# PIN gene effects on adventitious root formation in Arabidopsis

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In micropropagation, the major obstacle is poor adventitious root formation (ARF). In this paper, 30 μM IAA was applied to flower stem and hypocotyl of 6 different types of Arabidopsis thaliana (Col-0, pin1, pin2, pin3, pin4, pin7) to determine which PIN gene plays major role during ARF. Five different concentration (0, 3, 10, 30,100μM) of IAA were applied to the flower stem and hypocotyl of Arabidopsis thaliana to determine the optimum concentration for each PIN gene in ARF. And 3 different types of IAA, IBA pulses (24h, 48h, 72h) were applied to flower stem of Arabidopsis thaliana Col-0 to detect which stage of auxin application is the most important for ARF. The result shows that PIN1 plays an important role in ARF of Arabidopsis hypocotyl, while PIN3, PIN4 and PIN7 significantly influence ARF of Arabidopsis flower stem. The optimum IAA concentration in ARF of Arabidopsis hypocotyl is 10μM for pin1 and pin2, while in flower stem is 30μM for pin7 and 100μM for pin4. The application of auxin pulses in first 24hrs is the most important for ARF of Arabidopsis flower stem.

Key words: adventitious root formation; auxin; PIN gene; flower stem; hypocotyl; auxin pulses.

# Introduction

**Adventitious root formation**

Adventitious root formation (ARF) represents a developmental change of somatic cells in differentiated organs. During this process, differentiated cells undergo dedifferentiation and finally they develop into a new functional organ, a root. The molecular, biochemical, physiological and morphological processes that occur during this developmental change, are interesting topics for scientific research. On the other hand, ARF has also a major economic relevance. In horticultural industry, more than 70% of the propagation systems depends on successful rooting of cuttings (Davies et al., 1994) and many losses take place because cuttings fail to root. For instance, in The Netherlands about 25% of nursery crops and 5% ornamental crops cuttings don't form roots, and when adding these losses to the losses by poor root systems and the extensive use of chemicals for protection from fungal and bacterial attacks, the losses might be US $ 50 million per year (De Klerk et al., 1999). In this way, a novel discovery in rooting research may bring huge financial interest. This applies to rooting of conventional cuttings in soil, but in tissue culture applications, ARF is also one of the most important processes.

The biggest breakthrough in research on ARF was the discovery of the auxin indole-3-acetic acid (IAA) and its rhizogenic activity (Thimann and Went, 1934.) IAA is a natural endogenous hormone that is produced in the apical area of plants. It can be transported basipetaly but is also effective when applied via the cut surface (Hitchcock and Zimmerman, 1936). Simultaneously, artificial auxins, e.g., indole-3-butyric acid (IBA) and α-naphthylacetic acid (NAA) were synthesized chemically, and their ability for root induction was tested and proven (Zimmerman and Wilcoxon, 1935).

Later on, the different functions of auxin in different steps of ARF developmental processes were discovered. In the earlier step, auxin is act as a rooting inducer (De Klerk, 1995), and in the later step, auxin is functioning as an inhibitor and blocks the growth of roots (Thimann, 1936). The first 24 hours during the rooting process is called dedifferentiation period. In this period, the micro cuttings are not very sensitive to auxin (De Klerk et al., 1999). Between 24 and 72 hours (sometimes 96 hours, depends on the cultivar), the plant tissue shows highly sensitivity to auxin, many roots formation take place in the root primordia (Mitsuhashi et al., 1969), this period is called induction period. After 96 hours, the root division and elongation becomes visible to the naked eye, this period is called emergence period. In this period the plant tissue sensitivity to auxin is decreasing sharply, and the favourable auxin concentration in last period is inhibitory in this period (Attfield and Evans, 1991).

