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A Whole-plant Approach to Identifying Sites of Auxin Biosynthesis in Populus

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Abstract

The plant hormone auxin regulates many processes throughout plant growth and development. Aboveground auxin biosynthesis is thought to occur exclusively in the shoot apex, where it then moves via polar auxin transport (PAT) down through developing vasculature in the stem. In contrast to the canonical view of the shoot apex as the sole site of auxin production, there is some evidence to suggest that other tissues contribute auxin to the stem. Woody plants in particular may require additional sources of auxin to support the extensive vascular development in the woody stem, but very little is known about whole-plant auxin dynamics outside of herbaceous (i.e., non-woody) plants. A series of experiments were conducted using the model woody plant hybrid poplar (*Populus tremula* x *alba*) in order to better understand the sources of auxin that supply the developing stem. First, shoot apices were removed and the auxin content of developing xylem at three positions along the stem was determined after 48 hours. Removal of the apex led to a 40% reduction in the auxin content of developing xylem located 30 cm beneath the apex but had no effect further down, suggesting either that PAT is extremely slow, or that mature leaves contribute auxin directly to the developing xylem. Plants were then defoliated along 75% of the lowermost stem. Interestingly, defoliation reduced auxin levels in the apex by about 50% but had no effect on auxin levels in the developing xylem further down the stem. However, analysis of a transformant expressing an auxin-responsive reporter construct (DR5:GUS) showed that cells associated with the vasculature (primary xylem parenchyma; PXP) in the petioles of mature leaves were indeed auxin-responsive. Application of the auxin transport inhibitor NPA to petioles confirmed that PAT from leaf to stem is occurring, as auxin levels "downstream" from the site of NPA application were reduced by about 60%. In addition, qRT-PCR analysis showed that the auxin biosynthetic gene *YUCCA1* is expressed in mature leaves, albeit it at low levels. Despite this evidence for the production of auxin and its export by mature leaves, more extensive application of NPA to petioles had no effect on the auxin content of PXP or developing xylem in the stem, suggesting that the auxin moving through the petiole may not enter the stem. If all of the auxin in the stem is indeed derived from the shoot apex, transport through the stem must be exceptionally slow, as the levels of auxin in the apex are much lower than in the developing xylem. Transport rates this slow would imply that auxin is remarkably long-lived, given the distance it must travel in a large woody stem. These findings underscore the need for a better understanding of the rate and mechanism of auxin turnover, in addition to its transport and biosynthesis.

Introduction

The phytohormone auxin (indole-3-acetic acid, IAA; Figure 1) is a critical regulator of many processes throughout plant growth and development. The effects of auxin on plants have been studied for well over a century, long before the molecular identity of the compound was elucidated (Kramer and Went, 1949). In a series of experiment by Darwin, he demonstrated that a coleoptile was able to bend toward a light source, and that this was mediated by a signal derived from the shoot tip (Darwin and Darwin, 1880). Today, there is an increased understanding of auxin's molecular and biochemical role in key developmental events like vascular differentiation and embryonic development, in addition to tropic responses (i.e., movement) induced by environmental stimuli (Robert and Friml, 2009). At the tissue level, many of these effects are due to auxin transport throughout the plant and the creation of local auxin concentration gradients (Uggla, et al., 1996). These auxin gradients have been shown to be important for the development of plant vasculature, the tissues responsible for the transport of water and sugars throughout the plant (Schrader, et al., 2003). This makes auxin particularly important for woody plants, as their stems are essentially giant vascular organs. There are still many questions about which tissues are actually synthesizing auxin and how it is brought to the developing vasculature. In an effort to address this, I used the model system *Populus* to identify sources of auxin that may contribute to developing vascular tissues in a woody stem.

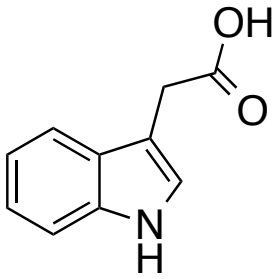

Figure 1. The chemical structure of indole-3-acetic acid (IAA), the most important member of the auxin class of phytohormones.

Auxin is transported in plants via two major routes and controls cellular gene expression

Auxin can be transported in plants via short distance, cell-to-cell polar transport, or over long distances through the phloem. Auxin found in the cell wall space is uncharged due to the relatively acidic pH of the apoplast, and can thus readily diffuse across the plasma membrane into the cell, where it affects gene expression (Robert and Friml, 2009). Once inside the relatively neutral cytoplasm of a plant cell, auxin exists in its charged form and thus cannot passively diffuse back across the plasma membrane (Robert and Friml, 2009). Because of this, the movement of auxin out of the cell must be facilitated by membrane-bound carrier proteins such as the pin-formed (PIN) proteins, which are asymmetrically localized in the plasma membrane and give directionality to auxin efflux (Friml, 2003). Other groups of proteins have also been implicated in the transport of auxin across cell membranes, however the PINs are thought to be the most important for driving polar auxin transport in plants (Blakeslee, et al., 2005).

In contrast to this polar, cell-to-cell transport, auxin can also move via the phloem. The phloem is the vascular tissue responsible for carrying photosynthate made by mature leaves to immature (e.g., shoot apices) and non-photosynthetic tissues in order to power their cellular processes. The form of auxin moving in the phloem is also distinct from the "free" form that travels via polar transport and affects gene expression. Auxin in phloem exists in a conjugated form and is covalently-linked to amino acids or sugars through amide or ester bonds respectively (Korasick, et al., 2013). Conjugates are not biologically active and are thought to serve as storage forms of auxin; in some cases, conjugates serve as a first step in an auxin degradation pathway (Ludwig-Müller, 2011), in other cases, when the phloem's contents are unloaded in

tissues such as shoot apices, conjugates can be hydrolyzed back to the active free auxin and be fed into a polar transport route (Cambridge and Morris, 1996).

Auxin has been shown to regulate the expression of many genes including those involved in metabolism, signal transduction, and development (Lee, et al., 2009). Biologically active, free auxin (e.g., IAA) affects gene expression rather indirectly by targeting the AUX/IAA family of transcriptional repressors, proteins that normally bind to and inhibit auxin responsive genes, for degradation (Reed, 2001; Tiwari, et al., 2003). Once inside the cell, IAA complexes with the F-box TIR1 protein, which then promotes degradation of the AUX/IAA repressors by SCF ubiquitin-ligase (Kepinski and Leyser, 2004; Tan, et al., 2007). This allows transcription factors, termed auxin response factors, or ARFs, to initiate transcription (Tiwari, et al., 2003). The ARF DNA binding motif has also led to the development of the synthetic auxin-responsive DR5 promoter, which has found wide utility in the creation of reporter constructs used to identify tissues that are active in auxin signaling (Ulmasov, et al., 1997; Friml, et al., 2002). This promoter can be used to drive the expression of reporter genes such as GUS or GFP to determine the pattern of auxin response across different tissues (Sabatini, et al., 1999; Spicer, et al., 2013).

Aboveground auxin production is thought to occur in developing shoot tips

The shoot tip has long been implicated in auxin production and is now regarded as the primary aboveground source tissue (Figure 2). This observation is strongly supported in classical work that describes a diffusible "growth signal", later determined to be IAA, derived from the shoot tip that could restore growth in decapitated stems (Went, 1926). This observation led to the development of a popular bioassay that took advantage of the bending of decapitated *Avena sativa* (oat) coleoptiles in response to the amount of "growth signal" applied to the cut surface

(Thimann and Bonner, 1933). Using this assay, it was shown that high concentrations of auxin could be found in shoot tips and developing leaves of herbaceous plants (Avery, 1935). The inhibitory effect of auxin on the growth of axillary buds gave further support to the idea of shoots as the primary source tissue. Removing the shoot tip was found to trigger lateral bud emergence, while replacement of the shoot tip with an auxin source maintained bud inhibition (Snow, 1931; Thimann and Skoog, 1934; Thimann, 1937). However, there was some evidence to suggest that the source tissues extended further below the apical bud, as removing developing leaves below the apex led to increased lateral branching (Delisle, 1937).

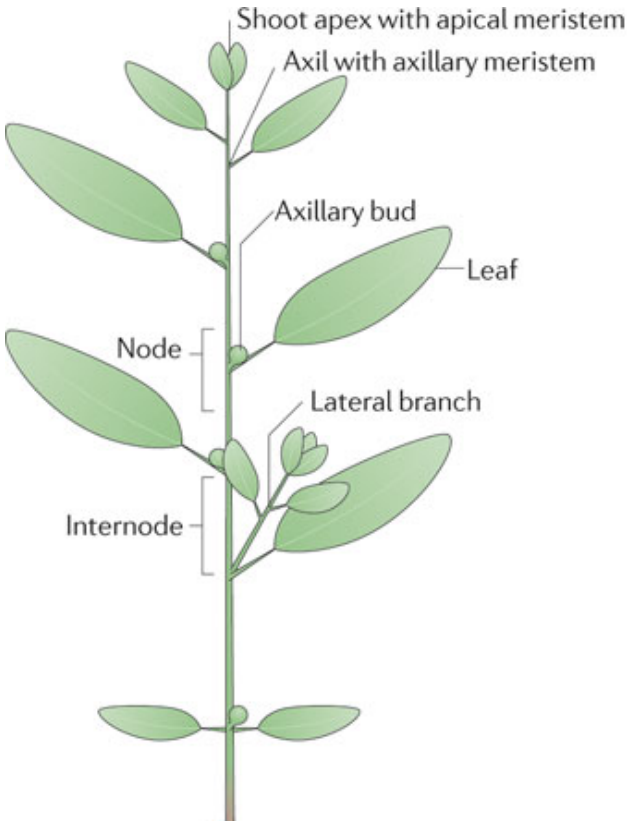

Figure 2. Image from Domagalska and Leyser (2011) depicting the locations of the shoot apex, axillary buds, nodes, and internodes in plants. The shoot apex is thought to be the primary site of aboveground auxin biosynthesis.

In all, a comprehensive understanding of which tissues synthesize auxin in plants is still lacking. Snow (1929) raised a valid point about the terminology used to refer to apical auxin-producing tissues. He wrote, "It has so far been usual to refer to the inhibiting region by the rather vague expression 'tip' or 'apex of the shoot' which naturally includes the young leaves of the bud as well as the stem apex." Although this was over 80 years ago, this ambiguity in the identity of the main auxin biosynthetic region has only increased in more recent literature. In modern molecular work, auxin biosynthesis is often described as occurring in the shoot apical meristem (Vernoux, et al., 2010) or leaf primordia (Aloni, et al., 2003). This idea is supported by work in *Arabidopsis* that has shown auxin biosynthesis in the leaf primordia is responsible for vein patterning in the developing leaf (Aloni, 2001), and is important for the maintenance of the shoot apical meristem (Eklund, et al., 2010). In contrast, biochemical analyses in *Arabidopsis* seedlings have suggested that expanding leaves are also important sites for auxin biosynthesis in addition to the shoot tip (Ljung, et al., 2001). There is also limited evidence that older leaves are active in auxin biosynthesis and can export free auxin out through the petiole. Measurable levels of free auxin exist in the expanding leaves of tobacco (Ljung, et al., 2001; Figure 3) and in the fully expanded leaves of pea, the latter of which have been shown to synthesize auxin and export conjugates via the phloem (Cambridge and Morris, 1996; Jager, et al., 2007). There is even evidence in early work that mature leaves are capable of suppressing lateral bud activation when the apex is removed, which suggests that these leaves may synthesize and export auxin (Snow, 1929).

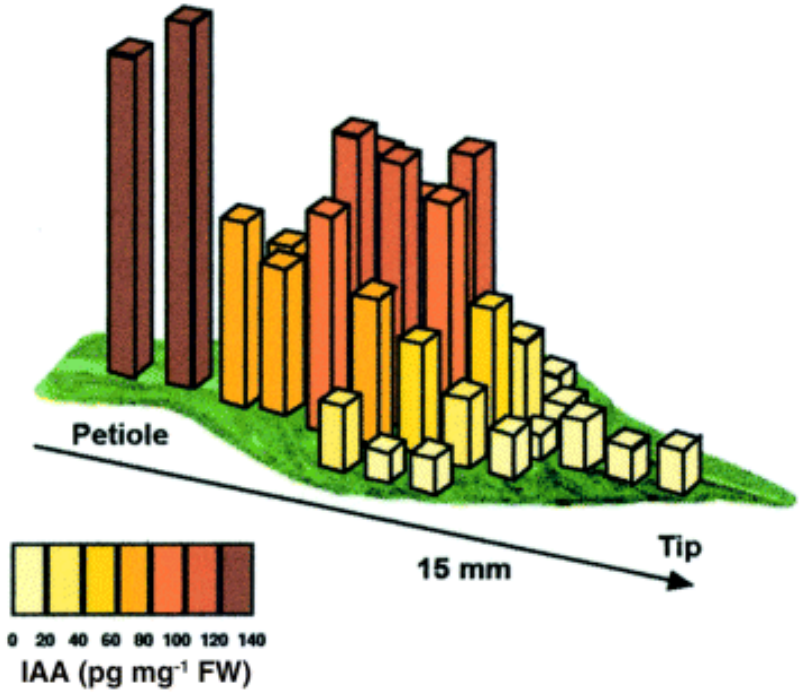

Figure 3. Image from Ljung et al. (2001) depicting auxin concentrations present throughout the mesophyll cells of an expanding tobacco leaf. The IAA content notably increases further toward the base of the leaf and is highest in the petiole.

