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The Effect of PIN Reduction in Populus: The Search for the Missing Phenotype

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The Effect of PIN Reduction in *Populus*: The Search for the Missing Phenotype

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Abstract

The plant hormone auxin is involved in virtually every aspect of plant growth and development. Because of its vital role in plant physiology, it is transported through controlled mechanisms. The PIN family of efflux carrier proteins are highly involved in this transport, shuttling molecules of auxin from cell to cell. This PIN-driven transport of auxin guides the differentiation of plant vasculature. In this study, I used a clonally-propagated line of the woody plant species *Populus tremula* x *alba* that had been genetically altered to induce an RNAi response against genes coding for the PIN proteins. These plants were shown to have reduced auxin transport rates, however, there was no difference when compared to wild-type in the external phenotype. First, reduction of PIN gene expression was confirmed in this *Populus* line through qRT-PCR, having been reduced by nearly 80% in shoot apices and over 90% in developing xylem. But despite this reduction of auxin transport, there was still no observable phenotype. It was then considered that this phenotype may be revealed if auxin transport rates were altered in localized areas of a single plant. Thus, this line of *Populus* was then used in reciprocal grafting experiments, in which RNAi line plants and wild-type plants were used alternatively as either scions or rootstocks. This resulted in whole plants with localized areas of reduced auxin transport. qRT-PCR was conducted on the scion and rootstocks of these plants to confirm that the RNAi signal is not graft transmissible, PIN expression in the RNAi plant segments having been reduced by over 65%, regardless of position (scion or rootstock). The vascular anatomy was then analyzed above and below the graft site. Again, regardless of the position of the RNAi line plant segment, digital image analysis revealed negligible difference in vessel size and distribution within developing xylem. These results may speak to the efficiency of homeostatic auxin mechanisms, which may have regulated the alterations in auxin transport such that the phenotype remained consistent with wild-type.

Chapter I: Introduction

Auxin was the first ever hormone to be studied in plants, and history has proven it to be one of the most important. Since the initial discovery of its involvement in phototropism (through a series of experiments set in motion by Charles Darwin himself), auxin has been found to be involved in virtually every aspect of plant growth and development (Davies 1995, Went 1928). Because of this universal and eclectic importance to all of plant life, the investigation of molecular mechanisms involved in transporting auxin from cell to cell is an area of great significance and interest. The first step to understanding any molecular mechanism is determining the genes and proteins involved. One method that can be utilized to study these molecular components is RNA interference (RNAi). This technique allows for the targeted degradation of mRNA transcripts in order to disrupt or reduce specific gene expression (Fire et al. 1998). The process of auxin transport involves numerous genes and their protein products, the disruption of which can lead to a deeper understanding of the function and overall process of auxin transport. The PIN family of proteins is perhaps the most essential to auxin transport, as they function to direct the movement of auxin molecules as they exit the cell. In this study, I research the role of PIN proteins in *Populus* tree development using RNAi.

Auxin

As mentioned, Darwin himself was among the first scientists to consider auxin signaling in plants, however, he did so without even knowing what auxin was. Plants had long been known to exhibit phototropism: the ability to grow or move toward light. Darwin was interested in the

mechanism behind this survival technique and conducted a study on phototropism in 1880 (Darwin 1880). His results showed that when the tips of a seedling sprout are removed or covered during growth, the sprout is unable to grow toward a light source, as it normally would. He concluded that there must be some signal in the tip that responds to light and travels through the plant such that it grows accordingly (Holland et al 2009). It wasn't until almost 50 years later that this signal was isolated and given the name auxin, a hormone that has since been found to be indole-3-acetic acid (IAA), and the term "auxin" has been broadened to include any compounds with abilities similar to those of IAA (Fig. I.1), such as the promotion of growth and various developmental processes within plants.

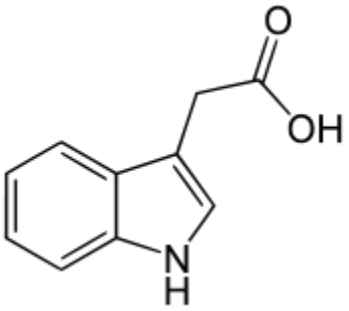

Figure I.1. The structure of indole-3-acetic acid (IAA), the most common naturally occurring compound from the auxin family of hormones.

Auxin functions to affect plant development by acting in an inducible gene transcription system (Santer et al. 2009). This is a system in which the target gene or genes are repressed as their default state and are only activated in the presence of an inducer molecule. The target genes, in this case, are those that express proteins involved in cell division, or cell elongation. These genes are activated by transcription factor proteins known as auxin response factors (ARFs). In order to activate their target genes, ARFs must bind to each other in pairs known as

dimers, but under normal conditions (in the absence of auxin) ARFs are repressed by AUX/IAA proteins, which bind to ARFs such that they cannot bind to each other and form these dimers (Fig. I.2). Auxin enters the cell and binds to a specific receptor protein complex, activating it (Santer et al. 2009). This complex then targets the AUX/IAA repressor proteins for degradation. The ARFs are then free to dimerize and activate the growth genes (Hagen et al 2002).

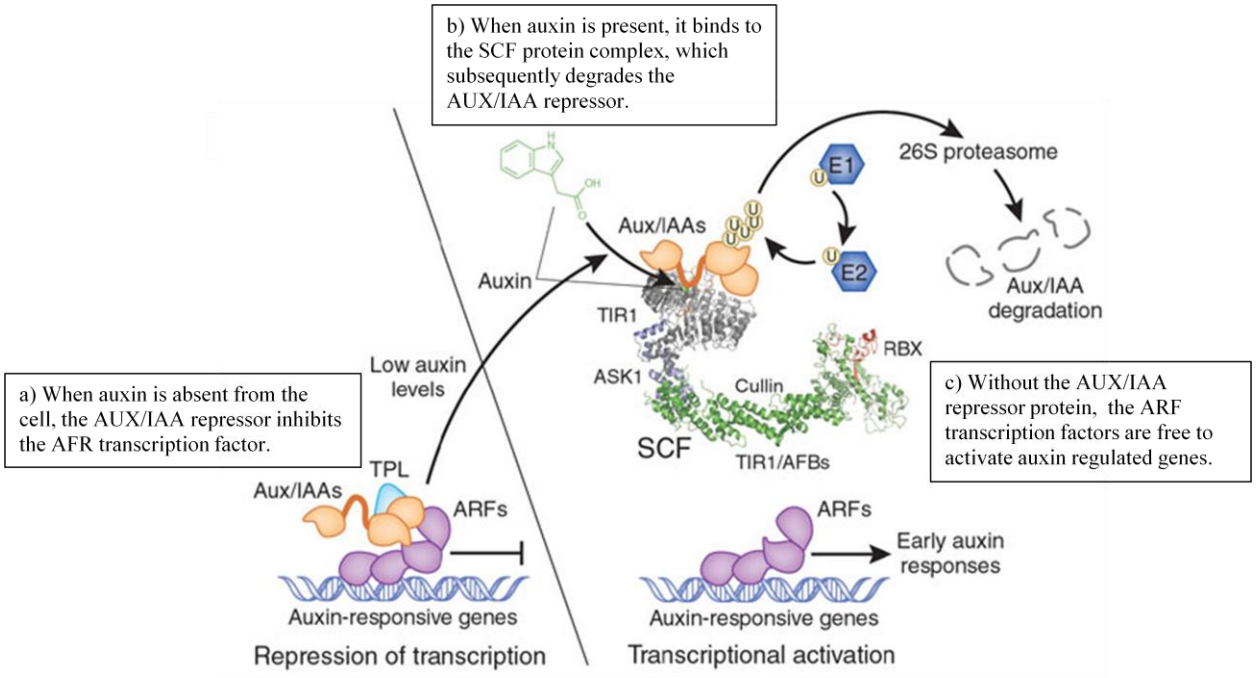

Figure I.2. Signaling cascade that leads to the activation of auxin-regulated genes. (Reprinted from Santer et al. 2009 with permission of the publisher)

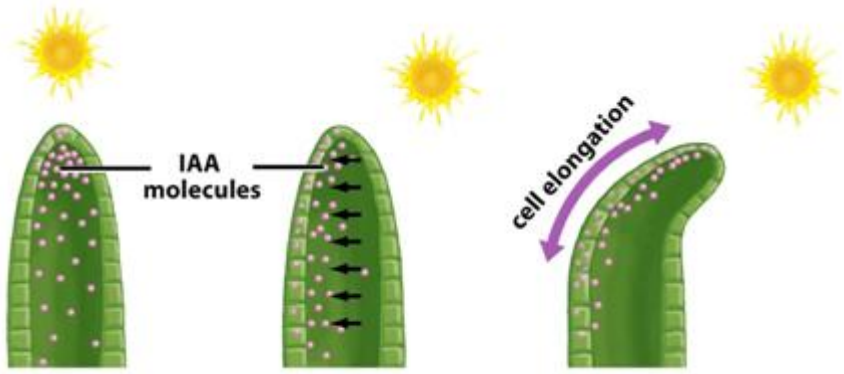

Figure I.3. Simplified diagram suggesting how the specific transport of IAA is involved in growth strategies. The IAA molecules induce the cells on the left side of the plant to elongate such that the plant bends toward the sun.

This complex process of molecular signaling is coupled to an equally complex process of auxin transport. It is extremely beneficial for a plant to be able to specifically control the delivery of auxin to its cells such that it can control which tissues are actively growing. For example, when a plant uses the previously described strategy of phototropism, auxin must be specifically transported to the proper cells, which will then grow in such a way that causes the plant to move toward the sunlight (Fig. I.3). This is only one example of many instances in which a plant would need to specifically distribute auxin. Therefore, plants have a variety of molecular structures and processes that allow for the strategic distribution of auxin throughout the plant. When it comes to controlling the direction of auxin flow, the PIN family of proteins are perhaps the most important molecular members of this strategic distribution (Petrasek et al. 2009).

Polar Auxin Transport and the PIN Proteins

Darwin was correct in his conclusion that the tip is the essential site of growth signaling. Although some auxin is synthesized in the roots and leaves of plants, most of the hormone is synthesized at the tip, in the shoot apical bud (Blakeslee 2005). This localization of auxin is why the majority of auxin transport occurs basipetally—from the apical tip of the plant downward to

the basal roots. This unidirectional flow of auxin that is driven by transmembrane proteins is known as polar auxin transport (PAT) (Fig. I.4).

