phenotype in these mutants. This might indicate that the functional redundancy reported for PIN-proteins in LR formation in *Arabidopsis* (Benková *et al.*, 2003) and tobacco cell cultures (Petrášek *et al.*, 2006) does not exist in AR formation in hypocotyl explants.

Similarly, an increase in auxin concentration either has no effect (*pin7*) or slightly improved, albeit not significantly, rooting of FS explants in *pin2*, *pin3* and *pin4* mutants. This might be because of either lack of functional redundancy among PIN-proteins or the involvement of other mechanisms in regulating the activity of PINs including transcriptional and post-transcriptional regulation as well as regulation of PINs polarity. For example, Vieten *et al.* have shown that the abundance of PIN7:GFP and PIN2:GFP decreases at higher auxin concentrations (Vieten *et al.*, 2005). It has also been shown that the degradation of PIN2 is regulated by auxin levels (Sieberer *et al.*, 2000). Therefore, although auxin itself influence the expression of PIN genes (Krecek *et al.*, 2009; Vieten *et al.*, 2005), at higher concentration it also affects their stability and abundance.

Despite the fact that *PIN1* has been reported as the major regulator of shoot-derived organ formation such as leaves, flowers with different floral organs, and ovules (Benková *et al.*, 2003), we did not observe a significant role for this export protein in the regulating AR formation in FS explants. This can be explained by the findings of

Jones *et al.* (2005) that showed that FS in both *pin1-1* and *WT* plants contain essentially similar levels of IAA. They suggested that despite impaired IAA transport in *pin1-1* mutant, most of the free IAA in mutant stems does not originate from the top of the ‘‘pin’’, but rather from another source, possibly the rosette leaves. This similar level of free endogenous IAA could, therefore, account for nearly similar rooting response of FS in *pin1* and *WT* plants in our study.

Developmental stages in which PIN1 and PIN2 are expressed during AR formation in hypocotyl explants

Histological analysis in chloral hydrate-cleared hypocotyls of *pin1*, *pin2* and *WT* plants indicated a role for *PIN1* and *PIN2* during different stages of AR formation. Lowest number of emerged ARs in *pin1* mutants compared to *WT* and *pin2* plants indicated that this gene might be involved during either emergence or during the induction stage. However, when the number of arrested root primordia was taken into account, the total root number was still significantly different from *WT* and *pin2* plants indicating that *PIN1* is mainly involved during induction stage. Nonetheless, a portion of hypocotyls in *pin1* (35%) contained arrested root primordia that implies another role for *PIN1* during the later stage of AR formation. This might be related to its role in vascular connection or in root emergence and outgrowth. Della Rovere *et al.*, (2013) have recently shown that PIN1 mediated auxin transport toward the AR tip is essential for the establishment of quiescent center cells (QC), providing auxin maxima in these cells and allowing their indeterminate growth. We also studied *in situ* expression of *PIN1* at different time points during AR formation in hypocotyl explants. Similar to Della Rovere *et al.* (2013), we observed that during the later primordia formation stages, *PIN1* expression pattern changes and becomes restricted to the central cell files (Supplementary Fig. S3).

In hypocotyls of *pin2* plants, the total number of induced ARs was not affected while compared to the *WT* plants significantly more arrested root primordia occurred. *In situ* expression pattern of *PIN2* showed that *PIN2* is only expressed in new ARs and not in the hypocotyl and its expression is limited to epidermis and cortex cells

(Supplementary Fig. S3D and E). This is similar to what has been reported in the root (Benková *et al.*, 2003). Our observations point to the importance of *PIN2* during the later stage of AR formation, emergence and outgrowth. This is most likely to remove the excess of auxin and facilitate the outgrowth similar to the role suggested for *PIN2* during LR formation (Benková *et al.*, 2003).

Temporal expression patterns of selected PIN genes in FS explants of WT plants

Comparing the rooting response of FS explants excised from different single *pin* mutants and *WT* plants showed that different *PIN* genes (*PIN2*, *PIN3*, *PIN4* and *PIN7*) are involved in the control of AR formation. Higher complexity of FS structure compared to hypocotyl explants made it difficult to perform histological analysis. Therefore, to gather further information about the possible role of these genes we evaluated their expression at different time points after auxin treatment. Increase in *PIN2* transcript levels from 72 h onward indicates the involvement of this gene during later stages of AR formation which might be similar to the role we suggested for *PIN2* in hypocotyl explants. In addition, our former results (Chapter 2) showed that in *Arabidopsis* FS explants the first 72 h after explant excision are essential for auxin to induce the ARs. Higher levels of *PIN2* transcripts after this period may imply the involvement of this gene not during induction but for emergence and outgrowth.

For *PIN3* and *PIN7*, however, an opposite trend was observed. Increase in their transcript level soon after auxin treatment and up to 48 h point their involvement during the early stages of AR formation. Available data on *in situ* expression of *PIN3* helps explaining the role of this gene during AR formation. Friml *et al.* (2002a) by performing staining experiments in transgenic lines (*PIN3::GUS*) showed that the expression of *PIN3* is associated with starch sheath cells around the vasculature. In addition, findings of Welander *et al.* (2014) illustrated that starch sheath cells adjacent to phloem are the origin of ARs in FS explants. These observations may, therefore, depict a better image for the mode of action of *PIN3*. Expression of *PIN3* in starch sheath cells during the early stage after auxin treatment provides the required auxin for initiation and induction of new ARP.

The expression pattern of *PIN4* might indicate the involvement of this gene during both early and later stages of AR formation. These assumptions, however, need further investigations to fully address the role of these genes and their interactions during AR formation.

Conclusions

AR formation is an important step for clonal multiplication. Physiological and genetic studies have started to scrutinize specific factors controlling the development of ARs. The results described in this study indicate the importance of PAT system for AR formation. PIN-proteins are also shown to play a major role during AR formation in an explant specific manner. Nonetheless, PIN2 showed importance in both explant types tested here in this study.

Our findings together with the findings of Sukumar *et al.* (2013) on the role of another class of efflux transporters (ABCBs) during AR formation indicate that efflux carriers are major regulators during different stages in AR formation. However, the importance of influx carriers should not be overlooked as it has been reported that members of these transporters *e.g.*, AUX1 and LAX3 are respectively important for LR initiation and emergence. Therefore, it seems that influx carriers may also play a role during AR formation. A support for this is the finding of Della Rovere *et al.* (2013) that indicated a role for *LAX3* genes during AR development. Further studies are therefore necessary to fully elucidate the role of different auxin transporters and their cross–regulation during AR formation.

Acknowledgments

We would like to thank Dr. R. Offringa (Department of Molecular and Developmental Genetics, Leiden University, The Netherlands) for their generosity in providing the mutants and transgenic lines used in this study.

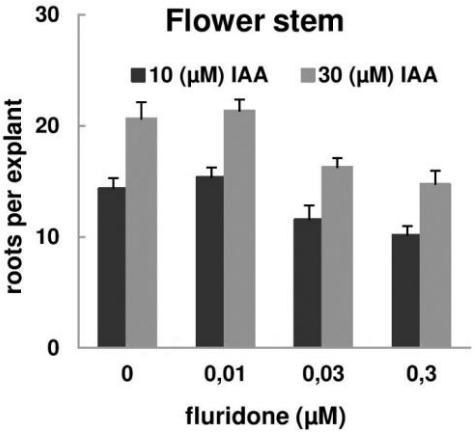
Supplementary documents

Supplementary Fig. S1. Rooting of FS explants with various concentrations of fluridone.

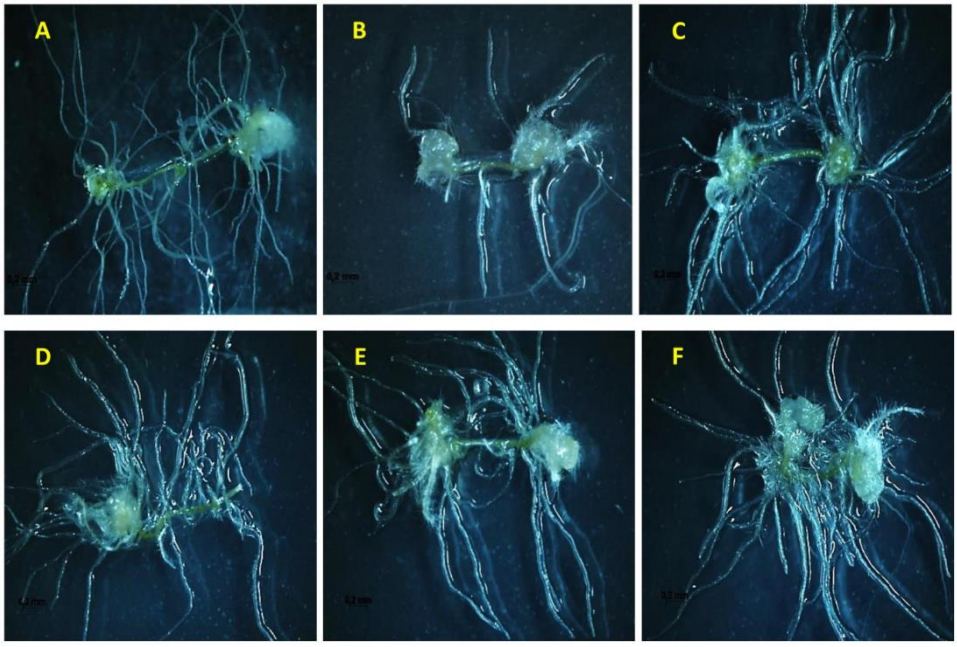
Supplementary Fig. S2. Rooting of hypocotyls in *WT* plants and single pin mutants treated with IAA.

Supplementary Fig. S3. *In situ* expression of *PIN1* and *PIN2* in hypocotyls at different time points after IAA application.

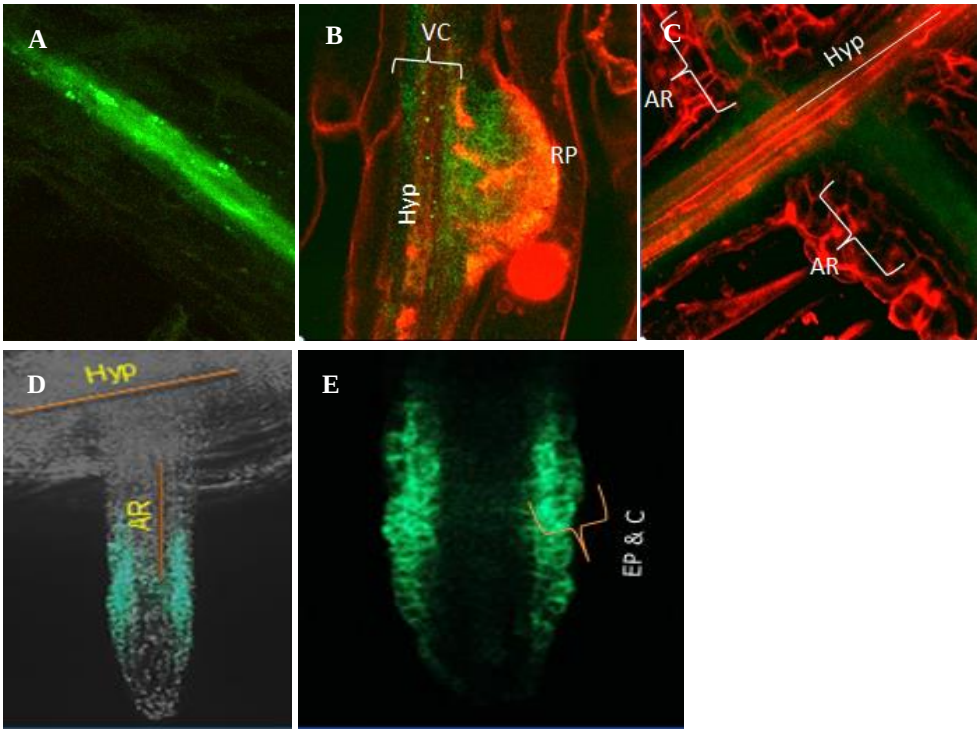
Supplementary table S1. List of primers used in this study.



Supplementary Fig. S1. Rooting of FS explants under the constant concentration of IAA (10 and 30 µM) and different concentrations of fluridone.



Supplementary Fig. S2. Rooting of hypocotyls in A) *WT*, B) *pin1*, C) *pin2*, D) *pin3*, E) *pin4* and F) *pin7* mutants treated with IAA (30 µM).



Supplementary Fig. S3. Expression of *PIN1* in hypocotyls treated with IAA A) 1 day, B) 6 days and C) 9 days after explant excision. Expression of *PIN2* during AR formation in hypocotyl treated with IAA 9 days after explant excision (D and E). Adventitious root (AR), hypocotyl (Hyp), roots primordia (RP), vascular cylinder (VC), cortex (C), and epidermis (EP). Propidium iodide staining is indicated by red and GFP fluorescence by green.

Supplementary Table 1S. List of primers used in this study.

Gene ID	AGI ID	Forward primer (5'→3')	Reverse primer (5'→3')
ACT2	*At3g18780*	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC
PIN2	*At5g57090*	TTACCACTTCCTCGCTGCTG	GCTAAACGCCTGCCAAAGAA
PIN3	*At1g70940*	GCTCATGTGAAACTGGAACAAG	TCTTTGATTAGGTTCGGGTAACTC
PIN4	*At2g01420*	CCGTTCAATCTTCTCGTGGT	TCTCTTGCAGTTGCTGTTGG
PIN7	*At1g23080*	TGTGATGCTCCATTCAAGACTACC	TCCACTTCATCTCCTCAAACAATC

Chapter 4

Azacytidine and miR156 promote rooting in adult but not in juvenile *Arabidopsis* tissues

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Submitted

Abstract

Poor adventitious root (AR) formation is a major obstacle in micropropagation and conventional vegetative propagation of many crops. It is affected by many endogenous and exogenous factors. With respect to endogenous factors, the phase change from juvenile to adult has a major influence on AR formation and rooting is usually much reduced or even fully inhibited in adult tissue. It has been reported that the phase change is characterized by an increase in DNA methylation and a decrease in the expression of microRNA156 (miR156). In this paper, we examined the effect of azacytidine (AzaC) and miR156 in adult and juvenile *Arabidopsis* tissues. We monitored AR formation. This enables to determine the ontogenetic status on the tissue/cell level and is preferable to the distinctive characteristics used in other studies, *viz.*, flowering and/or leaf morphology that can only be used when the organs concerned occur. Overexpression of miR156 promoted only the rooting of adult tissues indicating that the phase change-associated loss in tissues' competence to develop ARs is also under the control of miR156. AzaC inhibits DNA methylation during DNA replication. AzaC treatment also promoted AR formation in nonjuvenile tissues but had no or little effect in juvenile tissues. AzaC addition during seedling growth (by which all tissues become hypomethylated) or during the rooting treatment (by which only those cells become hypomethylated that are generated after taking the explant) are both effective in the promotion of rooting. An AzaC treatment may be useful in tissue culture for crops that are recalcitrant to root.

Key words: Adventitious root formation, hypomethylation, phase change, juvenile, miR156, *Arabidopsis thaliana*

Abbreviations: adventitious root (AR), 5-Azacytidine (AzaC), flower stem (FS), 5-methylcytosine (5-mC), microRNA (miRNA), rosette leaves (RL).

Introduction

Most ornamental and woody crops and various food crops (potato, banana, cassava) are propagated by vegetative propagation. This usually involves the excision of a cutting from a parent plant. Evidently, the cutting should develop new roots, a process referred to as adventitious root (AR) formation. AR formation can be easy, difficult or impossible to achieve. The process is influenced by numerous environmental and endogenous factors. Aging is one of the most pivotal endogenous factors (Diaz-Sala, 2014).

In plants, three types of aging occur, *viz.*, chronological, physiological and ontogenetic aging (Wendling *et al*., 2014a; Fortanier and Jonkers, 1976). Chronological aging refers to the time after the 'birth' of an individual or an organ. Physiological aging denotes "growing old" and loss of vigor. For this type of aging usually the term senescence is used. Ontogenetic aging indicates the transition to the next developmental stage, in the present context the phase change from juvenile to adult. Phase change was studied initially in woody species, but also occurs in herbaceous species. Here it is generally shorter in duration and the morphological and physiological changes associated with the phase transition are less distinct (Hackett, 1985). Phase change is not a one-way process: mature plants may be rejuvenated *in vitro* by repeated subculturing, and *ex vitro* by repeated pruning or by sequential grafting of adult scions onto juvenile rootstocks (Wendling *et al*., 2014b). Tissues maintain the ontogenetic state that they had at the time they were generated. Tissues near the base of the tree are juvenile. This region is known as the "cone of juvenility" (Fortanier and Jonkers, 1976). Accordingly, juvenile leaves occur on sucker shoots generated from the base of adult trees (Garcia *et al*., 2000). Mature tissues occur near the apical meristems of adult trees.

Initially, research on the mechanisms underlying phase change was done at the anatomical and morphological levels. Later, it was attempted to find biochemical and physiological features, especially with respect to distinctive hormones. However, clear and consistent differences between juvenile and adult tissues were not found although in a number of species gibberellins seemed to be involved (Hackett, 1985). With the advent of molecular research, though, striking differences between juvenile and adult

tissues became apparent with respect to DNA-methylation and expression of miRNAs. The step from juvenile to adult coincides with increased methylation of DNA (Valledor *et al*., 2007). Although not absolutely consistent, increased DNA methylation (hypermethylation) at a locus correlates with a reduction in expression and may result in complete silencing (Grant-Downton and Dickinson, 2005). Possibly, DNA methylation may be the cause for the maturation-related decline of rooting observed in woody (Diaz-Sala *et al*., 1996; Ballester *et al*., 1999; Vidal *et al*., 2003) and herbaceous (De Vier and Geneve, 1997; Diaz-Sala et al., 2002; Rasmussen *et al*., 2015) plant species. In *Arabidopsis* it has been shown that DNA methylation increases throughout development (Ruiz-García *et al*., 2005). In addition, mutants at the *DDM1* (Decrease in DNA Methylation) locus and transgenes overexpressing antisense DNA methyltransferase *MET1* in *Arabidopsis* (Ronemus *et al*. 1996), both showing reduced DNA methylation level, exhibited a late-flowering phenotype (Kakutani 1997; Vongs *et al*. 1993). This indicates that transition to flowering stage is negatively correlated with the methylation state of DNA.

More recently, small RNAs (19–24-nucleotide RNAs) have received much attention. In particular, microRNA156 (miR156) has been identified as key component of the genetic control mechanisms that underlie plant phase changes. Wu and Poethig (2006) showed that in the juvenile phase, miR156 is highly expressed and decreases dramatically during vegetative phase change. This small RNA which is conserved throughout the plant kingdom (Axtell and Bowman, 2008), controls the expression of *SBP/SPL* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*) transcription factors (Wu and Poethig, 2006). Small interfering RNAs (siRNAs) from miRNA sites mediate DNA methylation of target genes (Chellappan *et al*., 2010). By monitoring flowering, it was concluded that increased expression of miR156 (by genetic engineering) delays the transition to the adult phase (Wu and Poethig, 2006; Chuck *et al*., 2007).