Further evidence for mature leaves serving as potential sources of auxin comes from work on leaf abscission (Morris and Small, 1990; Meir, et al., 2006), although it is interesting to note that there is a complete disconnect between this literature and work on whole-plant auxin dynamics. In the current model of leaf abscission, *all* leaves are thought to synthesize auxin and export it out through the petiole. There, it keeps the abscission zone insensitive to ethylene, and thus inactivated (Morris and Small, 1990; Meir, et al., 2006). The abscission zone is a region of thin-walled cells at the base of the petiole that allow for separation of a senescing leaf from the rest of the plant. This mechanism of regulation is critical because early abscission zone activation would lead to the loss of an otherwise healthy and metabolically active leaf. Experiments using auxin-overproduction mutants of *Arabidopsis* have shown that higher levels of auxin biosynthesis by the leaf are correlated with delayed senescence phenotypes (Kim, et al.,

2011). In addition, work looking at gene expression in the abscission zone in response to auxin has shown that the PIN transport protein inhibitor NPA (*N*-1-napthylphthalamic acid), when applied to the petiole, led to similar gene expression profiles as removing the leaf blade (Meir, et al., 2006). While mature leaves have been shown to synthesize and export auxin through the petiole as a way to regulate abscission, no work has sought to determine if a path exists for this auxin into the stem. Because of this, studies investigating the ultimate destination of the auxin present in the petioles of mature leaves will be critically important in bridging this gap in the literature.

Auxin is particularly important to vascular development in woody species

The patterning of vascular tissues in plants is an important product of auxin signaling and transport. This process begins with the channeling of auxin through increasingly narrow files of cells in the earliest developing leaf primordia. This specifies the location of the undifferentiated cells that will go on to form vascular tissue (Scarpella, et al., 2006). In woody plants, these undifferentiated cells unite to form a complete cylinder in more mature regions of the stem, creating the vascular cambium. The vascular cambium is responsible for the formation of secondary xylem and phloem (wood and inner bark respectively), and it has been shown that high concentrations of auxin are present in the cambium and developing vascular tissues (Figure 4). It is also well-established that auxin is transported downward through the cambium, and that the PIN proteins are responsible for guiding this auxin flow (Schrader, et al., 2003).

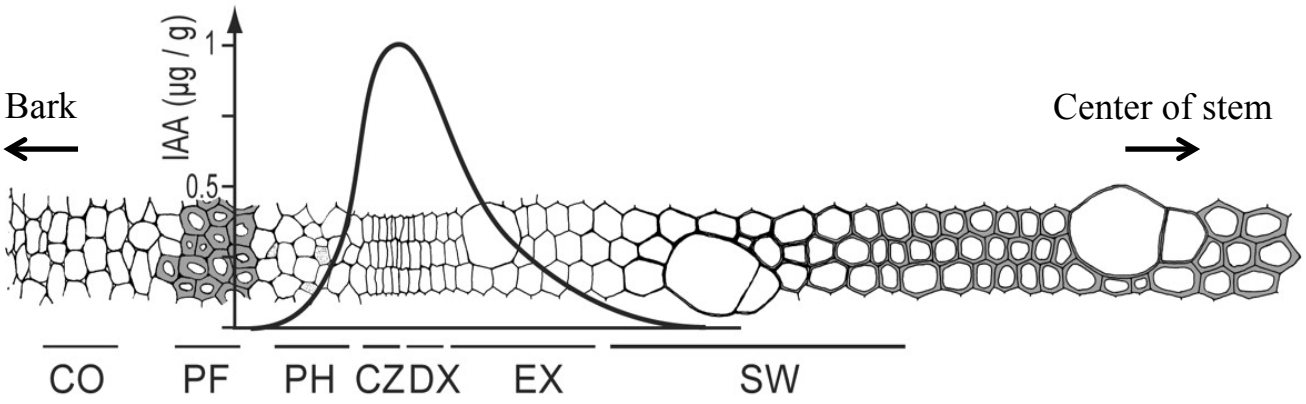

Figure 4. Figure from Schrader et al. (2003) that shows IAA concentrations across secondary vascular tissues in a *Populus* stem. PH- secondary phloem, CZ- cambial zone, DX- dividing secondary xylem, EX- expanding secondary xylem. The concentration of auxin is notably high in the cambial zone and the adjacent region of developing xylem and decreases as the vascular tissues mature.

Work in woody species supports the idea that apical tissues are the main source of auxin (Sundberg and Uggla, 1998), but also suggests that some tissues beneath the apex are capable of contributing auxin to the plant (Avery, et al., 1937). While it has been demonstrated that removal of the rapidly expanding shoot apex markedly reduces the auxin content of the cambial zone (Sundberg, et al., 2000), there is also evidence that defoliation leads to a decrease in the auxin content of the cambial zone in both angiosperm and conifer species (Savidge and Wareing, 1982; Rinne, et al., 1993). Interestingly, recent work has suggested that the auxin dynamics of the woody stem are more complex than simply basipetal (downward) transport through the cambial zone. Radioactive auxin transport assays have shown that woody stems are capable of transporting auxin laterally across different tissue types in addition to the predominating basipetal movement (Spicer, et al., 2013). In addition, the auxin-responsive reporter construct DR5:GUS together with these assays show that previously unknown cell types participate in auxin transport (Figure 5). In particular, primary xylem parenchyma cells surrounding the internal pith of the stem may function in auxin transport; strands of these cells depart the stem

and are connected to each leaf, making them a logical place to look for potential contribution of free auxin by more mature leaves (Spicer, et al., 2013). In order to understand where auxin is produced, it is important to also consider its biosynthesis.

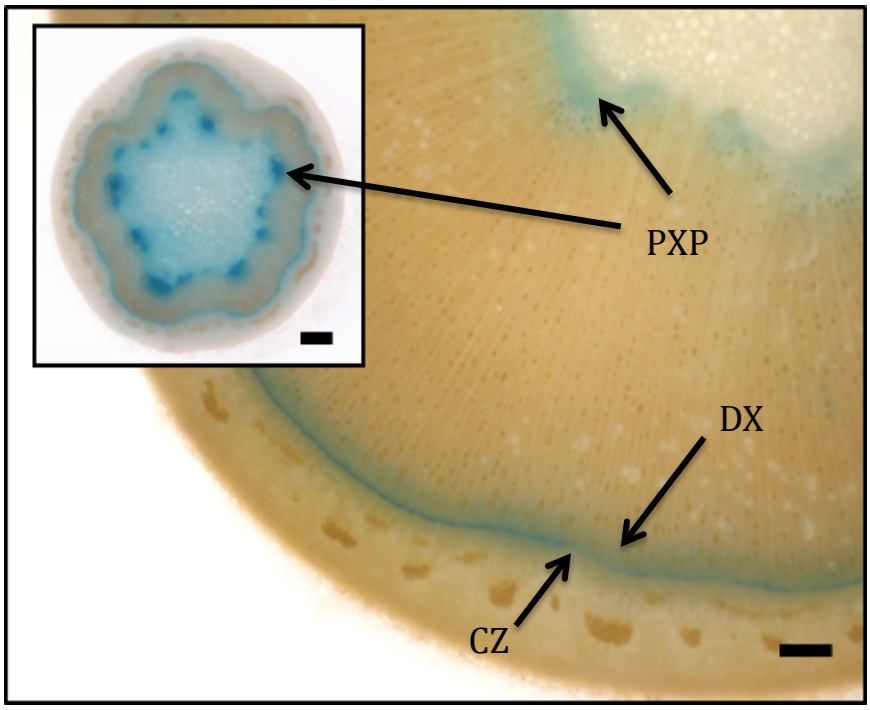

Figure 5. Image from Spicer et al. (2013) depicting stem sections of *Populus* transformed with the auxin-responsive DR5 promoter driving GUS expression. Arrows point to GUS expression (and hence an auxin response) in the cambial zone (CZ), developing xylem (DX) and primary xylem parenchyma (PXP) strands. PXP strands anastomose in the stem and three strands depart the stem together in each leaf trace. Scale bars represent 1 mm.

The major auxin biosynthetic pathway has recently been characterized

There are several compounds that make up the auxin-class of phytohormones. These include both endogenous molecules and those that have been developed as synthetic auxins, mainly for herbicide applications (e.g., 2,4-D). All of these compounds share particular structural features, namely an aromatic ring system bearing a fractional positive charge located about 5 Å away from a carbonyl oxygen, which allows them to be recognized by the auxin signaling machinery (Farrimond, et al., 1978). The primary auxin in plants, IAA, is synthesized through several

tryptophan-dependent pathways, but the one proceeding through an indole-3-pyruvic acid intermediate is now understood to be the most important. There is some evidence for the synthesis of IAA directly from indole, but the genetic and biochemical components of this putative pathway are largely unknown (Ouyang, et al., 2000; Pieck, et al., 2015). Only recently have the enzymes and intermediates of the Trp-dependent pathways been identified and their relative contributions to whole-plant auxin production determined. While much of the early work suggested that these pathways were all major contributors of free auxin, three (IAOx, TAM, and IAM) are now thought to play a less significant role (Tivendale, et al., 2014). One exception is the IAOx pathway, which has been shown to contribute to auxin production in *Arabidopsis* (Zhao, 2012). However, more recent work suggests this pathway is primarily used for the production of indole glucosinolates in *Arabidopsis* and other crucifers (Mikkelsen, et al., 2000; Sugawara, et al., 2009). Thus, it is now generally accepted that the IPyA pathway is responsible for most IAA biosynthesis in plants (Tivendale, et al., 2014). This two-step pathway proceeds from tryptophan through an indole-3-pyruvic acid (IPyA) intermediate before finally yielding IAA (Figure 6). The enzyme encoded by *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* / *TRYPTOPHAN AMINOTRANSFERASE RELATED* (*TAA1/TAR*) is responsible for the conversion of tryptophan to IPyA while the flavin-dependent monoxygenase encoded by the *YUCCA* gene family is responsible for producing IAA from IPyA (Stepanova, et al., 2008; Mashiguchi, et al., 2011).

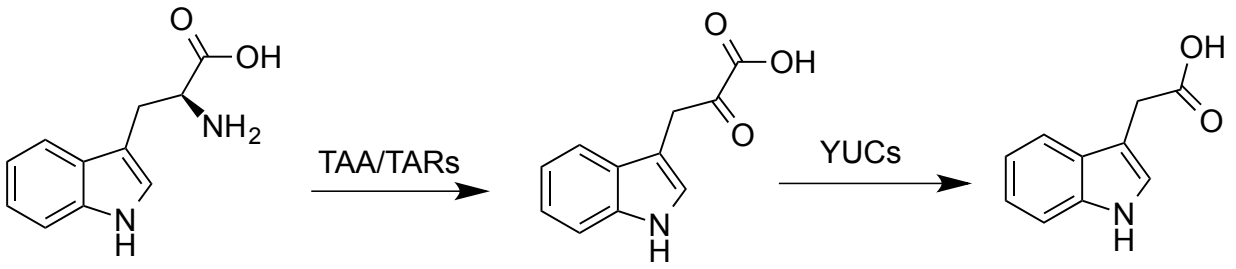

Figure 6. Diagram of the two-step IPyA auxin biosynthetic pathway. The TAA/TARs family of aminotransferases catalyze the conversion of tryptophan to indole-3-pyruvic acid (IPyA), then the YUCCA (YUC) family of flavin-dependent monooxygenases converts IPyA to IAA.