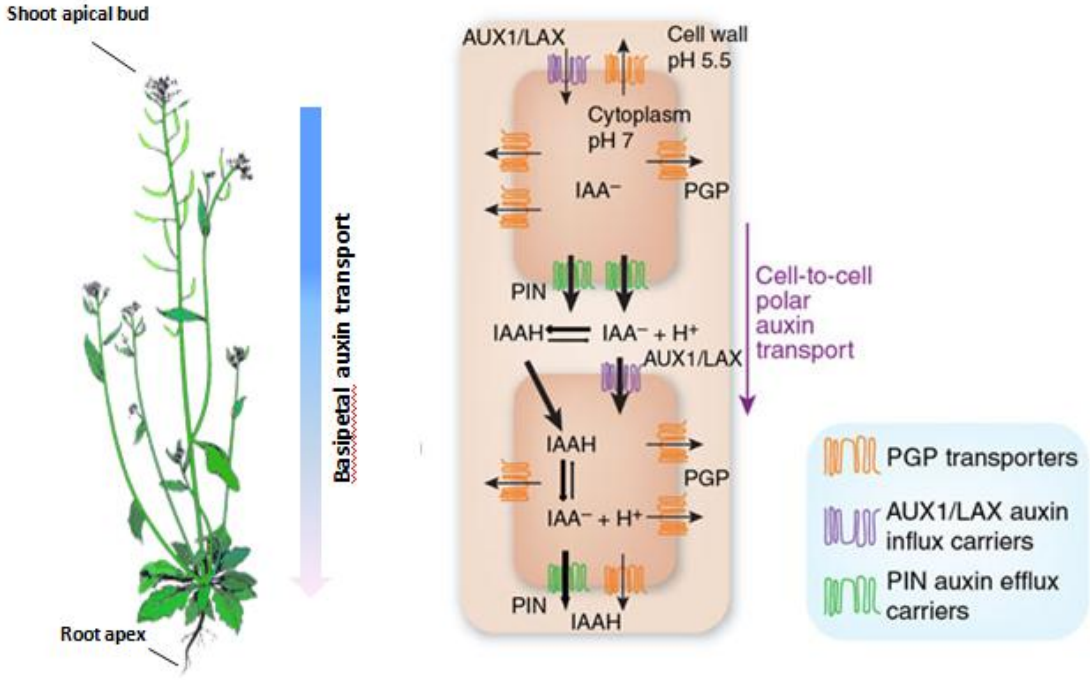

Figure I.4. Illustrations of polar auxin transport (PAT) on both the macro and cellular scale. (Reprinted from Helene et al. 2009 with permission of the publisher)

Although this unidirectional transport of auxin might seem like an effect of gravity or the result of simple diffusion, plants in fact utilize complex molecular processes to precisely direct the flow of auxin. Furthermore, PAT is essential for proper tissue development such as the formation of the vasculature and root system (Blilou 2005). In order to accurately maintain the direction of this flow, plants utilize the PIN family of proteins.

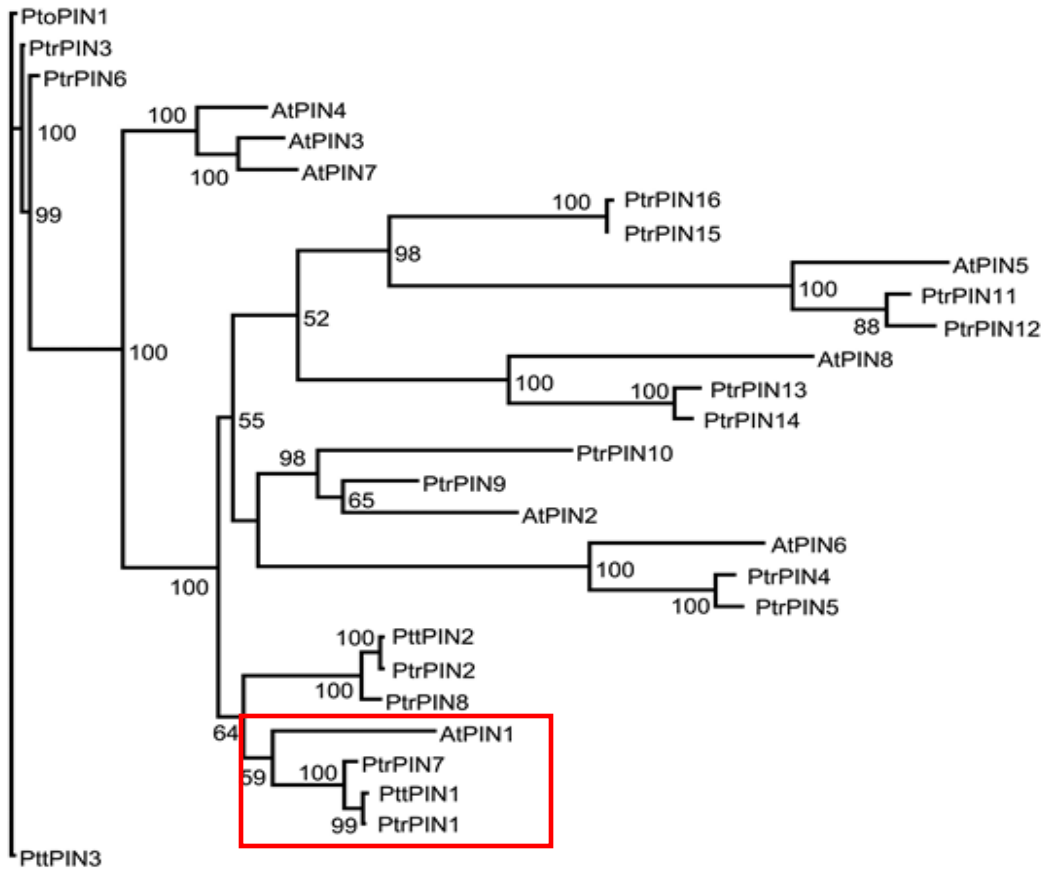

Figure I.5. Phylogeny of PIN proteins in both *Arabadopsis* (AtPINs) and *Populus* (PtrPINS), illustrating the redundancy and relatedness of PIN proteins. Phylogenetic relationship between PtrPIN1, PtrPIN7 and AtPIN1 highlighted (*source:* R. Spicer, unpublished data).

The PIN proteins were first characterized through the mutation of the then-undefined *pin1* gene in *Arabidopsis* (Krecek 2009). This caused mutant plants to develop pin-shaped appendages instead of proper flowers, and thus led to the protein family being named PIN-FORMED or PIN. PIN proteins are transmembrane, meaning they extend across the cell's membrane (Bililou 2005). This reflects the essential function of the PIN proteins, which is to allow the efflux of auxin across the membrane of a transport cell.

The PIN family of proteins is indeed a collection of many different types of PINs, all of which are involved in many instances of auxin transport. Not only does this allow for specific PINs to be involved in specific types of transport, but this variety of PINs is also in accordance

with the idea of redundancy: if the genes for one type of PIN become disrupted, there are still the other PINs to maintain overall function (Fig. I.5). Because of this redundancy, it's important when studying the effect of PIN protein disruption to target multiple PIN genes.

The PIN proteins work to shuttle IAA molecules which have been "trapped" within a cell due to an electrochemical gradient. As mentioned, the principal and most abundant auxin molecule is indole-3-acetic acid (IAA). When a molecule of auxin enters a plant cell, it does so by passive diffusion as the protonated, neutral molecule IAAH, or by uptake via the AUX1/LAX proteins (Swarup & Peret 2012). The pH inside of a plant cell is slightly more basic than on the outside, which has the effect of causing a neutral molecule of auxin to dissociate into its anionic form (IAA^-) once it enters the cytoplasm. When a bunch of negative IAA^- molecules accumulate within a cell, it causes disequilibrium in the charge between the inside of the cell and the outside. However, these negatively charged molecules cannot pass back through the plant cell membrane, which repels charged molecules, and thus they require a membrane transport protein to carry them across the lipid bi-layer (Li et al. 2005).

The PINs serve as these transport proteins, shuttling the negatively charged auxin molecules from the cytosol, across the plasma membrane, to the outside of the cell where they can be taken-up by the next cell in the longitudinal line. PIN proteins can thus determine the direction in which the auxin leaves the cell based on their position within the cell membrane. The reason why PIN proteins are largely responsible for PAT is because they are most often arranged on the basal side of the cell membrane such that the negatively charged auxin molecules can only exit downward, in the basipetal direction. The arrangement of PIN proteins, however, can be altered in instances when it is beneficial for auxin transport to deviate from standard basipetal movement (Galweiler et al. 1998).

So if these PIN proteins are the directors of auxin transport, and therefore the directors of growth and development, what happens when their levels are reduced? This is one of the questions that this study seeks to answer through the use of RNAi.

RNA interference

Although RNA interference is now a widely utilized strategy in the study of many organisms, it was in fact discovered in plants. In the 1990s, scientists sought to create an especially purple petunia by introducing more of the gene that coded for the purple pigment into the flowers. The result was petunias that were completely white, devoid of all pigment (Fig. I.6), and it was eventually determined that the reason was RNAi (Napoli et al. 1990).

Figure I.6. Petunias from Napoli et al. 1990. Wild-type on the left and the genetically modified flowers on the right (reprinted with permission of Napoli et al. 1990)

In many species, the RNAi response is initiated with the detection of long sections of double stranded RNA (dsRNA). This response begins with an enzyme called Dicer, which binds to the dsRNA molecule and cleaves it into smaller segments. These short fragments of dsRNA are known as short interfering RNAs (siRNAs). The siRNAs derived from the larger dsRNA then integrate into a protein complex called RNA-induced silencing complex (RISC). RISC next

uses the sequence of the siRNA to target complementary sequences of single stranded RNA (ssRNA). Once bound to a molecule of ssRNA (such as an mRNA), RISC will either cause degradation of that molecule, or simply prevent translation of that RNA sequence into a protein (Mello et al. 2004) (Fig. I.7). Therefore, when Napoli et al. were trying to cause an increase in purple pigment within those petunias, what they inadvertently accomplished was the creation of dsRNA molecules containing an RNA sequence complementary to that of the natural purple-pigment-protein mRNA precursor, which was subsequently targeted for degradation by the RNAi response such that no purple pigment was translated, leading to white flowers.

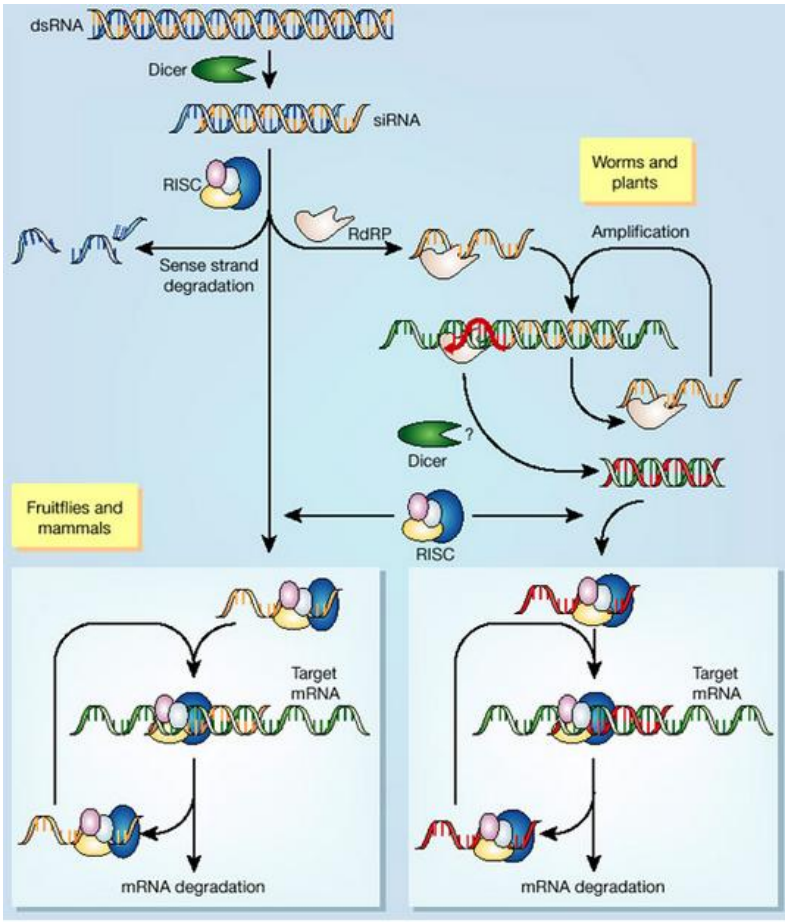

Figure I.7. Molecular process of RNAi. The process differs slightly between organisms, but the end result is always degradation of an mRNA transcript (reprinted from Novina and Sharp 2004 with permission of the publisher).