In the present paper we examine the role of methylation and miR156 in promoting the juvenile phase using AR formation as marker of juvenility. In other studies, flowering or leaf morphology are used as marker for the ontogenetic age, but ability to AR is more useful as it can be used to monitor the ontogenetic status of tissues and cells. It should also be noted that recalcitrance to root is a major problem in

horticulture and forestry and the present research may help to alleviate or even solve this problem.

Our experiments were carried out in tissue culture. The advantages of experimenting *in vitro* are that administration of compounds, the measurement of rootability and overall control are far more easy *in vitro* than *ex vitro*. Despite the superior possibilities for experimenting, phase change has only incidentally been examined in tissue culture. Langens-Gerrits *et al*. (2003) reported that sucrose accelerates vegetative phase change in lily, just as has been found in *Arabidopsis* (Yang *et al*., 2013; Yu *et al*., 2013). Ishimori *et al*. (2007) reported that cytokinin (CK) also promotes phase change in lily.

Materials and Methods

Plant materials

Arabidopsis thaliana (Col-0) seeds (Lehle Seeds, Round Rock, USA) were surface-sterilized with 70% (v/v) ethanol for one minute followed by 2% (w/v) sodium hypochlorite for 10 min. Then the seeds were washed three times for 10 min with sterilized distilled water. They were germinated in Petri-dishes or containers (80 mm high), depending on the explant type, using half-strength MS basal salt mixture including vitamins (Murashige and Skoog, 1962), 3% (w/v) sucrose and 0.7% (w/v) Micro-agar (Duchefa, Netherlands). To synchronize germination, the seeds were first stratified in the dark for 3 days at 4^{0}C. Then they were transferred to 20^{0}C under long day (16h light/8h dark) conditions (30 μmol m^{-2} s^{-1}, Philips TL33). Transformants *35S::MIM156* (expressing target mimicry from the constitutively active *35S* promoter with reduced miR156 activity) and *35S::MIR156* (over-expressing miR156) were a generous gift of Dr. R. Offringa (Department of Molecular and Developmental Genetics, Leiden University, The Netherlands).

Rooting treatment

We examined the rooting response of three types of explant, *viz*., 10 mm-long hypocotyl sections, 5-7 mm-long, node-free flower stem (FS) sections and excised

rosette leaves (RL) (including petioles) according to Massoumi and De Klerk (2013). Briefly, for hypocotyl explants the Petri dishes containing seeds were incubated vertically for 12 days in the dark. Then the seedlings were de-rooted and decapitated. Ten millimeter hypocotyl segments were excised and placed horizontally on the surface of the rooting medium. RL and FS explants were taken from 5 weeks-old-plants grown in containers (80 mm high) under 20^{0}C and long day (16 h light/8 h dark) conditions (30 µmol m^{-2} s^{-1}, Philips TL33). To examine the effect of the ontogenetic age, FS explants and RL were excised from different positions at the stem. Morphological markers were used to distinguish between juvenile and adult RL. According to Wu *et al.* (2009a), juvenile leaves (positioned low in the rosette) are round with smooth margins, and have no trichomes on the abaxial side. Adult leaves (positioned high in the rosette) are elongated, serrated, and produce many abaxial trichomes.

For an adequate comparison of rooting of RL and FS explants in wild-type (*Col-0*) plants and transgenic lines (*35S::MIR156* and *35S::MIM156*) explants were excised from the same position. To this end, FS explants were taken from lower 1.5 cm of the stem. For RL, only apical leaves showing adult characteristics were used. In both cases, the explants were distributed randomly amongst the replicates and hormonal conditions. IAA was added at a range of concentrations (0-100 µM). Temperature and light conditions were as descried above. Rooting was determined at the indicated times (12 and 21 days after culture establishment for hypocotyl and for leaf/FS explants, respectively) as percentage of rooted explants and as mean number of roots per explant. For each determination, 30 explants were used.

Azacytidine (AzaC) treatment

5-Azacytidine (Sigma Aldrich; CAS number 320-67-2) was used as hypomethylating agent. The explants were first treated with a range of AzaC concentrations (0-50 µM) to determine the optimum concentration and 10 µM of AzaC was used in all further experiments. As AzaC is unstable in aqueous solution, the medium was refreshed every two weeks when seedlings were grown in the presence of AzaC. Walker et al. (2012) report that in aqueous solution at room temperature 15% of the initial concentration is

lost after 9.6 h. Thus, after 2 weeks virtually all AzaC is lost. Calculations indicate that the mean concentration during the 2 weeks' period is 1.5 µM after an initial concentration of 10 µM. It was added either in combination with auxin during the rooting treatment (Fig. 2) or as donor plant pre-treatment from the germination stage onwards (Fig. 3A). To study the effect of AzaC on rooting of hypocotyl and FS explants, segments were excised from the same position on the plant in AzaC-treated and control.

Methylation studies and ELISA analysis

To check the DNA methylation status of plant materials cultured with or without AzaC, DNA was first extracted with DNeasy Plant Mini Kit from Qiagen according to the manufacturer instructions. Then for high-throughput detection of global 5-methylcytosine (5-mC) in DNA, extracted DNA was applied on 5-mC DNA ELISA Kit from Zymo Research according to the manufacturer instructions. In summary, the kit features a unique Anti-5-Methylcytosine monoclonal antibody that is both sensitive and specific for 5-mC. After color development, absorbance was measured at 405-450 nm using an ELISA plate reader. Positive and negative controls provided with this kit were used to generate a standard curve so that the 5-mC percentage in a DNA sample could be accurately quantified.

Statistics

For all rooting experiments, three repeats each containing 10 explants were used in each treatment. The means ± SE are given in the graphs. The significance of difference between root numbers was determined with a Student *t*-test and between the percentages with a χ^2- test. All experiments were carried out at least twice.

Results

Rooting of tissues at various ontogenetic ages

We reported previously, that at the optimal auxin concentration, hypocotyl segments regenerated about twice as much ARs as flower stem (FS) segments indicating the

occurrence of a juvenile-adult gradient in *Arabidopsis* plants (Massoumi and De Klerk, 2013 and Supplementary Fig. S1). This experiment suffers, however, from the pitfall that actually very different tissues are monitored (see Discussion). Therefore, we examined whether such gradient occurs when similar tissues are examined, *viz.*, rosette leaves (RL) taken from different positions (Fig. 1A). The ontogenetic stage of RL in *Arabidopsis* can be determined by means of various morphological markers (Wu *et al.*, 2009a).

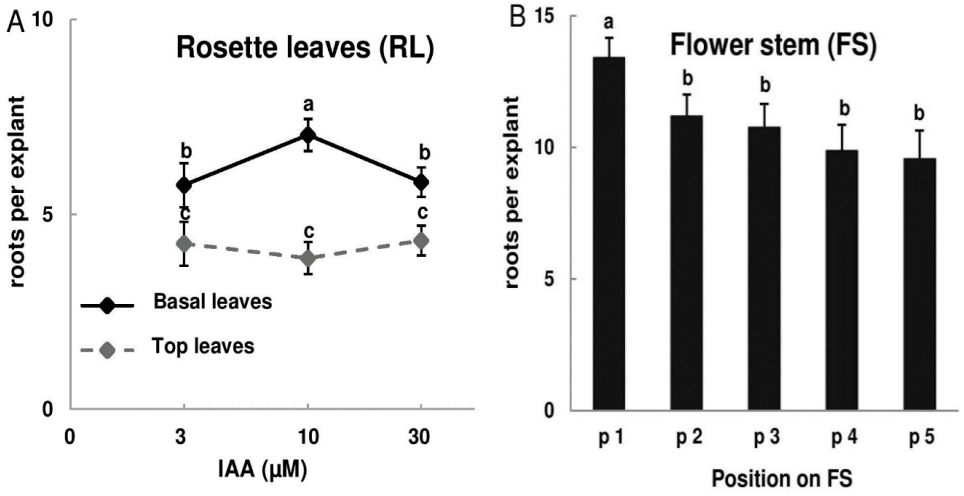


Fig. 1. Rooting of RL and FS explants taken from different position on the plant. A) Rooting of basal RL (with juvenile characteristics) and top RL (with adult characteristics) and B) rooting of stem segments excised from different positions at the flower stem [FS; p1 closest to the base (expectedly more juvenile), p5 closest to the top (expectedly more adult)]. Means are shown with SE. Different letters represent means that are significantly different at $P < 0.05$. Note that different scales are used in A and B.

Basal leaves produced significantly more ARs compared to the top leaves. A second experiment was done with FS segments, taken from different positions at the flower stem (Fig. 1B). The rooting response showed a gradual decline in explants excised closer to the top. The differences were significant for the explant closest to the base. Thus, in both types of explant rooting was significantly higher in explants taken

from a position closer to the base indicating the occurrence of the juvenile-adult gradient.

Azacytidine (AzaC)

When methylated DNA is duplicated, the newly produced DNA is methylated to the same extent. AzaC, a hypomethylating agent, does not interfere with existing methylated DNA but incorporates into DNA during DNA replication and thereby causes hypomethylation (Stresemann and Lyko, 2008). Thus, when AzaC is added at seed germination, all tissues in the developing seedling are hypomethylated. AzaC may also be added to explants of nontreated plantlets and in this case only DNA synthesized after excision is hypomethylated.

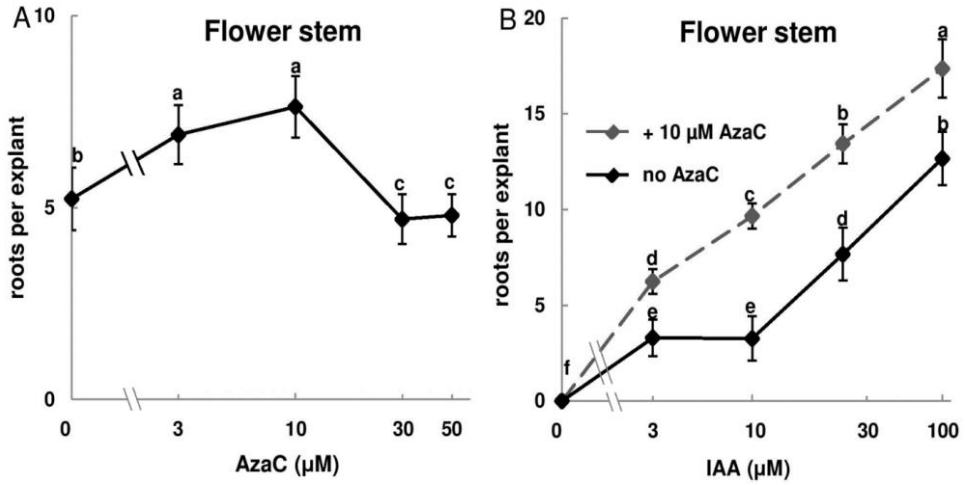


Fig. 2. Rooting of *Arabidopsis* FS segments when AzaC was added during rooting treatment. Rooting of *Arabidopsis* FS segments on medium with 30 µM IAA and increasing concentrations of AzaC (A), or medium with 10 µM AzaC and increasing concentrations of IAA (B). FS segments were taken from 5 weeks-old plants (lower 1.5 cm of the stem). Means are shown with SE. Different letters represent means that are significantly different at $P < 0.05$. Note that different scales are used in A and B. The X axes are plotted logarithmically.

We added AzaC in both ways. When added to hypocotyl explants cut from nontreated seedlings, AzaC did not influence rooting with the exception of 50 µM of AzaC (Supplementary Fig. S2). Hypocotyls taken from seedlings that were germinated and grown on AzaC, also produced the same number of roots as hypocotyls from nontreated seedlings (Supplementary Fig. S3). Hypocotyl tissue is very juvenile so the lack of a response was expected. The drop at 50 µM of AzaC may be caused by a toxic effect since the concentration is very high.

AzaC did promote rooting of FS segments cut from nontreated plants. To determine the optimum concentration of AzaC, increasing concentrations were added along with the optimum concentration of IAA (30µM). Ten µM of AzaC was the optimum concentration and increased the number of roots by 50% from 5 to 7.5 (P <0.05) (Fig. 2A). It should be noted that AzaC did act in spite of its great instability (*cf.* Walker *et al.*, 2012). We compared the rooting response of FS segments to various concentrations of IAA supplemented with or without AzaC (10 µM). AzaC did not change the shape of the dose response curve of IAA and promoted rooting at all IAA concentrations to a similar extent (Fig. 2B).

We also administered AzaC (10 µM) from the start of imbibition. Seedlings germinated on AzaC showed later phase transition compared to the control: they had a prolonged rosette stage and started bolting approximately 2-3 weeks later than the control. No other morphological differences were observed between seedlings germinated on AzaC and control ones. However, FS explants taken from plants germinated and grown on AzaC showed a much better rooting response, *viz.*, 12 vs. 7 ($P < 0.05$) (Fig. 3A).

We checked the methylation status of FS of control and AzaC-grown plants. We also examined hypocotyls. There was a huge difference between juvenile (hypocotyl) and adult plant material (11.9 versus 4.9 %) (Fig. 3B). In adult material germinated and grown on media containing AzaC (FS + AzaC) DNA methylation was significantly reduced ($P < 0.05$).

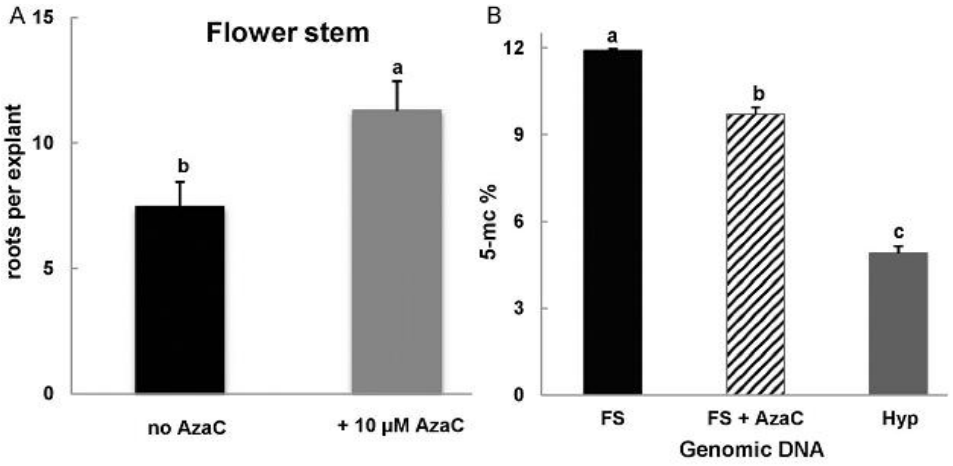


Fig. 3. Comparison of rooting response and methylation status of explants taken from plant germinated on medium supplemented with/without AzaC. A) Rooting of FS segments (lower 1.5cm of the stem) taken from 5 weeks-old plants germinated and grown on medium with/without AzaC. Rooting was evaluated under IAA (30 µM).

B) Global DNA methylation status of FS and hypocotyls (Hyp) in *Arabidopsis*. For the FS, control plants and plants grown with addition of 10 µM AzaC were examined.

Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$.

miR156

In the transition from the juvenile to the adult phase small RNAs, in particular miR156, seem to play a major role (Yu *et al*., 2015b). We studied rooting in three *Arabidopsis* lines *35S::MIM156* (a miR156 target site mimic construct which blocks the activity of miR156), *35S::MIR156 (*overexpressing miR156) and the wild-type (*Col-0*). Transition to adult occurred first in *35S::MIM156* followed by *Col-0* and *35S::MIR156,* respectively. Thus, the higher the level of miR156 expression, the longer the vegetative phase.

35S::MIR156 showed a much better growth in terms of number of RL and plant vigor compared to the other two genotypes (*Col-0* and *35S::MIM156*) (Fig. 4 and 5A). *Col-0* seedlings performed slightly better than *35S::MIM156* seedlings. The *35S::MIR156* seedlings remained longer in the rosette stage and developed substantially more RL; branching was strongly enhanced and the leaves showed more

juvenile characteristics (for a description, see before and Wu *et al*., 2009a, Fig. 4 and 5). Extra information regarding the comparison of juvenile characteristics of the leaves, number of adult and juvenile leaves is shown in Fig. 5A and B.

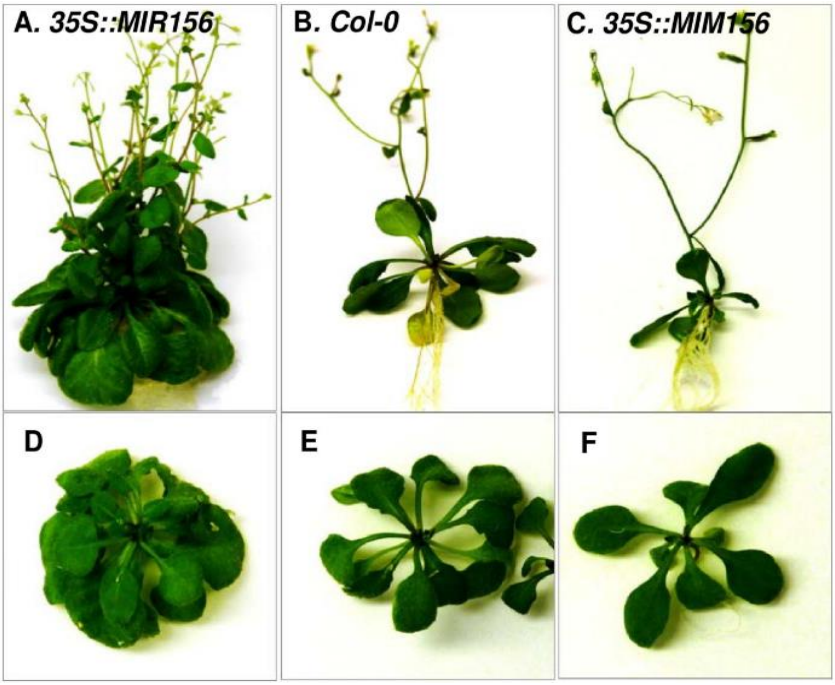


Fig. 4. Morphology of different genotypes grown under long day condition. Transgenic plants over-expressing miR156 (***35S::MIR156***) (A, D), wild-type (***Col-0***) (B, E) and plants overexpressing a target mimic of miR156, MIM156 (***35S::MIM156***) (C, F). Intact seedlings (A, B, C) and rosette parts (D, E, F) are shown.