Stable isotope feeding experiments have proved to be particularly useful in these studies and have helped identify many IAA intermediates and metabolites. Deuterium oxide (^2H_2O) feeding is one method that can be used to determine the *de novo* auxin biosynthetic capacity of specific tissues (Ljung, et al., 2001; Jager, et al., 2007). Deuterium atoms can be incorporated into the uncyclized intermediates early in the shikimate pathway during aromatic amino acid biosynthesis and then detected in various downstream IAA precursors and intermediates (Normanly, 2010). In addition, studies with labeled auxin precursors (e.g., D_5-tryptophan) have been useful in analyzing the contribution of the four described Trp-dependent auxin biosynthetic pathways (Sitbon, et al., 2000; Ludwig-Müller, et al., 2010; Mashiguchi, et al., 2011). While both methods allow for *in vivo* estimations of auxin biosynthetic activity, feeding directly with labeled precursors provides this estimate irrespective of whether aromatic amino acid biosynthesis occurs in that tissue. In terms of identifying locations of auxin biosynthesis, isotope-feeding assays have supported the role of the shoot apex in auxin production (Sitbon, et al., 2000; Bhalerao, et al., 2002), while also providing evidence for *de novo* biosynthesis in both expanding leaves of *Arabidopsis* (Ljung, et al., 2001) and mature leaves of pea (Jager, et al., 2007).

In the last several years, we've grown to understand that the YUCCAs catalyze the rate-limiting step of auxin biosynthesis through the IPyA pathway, making them particularly important for maintaining auxin homeostasis, and by extension, many facets of plant development (Mashiguchi, et al., 2011). When the YUCCA genes were first discovered, they were thought to function in the Trp-dependent TAM pathway and catalyze the conversion of tryptamine to *N*-hydroxytryptamine (Zhao, et al., 2001). This idea was later reconsidered in a series of papers that showed the YUCs were able to convert IPyA to IAA and also provided support for their role in a pathway along with TAA1 (Mashiguchi, et al., 2011; Stepanova, et al., 2011; Won, et al., 2011). The biochemical mechanism of this conversion has recently been described (Dai, et al., 2013), in addition to the identification of a small molecule inhibitor of the YUCCA proteins, termed Yucasin (Nishimura, et al., 2014). These advancements should enhance the molecular and biochemical tools available for the study of these important enzymes and will hopefully supplement our knowledge of their role in auxin biosynthesis.

Understanding the expression patterns and organization of the *YUCCA* gene family is also important to further defining the role of tissue-specific auxin biosynthesis in plant development. Some of this work is starting to be done in *Arabidopsis*, which has 11 YUCCA genes (Ye, et al., 2009). In particular, *YUCCA1*, *2*, *4*, and *6* have been shown to be important for vascular development in the leaves (Cheng, et al., 2007). In *Populus*, 12 YUCCA genes have been identified, but there is only minimal work looking at how their expression differs across tissues, especially during vascular development. A study done by Ye et al. (2009) generated tissue-specific expression profiles for *in vitro* grown *Populus* plantlets and illustrated that there are significant differences in expression across tissues. However, given that these studies were conducted on plants grown in culture that were only a few weeks old, it is difficult to extrapolate

the results to what might be observed in a mature tree. Given the importance of the YUCCA genes in auxin biosynthesis, working to identify their expression patterns during different developmental processes will be critical to advancing our understanding of auxin's role in plant growth.

Motivation for current work

Modern scientific advances have produced a wide range of molecular and biochemical tools for the study of auxin and its role in plant growth and development. However, our knowledge of this area is largely based on small herbaceous plants (namely *Arabidopsis*) with much less known about auxin dynamics in woody species. It is well established that in woody plants, auxin flux through the vascular cambium is necessary for the development of secondary vascular tissues. Although the source of this auxin is assumed to be the shoot apex, there are pieces of evidence to suggest that other tissues might contribute as well. In light of this, the work described here seeks to improve our understanding of potential sources of auxin in woody plants and how they might contribute to a developing stem. Specific questions addressed by the work presented here include: does the apex supply all of the auxin to the cambial zone and developing xylem, or do other tissues contribute? Is there evidence for auxin transport in mature leaves, and if so, through what tissues is it moving? What is the likely pathway for mature leaf-derived auxin into the stem? In addition to the fundamental merits, an improved understanding of how wood production is regulated by plants serves as an important practical outcome of this work.

Materials and Methods

Plant Material and Growth Conditions

All experiments were conducted using the INRA 717-1B4 *Populus tremula* x *alba* hybrid clone as well as a transformant of this clone containing the auxin-responsive DR5 promoter driving GUS expression (henceforth referred to as PtaDR5). Plantlets from both lines were propagated every four to six weeks in sterile culture on half-strength MS media (pH = 6.0) supplemented with 2% sucrose, 0.25 mg/mL MES, 0.4 mg/mL glycine, and 0.2 mg/mL myo-inositol. Culture vessels were maintained in an incubator at 24 °C under 16-hour days with 50 μmol m^{-2} s^{-1} from both cool white and full spectrum fluorescent bulbs. Three-week-old plantlets were subsequently transferred to Fafard ® Growing Mix supplemented with a slow-release fertilizer and grown in a greenhouse. Temperatures in the greenhouse ranged from 24 °C to 30 °C and a 16-hour day was maintained with metal halide lamps as needed.

All plants were transferred to 7L pots and grown for approximately four months at which time they were about 1.5 meters in height. Leaf numbers for each plant were determined based on a leaf plastochron index to standardize the developmental stage of stem and leaf material (Ford, 1982). Specifically, the “apex” was defined as the tight cluster of leaves where visible internodes could no longer be distinguished with the naked eye. The leaf directly beneath the apex was classified as leaf 1, and was approximately 1.5 cm long with one-third of the leaf margin still remaining curled. Leaves were numbered sequentially (e.g., leaf 1, leaf 2, etc.) from the apex to the base of the plant. Most experimental plants had between 40 and 50 leaves.

Manipulation Experiments

Shoot Tip Removal

In order to better understand the extent to which the apex supplies auxin to the developing xylem in the cambial zone, wild-type 717-1B4 plants were decapitated with a razor blade in the internode just above leaf one (i.e., the entire apex as defined above was removed). Developing xylem was collected 48 hours post-decapitation at three positions along the stem located 30, 60 and 100 cm beneath the apex. To obtain developing xylem tissue, plants were cut off at the base, the bark was peeled back over the sampling region (5-20 cm in length), and the exposed surface was scraped with a razor blade. Because developing xylem in the cambial zone is soft, it is easily separated from mature xylem in this way. To account for the differences in stem diameter at the different positions and still collect a comparable volume of tissue at the same developmental stage, developing xylem was collected along a 20 cm length for the uppermost position (i.e., where the stems are most narrow), a 10 cm length for the middle position, and a 5 cm length for the most basal position (i.e., where stems are the widest). Following collection, the tissue was gently mixed to ensure homogenization, and divided into 100 mg aliquots before being flash-frozen in LN2 and stored at -80 °C until IAA analysis.

Defoliation

A combination of greenhouse-grown 717-1B4 and PtaDR5 plants were partially defoliated in order to determine whether mature leaves contribute auxin to developing stem tissues. All leaves starting with leaf 8 were removed with a razor blade by cutting about 5 mm from where the petiole met the stem. Leaves 8, 16, and 24 were frozen in LN2 for RNA isolation and subsequent *YUCCA1* gene expression studies using qRT-PCR (see below). After 48 hours,

apices were harvested and developing xylem was obtained from the lowermost 10 cm of each stem. All of the developing xylem was collected and gently mixed before being divided into 100 mg aliquots. All tissue was flash-frozen in LN2 and stored at -80 °C prior to IAA analysis.

Petioles and segments of stem approximately 10 nodes beneath the apex were also harvested from control and defoliated PtaDR5 plants for GUS staining. Fresh tissue was incubated for 6 hours at 37 °C in X-Gluc solution containing 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide. Petiole segments were embedded in 5% agarose blocks and sectioned at 50 – 100 μm using a vibratome, while stem segments including a leaf trace were split down the center using a razor blade. Sections were viewed on an inverted Nikon TE2000 at 100x and 200x and photographed with a QImaging Retiga 2000R digital camera.

Single-Petiole and Whole-Plant NPA Application

The polar auxin transport inhibitor NPA (*N*-1-naphthylphthalamic acid) was applied to the petioles of leaves 10 and 20 in 717-1B4 and PtaDR5 plants to determine: (i) if auxin present in the petioles of mature leaves is moving by polar auxin transport, and (ii) the direction of auxin transport through the petiole. In an additional experiment, NPA was applied to most of the mature leaf petioles to determine whether inhibition of auxin transport from mature leaves affects delivery of auxin to stem tissues. For both sets of experiments, a lanolin paste containing 30 mM NPA (dissolved in DMSO) was applied in a 0.25 cm-wide ring to the center of each petiole. Control plants had a lanolin paste containing an identical volume of DMSO applied to petioles in the same manner. To determine the directionality of polar auxin transport in the petiole, NPA was applied to the petioles of leaves 10 and 20. Leaf 10 was still expanding, while leaf 20 had already reached full expansion. After five days, petiole pieces 2 cm in length were

harvested from positions both proximal (stem-side) and distal (leaf-side) to the site of NPA application (Figure 7). This tissue was then frozen in LN2 and stored at -80 °C prior to IAA analysis.

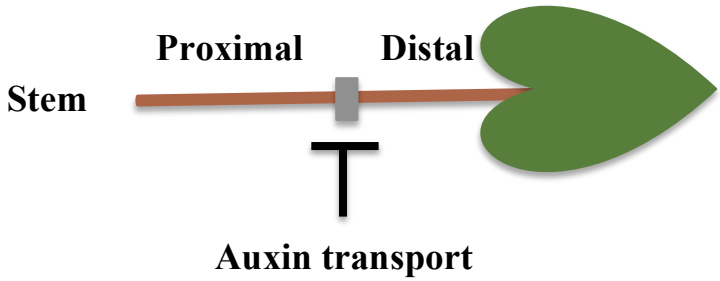

Figure 7. Depiction of experimental setup for the application of the auxin transport inhibitor NPA to the petioles of leaves 10 and 20. NPA was applied to the center of each petiole and after five days, the petioles pieces both proximal (stem-side) and distal (leaf-side) to the site of application were harvested for IAA analysis.

In order to determine whether blockage of auxin transport from leaves affects the auxin content of developing xylem and/or primary xylem parenchyma, NPA in lanolin was applied as described above starting with the first fully expanded leaf (typically leaf 13) and continuing down to the leaves at the base of the stem. On average, 35 leaves were treated per plant. After five days, apices were collected and developing xylem was obtained from a 10-cm-long-region near the base of each stem. All of the developing xylem from this region was collected and gently mixed before being divided into 100 mg aliquots for IAA analysis. Primary xylem parenchyma (PXP) tissue was also collected from the same 10-cm stem piece. Briefly, stem pieces that had been pre-frozen in LN2 were split into four 2.5-cm-long cylinders and then cut in half longitudinally using a hammer and razor blade. PXP was obtained by scraping the poles of green tissue surrounding the pith from each half-cylinder with a gouging tool taking care to minimize the collection of mature secondary xylem tissue. Tissue was placed into pre-weighed cryotubes and the mass of tissue was determined. All tissue was flash-frozen in LN2 and stored at -80 °C prior to IAA analysis.

IAA Extraction and Quantification

IAA was quantified from plant tissue using LC-MS/MS based on the methods described in Barkawi et al. (2010) using a $^{13}C_6$-IAA internal standard (Cohen, et al., 1986). Briefly, plant tissue that had been pre-frozen in LN2 was combined with a homogenization buffer containing 65% isopropanol 35% 0.2 M imidazole pH 7.0 with a known amount (typically 10 ng per 100 mg sample) of $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and homogenized in a mini bead mill with two tungsten carbide beads. Homogenized samples were then left to extract for one hour on ice. Following incubation, the samples were centrifuged and the supernatant was removed and stored at -80 °C.

To conduct the IAA extractions, an aliquot of the supernatant (250 uL) was diluted ten-fold with MS-grade H_2O and applied to a conditioned NH2 column. The columns were then washed with equal volumes of hexane, ethyl acetate, acetonitrile, and methanol before the IAA was eluted in 0.25% phosphoric acid. The pH of the eluate was brought to between 3.0 and 3.5 using 0.1 M succinic acid pH 6.0, and the samples were then applied to a pre-conditioned polymethylmethacrylate epoxide resin column (BioRad, Hercules, CA, USA). The columns were subsequently washed with an epoxide loading buffer (5:1 0.25 % phosphoric acid : 0.1 M succinic acid pH 6). Samples were eluted in methanol and trace H_2O was removed with the addition of anhydrous Na_2SO_4 followed by a short incubation. Samples were then methylated with ethereal diazomethane in diethyl ether at room temperature in the hood for 30 minutes and then reduced to dryness under N_2. Samples were resuspended in 100 μL 5% (v/v) MeOH 1% (v/v) acetic acid and stored at -20 °C until quantification.