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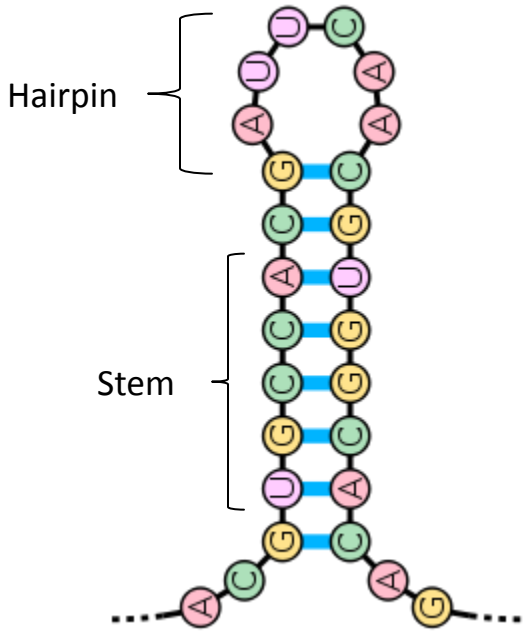


Figure I.8. Structure of a hairpin RNA (hpRNA)

In animals, inducing an RNAi response is relatively easy, as it simply requires is an injection of dsRNA containing a sequence complementary to the mRNA transcript to be silenced (Fire et al. 1998). This type of induction of the RNAi signal is not possible in plants because of the cell wall, which provides a barrier against exterior dsRNA. The most efficient and effective method of causing a specific RNAi reaction in plants is to transform them by inserting a gene containing two essential sequences of DNA: a primary sequence that will transcribe into an RNA molecule that is complementary to some part of the target mRNA, and a secondary sequence that is complementary to the primary sequence. This has the effect of creating a hairpin RNA (hpRNA), because, once transcribed, the two complementary sequences within this one long sequence will bind to one another, causing the RNA molecule to fold at the intron, which forms a loop that causes the entire structure to be in a sort of hairpin shape (Fig. I.8). This hpRNA molecule will be transported into the cytoplasm and recognized as a dsRNA by Dicer. RNAi will

commence, ultimately resulting in RISC incorporating the siRNA derivative to target the mRNA precursor of interest and silence it (Mansoor et al. 2007).

This technique can be used to effectively create knock-down versions of plants, such that one is able to study the importance of a particular protein by genetically engineering a plant line to have reduced levels of a specific protein. The PIN proteins are a prime example of a good target for RNAi, for they seem to be of great importance for plant development, and would thus theoretically provide an interesting phenotype for study when reduced by RNAi.

Another interesting question involving RNAi and plants is that of graft transmissibility. If a plant genetically modified to produce an RNAi signal was grafted onto a wild-type plant, would that signal be transmitted through the graft and cause the reduction of the target protein in the wild type as well? The answer to this question would hold implications about the RNAi function within plants, and perhaps lead to efficient techniques for inducing an RNAi signal into wildtype plants.

Auxin and Vascular Development

Plant vasculature is the system of conducting tissues (xylem and phloem) that transport water, sugar and mineral nutrients to the cells of the plant. The most evident example of this is venation in leaves—the branching configuration of veins within leaves that are visible to the naked eye. Many scientists have focused on this highly organized vascular scheme, and have found that auxin plays a major role in the development of these tissues. The exact mechanism of this development is not known for certain, but the prevailing theory resulting from and supported

by these studies is known as the canalization hypothesis (Sachs 1991), which involves both auxin and the PIN proteins.

This model of vascular development holds that leaf development begins at locations with very high concentrations of auxin known as the initiation points. The PIN1 proteins (which are the only PIN proteins expressed at this point in development) are specifically localized within the cells at these initiation points such that auxin flow is directed through the center of the developing leaf (Rolland-Lagan et al. 2005). Auxin induces both the growth and differentiation of these cells such that this specifically directed flow of auxin during leaf development defines the position of the large mid-vein found in the middle of most leaves (Scarpella 2006). The canalization hypothesis specifically holds that this auxin signal gets "canalized", or narrowed into thin strands of cells, which define both the polarity of the cells and the branching venation of leaves (Sachs 1991).

The majority of research in this area has focused on herbaceous plants, and in particular, leaves of the model organism *Arabidopsis thaliana*. In woody plants, the stem represents a novel site of extensive vascular development, and it is not known to what extent the canalization of auxin via the PIN proteins is involved in this process. Studies using RNAi to reduce PIN expression have not yet been conducted in a woody species. The tree species *Populus trichocarpa* was the first woody plant to have a complete genome sequence published (Tuskan et el. 2006) and thus provides a good model for the study of woody plants. I use a clonally-propagated line of *Populus tremula* x *alba* that has been transformed to have reduced expression of these essential PIN proteins via RNAi. More specifically, I use this line of *Populus* to examine the phenotype caused by the reduction of PIN expression using reciprocal grafts and vessel

distribution analysis. This study seeks to confirm that RNAi can be used to effectively reduce PIN protein levels in *Populus*, and subsequently confirm that these genetically modified *Populus* plants can then be used to create plants with localized areas of varying auxin transport rate through grafting. In creating these grafts, this study will then answer the question of how the morphological phenotype is altered by changes in auxin transport rates, through the examination of xylem anatomy. This study will also examine whether the effects of this genetic alteration can spread from one of these experimental *Populus* plants to a wild-type when the former is grafted to the latter. Not only will these results hold implications about the importance of the PIN proteins, but the data will also provide an examination of the graft transmissibility of RNAi signals, something which has never before been studied in trees.

Citations

Blakeslee J, Peer W, Murphy A. 2005. Auxin transport. Curr Opin in Plant Bio 8: 494-500.

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme M, Scheres B. 2005. The PIN auxin efflux facilitator network controls growth and Patterning in arabidopsis roots. Nature. 433: 39-44.

Davies P. 1995. Plant hormones physiology, biochemistry and molecular biology Dordrecht (Germany): Kluwer Academic. p.1-12.

Fire A. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391: 806–811

Galweiler L, Guan C, Muller A. 1998. Regulation of polar auxin transport by AtPIN1 in arabidopsis vascular tissue. Science. 282: 2226-230.

Hagen G, Guilfoyle T. 2002. Auxin-responsive gene expression: genes, promoters and regulatory

factors. Plant Mol. Biol. 49: 373–385.

Holland J, Roberts D, Liscum E. 2009. Understanding phototropism: from Darwin to today. J Exp Bot. 60: 1969-978.

Křeček P, Skůpa P, Libus K, Naramoto S, Tejos R, Friml J, Zažímalová E. The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biol. 10: 249-262.

Li J. 2005. Arabidopsis H -PPase AVP1 regulates auxin-mediated organ development. Science 310: 121-25.

Mello C, Conte D. 2004 Revealing the world of RNA interference. Nature. 431: 338-42.

Mansoor S, Amin I, Hussain M, Zafar Y, Briddon R. 2006. Engineering novel traits in plants through RNA interference. Trends Plant Sci. 11: 559-65.

Napoli C. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in Trans. Plant Cell. 2: 279-89.

Novina C, Sharp P. 2004. The RNAi revolution. Nature. 430: 161-64.

Petrasek J. 2006. PIN proteins perform a rate-limiting function in cellular auxin efflux. Science. 312: 914-18.

Hélène R, Friml J. 2009. Auxin and other signals on the move in plants. Nature Chem Biol. 5: 325-32.

Rolland-Lagan A, Prusinkiewicz P. 2005. Reviewing models of auxin canalization in the context of leaf vein pattern formation in Arabidopsis. Plant. 44: 854–865.

Sachs, T. 1991. Cell polarity and tissue patterning in plants. Development.1:83–93.

Santner A, Calderon-Villalobos L, Estelle M. 2009. Plant hormones are versatile chemical regulators of plant growth. Nature Chem Biol. 5: 301-307.

Scarpella E. 2006. Control of leaf vascular patterning by polar auxin transport. Genes Dev. 20: 1015-027.

Swarup R, Péret B. 2012. AUX/LAX family of auxin influx carriers—an overview. Front Plant Sci. 3:255

Tuskan G, Difazio S, Jansson S. 2006. The genome of black cottonwood, populus trichocarpa (Torr. & Gray). Science. 313: 1596-604.

Went F, Wushstoff W. 1928. Rec. Trav. Bot. Neerl. 25: 1–116.

Chapter II

Introduction

The plant hormone auxin is involved with virtually every aspect of plant growth and development (Davies 1995, Went 1928), and thus its transportation throughout various cell tissues is an area of great interest. The PIN family of proteins is responsible for auxin's transport from cell to cell, and includes transmembrane efflux carrier proteins which shuttle negatively charged IAA (auxin) molecules from the cytoplasm out toward the next cell in a basipetal progression (Petrasek et al. 2006). Of the many varieties of PIN proteins, PIN1 is the most well studied in plants, and is the protein most known to be involved with transport of auxin from the plant tip down to the roots, a process known as polar auxin transport or PAT (Wisnewska et al. 2006).

One way to confirm the importance of the PIN proteins in plant development is to reduce their levels. This would greatly alter the transport of auxin throughout the cell. Studies involving mutants of the PIN1 gene have shown disruption of PAT, and disruption of vascular organization as a result (Galweiler et al. 1998). A different method which could be used to modify PIN function would be to induce an RNA interference, or RNAi response. RNAi is a natural mechanism through which both plants and animals post-transcriptionally silence genes by targeting specific mRNA transcripts for degradation. This mechanism can be induced by transforming an organism such that it transcribes a sequence of dsRNA containing a sequence complementary to mRNA of interest.