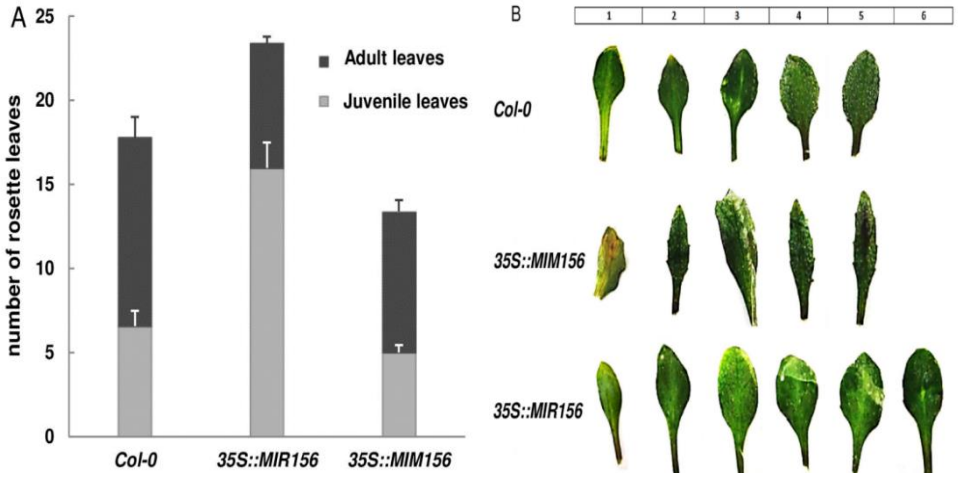


Fig. 5. Phenotypic analysis of transformants *35S::MIR156*, *35S::MIM156*, and wild-type (*Col-0*) plants. Number of juvenile/adult leaves in the rosettes (A) and morphology of leaves taken from different positions in the rosette (B) of two transgenic lines (*35S::MIR156, 35S::MIM156*) and *Col-0* plants. These plants were grown at long day condition.

Hypocotyls from two *Arabidopsis* transgenic lines *35S::MIM156*, *35S::MIR156* and *Col*-0 showed the same rooting response to IAA indicating that as long as tissues are in the very juvenile stage, the rooting response is not affected by the level of miR156 (Fig. 6A). Auxin response curves of top RL (Fig. 6B) and FS (Fig. 6C) explants show that the rooting potential depended on the miR156 expression level. With respect to RL (Fig. 6B), although *35S::MIR156* produced highest number of ARs, it was only slightly higher than *Col-0*. The rooting response of *35S::MIM156* RL was very limited when compared to *35S::MIR156* explants.

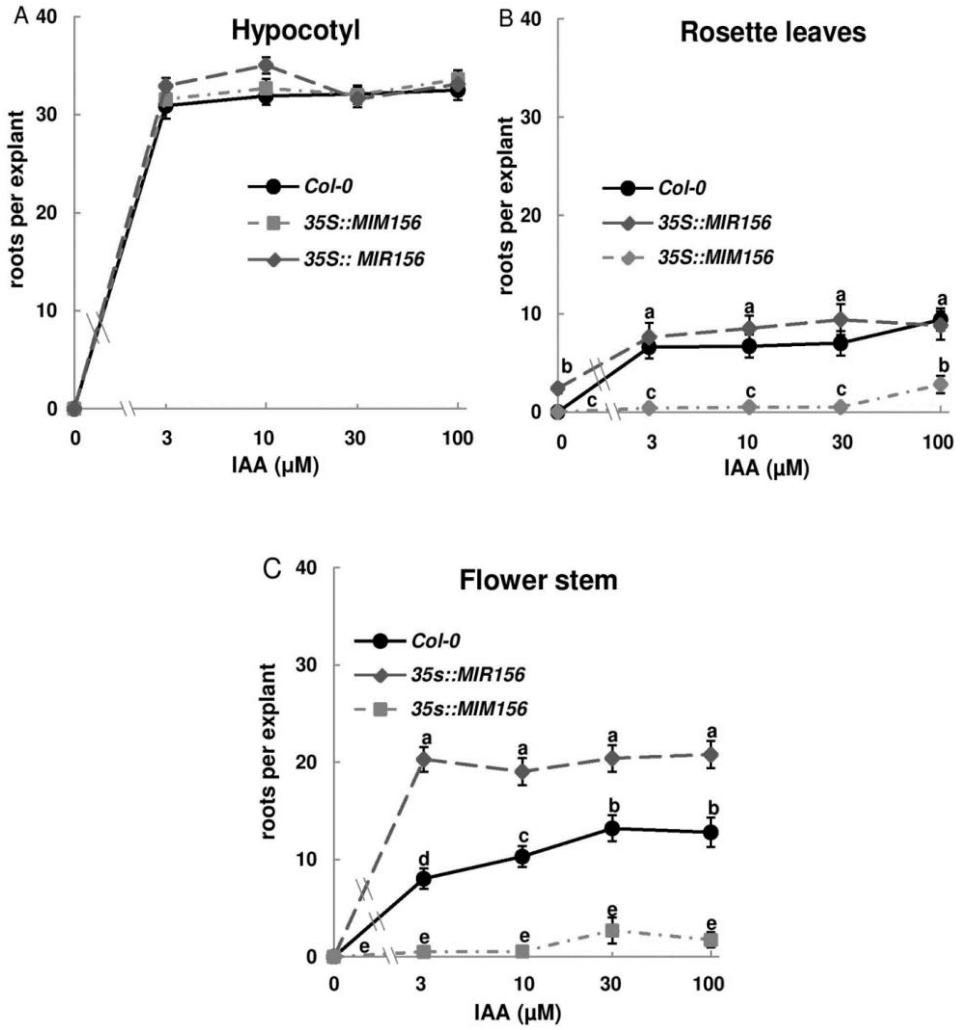


Fig. 6. Rooting of three explant types in *35S::MIR156*, *35S::MIM156* and *Col-0* plants. Hypocotyl (A), rosette (B) leaves and FS explants (C). Hypocotyl segments were taken from 12 days-old seedlings. FS and RL were taken from 5 weeks-old plants. To solely attribute the observed difference to the genotype, explants were taken from the same position on the plant. To this end, FS explants were taken from lower 1.5 cm of the stem. For RL, only apical leaves showing adults characteristics were used. In both cases, the explants were distributed randomly amongst the replicates and hormonal conditions. Means are shown with SE. Different letters represent means that are significantly different at $P < 0.05$. The X axes are plotted logarithmically.

We hypothesized that perhaps chronological age of FS explant in these lines are different as they transit to flowering stage at different time points (*35S::MIM156* earliest and *35S::MIR156* latest transition). To check this possibility, we evaluated the rooting response of FS from two transgenic lines against *Col-0* two weeks after the appearance of FS initials. In this way, at the time of experiment the FS are chronologically at the same age. The results showed that *35S::MIR156* and *35S::MIM156* still produces the highest and lowest number of ARs, respectively. *Col-0* is intermediate (Fig. 7).

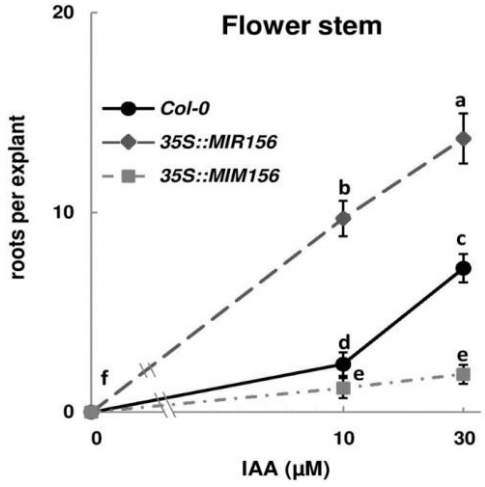


Fig. 7. Rooting of FS explants from the same chronological stage taken from two transgenic lines (*35S::MIR156*, *35S::MIM156*) and *Col-0* plants expressing different level of miR156. To solely attribute the observed difference to the genotype, explants were taken from the same position (lower 1.5 cm of the stem). Different concentrations of IAA were used. Means are shown with SE. Different letters represent means that are significantly different at $P < 0.05$. The X axes are plotted logarithmically.

Discussion

Juvenile and adult tissues in Arabidopsis plants

Higher plants pass through four distinct developmental phases, *viz.*, the embryonic, juvenile vegetative, adult vegetative, and adult reproductive phase (Poethig, 1990). The transition from the juvenile to the adult phase is referred to as phase change, maturation or ontogenetic aging. Associated with this transition are progressive changes in the morphology and physiology, including leaf shape and thickness, phyllotaxis, thorniness, shoot orientation, and the ability to form adventitious roots and buds (Hackett, 1985). During the generation of an adult tree, characteristics associated with juvenility are maintained in the tissues located in the basal portion (cone of juvenility) whereas only tissues in the upper part are adult. Thus, woody plants display a gradient of juvenile to mature tissue in the aboveground portion (Hackett, 1985). Accordingly, for initiation in tissue culture often epicormic shoots are being used. These are shoots generated by the outgrowth of dormant axillary meristems that have been initiated in the juvenile stage (Meier *et al*., 2012). These shoots often display high rootability.

In *Arabidopsis* plants, an ontogenetic gradient has been observed for RL as judged on the morphology (Wu *et al*., 2009a) and AR formation (Fig. 1A). We reported previously that FS of *Arabidopsis* representing mature plant parts develop much less ARs than hypocotyls representing juvenile parts (Massoumi and De Klerk, 2013; and Fig. S1). This difference may be caused by difference in ontogenetic age. However, *Arabidopsis* hypocotyls have a root-like structure (Goldfarb *et al*., 1998): they have pericycle cells that provide the founder cells for lateral root development in roots. So, it could be argued that the use of these explants in research of AR formation is erroneous because of the high similarity to lateral root formation. Still, many reports concerned with studying hypocotyls and root formation refer to this process as adventitious rooting (Falasca *et al*., 2004; Rasmussen *et al*., 2012b).

To obtain a more accurate picture of the effect of ontogenetic age on AR formation we investigated the rooting response of RL with different ontogenetic age as well as stem segments excised from different position at the FS. The correlation

between high AR formation and a low position (closer to the base) is shown in Fig. 1. It should be noted that the tissues at a low position are chronologically older than those on a high position. So, basal tissues root better in spite of their older chronological age. Similarly, Vidal *et al.* (2003) reported a better rootability of *in vitro* derived oak shoots taken from the basal part compared to those obtained from the crown. Various other articles report that juvenile plant material shows higher rootability compared to adult material (De Vier and Geneve, 1997; Diaz-Sala *et al.*, 2002; Rasmussen *et al.*, 2015). Together these observations show the occurrence of an ontogeny-related gradient in *Arabidopsis* plants. The presence of juvenile and adult tissues in the same plant enables a critical examination of the effects of (de)methylation and miR156 expression.

Methylation

The global methylation status of DNA in juvenile and mature tissues has been measured by a number of researchers. The degree of genomic DNA methylation, particularly in meristematic tissues, is related to the phase change in among others *Pinus radiata* (Fraga *et al*., 2002b), *Castanea sativa* (Hasbun *et al*., 2007), and *Sequoia sempervirens* (Huang *et al*., 2012). An opposite trend, however, has been reported in some other woody crops (Baurens *et al*., 2004; Monteuuis *et al*., 2008). Here, it should be noted that phase change is not the only developmental process influencing methylation and the effect of environmental or other developmental conditions on methylation may be the reason why these authors found that juvenile tissues are hypermethylated. DNA methylation has been shown in a variety of processes. Dormant buds have a very high extent of methylation (Hasbun *et al.*, 2007) and stress may alter the methylation status (Gutzat and Mittelsten Scheid, 2012; Omidvar and Fellner, 2015). In the case of Baurens *et al.* (2004), the medium might very well have been exhausted and the plant tissue might suffer from starvation stress or might have developed dormancy.

By methylation of DNA, gene expression is reduced (Saze *et al*., 2012). Thus, increased methylation in adult tissues causes reduced gene expression. In mammalian cells, another hypomethylating drug, 5-Aza-2′-deoxycytidine, has been reported to induce expression of silenced genes by demethylation of specific genome regions and

by affecting histone methylation (Zheng *et al*., 2012). The measurements of the extent of methylation concern global methylation status of genomic DNA. For individual genes, the methylation-behavior may be opposite. In *Eucalyptus grandis*, 245 and 363 transcripts were overexpressed in mature and juvenile cuttings, respectively (Abu-Abied *et al*., 2012). So generally more genes are activated in juvenile tissue but still a great number of genes is activated and presumably not methylated in mature or older tissues.

By adding AzaC, a hypomethylating agent, global hypomethylation of genomic DNA is brought about (Solís *et al*., 2015). AzaC incorporates into DNA during DNA replication, thus most effect is expected when it is added in periods with lots of cell divisions, *e.g*., when the tissues from where the adventitious roots will regenerate are being formed. This was indeed observed in this study. AzaC was applied during seed germination and seedling growth which should result in seedlings with reduced methylation in all tissues. In addition, AzaC was added during the rooting treatment itself using explants excised from plants with 'normal' extent of methylation. In terms of time of application, both methods increased the numbers of ARs to a similar extent (*ca*. 50%, Figs 3 and 4). As expected, AzaC had no effect on very juvenile tissues such as hypocotyls. The stimulation of rooting by AzaC when administered during the rooting treatment suggests that the rhizogenic effect of auxin occurs some time after taking the explant, a period during which cell divisions occur (that result in hypomethylated DNA when AzaC had been added simultaneously). This is in line with the supposed time of action of auxin, *viz.*, starting 24 h after excision (De Klerk *et al.*, 1995). In the current study, the effect of AzaC on rooting has been examined critically, in particular taking into account the timing of its action. AzaC may be used in practical micropropagation both to obtain shoots that are capable of rooting and to produce shoots to set up juvenile cultures.

Our result that adult plant materials (FS) have higher DNA methylation status compared to juvenile one (hypocotyl) is in accordance with previously reported data indicating that different organs show different methylation patterns in species such as tomato (Messeguer *et al*., 1991), rice (Xiong *et al*., 1999) and *Silene latifolia* (Zluvova *et al*., 2001), *Arabidopsis* (Ruiz-García *et al*., 2005), and among different

developmental phases in *Pinus* (Fraga *et al*., 2002a) and *Prunus* (Bitonti *et al*., 2002) with a trend towards increasing DNA methylation during plant development. Here, we showed that aging coincides with increased DNA methylation and application of AzaC can erase a part of the epigenetic marks and consequently promote capacity to form ARs. The ability of plants to redirect development is a prerequisite of adventitious regeneration and it requires that cells erase at least a part of existing epigenetic marks (Smulders and De Klerk, 2011). However, there are differences in regeneration capacity between genotypes and it depends on how fast and how easy these epigenetic markers are erased or reprogrammed (Smulders and De Klerk, 2011). Different factors, including cell type, developmental age and physiological age may influence reprogramming process (Grafi, 2004).

miR156

MicroRNAs (miRNAs) are small RNA molecules consisting of 20- to 24-nucleotide that modulate gene expression at the posttranscriptional level. The expression of miR156 correlates with the juvenile state in *Arabidopsis* (Wu *et al*., 2009a; Wu and Poethig, 2006). Also in other species, miR156 is expressed at high levels in seedlings and at reduced levels in mature plants (Chuck *et al*., 2007; Zhang *et al*., 2011). The involvement of miR156 in the phase change was shown by the phenotype of plants overexpressing this miRNA under the regulation of a strong constitutive promoter. They displayed a prolonged juvenile phase, increased branching, accelerated leaf production, and delayed flowering. This was found in among other *Arabidopsis* (Wu *et al*., 2009a; Wu and Poethig, 2006), poplar (Wang *et al*., 2011), tomato (Zhang *et al*., 2011), and *Torenia fournieri* (Shikata *et al*., 2012). In *Arabidopsis*, inactivation of miR156 with a target site mimic (*35S::MIM156*) produced the opposite phenotype: these plants expressed adult leaf traits precociously, and flowered with less leaves than normal (Franco-Zorrilla *et al*., 2007; Todesco *et al*., 2010; Wu *et al*., 2009a). Thus, miR156 was found to be closely associated with the juvenile phase, but it also has other functions. A bushy architecture is a common phenotype in miR156-overexpressing plants including *Arabidopsis*, maize, rice, and tomato (Chuck *et al*.,

2007; Schwab *et al*., 2005; Schwarz *et al*., 2008; Wu and Poethig, 2006; Xie *et al*., 2006; Zhang *et al*., 2011).

The rooting of the various explants, *viz.*, hypocotyls, leaves and FS excised from transgenic plants overexpressing miR156 or with a target site mimic (reduced in active miR156) showed the expected response including high or low adventitious rooting, respectively. In addition, in *35S::MIR156* the FS response curve for auxin had shifted to the left indicating that its explants shared a higher responsiveness towards applied auxin compared to explants derived from *35S::MIM156* and *Col-0.* In *35S::MIM156* with negligible rooting, we still observed formation of callus at the cut surface. This is in accordance with previous studies in which reactivation of cell division in response to exogenous auxin was reported in both rooting competent and incompetent cuttings (Ballester *et al*., 1999). It was found in our experiments that the rooting response in FS explants still depended on the levels of miR156 even though in FS the phase change had already been taken place albeit at a later or earlier time. It seems that better rooting response of FS in *35S::MIR156* might be related to either higher competence of the cells or slower decline of juvenile response. It could also be related to miR156 interaction with epigenetic DNA-methylation for RNA-directed DNA methylation. This function has been reported for many small RNAs, but is an open question for miR156. The uncoupling of ontogenetic age, miR156 levels and AR response suggested by our experiments needs to be studied further.

All these results indicate a positive correlation between the expression level of miR156 and AR formation potential in *Arabidopsis*. In two studies, overexpression of miR156 resulted in multiple vegetative and reproductive trait alterations among which increase in aerial stem roots in tomato (Zhang *et al*., 2011) and prop root in maize (Chuck *et al*., 2007). Nonetheless, contradicting results were reported in a recent study in which no correlation was reported between the switch of miR156 with miR172 expression in the stems and the loss of rooting ability in *E. grandis* and *E. brachyphylla* (Levy *et al*., 2014). An explanation is that the authors have analyzed the expression of microRNAs in the stem while in the previous studies leaves or shoot apices were examined (Wang *et al*., 2011; Wu *et al*., 2009a). Study of the expression pattern of miR156 in wild-type tomato plants showed that it was abundant in buds and

leaves, moderately expressed in flowers, fruits and roots, and barely detectable in stems (Zhang *et al*., 2011).

In general, microRNAs control posttranscriptional mRNA stability, translation or target epigenetic modification to specific regions of the genome by complementarily binding to target nucleic acids (Brodersen and Voinnet, 2009) and are therefore involved in the regulations associated with plant development (Brodersen and Voinnet, 2009). It has been reported that SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors are the main target of miR156 and this pathway mediates the morphological and physiological changes associated with phase transition (Poethig, 2010). Whether the increased AR formation potential upon miR156 overexpression is because of increased juvenile characteristics via its downstream pathways still needs to be studied in more detail. Recently, Yu *et al*. (2015a) showed that *Arabidopsis* plants overexpressing miR156 produce more lateral roots than plants overexpressing its target mimic, MIM156, indicating a role for miR156 in lateral root development. Further, they showed that promotive effect of miR156 is via its target genes, *SPL*, with *SPL10* playing a dominant role. It may indicate a similar regulatory pathway in AR formation. However, further investigations are needed to fully illustrate downstream pathways regulated by miR156 including SPLs and their targets to understand the molecular link between miR156/SPLs and AR formation. One possibility as has been suggested for lateral root formation is miR156/SPL10 pathway. In this context and if similar mechanism is involved in AR formation, overexpression of *SPL10* or its target miR172 (Wu *et al*., 2009a) are both expected to negatively influence AR formation.

