The roles of *SHORT INTERNODES/STYLISH* genes, regulators of auxin homeostasis, during leaf vein development in *Arabidopsis thaliana*

by

Tammy Kim Baylis

B.Sc. (Hons.), Simon Fraser University, 2010

Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

Master of Science

in the
Department of Biological Sciences
Faculty of Science

Simon Fraser University

Spring 2012

Approval

Name: **Tammy Kim Baylis**

Degree: **Master of Science (Biological Sciences)**

Title of Thesis: ***The roles of* SHORT INTERNODES/STYLISH *genes, regulators of auxin homeostasis, during leaf vein development in* Arabidopsis thaliana**

Examining Committee:

Chair: Gordon Rintoul, Associate Professor

Jim Mattsson
Senior Supervisor
Associate Professor

Sherryl Bisgrove
Supervisor
Assistant Professor

Harald Hutter
Supervisor
Professor

Enrico Scarpella
External Examiner
Associate Professor, Department of Biological Sciences
University of Alberta

Date Defended: April 20, 2012

Partial Copyright Licence

Abstract

Leaves require extensive venation systems to transport fixed carbon and water. While it is known that auxin can induce vascular differentiation, the mechanisms behind leaf vein patterning are poorly understood. Here I have assessed the roles of *SHI/STY*, *YUCCA*, and *TAA1* genes in leaf vein development. These genes have been linked to auxin biosynthesis. I have found that *SHI*, *STY1*, *STY2*, *SRS5*, *YUC1*, *YUC2*, *YUC4*, and *TAA1* are primarily expressed at the apical, basal, and marginal domains of leaf primordia in *Arabidopsis thaliana*, with little expression at sites of vein formation. *shi/sty* mutant analyses nevertheless revealed venation phenotypes, most notably in cotyledons. In addition, some *SHI/STY* expression patterns shift with auxin transport inhibition and exogenous auxin application treatments. Taken altogether, I hypothesize that SHI/STY members influence leaf vein patterning through local regulation of auxin biosynthesis from the margins of leaf blades.

Keywords: *Arabidopsis thaliana*; leaf vascular patterning; auxin; SHORT INTERNODES/STYLISH; YUCCA; TAA1

Dedication

I dedicate this to my mother, Kim, for your ongoing love, support, and strength through all of life's adventures. Thank you for being my inspiration and for teaching me to value life, nature, and knowledge.

I dedicate this also to my grandmother, C.C. Yu, for your bravery, generosity, and unconditional love for everyone in our big family throughout your many years. May you rest in peace.

Acknowledgements

I express my sincere gratitude to everyone who has somehow contributed to this thesis. My personal thanks go to:

My senior supervisor, Jim Mattsson, for providing me with the tools, space, and training I wielded to explore new concepts, and for showing unrivalled enthusiasm and support through thick and thin.

My supervisors Sherryl Bisgrove and Harald Hutter for providing additional lab resources as well as much-welcomed assistance and inspiration.

My external examiner, Enrico Scarpella, for graciously accepting the invitation to advise me during the final stages of my degree.

The chair of my thesis defense, Gordon Rintoul, for kindly taking the time to facilitate the particulars of my examination.

My supervisor and my collaborators at the Swedish University of Agricultural Sciences, Eva Sundberg, Izabela Cierlik, Veronika Ståldal, and many others, for providing me with plant materials, lab space and apparatus, and guidance.

Yunde Zhao, Nina Fedoroff, and Joanne Chory, for kindly donating their plant materials to my research.

Our visiting plant hormone specialist, Roni Aloni, for sharing your wisdom.

NSERC, the Mutual Fire Insurance Company of British Columbia, the H.R. MacCarthy Memorial Education Fund, and the SFU Department of Biological Sciences for providing me with financial support.

Members of the Mattsson lab, past and present, for each of your expertises, contributions to lab tasks and discussions, and senses of humour: Aktari Begum,

Mathias Schuetz, Adam Foster, Muhammad Arshad, Dua'a Riyal, Lotta Pellonperä, Ryan Thomas, Shelley Abercromby, Kathy Bayat, Lily Teng, Lucy Woodhead, Taylor Hardy, Mario Fidanza, and Lorenz Nierves.

Other researchers and friends in the department, for helpful tips and stories: Tarek Suliman, Shannon Squires, and Saeid Shahidi.

My best friend, Dustin, for guiding me, being there for me, and showing me how bright the future will be.

My parents and my brother, for your full support and for always believing in me. I made it here because of you.

Table of Contents

List of Figures

List of Tables

List of Supplemental Figures

1. Introduction

Typical plant leaves have evolved to provide large surface areas for maximal absorbance of light quanta in the process of photosynthesis. These natural solar panels, including the embryonic leaves called cotyledons, require highly developed venation systems to collect fixed carbon in the form of sugar for transport to other organs and also to distribute water from the roots to throughout the leaf blade. In the model plant *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*), a primary midvein is located in the centre of the leaf along the leaf's apical-basal (tip-to-base) axis (Figure 1). The midvein is connected to secondary vein loops, which often reflect the shape of the leaf. Rosette leaves, the first leaves to develop in *Arabidopsis* after seed germination, may contain small tertiary veins that are connected to secondary veins as well as smaller quaternary veinlets, both of which can be free-ended (Nelson & Dengler, 1997; Dengler & Kang, 2001). Each vein is comprised of bundles of vascular tissues known as xylem and phloem, which transport water with solutes and nutrients, respectively. Xylem and phloem exist, respectively, as simple tracheary elements (TEs) and sieve cells, or as more complex vessel elements and sieve tube elements.

The vascular systems of cotyledons and rosette leaves develop simultaneously with the rest of the leaf blade's growth as described previously in *Arabidopsis* (Mansfield & Briarty, 1991; West & Harada 1993; Goldberg et al., 1994; Donelly et al., 1999; Mattsson et al., 1999). Leaf veins, epidermal tissues, and the mesophyll cells of photosynthesis all result from the growth and primary differentiation of pluripotent shoot apical meristematic (SAM) cells. In the case of the leaf veins, SAM cells first acquire preprocambial cell determination. These cells are not easily discerned by their slightly different cell shape from surrounding ground meristem cells, the subepidermal cells of developing leaves (Foster, 1952; Esau, 1965). However, they can be visualized using preprocambial gene expression markers, such as the auxin response reporter DR5 (see section 1.1; Mattsson et al., 2003) the auxin response factor *MONOPTEROS* (*MP*, see section 1.1.5; Wenzel et al., 2007), or the *HD-ZIP III* gene *ATHB8* (see section 1.2.2;

Scarpella et al., 2004; Kang & Dengler, 2004). Preprocambial cells then divide and differentiate into procambial cells, the narrowed and interconnected precursors of mature vascular cells. This is followed by the differentiation of procambium into xylem and phloem (Sachs, 1981; Northcote, 1995). Part of this requires the development and thickening of plant secondary cell walls that lie just outside the plasma membrane and primary cell walls. Composed primarily of cellulose, lignin, and other polysaccharides, secondary walls allow for vascular cells to withstand high levels of tension from the water transpiration stream.

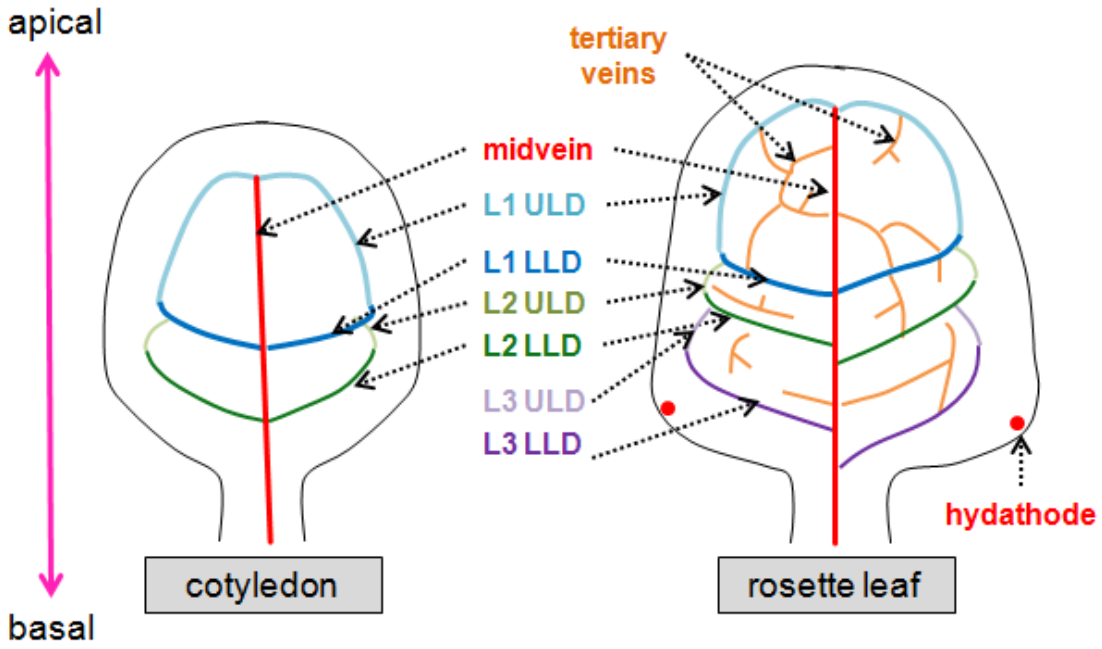


Figure 1. ***Classification of the vein orders in* Arabidopsis *cotyledons and rosette leaves***

The axis on the left indicates the apical and basal positional gradient of cotyledons and rosette leaves. The red line in the centre of each leaf is the midvein. The secondary vein loops each consist of two sections that are determined by separate developmental patterning events (see section 1.2.1). Light blue: upper-loop domain (ULD) of L1 secondary (2°) vein. Dark blue: lower-loop domain (LLD) of L1 2° vein. Light green: ULD of L2 2° vein. Dark green: LLD of L2 2° vein. Light violet: ULD of L3 2° vein. Dark violet: LLD of L3 2° vein. Note that cotyledons typically only have L1 and L2 2° vein pairs, whereas rosette leaves tend to have three or more pairs. Orange: closed and open-ended tertiary veins. In between the tertiary veins are very small quaternary veinlets that can only be seen at high magnification.

The beginning of the development of each leaf is marked by the emergence of bulges of cells called the cotyledonary or leaf primordia flanking the SAM. Already at this stage, some preprocambial cells can be identified at the center of the primordium, probably as a consequence of cell division planes along the apical-basal axis (Mattsson et al., 1999). These cells proliferate and elongate to produce a procambial midvein. The primordium then grows primarily along the apical-basal axis, generating a partially flattened, bilateral structure–the first indication of a leaf-like structure. At the same time, secondary procambial strands appear along the leaf margins, followed by the subsequent formation of tertiary and quaternary procambial veins throughout the leaf lamina. By leaf maturity, all procambial cells will have differentiated into xylem, phloem and other vascular-associated cells such as vascular parenchyma, fibers, companion cells, and idioblasts (Nelson & Dengler, 1997; Donnelly et al., 1999, Wenzel et al., 2008). The remaining ground meristem cells differentiate into photosynthetic spongy mesophyll and pallisade cells (Mansfield & Briarty, 1991; Donelly et al., 1999; Dengler & Tsukaya, 2001). It should be noted that TEs of the xylem are used to mark total vascular tissue maturity on account of their easily identified secondary cell wall structures of spiral or coaxial rings (Sachs, 1989; Sieburth, 1999).

While some of the morphological changes involved in leaf vein patterning have been documented, we do not fully understand the underlying cellular and molecular mechanisms of this aspect of plant development. Post-embryonic tissue patterning in plants is presumably influenced by position-dependent intercellular signalling mechanisms (reviewed in Benková et al., 2010), but the identities, spatial localizations, and timing of required components in developmental signal cascades remain elusive. Several approaches are still being taken to comprehend the natural formation of intricate leaf vein systems.

1.1. The phytohormone auxin

Plant hormones are natural compounds that in very low levels elicit plant responses. Also known as phytohormones, they can be synthesized by multiple tissue types and also in subsets of cells within each tissue. In line with this, plant hormones can act not only on the cells from which the molecules were synthesized, i.e. cell-

autonomously, but also they can also affect distant organs and tissues (non-cell autonomously; Davies, 2010). Several different hormones exist within plants, including auxins, gibberellins, cytokinins, ethylene, abscisic acid, strigolactones, and brassinosteroids. These phytohormones have been implicated in the regulation of cell growth, cell differentiation, and physiological events such as seed germination, organ initiation, and senescence. Two other principle hormones, jasmonates and salicyclic acid, are associated with plant chemical defence.

Auxins are perhaps the most studied of the plant hormones. Some examples of *Arabidopsis* physiological processes in which they are involved are the establishment of embryonic apical-basal (shoot to root) polarity, maintenance of apical dominance, lateral root development, flower development (including the female portion, the gynoecium), leaf development, and vascular tissue patterning (reviewed in Fukuda, 2004; Benjamins & Scheres, 2008; Rolland-Lagan 2008; Chandler, 2009; Möller & Weijers, 2009; Lau et al., 2010; Scarpella & Helariutta, 2010). A number of molecules found in plants are classified as auxins for their activities, such as 4-chloro-indole-3-acetic acid (4-Cl-IAA), indole-3-butyric acid (IBA), and phenylacetic acid (PAA; Woodward & Bartel, 2005). Some synthetic compounds, like 1-naphthalacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), can also act as auxins (Woodward & Bartel, 2005). The most abundant of the endogenous auxins, however, is indole-3-acetic acid (IAA). IAA is the auxin that will be discussed in this thesis. A method commonly used to detect areas of free IAA response in plants is analysis of the reporter gene DR5. The construct *DR5::GUS* consists of a composite promoter of seven tandem repeats of the auxin response element TGTCTC motif and a 35S minimal promoter (Ulmasov et al., 1997), fused to the *Esherichia coli* β-glucoronidase gene (GUS) to mark localized DR5 expression (Jefferson et al., 1987).

Evidence supports the hypothesis that active auxin induces developmental cell fate and pattern formation through directional auxin flow, and the unequal distribution of auxin activity and subsequent auxin responses (Went, 1974; Badescu & Napier, 2006; Dubrovsky et al., 2008; Santner & Estelle, 2009). In classical experiments, for example, the application of auxin to cut *Coleus* and tomato stems induces the differentiation of continuous vascular cells connecting to pre-existing vasculature through basal auxin flow towards the roots (LaMotte & Jacobs, 1963; Thompson & Jacobs, 1965; Jacobs, 1970;

Sachs, 1981). Similarly, the exogenous application of auxin to immature portions of leaf primordia correlates with the formation of extra leaf veins (Scarpella et al., 2006).

Local auxin maxima are tissue domains of high free auxin activity which could be required and sufficient for the initiation of leaf primordia (Reinhardt et al., 2003), cotyledonary primordia (Benková et al., 2003) floral primordia (Benková et al., 2003; Heisler et al., 2005), and lateral root primordia (Benková et al., 2003; Dubrovsky et al., 2008). The proper establishment of leaf venation systems may also require auxin maxima within the developing leaf blade, established by polar auxin flow (Aloni et al., 2003; Mattsson et al., 2003; Hay et al., 2006; Scarpella et al., 2006; Wenzel et al., 2007). Local auxin levels are influenced by mechanisms such as auxin transport, biosynthesis, conjugation, and degradation. How each of these components contributes to the formation and maintenance of local auxin maxima during leaf vein patterning is not well known.

1.1.1. Auxin biosynthesis

The auxin IAA has traditionally been proposed to be synthesized by multiple overlapping pathways in plants. Four proposed pathways are dependent on the substrate tryptophan (Trp-dependent): the indole-3-acetaldoxime (IAOx) pathway, the YUCCA (YUC) pathway, the indole-3-pyruvic acid (IPA) pathway, and the indole-3-acetamide (IAM) pathway (reviewed in Zhao, 2008, 2010; Lehmann et al., 2010). Another proposed biosynthesis pathway is Trp-independent. However, the determination of well-defined auxin biosynthesis pathways using genetic and biochemical approaches has been challenging because of the functional redundancy of the involved enzymes and metabolites. This could be further confounded by the natural recycling of IAA from less active IAA conjugate metabolites existing in plant cells (see section 1.1.3; Woodward & Bartel, 2005). As such, the genetic bases and physiological significances of the above five pathways have not been well-established. Redundancy in auxin biosynthesis has likely evolved as a protective mechanism against spurious mutations in the enzymes that contribute to a phytohormone involved in so many vital physiological mechanisms.

Recently, it has been suggested that a single Trp-dependent pathway is responsible for the majority of IAA biosynthesis in *Arabidopsis*. Mashiguchi et al. (2011) and Won et al. (2011) have found genetic, enzymatic, and metabolite-based evidence that Trp is converted to IPA which is then converted to IAA (Figure 2). The first step is mediated by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1; renamed after characterizing the corresponding alleles *weak ethylene insensitive8* (*wei8*) and *shade avoidance3* (*sav3*)) and two of its TAA1-related *Arabidopsis* homologs (TAR1 and TAR2), which have roles in shade response, ethylene interactions, embryogenesis, flower development, and vascular patterning (Stepanova et al., 2008; Tao et al., 2008). Phenotypic analyses revealed double and triple *wei8/tar* mutants with decreased numbers of cotyledon and rosette leaf secondary and tertiary veins (Stepanova et al., 2008). One particular triple mutant, *wei8 tar1-1 tar2-1*, completely lacked cotyledon vasculature and were often seedling-lethal (Stepanova et al., 2008), resembling *mp* phenotypes (see section 1.1.5; Hardtke & Berleth, 1998). Overall, double and triple *wei8/tar* mutants showed other hallmarks of decreased auxin-related phenotypes: reduced gravitropic response, shortened or missing roots and hypocotyls (the stems of young seedlings; Stepanova et al., 2008). In double and triple mutants that survived to the flowering stage, reduced plant stature, decreased apical dominance, abnormal flowers, and reduced fertility were observed (Stepanova et al., 2008). Hypocotyl elongation assays, DR5 analyses, IAA measurements of *wei8 tar2* mutants, and global expression analyses of auxin-responsive genes in *sav3* mutants further suggest that TAA1/TAR members contribute to normal auxin levels (Stepanova et al., 2008; Tao et al., 2008). The mild phenotypes of single *wei8/tar* mutants suggests members of the TAA1/TAR family have redundant functions (Stepanova et al., 2008; Tao et al., 2008).