**The role of Polar Auxin Transport (PAT) in ARF**

The elongation of the stems to cope with shading, bending of the leaves to optimize light perception, as well as redirection of root growth for water and nutrients, or blocked by hard objects from the soil: they all require the auxin concentrated in specific location of a plant tissue (Whippo and Hangarter, 2006; Richter et al, 2009). Actually, this kind of polar auxin transport is guiding the plant developmental programs from the very beginning of plant's life since embryogenesis (Grunewald and Friml, 2010). Firstly, auxin accumulates in the upper apical cell after the zygote initial division. Then, it accumulates in

the uppermost suspensor cell for future development of a root pole (Friml et al, 2003). After germination, the polarity establishment and organ initiation are still controlled by PAT, for instance, the formation and outgrowth of adventitious and lateral roots (Benkova et al, 2003; Dubrovsky et al, 2008; Swarup et al, 2008; Peretet al, 2009). The morphogenesis of adventitious and lateral root meristems are similar as the formation of original root meristem. After the plant tissue in vitro condition redifferentiates into root meristem, it follows a same pattern as normal root formation.

The specific directed auxin flow requires specialized auxin transporters. Recently, auxin transporters have been hot research topics. Three families of transport proteins are involved in PAT, namely ATP-binding cassette (ABC) superfamily, PIN proteins family (auxin efflux carrier) (Zazımalovaet al., 2010) and auxin influx carriers. In ABC superfamily, the most representative members are ABCB1, 4 and 19. They regulate the auxin efflux in plant and non-plant systems (Geisler et al, 2005; Petraseket al, 2006; Cho et al, 2007). ABC superfamily transporters were reported to be more functional in controlling the overall auxin quantity in the cell rather than local differences, since they are located more symmetrically in the cell (Mravec et al, 2008).

**PIN proteins**

The PIN proteins are transcriptions and expressions of PIN Gene family. They are asymmetrically located in multicellular plant cells (Figure 1), and their polarity leads to the directionality of intercellular auxin flow (Křeček et al., 2009). When inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) or 1-N-Naphthylphthalamic acid (NPA) are applied to the plant cuttings, ARF is also blocked (Guerrero et al., 1999). Furthermore, TIBA and NPA have been proven to be effective on PIN proteins directly (Li et al., 2012). It also proves the importance of PIN proteins in auxin transportation.

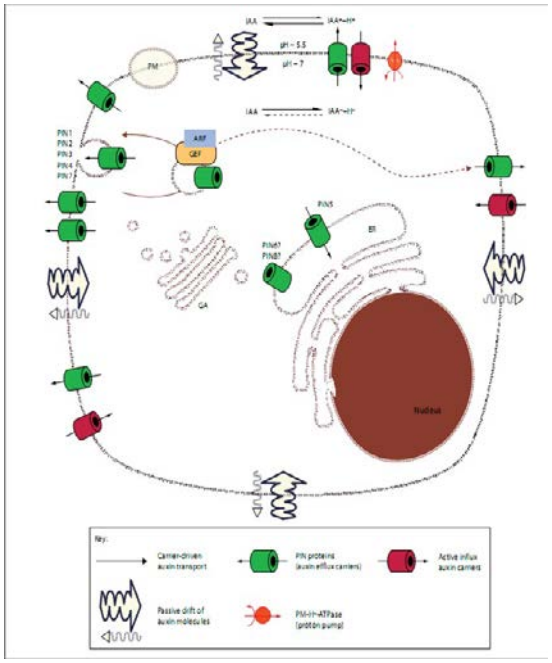

Figure 1. The location of different pin proteins (Krecek et al., 2009).

The first PIN mutant *pin 1* was found in the 1950s in *Arabidopsis* (Goto et al., 1987). The PIN mutants show a genetic expression failure in specific PIN proteins. So far, eight pins (*PIN 1-8*) were discovered in *Arabidopsis* (Tanaka et al., 2006; Vieten et al., 2007; Zazımalovaet al., 2007). The PIN gene effect on lateral root formation (LRF) is quite clear, and detailed information of the specific function of each pins can be read in literature (Figure 2). But literature about ARF is rare to be found.