This experiment utilized a genetically modified line of the model woodyplant *Populus.* Previously, the use of RNAi to reduce PIN expression has only been tested in herbaceous species of plants, specifically Arabidopsis (Friml et al. 2003, Lee et al. 2012). This technique has yet to

be used alter auxin transport in a woody species. This Populus line was modified to induce an RNAi response to degrade the PIN mRNA. The result was a reduction in PIN1 and PIN7 expression levels by about 80% in one genetic line (R. Spicer, unpublished data). Although the reduction of auxin transport was confirmed through radiolabeled auxin (^{3}H-IAA) transport assays, there was no noticeable change in phenotype, including growth rate and external morphology (R. Spicer, unpublished data). The RNAi line with the most extreme reduction in IAA transport rate had not yet been tested for reduced PIN gene expression however, nor had PIN gene expression reduction been compared in different tissues. In this experiment qRT-PCR was utilized to confirm the reduction of PIN expression in these RNAi lines of *Populus* using two different tissue types.

Materials and Methods

Plant Material

Eight biological replicates each were prepared from both the wild-type line and the RNAi line. Plants were subcloned in sterile tissue culture from previously existing RNAi and wild-type lines. They were *in vitro* grown in a solid agar medium for 4 weeks, then transferred to soil. Transfer included removal of plant and roots from agar medium and replanting in potted soil. The plants were stored in humidified ziplock bags under fluorescent lights for a 2 week transition period, during which the roots became established in the soil. Plants were then removed from ziplock storage, re-potted in larger pots and placed in a green house. Ten of these plants (five from the wild-type line and five from the RNAi line) were then allowed to grow for nine weeks before being harvested for shoot apex collection. The remaining six plants (three wild-type line,

three RNAi line) were grown for six months before being harvested for developing xylem collection.

To collect shoot apices, plants were harvested by cutting just under the 4th petiole beneath the shoot-tip. These shoots were then immediately plunge-frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The ground plant material was separated into aliquots of approximately 100 mg (about 0.25 ml) and stored at -80°C for use in qRT-PCR experiments.

Collection of developing xylem involved the removal of all leaves and cutting the plant at the base. Bark was peeled away to reveal the underlying developing xylem. Developing xylem was scraped from the stem onto aluminum foil using a razor blade. Both foil and razor blades were RNase-free. Consistent force was used around circumference of the stem to ensure consistent xylem collection. After scraping was complete, the tissue was folded into foil packet and immediately plunge-frozen in liquid nitrogen and stored at -80^{o}C. Tissue was subsequently ground into a fine powder via mortar and pestle, separated into approximately 100 mg aliquots in 1.7 ml conical eppendorf tubes and restored at -80^{o}C

Quantitative Real Time PCR

RNA extraction was carried out on both sets of tissue on a clean, RNAase-free bench, and using all RNAase-free materials. RNA was extracted from these samples using a SIGMA Spectrum Plant Total RNA™ kit according to the manufacturer's instructions. The cells of each approximately 100 mg aliquot of frozen plant tissue were lysed and filtered and RNA was collected in two 40 uL elutions pooled together. One uL of each of these elutions was run on a 1.5% agarose gel to check for RNA fragmentation, and re-extraction was carried out on those samples for which fragmentation was noted. The remainder of each of these elutions was stored at -80^{o}C.

Trace genomic DNA was removed by DNase treatment. All materials were kept on ice, and treatment was carried out according the manufacturer's instructions of the Ambion Turbo DNase kit, with the following alterations: 2 uL of DNase was added to the initial extract instead of the instructed 1 uL, and 10 uL of DNase inactivation reagent was added instead of the instructed 8 uL. Twenty uL of each RNA extract was used, and the resulting solutions of DNase free RNA were 100 uL in volume. One uL of each DNase treated RNA sample was aliquoted into a new tube for quantification. The rest of each sample was stored at -80°C.

The appropriate concentration for the RT reaction is between 50-200 ng/uL. RNA quantification was carried out in order to prepare for the RT reaction. Quantification was carried out using an Invitrogen Quibit Fluorometer™ according to the manufacturer's instructions. In mixing the solutions, bubble formation was avoided to assure accurate fluorescent readings and concentration was recorded in ng/mL. The average concentration of RNA was approximately170 ng/uL.

cDNA generation was carried out according to the protocol provided by the Invitrogen First Strand cDNA kit™ using SuperScript III with the following minor alterations: 4 uL of 5x RT buffer was added to each reaction instead of 2 uL, RNase out was not used, after the addition of the RT buffer and 0.1M DTT each reaction was incubated at 42°C instead of 50°C, and each reaction was deactivated at 70°C instead of 85°C. Each kit component was thawed on ice, having been stored at -20°C, and mixed and briefly centrifuged before use. Oligo(dT)s were used in this priming method, rather than random hexamers. This process allowed 10 uL of each RNA sample to be converted into 22 uL of cDNA. To avoid DNA damaging freeze thaw cycles, and because both Qubit ssDNA quantification and each qPCR reaction only require 1uL of cDNA, 2uL aliquots from each cDNA sample were taken, and the rest of each sample was stored at -80°C.

Qubit quantification of the cDNA was carried out using the same procedure as quantification of RNA using the Invitrogen Quibit Fluorometer^{TM} set to measure ssDNA. The average concentration of cDNA, discounting some outliers, was ~50 ng/mL.

Each qRT-PCR reaction was carried out according the manufacturer's instructions of the iCycler iQ. The genes of interest were PIN1 and PIN7. The housekeeping/normalizer gene used for quantification of the shoot apices genetic material was TUA (α-tubulin), and, upon refinement, the two normalizer genes were used for quantification of the developing xylem genetic material - TUA and UBQ (ubiquitin). Each cDNA sample was utilized separately in eight different reactions, one reaction for each primer pair corresponding to one of those 5 genes. Each reaction had three technical replicate, and for each primer pair, a no-template-control reaction was prepared using DNase-free H2O instead of cDNA. Once the appropriate wells were filled, the plate was then vortexed for ~80 seconds, followed by 6 minutes of spinning in a plate spinner at 4,000 rpm. This functioned to remove bubbles that could interfere with measurement of the fluorescent signal during the qPCR reaction.

The iCycler protocol began with a 95^{o}C initial denaturing step for 10 minutes, followed by 40 cycles of denaturation at 95^{o}C for 30 seconds, 60^{o}C for 25 seconds and 72^{o}C for 25 seconds. Fluorescent data was collected at the end of each one of these cycles. Once these 40 cycles were completed the temperature was raised to 95 ^{o}C for 1 minute, then lowered to 60°C for 1 minute. Melt curve data was then collected at the end of each of 80 cycles, during which the temperature began at 60^{o}C and was raised by 0.5^{o}C each cycle. The reactions were then held at 20^{o}C. C_t values were recorded and C_t values of technical replicates were all within +/- 0.2 C_t units. The melting curves were observed to check for primer dimers. Expression values for the PIN genes were normalized by reference gene expression levels through calculating Q. Q was

calculated using the following equation: $Q = {2^{C_t NORM}}/{2^{C_t\ PIN}}$ In instances where more than one reference gene was used, Q was calculated using the geometric mean of the C_t values of these normalizers. Paired t-tests were conducted for each treatment, and standard error was calculated.

Results and Discussion

These results provide confirmation that, despite the lack of noticeable phenotypic change in the RNAi line plants, PIN expression is indeed being reduced. The reduction of PIN1 expression is highly significant in both shoot apices and developing xylem tissue, with expression levels being reduced by over 50% in the shoot apices (p-value < 0.01, two-tailed t-test, n = 5), and over 90% in the developing xylem (p-value < 0.01, two-tailed t-test, n =4) (Fig. II.1). The reason behind the difference in expression levels of PIN1 and PIN7 between shoot apices and developing xylem is not known, nor is it known whether this represents a true difference in the degree of gene silencing between tissue types. One potential explanation for this dramatic difference in reduction may be that developing xylem, which is known to contain a high concentration of IAA (Schrader et al 2003; Spicer et al 2013), functions primarily in auxin transport, whereas shoot apices have many diverse functions and would thus contain a varied pool of mRNA precursors for a large variety of proteins. Developing xylem, being more specialized for auxin transport, would have an mRNA pool that is less varied, and much more enriched with PIN proteins in comparison to an entire shoot apex. This is represented in the comparison of the wild-type controls, which shows that PIN1 expression in developing xylem is twice that of PIN1 expression in shoot apices. Because the developing xylem has such a highly enriched collection of PIN mRNA precursors, the tissue would logically be more susceptible to a

reduction of PIN1 expression via the post-trancriptional silencing mechanism RNAi, as represented in Figure II.1.