The second step in the main auxin biosynthesis pathway is the conversion of IPA to IAA, which is facilitated by at least some studied members of the YUC family of flavin monooxygenases (Zhao et al., 2001). Of the 11 YUC members of *Arabidopsis*, YUC1, 2, 4, 6, 10, and 11 have been attributed to partially overlapping functions that are crucial for embryogenesis, seedling development, flower development, and vascular patterning (Cheng et al., 2006, 2007). Gain-of-function *yuc1D* mutant seedlings display symptoms of elevated auxin such as long hypocotyls, epinastic (downwardly-bent) cotyledons, and

elongated petioles (the stalks of leaves connecting them to the hypocotyl; Zhao et al., 2001). Free IAA measurements of *yuc1D* seedlings and phenotypic masking by increased IAA conjugation support the hypothesis that *yuc1D* overproduces auxin (Zhao et al., 2001). Meanwhile, loss-of-function analyses revealed reduced plant stature, decreased apical dominance, deformed flowers, and reduced fertility in certain combinations of *yuc1/2/4/6* loss-of-function double and higher order mutants while single *yuc* mutants show almost no abnormalities (Cheng et al., 2006). Multiple mutants also have misshapen rosette leaves with decreased secondary and tertiary veins (Cheng et al., 2006). The *yuc1 yuc4 yuc10 yuc11* quadruple mutant—like the *wei8 tar1-1 tar2-1* mutant—was sometimes seedling lethal with cotyledons lacking vascular tissue and diminished hypocotyls and roots (Cheng et al., 2007). Partially redundant roles of these YUCs in maintaining normal auxin levels have been highlighted in DR5 analyses in multiple *yuc* mutant backgrounds and tissue-specific mutant phenotype rescue by the bacterial auxin biosynthesis gene iaaM (Cheng et al., 2006). In support of these findings is the synergy of YUCs with the auxin efflux protein PIN-FORMED1 (PIN1; see section 1.1.4), according to severe *yuc1 yuc4 pin1* mutants completely lacking leaves (Cheng et al., 2007).

The similar auxin-deficient phenotypes of *taa1/tar* and *yuc* double mutants were confirmed to be phenocopies upon recent analyses (Won et al., 2011). As well, *wei8 tar 2-1 yuc1 yuc4* plants showed more severe hypocotyl and root defects than either *wei8 tar2-1* or *yuc1 yuc4* plants, providing evidence of TAA1/TAR and YUC synergy (Won et al., 2011). TAA1 likely acts upstream of YUCs in a single pathway, suggested by different IPA content measurements between *wei8 tar2-2* and *yuc1 yuc2 yuc6* (Mashiguchi et al., 2011; Won et al., 2011). Evidence also suggests that YUCs catalyze the rate-limiting step in IAA biosynthesis (Zhao et al., 2001), as TAA1 overexpression does not result in a gain-of-function while *yuc1D* does (Tao et al., 2008; Mashiguchi et al., 2011). Both *TAA1* and some *YUC* members are expressed in the apical domains of young leaf primordia (Cheng et al., 2006, 2008; Tao et al., 2008), suggesting that they act locally in auxin biosynthesis. This also suggests that the expression of these genes may be tightly controlled by endogenous and/or exogenous developmental signals.

1.1.2. Regulation of auxin biosynthesis: the SHI/STY *family*

A few genes have been found to participate in the regulation of the main pathway of auxin biosynthesis in *Arabidopsis*. TFs have not yet been identified in the regulation of *TAA1/TAR* gene expression, but potential candidates have been found for some *YUC* gene members (Figure 2; reviewed in Zhao, 2010). The NGATHAs (NGAs) are suggested to be activators of *YUC2* and *YUC4* in the developing gynoecium (Alvarez et al., 2009; Trigueros et al., 2009). Mutations in two or three of the *NGA* loci are associated with deformed gynoecia (Alvarez et al., 2009) while YUC2 and YUC4::GUS expression is reduced in artificial miRNA lines targeting four *NGA* members (Trigueros et al., 2009). LEAFY COTYLEDON2 (LEC2) is also a possible activator of auxin biosynthesis through *YUC2* targeting and the potentially direct targeting of *YUC4* (Stone et al., 2008). Expressed normally during embryogenesis, mutations in *LEC2* correlate with cotyledons having trichomes, more complex vascular patterns, and degenerated apical regions (Meinke et al., 1994; Stone et al., 2001). A dexamethosone (Dex)-inducible expression system using a fusion construct containing *LEC2* and the steroid-binding domain of a rat glucocorticoid receptor (*35S::LEC2-GR*) was used to show elevated *YUC2* and *YUC4* expression upon *LEC2* induction (Stone et al., 2008). In addition, chromatin immunopreciptation (ChIP) studies suggest that LEC2 binds to *YUC4*, though not *YUC2* (Stone et al., 2008).

An *Arabidopsis* homolog of metazoan *HETEROCHROMATIN PROTEIN1* (*HP1*) named *TERMINAL FLOWER2* (*TFL2*) participates in auxin biosynthesis regulation, perhaps by transcriptional control of multiple *YUCs* (Rizzardi et al., 2011). DR5 expression and young rosette IAA content are both reduced in *tfl2* seedlings (Rizzardi et al., 2011). Decreased *YUC5, 8,* and *9* expression is also reduced in *tfl2*, with TFL2 localizing to the latter two *YUCs* in ChIP experiments (Rizzardi et al., 2011). A candidate negative regulator of *YUCs* is SPOROCYTELESS (SPL), which shows an overexpressor phenotype similar to that of multiple *yuc* mutants (Cheng et al., 2006; Li et al., 2008). *YUC2* and *YUC6* expression—analyzed using GUS reporter constructs and quantitative reverse transcription-PCR (qRT-PCR)—was reduced in dominant *spl-D* seedlings (Li et al., 2008).

The genes I have chosen to characterize for the bulk of my thesis are members of the *SHORT INTERNODES/STYLISH* (*SHI/STY*) gene family. STYLISH1 (STY1) has been established as a DNA-binding transcriptional activator of *YUC4* after real-time PCR of *YUC4* and IAA content/biosynthesis measurements following *35S::STY1-GR* induction under normal growth conditions (Sohlberg et al., 2006; Ståldal et al., 2008) and after *35S::STY1-GR* induction in the presence of cyclohexamide (CHX), an inhibitor of protein translation (Eklund et al., 2010). *YUC8* transcription, but not transcription of the remaining eight *YUC* family members, is also activated by *35S::STY1-GR* induction (Eklund et al., 2010). The two highly conserved regions of the nine active *SHI/STY* family members in *Arabidopsis*, a 43-amino acid RING-like zinc finger domain and a more C-terminal, unique IGGH domain, suggest all SHI/STY members may be involved in macromolecule binding and nuclear localization (Fridborg et al., 2001; Kuusk et al., 2002, 2006; Eklund et al., 2010). Several members have been found to redundantly regulate the development of gynoecia and anthers on account of the defects observed in *shi/sty* multiple mutants (Fridborg et al., 1999; Kuusk et al., 2002, 2006). Multiple *SHI/STY* family members may, therefore, act in the developmental regulation of auxin biosynthesis at least in part by the transcriptional control of *YUC* members. Like YUC and TAA1/TAR family members, SHI/STY family members also display at least partial functional redundancy (Fridborg et al., 2001; Kuusk et al., 2002, 2006; Sohlberg et al., 2006; Cheng et al., 2006, 2007; Stepanova et al., 2008).

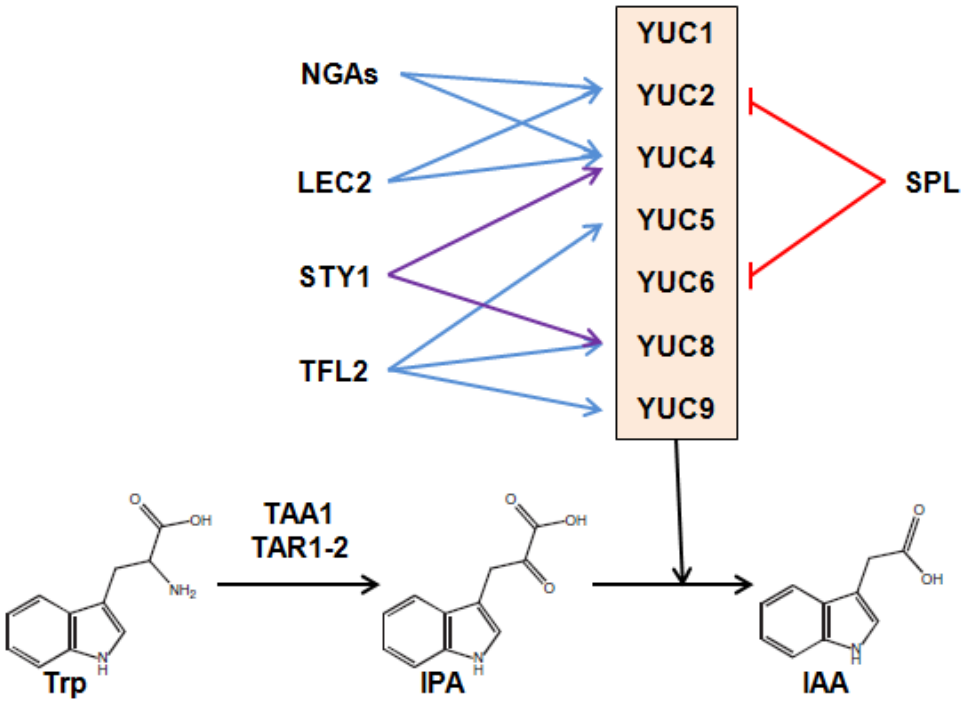


Figure 2. Regulators of the two-step auxin biosynthesis pathway

Steps in auxin biosynthesis are shown using black arrows. Positive regulation is marked by blue arrows; negative regulation is shown in red. The positive transcriptional regulation of *YUC*s by STY1 is shown in violet. Note that the only YUC members shown are those attributed to leaf development and/or those with characterized upstream regulators. Adapted from Won et al., 2011 (p. 18519, Figure 1b).

1.1.3. Auxin conjugation, degradation, & homeostasis

The unequal distribution of the active auxin IAA throughout the plant is important for development as I have mentioned previously. Much of the establishment and maintenance of this is thought to be the work of polar auxin transport (PAT; reviewed in Petrášek et al., 2011), which I will discuss in section 1.1.4. Evidence suggests that auxin homeostasis could also contribute to controlling auxin levels, though how the involved mechanisms act at the local level has not been explored. Free IAA is estimated to account for only 1% of total higher plant IAAs whereas the rest are amino acid or sugar conjugate forms (Normanly et al., 1993; Tam et al., 2000; Park et al., 2001; Pollmann et al., 2002). These conjugates may function in IAA storage, transport, compartmentalization, protection against degradation, and the decrease excessive IAA levels (Cohen & Bandurski, 1982; Östin et al., 1998). It has been hypothesized recently

that IAA could also function as a “tag” for amino acids to direct intracellular protein trafficking or protein-protein interactions (Normanly, 2009).

Amino acid conjugates include IAA molecules with amide linkages to single residues, such as IAA-Asp, and IAA-Leu. Only low levels (~17% of total IAAs) of these are found in *Arabidopsis* seeds and seedlings (Tam et al., 2000; Kowalczyk & Sandberg, 2001; Park et al., 2001; Rampey et al., 2004;) while it is suggested that IAAs linked to uncharacterized larger peptides are also present in larger quantities (~78% of total IAAs; Park et al., 2001; Ljung et al., 2002; Seidel et al., 2006). Seven of the 19 auxin-inducible GH3 proteins in *Arabidopsis* catalyze the synthesis of IAA-amide conjugates (Hagen & Guilfoyle, 1985; Staswick et al., 2005). IAA conjugation to Asp and Glu residues is increased upon exogenous auxin treatment (Östin et al., 1998). *GH3.6* overexpression results in seedlings with symptoms of increased auxin like the *yuc1D* gain-of-function mutant (Nakazawa et al., 2001; Zhao et al., 2001) and resistance of root growth inhibition to exogenous auxin application (Staswick et al., 2005). *gh3.6* loss-of-function mutants show increased sensitivity to IAA (Staswick et al., 2005). These findings support the proposed role of auxin conjugate synthesis in the control of free IAA levels.

IAA-sugar conjugates typically contain ester linkages to, for example, glucose or *myo*-inositol (Cohen & Bandurski, 1982; Normanly, 1997; Normanly & Bartel, 1999). Sugar-linked conjugates make up ~4% of total IAA content in *Arabidopsis* seeds (Park et al., 2001). A large multigene family involved in the synthesis of IAA-glucose are the UDP-glucosyltransferases (UGTs; Jackson et al., 2001). It is thought that IAA-glucose may be used to synthesize IAA-*myo*-inositol (Kowalczyk et al., 2003), potentially as part of another mechanism to control free IAA levels. Two other types of IAA-conjugates are IBA (a less active auxin mentioned previously) and methylated IAA (MeIAA). These are synthesized from IAA in reversible oxidation or carboxyl methyl-transfer reactions, respectively (reviewed in Chandler, 2009; Ludwig-Müller, 2011). IBA can undergo additional reversible reactions to form IBA amide or sugar conjugates (reviewed in Ludwig-Müller, 2011).

The preservation of auxin could be of particular biological importance in germinating seeds and developing young seedlings, including the growth of cotyledons, which require rapidly utilizable auxin reserves for growth (Ljung et al., 2002; Woodward

& Bartel, 2005). Active IAA for signalling in young seedlings can by formed by the hydrolysis of IAA conjugates, supported by the observations of amide conjugates serving as slow-release auxin sources in plant tissue culture (Magnus et al., 1992). *Arabidopsis* ILR1 facilitates the hydrolysis of IAA-Leu and IAA-Phe, a gene identified by a screen for mutants resistant to IAA-Leu-supplemented growth media (Bartel & Fink 1995). Similarly, IAR3 facilitates the hydrolysis of IAA-Ala (Davies et al., 1999). IAA-glucose is hydrolyzed by the IAA-glucose hydrolase (reviewed in Woodward & Bartel, 2005).

The ability to control IAA degradation is also important for plants as a means to control auxin levels, since free IAA is a signalling molecule found at low concentrations. The major pathway by which IAA degradation occurs in *Arabidopsis* is by the non-decarboxylative oxidation of the indole ring of IAA (Östin et al., 1998). Metabolic profiling using mass spectrophotometry techniques on plant extracts that were fed radiolabelled IAA suggests that free, unconjugated IAA can undergo oxidative steps. Free IAA is first inactivated by oxidation to the intermediate OxIAA, followed by conjugation to hexose (Östin et al., 1998). The amino acid conjugates IAAsp and IAGlu, possibly synthesized via GH3-like enzymes, may also undergo irreversible oxidation in IAA catabolism (Östin et al., 1998).

1.1.4. Auxin transport

The importance of polar auxin transport (PAT) in plant development, which I will discuss later in this section, has been acknowledged for nearly a century (reviewed in Morris et al., 2010). In general, auxin is transported by two different mechanisms. In the first, auxin in mature plants can be transported passively over long distances through phloem tissues. This mass translocation occurs in a source-to-sink direction, typically from the plant shoot where auxin has accumulated and towards the root, but not in a strictly polar manner. It is unlikely that auxin is exported by other tissues as only small amounts of IAA have been detected in the xylem, epidermis, cortex, and pith of mature stems in various species (reviewed in Goldsmith, 1977). Over both long and shorter distances, the second mechanism of active transport occurs through other cell types using appropriate cellular machinery, albeit at a slower rate than bulk phloem transport (Goldsmith, 1977). This system consists of intercellular PAT from the young shoot tissues and leaves towards the root.

The following chemiosmotic model of polar diffusion suggests that PAT is a thermodynamically favourable phenomenon but it requires a secondary energy expenditure to maintain transmembrane pH gradients among individual cells (Rubery & Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). Passive diffusion of undissociated, lipophilic IAA molecules into a plant cell likely occurs with ease. Compared to the slightly acidic extracellular zone (pH 5.5), the neutral pH of the cytosol (pH 7) favours the dissociation of most cytosolic IAA into hydrophilic IAA^- molecules and H^+ that become "trapped" in the cell. IAA dissociation lowers cytosolic intact IAA content, keeping a concentration gradient which favours the inward diffusion of IAA so long as the pH gradient persists. This is supported by early findings of increased IAA uptake by seedlings in media of increased acidity and pH-dependent cellular permeability studies (Raven, 1975; Goldsmith, 1977). A number of H^+-ATPase and H^+-PPase protein pumps have since been identified in *Arabidopsis* (Sze et al., 1999; Palmgren, 2001) with one so far that has been attributed to regulating apoplast (plant extracellular space) acidity and PAT (Li et al., 2005). AVP1, a H+-PPase traditionally viewed as a vacuolar pump (Sarafian et al., 1992), likely contributes to extruding cytosolic H^+ and mediating the activities of H^+-ATPases at the plasma membrane (Li et al., 2005).

The polar secretion model suggests that an asymmetric distribution of energy-dependent auxin carriers in the cell contributes to PAT (Rubery & Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). In essence, the distinct subcellular localizations of auxin influx and efflux carrier proteins—involved in the respective uptake into and expulsion of auxin from the cell—are hypothesized to enhance intercellular IAA movement. This strategy is likely most influential for transport of low levels of auxin as a signalling molecule in smaller, developing seedlings, particularly when hydrophilic IAA^- anions need to be actively exported from the cytosol. The importance of efflux and influx carriers to plant development is observed in wildtype *Arabidopsis* subjected to chemical inhibition of PAT by compounds such as by N-(1-naphthyl)phthalamic acid (NPA), 2,3,5-triiodobenzoic acid (TIBA), or 9-hydroxyfluorene-9-carboxylic acid (HFCA). Seeds germinated on media containing each of these inhibitors experienced reduced growth, elongated inflorescences without flowers, reduced root systems, and small, abnormally oriented leaves (Okada et al., 1991; Mattsson et al., 1999). Sections of these inflorescence stems that are adjacent to leaves contain massive radial xylem

proliferations of vascular tissues within the xylem bundles (Gälweiler et al., 1998). Close inspections of inhibitor-grown rosette leaves revealed several alterations in leaf vascular organization, increasing in severity with the concentration of chemical inhibitor applied (Mattsson et al., 1999; Sieburth, 1999). Most veins were broader than those of control plants, containing more cells that were often improperly aligned. Midveins were often bifurcated/skewed (split into two strands approaching the apical end) or replaced with several parallel strands. Distinct secondary and tertiary veins were increased in number and connected to a broad band of proliferations of TEs at the apical margin. As well, the vascular tissues connecting the leaf vein system to the hypocotyls were sometimes absent. Meanwhile, the cotyledons of inhibitor-treated wildtype *Arabidopsis* contained normal venation patterning but with thicker veins (Mattsson et al., 1999; Sieburth, 1999). This is congruent with cotyledon vein patterning occurring mostly during embryogenesis.