Quantification was performed using LC-MS/MS on a Thermo Finnegan Surveyor HPLC and an LTQ XL™ Linear Ion Trap Mass Spectrometer operating in positive ESI mode. The

HPLC column was a reverse-phase C-18 column (15-cm-long Thermo Fisher Hypersil GOLD™, 1 mm diameter, 3 μm particle size with a 5 mm guard column). Twenty microliter injections were separated over a 45 minute run at a flow rate of 90 μL/min. A linear gradient of 0.1% formic acid in water (FA) and 0.1% formic acid in acetonitrile (ACN) was run as follows: 95% FA:5% ACN for five minutes, increased to 20% FA:80% ACN over 30 minutes, held at 20% FA:80% ACN for three minutes, re-equilibrated to 95% FA:5% ACN over two minutes, and held at 95% FA:5% ACN for 8 minutes.

Samples were introduced into the ESI source held at 250 °C with a capillary voltage of 27 kV, a source voltage of 5 kV, and a sheath gas (N_2) flow rate of 21 (arbitrary units). The mass spectrometer was operated in MRM mode, with CID using helium gas at a normalized collision energy of 35%. The following transitions were monitored: 190 m/z to 129.0 – 131.0 for endogenous IAA and 196 m/z to 135.0 – 137.0 for the $^{13}C_6$-IAA internal standard. Peaks for endogenous and $^{13}C_6$-IAA were integrated using the Genesis™ algorithm. The amount of auxin present in the original tissue sample was calculated using the isotope dilution equation (Rittenberg and Foster, 1940) where x is the amount of $^{13}C_6$-IAA added, C_o is its isotopic enrichment, C_f is the isotopic enrichment of both the standard and endogenous IAA, and R is a correction factor (R = 1.13) for the natural abundance of ^{13}C isotopes of IAA:

$$y = \frac{\left(\frac{C_o}{C_f} - 1\right)x}{R}$$

YUCCA gene expression via qRT-PCR

Expression levels of *YUCCA1* (*YUC1*) in *Populus* leaves of different ages were determined via quantitative real-time PCR (qRT-PCR). Leaves 8, 16, and 24 were collected from four individuals (four-month-old, greenhouse grown 717-1B4; see above) and immediately frozen in

LN2. Entire leaves were ground to a fine powder in a mortar and pestle with LN2 and stored at -80 °C prior to RNA extraction.

RNA isolation and cDNA synthesis

RNA was extracted with the Spectrum Plant Total RNA Kit™ with on-column DNAse digestion (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. Total RNA was eluted in 40 µL of elution buffer and then quantified using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA). RNA quality was also checked using gel electrophoresis with 1.5 µL RNA. All RNA was stored at -80 °C until the reverse transcriptase reactions could be run. First-strand cDNA was synthesized from approximately 1.5 µg total RNA with SuperScriptIII reverse transcriptase and oligo$(dT)_{20}$ primers (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was then treated with RNAseH (Life Technologies, Carlsbad, CA, USA) and quantified using a Qubit Fluorometer. All cDNA was stored at -20 °C.

qRT-PCR

qRT-PCR was conducted on a CFX Connect™ RealTime system using iTAQ™ Universal SYBR ® Green Supermix (Biorad, Hercules, CA, USA). Reactions were carried out in a total volume of 20 µL with between 5 - 40 ng cDNA and a primer concentration of 10 µM. Primer sets for *YUC1* and *TUA2* (alpha 2-4 tubulin; used here as a reference gene) are listed in Table 1. Four technical replicates were run per gene per individual in addition to three no-template-controls (NTC) per gene. Following loading, plates were spun down briefly in a mini plate spinner to collect the reactions. For each qRT-PCR experiment, the following conditions were

used: initial denaturation step at 95 °C for 30 seconds followed by 37 cycles of 5 seconds at 95 °C (denaturation) and 30 seconds at 53 °C (annealing and extension). Florescence was measured at the end of each cycle. Following the last amplification cycle, melt curve analysis was performed in which the temperature was raised from 55 °C to 95 °C in 0.5 °C increments with florescence measured after each incremental increase. The relative quantity to the reference gene (Q) was calculated using the following equation:

$$Q = \frac{(2)^{Ct_{ref}}}{(2)^{Ct_{GOI}}}$$

where "2" represents the efficiency of amplification based on a theoretical doubling with each amplification cycle, and Ct represents the cycle number at which each gene crossed a pre-determined threshold fluorescence level. The efficiency of amplification for both primer pairs was tested with a standard curve analysis using a ten-fold dilution series spanning four orders of magnitude. In both cases, the efficiency was found to be equal to 2 (1.95 for *YUC1* and 1.95 for *TUA2*) such that correcting for differences in efficiency was not necessary. Ct_{ref} and Ct_{GOI} were calculated as the average of four technical replicates.

Table 1. List of primers used for qRT-PCR experiments

Gene	Direction	Sequence (5' to 3')	T_m (°C)	Amplicon (bp)
TUA2 RT-F1	Forward	CCTACTGTAGTACCTGGGGGTG	58	230
TUA2 RT-R1	Reverse	CCAACTTCCTCGTAATCCTTCTCA	56	
YUC1 RT-F1	Forward	GGAACAGCCTCTGATGCTGTG	53	91
YUC1 RT-R1	Reverse	ATGGGAATTGCAAGATTTATCGT	58	

Statistical Analyses

Two-tailed t-tests were used to compare the IAA contents of tissue from treated (decapitated, defoliated) and control trees. Paired two-tailed t-tests were used to compare the IAA contents of petiole tissue on either side of the site of NPA application (i.e., samples were paired within each petiole).

Results

Changes in the auxin content of developing xylem in response to decapitation

To determine the relative contribution of the shoot apex to the auxin content of the cambial zone, auxin levels in the developing xylem of decapitated plants were measured 48 hours following removal of the apex. Developing xylem was sampled at upper, middle and lower positions located 30, 60 and 100 cm beneath the apex respectively. Decapitation led to a 40% reduction in the auxin content of developing xylem from the upper sampling position compared to control ($p < 0.05$, two-tailed t-test, $n = 6$), whereas the auxin content of the middle and base positions remained unaffected ($p = 0.8$ for both positions; Figure 8). These data support the assumption that the apex is a major source of auxin in the stem and also provide an approximation of the rate of auxin transport moving down the stem. Given that evidence of a reduction in auxin levels following removal of the apex was observed 48 hours post-decapitation 30 cm beneath the apex, but not 60 cm beneath, this suggests that the rate of auxin transport from the apex is around 2 mm/hr.

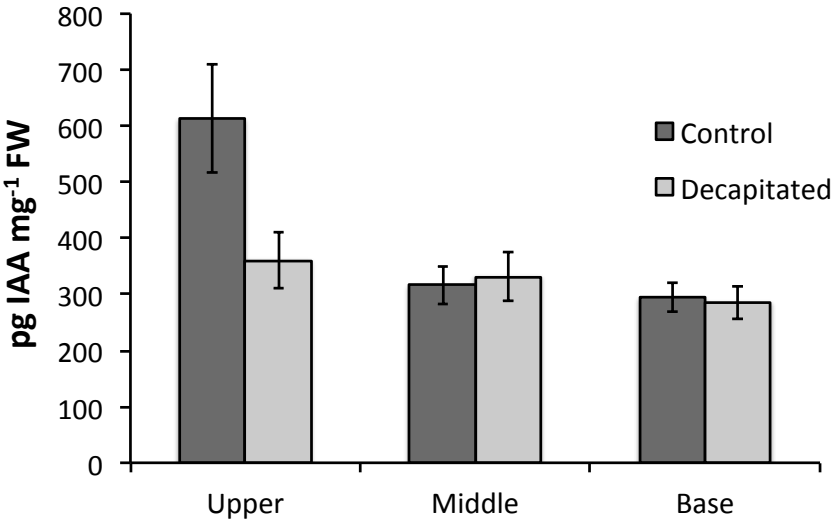

Figure 8. Mean IAA content of developing xylem from three positions collected 48 hours following the removal of the apex. The three positions corresponded to 30 (upper), 60 (middle), and 100 cm (base) below the apex. Decapitation significantly reduced the auxin content of the developing xylem at the uppermost position by about 40%, however no effect was observed at the more basal positions. Error bars are ± SE.

Changes in the auxin content of stem tissues following defoliation

In order to determine if auxin produced by mature leaves might contribute to the developing xylem, plants were defoliated starting at the eighth leaf beneath the apex and the auxin content of the developing xylem and apices was measured after 48 hours. Removal of mature leaves did not affect the auxin content of the developing xylem ($p = 0.1$, two-tailed t-test), but it led to a highly significant reduction in the amount of auxin in the shoot apex by about 50% compared to control ($p < 0.001$, two-tailed t-test, $n = 7$; Figure 9).

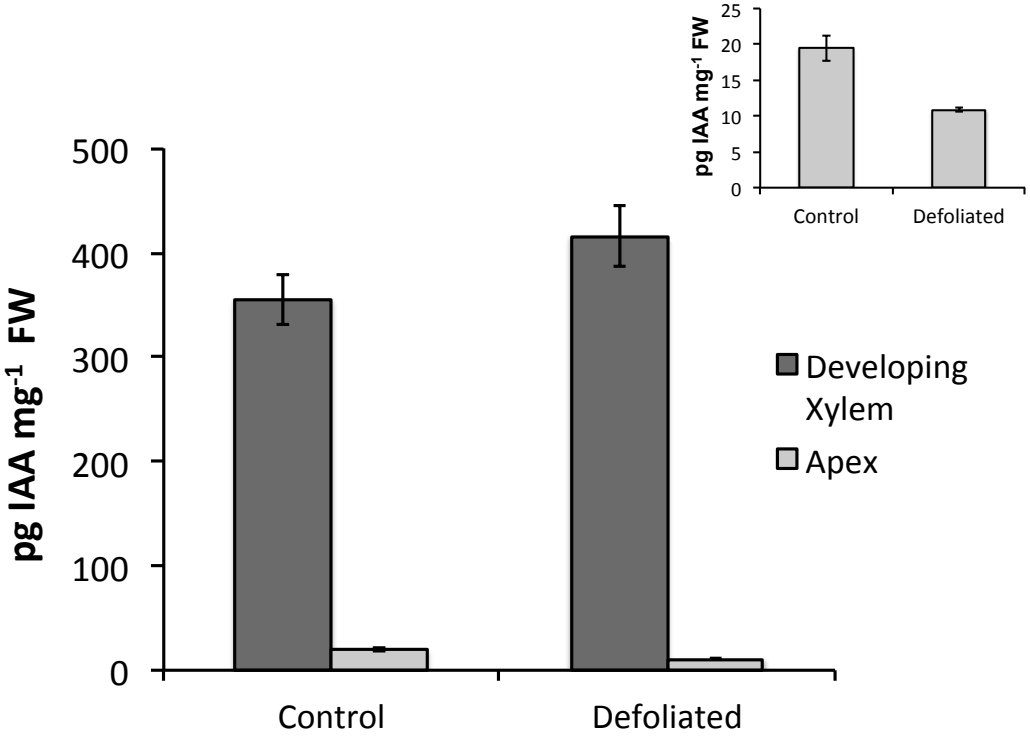

Figure 9. Mean IAA content in developing xylem and shoot apices for control and defoliated plants, with apices reproduced in inset for scaling purposes. Plants were completely defoliated starting from leaf eight downward and tissues were harvested 48 hours post-defoliation. The IAA content of the developing xylem was not affected by defoliation, however the IAA content of the apex was significantly reduced relative to control (inset). Error bars are ± SE.

GUS staining of petioles from PtaDR5 plants was also done to determine what tissues appear capable of responding to auxin. GUS expression was observed only within the petiole vascular bundles, with expression restricted to the primary xylem parenchyma (PXP) cells that are interspersed among the most internal xylem vessels (Figure 10). In addition, stem segments from control and defoliated PtaDR5 plants were split down the center to determine if the GUS signal persists following removal of the leaf blade. The GUS signal is present at the base of the petiole and adjacent region of the stem only in plants to which the leaf blade remained attached, and is absent from this region in defoliated plants (Figure 11).