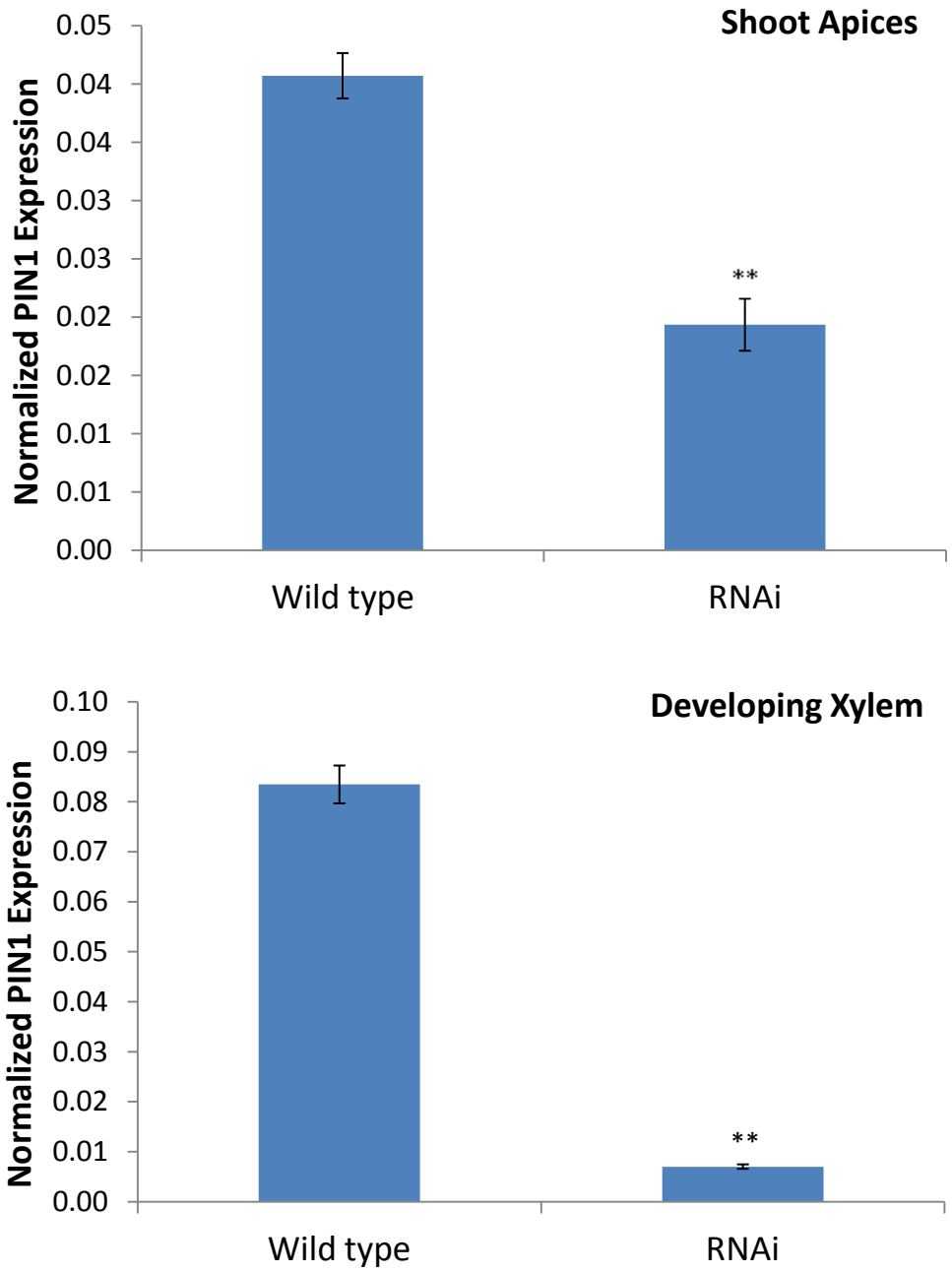
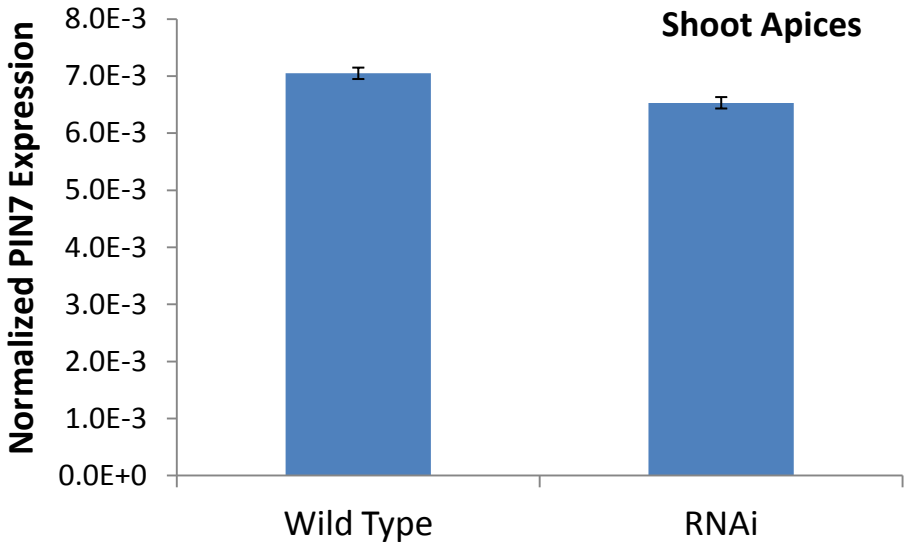


Figure II.1. Normalized PIN1 expression in both wild type and RNAi treated plants. Plant material used for quantification was taken both from both whole shoot apices and developing xylem tissue. **=highly significant. The p-value when comparing the RNAi expression of PIN1 to that of the control was less than 0.01 for both tissue types. For both wild-type and RNAi treatments from which shoot apices were collected n = 5. For both wild-type and RNAi treatment from which developing xylem was collected n = 3. Mean +/- standard error shown.

In contrast to PIN1, PIN7 was not found to be significantly reduced in the shoot apices taken from RNAi line plants (Fig. II.2), with a reduction of just over 7% in comparison with expression in the wild type line (p-value > 0.05, two-tailed t-test, n = 5). In the developing xylem tissue, however, PIN7 expression was reduced with high significance (Fig. 2b) with nearly 80% reduction between the RNAi line and the wild type line (p-value < 0.01). PIN7 is expressed at very low levels, with C_t values that approach negligible expression (>30 C_t units), and its exact involvement with auxin transport is largely unknown. However, PIN7 is the closest related PIN to PIN1(Fig. I.1) with a very similar sequence. It's logical that the RNAi response that reduces PIN1 would reduce PIN7, and thus this data provides a nice confirmation of the efficacy of the RNAi technique.



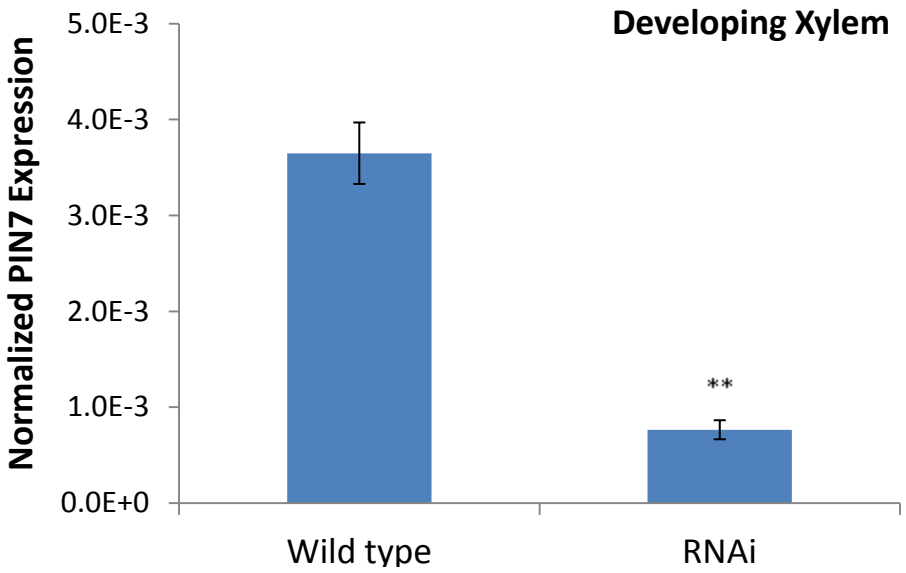


Figure II.2. Normalized PIN7 expression in both wild type and RNAi treated plants. Plant material used for quantification was taken both from both whole shoot apices and developing xylem tissue. *= significant. The p-value when comparing the RNAi expression of PIN1 to that of the control was less than 0.05 for developing xylem. For both wild-type and RNAi treatments from which shoot apices were collected n = 5. For both wild-type and RNAi treatment from which developing xylem was collected n = 3. Mean +/- standard error shown.

With this confirmation of RNAi-based reduction of PIN protein gene expression, future experimentation with this RNAi line can move forward to determine how this reduction in auxin transport caused by the reduction in PIN expression is affecting the physiology of the plants. Furthermore, these results indicate developing xylem to be the most promising tissue type for further study of the RNAi effect, especially if PIN7 is to be a continued consideration.

Citations

Davies P. 1995 Plant hormones physiology, biochemistry and molecular biology Dordrecht (Germany): Kluwer Academic. p.1-12.

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G. Efflux-dependent auxin gradients establish the apical–basal axis of Arabidopsis. Nature. 426: 147-53.

Galweiler L, Guan C, Muller A. 1998. Regulation of polar auxin transport by AtPIN1 in arabidopsis vascular tissue. Science. 282: 2226-230.

Lee R, Kim S, Kim H, Hong J, Ryu S, Lee S, Ganguly A, Cho H. 2010. Phospholipase A2 is required for pin-formed protein trafficking to the plasma membrane in the Arabidopsis root. Plant Cell. 22: 1812-1825.

Napoli C. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell. 2: 279-89.

Petrasek J. 2006. PIN proteins perform a rate-limiting function in cellular auxin efflux. Science. 312: 914-18.

Schrader J. 2003. Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. Nat Acad Sci. 100: 10096-10101.

Spicer R. 2014. Symplasmic networks in secondary vascular tissues: parenchyma distribution and activity supporting long distance transport. J Exp Bot. 65:1829-48.

Wisniewska J. 2006. Polar PIN localization directs auxin flow in plants. Science. 312: 883.

Went F. 1928. Wushstoff und wachstum. Rec. Trav. Bot. Neerl. 25: 1–116.

Chapter III

Introduction

A major role of the hormone auxin in plant growth and development is in directing vascular differentiation (Fukada et al. 2004, Scarpella et al. 2010). This is the specialization and organization of long-distance transport cells such as vessels and sieve tubes, which transport water and carbohydrates throughout the plant, respectively. One theory as to how auxin affects vascular differentiation is the canalization hypothesis (Sachs 1969). This is the widely-accepted concept that auxin works in a feedback loop when traveling through transport cells, such that vascular differentiation and auxin flow enhance one another: cells that differentiate into vascular cells transport auxin more efficiently, and auxin reflexively induces differentiation of vascular cells (Sachs, 1981). The effect is that the flow of auxin is "canalized" into a narrow (i.e., one- to several-cells-wide) stream, as the flow itself induces the cells to be more efficient auxin transporters. Sachs developed this concept through observations of plant physiology, observations which have since been corroborated by the study of the PIN proteins (Vieten et al 2007, Merks 2007).

Because this flow of auxin is so important to vascular differentiation, the alteration of auxin transport and hence its concentration can have major effects on vascular anatomy. In normal xylem anatomy, vessels cells are large conductors of water surrounded by much smaller fiber cells which aid in mechanical support. Figure III.1 shows how the external application of a high concentration of the auxin transport inhibitor NPA (N-1-naphthylphthalamic acid) affects xylem structure, causing the large vessel cells to shrink and reorganize into bands of small fiber-like cells (Spicer, unpublished data). However, in *Populus* plants in which auxin transport rate is reduced in the whole plant there is no phenotype (Chapter I). NPA slows auxin transport in a

small region, and perhaps this localized area of slowed auxin transport is more inductive of an alternate phenotype. This, however, is an extreme example of this effect, as the concentration of the applied auxin transport inhibitor NPA was extraordinarily high. Furthermore, due to this auxin inhibitor being applied externally, the inhibition likely spread throughout the plant via the phloem. A more ideal means of comparing the effect of auxin transport on vascular structure would be to create localized areas of varying auxin transport rates and/or concentration within a single plant.

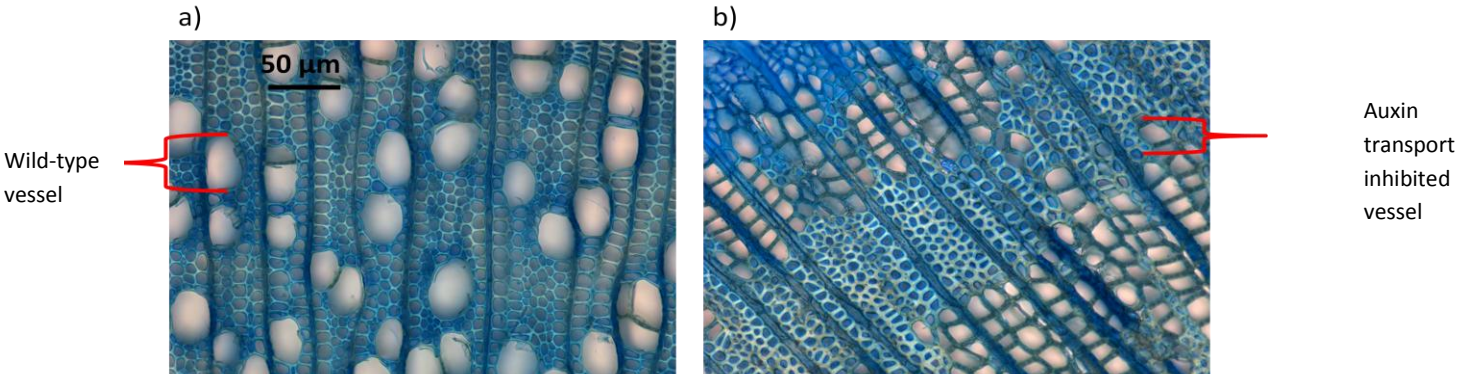


Figure III.1. Images of secondary xylem, stained with 0.1% toluene, from *Populus* trees. 100x magnification. a) wild type section. b) Section from a plant externally treated with a high concentration of auxin transport inhibitor NPA. Note the small vessel sizes.