The same developmental defects of *Arabidopsis* with chemically inhibited PAT have been observed in single *pin-formed* (*pin1*) mutants (Okada et al., 1991; Mattsson et al., 1999), which are named for the knitting needle-shaped inflorescences that lack flowers (Haughn and Somerville, 1988). In addition, *pin1* mutants display aberrant cotyledon numbers and fused cotyledons, with proliferations of vascular elements at the cotyledon margins and radially distributed veins across cotyledon lamina (Aida et al., 2002; Furutani et al., 2004) suggesting PAT is disrupted even during embryogenesis. The leaf and cotyledon vascular patterning defects of both chemically inhibited wildtype and control-treated *pin1* mutants are hypothesized to result from trapped auxin unable to drain from the leaf margins, as DR5 expression is widespread at margins of NPA-treated primordia (Mattsson et al., 1999, 2003). Molecular analyses suggest that *PIN1* encodes a transmembrane protein involved in auxin efflux (Gälweiler et al., 1998). Its expression is localized at the basal ends of parenchymatous xylem cells in the *Arabidopsis* influorescence stem (Gälweiler et al., 1998). Interestingly, *PIN1* is also expressed early in leaf development along discrete, narrow cell files that correlate with the locations of incipient leaf veins (Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). Altogether, this suggests that auxin efflux, influenced by PIN1, is an important component of PAT in proper leaf vascular patterning, which I discuss in section 1.2.1.

A few regulators of PIN1 have been characterized so far. PINOID (PID) positively regulates PAT by regulating the subcellular localization of PIN1 (Friml et al.,

2004; Lee and Cho, 2006). The defects in leaf formation of *pid* mutants are similar to those of *pin1* mutants, highlighting the importance of PID in proper organ development (Bennett et al., 1995; Benjamins et al., 2001). As a Ser-Thr kinase, PID likely promotes apical polar PIN targeting through phosphorylation of a conserved PIN site rich in serine residues (Friml et al., 2004; Huang et al., 2010; Zhang et al., 2010). This reversible mechanism may be mediated by the antagonistic action of protein phosphatase 2A (PP2A), a protein whose function is required for normal *Arabidopsis* root and cotyledon establishment and normal PIN basal localization (Michniewicz et al., 2007; Klein-Vehn et al., 2009). PP2A localizes to the cytosol and ER of several plant cell types as well as at the basal faces of cells, which in part coincides with PINs (Michniewicz et al., 2007). An endosomal regulator of vesicle budding, GNOM (GN), is also required for embryogenesis and organogenesis, on account of the collapsed root apical meristem and leaf vascular defects of *gn* mutants (Mayer et al., 1993; Shevell et al., 1994; Geldner et al., 2004). GN is a guanine nucleotide exchange factor for adenosyl ribosylation factors (ARF-GEF) that is localized at the endosomes and at least partially mediates PIN recycling to the basal face of the cell (Geldner et al., 2003; Kleine-Vehn et al., 2008).

Evidence suggests that PID, PP2A, and GN are involved in coordinated mechanisms to establish the polar subcellular localization of PIN proteins (Klein-Vehn et al., 2009). Colocalization of PIN2 and PID primarily at the apical cell faces of root epidermal cells suggests that PID regulates PIN residence at this site by phosphorylation (Michniewicz et al., 2007; Klein-Vehn et al., 2009). The overexpression of PID leads to a collapsed root phenotype, limited root auxin distribution, and PIN polarity defects that are similar to those of a mutant of reduced GN function, suggesting that PID and GN play antagonistic roles in PIN localization and activity (Benjamins et al., 2001; Geldner et al., 2004; Klein-Vehn et al., 2009). Analyses of subcellular locations of PID, PP2A, and GN under normal conditions and conditions where vesicle trafficking is inhibited by brefeldin A (BFA) suggest that GN facilitates basal PIN recycling independently of PID activity, while PID and PP2A contribute to apical PIN targeting independently of GN (Klein-Vehn et al., 2009). While these two teams of proteins probably do not immediately interact with one another, it is hypothesized that the sorting of PIN proteins to either the apical or basal cell face is dependent on PID phosphorylation (Klein-Vehn et al., 2009).

An *Arabidopsis* mutant first attributed to a reduced sensitivity to IAA or 2,4-D application and altered root gravitropic response was named *auxin resistant1* (*aux1*; Maher & Martindale, 1980; Pickett et al., 1990). Further physiological analyses of the *aux1* mutant revealed that the phenotype shows a delayed response to IAA (Evans et al., 1994) and *aux1* is not resistant to the application of NAA (Yamamoto & Yamamoto, 1998), an auxin more permeable to the plasma membrane than IAA and 2,4-D (McCready, 1963; McCready & Jacobs, 1963). Rather, the *aux1* root apical zone is deficient in auxin uptake (Marchant et al., 1999) suggesting AUX1 operates in the auxin transport required for normal directional root growth instead of auxin signalling. Characterization of the *AUX1* gene suggests that it encodes a plasma membrane-spanning, auxin influx carrier protein (Bennett et al., 1996; Yamamoto & Yamamoto, 1998; Marchant et al., 1999). The expression of *AUX1* has been localized to shoot epidermal cells, root epidermal cells and the root cap (Reinhardt et al., 2003; Swarup et al., 2001, 2005) while its three *LIKE AUX* (*LAX*) related sequences have been localized to the mature stele (vascular cylinder) of *Arabidopsis* roots (Parry et al., 2001; Swarup et al., 2008).

AUX1/LAX members have more recently been suggested to be involved in leaf development. *AUX1* is expressed in the surface layer of the SAM (L_1 layer) and subcellularly localized to lateral cell faces as well as the faces where PIN1 is expressed (see section 1.2.1), suggesting AUX1 may contribute to auxin accumulation in leaf growth (Reinhardt et al, 2003) by potentially stabilizing auxin maxima generated by PIN1 (Heisler & Jönsson, 2006). The *aux1* single mutant shows no leaf phenotype (Reinhardt et al., 2003), but the *aux1 lax1 lax2 lax2* quadruple mutant shows elongated, downward-twisted rosette leaves initiated at abnormal angles, suggesting functional redundancy of *AUX1/LAX* genes (Bainbridge et al., 2008). The *pin1 aux1* mutant forms oversized primordia upon exogenous auxin application, suggesting that the organ-limiting function for AUX1 in development is masked by rate-limiting PIN1 action in leaf development (Reinhardt et al., 2003). Potentially, AUX1/LAX function may influence the focus of auxin flow, as PIN1 expression and subcellular localization is slightly disorganized in *aux1 lax1 lax2 lax2* quadruple mutants (Bainbridge et al., 2008).

Other proteins involved in the intercellular transport of auxin are P-glycoproteins of the ABCB transporter family (ABCB/PGP). This subfamily contains *Arabidopsis*

members ABCB1 and ABCB19 that are involved in auxin efflux (Yang & Murphy, 2009). ABCB1 and ABCB19 likely function in a coordinated fashion with PIN1 by participating independently in the transport of auxin while also enhancing PIN1 stability, as these ABCBs and PIN1 partially overlap in subcellular localization and expression during embryogenesis (Bandyopadhyay et al., 2006; Petrášek et al. 2006; Blakeslee et al., 2007; Mravec et al., 2008; Titapiwatanuakun et al., 2009). As well, ABCB1, ABCB19, and PIN1 act synergistically based on *pin1* phenotype enhancement in the *abcb1 abcb19* background (Blakeslee et al., 2007; Mravec et al., 2008). Double *abcb1 abcb19* mutants alone show more exaggerated dwarfism and curled leaf phenotypes and decreased basipetal auxin transport than corresponding single mutants, suggesting overlapping functions for both members in PAT (Noh et al., 2001; Geisler et al., 2005). It is suggested therefore that ABCB1 and ABCB19, and possibly other ABCBs, play roles in PAT-mediated organogenesis and embryogenesis (Blakeslee et al., 2007; Mravec et al., 2008), but the local roles of ABCB proteins specific to leaf vein patterning are not yet known.

1.1.5. Auxin signalling

The responses of plant cells to phytohormones presumably require the interpretation of signals and the appropriate cell reactions, such as the transcription of genes. These actions, while considering the differential distributions of auxin across plant tissues, can result in distinct zones of auxin-triggered growth and development (reviewed in Tanaka et al., 2006). Auxin signalling involves three protein families that have so far been characterized.

The auxin response factors, or ARFs, are a 23-member family of transcription factors (TFs) in auxin perception (Guilfoyle & Hagen, 2007). Recent evidence supports the hypothesis that different ARFs contribute partially redundantly but uniquely to *Arabidopsis* auxin responses in development (Rademacher et al., 2011). ARFs most likely operate in auxin response by binding to TGTCTC auxin response elements in the promoters of auxin response genes to activate their expression (Ulmasov et al., 1997; Guilfoyle & Hagen, 2001). Meanwhile the post-translational activities of ARFs are repressed by interactions with the AUXIN (Aux)/IAA proteins—short-lived negative regulators of auxin signalling—to form heterodimers (see below; Kim et al., 1997; Tiwari

et al., 2003; Weijers et al., 2005a). One ARF I have already mentioned, MONOPTEROS (MP)/ARF5 is required for leaf vein patterning and apical-basal patterning of *Arabidopsis* embryos (Berleth & Jürgens, 1993; Hardtke & Berleth, 1998; Przemeck et al., 1997). *MP* is expressed in the leaf vascular tissues at all stages of patterning, from before procambial sites are established to vascular maturation (Wenzel et al., 2007), and strong *mp* alleles have extremely reduced vascular systems and missing hypocotyls and roots (Berleth and Juergens, 1993; Przemeck et al., 1996). The regulation of transcription of *PIN1* may be regulated by MP (Mattsson et al., 2003; Wenzel et al., 2007) and also other TFs in organ initiation, such as the APETALA2 putative TF DORNRÖSCHEN (DRN) and the mobile TF TARGET OF MONOPTEROS7 (TMO7) (Cole et al., 2009; Schlereth et al., 2010).

The 29 *Aux/IAA* gene members as I have mentioned are repressors of auxin response gene transcription. In addition to heterodimerizing with ARFs to prevent gene expression, Aux/IAA proteins may recruit co-repressors to establish multi-protein repressor complexes (Szemenyei et al., 2008; Santner & Estelle, 2009). High levels of auxin promote the protease-mediated degradation of Aux/IAAs, resulting in a release of ARF from inhibition and the transcription of auxin response genes (reviewed in Dreher et al., 2006). Therefore it is suggested that Aux/IAA-mediated repression occurs under low auxin conditions (Ulmasov et al., 1997; Tiwari et al., 2003). One characterized Aux/IAA of interest is BODENLOS (BDL)/IAA12. The strong *mp* phenotype has been observed in gain-of-function *bdl* mutants, attributed to the expression of stabilized versions of BDL proteins (Hamann et al., 1999, 2002). Coexpression of the two genes, yeast two-hybrid interaction assays, and genetic analyses suggest that BDL interacts with MP to interfere with its transcriptional activation of auxin response genes (Hamann et al., 2002; Weijers et al., 2005b).

The mechanism by which Aux/IAA proteins are degraded at high concentrations of auxin is likely influenced by Skp1/Cullin/F-box-TIR1 E3 ubiquitin protein ligases (SCF^{TIR}; Gray et al., 2001) This turnover depends on direct interactions of the F-box proteins with Aux/IAAs, presumably to ubiquitinate them (Gray et al, 2001; Dharmasiri et al., 2003; Kepinski & Leyser, 2004). One characterized F-box protein family is AUXIN TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB), consisting of five members (Kepinski & Leyser, 2004; Dharmasiri et al.,

2005a, 2005b). The binding of active auxins to TIR1 increases TIR1-Aux/IAA interaction stability, which is a likely mechanism by which auxin promotes Aux/IAA degradation (Kepinski & Leyser, 2004; Dharmasiri et al., 2005a; Tan et al., 2007). As such, TIR1 and its related proteins are considered to be auxin receptors, possibly with partially overlapping but distinct biological roles (Parry et al., 2009). For example, some TIR1/AFB members are expressed in leaves and other organs and are suggested to play roles in leaf morphogenesis (Dharmasiri et al., 2005b). Triple and quadruple *tir1/afb* mutant seedlings range in phenotype from roots displaying altered gravitropic response to *mp*-like phenotypes, while those that survive this stage have rosettes with small, curled leaves; single and double *tir1/afb* mutants appeared more like the wildtype (Dharmasiri et al., 2005b). One notable Cullin protein, AUXIN-RESISTANT6 (AXR6), has been characterized as a necessary component in leaf vascular development and embryogenesis (Hobbie et al., 2000; Hellmann et al., 2003). Some *axr6* alleles are associated with *mp*-like phenotypes (Hobbie et al., 2000), highlighting the importance of multiple SCF^{TIR} components in auxin response and plant development.

1.2. A working model for leaf vein development

1.2.1. The canalization of auxin flow in early vein patterning

The formation of vascular strands in leaf primordia is hypothesized to be triggered by the unique action of directional auxin transport and shifts in the locations and relative strengths of local auxin maxima. In particular, the auxin-flow canalization hypothesis provides an underlying principle in these processes (Sachs, 1981). A positive feedback mechanism is proposed by this hypothesis where auxin produced in young leaf primordia initially diffuses towards the roots. This induces an increase in PAT through only certain queues of cells while draining auxin from adjacent cells. This concentration of auxin flow may ultimately trigger the differentiation of procambial and mature vascular cells.

Several lines of evidence support the auxin canalization model of leaf vein patterning in *Arabidopsis*. Active auxin is the only known molecule that alone can trigger vascular strand formation and vascular cell differentiation (Sachs 1981; Berleth et al.,

2000); it is a simple signal that activates a developmental response. Molecular studies strongly suggest that auxin biosynthesis occurs primarily at developing apical organs and auxin transport is aimed towards the roots (reviewed in Aloni et al., 2003). As I mentioned previously, chemical and genetic (i.e. via *pin1* mutation) inhibition of auxin transport can lead to compromised leaf organogenesis and proliferations of vascular tissues at cotyledon and leaf margins, as opposed to the more evenly distributed veins found in normal leaves (Okada et al., 1991; Galweiler et al., 1998; Mattsson et al., 1999; Sieburth, 1999; Aida et al., 2002; Furutani et al., 2004;). Considering also the subcellular localizations of PIN1 proteins along cell lines that will differentiate into procambium in either normal or NPA-treated seedlings (Scarpella et al., 2006; Wenzel et al., 2007), and that exogenous application can evoke an increase in PIN1 expression and patterning of extra veins (Scarpella et al., 2006), PIN1 proteins are may be highly influential in the gradual concentration of directional leaf auxin transport and vascular cell selection.

There are several lines of evidence that local accumulation of auxin triggers leaf formation. First, chemical inhibition of PAT inhibits the initiation of new leaf primordia in tomato plants, while local IAA application to the shoot apices of these treated plants can induce leaf formation only at the flanks of the SAM (Reinhardt et al., 2000). Second, IAA application to developing primordia of normal tomato plants can enlarge basal primordia regions and IAA application to regions where primordia ordinarily would not form is associated with ectopic and sometimes fused primordia formation, except for in close proximity to pre-existing primordia (Reinhardt et al., 2000). The positioning of tomato leaves is also affected by NPA, as primordia initiation does not occur on shoot faces where NPA is applied (Reinhardt et al., 2000). Third, local application of IAA to *pin1* mutant *Arabidopsis* stems correlates with the development of normal rosette leaves and inflorescence primordia depending on plant age (Reinhardt et al., 2000, 2003), as opposed to *pin1* mutants normally having deformed leaves and missing inflorescences (Okada et al., 1991). In *pin1* mutants crossed with *leafy* (*lfy*), corresponding to a floral marker gene in which mutations are associated with shoots in place of inflorescences and flowers consisting of leaf-like sepal organs (Weigel et al., 1992), IAA application can trigger true cauline (above the rosette) leaf formation (Reinhardt et al., 2003). Fourth, *PIN1* is expressed in the epidermis near the SAM and asymmetrically localized at certain

faces of cells in such a way that suggests auxin flows to and accumulates at the centres of young primordia (Reinhardt et al., 2003). In inflorescence apices, which show similar *PIN1* expression patterns and phyllotactic patterning as vegetative meristems, *PIN1* precedes the expression of *LFY* (Reinhardt et al., 2003; Heisler et al., 2005). Fifth, spatio-temporal analyses of dynamic *PIN1* and DR5 expression suggest that as primordia grow, PIN1 orientations shift polarity to alter free auxin distribution, perhaps to provide positional position information to future organ boundaries and faces (Heisler et al., 2005). Sixth, the expected *PIN1* expression pattern during leaf initiation is disrupted in *pin1*, *pid*, and *mp* backgrounds (Reinhardt et al., 2003), in congruence with the organ outgrowth defects seen in all three mutants and the roles of all three genes in influencing auxin levels (Okada et al., 1991; Bennett et al., 1995; Przemeck et al., 1996).

The current molecular model for auxin-induced leaf vein patterning is as follows (Figure 3). After a leaf or cotyledonary primordium is initiated by auxin, it is hypothesized that midvein procambium cells act early as an auxin sink, depleting auxin from surrounding tissues by way of epidermal PIN1 proteins oriented towards the primordium centre, where there may be an auxin maximum (Figure 3a; Friml et al., 2003; Reinhardt et al., 2003; Scarpella et al., 2006; Weijers et al., 2005a; Lewis et al., 2009). The perception of auxin throughout these processes may require MP to activate local *PIN1* expression in the presence of auxin (Hardtke & Berleth, 1998; Mattsson et al., 2003; Reinhardt et al., 2003; Wenzel et al., 2007) as well as PID, PP2A, and GN for PIN1 subcellular localization (Klein-Vehn et al., 2009). Free auxin may be distributed at the leaf margins and incipient vein sites, as suggested by DR5 localization (Figure 3; Mattsson et al., 2003). As the primordium expands, the apical tip of the leaf or cotyledonary primordium shows high DR5 activity (Mattsson et al., 2003; Lewis et al., 2009). This is likely not an indication of a spatial limitation in primordium auxin response to this region because 2,4-D treated *Arabidopsis* primordia show nearly homogenous DR5 expression (Mattsson et al., 2003). Rather, limited but strong DR5 expression in normal primordia may be due to accumulated free auxin and/or domain-specific auxin biosynthesis, but whichever occurs first is not currently known. Either way, DR5 at the apical tip correlates with widely expressed epidermal PIN1 proteins changing their localization to transport auxin towards the SAM after meeting at a convergence point (Figure 3a; Heisler et al., 2005; Scarpella et al., 2006; Wenzel et al., 2007). This apical

epidermal convergence point is associated with subepidermal expression of PIN1 that extends towards the leaf base (basally) along preprocambium, the cells that will become midvein procambium (Figure 3a; Friml et al., 2003; Lewis et al., 2009; Scarpella et al., 2006; Wenzel et al., 2007).