Conclusions

There has been extensive research about the biochemical characteristics of the phase change from juvenile to adult. In these studies the major characteristic used to validate the phase change was flowering. In the present study, we investigated phase change with AR formation as distinctive characteristic. We showed in *Arabidopsis* that phase change and changes in DNA methylation and in the expression of miR156 are closely related with the ability of tissues to form ARs. Generally, after flowering, the loss of

rooting is considered as the second major characteristic of the phase change from juvenile to adult. Finally, it should be noted that the promotion of rooting by treatment of shoots with AzaC either before the exposure to auxin or in combination with auxin during rooting treatment may be well used in practice.

Acknowledgments

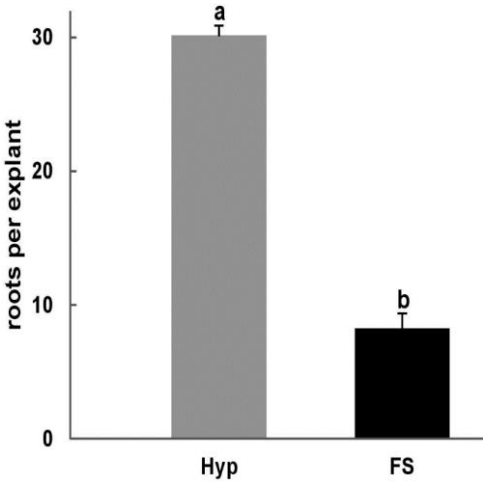
We would like to thank Dr. R. Offringa and O. Karami (Department of Molecular and Developmental Genetics, Leiden University, The Netherlands) for their generosity in providing the *35S::MIM156* and *35S::MIR156* transgenic lines.

Supplementary documents

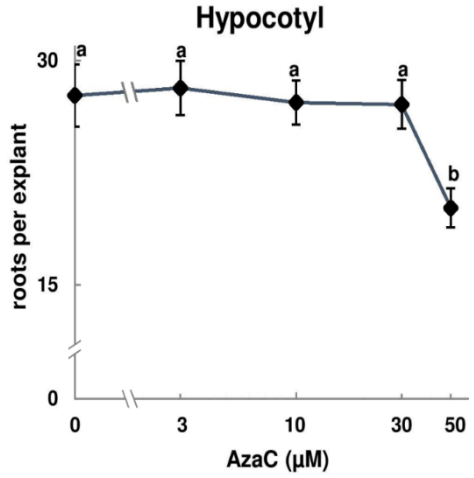
Supplementary Fig. S1. Rooting of hypocotyl (juvenile) versus Flower stem (adult) explants.

Supplementary Fig. S2. Rooting of hypocotyl segments on medium with constant IAA concentration and increasing concentrations of AzaC.

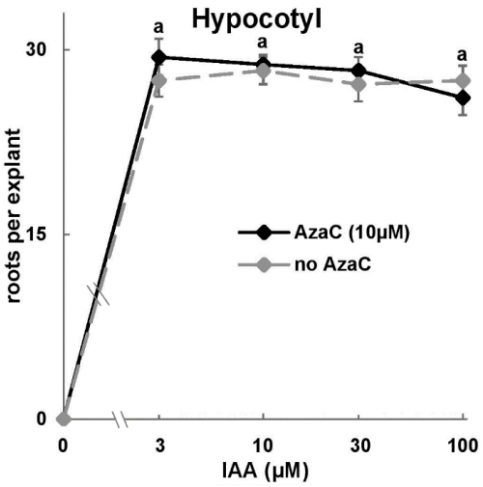
Supplementary Fig. S3. Rooting of hypocotyl segments excised from seedlings germinated on medium supplemented with/without AzaC.



Supplementary Fig. S1. Rooting of hypocotyl (juvenile) versus FS (adult) explants under IAA (30 µM). Hypocotyl segments (Hyp) were taken from 12 days-old seedlings and FS segments were taken from 5 weeks-old plants (lower 1,5 cm of the stem). Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$.



Supplementary Fig. S2. Rooting of *Arabidopsis* hypocotyl segments on medium with 30 µM IAA and increasing concentrations of AzaC. Hypocotyl segments were taken from 12 days-old seedlings. Means are shown with SE. Different letters represent means that are significantly different at $P < 0.05$. The X axis is plotted logarithmically.



Supplementary Fig. S3. Rooting of *Arabidopsis* hypocotyl segments excised from seedlings germinated on medium supplemented with/without AzaC (10 µM). Hypocotyl segments were taken from 12 days-old seedling. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$. Scales in the X axes are logarithmic.

Chapter 5

Etiolation and flooding, two pre-treatments of donor plants that enhance the capability of *Arabidopsis* tissues to root

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Abstract

The success of rooting treatments depends on the treatment itself and on the capability of the cuttings to root. We have examined in *Arabidopsis* two donor plants pre-treatments that enhance the capability to root in some crops, *viz.*, etiolation and flooding. Flooding is equivalent to the double layer technique, a tissue culture method developed some 30 years ago in which a layer of liquid medium is put on top of semi-solid medium. Both had a significant effect. In etiolation, promotion may be brought about by enhanced polar auxin transport and in flooding by ethylene accumulation and by the formation of secondary phloem. Both pre-treatments lower the endogenous sucrose level. As low sucrose favors the juvenile state and juvenile tissues are well known to have a higher capability to root, the low sucrose levels may also play a role.

Key words: Adventitious root formation, *Arabidopsis thaliana*, donor plant pre-treatment, etiolation, flooding

Abbreviations: adventitious root (AR), flower stem (FS), endogenous soluble carbohydrates (ESCs), gibberellic acid (GA), strigolactone (SL)

Introduction

Vegetative propagation is widely used in agriculture, horticulture and forestry to multiply elite plants selected from natural populations and breeding programs (Hartmann *et al.*, 2011). Since cuttings without roots are used as propagules, adventitious root (AR) formation is indispensable for vegetative propagation (De Klerk *et al.*, 1999b). ARs are initiated from differentiated cells. They occur often in normal development, in particular in monocotyledonous plants, or may be induced *e.g.*, by wounding or hormone application (De Klerk *et al.*, 1999a). In commercial propagation, treatment with auxin is the common way to induce ARs. There are other treatments but none is so broadly applicable and reliable as application of auxin. The other treatments include donor plant pre-treatment and adaptation of the rooting treatment itself. In our research on AR formation in *Arabidopsis* we have examined in the context of pre-treatment rejuvenation (Chapter 4). In the present chapter we deal with etiolation and stem elongation and with flooding, the other rooting promoting pre-treatments that have been reported (De Klerk, 2002b).

Light is one of the physical factors that shape plant development (Alabadi and Blazquez, 2009). When applied during the rooting treatment, light (quality, intensity and duration) influences the rooting of cuttings (*e.g.*, Daud *et al.*, 2013). Light also influences rootability of cuttings when the donor plant is treated. Keeping donor plants for some period (weeks) in the dark, usually referred to as etiolation, often improves the rootability of cuttings (Hammerschlag *et al.*, 1987; Klopotek *et al.*, 2010; Koukourikou-Petridou, 1998; Shi and Brewbaker, 2006). Researchers have attempted to relate the effect of etiolation with anatomical, physiological and molecular changes (Maynard and Bassuk, 1988; Haissig and Davis, 1994; Hartmann *et al.*, 2011; Sorin *et al.*, 2005) but the mechanism is still not understood. A complicating factor is the broad spectrum of roles that sucrose the product of photosynthesis, plays: energy source, building block and signal molecule. With respect to plant hormones, it was initially believed that brassinosteroids play a key role but this has been refuted (review in Symons and Reid, 2003). It has been suggested that GA_1 plays such role: after exposure of de-etiolated seedlings to light, there is an inhibition of stem growth caused in part by a rapid drop in GA_1.

Apart from rejuvenation and etiolation, flooding also enhances rootability (Voesenek and Sasidharan, 2013). This is mediated by an accumulation of endogenous ethylene brought about by a reduction in gas release from submerged tissue (Visser *et al.*, 1996). It should be noted that the diffusion rate of gases in water is 10,000 times slower than in air (Jackson, 1985). Double layer (a layer of liquid medium on top of the semi-solid medium) is the tissue culture equivalent of flooding. The effect of double layer on rooting has only been examined occasionally and a strong increase was observed (De Klerk, 2002b).

In the present study we investigate the effect of etiolation and flooding/double layer culture on rootability of *Arabidopsis* explants cultured *in vitro*.

Materials and methods

Plant materials

Arabidopsis thaliana (Col-0) seeds (Lehle Seeds, Round Rock, USA) were surface-sterilized with 70% (v/v) ethanol for one minute followed by sodium hypochlorite 2% (w/v) for 10 min. Then the seeds were washed three times for 10 min with sterilized distilled water. They were germinated in Petri dishes or containers (depending on the explant type) using half-strength MS basal salt mixture including vitamins (Murashige and Skoog, 1962), 3% (w/v) sucrose and 0.7% (w/v) Micro-agar (Duchefa, Netherlands). To synchronize germination, the seeds were first stratified in the dark for 3 days at 4^{o}C. Then they were transferred to 20^{o}C under long day (16 h light/8 h dark) conditions (30 μmol m^{-2} s^{-1}, Philips TL33).

Etiolation experiment

The experiments were performed with segments excised from hypocotyl and flower stem (FS). Hypocotyl segments were excised from etiolated seedlings. We kept the Petri dishes containing seeds in the growth chamber in a vertical position in the dark for 12 days. In this way, seedlings did grow alongside the medium surface. Seeds were also germinated on these conditions with 9 days darkness followed by 3 days light, 6 days darkness followed by 6 days light, 3 days darkness followed by 9 days light, and

12 days light. The light condition was long day (16 h light/8 h dark) (30 µmol m^{-2} s^{-1}, Philips TL33). After that, 5-10 mm-long hypocotyl segments were taken from the 12 days-old seedlings and their rooting responses were evaluated.

For FS, seedlings were germinated and allowed to grow in darkness for 12 weeks in plastic containers. As control, plantlets were cultured in long day condition. The rooting responses of 5-7 mm-long FS segments taken from these two groups were compared.

Flooding experiment

For FS explants, after four weeks, when the donor plants were fully developed, a layer of liquid half-strength MS medium (60 ml equal to 6-7 mm) was added on top of the semi-solid MS medium for one week. Then the rooting of 5-7 mm explants excised from FSs of flooded and control plants was evaluated.

For hypocotyl explants, seeds were first germinated and allowed to develop into seedlings in darkness for 6 days in plastic containers. Darkness was used to elongate the hypocotyls as they are otherwise very short and difficult to work with. The flooding treatment lasted one week. After that, the rooting responses of 10 mm segments were compared.

Rooting treatment

Depending on the explant type, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was used as auxin. Considering that most of the auxins are sensitive to photo-oxidation and auxins are only required during the first few days after explant excision (De Klerk *et al.*, 1989), the cultures were kept in darkness during rooting treatment for one week to avoid the photo-oxidation of applied auxins and after that the explants were transferred into hormone-free MS medium and into the light. Rooting was determined at the indicated times as percentage of rooted explants and as mean number of roots per explant.

Histological analysis

FS segments of flooded and control *Arabidopsis* plants were fixed in 5% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. Plant materials were then rinsed four times (15 min each) in 0.1 M phosphate buffer (pH 7.2) followed by four times (15 min each) rinsing in demi-water. Then the materials were dehydrated in a gradient series of ethanol (v/v: 10, 30 and 50% each for 15 min, 70, 90% and absolute ethanol for 2 h each step) before processing further with glycol-methacrylate-based resin (Technovit 7100, Heraeus-Kulzer Technik, Germany). Infiltration in Technovit was performed according to the manufacturer's instruction. Sections (5 µm thick) were cut with a rotary microtome, mounted onto glass slides, dried on a heater (60 °C) and stained with 0.25% (w/v) toluidine blue in distilled water.

Carbohydrate analysis

Depending on the experiment, plant materials (hypocotyl and/or FS) were oven-dried at 68 °C for 2–3 days, ground with a mortar and pistil. Hot ethanol was used for soluble sugar extraction. For each condition, five samples (with the same initial weight) were extracted three times with 5 ml 80% (v/v) ethanol, by boiling the samples in glass tubes capped with glass marbles in a 95 °C water bath for 10 min each. After each extraction, the tubes were centrifuged at 2500 rpm for 5 min, the supernatants of the three extractions were combined for sugar analysis and evaporated to dryness. Endogenous soluble carbohydrates (ESCs) were determined as fructose, sucrose and glucose equivalents by the Anthrone method (Yemm and Willis, 1954). Absorption was measured at 620 nm on a Beckman DU-50 Spectrophotometer. Absorption of the samples with known concentrations of sugars was measured to generate a standard curve so that the amount of ESCs could be accurately quantified.

Quantitative Real-Time PCR

Per treatment 200 hypocotyls were harvested, pooled and ground to fine powder in liquid nitrogen. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and subjected to a treatment with RNase-free DNase I (Qiagen)

following the manufacturer's instructions. The extracted RNA served as template for the synthesis of single-stranded cDNA templates with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Quantitative Real-Time PCR (qRT-PCR) was performed using the SYBR Green Supermix with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). All qRT-PCR assays were performed as follows: 95 °C for 3min, 40 cycles of 95 °C for 10s, 55 °C for 30s. At the end of the PCR, the temperature was increased from 55 °C to 95 °C to generate the melting curve. The expression of the following genes was measured: *more axillary growth 1 through 4 (MAX1: At2g26170; MAX2: At2g42620; MAX3: At2g44990; MAX4: At4g32810), auxin signaling F-box1 and 2* (*AFB1*: *At4g03190; AFB2: At3g26810*) and *transport inhibitor response 1* (*TIR1: At3g62980*). The primer pairs used for qRT-PCR are shown in Supplementary Table S1. The relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001); the expression levels of genes of interest were normalized to the expression level of the gene *actin-2* (*ACT2: At3g18780*).

Statistics

For all rooting experiments, three repeats each containing 10 explants were used in each treatment. The means ± SE are given in the graphs. The significance of difference between root numbers was determined with a Student *t*-test and between the percentages with a χ^2- test. All experiments were carried out at least twice.

Results

Etiolated donor plants

Differences caused by light and dark conditions

Dark and light grown seedlings showed substantial morphological differences. Seedlings that had been grown in continuous darkness developed the longest hypocotyls (~30 mm). With an increase in the number of days exposed to the light, the length of hypocotyls decreased and reached the shortest value (~5 mm) in 12 days

light condition. In addition, absence of chlorophyll was observed when the seedlings had developed in total darkness. The chlorophyll content increased with the number of days of exposure to the light. At the end of the pre-treatment period (12 days) and before the start of the rooting treatment, in some of the hypocotyls grown in darkness (12 days) root initials were visible.

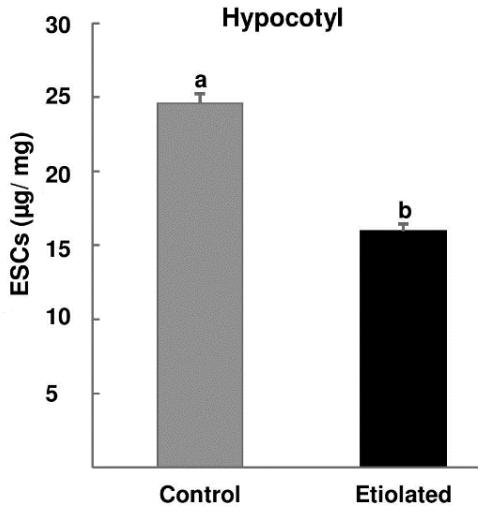


Fig. 1. The level of endogenous soluble carbohydrates (ESCs) in hypocotyl explants grown in dark (12 days) and light (12 days). Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.001$.

The level of endogenous soluble carbohydrates (ESCs) in light grown and etiolated hypocotyl samples is shown in Fig. 1. Measurements were performed just prior to rooting treatment. The results showed that etiolation significantly ($P < 0.001$) decreased the amount of ESCs.

Rooting of etiolated tissues

The rooting response of *Arabidopsis* hypocotyls after increasing periods of exposure to light is presented in Fig. 2A. The highest rooting response (100 % with an average of 5.6 roots per explant) was observed when the hypocotyl explants were kept for 12 days in darkness. The increased competence for rooting was also evident from the speed of

rooting (7 vs. 9 d after explant excision, data not shown). Increase of the number of days with light reduced rooting and the lowest rooting response (2.8 roots per explant) was observed in hypocotyl segments excised from seedlings that had grown 12 days in light.

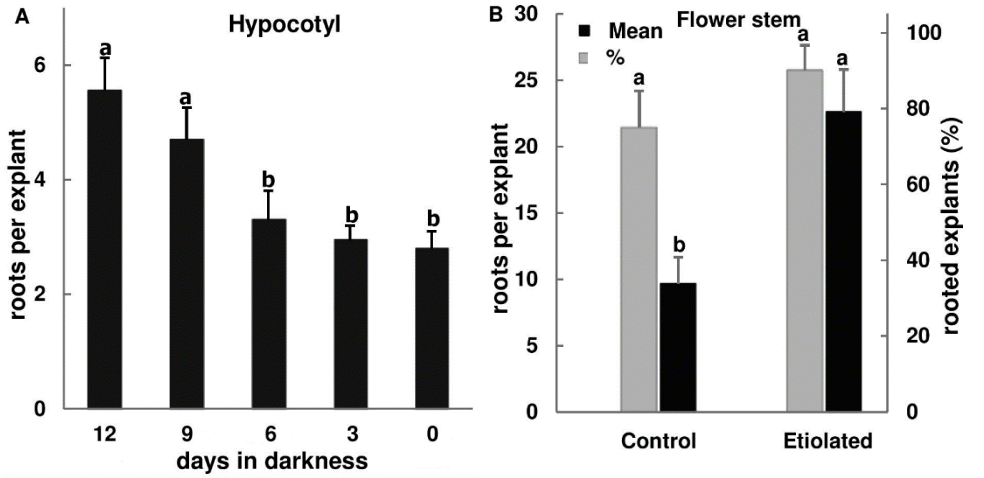


Fig. 2. Rooting of *Arabidopsis'* explants after etiolation pre-treatment. A) Rooting at 10 µM IBA of hypocotyl explants after increasing periods of exposure to light. Different letters represent means that are significantly different at $P < 0.05$. B) Rooting at 30 µM IAA of FS segments grown in darkness or light for 12 weeks. Different letters represent means that are significantly different at $P < 0.002$. Means across replicates are presented with SE. Note that different scales are used in A and B.