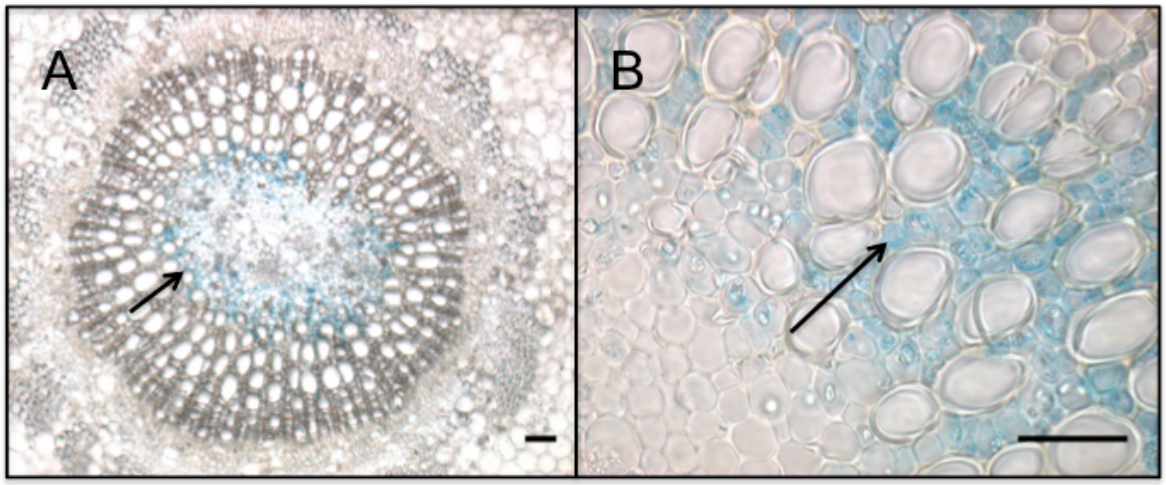

Figure 10. Cross-sections of petioles from PtaDR5 plants located 10 nodes beneath the apex. GUS expression, which is indicative of an auxin response, is found only in the vascular bundles of the petiole (A). Within the bundle, the auxin response is restricted to the primary xylem parenchyma cells (arrows). These cells are associated with the most internal primary xylem vessels. Scale bars represent 100 μm.

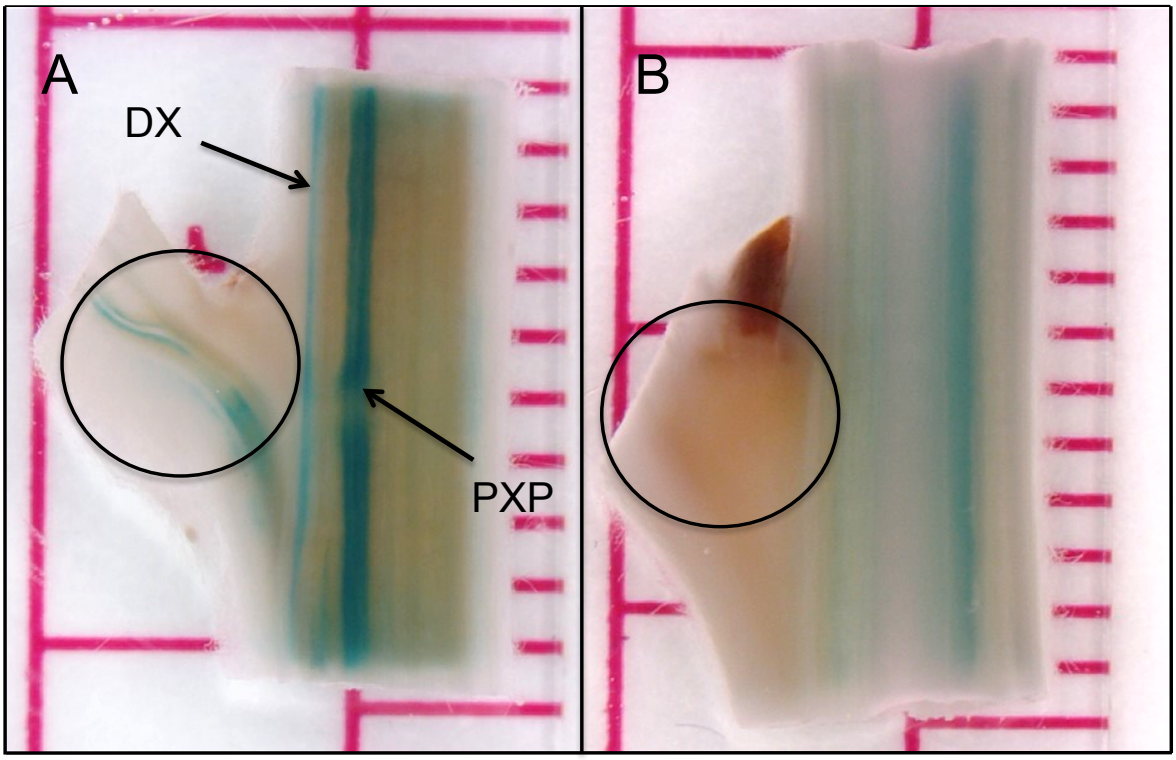

Figure 11. DR5:GUS expression in stem pieces indicating an auxin response in control (A) and defoliated (B) plants 10 nodes beneath the apex. The GUS signal is present in the petiole stump and surrounding tissue of control plants, but is notably absent in defoliated plants (circles). In defoliated plants, there is no evidence for the GUS signal in the petiole stump or in the surrounding stem tissue. Note that GUS expression is qualitative and not quantitative, as staining intensity is a function of the rate of substrate diffusion through tissue, etc. The faint GUS expression shown in the developing xylem (DX) and primary xylem parenchyma (PXP) of defoliated plants (B) did not translate into reduced auxin levels in those tissues compared to control. Ruler in the background showing mm scale.

Effects of NPA on petiolar auxin transport and delivery to stem tissues

When removal of mature leaves did not decrease the auxin content of the developing xylem, the auxin transport inhibitor NPA was applied to petioles in an effort to determine if polar auxin transport occurs through these tissues. NPA applied to petioles of two different ages (leaf 10 and leaf 20) reduced auxin content proximal to the site of application by about 60% in leaves 10 and 20 ($p < 0.01$ for both leaf ages, paired t-test, $n = 7$; Figure 12). There was no evidence at either leaf age for an effect of the delivery medium (i.e., DMSO in lanolin), or for any natural variation in the auxin content between positions proximal and distal to the site of NPA application, in the petioles of control leaves ($p = 0.7$ for both leaf ages, paired t-test). Interestingly, while auxin levels were significantly decreased proximal to the site of NPA application, there was no evidence for an increase on the distal side in leaves 10 ($p = 0.4$, two-tailed t-test) or 20 ($p = 0.08$, two-tailed t-test).

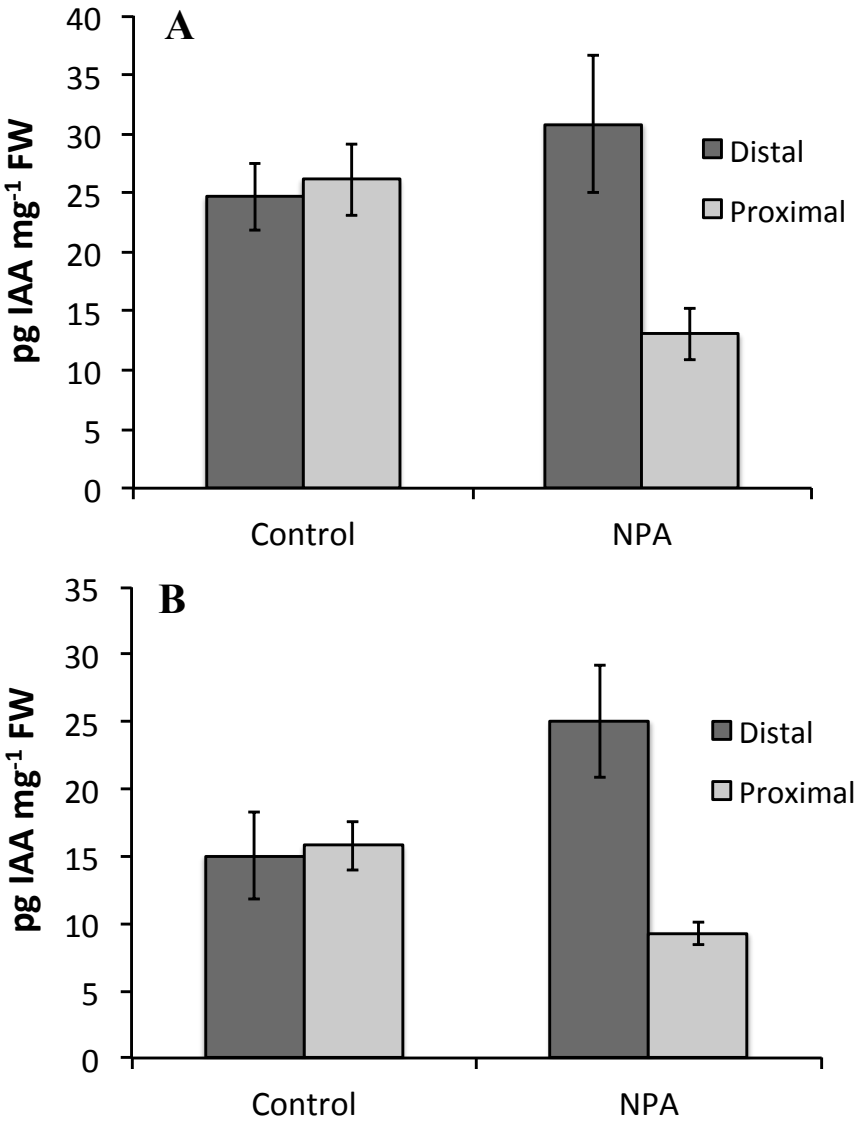

Figure 12. Mean IAA content in petiole segments on either side of a region treated with the auxin transport inhibitor NPA. NPA (30 mM) was applied in lanolin in a 0.25 cm-wide ring to the central region of the petiole of leaves 10 and 20. After five days, petiole segments were collected both distal (leaf-side) and proximal (stem-side) to the site of NPA application and IAA was quantified. The presence of NPA led to a significant reduction in the auxin content of the proximal petiole piece relative to distal for both leaf 10 (A) and 20 (B). Error bars are ± 1 SE.

In a separate experiment, NPA was applied to the petioles of all leaves beneath the first fully expanded leaf to determine if selectively blocking auxin transport from petioles leads to decreases in the auxin content of stem tissues. NPA failed to reduce the auxin content of developing xylem ($p = 0.3$, two-tailed t-test) or primary xylem parenchyma ($p = 0.8$, two-tailed t-

test) compared to control (Figure 13). In addition, there was no evidence of any effect on the auxin content of the apices between control and treated plants ($p = 0.9$, two-tailed t-test).

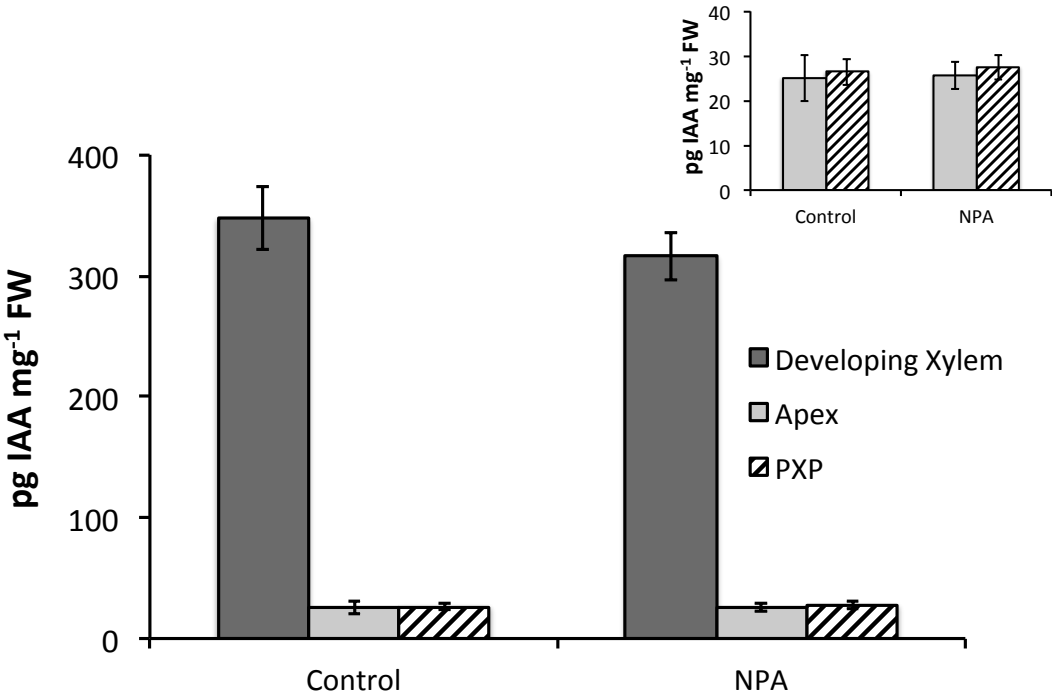

Figure 13. Mean IAA content in developing xylem, apices, and primary xylem parenchyma (PXP) in response to application of the auxin transport inhibitor NPA to the petioles of mature leaves. NPA (30 mM) was applied in a 0.25 cm-wide ring to the center of all petioles starting with the first fully expanded leaf. Developing xylem, apices, and PXP were collected after five days and analyzed for IAA content. Application of NPA had no effect on the auxin content in any of the tissues measured. Error bars are ± 1 SE.