It is hypothesized that at some point in the more developed primordium, future sites of water-secreting glands at the leaf margins called hydathodes (Figure 1) somehow become the next primary sites of free auxin accumulation by polar auxin transport, followed by auxin biosynthesis to become new auxin sources before hydathode differentiation (Figure 3b; Aloni, 2001; Aloni et al., 2003; Cheng et al., 2006). This correlates with the appearance of PIN1-associated convergence points at the leaf margins and subepidermal PIN1 expression that concentrates into the cell files that become the preprocambium of lower-loop domains (LLDs) of the first pair (L1) of secondary veins (Figure 3b; Scarpella et al., 2006; Wenzel et al., 2007). The upper-loop domains (ULDs) of the L1s are initiated as extensions and narrowing of future-LLD PIN1 expression towards the apical portion of the midvein, which at this point in time may still be an auxin convergence point (Figure 3b; Sawchuk et al., 2007; Scarpella et al., 2006; Wenzel et al., 2007). The midvein and secondary vein procambium then displays DR5 activity suggesting auxin acts during vein differentiation (Figure 3b; Mattsson et al., 2003). The formation of the next secondary vein pairs that are more basal on the leaf (L2, L3, L4, etc) may depend on localized auxin biosynthesis at the margins, but not on auxin maxima at the apical primordium tip (Figure 3c, d; Cheng et al., 2006; Sawchuk et al., 2007). Higher order (tertiary and quaternary) veins in leaves formed post-embryonically appear less predictably and without epidermal PIN1 expression, but still in association with subepidermal PIN1 expression that is continuous with pre-existing domains of PIN1 expression or mature vascular cells marked by DR5 expression (Mattsson et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007).

Major questions for this model to which I have already alluded include whether or not auxin biosynthesis maxima play important roles in vein patterning. The synergy of YUCs with PIN1 and AUX1 suggest that both local auxin biosynthesis and auxin transport are required for normal leaf development (Cheng et al., 2007). As I have mentioned, *pin1* and *yuc1 yuc4* mutants both have defects in vascular tissue patterning (Gälweiler et al., 1998; Cheng et al., 2006), but *yuc1 yuc4 pin1* triple mutants fail to form

leaves (Cheng et al., 2007). This severe defect is phenocopied in *yuc1 yuc2 yuc4 yuc6 aux1* quintuple mutants suggesting that auxin influx is also important to leaf development (Cheng et al., 2007). However, how exactly these three gene families contribute to vein patterning is not known, i.e. if the mutant defects observed are due to altered genetic epistasis, decreased auxin maxima, disrupted PAT, or some combination of factors (Cheng et al., 2007). As well, the ways by which these potential mechanisms are regulated must also be explored.

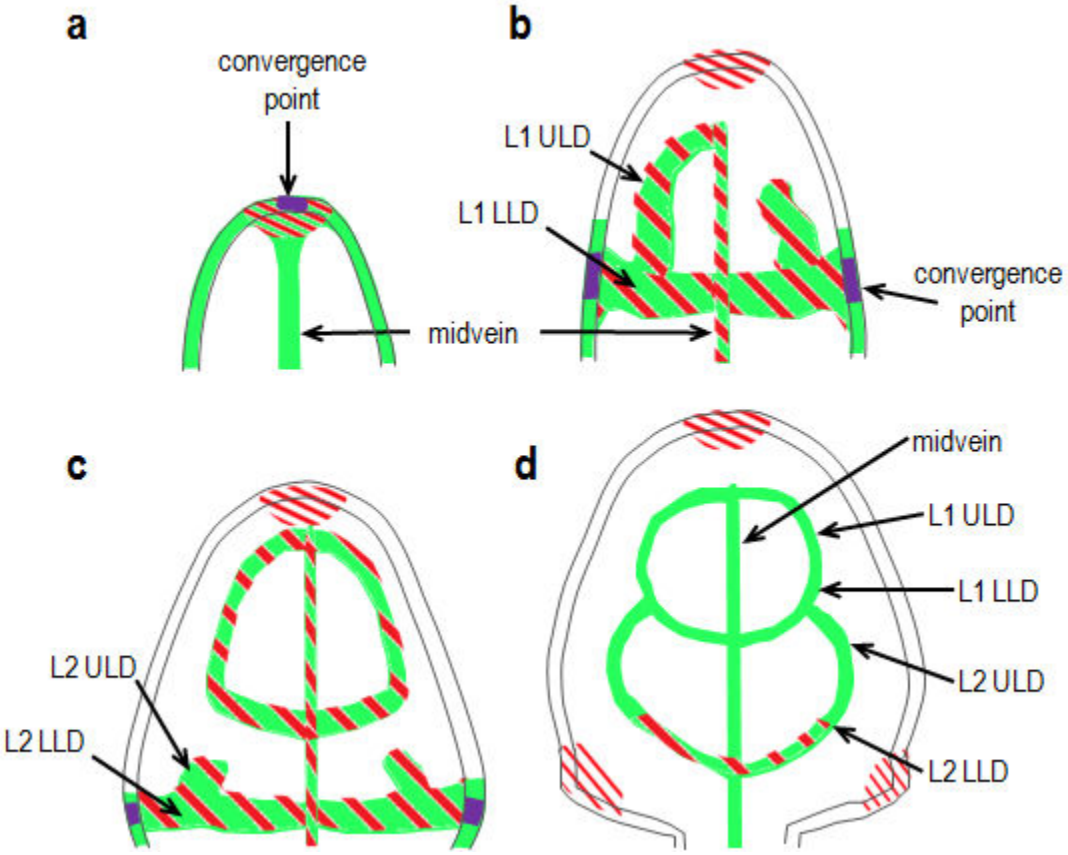


Figure 3. PIN1 expression in a module for major vein formation

Evidence suggests that PIN1 expression (green) is involved in a regulatory loop with auxin-induced leaf vein patterning. Red hashed areas mark the distribution of auxin at the margins and developing vascular tissues. Violet areas in the leaf margins indicate epidermal zones of auxin convergence by PIN1 transport. These areas may coincide with auxin biosynthesis maxima. a: The first convergence point occurs at the apical leaf tip. Auxin internalization and flow from this point initiates midvein formation. Auxin continues to flow towards the leaf base throughout vein patterning. Note that PIN1 is also expressed throughout the primordium epidermis at this stage (not shown). b: The apical convergence point diminishes and lateral convergence points emerge at the leaf margins. These points are correlated with the internalization of auxin fluxes and the concentration of PIN1 expression into linear domains that mark the lower-loop domains (LLDs) of the L1 2° veins. Associated with this is the appearance of the L1 upper-loop domains (ULDs), c: The first lateral convergence points diminish and newer ones emerge at more basal positions at the leaf margins. In a similar manner as the L1 veins, the L2 vein loops start to pattern. Also, PIN1 expression at the incipient L1 ULD and LLD narrow into finer domains. d: The final stages of leaf vein patterning include the expression of PIN1 in narrow cell files corresponding to all veins, to presumably continue to facilitate polar auxin transport. Only PIN1 expression corresponding to major vein orders (midvein and 2°) are shown. Adapted from Mattsson et al., 2003 (p. 1330, Figure 2), Scarpella et al., 2006 (p. 1024, Figure 7), and Wenzel et al., 2007 (p. 394, Figure 9).

1.2.2. Factors in vascular bundle formation

High free auxin levels in narrow files of leaf ground meristem cells likely activate the expression of genes required for procambial fate determination. High levels of DR5 expression in isodiametric preprocambial cells precede establishment of preprocambial cells (Mattsson et al., 2003). Since auxin is a known inducer of vascular strand formation, it is possible that the preprocambial accumulation of auxin is the event that triggers procambial fate specification and differentiation, indicating that auxin canalization may result in tube-shaped procambial cells (Figure 3). Evidence from Mattsson et al. (1999, 2003, unpublished) suggests that procambial fate begins with cell division through planes along the axis of the incipient procambial strands. Then cell elongation in an ordered, coordinated fashion occurs along each entire preprocambial bundle simultaneously (Donnelly et al., 1999; Kang & Dengler, 2002; Sawchuk et al., 2007). The procambial midvein forms first, dividing as mentioned then elongating from the primordium base, then extending towards the apical end (Donelly et al., 1999; Mattsson et al., 1999, 2003, unpublished). Secondary and high order procambial cells divide and elongate starting from their apical ends and ending at their basal ends (Nelson & Dengler, 1997; Donnelly et al., 1999), with the exception of the L1 secondary vein procambium which differentiates simultaneously (Sawchuk et al., 2007). A few of what may be a large number of genetic factors have been characterized in their contributions to leaf vein differentiation, including functions in preprocambial state acquisition and procambial differentiation.

Leaves have adaxial-abaxial polarity based on which face of the planar leaf blade is closer to the SAM. At leaf maturity, typically the more apical leaf surface is the adaxial side while the underside of the leaf is considered abaxial. The class III homeodomain-leucine-zipper-containing family of TFs (HD-ZIP III) have roles in establishing adaxial face identity in an antagonistic relationship with the KANADI (KAN) transcriptional regulators, which contribute to abaxial identity procurement. In particular, the normal radial orientation of xylem and phloem within each mature vascular bundle requires the normal balance of HD-ZIP III and KAN activity; *hd-zip iii* mutants typically have adaxialized lateral organs with xylem surrounding phloem in the vascular bundles, while *kan* mutants have phloem-encircled xylem within the veins of abaxialized leaves (reviewed in Carlsbecker & Helariutta, 2005). Thus, the HD-ZIP III members

PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) may promote xylem identity of cells whereas KAN1-3 may promote phloem identity (reviewed in Caño-Delgado et al., 2010). Though the exact mechanisms are not yet known, *HD-ZIP III* and *KAN* expression coordinates with the activity of a microRNA in vascular development, miRNA165/166. A predicted sterol/lipid-binding START domain in *HD-ZIP III* mRNAs is targeted by miRNA165/166 for degradation (Kim et al., 2005; Zhong & Ye, 2007) while KAN members might somehow promote the accumulation of miRNA165/166 to negatively regulate *HD-ZIP III* expression (Bowman, 2004; Engstrom et al., 2004).

The expression of one *HD-ZIP III* gene *ATHB8* marks preprocambial cells in leaf primordia that extend from pre-established vascular cells (Kang & Dengler, 2004; Scarpella et al., 2004). *ATHB8* expression is spatially very similar to that of PIN1 along narrow cell files, but PIN1 expression temporally precedes that of ATHB8 suggesting that auxin initiates the formation of leaf veins (Baima et al., 1995; 2001; Sawchuk et al., 2007). In support of this, MP directly controls the expression of *ATHB8* in the leaf through an auxin response element in the *ATHB8* promoter (Donner et al., 2009; Ilegems et al., 2010). Biologically, ATHB8 acts as a stabilizer of preprocambial identity against disturbances in auxin transport, allowing only for the differentiation of narrow lines of procambial cells (Donner et al., 2009). This is a transient role for ATHB8 proteins and, accordingly, the *athb8* mutant phenotype is fairly weak, having near-normal leaf venation patterning (Donner et al., 2009).

The *vascular network3/scarface* (*van3/sfc*) mutant of *Arabidopsis* was isolated in two independent mutant screens for defects in continuous vascular systems (Koizumi et al., 2000; Deyholos et al., 2000). The *van3/sfc* phenotype consists of a short root and networks of leaf vein fragments that appear to have formed along the expected domains of normal vein patterning (Koizumi et al., 2000; Deyholos et al., 2000). It was initially postulated that this phenotype cannot be accounted for if the canalization of auxin is responsible for normal leaf vein patterning (Fukuda, 2004). The reaction-diffusion prepattern hypothesis was favoured instead, where differential diffusion rates of activating and inhibiting substances (e.g. auxin) influence the autocatalytic genes required for predetermined pattern formation of the leaf (Meinhardt, 1996; Nelson & Dengler, 1997). VAN3, therefore, was hypothesized to be one of the autocatalytic genes in this model, as disconnects in veins could have been a result of gaps in pattern

formation (Fukuda, 2004). However, later experiments with *VAN3/SFC* contributed to a different interpretation where defective canalization was hypothesized to be behind the *van3/sfc* phenotype (Scarpella et al., 2006). *VAN3/SFC* encodes an ADP ribosylation factor GTPase activating protein (ARF-GAP; Koizumi et al., 2005; Sieburth et al., 2006), a factor needed for vesicle protein uncoating before target membrane vesicle fusion (Chavrier & Goud, 1999). As well, the expression of *PIN1* along future leaf preprocambium in *van3/sfc* was found to be initially the same as wildtype (Scarpella et al., 2006). This, in addition to *VAN3/SFC* expression being localized to vascular cells, suggests a special molecular function exists for VAN3/SFC proteins in influencing auxin transport in vascular development (Scarpella et al., 2006; Naramoto et al., 2009). Taken together, the accepted hypothesis for mechanisms behind leaf vascular patterning is still the auxin canalization hypothesis (Sachs, 1981) as VAN3/SFC is required to uphold the continuity of PIN1 expression in discrete cell files during leaf primordium procambial differentiation (Naramoto et al., 2009; Scarpella et al., 2006). Subepidermal ground meristem cells tagged by canalized auxin flow in preprocambial state acquisition undergo cell divisions and elongation to produce narrow procambial cells, therefore PIN1 proteins may need to change their subcellular localizations to ensure that auxin is transported in the same overall direction as before divisions occurred (Geldner et al., 2001; Scarpella & Helariutta, 2010). VAN3/SFC may have a temporally specific role in regulating the proper transport and subcellular localization of PIN1 proteins during preprocambial selection and procambial differentiation.

Two genes with unknown molecular function but known roles in vascular differentiation are also worth mentioning here. Normal *BYPASS1* (*BPS1*) expression is required to prevent the constitutive production of some mobile, root-derived signal that inhibits proper root and shoot development, including proper leaf vein patterning (Van Norman et al., 2004). The signal, which is likely a carotenoid-derived compound, may interfere with auxin signalling (Van Norman et al., 2004; Van Norman & Sieburth, 2007). But recently, BPS1 and two related genes, BPS2 and BPS3, have been suggested to be involved in *Arabidopsis* embryogenesis in a functionally redundant manner due to increasingly diminished shoots and roots in multiple mutants (Lee et al., 2012). Interestingly, *bps* mutants show near-normal expression of *PIN1* and auxin response, suggesting that BPS members operate in a developmental mechanism independent of

auxin (Lee et al., 2012). Another gene called *NO VEIN* (*NOV*) encodes a nuclear protein in which mutations result in poor leaf and cotyledon outgrowth and leaf laminae almost completely lacking veins (Tsugeki et al., 2009). This correlates with a failure to maintain *MP* and therefore *PIN1* expression in the *nov* background, suggesting NOV somehow mediates auxin-dependent vein patterning (Tsugeki et al., 2009, 2010).

1.2.3. Regulators of vascular cell differentiation

Xylem and phloem maturation must occur in differentiated procambial domains before these cells can function in long-distance water, solute, and nutrient transport. Xylem TEs, for example, undergo secondary cell wall thickening and programmed cell death (PCD) to become structurally robust hollow cylinders. I will describe a few plant regulators that have been described for their specific roles in xylem differentiation. Knowledge of the regulation of phloem cell specification is not as extensive and will not be discussed.

In *Zinnia elegans* plant mesophyll cell culture, transdifferentiation into TEs can be activated by auxin and cytokinin application (Fukuda & Komamine, 1980). From these experiments, the diffusible arabinogalactan protein xylogen was isolated and characterized as a mediator of TE differentiation (Motose et al., 2001a, 2001b, 2004). Two *Arabidopsis* genes encoding for xylogen, *A. THALIANA XYLOGEN PROTEIN1* and *2* (*AtXYP1 & 2*), function redundantly in the differentiation of complete, interconnected xylem networks in cotyledons, leaves, and roots (Motose et al., 2004). Xylogen is likely a coordinator rather than an essential determinant of all xylem differentiation, however, since xylogen deficiency experiments *in planta* still resulted in formation of some xylem tissues (Motose et al., 2004). Xylogen activity may be post-transcriptionally activated by the synergy of auxin and cytokinin (Motose et al., 2004), suggesting that canalized auxin flow through incipient leaf veins is required from cell selection until cell differentiation.

From the above described cell culture system in *Zinnia* and *Arabidopsis*, microarray analyses suggested that the expression of more genes are induced during xylem differentiation (Demura et al., 2002; Kubo et al., 2005). These include two members of the NO APICAL MERISTEM/ATAF1&2/CUP-SHAPED COTYLEDON2 (NAC) domain TF superfamily, VASCULAR-RELATED NAC-DOMAIN PROTEIN6 and 7

(VND6 & 7). Like *AtXYP1* and *AtXYP2*, the expression of *VND6* and *VND7* are regulated by activated by auxin and cytokinin as well as by brassinosteroid plant hormones (Kubo et al., 2005). Ectopic expression analyses suggest that VND6 regulates metaxylem (later-forming, wide xylem cells) formation whereas VND7 regulates protoxylem (the first-appearing narrow vessels) formation in *Arabidopsis* leaves and roots, though genetic redundancy is likely a factor on account of a lack of phenotype from either corresponding knockout mutant (Kubo et al., 2005). Use of a dominant repression system for VND6 or VND7 does inhibit metaxylem and protoxylem formation in the roots, respectively (Kubo et al., 2005; Yamaguchi et al., 2008). The crucial roles of VND6 and VND7 are further highlighted in their posttranslational induction in *Arabidopsis* seedlings, tobacco cell culture, and poplar trees, which resulted in transdifferentiation of non-vascular cells into thick-walled xylem cells (Yamaguchi et al., 2010). Further microarray analyses suggest that VND6 and VND7 are direct and indirect regulators of genes and TFs required for PCD and secondary wall material deposition in xylem cells (Caño-Delgado et al., 2010; Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). Also, localization studies suggest that VND6 and VND7 are expressed in different types of vascular tissue cells (Yamaguchi et al., 2008; Ohashi-Ito et al., 2010). Taken together, these TFs are considered master regulators of xylem identity specification.

1.3. Other hormones in leaf vascular patterning

This thesis focuses on the role of one phytohormone, auxin, in leaf vascular development. It should be noted, however, that the activities of some other hormones can also influence vascular patterning. Some instances of “cross-talk” between auxin and other plant hormones in developmental processes have also been suggested. That is, molecular pathways involving auxin can be interdependent on pathways including the action of another phytohormone, forming signalling networks that affect cellular and morphological responses.