NPA acts as an auxin transport inhibitor through the inhibition of the PIN protein family of auxin efflux carriers (Murphy et al. 2002). This specific effect of NPA could be reproduced through genetic modification, creating a plant that endogenously reduces PIN proteins. This could be accomplished by creating a line of plants genetically altered to induce an RNA interference response against mRNA precursors of the PINs (Friml et al. 2003, Lee et al. 2012). The method would result in an RNAi line of plants with a slowed rate of auxin transport (Chapter 1). With both this altered line and the wild-type from which it was derived, grafting could then be used to achieve localized areas of varying auxin transport within one plant.

Grafting is the fusion of plant parts so that vascular continuity is established between them (Mudge 2009). The simplest way to make a graft is through a cleft-wedge cut, wherein a scion is cut with a point and wedged into a rootstock (Fig. III.2). The scion and the rootstock then fuse through wounding responses and function as a single plant. Grafting is often used for plant propagation and growth control, but also for the scientific study of signaling events from a genetic, molecular and physiological standpoint (Pina et al. 2005). These studies often involve a genetically modified plant being grafted onto a wild type in order to observe the effect (Mapelli et al. 1992, Lin et al. 2008).

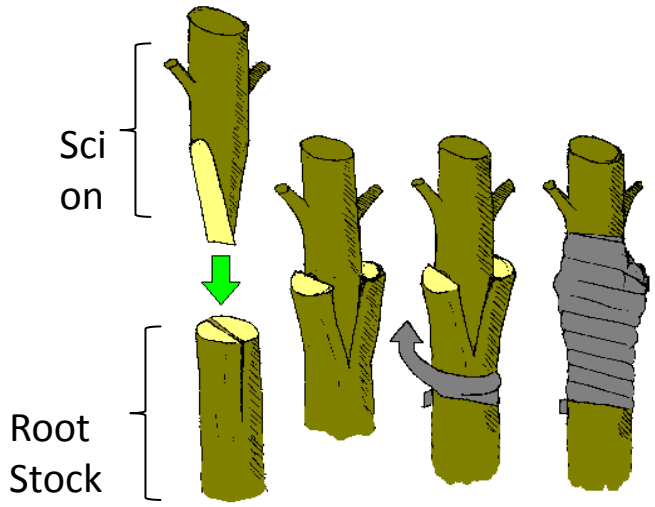


Figure III.2. Illustration of the process of wedge grafting. (source: togetherfarm.org)

The combination of RNAi and grafting can thus be used to engineer plants with localized areas of varying auxin transport rates. The only potential issue with this technique is the fact that in many cases the signal triggering an RNAi response has been shown to move between the rootstock and scion - that is, the RNAi signal is graft transmissible. Experiments with tobacco, Arabidopsis and tomato plants have shown that the mRNA degradation effect of RNAi can indeed spread from an RNAi line plant to a wild type plant through grafting (Table 1). For

example, if an RNAi line plant with reduced auxin transport is grafted onto a wild type plant, the reduced transport rate could spread to the wild type plant section. However, the graft transmissibility of an RNAi signal has never been tested in a woody plant.

Plant Species	RNAi Transmissibility	Paper
Nicotiana tabacum	Transmissible: Rootstock to Scion	Palauqui et al. 1997
Nicotiana benthamiana	Transmissible: Bidirectional	Vionnet et al. 1998
Nicotiana benthamiana	Transmissible: Bidirectional	Sonoda et al. 2000
Solanum lycopersicum	Transmissible: Bidirections	Shaharuddin et al. 2006
Nicotiana benthamiana	Transmissible: Rootstock to Scion	Tournier et al. 2006
Arabidopsis thaliana	Transmissible: Rootstock to Scion	Brosnen et al. 2007

Table 1. List of papers which studied graft transmissibility of RNAi signals, with corresponding plant species and result of transmissibility tests listed.

For this study, a single RNAi line of the woody *Populus* trees was used. These plants have been transformed such that an RNAi response is induced against the mRNA precursors for the PIN proteins. The reduction of PIN1 and PIN7 gene expression in developing xylem (by 91% and 79% respectively) was confirmed (Fig. II.1), and radiolabeled auxin (^{3}H-IAA) assays demonstrated that auxin transport is indeed reduced by up to 70% (R. Spicer, unpublished data). Reciprocal grafts were constructed from these RNAi plant lines and the wild type plant lines from which they were derived. Developing xylem from the scion and the rootstock of these grafts was subjected to qRT-PCR to test for graft transmissibility of the RNAi, and sections from above and below the graft were taken to analyze the effect of differing rates of auxin transport on vessel size and distribution.

Materials and Methods

All plants for use in grafting experiments were subcloned using sterile technique and transferred to soil as described in Chapter II. Twelve plants were prepared to be used for qRT-PCR (six RNAi line plants and six wild-type plants), and 32 plants were prepared to be used for

observing anatomy (nine RNAi plants and thirteen wild-type plants). Fifteen of these plants were from the RNAi line and 19 were from the wild type line. The plants to be used for qRT-PCR analysis were grown for between four and five months, and the plants to be sectioned for analyzing vessel size and distribution in xylem anatomy were grown for approximately three months. All plants were to be used as rootstocks in reciprocal grafts.

Rootstocks were prepared for grafting by removing the top of the plant approximately 15-20 cm from the base, just under the nearest petiole. A razor blade was then used to make a single vertical slit, or cleft approximately 3 cm long down into the stem. Defoliated stem segments, cut to be 4-5 cmlong, served as scions and were prepared from plant sections of previously harvested wild type and RNAi line plants. Each scion was prepared such that its diameter was approximately equal to that of the rootstock. Graft unions were then formed by cutting the bottom end of the scion with a razor blade into a wedge approximately 1-cm-long and inserting that wedge into the cleft (Fig. III.1). Parafilm was then immediately wrapped around the plant such that the point of intersection between the wedge and the cleft and the entire scion were covered.

The twelve plants to be used for qRT-PCR analysis underwent this grafting technique such that there were four grafted plants for each of three treatments: RNAi line scions grafted onto wild-type (wt) rootstocks (RNAi/wt), wild-type scions grafted onto RNAi line rootstocks (wt/RNAi), and RNAi line scions grafted onto RNAi line rootstocks (RNAi/RNAi) as a control (a shortage of wt plants prevented the creation of wt/wt controls). The 32 plants to be sectioned and used for xylem anatomy analysis were also separated into the same three treatments: eight RNAi /RNAi, 17 wt/RNAi and seven RNAi/wt. Grafted plants were allowed to grow for approximately 10 weeks before being defoliated and harvested.

For the plants to be subject to qRT-PCR, equal length segments about 20 cm long were taken from above and below the graft. Developing xylem was collected using the technique described in Chapter II. RNA extraction, cDNA generation and qRT-PCR were conducted on all samples according to the methods described in Chapter II. The genes of interest were PIN1 and PIN7, and the geometric mean of three normalizers - ACT, UBQ and TUA - was used for each sample.

For the plants to be used to analyze xylem anatomy, the stems were cut approximately 3 cm above and below the graft. Each graft segment was stored in 70% EtOH. Four 20-micron-thick tissue sections were taken from approximately one cm above and below the graft with a sliding microtome. Each section was stained with 0.1% safranin-o and mounted onto a slide. Ten photographs were taken at 100x magnification for each position (i.e., above and below) within the outer one-third of the stem cross section, and distributed randomly throughout the four sections. Images were then processed in ImageJ to automatically measure the area of the vessel lumens. A thresholding function was used to highlight vessel lumens (Fig. III.3). A pre-determined range of lumen areas of 300-3000 square microns was used to distinguish fibers (<300 μm^2) from vessels. Twenty bins were created across this range, and the number of vessels within each bin was counted by using the Analyze Particles function.

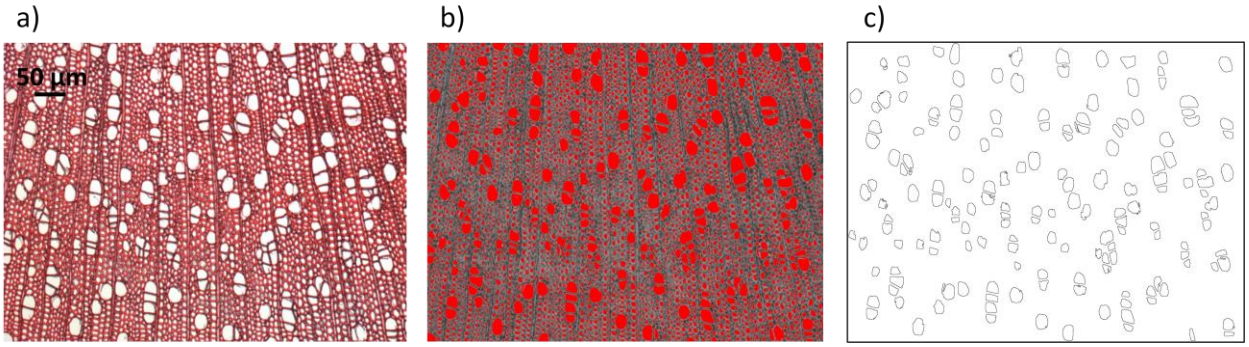


Figure III.3. Progression of ImageJ image processing. a) Original image of safranin-o stained Populus section used to identify vessels and measure vessel diameter. 100x magnification. b) Thresholded image of xylem highlighting vessel (and fiber) lumens in red. c) Mask of image showing all the vessels recognized by ImageJ (note that although fiber lumens were highlighted in red, only vessels were counted by ImageJ. The lumen cross-sectional area of each of these vessels was measured by the program.

Results and Discussion:

The qRT-PCR revealed that both PIN1 and PIN7 expression in the RNAi graft segments were comparable across the three treatments, regardless of position (Fig. III.4). Similarly, both PIN1 and PIN7 expression were found to be higher in the wild-type graft segments than the RNAi graft segments, again regardless of position. This indicates that the RNAi signal in Populus is not graft transmissible, which is in contrast to results from studies using herbaceous plants (Table 1).