Similar results were obtained with FS segments. We germinated seeds and grew them for 12 weeks in the dark. The dark grown plants were etiolated (white and strongly elongated). In addition, compared to light-grown donor plants (control) that have a single FS and few lateral branches, massive production of axillary branches occurred. Aerial leaves were abnormally developed compared to light grown ones and rosette leaves (RL) showed the characteristics of juvenile leaves (Wu *et al.*, 2009a; data not shown). Segments excised from FS of etiolated donor plants regenerated more roots per explant (~23 vs.10, $P < 0.002$). The percentage of rooted explants was also higher but the difference was not significant (90% vs. 75%, $P = 0.16$) (Fig. 2B).

Gene expression in control and etiolated hypocotyl explants

The abundance of lateral shoots in dark-grown plants resembles the response of *max* (*more axillary growth*) mutants. These mutants are characterized by increased outgrowth of axillary buds caused by a defect in strigolactone (SL) synthesis (Stirnberg *et al.*, 2002) and by increased AR formation (Rasmussen *et al.*, 2012b). We measured *MAX* expression in the light and in the dark and found it to be decreased in the dark (Fig. 3).

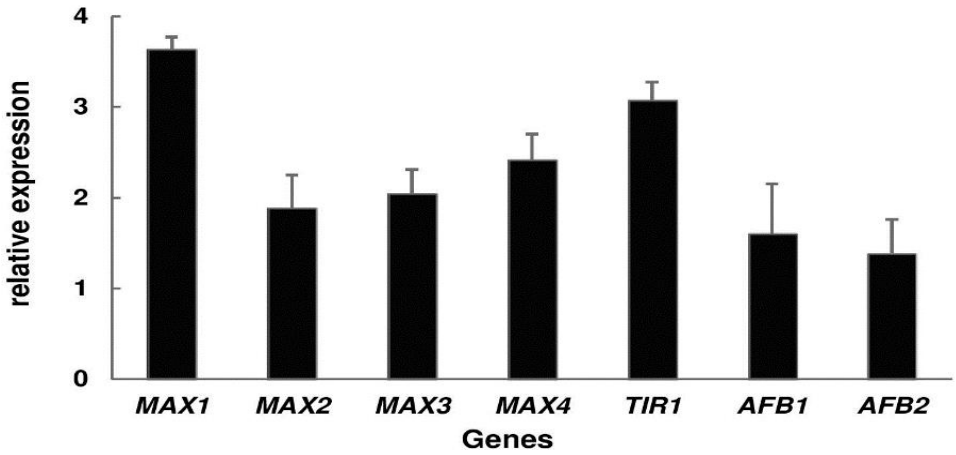


Fig. 3. Expression of strigolactone biosynthesis genes, signaling genes as well as auxin response genes, comparing hypocotyls of light grown versus those of etiolated seedlings. Total RNA was extracted from complete seedlings 12 days after germination and seedling growth in two different light conditions (12 days dark vs. 12d light). Relative expression levels of indicated genes were quantified by qRT-PCR and normalized to *Act2* levels. Each value is the mean ± SE of three biological and three technical replicates and are presented as expression fold change.

This would cause a reduction of the SL level in the dark and thereby an increased AR formation. In addition, we were also interested to see if the better rooting response of etiolated seedlings is because of change in auxin signaling. Auxin signaling happens through the SCF^{TIR1}-Aux/IAA-ARF pathway. In *Arabidopsis* TIR1 (TRANSPORT INHIBITOR RESISTANT 1) and AFBs (AUXIN SIGNALING F-BOX PROTEIN 1 through 5 (AFB1–5)) are F-box components of a nuclear SCF-type E3 ubiquitin ligase, which target the Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE)

proteins for degradation (Gray *et al.*, 2001; Petroski and Deshaies, 2005; dos Santos Maraschin *et al.*, 2009). Therefore, the expression of SL biosynthesis and signaling genes (*MAX1*, *MAX2*, *MAX3*, and *MAX4*) as well as *TIR1*, *AFB1* and *AFB2*, was analyzed by qRT-PCR. ARFs (AUXIN RESPONSE FACTORS) were excluded from this study as changes in their expression by light have been addressed earlier (Gutierrez *et al.*, 2009).

All genes showed a level of up-regulation under light conditions. Highest up-regulation was observed in *MAX1* and TIR1 ($\geq$ 3 fold) followed by nearly two-fold up-regulation in *MAX4*, *MAX3* and *MAX2* (Fig. 3). The up-regulation of *AFB1* and *AFB2* genes were less pronounced (1.4 and 1.6-fold, not significantly different).

The effect of applying flooding as pre-treatment on AR formation

Morphology, anatomy and endogenous soluble carbohydrates (ESCs)

Major morphological differences were observed between flooding-treated and control donor plants. Flooded donor plants showed stronger vigor (Fig. 4). Their FS was thicker, the leaves (both rosette and aerial) were larger and instead of a single FS, flooded donor plants did generate additional FSs ($\sim$ 2-3).

The anatomical structure of *Arabidopsis* FSs has been addressed previously. The FS consists of one row of epidermis cells as outermost layer and a few rows of cortex cells. The innermost cortical cell layer has been reported as a starch sheath. In the center of the FS interfascicular tissues, phloem and xylem are present which are separated by procambial cells. Protoxylem with parenchyma constitutes the innermost part of the vascular bundle (Fig. 5C). We carried out a microscopic analysis just before the start of the rooting treatment. Results are shown in Fig. 5. The obvious difference is the larger diameter of cross sections of flooded stems (nearly two times). Comparing the cross section of FS explants taken from flooding-treated (Fig. 5D & E) and control (Fig. 5A & B) donor plants points to the formation of secondary phloem in FS of flooding-treated donor plants (arrow head in Fig. 5D & E).

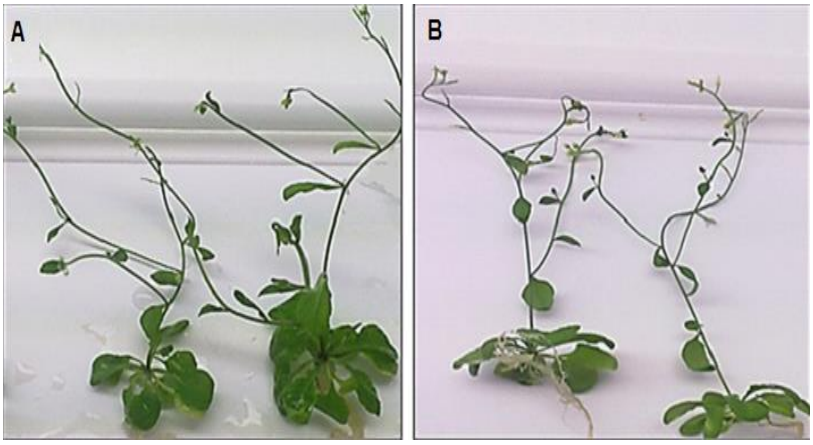


Fig. 4. Vegetative performance of flooding-treated and nontreated (control) *Arabidopsis* donor plants. Flooding was applied for one week. A) Flooding-treated, B) Control.

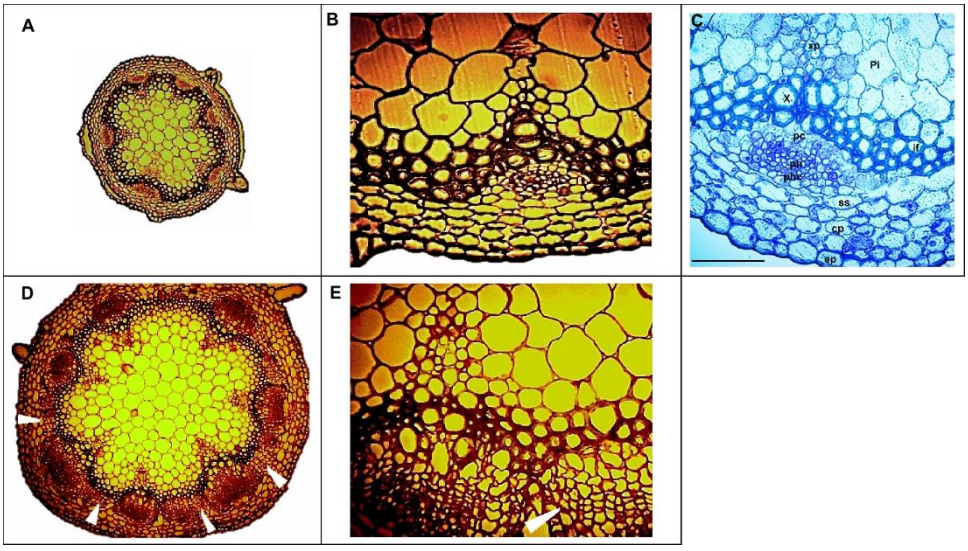


Fig. 5. Anatomy of flooding-treated (D & E) and nontreated (A & B) *Arabidopsis'* FSs. The duration of the flooding treatment was one week. Different layer in the FS of *Arabidopsis* are illustrated in C. Arrow heads point the secondary phloems. Scale bar is 50 µm and applies to all pictures. cortical parenchyma (cp), epidermis (ep), interfascicular fibers (if), procambium (pc), phloem (ph), phloem cap (phc), pith (pi), protoxylem (px), root primordia (rp), starch sheath (ss), xylem (x), xylem parenchyma (xp).

We measured the level of ESCs in FS explants right after flooding treatment before the start of rooting experiment. The results showed that the level of ESCs in FS of flooded seedling was significantly ($P < 0.02$) lower than in nontreated ones (Fig. 6).

Rooting

The rooting response of explants excised from hypocotyl and FSs after one week flooding seedlings/donor plants is shown in Fig. 7. Hypocotyls excised from flooded seedlings produced significantly more roots ($P < 0.01$) (Fig. 7A.) Furthermore, rooting started earlier (6 vs. 9 d after explant excision, data not shown).

Flooding of donor plants increased significantly ($P < 0.05$) the rooting response of FS explants compared to the control ones (Fig. 7B).

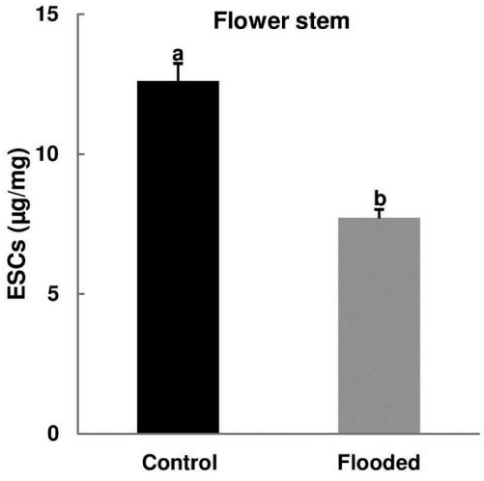


Fig. 6. The level of endogenous soluble carbohydrates (ESCs) in FS explants taken from flooding-treated and control (nontreated) donor plants. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.02$.

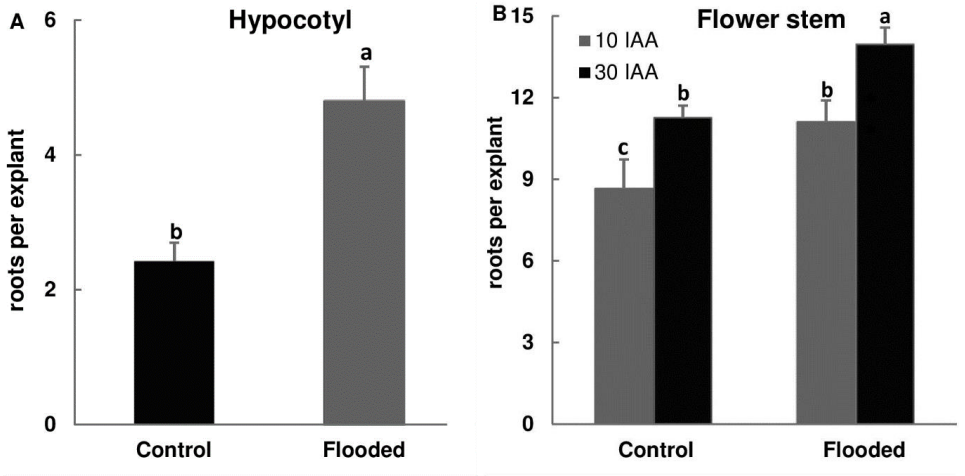


Fig. 7. The rooting response of two *Arabidopsis* explant types after one week treatment of seedlings/donor plants with flooding. A) Rooting of hypocotyls at 10 μM IBA. Different letters represent means that are significantly different at $P < 0.01$. B) Rooting of FSs at IAA (10 and 30 μM). Different letters represent means that are significantly different at $P < 0.05$. Means across replicates are presented with SE. Note that different scales are used in A and B.

Discussion

Adventitious rooting is an essential, inevitable step in vegetative propagation. The failure of cuttings to regenerate roots is a main problem in horticulture. There are two major pathways to improve rooting. The first is to improve the rooting process itself. In this approach, all hormones and all different auxins have been examined in many publications. Unfortunately, no substantial progress has been made and the treatment with auxin developed by Thimann and Went in the 1930s remains the best option. The second pathway is to improve rootability of the cuttings. This approach involves pre-treatments of donor plants and has received less attention. Three methods have emerged, namely, rejuvenation, etiolation and flooding. Rejuvenation is dealt with in the chapter on ontogenetic change (chapter 4) and the other two in the present chapter.

Etiolation

Both seedlings and adult plants were etiolated by culturing them for extended periods (12 days and 12 weeks, respectively) in the dark. We observed the expected

morphological changes, *viz.*, strong elongation and complete whitening of tissues. Rooting of both hypocotyl and FS segments excised from etiolated plants was strongly enhanced. Actually, hypocotyls of etiolated seedlings (12 and 9 days dark) had already started root formation at the time of explant excision, whereas no AR had been formed on hypocotyls of nontreated seedlings. In addition, during the rooting treatment, ARs in hypocotyls of etiolated seedlings emerged two days earlier than in hypocotyl sections excised from nonetiolated seedlings. Similarly, Klopotek *et al.* (2010) reported that in petunia cuttings root meristem formation had already started during the dark treatment and was enhanced during the rooting period.

It should be stressed that the promotion of rooting by darkness depends on the proper timing of the dark treatment which should be given before the auxin treatment. For example, Tyburski and Tretyn (2004) reported that exposure to continuous light compared to dark increased AR formation in tomato hypocotyls. They gave the light treatment together with the auxin treatment. We observed in apple microcuttings that depending on the timing of the treatment, etiolation had a positive or negative effect. Its application during donor plant preparation increased further rooting response of the cuttings whereas during the rooting treatment it negatively influenced the rooting response (Supplementary Fig. S2).

Why is rooting enhanced in etiolated stem tissues? This may be caused by enhanced auxin transport. We observed that in etiolated seedlings apical dominance is reduced. In *Arabidopsis* it has been shown that increased auxin transport, brought about by mutation of the MAX genes leads to increased branching (Bennett *et al.*, 2006). In these mutants, the synthesis of SL is greatly diminished. SL is thus an endogenous inhibitor of polar auxin transport. We observed that when SL is reduced by the carotenoid biosynthesis inhibitor, fluridone, rooting is also increased (chapter 3). Similarly, it has been reported that fluridone treatment increases rooting of different plant species (Rasmussen *et al.*, 2012a). We showed that in the light, the expression of SL biosynthesis (*MAX1*, *MAX3* and *MAX4*) and signaling (*MAX2*) genes is increased. Therefore, the synthesis of SL will also be increased in the light. SL reduces auxin transport and since SL is reduced in the dark there will be more auxin transport which is favorable for rooting.

Although, we observed increased rooting response by etiolation, up-regulation of *TIR1* by light seems contrasting as increase in its expression may result in more auxin signaling, activation of downstream pathways and consequently formation of more root. However, it is not always the case and the involvement of other regulatory mechanisms *e.g.,* post-transcriptional regulation of TIR/AFBs which restrict their spatial protein expression levels should also be taken into account. We did observe that in etiolated hypocotyls root formation had already been started at the time of explant excision. This may indicate that in the dark, cells become competent and root initials are formed. Probably from this stage onward, root initials just need an external signal for further growth. If this is true, the up-regulation of *TIR1* in the light is then justified. Gutierrez *et al.* (2009) have also found that ARF6 and 8, positive regulators of AR formation, are positively regulated by light.

There is a second possible mechanism for better rooting response of explants taken from etiolated seedling compared to nontreated ones. AR formation is known as a high-energy demanding process. Carbohydrates are the principle source of energy and structural elements. Indeed, a reduction of sucrose during the rooting treatment leads to a reduction of rooting (De Klerk and Calamar, 2002). Here we observed a negative influence of etiolation on the level of ESCs possibly caused by the absence of photosynthesis and by the extra use of building blocks because of stem elongation. This will cause a lower level of sucrose during the rooting treatment even though this may be compensated by uptake of sucrose from the rooting medium. So if etiolation has an effect via sucrose levels, at first sight a reduction of rooting would be expected. In etiolated tissues, though, an increase was observed. This may be related to the role of sucrose as a signaling molecule (Smeekens, 2000; Price *et al.*, 2004). Wu and Poethig (2006) showed that the transition of juvenile to mature phase is stimulated by a high endogenous sucrose level. This seems to be a general mechanism as the same was found in lily (Langens-Gerrits *et al.*, 2003). In the juvenile phase, miR156 is highly expressed and its expression decreases dramatically during vegetative phase change. It has also been shown that supplying *Arabidopsis* plant with sugar reduces the level of miR156 while sugar deprivation increases their expression. Removing leaves and reduced photosynthesis also lead to increased miR156 level and consequently delays

the juvenile-to-adult transition (Yang *et al.*, 2013 and Supplementary Fig. S1). Considering these facts and our own results (chapter 4, PhD thesis) indicating that maturation related loss in AR formation is under the influence of miR156, an additional role for sugar in increasing AR formation can be hypothesized. We speculate here that reduced carbohydrate content during dark exposure increases the level of miR156 leading to rejuvenation of donor plants and consequently increases AR formation potential.

Flooding treatment of donor plants

We also applied flooding as a pre-treatment of donor plants. The plantlets were cultured on a double layer: a layer of liquid medium of ca. 6-7 mm was added on top of the semi-solid medium. It first should be noted that the growth was much enhanced as is usually observed for double layer. To our knowledge, genuine flooding *ex vitro* never results in an increase of growth (*e.g.*, Maurenza *et al.*, 2012). The enhancement of growth *in vitro* is probably related to a better nutrient uptake from liquid medium than from semi-solid medium.