1.3.1. Gibberellic acid

Gibberellic acid (GA) or the gibberellins are diterpenoid acid phytohormones. Exogenous application of GA to the decapitated or wounded stems of various species has no effect on the differentiation of new vascular cells. When GA is applied in combination with auxin, however, twice as many new vascular fibres appear as in stems treated with IAA alone—the result of a synergy between both phytohormones (Digby & Wareing, 1966; Aloni, 1979). Auxin is hypothesized to be a limiting factor in vascular bundle induction while GA promotes vascular cell differentiation and elongation of secondary xylem and phloem fibres (Digby & Wareing, 1966; Aloni, 1979). GA contributions to plant physiological phenomenon such as cambial activity and vascular cell morphology have been documented (reviewed in Aloni, 2001; Elo et al., 2009) while some of the underlying molecular mechanisms involved have also begun to been explored. For example, the enzyme GA 2-oxidase catabolises active GAs as a major mechanism in their inactivation (Martin et al., 1999; Thomas et al., 1999; Rieu et al., 2008). The overexpression of *GA 2-oxidase* is associated with a reduction in GA levels and a dwarf phenotype (Schomburg et al., 2003). Recently, the silencing of the *GA 2-oxidase* has been shown to induce fibre production in tobacco, suggesting that this enzyme regulates vascular differentiation through regulation of GA levels (Dayan et al., 2010). As well, evidence suggests that developing leaves produce a mobile GA-type signal to influence fibre formation and stem elongation through its basipetal flow, akin to PAT in vascular patterning (Dayan et al., 2012). These molecular players suggest that GA has an important role as a signal in vascular growth, but a role for the GA network in leaf vascular patterning has yet to be explored.

1.3.2. Brassinosteroids

The brassinosteroids (BRs) are phytohormones characterized by their steroid-containing molecular structures. In the same *Zinnia* mesophyll cell culture system I mentioned previously, differentiation into TEs can be activated by auxin and cytokinin application (Fukuda & Komamine, 1980). Prior to TE formation in this system, BR levels increased (Yamamoto et al., 2001) as well as the transcript levels of some *HD-ZIPIII* genes (Ohashi-Ito et al., 2002). In fact, BR application is sufficient to trigger TE transdifferentiation in Jerusalem artichoke tuber explants (Clouse & Zurek, 1991) while

the suppression of TE differentiation by uniconazole was rescued by BR application (Yamamoto et al., 1997). It is suggested that BR promotes xylem differentiation by enhancing the expression of *HD-ZIP III* members, and that some of these targets also promote BR signalling (Yamamoto et al., 1997; Ohashi-Ito et al., 2005; Hardtke et al., 2007; Dettmer et al., 2009). Appropriately, *Arabidopsis* mutants with decreased BR biosynthesis show decreased stem vascular tissue formation (Szekeres et al., 1996; Choe et al., 1999). The BR receptor proteins BRASSINOSTEROID INSENSITIVE-LIKE1 and 3 (BRL1 & 3) are expressed in provascular tissues of all *Arabidopsis* organs and are required for shoot vascular bundle patterning (Caño-Delgado et al., 2004). Other components of BR signalling and coordination between BR and auxin have also been characterized in proper shoot bundle development and spacing (Ibañes et al., 2009; Caño-Delgado et al., 2010).

One known BR signalling protein VASCULAR HIGHWAY1 (VH1)/BRL2, a homolog of BRL1 and BRL3, has been attributed to a role in cotyledon vein patterning due to a mutant phenotype with reduced or gapped venation (Clay & Nelson, 2002; Caño-Delgado et al., 2004; Nakamura et al., 2006; Ceserani et al., 2009). VH1/BRL2 may act in the responses to both auxin and BR in establishing domains of incipient leaf vein formation, possibly through BR perception and influence on TIR1-Aux/IAA interaction stability (Ceserani et al., 2009). Other genetic and molecular factors underlying BR influences on leaf vascular patterning have yet to be characterized.

1.3.3. Cytokinins

The cytokinins are a group of molecules traditionally classified by their ability to promote plant cell division. Some of the molecular mechanisms behind the influences of cytokinin on root vascular development, SAM maintenance, and root meristem maintenance have been characterized (Moubayidin et al., 2009; Scarpella & Helariutta, 2010; Caño-Delago et al., 2010; Bishopp et al., 2011). In the interest of this thesis, I will discuss what has been learned about cytokinins in leaf vein patterning.

As with GA, the application of cytokinin alone cannot induce fibre differentiation. Only in combination with IAA and GA can cytokinin influence vascular development in hypocotyls explants (Aloni, 1982), suggesting that auxin is the limiting trigger in vein

patterning while cytokinin is a controlling factor in TE differentiation. In the leaves, ACAULIS5/THICKVEIN (ACL5/TKV) may be involved in vascular development through regulation of PAT, as the thickened leaf veins of *acl5/tkv* mutants suggest mild auxin transport inhibition is at hand (Clay & Nelson, 2005). *acl5/tkv* mutants are hypersensitive to exogenous cytokinin, showing severely reduced root growth relative to the wildtype (Clay & Nelson, 2005). These mutants also fail to produce thermospermine, a polyamine attributed to xylem vessel element differentiation through the prevention of premature PCD (Kakehi et al., 2008; Muñiz et al., 2008). This suggests that ACL5/TKV functions in the synthesis of thermospermine in vascular differentiation, while somehow influencing or being influenced by auxin and cytokinin activities.

The *wooden leg* (*wol*) mutant of *Arabidopsis* is named for a seedling phenotype of reduced cell number and vascular tissues containing only xylem (Scheres et al., 1995). The *WOL* locus turns out to be the same as *ARABIDOPSIS HISTIDINE KINASE4/CYTOKININ RESPONSE1* (*AHK4/CRE1/WOL*; Mähönen et al., 2000). This and two other kinases, AHK2 and AHK3, are involved in cytokinin reception and signal transduction (Yamada et al., 2001; Nishimura et al., 2004). In the interest of leaf vein development, it should be noted that certain multiple *ahk* mutant combinations result in fewer, smaller, aberrantly-shaped rosette leaves containing fewer higher order veins (Nishimura et al., 2004). This has been hypothesized to be a result of cell cycle inhibition through defective cytokinin signalling, as cell number was also decreased in the SAMs of the *ahk* mutant plants (Nishimura et al., 2004).

Another gene that potentially affects leaf vein patterning is *POLARIS* (*PLS*). Reduced leaf venation has been observed in *pls* mutants, which are hyperresponsive to cytokinin and less responsive to auxin application (Casson et al., 2002). It is hypothesized that *PLS* somehow functions through cytokinin and auxin homeostasis but more experiments are needed to explore this possibility.

1.4. Research objectives

The central aim of my work is to investigate the contributions of local biosynthesis maxima of the plant hormone auxin to leaf vein patterning in the model

organism *Arabidopsis thaliana*. Specifically, my goal is to investigate the leaf-specific roles of the *SHI/STY* gene family in the regulation of auxin biosynthesis. This thesis describes my studies of the effects of *SHI/STY* gene expression on leaf vein patterns as well as the interactions between *SHI/STY* and local auxin biosynthesis maxima. The enclosed research manuscript in Chapter Two, prepared for submission to a scientific journal, summarizes the bulk of my work. Chapter Three includes additional research I have done on *YUC*s and *TAA1*, relevant genes in auxin biosynthesis. Interpretations and hypotheses based on the results of Chapter Two and Three combined are written in Chapter Four.

2. The roles of SHORT INTERNODES/STYLISH, regulators of auxin homeostasis, during leaf vein development in *Arabidopsis thaliana*

Tammy Baylis[1], Izabela Cierlik[2], Eva Sundberg[2], Jim Mattsson[1]

[1] Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., V5A 1S6, Canada

[2] Department of Plant Biology and Forest Genetics, Uppsala BioCenter, Linnean Centre of Plant Biology in Uppsala, Swedish University of Agricultural Sciences, PO-Box 7080, SE-750 07 Uppsala, Sweden

2.1. Abstract

Leaves depend on highly developed venation systems to collect fixed carbon for transport to other organs and to distribute water throughout the leaf blade. Although it is well known that auxin can induce vascular differentiation, the mechanisms behind leaf vein patterning are still veiled in some mystery. This is particularly the case for those aspects involved in auxin biosynthesis. Here we have assessed the roles of five members of the *SHORT INTERNODES*/*STYLISH* (*SHI/STY*) gene family in leaf vein development. These genes encode transcriptional regulators linked to auxin homeostasis, with activities characterized primarily in gynoecia. We found that four *SHI/STY* genes, *SHI*, *STY1*, *STY2* and *SRS5*, are primarily expressed at the apical domains, basal domains, and margins of developing *Arabidopsis* cotyledon and leaf primordia, with little or no expression at sites of vein formation. Mutant analysis nevertheless revealed leaf venation phenotypes, most notably in cotyledons. The distal peg venation phenotype, seen in *shi/sty* single and multiple mutants, is an ectopic file or proliferation of vascular elements seen at the apical ends of cotyledons and rosette leaves. Bifurcated/skewed midveins and secondary vein defects are also seen in double, triple, and quintuple *shi/sty* mutant cotyledons. In addition, the expression patterns of some *SHI/STY* genes shift with auxin transport inhibition and exogenous auxin treatments. Taken together, the data imply that SHI/STY members play a role in leaf vein patterning, likely through local regulation of auxin synthesis from sites in the margin of the leaf blade.

2.2. Introduction

Plant leaves require highly developed venation systems to collect products of photosynthesis for transport and also to distribute water throughout the leaf blade. In the model plant *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*), the venation systems of the embryonic cotyledons and the post-embryonically formed rosette leaves begin to develop simultaneously with the rest of the leaf tissue, emerging as bulges called leaf primordia (Mansfield & Briarty, 1991; West & Harada 1993; Goldberg et al., 1994; Donelly et al., 1999). Leaf vascular tissues begin differentiation as precursor cells known as procambium, which shape into at least one midvein, several pairs of secondary veins, and a highly variable pattern of tertiary and quaternary veinlets before leaf maturity (Donelly et al., 1999; Sieburth, 1999; Steynen & Schultz, 2003).

Auxin serves as an inducer of vascular cell fate and vascular differentiation (reviewed in Fukuda, 2004). As such, auxin and active, polar auxin transport (PAT) appear to regulate the patterns and extent of vascular bundles and veins in plants. Based on numerous experiments testing the interactions between applied auxin, endogenous auxin, and tissue polarity on the pattern of trans-differentiation of stem parenchyma into vessel elements, Sachs (1981 and 1989) proposed the canalization of signal flow hypothesis as a mechanism for vascular patterning. This hypothesis states that initially diffuse distributions of auxin are drained by pre-existing vascular tissues, establishing preferred canals of flow that are gradually narrowed by a positive feedback mechanism. This creates specialized files of cells undergoing PAT and, ultimately, differentiated vascular tissues while draining surrounding cells of inductive auxin. There is now evidence on multiple levels in support of this process, especially in the development of leaf veins in Arabidopsis. For example, pharmacological inhibition of PAT in developing seedlings leads to enhanced leaf venation and defective connections between vessel elements, in line with reduced canalization of auxin flow (Mattsson et al., 1999; Sieburth, 1999). The changes in vein distribution are preceded by similar changes in expression patterns of *DR5::GUS* (DR5), a marker construct containing a composite promoter of seven tandem repeats of the auxin response element TGTCTC motif and a 35S minimal promoter (Ulmasov et al., 1997), providing visual evidence for correlations between auxin distribution or responses with vascular differentiation (Mattsson et al.,

2003). In addition, gradual refinement of both the expression of the auxin efflux carrier PIN1 into sites of vein formation and its subcellular protein localization indicative of increasingly polarized auxin transport provides additional evidence in support of the canalization of signal flow hypothesis (Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007).

Local auxin biosynthesis may also contribute to leaf vein development. The partially functionally redundant *YUCCA* (*YUC*) gene family of 11 members, encoding flavin monooxygenases, play roles in a rate-limiting step of Trp-dependent auxin biosynthesis in leaves (Zhao et al., 2001; Cheng et al, 2006, 2007). Three *YUC* members have unique expression patterns in young leaves and multiple *yuc* mutants display severe leaf venation defects along with decreased auxin levels (Cheng et al, 2006). STYLISH1 (STY1), of the *SHORT INTERNODES/STYLISH* (*SHI/STY*) gene family, has been found to positively regulate the transcription of *YUC4* (Sohlberg et al., 2006; Eklund et al., 2010). The *SHI/STY* family, with its conserved 43-amino acid RING-like zinc finger domain and a more C-terminal, unique IGGH domain, may include several direct or indirect activators of auxin-related leaf and style developmental processes on account of *shi/sty* mutant phenotype sensitivity to PAT inhibition and evidence for STY1 binding to and affecting other genes (Fridborg et al., 1999, 2001; Kuusk et al., 2002, 2006; Sohlberg et al., 2006; Eklund et al., 2010; Ståldal et al., 2012). However, it is not known if nor how the *SHI/STY* and *YUC* family members coordinate and contribute to the auxin-faciliated mechanisms underlying leaf vascular development.

In this paper, we sought to identify the functional roles of *SHI*/*STY* genes in leaf vascular development. We first mapped the spatial and temporal expression of *SHI*/*STY* genes during cotyledon and leaf vein development and also tested whether expression patterns were influenced by alterations in auxin distribution. Finally, we analyzed the vascular architecture phenotypes of *shi/sty* mutants. The expression analyses suggest a role of these genes in embryo, cotyledon, and leaf development. Analysis of mutants revealed defective venation patterns primarily in cotyledons. Since expression patterns and phenotypic defects do not necessarily overlap spatially, it appears that SHI/STY genes may act cell non-autonomously in vein formation, possibly by influencing auxin production.

2.3. Results

2.3.1. SHI/STY *genes are expressed in incipient cotyledon primordia and apices of developing cotyledons*

We mapped the expression patterns of the four *SHI/STY* family members *SHI*, *STY1*, *STY2*, and *SHI-RELATED SEQUENCE5* (*SRS5*) by the use of previously described promoter-GUS constructs (Fridborg et al., 2001; Kuusk et al., 2002, 2006), Embryos were first dissected from ovules then assayed for GUS expression.

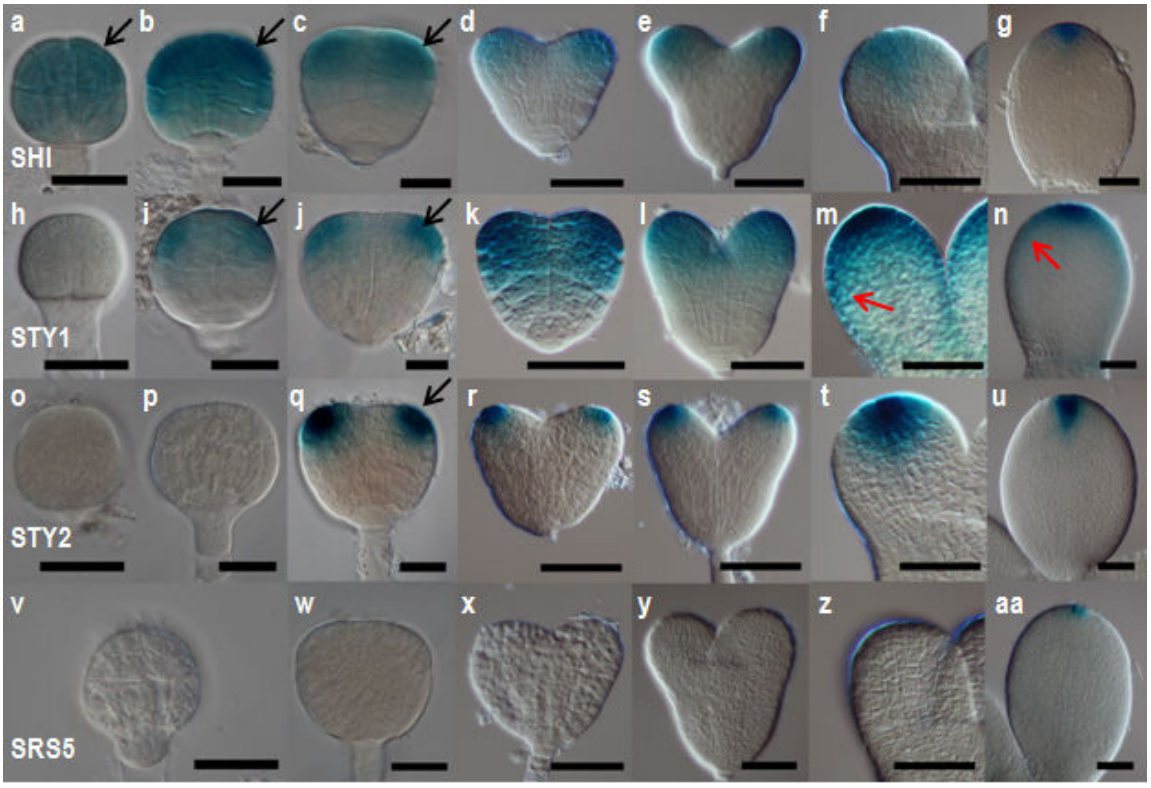


Figure 4. **SHI/STY-promoter::GUS *expression in* Arabidopsis *embryo development***

All frames are whole specimen views except for the two rightmost columns, which are planar median views of the cotyledons only. Columns from the left to the right contain different stages in embryogenesis: globular, triangle/transition, heart, and the cotyledons of torpedo stages. Each row contains representative samples for each marker line: SHI (a-g), STY1 (h-n), STY2 (o-u), and SRS5 (v-aa). Black arrows mark expression at points of incipient cotyledon formation; red arrows mark expression at leaf margins. Scale bars = 20 μm in the three leftmost columns; 50 μm in all other columns.

We found that *SHI* is expressed already in the globular stage throughout the apical and central domains of the embryo proper (Figure 4a, b). In the triangular embryo, *SHI* is expressed primarily at sites of cotyledon primordia (Figure 4c), which is the exclusive site of expression in heart-stage embryos (Figure 4d, e). As the cotyledons develop, *SHI* expression is gradually confined to the apex of cotyledons (Figure 4f, g). *STY1* is also expressed early in embryonic development, but after *SHI* beginning at the late globular stage (Figure 4i); no *STY1::GUS* expression could be detected after more than 24 h of staining at the early globular stage (Figure 4h). At the onset, *STY1* expression also appears to be more restricted than *SHI*, being limited to cells at the sites of incipient cotyledon primordia (Figure 4i). At the heart stage, *STY1* is expressed across larger domains in the developing cotyledons (Figure 4k) before being restricted again to the cotyledon apex and margins near leaf maturity (Figure 4l-n). *STY2* expression appears only at the triangular stage, again localized to sites of emerging cotyledon primordia (Figure 4q). From heart-stage embryo and onwards, *STY2* expression is confined to the apices of cotyledon primordia (Figure 4r-u). *SRS5* expression does not begin until very late in embryo development when the cotyledons are almost mature. We observed *SRS5* expression at a few cells at the apical end of the cotyledon (Figure 4aa). In summary, *SHI*, *STY1*, and *STY2* are all expressed in areas of incipient cotyledon formation (black arrows, Figure 4a-c, i-j, q), and are later restricted to various degrees to the apices of cotyledon primordia as they develop.