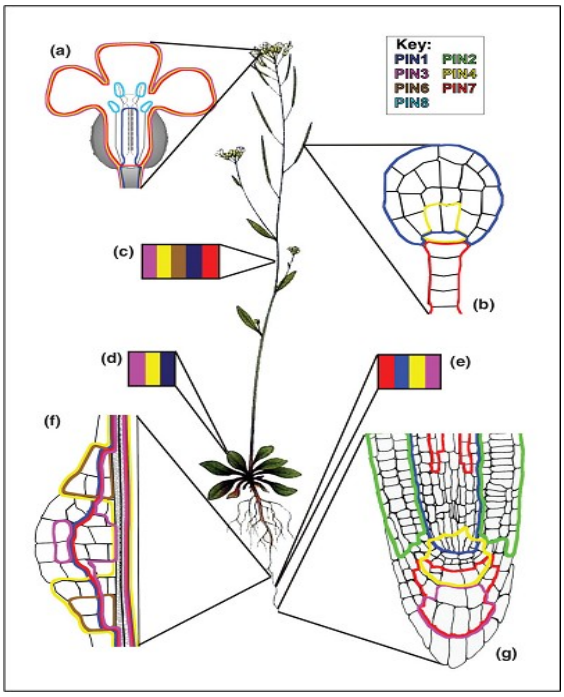


Figure 2. Expression map of Arabidopsis thaliana PIN genes compiled from both promoter activity data and protein localization. Each PIN gene-expression domain is marked out by a colored line (see key in upper right corner. The organs depicted are (a) flower; (b) embryo (late globular stage); (c) stem; (d) rosette leaf; (e) mature part of the primary root; (f) lateral root primordium (stage 5); (g) root tip. The figure is based on the data from (Friml, J. et al., 2002a; Friml, J. et al., 2002b; Friml, J. et al., 2003; Benkov, E.et al., 2003; Vieten, A. et al., 2005; Hruz, T. et al., 2008; ) Note that *PIN5* expression is not depicted, as it is expressed weakly throughout the aerial part of the plant with maxima in the hypocotyl, the guard cells of stomata, and cauline leaves (Krecek et al., 2009).

**ARF in *Arabidopsis***

*Arabidopsis* is the model plant that represents for dicotyledonous herbs. It has the advantage of wide distribution, easy to plant and fast growing (Al-Shehbaz, 2002). The micro-cuttings of its flower, flower stem,

leave, hypocotyl and many other parts are commonly used for different purpose in tissue culture research. Since Arabidopsis is a rosette plant and produce very short hypocotyl, for rooting test, we decided to use etiolated hypocotyl in order to create elongated hypocotyl. Although there are many discussions about whether the root formation from the hypocotyl is real ARF or not. Microscopic analysis of hypocotyl explants have shown that it has root like structure (having pericycle layer), (Konishi and Sugiyama, 2003) and therefore newly formed roots can be regarded as lateral roots, but according to other definitions, since it is an above ground organ, those roots which come out of it are adventitious roots.

**The Aim of this research**

Due to the different sensitivity to the auxin treatment in different time period, we setup different auxin pulse in our research. We use *Arabidopsis* hypocotyl and flower stems of pin mutants to test the effects of PIN gene on ARF in *Arabidopsis* micro-cuttings. In order to compare with the etiolated hypocotyl, we also selected flower stem, which is morphologically very similar to stems, to do the ARF test.

# Material and Methods

Medium preparation:

1. Germination medium: For seed germination, MS half strength with 3% sucrose and 0.7% micro agar was used. The pH was set at 5.6± 0.1 before autoclaving.

2. Rooting medium: Based on desired auxin type and concentration, different amount of 1mg/L stock solution were added to MS medium (Table 1). The plant materials were firstly treated with desired auxin concentration and kept in dark for one week. This step is really crucial because previous researches have proven that auxin is only important during the first few days and later on would be inhibitory. Apart from that, many auxin types are sensitive to photo oxidation. Therefore, after auxin treatment plant material must be transferred to the light and auxin free medium.

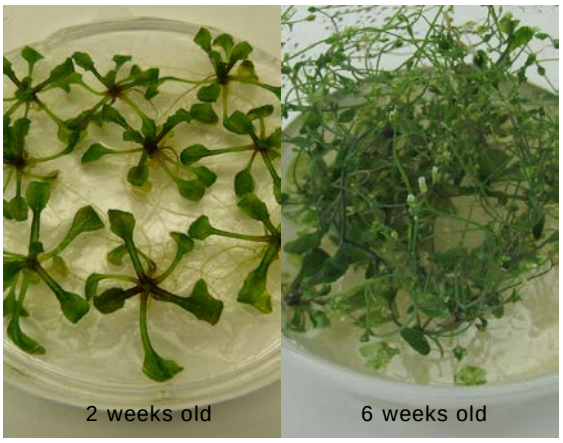


Figure 3. *Arabidopsis* grow in containers.