In addition to morphological changes, a flooding pre-treatment increased rooting response of excised hypocotyl and FS segments. This was evident at the time of explant excision (prior to rooting treatment) when some of the hypocotyls had already started rooting, similar to what we observed after etiolation treatment. Vidoz *et al.* (2010) also reported that 24 h after submergence of tomato plants, the root primordia had already formed and by 48 h they reached the epidermis layer. In control plants however, no emerged ARs were observed even after 7 days. The replacement of the original root system with ARs from the stems in flooded plants has also been observed in other species such as *Rumex palustris* Sm. (Visser *et al.*, 1996), deepwater rice (Mergemann and Sauter, 2000), the perennial wetland species *Cotula coronopifolia* and *Meionectes brownii* (Rich *et al.*, 2012) and in *Larix laricina* (tamarack) (Calvo-Polanco *et al.*, 2012). Thus, initiating organogenesis to replace the original root system with ARs seems to be an adaptive response to the stress situation.

In order to get better insight about the better rooting performance of explants taken from flooded donor plants we performed a microscopic analysis in FS. The

results pointed to the formation of secondary phloem in explants excised from flooded plants. Our previous anatomical studies in FS explants at different time points after auxin treatment (chapter 2, PhD thesis) and the findings of Welander *et al.* (2014) showed that starch sheath cells adjacent to the phloem are the main origins of ARs. It seems, therefore, that the formation of secondary phloem in flooded explants increases the area in which these cells are adjacent to the phloem parts. This consequently increases the chances of root initials being formed.

In addition to microscopic analysis, our study showed that the level of ESCs is influenced by flooding. Just as in the etiolation treatment, this may promote the capacity to root by promoting the juvenile state.

Conclusions

Adventitious root (AR) formation is influenced by numerous environmental and endogenous factors. Among other environmental changes, flooding and change in the quality/intensity of light have been shown to influence the *ex vitro* rooting of the cuttings. In this study, we studied the effect of two donor plant pre-treatments, flooding and etiolation, on subsequent *in vitro* rooting of *Arabidopsis* tissues (hypocotyl and FS explants). Our results showed that these two pre-treatments can be used as efficient ways to increase AR formation. In addition to *Arabidopsis*, we performed similar experiments in apple as a model for woody crops to see whether these findings can be translated into other species. Nearly, similar results were observed (Supplementary Fig. S2 and S3). This provided indications for the wider applicability of these techniques in increasing AR formation in other crops. We provided further evidence of how environmental conditions can affect the physiological and biochemical quality of donor plants and consequently influence the rooting capacity of the cuttings.

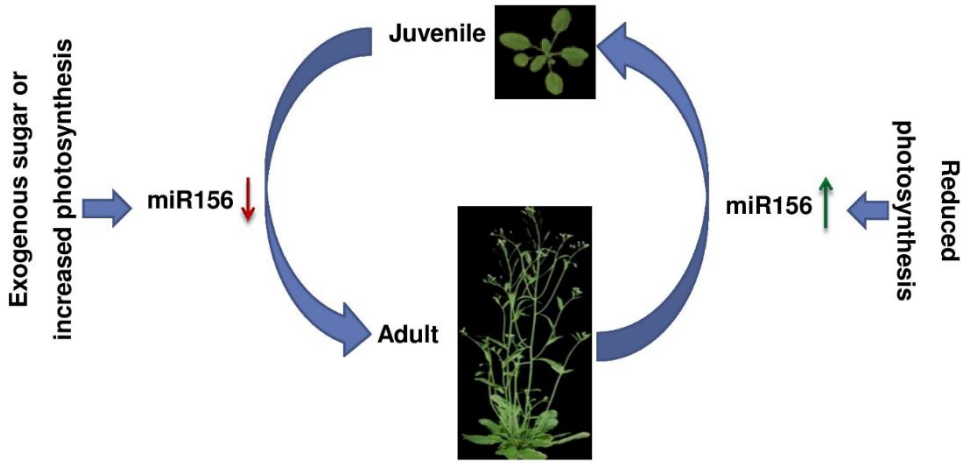
Supplementary documents

Supplementary Fig. S1. Schematic figure of how phase change is regulated by miR156 and how the level of miR156 is influenced by endogenous and exogenous factors in *Arabidopsis thaliana*.

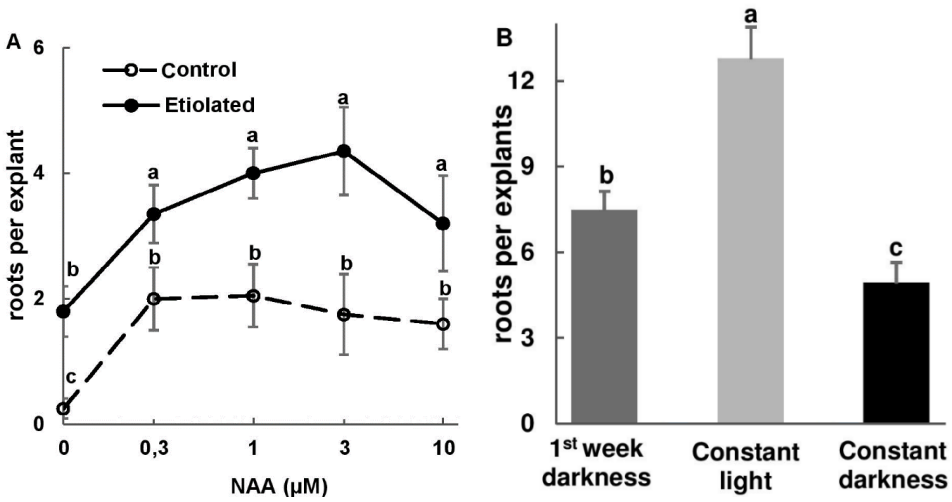
Supplementary Fig. S2. Effect of etiolation during donor plant preparation or during auxin treatment on rooting of apple micro cutting.

Supplementary Fig. S3. Effect of flooding pre-treatment on vegetative vigor of apple donor plants and further rooting of micro cuttings.

Supplementary Table 1S. Primers sequences used for Quantitative Real-Time PCR analysis.

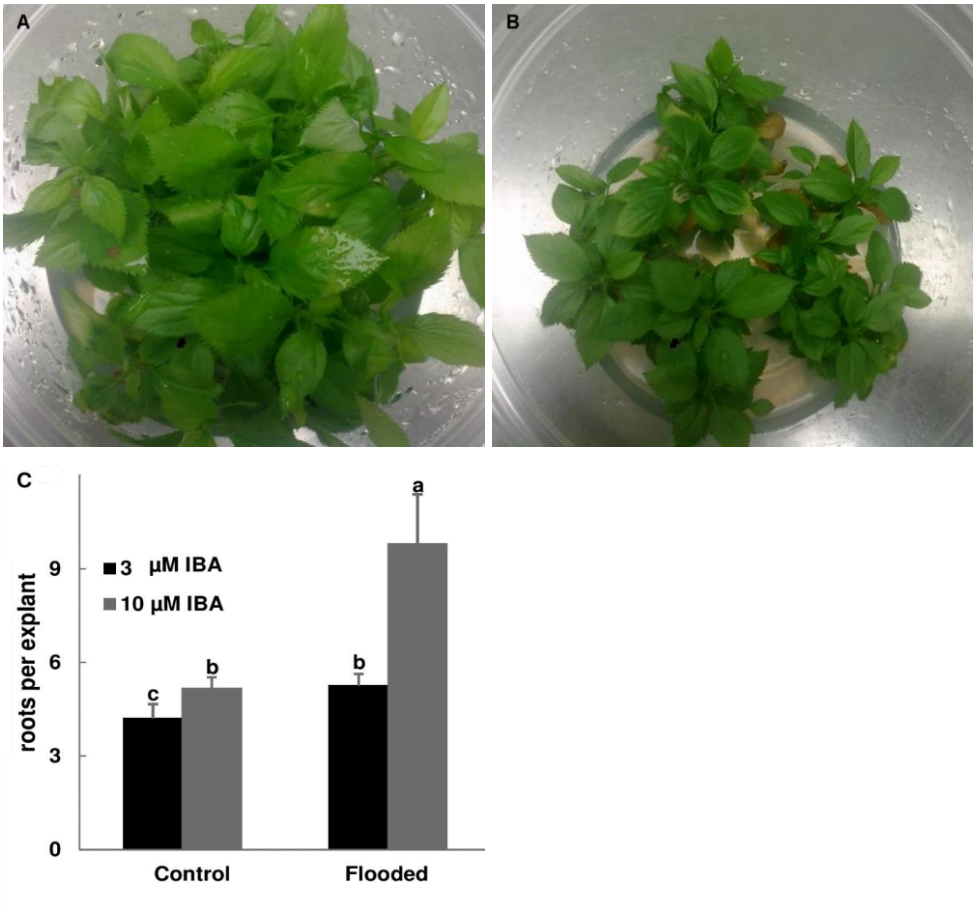


Supplementary Fig. S1. Schematic figure of how phase change is regulated by miR156 and how the level of miR156 is influenced by endogenous and exogenous factors in *Arabidopsis thaliana*. The model has been deduced from recent research (Yang *et al.*, 2013; Yu *et al.*, 2013). Red upside down arrow: reduced level, green upright arrow: increased level.



Supplementary Fig. S2. Effect of etiolation during donor plant preparation or during auxin on rooting of apple micro cutting. A) Rooting of micro cutting after 2 weeks etiolation of donor plants. B) Rooting of micro cutting while different light/dark was applied during rooting treatment. The results suggest both positive and negative effect for etiolation depending on the

time of application. Its application during donor plant preparation increased further rooting response of the cuttings whereas during the rooting treatment it negatively influenced the rooting response. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$.



Supplementary Fig. S3. Effect of flooding pre-treatment on vegetative vigor of apple donor plants and further rooting of micro cuttings. Flooding pre-treatment was applied to 4 weeks-old donor plants for one week. A) Flooding pre-treated apple donor plants for one week, B) nontreated (control) apple donor plants. C) Rooting of apple micro cutting taken from flooded and control donor plants. Means across replicates are presented with SE. Different letters represent means that are significantly different at $P < 0.05$.

Supplementary Table S1. Primers sequences used for Quantitative Real-Time PCR analysis.

Gene ID	AGI ID	Forward primer (5'→3')	Reverse primer (5'→3')
ACT2	*At3g18780*	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC
AFB1	*At4g03190*	GTGCTTCCGTCTCTGTGTGA	GAAGACCAGAGACGGAGAGC
AFB2	*At3g26810*	GGTTGGGACAAGAATGGATG	CGGAAGACGACCAATCAGAA
MAX1	*At2g26170*	GTCCAACCGCTATGCCTCTA	GCTGAGATTGGGGAAGGAAT
MAX2	*At2g42620*	GTGCCTGACTTTGAGGAAGC	CGGCTACACGAACCAACTCT
MAX3	*At2g44990*	CCTCTAAACGGGTGGAACAA	CTCCGGTAGACCAAGTACGG
MAX4	*At4g32810*	GCGGTGACGGAGAATTATGT	GGGACACCACTCGAACTTGTA
TIR1	*At3g62980*	CCTCTGGGTGCTTGACTACA	ACGGAAACACTCTCAGCTCG

Chapter 6

General Discussion

Vegetative propagation is widely used in horticulture and forestry for multiplication of elite plants obtained in breeding programs or selected from natural populations (Hartmann *et al.*, 2011). It is highly dependent on adventitious root (AR) formation as the capacity of plants to establish themselves successfully depends mainly on the ability to form new roots. The process of AR formation, which is itself influenced by many endogenous and exogenous factors, depending on the plant species, can be easy, difficult or impossible to achieve. Poor or lack of AR formation in many plant species has severe economic consequences. It can lead to losses of up to 25% of the nursery crops in Dutch horticultural industry (De Klerk *et al.*, 1999b). During AR formation differentiated somatic cells transit into a new developmental pathway. The mechanisms underlying this switch are highly interesting from the scientific point of view. However, many aspects of this transition are unclear and elucidation of these aspects require further investigations.

The aim of the research presented in this thesis was to investigate the influence of different factors on AR formation and to describe the mechanisms via which each of these factors exert their effect. In this chapter, the most important results are recapitalized with emphasis on polar auxin transport (PAT), plant age and the influence of some donor plant preparation techniques. Finally, future prospects of this research are discussed.

A role for auxin in AR formation

Auxin is the most studied phytohormone and participates in a variety of developmental processes. Distribution of auxin, during early stages of plant development, mediates embryo patterning (reviewed in Möller and Weijers, 2009). In addition, auxin is also required for other developmental events in which establishment of new meristem identity is necessary *e.g.*, during flower development (Krizek, 2011) and lateral root (LR) formation (Dubrovsky *et al.*, 2008). The endogenous levels of auxin, regulated by biosynthesis and metabolism as well as the transport throughout the plant are affected by genetic and environmental factors (Han *et al.*, 2009). By PAT, auxin gradients are formed throughout the entire length of the plant and these gradients are involved in *de novo* organogenesis, such as leaves and roots.

Just as in stems, in all other types of tissue in *Arabidopsis* (Ludwig-Müller *et al.,* 2005) application of auxin results in the stimulation of AR formation. Similarly, application of auxin to cuttings has a consistent effect across many plant species *i.e.,* induction of ARs (reviewed in Oinam *et al.,* 2011; Pijut *et al.,* 2011) indicating a key role for auxin in AR formation. Different factors, *i.e.,* uptake, transport, oxidation and conjugation influence the actual concentration of free auxin that reaches the target cells. PAT has been shown to be intimately related with AR formation as in many cases application of PAT inhibitors negatively influences AR formation (Diaz-Sala *et al.,* 1996; Tybruski and Tretyn, 2004; Ahkami *et al.,* 2013). However, precise information on the molecular network controlling PAT during this process is lacking and therefore was one of the research targets of this study.

Development of a model system in *Arabidopsis*

An approach using reverse genetics has been crucial in identifying components of PAT during different stages of LR and crown root development in *Arabidopsis* and rice (Coudert *et al.,* 2010; Lavenus *et al.,* 2013). Accordingly, we also utilized this technique to identify the role of members of PIN family of transporters during AR formation on hypocotyl and flower stem (FS) explants (**Chapter 3**). However, an efficient AR model system in *Arabidopsis* was lacking and establishment of such a system was therefore needed prior to mutant analysis. To this end, in **Chapter 2**, we examined the rooting response of various explant types *viz.,* hypocotyl, FS and rosette leaves (RL) to different auxin types (IAA, IBA and NAA). Our results showed that different plant organs or tissues have different rooting responses, *e.g.,* highest rooting response was observed in hypocotyl explants whereas RL produced the lowest number of roots. This may be related to different factors including age (physiological/ontogenetic age) (Ballester *et al.,* 1999; Vidal *et al.,* 2003), endogenous levels of phytohormones (most importantly auxin) (Malamy 2005; Osmont *et al.,* 2007), vascular patterning (Bellini *et al.,* 2014) and distance from the root system (Dick and Leakey, 2006; Leakey, 2004). The better rooting response of hypocotyl explants, for instance, may be because of its ontogenetic age (they are juvenile while in

FS explants transition to adult stage has occurred) or the presence of pericycle-like cell layer (predestined cells to form ARs reminiscent to that in LR formation).

In addition, various auxins have also caused different rooting responses. We determined IBA and IAA as the auxins of choice for *in vitro* rooting of *Arabidopsis* hypocotyls (**Chapter 2**, Fig. 4A) and IAA for *in vitro* rooting of both RL and FS explants (**Chapter 2**, Figs. 1 and 2). The reasons for the differences in effectiveness amongst the various auxins may be related to the different uptake, metabolism and transport of the three tested auxins. On the other hand, different affinities for the auxin receptors induce a different signal transduction (Verstraeten *et al.,* 2013). Moreover, the physiological status or receptivity of the target cells may also play a role in the organogenic response (Verstraeten *et al.,* 2013).

By monitoring the root formation at different positions on an explant, we found that in both hypocotyl and FS explants the basal ends generate more ARs compared to the apical ends. The better rooting response of basal versus apical ends might be related to the PAT. In our rooting system, hypocotyl and FS explants are positioned horizontally on the medium surface and both ends are in contact with the same level of auxin. It seems therefore that change over the time in concentration of auxin is the signal rather than concentration of auxin that both ends are in contact with. During LR initiation, accumulation of auxin in the pericycle has been shown as a signal to convert a pericycle cell to a founder pericycle cell (Dubrovsky *et al.*, 2008). In addition, Laskowski *et al.* (2008), with modeling of auxin transport in root on the stretch convex side of the bend, showed that the larger cells (at the bend) act as a stronger auxin sink. Subsequently, these cells accumulate sufficient auxin to trigger LR formation.

In addition to the establishment of a model system, in **Chapter 2** we determined the timing of successive phases during AR formation based on sensitivity to auxin and cytokinin. This is very important for many reasons. For instance, molecular studies aimed at elucidating the changes in expression of genes involved in induction and initiation stage can benefit much from these timings. Our results (**Chapter 2**, Fig. 6) indicated that induction occurs at 24 h after explant excision and the presence of auxin for at least 72 h is vital for AR formation in FS explants. Histological observations (**Chapter 2**, Fig. 7) have also determined that mainly the starch sheath cells adjacent to

phloem and to a lesser extent epidermis cells are the origins of ARs in FS explants. In the course of our research other researchers found similar results which do support our findings (Verstraeten *et al.*, 2013; Welander *et al.*, 2014). These findings are essential for cell-specific transcriptional profiling to investigate the early events that happen in root initials. Application of specific techniques *e.g.*, laser capture microdissection (LCM) and fluorescence activated cell sorting (FACS) will help performing cell-specific transcriptional profiling.

The role of PAT during AR formation in *Arabidopsis* hypocotyl and FS explants

After establishing a model system for AR formation in *Arabidopsis*, we performed in **Chapter 3** a detailed study to unravel the molecular mechanisms controlling PAT during AR formation in hypocotyl and FS explants. Application of PAT inhibitors, TIBA (2,3,5-triiodobenzoic acid) and NPA (1-N-Naphthylphthalamic acid), negatively affected the rooting response of both hypocotyl and FS explants (**Chapter 3**, Fig. 1). A positive role for PAT during AR formation was evident from our earlier observations (**Chapter 2**, Fig. 5 and Supplementary Fig. S3) in which the basal end of stem cuttings compared to the apical ends showed a higher tendency to form ARs. It has been reported that members of influx carriers (AUX1 and LAX3) as well as efflux carriers (PINs) are important for different stages of LR formation (Marchant *et al.*, 2002; Benkova *et al.*, 2003; Swarup *et al.*, 2008). We utilized a reverse genetics approach to unravel the role of the PIN family of transporters during AR formation in hypocotyl and FS explants.

We observed that hypocotyls in *pin1* and *pin2* mutants produced substantially less ARs compared to wild-type (*WT*) plants (**Chapter 3**, Fig. 4A) indicating a major role for PIN1- and PIN2-proteins in controlling AR formation in hypocotyl explants. In FS explants however, we showed that other PIN-proteins (PIN2, PIN3, PIN4 and PIN7) play a role in AR formation (**Chapter 3**, Fig. 4B). Our results also imply that the effect of PIN-proteins in AR formation is explant-specific, but that PIN2 is a main regulator of auxin transport in both explants.