2.3.2. SHI/STY *genes are also expressed in incipient leaf primordia, leaf apices, and incipient hydathodes*

We also mapped the expression of the same four *SHI*/*STY* reporter lines in developing rosette leaves. *SHI* expression was detected at the apical portion of the first leaf primordium, but only in early stages, 3 to 5 days after germination (DAG; Figure 5a-c). Expression was also detected later in forming hydathodes (Figure 5e, f). *STY1* expression was observed at the apices of leaf primordia from 3 to 7 DAG (Figure 5g-k). Differently from SHI, *STY1* expression was also detected along the margins of 5 DAG primordia (Figure 5i), and later the along the lower margins and at the base of primordia (Figure 5j-l). *STY2* displayed apical expression throughout leaf primordia development (Figure 5m-r). Interestingly, *STY2* also appears to be expressed internally at the site of the incipient midvein from 3 to 5 DAG (Figure 5 m-o). *STY2* expression was also

detected in the hydathodes near leaf maturity (Figure 5r). *SRS5* expression was not observed until approximately 6 DAG through to leaf maturity, and only in the apical portion of the leaf blade (Figure 5w, x). We also observed similar expression patterns for these genes during the development of the third leaf (Supplemental Figure 1).

In summary, we observed similar expression patterns in cotyledon and leaf development, with expression in incipient primordia followed by expression localized to the apices of primordia, and the forming hydathodes of rosette leaves. In addition, *STY1* also had unique expression at the base of leaf primordia (Figure 4m; Figure 5j-l), and *STY2* was expressed at the site of incipent midveins of rosette leaf primordia (Figure 5m-o).

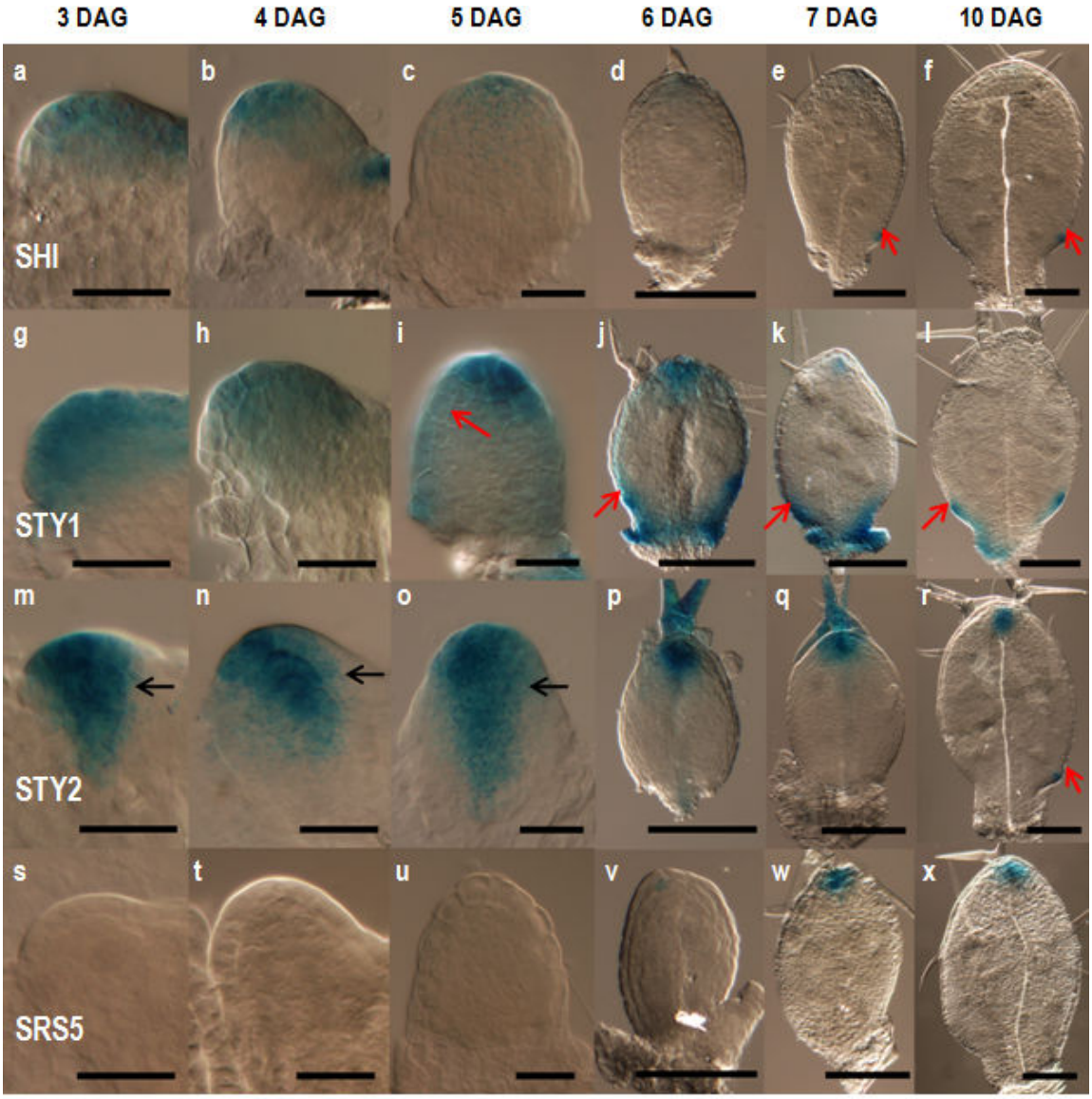


Figure 5. **SHI/STY-promoter::GUS *expression in* Arabidopsis *first/second rosette leaf development***

All frames are planar median views except for 3 and 4 DAG, which are lateral median views. Columns from the left to the right contain leaf primordia at different stages in development, from youngest to oldest. Each row contains representative samples for each marker line: SHI (a-f), STY1 (g-l), STY2 (m-r), and SRS5 (s-x). Black arrows indicate expression at areas of incipient midvein formation; red arrows mark expression at leaf margins. DAG: days after germination. Scale bars = 20 μm at 3-5 DAG; 100 μm at 6-10 DAG.

2.3.3. shi/sty *cotyledons have defective venation patterns*

Since the expression of the four *SHI*/*STY* genes in developing cotyledon primordia overlaps with auxin response maxima implicated in venation patterning (Aloni

et al., Mattsson et al., 2003), we assessed mutants in these genes (Fridborg et al., 2001; Kuusk et al., 2002, 2006) for defective cotyledon venation patterns. The vascular architecture of wildtype cotyledons consists of one midvein (1°) and four secondary (2°) vein loops (Figure 6a; Table 1). Secondary vein loops typically connect the midvein to another 2° loop without interruption. However, the basal secondary veins frequently lack more or less of their basal ends (Figure 6a; Supplemental Figure 2), a phenomenon we have called lower-loop domain (LLD) defects in accordance with developmental nomenclature by Scarpella et al. (2006). This type of defect correlates with a larger arch of the corresponding apical secondary vein (Figure 6a).

We began characterizing *shi/sty* mutants by counting the number of secondary veins in cotyledons, regardless whether they were complete or not. All single *shi*/*sty* mutant and *sty1-1 shi-3* double mutant cotyledons have the same number of 2° vein loops as their respective ecotype wildtypes. However, *sty1-1 sty2-1* double mutant, *sty1-1 sty2-1 shi-3* triple mutant, and *sty1-1 sty2-1 shi-3 lrp1 srs5-1* quintuple mutant cotyledons (*LRP1*, the *SHI/STY* member *LATERAL ROOT PRIMORDIUM1*; Smith & Federoff, 1995; Kuusk et al., 2006) have significantly fewer 2° veins loops compared to all ecotype wildtypes (Table 1; Figure 6k, l). As a consequence, the number of areoles—areas of the leaf blade completely surrounded by vascular tissues—are also reduced (Table 1). There is evidence that each secondary vein is formed as two fragments: one upper-loop domain (ULD) and one LLD. The patterning of each fragment is directed from an auxin convergence point at the primordium margin (Scarpella et al., 2006), resulting in the approximate vein domains indicated in Figure 6b. While LLD defects are common (Supplemental Figure 2), ULD defects are rare in wildtype cotyledons (Figure 7). In the *shi/sty* mutants, the frequency of ULD defects is elevated, reaching significantly higher levels in *sty1-1 sty2-1* double, triple, and quintuple mutant combinations (Figure 7; see Figure 6k, l for examples). This is indicative of a dose-response dependence of ULD formation on SHI/STY activity.

We observed a similar dose-response dependence for two other defect types in *shi/sty* mutants. The midvein runs fairly straight in wildtype cotyledons, ending close to the cotyledon apex (Figure 6a-c). In shi/sty mutants and mutant combinations, the midvein frequently appears to have bifurcated/skewed some distance before the apex, and it is no longer clear what is the apical end of the midvein (Figure 6i-l).

Bifurcated/skewed midveins occur in double or higher mutant cotyledons at frequencies significantly different from the wildtypes (Figure 8).

A unique feature of *shi*/*sty* mutant leaf vasculature is an extra vein at the cotyledon apex which is not necessarily an extension of the midvein. We refer to this extension as the distal peg (Figure 6e, f, i-l; Figure 9; Figure 10). It is rare in wildtype cotyledons but found frequently in the cotyledons of *sty1-1* and *sty2-1* single mutants, *sty1-1 sty2-1* and *sty1-1 shi-3* double mutants, and especially in triple mutants and quintuple mutants (Figure 9). Distal pegs do occur in *shi-3*, *srs5-1*, and *lrp1* single mutant cotyledons, but these frequencies do not differ significantly from the wildtype ecotypes (Figure 9). We wanted to determine whether this defect was caused by either distance (a): a displacement of secondary vein connections to the midvein towards the leaf base, or; distance (b): an extension of the midvein closer to the cotyledon apex (see e.g. Figure 10c). We measured these distances in genotypes with the most consistent distal peg phenotypes (*sty1-1 shi-3*, *sty1-1 sty2-1*, triple mutant; Figure 9) and their corresponding ecotype wildtypes (Col, Ws; see Materials and Methods). In the absence of a distal peg, such as in wildtype cotyledons, both distances (a) and (b) were equal. We found that distance (a) is the same for both ecotype wildtypes as well as for *sty1-1 shi-3*, but significantly larger for *sty1-1 sty2-1* and the triple mutant (Figure 10a), suggesting that secondary vein connections are basally displaced. Distance (b) is the same for both ecotypes and *sty1-1 sty2-1* (Figure 10b). This distance is significantly larger for the triple mutant indicating that, even with the distal peg phenotype, the vascular system of this phenotype does not sufficiently cover the apical portion of the cotyledon blade (Figure 10c). However, distance (b) is significantly smaller for *sty1-1 shi-3* than in wildtype cotyledons. In other words, this specific mutant combination results in an extension of the vascular system towards the apical end of the leaf (Figure 10c). In summary, we found that the two options (a) and (b) were not mutually exclusive, and the tendency was that the apical ends of secondary veins as well as apical end of the distal peg were displaced towards the basal end of leaves with increasing *shi/sty* loss of function. The exception was the *sty1-1 shi-3* combination, as it resulted in the distal peg reaching closer to the cotyledon apex (Figure 10c).

We also scored *shi/sty* mutant cotyledons for additional venation criteria as described by Steynen and Schultz (2003). The scores show that cotyledons of some

shi/sty single and multiple mutants display elevated frequencies of free vein ends and vascular islands (short satellite fragments of differentiated vascular tissue), which are markers of increased venation disconnect (Table 1).

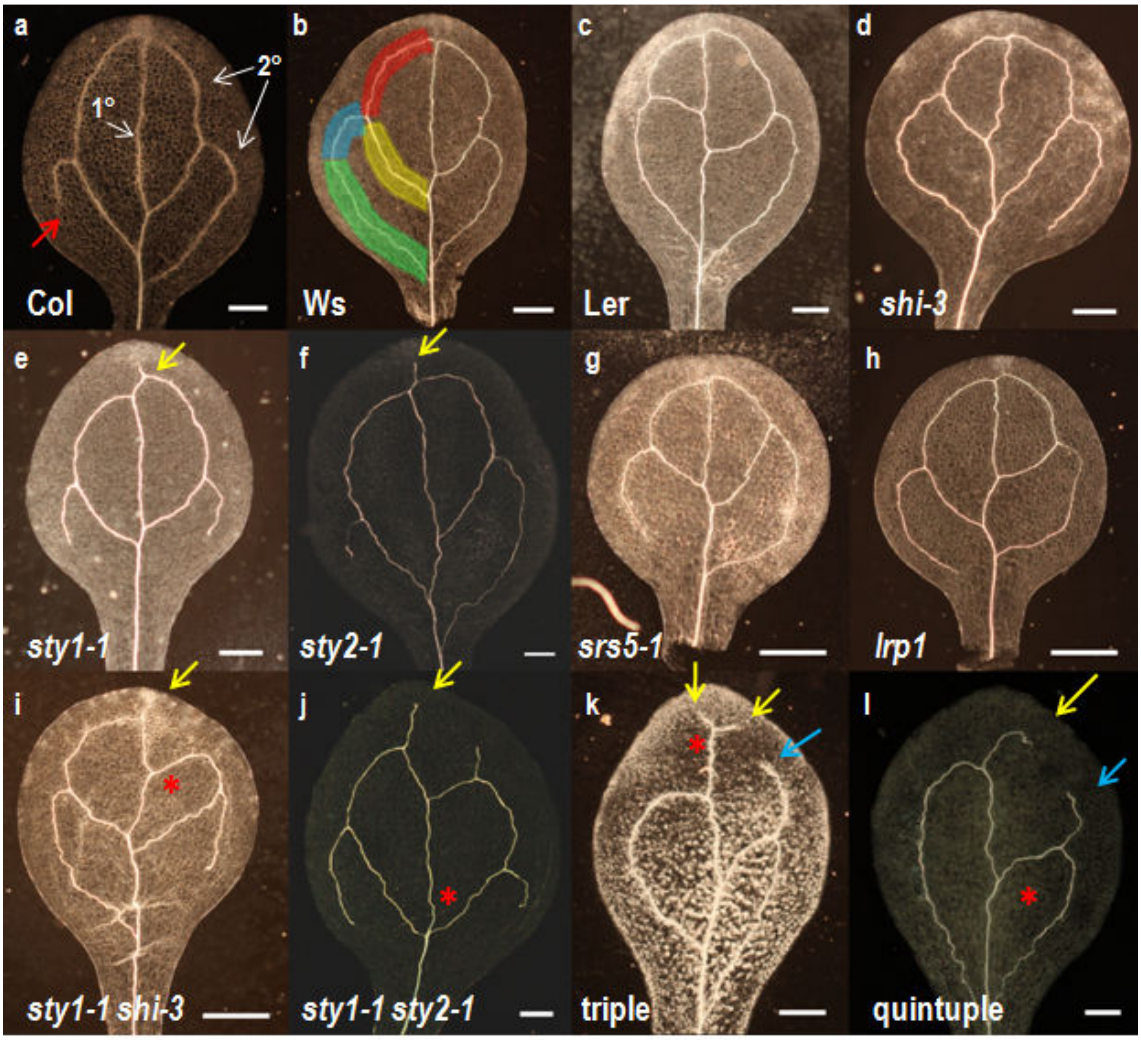


Figure 6. ***Venation patterns of shi/sty mutant* Arabidopsis *cotyledons at 14 DAG***

Each frame contains a representative cotyledon of each genotype. The 1° and 2° vein orders are shown in Col (a) as an example. Also shown, using Col as an example, is a red arrow indicating a lower-loop domain (LLD) defect in a secondary vein, which is found in both wildtypes and mutants. Secondary veins in Ws (b) have been highlighted to show examples of the L1 upper-loop domain (ULD; red), L1 LLD (yellow), L2 ULD (blue), and L2 LLD (green). Yellow arrows mark distal pegs of the vascular systems. Blue arrows mark defects in upper-loop domains (ULDs) of secondary veins. Red asterisks mark bifurcations in midveins. Triple: *sty1-1 sty2-1 shi-3*. Quintuple: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Scale bars = 500 μm.

Table 1. ***Vascular pattern characteristics of* shi/sty *mutant* Arabidopsis *cotyledons***

	Secondary Veins	Areoles	Branch Points	Total Free Ends	Vascular Islands
Col (65)	4.0 ± 0.2	3.3 ± 0.8	5.9 ± 0.9	1.0 ± 1.2	2%
Ws (65)	3.8 ± 0.4	3.2 ± 0.9	5.8 ± 1.2	1.2 ± 1.0	2%
Ler (36)	3.9 ± 0.3	3.5 ± 0.8	6.1 ± 1.0	0.9 ± 0.9	0%
***shi-3* (45)**	4.0 ± 0.4	3.2 ± 0.9	6.6 ± 1.4	1.8 ± 1.3 °	7%
***sty1-1* (36)**	4.0 ± 0.6	2.8 ± 0.9	5.6 ± 1.1	2.1 ± 1.0 °	3%
***sty2-1* (53)**	3.9 ± 0.4	2.8 ± 0.7 *	5.8 ± 1.1	2.0 ± 1.1 °	2%
***srs5-1* (39)**	3.9 ± 0.3	3.0 ± 0.9	5.5 ± 0.8	1.2 ± 1.0	0%
***lrp1* (37)**	4.0 ± 0.3	3.2 ± 0.8	5.6 ± 0.9	1.1 ± 0.9	3%
***sty1-1 shi-3* (44)**	3.8 ± 0.6	2.9 ± 0.8	5.7 ± 1.3	2.1 ± 1.1 °	5%
***sty1-1 sty2-1* (73)**	**3.3 ± 0.8 ***	**2.2 ± 0.6 ***	**4.9 ± 1.4 ***	2.2 ± 1.3 °	8%
triple (31)	**2.5 ± 0.6 ***	**1.8 ± 0.5 ***	**3.6 ± 1.0 ***	1.4 ± 1.2	3%
quintuple (58)	**2.6 ± 0.7 ***	**1.9 ± 0.5 ***	**3.7 ± 1.1 ***	1.4 ± 1.1	3%

Numbers in brackets represent the numbers of cotyledons scored. Values are means ± standard deviation for the entire cotyledon blades excluding the petioles, except for vascular islands (VIs), where values represent the percentages of all cotyledons with VIs occurring at least once. Results are a summary of multiple pairwise Student's t-tests (α = 0.01). Triple: *sty1-1 sty2-1 shi-3*. Quintuple: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Single asterisks (*) indicate that the mutant is significantly smaller than all three ecotype wildtypes, with bolded values highlighting that several vein markers for those genotypes are significantly smaller. Hollow circles (°) indicate that the mutant is significantly larger than all three ecotype wildtypes.