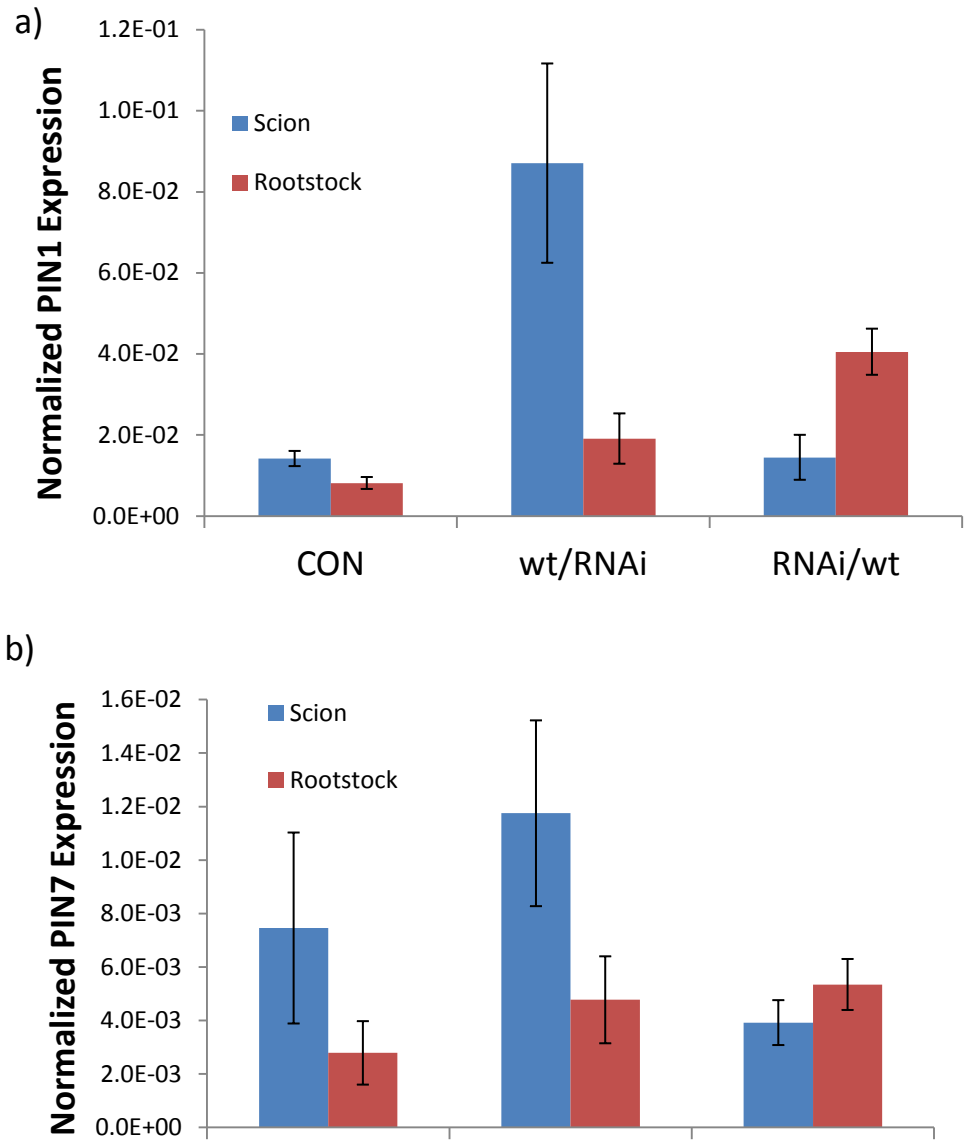


Figure III.4. Normalized PIN1 expression (a) and PIN7 expression (b) for all three graft treatments. Mean +/- standard error shown. n = 3 for control treatment, and n = 4 for both treatments including anRNAi line segment.

It is notable that expression of both PIN1 and PIN7 is higher in the scion of the control treatment. This effect seems to augment the difference in PIN expression between the wild type and RNAi plant segments when the wild type segment is in the scion position. This may be due to the relative age and/or growth rate of the scion versus the rootstock. In each graft, the scion was typically from a younger region of the plant shoot, and once grafted to the rootstock, was developing and growing at a comparatively increased rate. This would suggest a potential increase in PIN expression in accordance with the more rapidly dividing cells. Nevertheless, even when the RNAi segment was in the scion position, PIN expression was greatly reduced (Fig. III.4). PIN1, in fact, was reduced by 64% relative to wild-type in the RNAi segment of this treatment, while being reduced by 78% in the RNAi segment of the wt/RNAi treatment. These numbers compare favorably with the percent reduction of PIN1 found in the shoot apices and developing xylem analyzed in Chapter I (Fig. III.5). This level of comparability in PIN1 reduction shows that the grafting of RNAi line plants onto wild-type plants did not strongly reduce the effect of the induced RNAi response to PIN1 mRNA precursors.

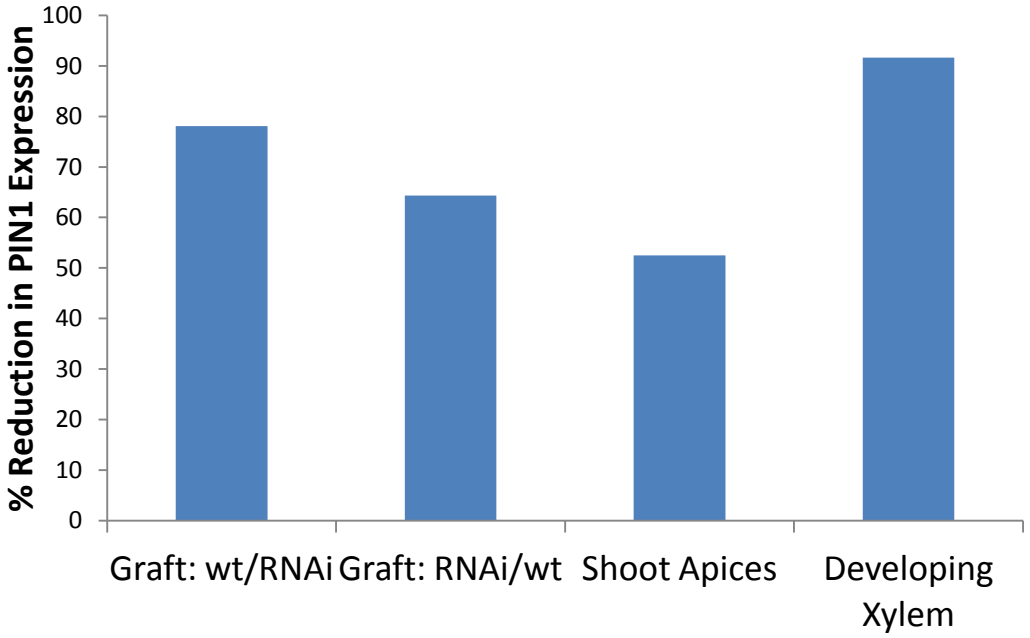


Figure III.5. Relative % reductions in PIN1 expression relative to wild-type in RNAi line tissue for the two graft treatments, as well as the shoot apices and developing xylem taken from non-grafted plants.

Furthermore, when the experimental graft treatments were compared to the control graft treatment, the differences in PIN1 expression between the scion and the rootstock were found to be highly significant for the RNAi/I treatment (p-value < 0.01; two-tailed t-test; n = 3) and significant in the wt/RNAi treatment (p-value < 0.05; two-tailed t-test; n = 4). These differences in comparison to the control were not found to be significant for PIN7, most likely due to the comparatively low level at which PIN7 is expressed, however, the expression levels still followed the same the same trends as PIN1 (Fig III.6) providing further evidence that the RNAi signal was not transmissible through the graft.

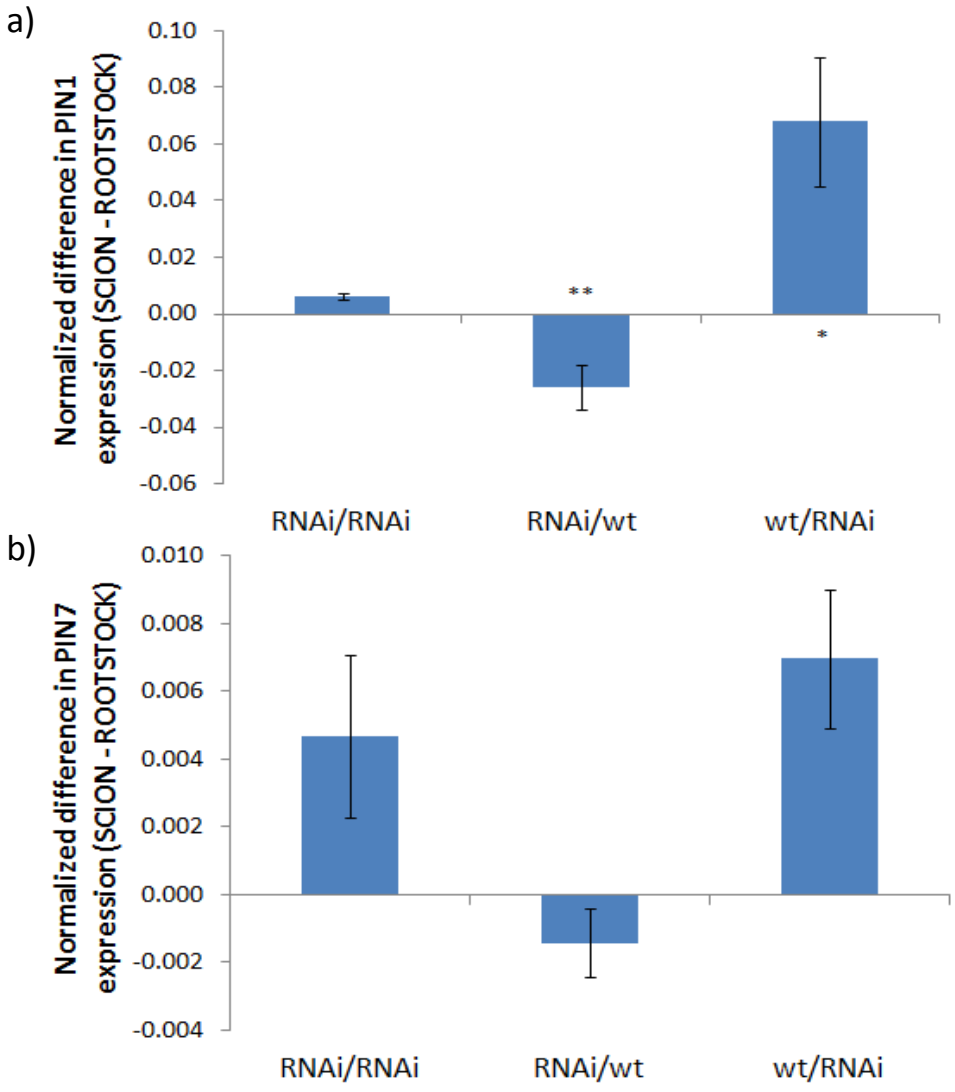


Figure III.6. Normalized expressions of both PIN1 (a) and PIN7 (b) represented as a difference in the expression level between the scion and the rootstock. *=significant (p-value < 0.05),**=highly significant (p-value < 0.01). For RNAi/RNAi treatment, n = 3. For RNAi/wt and wt/RNAi, n=4.