Characterization of *YUCCA1* expression in mature leaves

Expression levels of the auxin biosynthetic gene *YUCCA1* were determined for leaves of three different ages (8, 16 and 24) using qRT-PCR. The results of the analysis suggest that *YUCCA1* is expressed at measureable levels in leaves of all three ages, but that considerable variation exists as there failed to be significant differences in *YUCCA1* expression levels between leaves of different ages ($p > 0.2$ for all leaves, paired t-test, $n = 4$; Figure 14).

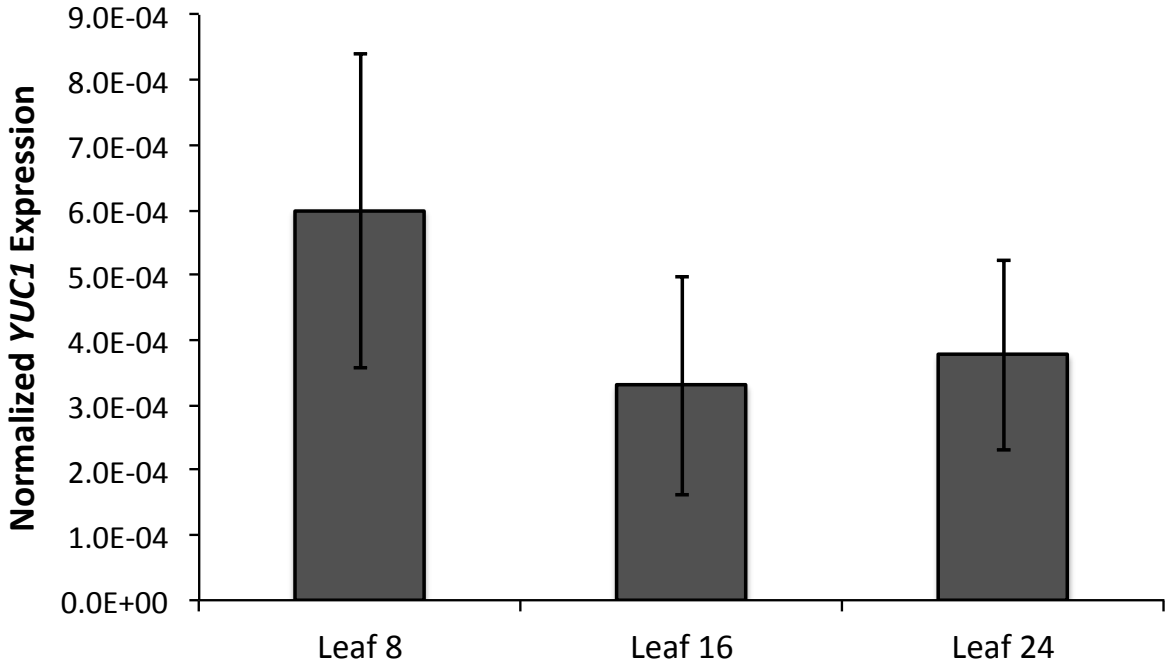

Figure 14. Normalized *YUC1* expression in leaves 8, 16, and 24 obtained from four greenhouse grown plants. While expression was detectable in all leaves sampled, there was considerable variation in *YUC1* expression even between leaves of the same age. Error bars are ± SE.

Discussion

Removal of the apex reduces but does not eliminate auxin in the developing xylem

The reduction in auxin content of the developing xylem from the uppermost position following decapitation supports the prevailing idea in the literature that most of the auxin moving through the cambial zone originates in the apex. It is interesting to note however, that removal of the apex had no effect on auxin content further down in the stem (Figure 8). It is possible that this is a product of the rate of auxin transport, and that any evidence of auxin depletion did not have sufficient time to reach the lower stem positions. While increasing the duration of the experiment would seem to be a logical next step, the expected growth of axillary buds following

decapitation would make it difficult to run the experiment for a longer period of time. Normally, auxin movement down from the apex keeps the buds that are located in leaf axils dormant (Leyser, 2003). Removing the apex reduces the amount of auxin moving down the stem, which causes these buds to begin growing. Once activated, the axillary shoots become sources of auxin that would likely raise the levels in developing xylem such that any depletion caused by apex removal would be eliminated. Thus, future iterations of this experiment would require the pre-emptive removal of axillary buds, which in turn could produce a wounding response that would have unknown effects on auxin dynamics in the stem.

The partial reduction (about 40%) in the upper position and lack of an effect further down could also be due to other tissues contributing to the auxin content of the developing xylem. Following removal of the apex, approximately eight leaves, all of which were still expanding, remained in the stem region above the uppermost (i.e., 30 cm) position. Some work has shown that young expanding leaves contribute to the whole-plant auxin pool in *Arabidopsis* (Ljung, et al., 2001), and it is possible that these same tissues supply auxin to the developing xylem in *Populus*. Further down the stem, mature leaves could also be contributing to the auxin found in developing xylem. Some studies have shown that mature leaves can be sources of auxin conjugates that are transported in the phloem (Cambridge and Morris, 1996; Jager, et al., 2007). Although there is currently no evidence that mature leaves export free auxin, the evidence that they produce conjugates suggests that they are capable of synthesizing IAA that might also be transported into the stem. In fact, radiolabeled auxin transport assays conducted in excised *Populus* stems have shown that there is a potential route of transport from the petioles of mature leaves into the stem that does not occur via the phloem (Spicer, et al., 2013), but it is not known if transport along this pathway actually occurs *in vivo*. The status of mature leaves as possible

sources of free auxin has not been well investigated, and the potential for these leaves to contribute auxin to the developing vasculature warrants further analysis.

Petioles of mature leaves contain auxin and show evidence of leaf-to-stem polar auxin transport

GUS staining in PtaDR5 plants provided strong evidence for the presence of auxin-responsive cells in the petioles of mature leaves, specifically the primary xylem parenchyma (PXP) cells at the center of each petiole vascular bundle (Figure 10). Prior work with GUS has shown that PXP cells are also present in the center of the stem (Figure 5) and continue to exhibit an auxin response as long as the leaves to which they are connected remain attached (Spicer, et al., 2013). Given that the primary xylem in the petiole (i.e., the water-conducting tissue that supplies the leaf) is functionally connected to the primary xylem in the stem (Larson, 1975), and that the PXP is adjacent to this tissue, it stands to reason that the PXP would be continuous as well. In addition, the fact that the GUS signal continues toward the stem in the control (Figure 11) further suggests a possible route of auxin transport into the stem. In light of this, evidence for polar auxin transport (PAT) in petioles was investigated using NPA.

Application of the auxin transport inhibitor NPA to the petioles of leaves 10 and 20 supported the presence of PAT in the petioles of leaves that were both expanding (leaf 10) and well past full expansion (leaf 20). NPA was able to block the transport of auxin through the petiole causing a depletion of IAA immediately adjacent to the site of application. The location of the depletion also provides strong evidence for the directionality of auxin transport through the petiole. Given that auxin levels were decreased only in the proximal (i.e., nearer to the stem) petiole section, this suggests that auxin transport occurs in the direction of the stem, making the

leaf blade the most likely site of auxin production. This finding is also supported by a body of work looking at the molecular basis for leaf abscission, which has demonstrated that auxin transport through the petiole is critical for keeping the abscission zone inactivated in non-senescing leaves (Morris and Small, 1990; Meir, et al., 2006). While this role for auxin in the petiole is well-understood, there is essentially no mention in the literature of its transport through the petiole or what happens after it reaches the stem. While it is possible that rapid turnover of IAA at the base of the petiole prevents any from entering the stem, the GUS signal in PtaDR5 plants can be seen departing the petiole base and entering the stem. In defoliated plants this signal was absent from the petiole base and surrounding stem region, but was clearly visible in the control (Figure 11).

In order to determine if auxin from mature leaves might contribute to stem tissues, including PXP, polar auxin transport through most of the petioles (average of 35 per plant) was blocked with NPA. This method was used over defoliation for two reasons. The first is that application of NPA to petioles led to a highly significant reduction (60%) in the auxin content of the stem-side region that would likely reduce the amount of auxin entering the stem. Secondly, NPA application is far less invasive and allowed the leaves to remain attached. While defoliation was sufficient to eliminate any GUS signal (and hence auxin activity) in the petiole base (Figure 11), it also led to a significant reduction in the auxin content of the shoot apex (Figure 9; see discussion below). This suggests that defoliation had a large effect on whole-plant auxin production and might make it difficult to identify any specific contribution by mature leaves. Interestingly however, reducing auxin transport in petioles with NPA had no effect on the developing xylem or stem PXP (Figure 13). This was particularly unexpected for the PXP because of the evidence for a connection between these cells in the petiole and in the stem.

These results suggest that either IAA moving through the petiole does not enter the stem at all, or that auxin might be synthesized along the entire length of the petiole such that the reduction in auxin levels following NPA application is only a local effect.

It is interesting to note that there was no evidence for elevated auxin levels distal to the site of NPA application, which would be expected if the normal flow of auxin through the petiole from the leaf blade were suddenly blocked. This may suggest that the levels were reduced by conjugation of IAA to amino acids, a process that occurs in response to increases in auxin levels (Ribnicky, et al., 1996; Barratt, et al., 1999; Staswick, et al., 2005). If PXP cells are able to conjugate auxin, it suggests that they may have an important role in maintaining auxin homeostasis and are not simply passive conduits for its transport.

***PtaYUCCA1* is expressed in mature leaves of different ages**

While the results of this experiment do not illustrate a clear connection between leaf age and *YUCCA1* expression, they do show that the gene is expressed at low levels in mature leaves. Evidence for the expression of auxin biosynthetic genes in the mature leaves of *Populus* has never been documented and supports the hypothesis that these leaves may be capable of making auxin. However, it is difficult to interpret the expression data for mature leaves without knowing the magnitude of *YUCCA1* expression in tissues that are known to synthesize auxin, namely, the apex. It was not possible to collect matched sets of leaves and apices in this study because the material was collected from plants nested within a larger experiment that required measuring the auxin content of the apices, and measurement of both auxin content and gene expression are mutually exclusive when working with small tissue samples. Now that expression of *YUCCA1*

in mature leaves has been established, it will be interesting to conduct a larger study of gene expression in different tissues.

In addition to comparing *YUCCA1* expression in expanding and mature leaves with apices, future qRT-PCR studies should include a larger number of replicate plants and consider within-leaf spatial variation in expression levels. The variation in expression levels even among leaves of the same age in the current study was quite large and suggests that an increased sample size is needed. Due to the significant time, effort and cost associated with qRT-PCR, it is not uncommon to rely on just three or four biological replicates. Four individuals were used in this study, which will likely be increased to six for future experiments. It is also worth considering the effect of tissue sampling on the variability in the data. Total RNA was collected from whole leaves, meaning that the RNA pool represented many different cell types, many of which are unlikely to be active in auxin biosynthesis. Given that auxin is largely transported through cells associated with vascular tissues, it is possible that auxin biosynthesis in leaves could be restricted to cells associated with the veins. Given that the veins make up only a fraction of the tissue in a whole leaf, they would potentially contribute only a small portion to the total leaf RNA used for the qRT-PCR. In future studies, a comparison of *YUCCA1* expression levels between leaf mesophyll and vein tissue would be useful in defining the cell types likely responsible for auxin biosynthesis in the leaf.