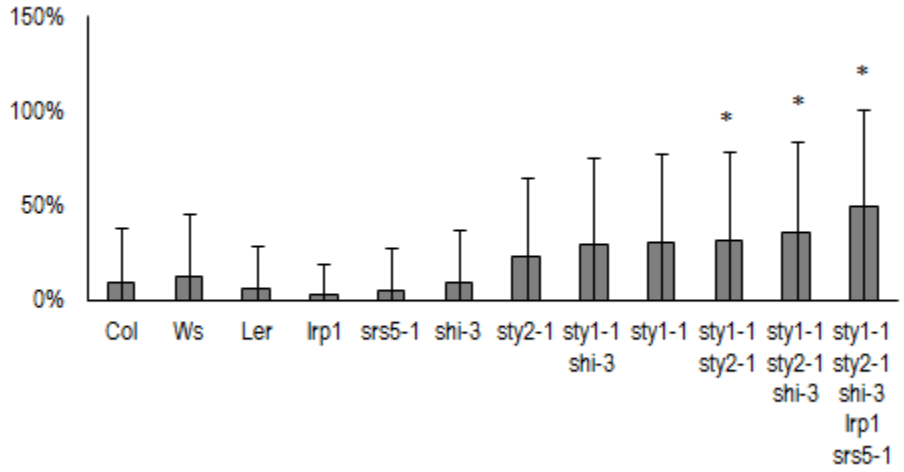


Figure 7. ***Frequencies of secondary vein upper-loop domain (ULD) defects in* shi/sty *mutant* Arabidopsis *cotyledons***

Means with errors bars showing ± standard deviation are shown. Single asterisks (*) indicate significant differences from the three ecotype wildtypes Col, Ws, and Ler (Student's t-tests, n: 31-73, α = 0.01).

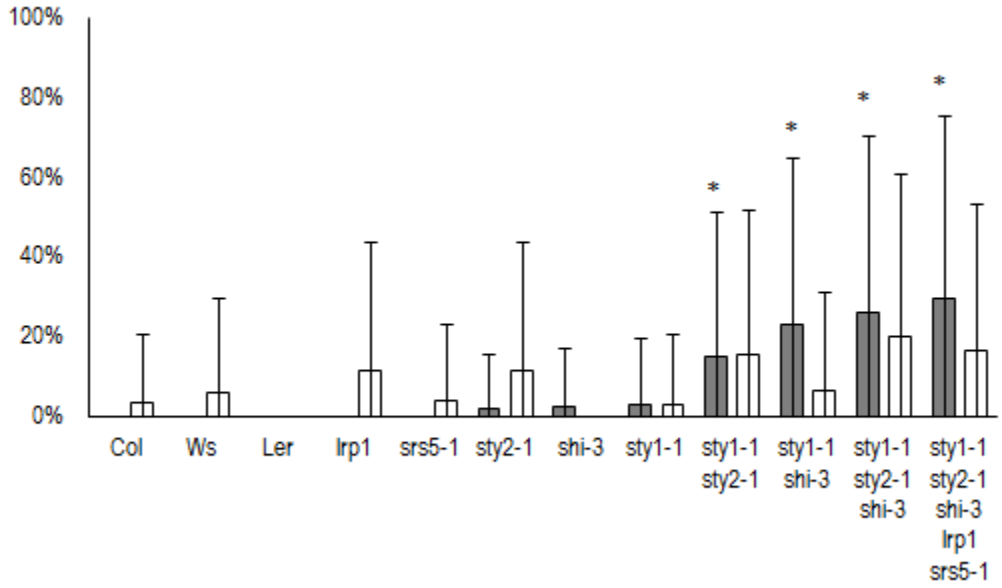


Figure 8. ***Frequences of bifurcated/skewed midveins in* shi/sty *mutant* Arabidopsis *cotyledons and first/second rosette leaves***

Means with error bars showing ± standard deviation are shown. Grey bars: cotyledons; white bars: first rosette leaves. Single asterisks (*) indicate significant differences from the three ecotype wildtypes Col, Ws, and Ler (Student's t-tests, n: 31-73 for cotyledons, n: 27-35 for rosette leaves,
α = 0.01).

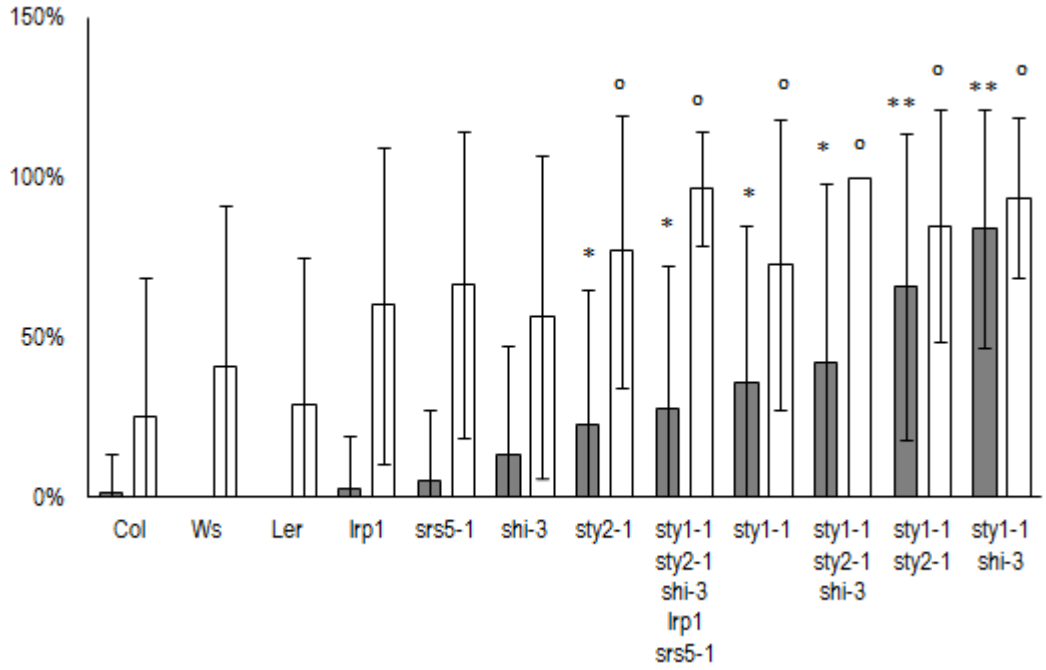


Figure 9. ***Frequencies of the distal peg phenotype in* shi/sty *mutant* Arabidopsis *cotyledons and first/second rosette leaves***

Means with errors bars showing ± standard deviation are shown. Grey bars: cotyledons; white bars: first rosette leaves. Single asterisks (*) indicate significant differences in cotyledons from the three ecotype wildtypes Col, Ws, and Ler, while double asterisks (**) indicate that the double mutant cotyledons are significantly different from related *shi/sty* single and multiple mutants. Hollow circles (^{o}) mark significant differences between mutant rosette leaves and the three ecotypes (Student's t-tests, n: 31-73 for cotyledons, n: 27-35 for rosette leaves, $\alpha = 0.01$).

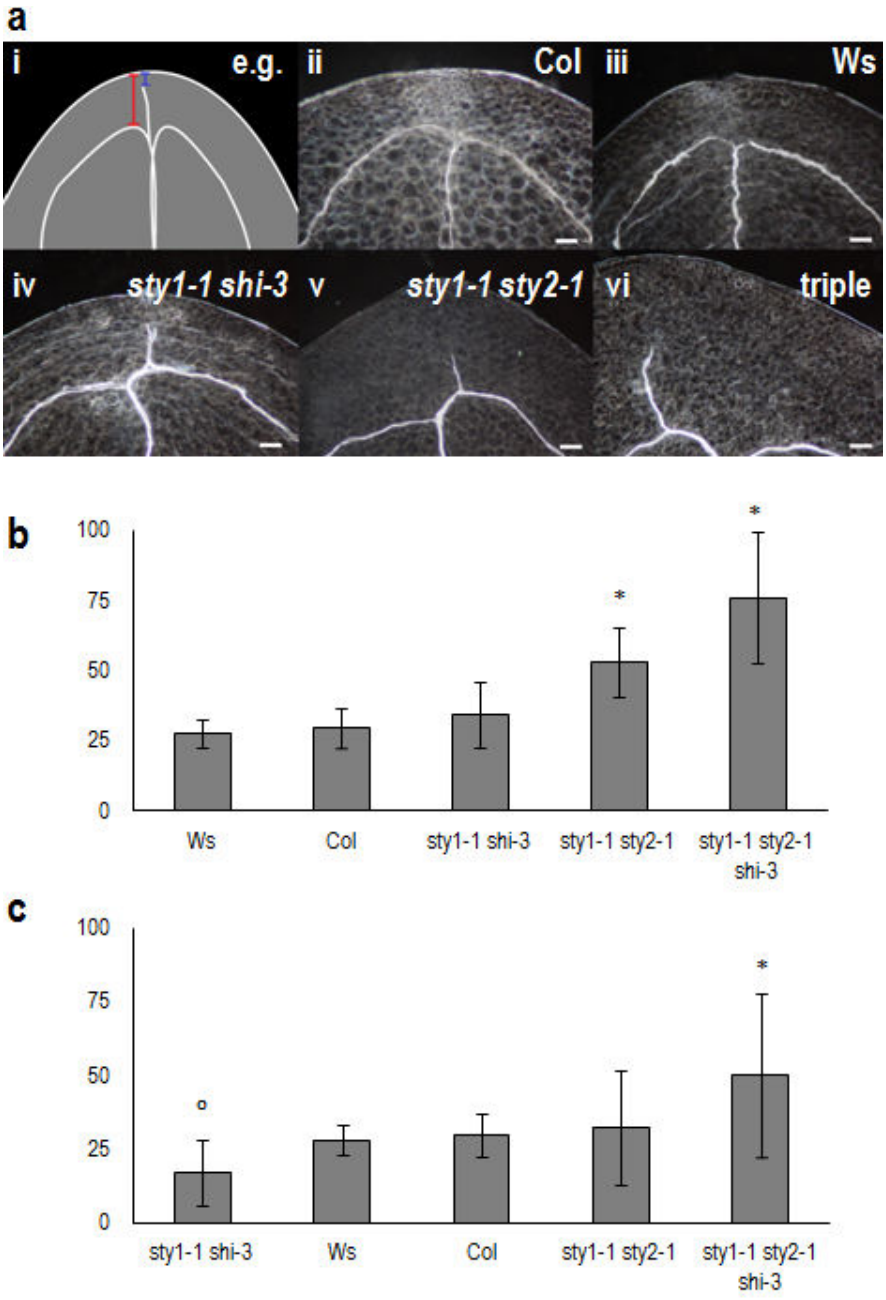


Figure 10. ***Distal peg analysis of*** **shi/sty** ***mutant*** **Arabidopsis** ***cotyledons***

a: Representative cotyledon distal peg phenotypes. i shows an example of a distance (a) measurement (red) and a distance (b) measurement (blue). Triple: *sty1-1 sty2-1 shi-3*. Scale bars = 100 µm. b and c: means in µm with error bars showing ± standard deviation are shown on the graphs. b: Distances between the most apical point of the secondary veins and the most apical pavement cells, distance (a). c: Distances between the most apical ends of the distal pegs and the most apical pavement cells, distance (b). Single asterisks (*) indicate distances significantly larger than both Col and Ws ecotype wildtypes; the hollow circle (^{o}) indicates a mean distance significantly shorter than both ecotypes analysed (Student's t-tests, n: 19-37, $\alpha = 0.01$).

2.3.4. shi/sty *mutants also have defects in rosette leaf venation patterns*

The venation of the first pair of true rosette leaves is more complex than that of cotyledons, consisting of a midvein with three 2° vein loops per half of the leaf blade in the wildtype (Figure 11a-c; Table 2). Rosette leaves also contain small tertiary (3°) and quaternary (4°) veinlets, which form various closed or open connections (Figure 11). In addition to the above criteria for cotyledons, we also scored for marginal free ends in the first leaves, which are those found nearest the leaf blade edge. Marginal free end counts do not include distal pegs, if they are present (see below).

Interestingly, all *shi/sty* rosette leaves have the same number of 2° vein loops as the three ecotype wildtypes (Table 2), unlike the significant reduction found in triple and quintuple mutant cotyledons (Table 1). There are, however, abnormal numbers of other rosette leaf venation markers in some *shi/sty* mutant combinations, indicating that they still have defective vein patterns. The triple and quintuple mutants show significantly higher numbers of marginal free ends (Table 2); the triple mutants also show a significantly higher number of total free ends. The *lrp1* single mutant shows significantly fewer areoles, branch points, and total free ends than the three wildtypes, showing that this genotype has less extensive rosette leaf venation. As well, only *lrp1* shows a high frequency of LLD defects (31.4%; Supplemental Figure 2), which are not common in wildtype rosette leaves. ULD defects do occur frequently in the first leaves of all wildtypes and *shi/sty* mutants, but there are no significant differences between these values (Supplemental Figure 4). Bifurcated/skewed midveins are also seen in mutant rosette leaves, but again not at a frequency significantly different from the wildtypes (Figure 8).

The distal peg is found infrequently in wildtype first leaves (Figure 9, Figure 11). It is found in *shi-3*, *srs5-1*, and *lrp1* single mutants but these frequencies do not differ significantly from the wildtypes (Figure 9). The distal peg is, however, found frequently in *sty1-1*, *sty2-1*, and all multiple mutants (Figure 9, Figure 11). We did not perform a detailed analysis of the distal peg phenotype for any rosette leaves as was done for the cotyledons because initial first leaf measurements showed that neither distance (a) nor distance (b) differed significantly between any genotypes. This suggests that any basal

displacement of vascular systems occurring in rosette leaves is minimal, and/or that the distal pegs in rosette leaves are short but thick proliferations of vascular elements (see *sty1-1, sty2-1, sty1-1 shi-3*; Figure 11e, f, i). In summary, the first rosette leaves of *shi/sty* single and multiple mutant combinations displayed several of the phenotypic defects observed in cotyledons, however at reduced penetrance. Rosette leaves differed in two major aspects. First, the *lrp1* single mutant displayed statistically significant defects, indicating that this gene plays a stronger role in rosette leaves than in cotyledons. Secondly, we observed an enhanced frequency of vascular islands in several *shi/sty* mutant combinations, indicating that the *SHI/STY* genes still play a significant role in the differentiation of continuous veins in rosette leaves (Table 2).

Statistical analyses were not performed for higher order leaves, but our casual observations suggest that triple and quintuple *shi/sty* mutant third leaves may also have more marginal free ends than the ecotype wildtypes (Supplemental Figure 3). These informal analyses also suggest that distal pegs and bifurcated/skewed midveins are not uncommon in higher order leaves as well (Supplemental Figure 3).

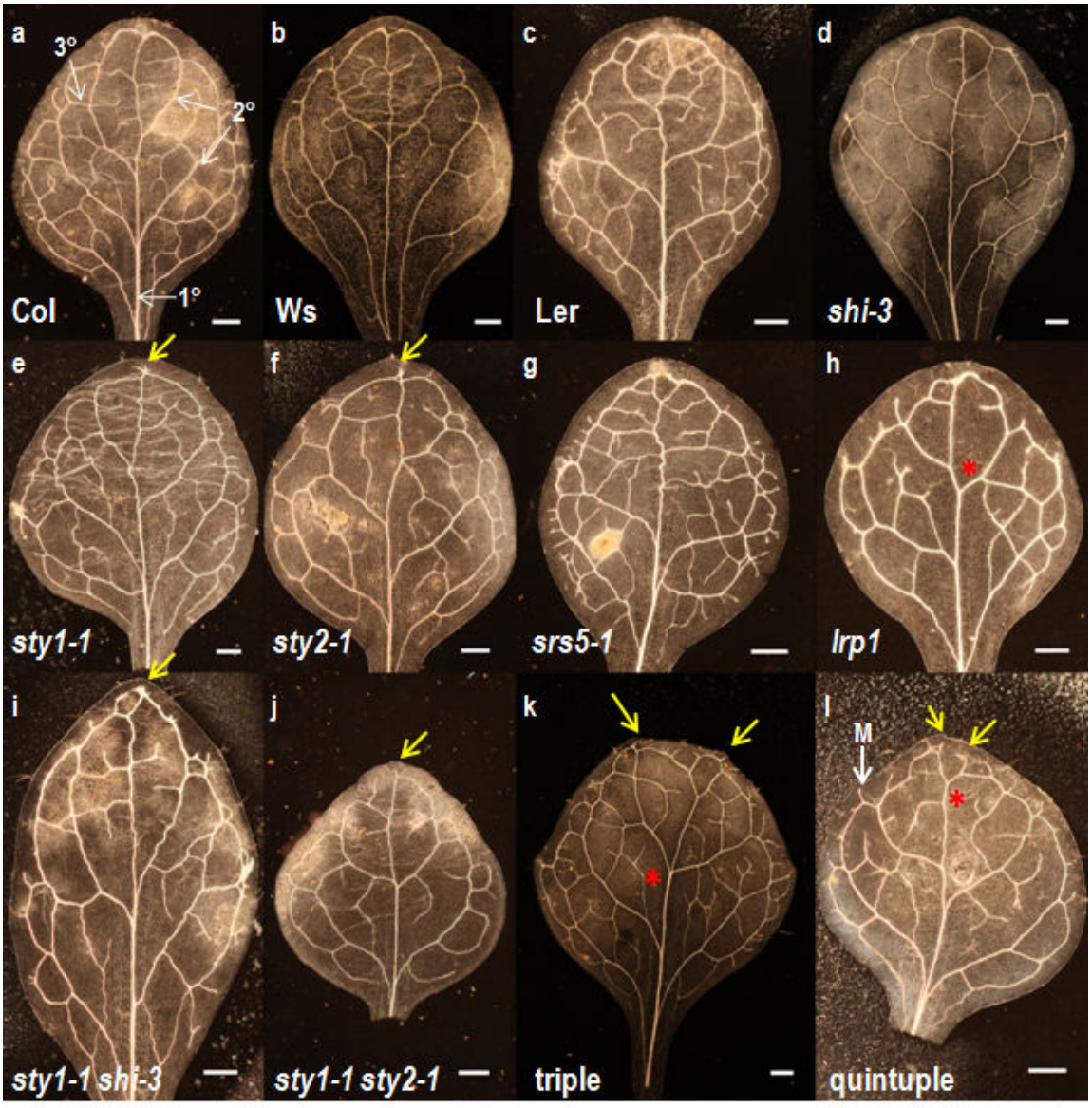


Figure 11. ***Venation patterns of* shi/sty *mutant* Arabidopsis *first/second rosette leaves at 21 DAG***

Each frame contains a representative leaf of each genotype. The 1°, 2°, and 3° vein orders are shown in Col (a) as an example (small 4° veins are not visible at this magnification). Yellow arrows mark distal pegs of vascular systems. Red asterisks mark bifurcations in midveins. M: marginal free end. Triple: *sty1-1 sty2-1 shi-3*. Quintuple: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Scale bars = 500 μm.