Benková *et al.* (2003) have reported that that PIN1 is the major regulator of shoot-derived organ formation. However, we did not observe such significant role for PIN1 in formation of ARs in FS explants. It is possible that most of the free IAA in FS of *pin1* mutants originates from another source like RL. This was suggested by Jones *et al.* (2005) who showed that the level of endogenous IAA in the FS of *pin1-1* and *WT* plants is similar. However, if this is the case, in FSs of the *pin2* mutant a similar level of free IAA is expected as in the *pin1* mutant. Nevertheless, the number of ARs is significantly reduced in FSs of *pin2* mutants. It is possible that in FSs of *pin2* mutants, the induction stage has not been affected, but instead a later stage of AR formation *i.e.*, emergence and outgrowth, is hampered.

We scored the roots from different developmental stages (emerged or arrested root primordia) in the hypocotyls of *pin1*, *pin2* and *WT* plants cleared with chloral hydrate (**Chapter 3**, Fig. 6). In *pin1* plants, compared to *pin2* and *WT* plants, the number of induced ARs was significantly less. This indicates that in hypocotyl explants, PIN1 is mainly important during the early stage of AR formation. In addition, we also observed a proportion of arrested root primordia in hypocotyls in *pin1* implying that PIN1 also plays a role during later stage of AR formation.

In hypocotyls of *pin2*, the numbers of induced ARs were nearly similar to the numbers in *WT* plants. However, the higher number of arrested root primordia in the hypocotyls of *pin2* mutants versus *WT* plants revealed that this protein (PIN2) is important during the later stage of AR formation, emergence and outgrowth. Based on our histological observations (**Chapter 3**, Fig. 6) and *in situ* expression of *PIN1* and *PIN2* in hypocotyls at different time points (**Chapter 3**, Supplementary Fig. S3), we describe a model that portrays the action of these genes during AR formation (Fig. 1). *PIN1* expression at vascular cylinder and pericycle layers causes transport of auxin in this region. Accumulation of auxin then determines the site of new ARs (Fig. 1A). This will lead to cell division and formation of new AR primordia (Fig. 1B) 6 days after auxin treatment. In this stage, the *PIN1* is expressed in all cells in the primordium. Nine days after auxin treatment, the newly formed AR primordium is at the stage of emergence and outgrowth (Fig. 1C). In this stage, expression of *PIN1* is restricted to the central cylinder and vascular connection has been established between newly

formed AR and the hypocotyl. *PIN2* expression was not detected in the hypocotyl, however, at a later stage (emergence and outgrowth), its expression was observed at epidermis and cortex layers of newly formed AR. We speculate that its expression at this stage is necessary for removing the excess of auxin from the root tip to facilitate root's outgrowth. This is similar to what has been previously reported for the role of *PIN2* during LR formation (Benková *et al.*, 2003).

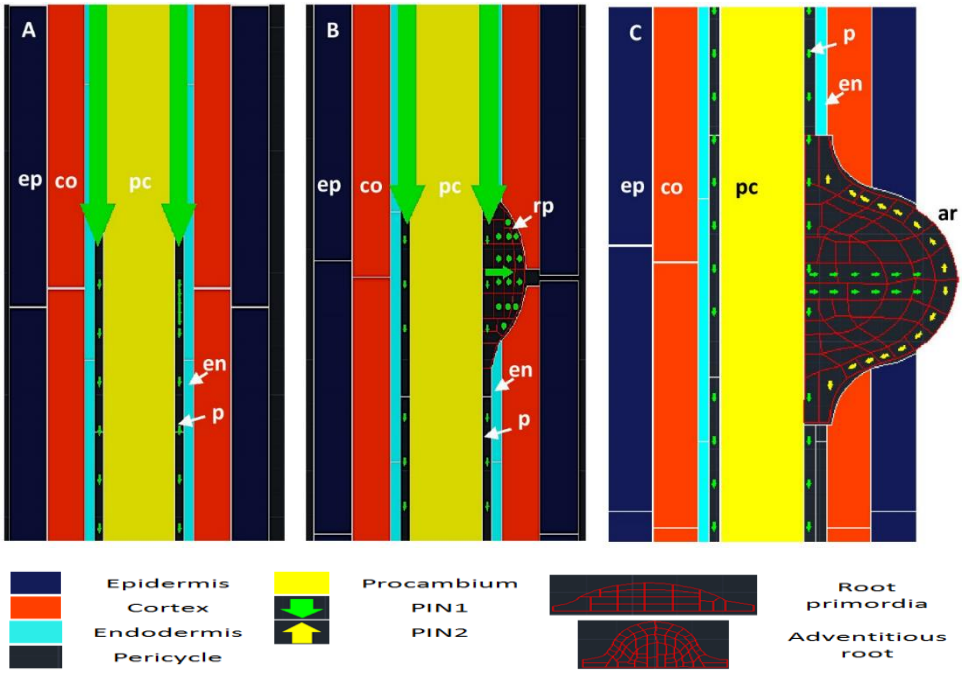


Fig. 1. Hypothetical model for the role of *PIN1* and *PIN2* during the process of AR formation in *Arabidopsis* hypocotyls. Longitudinal-section of hypocotyl 1 day A), 6 days B) and 9 days after auxin treatment are shown in this figure. adventitious root (ar), cortex (co), endodermis (en), epidermis (ep), pericycle (p), procambium (pc), root primordia (rp).

Our result, that PIN1 mediated auxin transport is required for AR formation in hypocotyl explants, is in line with the findings of Xu *et al.* (2005). They showed that in OsRNAi lines of PIN1 in rice the number of ARs is reduced. In addition, in rice a defect in GNOM (a known regulator of localization of auxin transport protein in

Arabidopsis) reduced the number of ARs. This is possibly because of altered localization of PIN-proteins (Liu *et al.*, 2009). With respect to the involvement of other efflux carriers, Sukumar *et al.* (2013) have shown that ABCB19 plays a significant role in AR formation in *Arabidopsis* hypocotyls. They observed that upon explant excision, ABCB19- and PIN1-mediated auxin transport increase. The involvement of auxin influx and efflux carriers during the mechanism of quiescent center (QC) cell establishment in AR tips of *Arabidopsis* has also been unraveled (Della Rovere *et al.*, 2013). These findings indicate that both influx and efflux carriers are intimately involved during different stages of AR formation.

In FS explants, its complex structure compared to hypocotyls made the histological analysis difficult. We utilized qRT-PCR to check the transcript levels of *PIN* genes in these explants at different time points (**Chapter 3**, Fig. 7). The results provided us with an indication about the timing of action and the involvement of these genes during AR formation. For instance, the transcript level of *PIN2* significantly increased at 120 h after auxin treatment. This may indicate the importance of this gene during later stages of AR formation (emergence), probably similar to what we suggested for its role during AR formation in hypocotyl explants. On the other hand, *PIN3* and *PIN7* transcript levels were highest at the early stage (12 h) whereas *PIN4* transcript was high both at early and later stages. Further investigations are required to completely elucidate the role of these genes. Nevertheless, previous studies have provided information that helps explaining the mode of action of some of these genes. For example, we suggest a role for *PIN3* gene during early stage of AR formation in providing auxin in the starch sheath cells that are the origins of new ARs.

Phase transition and its influence on AR formation

Transition from juvenile to adult, also known as phase transition, maturation or ontogenetic aging, is associated with progressive changes in the morphology and physiology, including leaf shape, phyllotaxis, shoot orientation, and the ability to form ARs (Hackett, 1985). Woody plants display a gradient of juvenile to mature tissue in the above ground portion (Hackett, 1985). The influence of phase transition on the

capacity of plant tissues to form AR has been shown mainly in tree species (Diaz-Sala *et al*., 1996; Ballester *et al*., 1999; Vidal *et al*., 2003).

The aim of this research was to obtain a more accurate picture of the effect of ontogenetic age on AR formation in herbaceous plants. To this end, in **Chapter 4**, we investigated the rooting response of RL of different ontogenetic age (based on their morphology) (**Chapter 4**, Fig. 1A), stem segments excised from different position at the FS (**Chapter 4**, Fig. 1B) as well as hypocotyl versus FS segments (**Chapter 4**, Supplementary Fig. S1). Our observations support the notion that an ontogeny-related gradient in AR formation potential in *Arabidopsis* plants does exist.

One of our hypotheses was that upon aging the methylation status of DNA increases which plays an integral role in regulating gene expression, as a gene that is methylated is silenced and cannot be transcribed (Grant-Downton and Dickinson 2005). Among the silenced genes some might be related to the mechanism controlling AR formation. To test that hypothesis, 5-azacytidine (AzaC), a hypomethylating agent, was applied both during the rooting treatment (short) and during seedling growth and development (long). The rooting response of the explants from different ontogenetic ages (hypocotyl and FS) was evaluated accordingly. The reason for application of AzaC during both rooting treatment and seedling development was that both stages contain massive cell division. Since AzaC incorporates into DNA during DNA replication, its effect is expected during both stages. When AzaC was applied during rooting treatment, it did not change the rooting response of hypocotyl explants (**Chapter 4**, Supplementary Fig. S2). This indicated that juvenile plant materials naturally have a low methylation status and treatment with AzaC cannot further reduce the level or cause additive effects. On the other hand, AzaC did increase the rooting response of FS explants (**Chapter 4**, Fig. 2).

Similar to addition of AzaC during the rooting treatment, application during seedling growth and development increased only rooting of adult plant materials (**Chapter 4**, Fig. 3) and not rooting of juvenile ones (**Chapter 4**, Supplementary Fig. S3). Evaluations concerning the changes in DNA methylation levels upon AzaC treatment (**Chapter 4**, Fig. 3B) confirmed that DNA methylation is reduced in FS

tissues after AzaC treatment; together this adds proof to our hypothesis that AR formation is negatively influenced by the methylation status of genomic DNA.

It has been reported that AzaC affects some cellular processes besides DNA methylation *e.g.,* protein and nucleotide synthesis (reviewed in Christman, 2002). However, we did not observe any phenotypic differences between AzaC treated and nontreated plants. Furthermore, our results that AzaC did not influence rooting of juvenile plant materials but increased that of mature ones as well as the results of methylation analysis showed that the increased rooting after AzaC treatment is mainly related to hypomethylation. Here we showed that AzaC treatment can be an effective method for *in vitro* rooting of cuttings especially those taken from plants that are recalcitrant to root.

In addition to a role for DNA methylation, we investigated in **Chapter 4**, the correlation between miR156 and AR formation. The level of miR156 determines the transition from juvenile to adult in *Arabidopsis* (Wu *et al.*, 2009a; Wu and Poethig, 2006). Also in other species, miR156 is expressed at high levels in seedlings and at reduced levels in mature plants (Chuck *et al.*, 2007, Zhang *et al.*, 2011). However, it is not clear whether maturation-related loss in AR formation is also under the influence of miR156. To address this question we performed further experiments. We evaluated the rooting response of juvenile and adult plant materials (hypocotyl, RL and FS) in three *Arabidopsis* lines expressing different levels of miR156 *viz.*, *35S::MIM156* (under), *35S::MIR156* (over) and *WT* plants. No obvious difference was observed in the rooting response of hypocotyl explants in these three lines (**Chapter 4**, Fig. 6A) indicating that as long as plant materials are at the juvenile stage the rooting response is not affected by the level of miR156. However, in adult plant materials (RL from vegetative adult stage and FS explants), explants obtained from *35S::MIR156* and *35S::MIM156* produced the highest and lowest number of roots respectively (**Chapter 4**, Fig. 6B and C). Explants taken from *WT* plants showed an intermediate response.

We observed that in *35S::MIR156* the FS response curve for auxin had shifted to the left. This indicates that in this plant FS cells have a higher responsiveness towards applied auxin compared to the explants excised from *35S::MIM156* and *WT* plants. Interestingly, despite negligible rooting of FS explants in *35S::MIM156*, formation of

callus was observed at the cut surface indicating that reactivation of cell division in response to the supply of exogenous auxin is not impaired. Therefore, in this case organized cell division seems to be missing as was suggested by Diaz-Sala *et al.* (1996) in loblolly pine.

Although in FS explants phase change had already occurred, we observed that in these explants the rooting response still depends on the levels of miR156. Better rooting response of FS in *35S::MIR156* might be related to either higher competence of the cells, slower decline of juvenile response or the interaction of miR156 with epigenetic DNA-methylation for RNA-directed DNA methylation. It is also possible that increased AR formation potential upon miR156 overexpression is a result of increased juvenile characteristics via its downstream pathways (regulation of SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) transcription factors as well as miR172 and MADS-box genes). However, this still needs to be studied in more detail. Recently, Yu *et al.* (2015a) showed that the overexpression of miR156 in *Arabidopsis* causes production of more LRs via its target genes (*SPL*) with *SPL10* playing a dominant role.

Our findings indicate that in *Arabidopsis* mature explants (FS and RL) there is a positive correlation between the expression level of miR156 and AR formation potential. Similarly, overexpression of miR156 resulted in multiple vegetative and reproductive trait alterations among which increase in aerial stem roots in tomato (Zhang *et al.*, 2011) and prop roots in maize (Chuck *et al.*, 2007).

The effect of donor plant pre-treatments on AR formation in *Arabidopsis*

Two major pathways to improve rooting are first to improve the rooting process itself and second to improve rootability of the cuttings. The latter approach includes pre-treatments of donor plants and different methods for that have emerged *i.e.*, rejuvenation, etiolation and flooding. In the previous section we dealt with rejuvenation. In the current section, we discuss the effect of etiolation and flooding as two donor plant pre-treatments.

In order to unravel the mechanism underlying the effect of etiolation, in **Chapter 5**, we first established a system in *Arabidopsis* hypocotyl and FS explants. Our results

showed that etiolation positively influences AR formation in both explant types (**Chapter 5**, Fig. 2). In addition, hypocotyls of etiolated seedlings had already started root formation at the time of explant excision, whereas no AR had been formed on hypocotyls of nontreated seedlings. Similarly, in petunia cuttings it has been reported that the formation of root meristems had already started during the dark treatment and was enhanced during the rooting period (Klopotek *et al.*, 2010).

In addition to the change in rooting response, morphology of the seedlings was affected when etiolated, in particular, reduced apical dominance and increased branching. This may be caused by changes in auxin transport. Similarly, it has been shown that in *Arabidopsis* mutation of the *MAX* genes impairs strigolactone (SL) biosynthesis. This consequently increases the transport of auxin and growth of axillary branches (Bennett *et al*., 2006). We have previously (**Chapter 3**) showed that when SL is reduced by a carotenoid biosynthesis inhibitor, fluridone, rooting is also increased. In order to see if there is any correlation between light/dark and observed phenotypic changes we checked the expression of genes involved in SL biosynthesis and signaling. The results showed that in light the expression of SL biosynthesis (*MAX1*, *MAX3* and *MAX4*) and signaling (*MAX2*) genes is increased (**Chapter 5**, Fig. 3). This may, therefore, be a reason for less rooting response of light grown seedlings versus etiolated ones.

There is, however, a second possible mechanism for better rooting response of explants taken from etiolated seedlings compared to nontreated ones. Carbohydrates are the principle source of energy and structural elements. Considering that AR formation is a high-energy demanding process, a reduction of sucrose during the rooting treatment leads to a reduction of rooting as was reported *e.g.*, by De Klerk and Calamar (2002). We measured the level of endogenous soluble carbohydrates (ESCs) in etiolated and nontreated seedlings (**Chapter 5**, Fig. 1). Negative influence of etiolation on the level of ESCs was observed which is probably caused by the absence of photosynthesis and by the extra use of building blocks (used for elongation). Despite decreased ESCs in etiolated seedlings, rooting response was significantly increased. This can be explained by the role of sucrose as a signaling molecule (Smeekens, 2000; Price *et al.*, 2004). It has been reported that transition of juvenile to mature phase is

stimulated by a high endogenous sucrose level (Wu and Poethig, 2006). In addition, sugar negatively influences the level of miR156 (Yang *et al.*, 2013). In the juvenile phase, miR156 is highly expressed and its expression decreases dramatically during vegetative phase change. We have shown (**Chapter 4**) that maturation-related loss in AR formation is under the influence of miR156. Considering these facts, we speculate that reduced carbohydrate content during dark exposure increases the level of miR156. This leads to rejuvenation or arresting of the ontogenetic aging of donor plants and consequently increases AR formation potential.

In **Chapter 5**, we also investigated the effect of flooding/double layer as another donor plant pre-treatment on subsequent rooting of hypocotyl and FS explants. We observed that in flooding-treated seedlings, the growth was much enhanced (**Chapter 5**, Fig. 4A). This is probably related to a better nutrient uptake from liquid medium than from semi-solid medium. Similar to etiolation pre-treatment, flooding increased rooting response of excised hypocotyl and FS segments (**Chapter 5**, Fig. 7). This was evident at the time of explant excision when some of the hypocotyls had already started rooting. This is similar to the findings of Vidoz *et al.* (2010) who reported that 24 h after submergence of tomato plants, the root primordia had already formed. It seems that formation of AR caused by flooding is an adaptive response to this stress situation in order to replace the original root system. To see if these finding can be translated into other crops, we also performed similar experiments in apple as a model for woody crops and nearly the same results were observed (**Chapter 5**, Supplementary Fig. S3). This provided indications for the wider applicability of this technique in increasing AR formation.

We performed a microscopic analysis in flooding treated and nontreated FS (**Chapter 5**, Fig. 5). We observed that in FS of flooded seedlings, secondary phloem is formed. Welander *et al.* (2014) showed that starch sheath cells neighboring the phloem are the main origins of ARs. It seems, therefore, that the formation of secondary phloem in flooded explants increases the area where these cells are adjacent to the phloem parts. This will consequently increase the chances of root initials being formed. There seems to be another reason for better rooting response of explants taken from flooded seedlings. Our study showed that the level of ESCs is negatively influenced by

flooding (**Chapter 5**, Fig. 6). Just as in the etiolation treatment, this may promote the capacity to root by promoting the juvenile state.

Conclusions and future prospects

AR formation which is a major step in vegetative propagation represents a switch of differentiated cells into a new developmental pathway. Despite numerous studies on AR formation, many aspects of AR formation are still unclear. The research presented in this thesis succeeds in assigning a role for auxin transport and some of its components, notably PIN-proteins, during different stages of AR formation. In addition, we provided evidence for a mechanism that regulates maturation-related loss in AR formation capacity. Furthermore, application of two pre-treatments of donor plants and their possible mode of action in increasing AR formation were discussed.

In this research, we established a model system for *in vitro* rooting of *Arabidopsis* by testing different explant types as well as various auxin types/concentrations. This is an efficient model system for further investigations of mutant/transgenic lines in order to elucidate the role of specific pathways in controlling AR formation. On the other hand, because of structural similarity of *Arabidopsis'* FSs to cuttings or *in vitro* micropropagated shoots, our model in FS explants seems relevant for woody species that are often vegetatively propagated by stem cuttings. In addition, we determined the timing of phases during adventitious rooting as well as origins of ARs in FS explants. These results are advantageous for cell specific transcriptional profiling via various techniques like LCM and FACS. This will lead to deciphering of the early events that occur in AR initials and distinguish them from surrounding cells.