In addition to comparing *YUCCA1* expression among different parts of the leaf, it is important to consider that other YUCCA genes might be expressed at higher levels in the mature leaves of *Populus*. The lack of any work examining tissue-specific roles for the various YUCCA genes in mature *Populus* made it difficult to select a particular candidate for this analysis. While *YUCCA1* was chosen because there is evidence for its role in leaf vascular development in

Arabidopsis (Ye, et al., 2009), it is possible that it may not be the primary gene used for auxin biosynthesis in mature leaves. Exploring the expression levels of other YUCCA genes in mature leaves could be used to identify those that are most highly expressed and thus most likely responsible for auxin biosynthesis in the leaf. Performing this analysis on all 12 *Populus* YUCCA genes would be a significant undertaking, but underscores the need for more work looking at tissue-specific expression patterns of the YUCCA genes in this system.

Extensive defoliation reduces the auxin content of the shoot apex

While defoliation failed to reduce the auxin content of developing xylem, a highly significant reduction (by about 50%) was observed in the shoot apex (Figure 9). This was likely due to changes in phloem transport to the apex following removal of mature leaves, as apices are dependent on these leaves for the production of important compounds (e.g., carbohydrates, amino acids and secondary compounds) that are sent to them via the phloem (Turgeon and Wolf, 2009). While decreased carbohydrate delivery is likely a contributing factor, it is also possible that the relationship is more direct, with mature leaves contributing auxin precursors and/or conjugates to the apex via the phloem.

The most obvious explanation for the reduction in apex auxin content is that removal of mature leaves reduced delivery of compounds to the apex that are needed to drive auxin biosynthesis. For instance, if delivery of sugars to the apex was reduced, the cells in the apex would be unable to generate enough ATP to power basic cellular process. Without sufficient sources of energy, rates of auxin biosynthesis would likely be reduced. Given that carbohydrates derived from photosynthesis are used as the primary source of carbon for the synthesis of various molecules, reduced delivery of photosynthate would also lower the abundance of basic carbon

skeletons in the apex. This would decrease the amount of raw material for the biosynthesis of new compounds by the apex. It is also possible that mature leaves could be actively providing auxin precursors to the apex. These could come in the form of tryptophan (the most basic auxin precursor) or other metabolites that are part of the auxin biosynthetic pathway.

Mature leaves may also play a more direct role in determining auxin levels in the apex by supplying auxin conjugates via the phloem. This idea was proposed by Cambridge and Morris, (1989) and asserts that mature leaves are actually a significant source of auxin in plants and supply a majority of the auxin to the apex in conjugated form via the phloem. Once in the apex, the conjugates can be hydrolyzed and sent to the stem via PAT (Jager, *et al*., 2007). In contrast, there is little reason to expect that mature leaves supply free (non-conjugated) auxin to apices. Free auxin has never been observed in the phloem, and would therefore have to move via PAT. Rates of PAT have been shown to range from 1.2 – 18 mm/hr (Kramer, et al., 2011), with the results of the decapitation experiment suggesting about 2 mm/hr in *Populus* stems. An auxin molecule would simply be unable to travel from the leaves to the apex, a distance of at least 50 cm, in just 48 hours. Similarly, NPA application led to significant reductions in PAT in petioles but had no effect on auxin levels in apices, even after five days (Figure 13).

It is likely that the decrease in apex auxin content following defoliation is actually due to a combination of the effects described above. Given the importance of photosynthate delivery to the apex and the interconnected nature of carbon metabolism, it is difficult to consider any one explanation in isolation. One particularly interesting example of this connectivity is the evidence for the role of carbohydrate signaling in regulating auxin biosynthesis. Transcript levels of auxin biosynthetic genes have been shown to increase in response to higher sugar concentrations, which could serve to connect a decrease in photosynthate delivery with the reduced apex auxin

content observed here (Mishra, et al., 2009; LeClere, et al., 2010). However, more work still needs to be done to determine the role of mature leaves in influencing auxin levels in the apex.

Conclusions

The lack of an effect on stem auxin levels following NPA application and defoliation suggests that auxin derived from mature leaves may not actually enter the stem. If leaves and the shoot apex are the only two possible sources of auxin, this would suggest that all of the auxin in the cambial zone and associated developing xylem is indeed derived from the apex. Although this is the prevailing assumption in the literature, the data presented here add a surprising twist to this model: the auxin content of the shoot apex is more than an order of magnitude *lower* than the auxin content of the developing xylem (Figure 15). The only way to generate this significant auxin differential would be if rates of auxin transport in the apex were much faster than those in the cambial zone. This could very well be the case, as rates of transport through the cambial zone actually appear to be quite slow. The rough estimate of 2 mm/hr, which is based on the distance down the stem that auxin depletion was detected 48 hours following removal of the apex, is much lower than many of the values reported in a comprehensive review of published auxin transport rates (Kramer, et al., 2011). However, further radiolabeled auxin transport assays will also need to be conducted due to the lack of data on auxin transport rates in woody plants. Most of the values reported in the literature are for young stems or branches, and no work has compared transport rates between the apex and cambial zone.

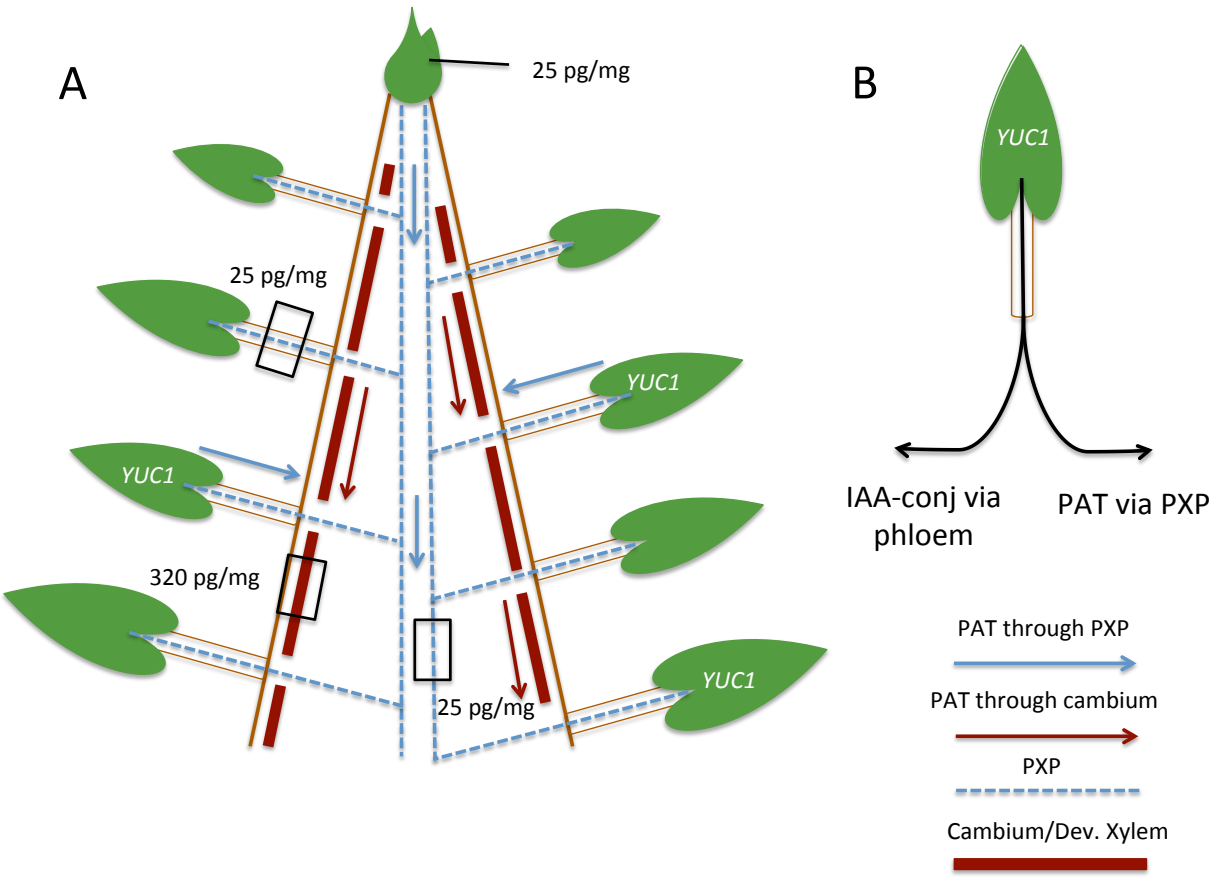

Figure 15. Schematic depicting the auxin dynamics in a woody *Populus* stem (A). Polar auxin transport (PAT) occurs through strands of primary xylem parenchyma (PXP) tissue surrounding the internal pith of the stem as well as through the cambial zone/developing xylem. Auxin moving through the cambial zone/developing xylem and PXP poles is thought to come from the apex, however there is the possibility that auxin produced by mature leaves may be fed into PXP in the stem. In addition, there is evidence for the expression of *YUCCA1*, an auxin biosynthetic gene, in mature leaves. Estimates of auxin concentrations (pg mg^{-1} fresh weight) in developing xylem and apices, as well as stem and petiole PXP are depicted. There are two routes through which auxin might be exported from mature leaves: cell-to-cell polar transport through petiole PXP or through the phloem as conjugated IAA (B).

Although shoot apices and leaves are the only tissues proposed to synthesize auxin in the aboveground shoot, it is also possible that cells within the stem itself are active in auxin biosynthesis. Given that PXP in the stem both contains auxin (Figure 15), and appears to transport it (Spicer, et al., 2013), it seems reasonable to ask whether these cells synthesize auxin

as well. If they do, they may be able to deliver it to the cambial zone via radially-oriented parenchyma cells. It is also curious given the high concentration of auxin in the cambial zone and developing xylem that there is no reference in the literature to these cells producing their *own* auxin. Although there are no documented cases of meristematic cells (i.e., stem cells, which make up the vascular cambium) producing auxin, it is not out of the question here. Of particular note is that the auxin level in the cambial zone does not drop to zero when trees are dormant and have lost their leaves, but rather, the distance over which that auxin is distributed radially is much more narrow - that is, the auxin is more concentrated near the stem cells of the vascular cambium (Sundberg, et al., 2000).

A better understanding of auxin turnover in *Populus* stems will be critical to determine whether transport rates alone can explain the differences in stem auxin content. Compared to work on auxin biosynthesis, very little is known about auxin catabolism in plants. This is particularly interesting with regard to large trees, as auxin moving through the developing xylem of a tree several meters tall would likely be subject to extensive degradation. Since auxin concentrations in developing xylem remain roughly the same along the length of the stem (Figure 15), it would suggest that other tissues may contribute auxin to the stem. However, it has been shown that in Scots pine, the vascular cambium has extremely low rates of auxin catabolism compared to other plant tissues, which supports the idea that all of the auxin moving down through the cambial zone is derived from the apex (Kramer and Ackelsberg, 2015).

Taken together, these results support the canonical view of the apex as the sole source of auxin in the stem. However, this requires a significant reduction in auxin transport rates in order to generate the significant difference in auxin concentration between apices and developing xylem. Interestingly, these results also support the less common view that mature leaves are

sites of auxin biosynthesis, as they both express the auxin biosynthetic gene *YUCCA1* and show evidence of auxin export from the leaf via PAT in the petiole. However, there is no indication that this auxin contributes to the cambial zone or stem PXP. Future work will seek to identify differences in auxin transport rates between apices and the stem and also determine the fate of auxin moving through the petioles of mature leaves.