For response curve experiment various concentration of auxin ( IAA) in a logarithmic way were used ( 0, 3, 10, 30 and 100 μM).

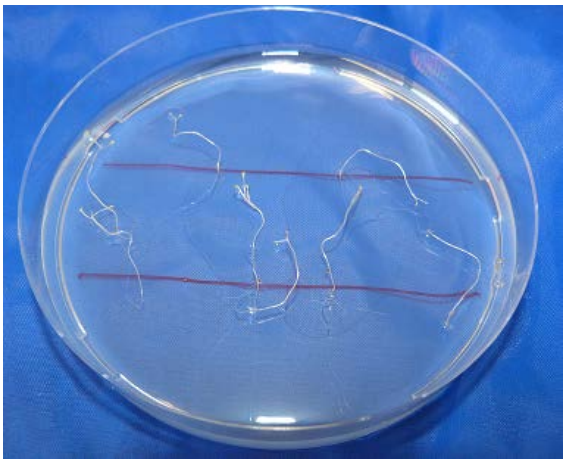

Figure 4. *Arabidopsis* grow in petri-dishes (12days old).

Plant material preparation:

1. Flower stem: *Arabidopsis* seeds were sterilized in 70% alcohol for 1 minutes, 2% sodium hypochlorite for 10 minutes and washed in sterilized demi water for 3 times, each time 10 minutes. After 4°C vernalization treatment for 3 days they were sowed in the germination medium transparent containers. From 4-6 weeks old plant material, flower stem were cut under the first internode and cut into 0.5-0.7 cm length node free segments. In each treatment 10 explants were placed horizontally on the medium surface, and 3 repeats/ treatment.

2. Hypocotyl: sterilized *Arabidopsis* seeds were sown in the 1/2 MS medium petri-dishes in way shown in figure 4 and petridishes were placed vertically in growth chamber. From 12 days old seedlings grown in the darkness, both root part and apical bud were cut off from hypocotyl and cut into 0.5-0.7 cm length segment and then were placed in the same way as flower stem on the medium surface.

Arabidopsis lines used: A. thaliana wild type Columbia Col-0, mutants *pin1, pin2, pin3, pin4, pin7.*

To have a constant suitable environmental condition, the plant materials were grown in the 20 ºC , 16h light/ 8h dark thermostatic chamber.

Treatments:

In order to establish a phase during which auxin is important to bring about rooting we applied 48hr and 72hr auxin pulses. The treatments were as follow:

48hr 100IBA pulses: Control, 0-48h, 24-72h, 48-96h, 72-120h, 96-144h. 72hr 30IAA pulses: Control, 0-72h, 24-96h, 48-120h, 72-144h. The plant material (flower stems) in control were treated for 144h in auxin. Rooting was determined as number of rooted explants and number of root per rooted explant in different interval at 10, 17 and 24 days after culture establishment. In the last date of the experiment, the photos of ARF were recorded by digital camera.

Mutant analysis:

Rooting response of different mutants indicated above was compared against their wild-type. All mutant were from *Col-0* background. For this experiment both hypocotyl and flower stem were tested as explants.

After first screening those mutant which showed negative significant response compared with control were targeted for next round of analysis. Then we tried to check their response curve and compare it with wild type. For response curve, various concentration of IAA (0, 3, 10, 30, 100 µM) were used.

# Results

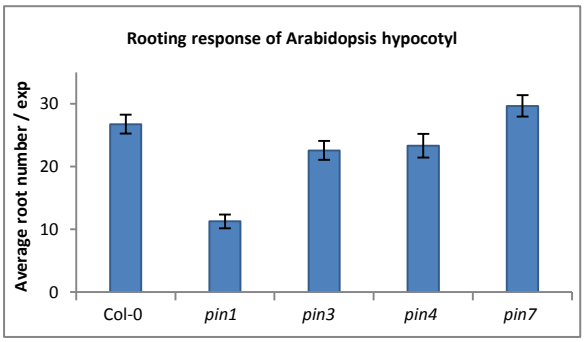


Figure 5. The rooting responses of *Arabidopsis* hypocotyl (Wild type Columbia Vs. various pin mutants) to 30 µM IAA.