This evidence suggests that both the RNAi/wt and wt/RNAi grafted plants should have localized areas of reduced auxin transport that could create regions with high concentrations of auxin in developing xylem. The logic behind this is that auxin transport is suddenly slowed when auxin flowing down a wild type scion enters cells in the RNAi rootstock with reduced auxin transport. Similarly, auxin being transported relatively slowly down an RNAi line scion would be suddenly exported much more quickly from the cells in the wild type line scion. Both instances suggest the pooling of auxin at the graft.

A local accumulation of auxin in the stem would be expected to affect xylem anatomy, however, the image analysis of the graft sections revealed that there is no discernible difference in overall vascular organization between the scion and rootstock in either graft treatment with an RNAi line segment (Fig. III.7a). Figure 3 in the Materials and Methods section shows the progression of the ImageJ processing, demonstrating how the program identified vessel lumens, and excluded fiber lumens. The data collected from this processing revealed that in each treatment there is a relatively consistent number of vessels per mm^2 between sections taken from the rootstock of each plant and sections taken from the scion (Fig. III.7a).

Furthermore, auxin pooling is known to result in the development of "small" vessels, which in this line of *Populus* corresponds to vessels with a diameter under 30 μm (Fig.III.1) (R. Spicer, personal communication). But in measuring the proportion of vessels under 30 μm per treatment, there was also found to be a negligible difference between the rootstock and the scion for each graft treatment (Fig. III.7b).

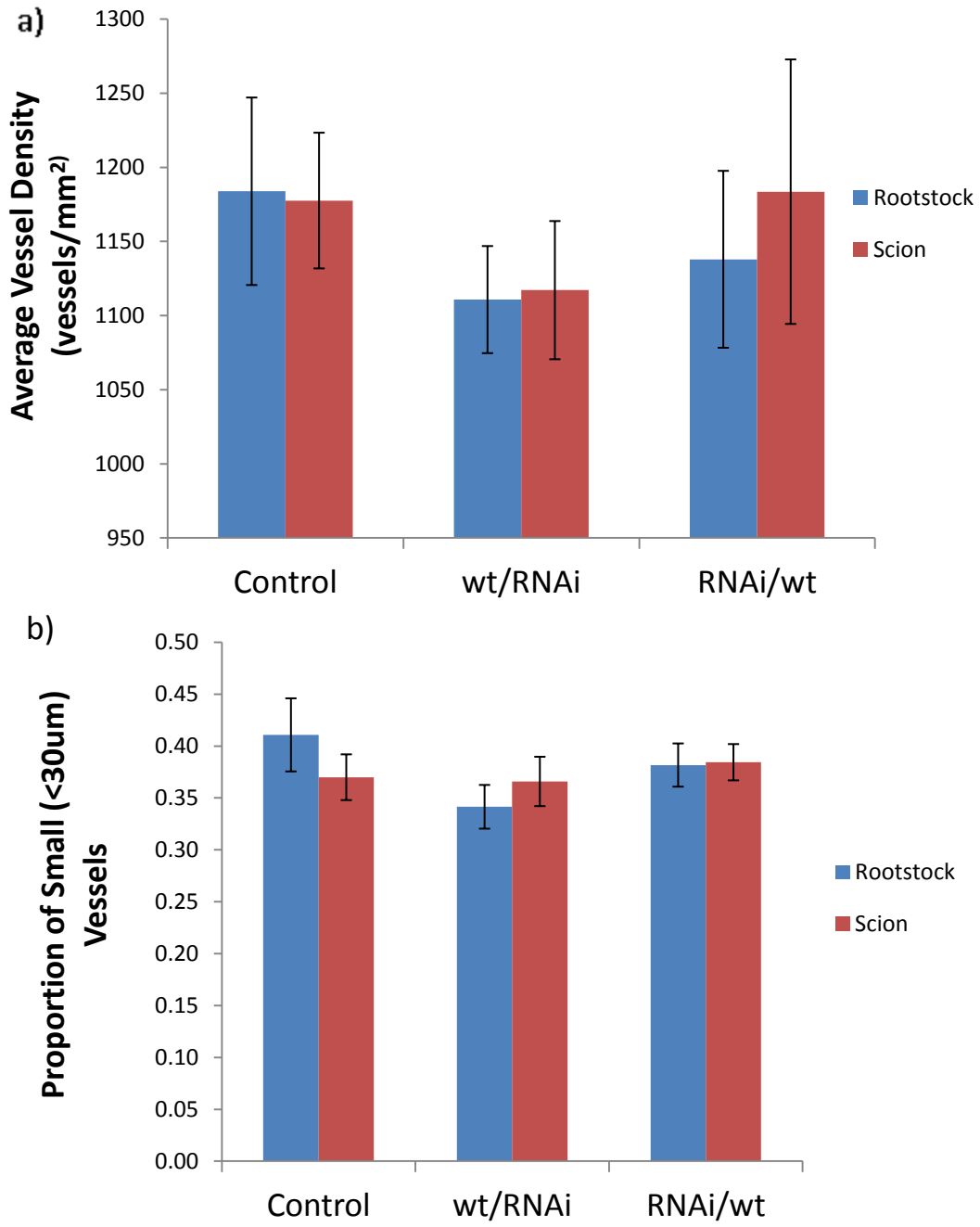


Figure III.7. Comparison of total vessel density between rootstock and scion in number of vessels per mm^2 (a), and comparison of the proportion of vessels less than 30 μm in diameter between rootstock and scion (b). For control treatment n=7, for wt/RNAi n = 17, and for RNAi/wt n = 5.

Although it was confirmed that there was no graft transmissibility of the RNAi signal between the scion and the rootstock in either of the experimental treatments, the evidence showed that the localized areas of varying auxin transport rates within one plant had no effect on

vessel anatomy. One potential explanation is that the plants have homeostatic mechanisms that reduce any effects caused by the difference in auxin transport rates, effectively regulating auxin levels in the tissue. Auxin is such a vital hormone for plant development that this data may emphasize how strictly it's regulated by the plant in order to maintain proper physiology and growth patterns.

Conclusions

We are left with the confirmation that PIN protein levels are indeed being reduced in these RNAi line *Populus* plants, and that this RNAi signal is not graft transmissible. But despite these confirmations, there seems to be neither an external phenotypic effect, nor an effect on vessel patterning. This is curious, as auxin and its transport via the PIN proteins is known to have major effect on plant anatomy (Blakeslee 2005, Scarpella 2006), and we have seen that the auxin transport inhibitor NPA has a very dramatic effect on vessel structure and organization (Fig. III.1). Even in the graft plants, which would logically have auxin pooling at the graft site, there is no indication of the expected higher proportion small vessels. What these results may most directly indicate is the degree to which *Populus* regulates auxin and auxin transport, maintaining a seemingly normal phenotype despite all these genetic and physiological alterations.

The most logical explanation for this lack of auxin pooling effects is that the plant has homeostatic mechanisms in place which are regulating auxin levels, i.e., despite stem segments of differing auxin transport capacity being fused, the concentration of auxin in the developing xylem remained constant. In employing homeostatic strategies, the plant is ensuring that its internal conditions remain stable, despite the changes that were made to the plant through RNAi and grafting. There are three broad means through which auxin homeostasis is maintained:

synthesis, degradation and conjugation (Rosquete 2012). Auxin synthesis involves a variety of precursors and pathways that are known to be differentially controlled in response to external factors (Korasick 2013). There are also multiple pathways involved in the oxidative degradation of auxin, which is selectively activated during plant development (Barcello et al. 1990). And auxin conjugation is a mechanism by which auxin is silenced by binding the IAA molecule to proteins and sugars, as only free IAA can initiate the pathway to activate auxin dependent genes (Seidel et al. 2006).

Because of these various means of auxin homeostasis, the most immediate future study would be to quantify the amount of auxin present at the graft site within the RNAi/wt and wt/RNAi plants using mass spectroscopy. This would allow us to more precisely note the differences in auxin transport between the scion and rootstock. To further explore this difference between the effects of RNAi reduced PIN levels and NPA inhibition of PINs, more NPA manipulations could be conducted, both on wild-type *Populus* plants and on the RNAi lines. These subsequent studies may lead to a much larger question of auxin's overall importance for xylem vessel patterning.

Citations

Barceló A, Pedreño MA, Ferrer MA, Sabater F, Muñoz R. 1990. Indole-3-methanol is the main product of the oxidation of indole-3-acetic acid catalyzed by two cytosolic basic isoperoxidases from Lupinus. Planta. 181:448-450

Bernstein E, Caudy A, Hannon G. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 409: 361-66.

Blakeslee J, Peer W, Murphy A. 2005. Auxin transport. Curr Opin in Plant Bio 8: 494-500.

Brosnan CA, Mitter N, Christie M, Smith NA, Waterhouse PM. 2007. Nuclear gene silencing directs reception of long-distance mRNA silencing in *Arabidopsis*. Proc Natl Acad Sci USA. 104: 14741–14746.

Fire A, Xu S, Montgomery M, Kostas S, Mello C. 2001. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 391: 806-811.

Ketting, R. F. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. Elegans*. Genes Dev. 15: 2654-2659.

Korasick DA, Enders TA, Strader LC. 2013. Auxin biosynthesis and storage forms. J Exp Bot. 64: 2541-2555.

Palauqui J, Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. 16: 4738-4745.

Pina A, Errea P. A review of new advances in mechanism of graft compatibility–incompatibility. Sci Hortic-Amsterdam. 106: 1-11.

Rosquete MR, Barbez E, Kleine-Vehn J. 2012. Cellular auxin homeostasis: gatekeeping is housekeeping. Mol Plant. 5: 772-786.

Scarpella E. 2006. Control of leaf vascular patterning by polar auxin transport. Genes Dev. 20: 1015-1027.

Shaharuddin A, Han Y, Li H, Grierson D. The mechanism of graft transmission of sense and antisense gene silencing in tomato plants. FEBS Lett. 580: 6579-586.

Seidel C, Walz A, Park S, Cohen JD, Ludwig-Muller J. 2006. Indole-3-acetic acid protein conjugates: Novel players in auxin homeostasis. Plant Biol. 8: 340-345.

Sonoda S, Nishiguchi M. 2000. Graft transmission of post-transcriptional gene silencing: target specificity for RNA degradation is transmissible between silenced and non-silenced plants, but not between silenced plants. Plant J. 21: 1-8.

Tournier B, Tabler M, Kalantidis K. 2006. Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants. Plant J. 47: 383–394.

Voinnet O, Vain P, Angell S, Baulcombe D. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell. 95: 177-87.

Zamore, Peter, Thomas Tuschi, Philip Sharp, and David Bartel. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 101: 25-33.