Literature Cited

Aloni R (2001) Foliar and Axial Aspects of Vascular Differentiation: Hypotheses and Evidence. J Plant Growth Regul **20(1):** 22-34

Aloni R, Schwalm K, Langhans M, Ullrich CI (2003) Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in *Arabidopsis*. Planta **216:** 841-853

Avery GS (1935) Differential distribution of a phytohormone in the developing leaf of Nicotiana, and its relation to polarized growth. Bull Torrey Bot Club **62:** 313-330

Avery GS, Burkholder PR, Creighton HB (1937) Production and distribution of growth hormone in shoots of *Aesculus* and *Malus*, and its probable role in stimulating cambial activity. Am J Bot **24:** 51-58

Barkawi LS, Tam Y, Tillman JA, Normanly J, Cohen JD (2010) A high-throughput method for the quantitative analysis of auxins. Nat Protoc **5:** 1609-1618

Barratt NM, Dong W, Gage DA, Magnus V, Town CD (1999) Metabolism of exogenous auxin by *Arabidopsis thaliana*: Identification of the conjugate N-(indol-3-ylacetyl)-glutamine and initiation of a mutant screen. Physiol Plantarum **105:** 207-217

Bhalerao RP, Eklöf J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. Plant J **29:** 325-332

Blakeslee JJ, Peer WA, Murphy AS (2005) Auxin transport. Curr Opin Plant Biol **8:** 494-500

Cambridge AP, Morris DA (1996) Transfer of exogenous auxin from the phloem to the polar auxin transport pathway in pea (*Pisum sativum* L.). Planta **199:** 583-588

Cheng Y, Dai X, Zhao Y (2007) Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. Plant Cell **19:** 2430-2439

Cohen JD, Baldi BG, Slovin JP (1986) C(6)-[Benzene Ring]-Indole-3-Acetic Acid: A New Internal Standard for Quantitative Mass Spectral Analysis of Indole-3-Acetic Acid in Plants. Plant Physiol **80:** 14-19

Dai X, Mashiguchi K, Chen Q, Kasahara H, Kamiya Y, Ojha S, DuBois J, Ballou D, Zhao Y (2013) The biochemical mechanism of auxin biosynthesis by an *Arabidopsis* YUCCA flavin-containing monooxygenase. J Biol Chem **288:** 1448-1457

Darwin C, Darwin F (1880) The power of movement in plants. John Murray, London

Delisle AL (1937) The influence of auxin on secondary branching in two species of Aster. Am J Bot **24:** 159-167

Domagalska MA, Leyser O (2011) Signal integration in the control of shoot branching. Nat Rev Mol Cell Biol **12:** 211-221

Eklund DM, Staldal V, Valsecchi I, Cierlik I, Eriksson C, Hiratsu K, Ohme-Takagi M, Sundstrom JF, Thelander M, Ezcurra I, Sundberg E (2010) The *Arabidopsis thaliana* STYLISH1 protein acts as a transcriptional activator regulating auxin biosynthesis. Plant Cell **22:** 349-363

Farrimond J, Elliott M, Clack D (1978) Charge separation as a component of the structural requirements for hormone activity. Nature **274:** 401-402

Ford H (1982) Leaf demography and the plastochron index. Biol J Linn Soc **17:** 361-373

Friml J, Wiśniewska J, Benková E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature **415:** 806-809

Friml J (2003) Auxin transport—shaping the plant. Curr Opin Plant Biol **6:** 7-12

Jager CE, Symons GM, Glancy NE, Reid JB, Ross JJ (2007) Evidence that the mature leaves contribute auxin to the immature tissues of pea (*Pisum sativum* L.). Planta **226:** 361-368

Kepinski S, Leyser O (2004) Auxin-induced SCFTIR1-Aux/IAA interaction involves stable modification of the SCFTIR1 complex. Proc Natl Acad Sci U S A **101:** 12381-12386

Kim JI, Murphy AS, Baek D, Lee SW, Yun DJ, Bressan RA, Narasimhan ML (2011) YUCCA6 over-expression demonstrates auxin function in delaying leaf senescence in *Arabidopsis thaliana*. J Exp Bot **62:** 3981-3992

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

Kramer EM, Ackelsberg EM (2015) Auxin metabolism rates and implications for plant development. Front Plant Sci **6:** 150

Kramer EM, Rutschow HL, Mabie SS (2011) AuxV: a database of auxin transport velocities. Trends Plant Sci **16:** 461-463

Kramer M, Went FW (1949) The Nature of the Auxin in Tomato Stem Tips. Plant Physiol **24:** 207-221

Larson PR (1975) Development and organization of the primary vascular system in *Populus deltoides* according to phyllotaxy. Am J Bot 1084-1099

LeClere S, Schmelz EA, Chourey PS (2010) Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. Plant Physiol **153:** 306-318

Lee DJ, Park JW, Lee HW, Kim J (2009) Genome-wide analysis of the auxin-responsive transcriptome downstream of iaa1 and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression. J Exp Bot **60:** 3935-3957

Leyser O (2003) Regulation of shoot branching by auxin. Trends Plant Sci **8:** 541-545

Ljung K, Bhalerao RP, Sandberg G (2001) Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. Plant J **28:** 465-474

Ludwig-Müller J (2011) Auxin conjugates: their role for plant development and in the evolution of land plants. J Exp Bot **62:** 1757-1773

Ludwig-Müller J, Denk K, Cohen JD, Quint M (2010) An inhibitor of tryptophan-dependent biosynthesis of indole-3-acetic acid alters seedling development in *Arabidopsis*. J Plant Growth Regul **29:** 242-248

Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M, Hanada A, Yaeno T, Shirasu K, Yao H, McSteen P, Zhao Y, Hayashi K, Kamiya Y, Kasahara H (2011) The main auxin biosynthesis pathway in Arabidopsis. Proc Natl Acad Sci U S A **108:** 18512-18517

Meir S, Hunter DA, Chen JC, Halaly V, Reid MS (2006) Molecular changes occurring during acquisition of abscission competence following auxin depletion in *Mirabilis jalapa*. Plant Physiol **141:** 1604-1616

Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79B2 from *Arabidopsis* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J Biol Chem **275:** 33712-33717

Mishra BS, Singh M, Aggrawal P, Laxmi A (2009) Glucose and auxin signaling interaction in controlling *Arabidopsis thaliana* seedlings root growth and development. PloS one **4:** e4502

Morris DA, Small DK (1990) Auxin transport and the regulation of petiole abscission in cotton (*Gossypium hirsutum* L.): A comparison of indol-3yl-acetic acid and phenylacetic acid. Plant Growth Regul **9:** 201-214

Nishimura T, Hayashi K, Suzuki H, Gyohda A, Takaoka C, Sakaguchi Y, Matsumoto S, Kasahara H, Sakai T, Kato J (2014) Yucasin is a potent inhibitor of YUCCA, a key enzyme in auxin biosynthesis. Plant J **77:** 352-366

Normanly J (2010) Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. Cold Spring Harb Perspect Biol **2(1):** a001594

Ouyang J, Shao X, Li J (2000) Indole-3-glycerol phosphate, a branchpoint of indole-3-acetic acid biosynthesis from the tryptophan biosynthetic pathway in *Arabidopsis thaliana*. Plant J **24:** 327-334

Pieck M, Yuan Y, Godfrey J, Fisher C, Zolj S, Thomas N, Wu C, Ramos J, Lee N, Normanly J, Celenza J (2015) Identification of an Arabidopsis Aminotransferase that Facilitates Tryptophan and Auxin Homeostasis. bioRxiv 013821

Reed JW (2001) Roles and activities of Aux/IAA proteins in *Arabidopsis*. Trends Plant Sci **6:** 420-425

Ribnicky DM, Ilic N, Cohen JD, Cooke TJ (1996) The Effects of Exogenous Auxins on Endogenous Indole-3-Acetic Acid Metabolism (The Implications for Carrot Somatic Embryogenesis). Plant Physiol **112:** 549-558

Rinne P, Tuominen H, Sundberg B (1993) Growth patterns and endogenous indole-3-acetic acid concentrations in current-year coppice shoots and seedlings of two *Betula* species. Physiol Plantarum **88:** 403-412

Rittenberg D, Foster G (1940) A new procedure for quantitative analysis by isotope dilution, with application to the determination of amino acids and fatty acids. J Biol Chem **133:** 737-744

Robert HS, Friml J (2009) Auxin and other signals on the move in plants. Nat Chem Biol **5:** 325-332

Sabatini S, Beis D, Wolkenfelt H, Murfett J, Guilfoyle T, Malamy J, Benfey P, Leyser O, Bechtold N, Weisbeek P (1999) An Auxin-Dependent Distal Organizer of Pattern and Polarity in the *Arabidopsis* Root. Cell **99:** 463-472

Savidge R, Wareing P (1982) Apparent auxin production and transport during winter in the nongrowing pine tree. Can J Bot **60:** 681-691

Scarpella E, Marcos D, Friml J, Berleth T (2006) Control of leaf vascular patterning by polar auxin transport. Genes Dev **20:** 1015-1027

Schrader J, Baba K, May ST, Palme K, Bennett M, Bhalerao RP, Sandberg G (2003) Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. Proc Natl Acad Sci U S A **100:** 10096-10101

Sitbon F, Åstot C, Edlund A, Crozier A, Sandberg G (2000) The relative importance of tryptophan-dependent and tryptophan-independent biosynthesis of indole-3-acetic acid in tobacco during vegetative growth. Planta **211:** 715-721

Snow R (1931) Experiments on Growth and Inhibition. Part II.--New Phenomena of Inhibition. Proc R Soc B **108:** 305-316

Snow R (1929) The young leaf as the inhibiting organ. New Phytol **28:** 345-358

Spicer R, Tisdale-Orr T, Talavera C (2013) Auxin-responsive DR5 promoter coupled with transport assays suggest separate but linked routes of auxin transport during woody stem development in *Populus*. PloS one **8:** e72499

Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, Suza W (2005) Characterization of an Arabidopsis Enzyme Family That Conjugates Amino Acids to Indole-3-Acetic Acid. Plant Cell **17:** 616-627

Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie D, Doležal K, Schlereth A, Jürgens G, Alonso JM (2008) *TAA1*-Mediated Auxin Biosynthesis Is Essential for Hormone Crosstalk and Plant Development. Cell **133:** 177-191

Stepanova AN, Yun J, Robles LM, Novak O, He W, Guo H, Ljung K, Alonso JM (2011) The *Arabidopsis* YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. Plant Cell **23:** 3961-3973

Sugawara S, Hishiyama S, Jikumaru Y, Hanada A, Nishimura T, Koshiba T, Zhao Y, Kamiya Y, Kasahara H (2009) Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in *Arabidopsis*. Proc Natl Acad Sci U S A **106:** 5430-5435

Sundberg B, Uggla C, Tuominen H (2000) Cambial growth and auxin gradients. In R Savidge, J Barnett, R Napier, eds, Cell & Molecular Biology of Wood Formation. BIOS Scientific Publishers Ltd, Oxford, pp 169-188

Sundberg B, Uggla C (1998) Origin and dynamics of indoleacetic acid under polar transport in *Pinus sylvestris*. Physiol Plantarum **104:** 22-29

Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446:** 640-645

Thimann KV (1937) On the nature of inhibitions caused by auxin. Am J Bot **24:** 407-412

Thimann KV, Bonner J (1933) The mechanism of the action of the growth substance of plants. Proc R Soc B **113:** 126-149

Thimann KV, Skoog F (1934) On the inhibition of bud development and other functions of growth substance in *Vicia faba*. Proc R Soc B **114:** 317-339

Tivendale ND, Ross JJ, Cohen JD (2014) The shifting paradigms of auxin biosynthesis. Trends Plant Sci **19:** 44-51

Tiwari SB, Hagen G, Guilfoyle T (2003) The roles of auxin response factor domains in auxin-responsive transcription. Plant Cell **15:** 533-543

Turgeon R, Wolf S (2009) Phloem transport: cellular pathways and molecular trafficking. Annu Rev Plant Biol **60:** 207-221

Uggla C, Moritz T, Sandberg G, Sundberg B (1996) Auxin as a positional signal in pattern formation in plants. Proc Natl Acad Sci U S A **93:** 9282-9286

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9:** 1963-1971

Vernoux T, Besnard F, Traas J (2010) Auxin at the shoot apical meristem. Cold Spring Harb Perspect Biol **2(4):** a001487

Went FW (1926) On growth-accelerating substances in the coleoptile of *Avena sativa*. Proc K Ned Akad Wet C **30:** 10-19

Won C, Shen X, Mashiguchi K, Zheng Z, Dai X, Cheng Y, Kasahara H, Kamiya Y, Chory J, Zhao Y (2011) Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF *ARABIDOPSIS* and YUCCAs in *Arabidopsis*. Proc Natl Acad Sci U S A **108:** 18518-18523

Ye X, Kang B, Osburn LD, Li Y, Max CZ (2009) Identification of the flavin-dependent monooxygenase-encoding YUCCA gene family in *Populus trichocarpa* and their expression in vegetative tissues and in response to hormone and environmental stresses. Plant Cell, Tissue and Organ Culture (PCTOC) **97:** 271-283

Zhao Y (2012) Auxin biosynthesis: a simple two-step pathway converts tryptophan to indole-3-acetic acid in plants. Mol Plant **5:** 334-338

Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J (2001) A Role for Flavin Monooxygenase-Like Enzymes in Auxin Biosynthesis. Science **291:** 306-309