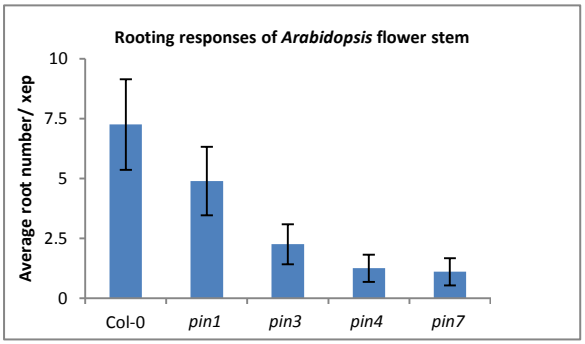


Figure 6. The rooting responses of *Arabidopsis* flower stem (Wild type Columbia Vs. various pin mutants) to 30 µM IAA.

**According to the explant type, different PIN genes play major role during ARF of *Arabidopsis* .**

We used the concentration of 30µM IAA since we have already tested several range of concentrations and we found this one as the optimum concentration for *Arabidopsis* Col-0 (wild type). We prepared *pin1*, *pin3*, *pin4* and *pin7* mutants as indicated above and we checked their rooting response to the constant concentration of 30 µM IAA. The rooting responses of hypocotyl shows that *PIN1* plays an important role in ARF of *Arabidopsis* hypocotyl (Figure 5). While the rooting responses of flower stem shows that *PIN3*, *PIN4* and *PIN7* are significantly influential in ARF of *Arabidopsis* flower stem (Figure 6).

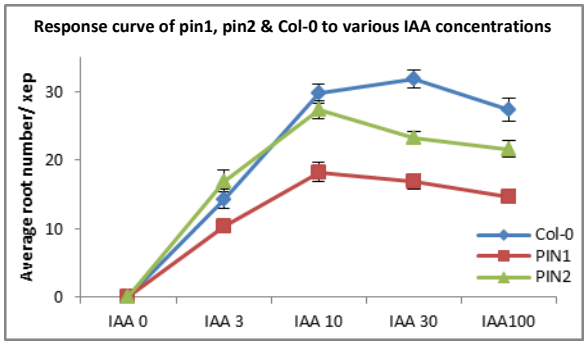


Figure 7. *Arabidopsis* hypocotyl rooting response curve of pin1, pin2 & Col-0 to various IAA concentrations

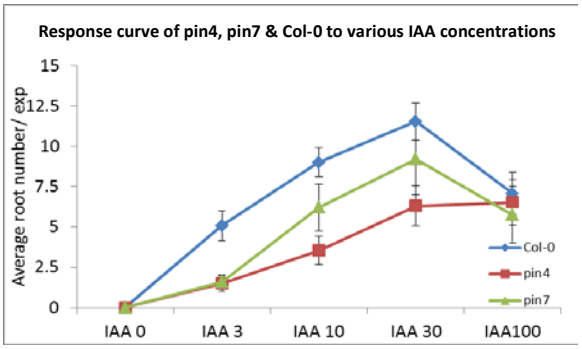


Figure 8. *Arabidopsis* flower stem rooting response curve of pin4, pin7 & Col-0 to various IAA concentrations

**The optimum IAA concentration in ARF of *Arabidopsis* hypocotyl**

As we found *PIN1* plays an important role in ARF of *Arabidopsis* hypocotyl, while *PIN3, PIN4, and PIN7* are significantly influential in ARF of *Arabidopsis* flower stem, we followed up with the response curve of these pin mutants to various IAA concentrations.

Since our *pin2* was prepared later on we added it to the response curve test. The average root number per explant of *pin1* and *pin2* is significantly different from the optimum IAA concentration of Col-0 in 30µM (Figure 7), *pin1* shows less roots than *pin2*. But at the optimum IAA concentration of *pin1* and *pin2* in 10µM, *pin1* shows the significant difference with Col-0 (control), but *pin2*

shows not. It indicates that the dysfunction of *pin1* and *pin2* proteins will significantly decrease the ARF of *Arabidopsis* hypocotyl, and *pin1* is more important in PAT than *pin2*.