In the study on PAT, our results showed that PAT is important for AR formation. We also showed that PIN-proteins play a major role during AR formation. Except for PIN2 that is important for adventitious rooting in both hypocotyl and FS explants, the role of other PIN-proteins seems to be explant-specific. Our results together with the findings of Sukumar *et al.* (2013), make it clear that efflux carriers are the major regulators during different stages of AR formation. However, the importance of influx carriers should also be taken into account. Further studies are therefore essential to

fully elucidate the role of different auxin transporters and their cross–regulation during different stage of AR formation.

In this study, we investigated the influence of phase change on AR formation. In earlier research on phase change, flowering has been used as major characteristic. Here, we used AR formation as distinctive characteristic and showed how it changes upon aging. We showed in *Arabidopsis* that the ability of tissues to form AR is closely related with phase change, changes in DNA methylation and in the expression of miR156. Despite the fact that in mature plant tissue phase transition has already occurred, the results showed that the level of miR156 still matters and this is possibly because of its downstream pathway similar to what has been reported for LR formation. Further studies are therefore needed to fully address the link between ontogenetic age, miR156 levels and its downstream pathway and AR response suggested by our experiments. On the other hand, since miR156 targets SPL transcription factors to mediate the morphological and physiological changes associated with phase transition (Poethig, 2010), it seems interesting to further investigate whether increased AR formation potential upon miR156 overexpression is regulated via its downstream pathways.

We showed that AzaC treatments during seedling growth (long) or during the rooting treatment (short) are both effective in the promotion of rooting. Moreover, we applied AzaC to apple microcuttings and the results confirmed our findings in *Arabidopsis*. An AzaC treatment can therefore be seen as a useful method in tissue culture for other crops especially those that are recalcitrant to root. We propose application of AzaC and other drugs with similar effect *e.g.*, 5-Aza-2′-deoxycytidine in horticultural practice to develop a protocol to increase rooting.

In this study, we also performed investigations to understand the mechanisms behind the role of etiolation and flooding as two donor plant pre-treatments in controlling AR formation. Our results showed that these two techniques can be used as an efficient way to increase AR formation in cuttings. Apart from this research that was performed in *Arabidopsis* as a model organism, we also performed similar experiments in apple as a representative for woody crops to see whether these finding can be extrapolated to other species. Nearly similar results were obtained (**Chapter 5**,

Supplementary Fig. S2 and S3). This provided indications for the wider applicability of these techniques in increasing AR formation in other crops. We provided further evidence of how environmental conditions can affect the physiological and biochemical quality of donor plants and consequently influence the rooting capacity of the cuttings. As further research, we propose studying the effect of different environmental factors during either donor plant preparation or during rooting treatment. One example would be the influence of different light intensities. Significant improvements have been achieved in the development of plant lighting. By the advent of light-emitting diodes (LEDs), the first testing of LEDs for plant growth was done at the University of Wisconsin (Bula *et al.*, 1991). Now, LED lights are more cost-effective than ever and their application in horticulture is becoming a trend. Therefore, we propose studying the effect of LED lights on further rooting of cuttings. This lighting system facilitate the application of different wavelengths and their effects on rooting of the cuttings.

We also propose to study the importance of photosynthesis (PS) for AR formation. Here we showed that etiolation can increase the rooting response when it is applied during the donor plant preparation stage. We observed similar results in apple microcuttings. Since the major effect of etiolation is lack of photosynthesis (PS), in apple microcuttings we applied PS inhibitors and promotors during rooting treatment (Massoumi *et al.*, unpublished observations). Our results indicate a positive role for PS during rooting treatment whereas it was inhibitory during donor plant preparation.

Finally, based on our observations (in *Arabidopsis* and apple), in the following model (Fig. 2), we summarize the involvement of various factors (positive or negative regulators) on AR formation in cuttings.

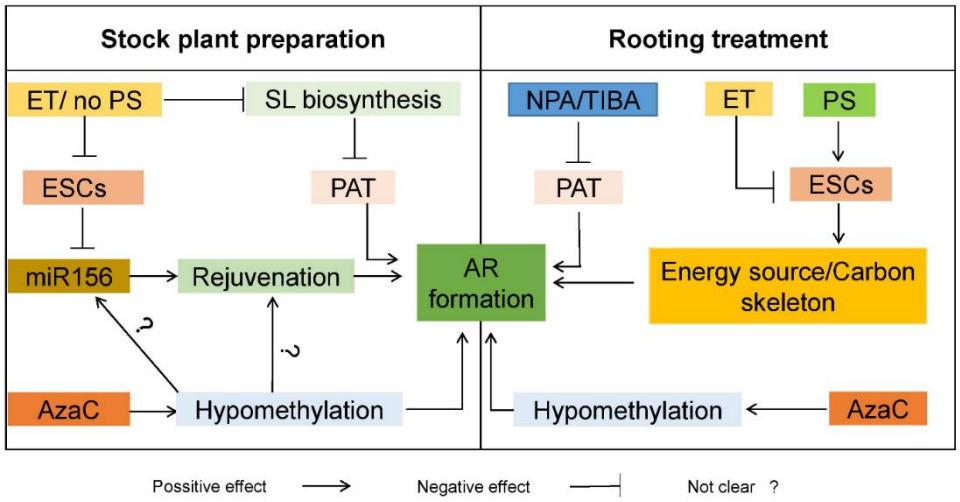


Fig. 2. Model representing the influence of different factors on AR formation in cuttings. These factors have been applied during the donor plant preparation or during rooting treatment stage. Adventitious root (AR), 5-azacytidine (AzaC), endogenous soluble carbohydrates (ESCs), ethylene (ET), microRNA 156 (mir156), 1-N-Naphthylphthalamic acid (NPA), polar auxin transport (PAT), photosynthesis (PS), strigolactone (SL), 2,3,5-triiodobenzoic acid (TIBA).

We found that the influence of these factors are specific to a certain stage *i.e.*, donor plant preparation and rooting treatment. During donor plant preparation, etiolation can negatively influence both SL biosynthesis and the amount of ESCs. Reduced SL biosynthesis can increase PAT and subsequently increase AR formation. On the other hand, reduced ESCs level will increase the level of miR156 (cause rejuvenation or prolonging the juvenile stage) and as a results rooting response increases. Application of AzaC at this stage causes hypomethylation of DNA and consequently an increase of adventitious rooting. Whether increase rooting is a result of rejuvenation or change in the expression of genes *e.g.*, MIR156 is unclear and further investigation is therefore needed.

During rooting treatment, etiolation or other factors (removing the leaves or CO_2 from the head space) that negatively influence photosynthesis cause reduction of ESCs level. We think, at this stage the role of ESCs maybe serving as energy source or as carbon skeleton. When sugar was removed from the media similar results were

observed. Application of AzaC at this stage caused DNA hypomethylation and as a result adventitious rooting was increased. Whether increase rooting is a result of change in the expression of genes (*e.g.*, adventitious rooting related genes) remains to be explored.

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Summary

Vegetative propagation is widely used in agriculture, horticulture and forestry to multiply elite plants selected from natural populations and breeding programs. In vegetative propagation, rooting (adventitious root formation, AR formation) is an indispensable step. If cuttings do not form roots, no plants are produced, which results in large financial losses. AR formation is a heritable quantitative trait controlled by multiple endogenous and exogenous factors. The plant hormone auxin plays a central role. Exogenous application of auxin is routinely used to promote the development of ARs on stem cuttings but is not always effective. Better understanding of mechanisms underlying AR formation is, therefore, needed to improve the rooting of cuttings, in particular, those taken from recalcitrant genotypes.

The research described in this thesis was carried out with the model plant *Arabidopsis*. The availability of numerous mutants and transgenic lines makes *Arabidopsis* a valuable model system for dissecting the molecular mechanisms involved in the control of diverse developmental processes. In the present research, basic and applied aspects of the followings were studied: (1) establishment of a model system for AR formation in *Arabidopsis*, (2) the role of polar auxin transport and PIN-proteins during AR formation in different tissues, (3) the influence of rejuvenation and ontogenetic aging on rooting, and (4) the effect of two donor plants pre-treatments that enhance the capability of *Arabidopsis* tissues to root.

In order to establish an efficient AR formation model system in *Arabidopsis*, we examined the rooting response of various explant types, *viz.*, hypocotyls, flower stems (FSs) and rosette leaves (RLs), to different auxins (IAA, IBA and NAA). The results showed that different plant organs and tissues have different rooting responses. The highest rooting response was observed in hypocotyl explants. Different types of auxin cause different rooting responses. IBA and IAA were determined as the auxins of choice for *in vitro* rooting of *Arabidopsis* hypocotyls and IAA for *in vitro* rooting of both RL and FS explants. We also found that in both hypocotyl and FS explants the basal ends generate more ARs compared to the apical ends likely because of polar auxin transport (PAT). We determined the timing of successive phases during AR formation. This was done based on the sensitivity of FS explants to auxin and cytokinin. We showed that induction occurs at 24 h after explant excision and the

presence of auxin for at least 72 h is essential for AR formation in FS explants. We also performed histological analysis. The results demonstrated that mainly the starch sheath cells adjacent to phloem and to a lesser extent epidermis cells are the origins of ARs in FS explants.

It was mentioned before that AR formation is influenced by different factors and that auxin plays a central role. Since PAT is one of the factors determining the amount of free auxin that reaches the target cells, we investigated the role of PAT and PIN-proteins during AR formation. A significant decrease in rooting response occurred by application of PAT inhibitors. This indicated a positive role for PAT during AR formation. Then a reverse genetics approach was performed to unravel the role of the PIN family of transporters during AR formation in hypocotyl and FS explants. The results implied that the effect of PIN-proteins on AR formation is explant-specific, but that PIN2 is a main regulator of auxin transport in both explants. In hypocotyl explants PIN1 and PIN2, and in FS explants PIN2, PIN3, PIN4 and PIN7 were shown to be important. Based on histological observations in hypocotyl explants, we proposed a role for PIN1 during early stage of AR formation, *i.e.,* induction. For PIN2, however, we suggested a role during later stage of AR formation, *i.e.,* emergence and outgrowth.

Phase transition, also referred to as maturation or ontogenetic aging, is associated with progressive changes in the morphology and physiology of plants. In order to get better insight into the effect of phase transition on the ability of tissue to form ARs and its underlying mechanisms we performed a detailed study. To this end, the rooting response of RL explants with different ontogenetic ages was investigated. Moreover, we examined the rooting response of FS explants (excised from different position at the FS) as well as hypocotyl segments. The results showed that there is an ontogeny-related gradient in AR formation potential in *Arabidopsis* plants. One of our hypotheses was that aging might increase the methylation status of DNA and this would consequently affect the expression of genes, among others those related to AR formation. To test that hypothesis, 5-azacytidine (AzaC), a hypomethylating agent, was applied both during the rooting treatment (short) and during seedling growth and development (long), and the rooting response of the explants from different ontogenetic ages (hypocotyl and FS) was evaluated. Application of AzaC during both

stages did not change the rooting response of hypocotyl explants but did increase that of FS explants. This indicates that juvenile plant materials naturally have a low methylation status and treatment with AzaC cannot further reduce the level. We also evaluated the DNA methylation levels upon AzaC treatment. The results confirmed that DNA methylation is reduced in FS tissues after AzaC treatment.

Our second hypothesis was that upon aging the level of miR156 decreases and this may also be associated with the ability of tissue to root. To address this, we evaluated the rooting response of juvenile and adult plant materials (hypocotyl, RL and FS) in three *Arabidopsis* lines expressing different levels of miR156, *viz.*, *35S::MIM156* (under), *35S::MIR156* (over) and wild-type (*WT*) plants. The results showed that as long as plant materials are at the juvenile stage the rooting response is not affected by the level of miR156. However, in adult plant materials (RL and FS explants), the level of miR156 matters and is positively correlated with the rooting response.

In addition to treatment with auxin (that is the common way of inducing AR formation in commercial propagation) there are other treatments to achieve rooting concerning pre-treatment of donor plants. In this research, we examined the effect of two donor plant pre-treatments, etiolation and flooding, on *in vitro* rooting of hypocotyl and FS explants. Our results showed that etiolation positively influences AR formation in both explants. We observed that hypocotyls of etiolated seedlings had already started root formation at the time of explant excision, whereas no AR had been formed on hypocotyls of nontreated seedlings. We further performed qRT-PCR to check the expression of genes involved in strigolactone (SL) biosynthesis and signaling. Our results showed that SL biosynthesis and signaling genes are upregulated in light grown hypocotyls. So, auxin transport is reduced in light grown hypocotyls. We also checked the changes in the level of endogenous soluble carbohydrates in dark- and light-grown explants. Etiolation reduced the levels of soluble carbohydrates. This lower level of carbohydrates is unfavorable for rooting as carbohydrates are a main source of energy and building blocks for AR formation. However, etiolation stimulated rooting in spite of the lower carbohydrate level. This may be related to the role of sucrose as a signaling molecule. Since it has been shown that level of miR156 is

decreased at a high level of sugar, we speculate that reduced carbohydrate content during dark exposure increases the level of miR156 leading to rejuvenation of donor plants and consequently increases AR formation potential.

We also investigated the effect of flooding as another donor plant pre-treatment on subsequent rooting of hypocotyl and FS explants. This pre-treatment influenced the morphology of the plant (stronger vigor and larger leaves) as well as the rooting (increased number of ARs). Histological observations in FSs of flooding-treated and nontreated seedlings showed that flooding pre-treatment induces the formation of secondary phloem in FS explants. Since starch sheath cells adjacent to the phloem have been shown as the main origins of ARs, we think that the formation of secondary phloem in flooding-treated explants increases the area where starch sheath cells are adjacent to the phloem. Because of this the chance of root initials being formed is increased. In addition to microscopic analysis, our results showed that the level of soluble carbohydrates is negatively influenced by flooding. Just as in the etiolation treatment, this may promote the capacity to root by promoting the juvenile state.

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About the author

Mehdi Massoumi was born on September 18th, 1982 in Esfahan, Iran. From 2001-2005 he studied Horticulture at Faculty of Agriculture in Isfahan University of Technology (IUT), Iran. In 2005, Mehdi graduated as top bachelor student and received a scholarship to attend Master program without entering the national exam. For his master, he studied Plant Breeding in IUT, Iran. He carried out research on micropropagation and karyotype analysis of Alstroemeria flower as his master thesis and graduated on 2008. After fulfilling his military obligations and working for a short period of time in his own business in landscape designing, in December 2010, he started PhD in the Plant Breeding Department, Ornamental, tissue culture and gene transfer group at Wageningen University. He focused on understanding the mechanisms underlying adventitious root formation in Arabidopsis. This thesis is the outcome of his PhD research work. Since March 2015, Mehdi has been working as a researcher and the head of Research and Development department at Euro-Tiss company, the Netherlands.

EPS Certificate

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Mehdi Massoumi Bagherabadi
Date: 6 September 2016
Group: Laboratory of Plant Breeding
University: Wageningen University & Research Centre

1) Start-up phase	*date*
▶ **First presentation of your project**	
Adventitious Root Formation Underlying Mechanisms and Applications	Apr 2011
▶ **writing or rewriting a project proposal**	
Adventitious root formation in horticultural crop underlying mechanisms and applications	Jan 2011
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Course 'Radiation Hygiene', level 5B	Dec 2011
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, Amesterdam University	Nov 30, 2012
EPS PhD student day, Leiden University	Nov 29, 2013
▶ **EPS theme symposia**	
EPS theme 3 Symposium 'Metabolism and Adaptation', Wageningen University	Feb 07, 2011
EPS theme 3 Symposium 'Metabolism and Adaptation', Utrecht University	Apr 26, 2012
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden University	Jan 17, 2013
EPS theme 3 Symposium 'Metabolism and Adaptation', University of Amsterdam	Mar 22, 2013
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden University	Jan 08, 2015
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen University	Jan 21, 2016
▶ **Lunteren days and other National Platforms**	
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 02-03, 2012
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 22-23, 2013
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 13-14, 2015
▶ **Seminars (series), workshops and symposia**	
PhD retreat UK 2012	Aug 14-17, 2012
Next Generation Plant Breeding conference- Wageningen University	Nov 11-14, 2012
Plant Research day (Plant Breeding)	Mar 08, 2011
New Developments in Plant Endomembrane Biology- VU Amsterdam	Jun 15, 2012
Seminar Prof. Yukihiro Sugimoto (Kobe University, Japan)	Oct 16, 2012
▶ **Seminar plus**	
▶ **International symposia and congresses**	
V Intern. Symp. on Acclimatization and Establishment of Micropropagated Plants, Nebraska City, USA	Oct 16-20, 2011
VII international Symposium on Root Development, Weimar, Germany	Sep 15-19, 2014
▶ **Presentations**	
Establishment of a Model System in Adventitious rooting of Arabidopsis Thaliana, ISHS congress, USA (Talk)	Oct 16-20, 2011
Polar Auxin Transport influences rooting in Apple and Arabidopsis, NGP Breeding- WUR, (Poster)	Nov 11-14, 2012
Adventitious Root Formation in Arabidopsis, Lunteren (Poster)	Apr 13-14, 2012
Mutant Analysis: Concerning The Role of PAT in Adventitious Root Formation, Weimar, Germany (Talk)	Sep 15-19, 2014
Reduced rooting after the transition from juvenile to adult is accompanied by decreased miR156 and increased DNA methylation in Arabidopsis. Lunteren (Poster)	Apr 13-14, 2015
Adventitious Root Formation in Arabidopsis: The role of PIN genes, Lunteren (Poster)	Jun 05, 2015
7- Roots of rooting; mechanisms underlying adventitious root formation, NVPW, Wageningen (Talk)	Jul 05, 2015
▶ **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
▶ **Excursions**	
Seed valley- Enkhuizen	Jun 23, 2011
Enza Zaden	2013
Subtotal Scientific Exposure	*18.7 credits**

3) In-Depth Studies	*date*
▶ **EPS courses or other PhD courses**	
Postgraduate course 'Introduction to R for Statistical Analysis'	Oct 24-25, 2011
Postgraduate course 'Bayesian Statistics"	Oct 27-28, 2011
Postgraduate course 'Microscopy and Spectroscopy in Food and Plant Science'	May 7-11, 2012
Postgraduate course 'Natural Variation in Plants'	Agu 21-24, 2012
Postgraduate course 'Bioinformatics-A User Approach'	Aug 26-30, 2013
Postgraduate course 'The Power of RNA Sequencing'	Dec 16-18, 2013
▶ **Journal club**	
▶ **Individual research training**	
Subtotal In-Depth Studies	*6.3 credits**

4) Personal development	*date*
▶ **Skill training courses**	
Information literacy PhD -EndNote introduction	June 12-13, 2012
English for IELTS- 45 hrs, of study	Aug 19-30, 2013
Project and Time Management	Jan 15 & 29- Feb 29, 2015
▶ **Organization of PhD students' day, course or conference**	
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**37,1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*