In flower stem, the average root number per explant of *pin4* is significantly different from the optimum IAA concentration of Col-0 in 30μM (Figure 8), but the optimum IAA concentration of *pin4* is 100μM. The optimum IAA concentration of pin7 is the same as Col-0, but the standard error is a bit too high, that the root number difference is not very significant when compare with control (Col-0). It indicates that the dysfunction of *pin4* protein will significantly decrease the ARF of *Arabidopsis* flower stem.

**Effect of auxin pulses in ARF of *Arabidopsis* flower stem**

In order to establish a phase during which auxin is important to bring about rooting we applied 48hr and 72hr auxin pulses to flower stem of *Arabidopsis* Col-0. The average root number per explant treated in 0-72h 30μM IAA pulses shows almost no difference from control, which treated in auxin for 144h (Figure 9). But the average root number per explant treated in 24-96h 30μM IAA pulses shows a significant decrease. It indicates that the first 24hrs culture in vitro is most important for ARF of *Arabidopsis* flower stem.

The average root number per explant treated in 0-48h 100IBA pulses shows a significant difference from control (Figure 10), while there is almost no difference between the average root number per explant treated in 0-48h, 24-72h, 48-96h 100IBA pulses. It indicates that 48h 100 IBA pulses might be too short for vitro *Arabidopsis* flower stem to absorb enough amount of auxin, although the concentration of auxin is much higher.

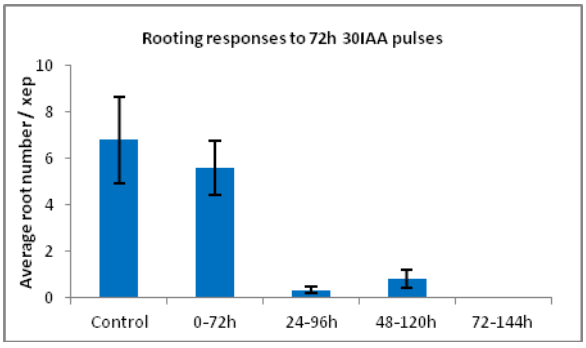


Figure 9. *Arabidopsis* flower stem rooting response to 72h 30IAA pulses

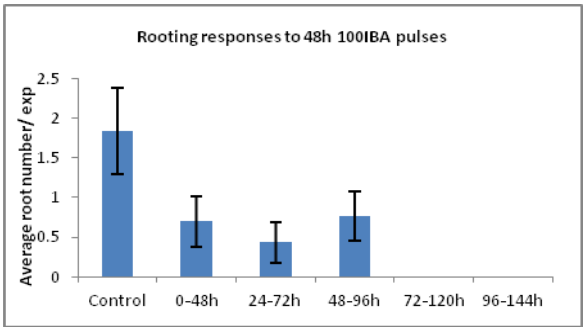


Figure 10. *Arabidopsis* flower stem rooting response to 48h 100IBA pulses

# Discussion

The rooting responses of hypocotyl shows that *pin1* has significant root reduction when compare with Col-0 (Figure 5). As *pin1* is the pin mutant which failed in expression of gene *PIN1*, and the absence of *PIN1* auxin transporter leads to the notable decreasing of newly formed root number, hence *PIN1* plays an important role in ARF of *Arabidopsis* hypocotyl. While the rooting responses of flower stem shows that *PIN3*, *PIN4* and *PIN7* are significantly influential in ARF of *Arabidopsis* flower stem (Figure 6). It is understandable that *PIN7* is not influential in ARF of *Arabidopsis* hypocotyl, because it is not expressed in the hypocotyl (Figure 2.). But *PIN1* is also expressed in flower stem while *PIN3*, *PIN4* are also expressed in hypocotyl. The result of different PIN genes show different importance in ARF is out of our expectation. It means a single PIN gene can be very determine in ARF of one part of the plant, but also can be unimportant in the other part of the same plant. This finding indicates that in different part of the *Arabidopsis* tissue, the mechanism of ARF might have some difference.

We setup the ARF experiment in flower stem because there are some discussions that arguing whether the root formation in hypocotyl can be regarded as ARF or it contains the lateral root primordia. Under the microscope, we found out most of the roots which formed in hypocotyl were originally from the central tissue. But in flower stem, a lot of roots were formed from the side tissue. We suppose that in hypocotyl the adventitious root primordia are mainly formed from the pericycle tissue, and in flower stem they are partly formed from the epidemic tissue. It might be different PIN genes are expressed in different tissue. For instance, *PIN1* is expressed in pericycle tissue and *PIN3*, *PIN4* are expressed in epidemic tissue. In order to make clear the adventitious root orientation, green florescent protein (GFP) can be introduced into plant tissue in the following research.

The response curve of hypocotyl (Figure 7.) and flower stem (Figure 8.) shows that the optimum IAA concentration for Col-0 is around 30μM. This is because auxin is toxic in excessive concentration. When the auxin concentration is higher than the maximum carrying load of the auxin transporters (pin proteins and other transporters), auxin will cause some damage to the plant

tissue. As a by-produce of this process, more ethylene will be generated. Ethylene is an inhibitor for ARF, it will cause the reduction of root formation under 100μM IAA concentration. In hypocotyl, the root number of both *pin1* and *pin2* show a significant decrease when compare with Col-0. But the optimum IAA concentration for *pin1* and *pin2* are around 10μM. It probably because with the absence of *PIN1* and *PIN2* expression, the maximum carrying load of auxin will also decrease. Then the auxin toxic concentration level will be lower.

In flower stem, *pin7* shows a significant decrease in root number when compare with Col-0 in all different IAA concentration, it proves again the importance of *PIN7*. The root number difference between *pin4* and Col-0 is even larger in 0, 3, 10, 30μM IAA concentration. But for *pin4*, the root number under 100μM IAA concentration is slightly higher than under 30μM, and the root number is even close to the root number of Col-0 under the same concentration. It suggests that under the excessive IAA concentration, the absence of *PIN4* expression might stimulate the overexpression of other PIN genes. To get more depth understanding of this mechanism, double mutants should be introduced in the following research.

In order to discover in which phase auxin is important for bring about rooting we setup the auxin pulses experiment. We gave 24h 30IAA pulses at first, but there is no significant root number difference between each treatment. Most of the flower stem formed no root or very few. It means auxin concentration within the flower stem is lower than the optimum concentration for rooting. We thought there can be two possibilities: 1. The 24h pulses is too short for the pin proteins to transport enough amount of auxin into the flower stem. 2. The concentration of auxin in medium is too low for the maximum transport efficiency of the pin proteins.

Hence we tried to give 48h 30IAA pulse and 24h 100IBA pulse. We failed again and finally we tried 72h 30IAA pulses (Figure 9.) and 48h 100IBA pulse (Figure 10.). It is quite clear that the longer pulse is far more important than the higher concentration. And the first 24h is the most important phase to apply the auxin treatment. It is quite understandable that the longer pulse is more important than the higher concentration. Because the plant tissue needs to maintain a certain level of auxin concentration for some hours to stimulate the formation of root primordia. But the excessive auxin concentration is toxic for the plant tissue. It is interesting that the first 24h is most important for auxin application. It indicates that there is some special auxin sensitivity in the first 24h for the plant tissue in vitro. It is possible that some substance synthesised in the wound reaction speed up the senescence of the plant tissue. It also possible that there is some signal substance been used during the first 24h for the plant tissue in vitro. This signal substance might break down with the wound reaction of the plant tissue. To get more understandings in this topic, the following research is suggested.

- Confocal imaging of *PIN-GFP* constructs will help to better interpret the role of these gene during the process of ARF.

- Study the rooting response of combined mutant (double, triple or more) and compare it with single mutant and wild type.

- Address the role of selected *PIN* genes to different stage of ARF.

# Conclusion

1.PIN genes play major role during ARF of *Arabidopsis*.
2.Different PIN genes have different importance in ARF of *Arabidopsis* according to the explant type.
3.The optimum auxin concentration for ARF of *Arabidopsis* is 30μM IAA.
4.The first 24h in vitro is most important for application of auxin for ARF of *Arabidopsis*.
5. The minimum auxin treatment time for ARF of *Arabidopsis* flower stem is 72h.

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