Table 2. ***Vascular pattern characteristics of* shi/sty *mutant* Arabidopsis *first/second rosette leaves***

	Secondary Veins	Areoles	Branch Points	Total Free Ends	Marginal Free Ends	Vascular Islands
Col (32)	3.0 ± 0.6	14.8 ± 7.6	35.3 ± 12.2	8.5 ± 3.3	2.3 ± 1.7	3%
Ws (34)	3.1 ± 0.7	11.6 ± 9.6	28.5 ± 15.0	7.3 ± 3.3	2.8 ± 1.6	9%
Ler (31)	3.2 ± 0.7	20.3 ± 12.3	42.0 ± 16.9	9.4 ± 4.1	3.0 ± 2.4	12%
***shi-3* (30)**	3.2 ± 0.7	14.0 ± 7.1	33.8 ± 11.7	7.4 ± 3.1	3.0 ± 1.9	10%
***sty1-1* (33)**	3.0 ± 0.7	11.1 ± 4.1	29.0 ± 7.9	7.6 ± 3.3	3.3 ± 2.0	9%
***sty2-1* (35)**	3.0 ± 0.6	9.7 ± 3.9	25.1 ± 7.3	6.6 ± 3.0	2.8 ± 1.6	11%
***srs5-1* (27)**	2.8 ± 0.5	8.3 ± 3.5	25.1 ± 7.5	7.7 ± 2.6	3.6 ± 1.8	19%
***lrp1* (35)**	2.6 ± 0.5	5.1 ± 3.1 *	16.1 ± 6.0 *	4.3 ± 2.4 *	2.4 ± 1.8	3%
***sty1-1 shi-3* (31)**	2.9 ± 0.5	8.8 ± 2.7	24.1 ± 5.9	6.2 ± 2.9	3.2 ± 1.9	19%
***sty1-1 sty2-1* (33)**	2.9 ± 0.5	9.1 ± 3.1	25.9 ± 6.9	7.0 ± 2.9	3.1 ± 1.7	6%
triple (30)	2.8 ± 0.7	14.0 ± 5.6	37.6 ± 10.7	11.6 ± 3.4 °	4.9 ± 2.4 °	7%
quintuple (31)	2.8 ± 0.6	10.8 ± 3.8	32.0 ± 9.4	9.8 ± 3.9	4.4 ± 2.4 °	25%

Numbers in brackets represent the numbers of rosette leaves scored. Values are means ± standard deviation for the half of the leaf blade as divided by the midvein (excluding the petioles), except for vascular islands (VIs), where values represent the percentages of all rosette leaves with VIs occurring at least once in the entire leaf blade. Results are a summary of multiple pairwise Student's t-tests (α = 0.01). Triple: *sty1-1 sty2-1 shi-3*. Quintuple: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Single asterisks (*) indicate that the mutant is significantly smaller than all three ecotype wildtypes. Hollow circles (°) indicate that the mutant is significantly larger than all three ecotype wildtypes.

2.3.5. Chemical auxin transport inhibition results in unfocused expression of SHI/STY *genes in rosette leaves*

Previous work has suggested that *SHI/STY* family members influence the regulation of auxin homeostasis in aerial organs (Kuusk et al., 2002, 2006; Sohlberg et al., 2006; Ståldal et al., 2008), and that *STY1* directly regulates *YUC4*-mediated auxin biosynthesis (Sohlberg et al., 2006; Eklund et al., 2010, 2011). Considering this, along with the expression patterns of *SHI*/*STY* in the leaves and the leaf vascular phenotypes of *shi/sty* mutants, we hypothesize that the *SHI*/*STY* family influences leaf vein development via regulation of auxin homeostasis. The expression of DR5 reflects auxin response sites in the leaf, which are suggested to influence the positioning of leaf

vascular strands (Aloni et al., 2003; Mattsson et al., 2003). Inhibition of auxin transport results in the local expression of DR5 at the leaf margin, probably as a consequence of auxin accumulation at this site as it also results in the proliferation of vascular tissues adjacent to the apical leaf margin (Mattsson et al. 2003).

To explore the possible relationships between local auxin levels and *SHI/STY* activity, we mapped the expression patterns of DR5 and our *SHI/STY* reporter constructs in the first pair of rosette leaves after seedlings were grown on media containing the auxin transport inhibitor, 1-N-naphthylphtalamic acid (NPA). Young leaves (4 DAG) of *SHI::GUS*, *STY2::GUS*, and *SRS5::GUS* show broadened domains of expression at the apical leaf margin on 1 µM and 10 µM NPA (0.05 µM and 0.1 µM NPA for *SHI* due to an apparent NPA hypersensitivity of this reporter construct in the Ler ecotype background) compared to those grown on control media (DMSO; Figure 12d-l). In particular, *SHI* even shows ectopic expression at the basal leaf margins. Similar results for these reporter lines are also seen in more mature leaves (7 DAG; Figure 13d-l), suggesting that the relationships between local auxin levels and *SHI/STY* family genes are not limited to an early time window in leaf development. Altered expression of *SHI*, *STY2* and *SRS5* (Figure 12d-l) coincides with broadened DR5 expression in the same position of the leaf with auxin transport inhibition (Figure 12a-c; Mattsson et al., 2003). This suggests that *SHI/STY* expression is, at least in part, influenced by auxin. If this is the case, while some *SHI/STY* family members directly and positively regulate *YUC* expression (Sohlberg et al., 2006; Eklund et al., 2010, 2011), then perhaps the *SHI/STY* family members studied here are potentially involved in a positive feedback loop that leads to the establishment of local auxin biosynthesis maxima.

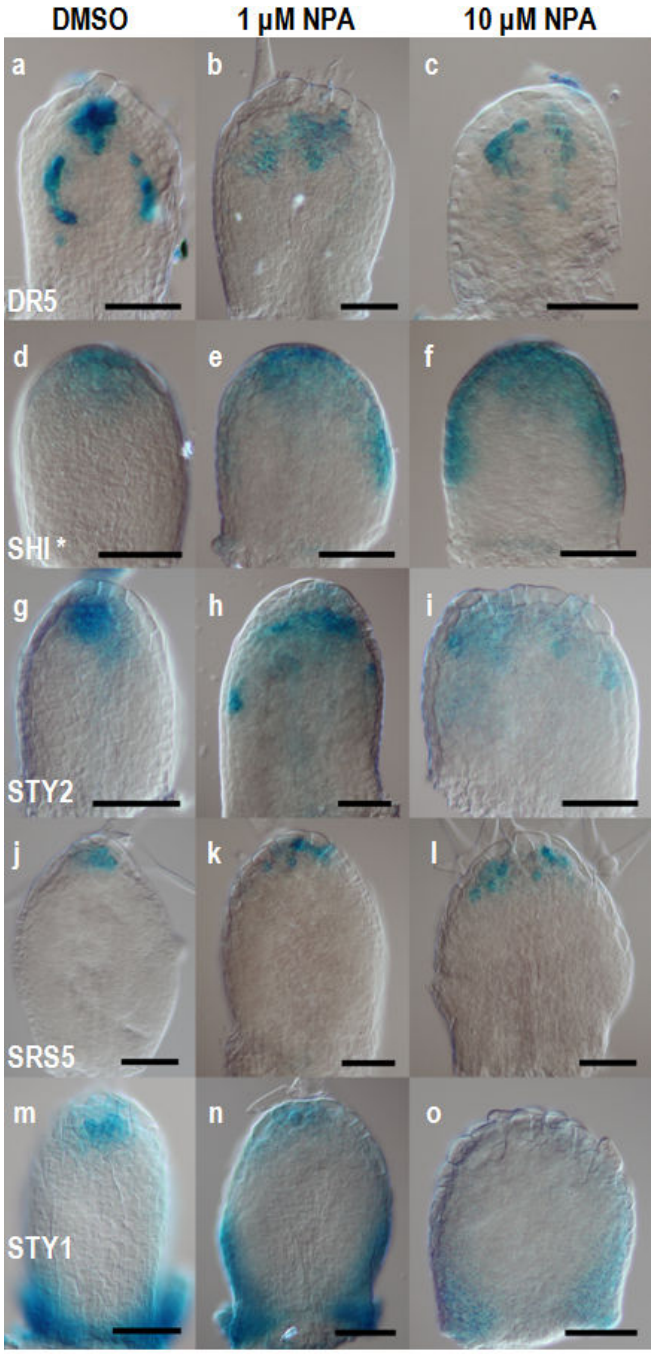


Figure 12. **SHI/STY-promoter::GUS *expression in* Arabidopsis *first/second rosette leaves at 4 DAG, after growth on media containing the auxin transport inhibitor NPA***

All frames are planar median views. Rows from the left to the right (e.g. a-c) contain representative samples for each marker line at different concentrations of 1-N-naphthylphtalamic acid (NPA). DMSO: control conditions. The asterisk indicates that *SHI::GUS* seedlings were grown on 0 µM, 0.05 µM, and 0.1 µM NPA due to an apparent NPA sensitivity of this particular construct and ecotype background (Ler). DAG: days after germination. Scale bars = 50 µm.

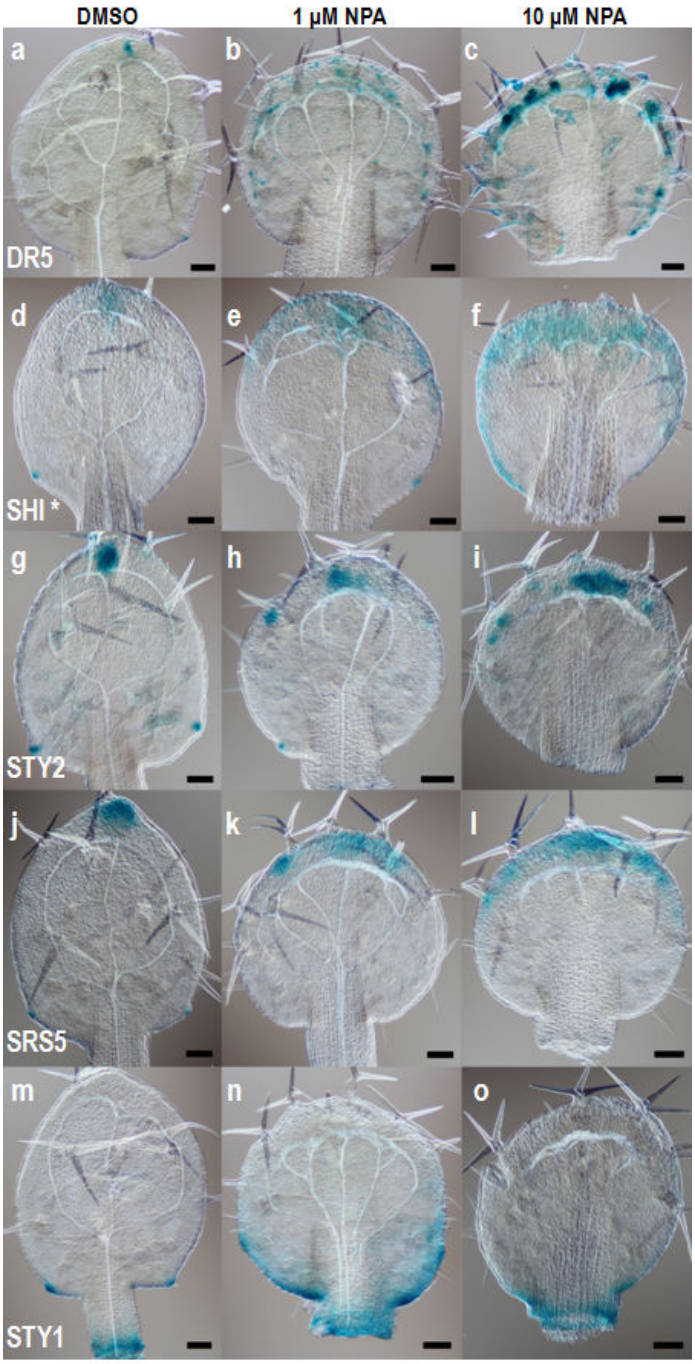


Figure 13. **SHI/STY-promoter::GUS *expression in* Arabidopsis *first/second rosette leaves at 7 DAG, after growth on media containing the auxin transport inhibitor NPA***

All frames are planar median views. Rows from the left to the right (e.g. a-c) contain representative samples for each marker line at different concentrations of 1-N-naphthylphtalamic acid (NPA). DMSO: control conditions. The asterisk indicates that *SHI::GUS* seedlings were grown on 0 µM, 0.05 µM, and 0.1 µM NPA due to an apparent NPA sensitivity of this particular construct and ecotype background (Ler). DAG: days after germination. Scale bars = 100 µm.

Unlike the other *SHI/STY* members studied, the expression of *STY1* at the apical leaf margin at 4 DAG and 7 DAG diminishes at 10 μM NPA (Figure 12m-o; Figure 13m-o). Instead, at 1 μM and 10 μM NPA, increased *STY1* expression is observed at the basal leaf margins (Figure 12n, o; Figure 13n, o). This is interesting because the expression of *STY1* does not overlap largely with that of DR5 under control conditions, except for the apical tips of young leaf primordia (Figure 12a, m) and the hydathodes of mature leaves (Figure 13a, m). Most *STY1* expression in leaf primordia approaching maturity is at the basal margins (Figure 5k, l). Our results suggest that auxin transport inhibition results in a relative increase of local free auxin at the apical end of the leaf, as well as a relative increase of local *STY1* expression, with very little overlap of the separate domains. Perhaps, like the other *SHI/STY* members studied, *STY1* is also involved in a positive feedback loop in the regulation of auxin biosynthesis in leaf primordia, but an additional component is required to transmit the increase in local auxin from the apical end towards the basal end and vice versa.

2.3.6. Exogenous auxin application can lead to ectopic expression of SHI/STY *genes in first rosette leaves*

To further investigate the nature of the relationships between auxin and the *SHI/STY* family members studied in leaf primordia, we wanted to observe the effects of exogenous auxin on the expression patterns of our reporter lines. It has been shown that incubation of young DR5 leaf primordia with synthetic, poorly transported (McCready, 1963; McCready & Jacobs, 1963) auxin 2,4-dichlorophenyoxy acetic acid (2,4-D) results in an expansion of DR5 expression (Mattsson et al., 2003; Figure 14a, b). If altered auxin distribution also alters the distribution of *SHI*, *STY1*, *STY2*, and *SRS5* as we hypothesized above, we expected the application of exogenous auxin to whole primordia to result in enlarged domains of *SHI*, *STY1*, *STY2*, and *SRS5* promoter - reporter expression. Indeed, the domain of *SHI* expression expanded with 2,4-D treatment as with NPA treatment (Figure 12d-f), from the apical end of the leaf primordium down along the apical and basal margins (Figure 14c, d). *STY1* expression with 2,4-D treatment also appeared somewhat similar to that of primordia treated with NPA (Figure 12m-o), with increased expression at the basal leaf margins (Figure 14e, f). But unlike treatment with NPA, expression persisted at the apical end of the primordia of *STY1* seedlings, and low levels of ectopic expression can also be seen in the rest of the

primordia (Figure 14e, f). With 2,4-D treatment, *STY2* was expressed ectopically in domains not previously observed under other growth conditions, at the basal margins (Figure 14g, h). These results support the notion that increased local auxin can upregulate local *SHI/STY* expression, but also suggest that only certain domains of cells within the leaf primordia are susceptible to this upregulation.

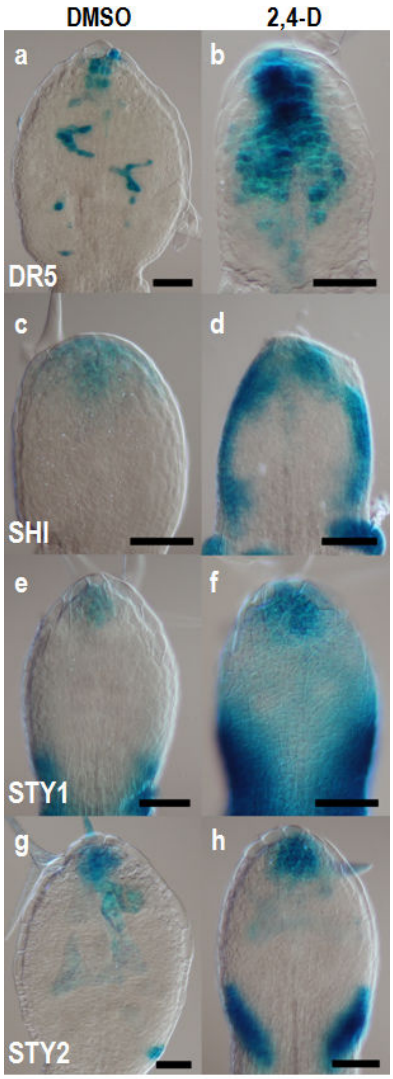


Figure 14. **SHI/STY-promoter::GUS *expression in* Arabidopsis *first/second rosette leaves at 5 DAG, after 16h treatment with the synthetic auxin 2,4-D***

All frames are planar median views. Rows from the left to the right (e.g. a-b) contain representative samples for each marker line at control (DMSO) or 40 µM 2,4-dichlorophenyoxy acetic acid (2,4-D). DAG: days after germination. Scale bars = 50 µm.

2.3.7. Quintuple mutant leaf venation patterns are neither hypersensitive nor hyposensitive to auxin transport inhibition

Several *shi/sty* mutant combinations show gynoecium phenotypes, including vascular tissue patterning defects, increase in severity upon inhibition of auxin transport (Kuusk et al., 2002, 2006; Sohlberg et al., 2006). This phenomenon occurs due to, at least in part, the normal role of *SHI/STY* in the regulation of auxin homeostasis (Kuusk et al., 2002, 2006; Sohlberg et al., 2006; Ståldal et al., 2008). Since we have evidence that *SHI/STY* family genes also control venation patterning in leaves and that they might do so through the regulation of auxin biosynthesis, we hypothesized that inhibiting auxin transport would enhance the defective leaf phenotypes of *shi/sty* mutants. We first compared the number of rosette leaves in 14 DAG Col, Ws, and quintuple mutants grown on control or NPA-containing media (see Materials and Methods). All genotypes showed reduced leaf numbers at 10 μM NPA, compared to their respective genotypes under control conditions (Figure 15), confirming that auxin transport inhibition affects the outgrowth of leaf primordia (Reinhardt et al., 2000, 2003). Interestingly, Ws had significantly fewer leaves than Col under all conditions except for 10 μM NPA (Figure 15). Meanwhile, the quintuple mutant had significantly fewer leaves than Col only at 10 μM NPA, but not fewer than Ws at any NPA concentration (Figure 15). This suggests that it is not necessarily the defective *SHI/STY* genes of the quintuple mutant that lead to increased leaf development impediment, but perhaps it is instead due other genetic factors specific to the Ws ecotype.

It has been observed previously that *pin1* mutant *Arabidopsis* and wildtype *Arabidopsis* seedlings treated with chemical PAT inhibitors can display high frequencies of fused rosette leaves, suggesting normal auxin distribution influenced by PAT is needed for the separation of leaf organs (Okada et al., 1991; Sieburth, 1999; Reinhardt et al., 2000, 2003). As part of our analyses of the effects of *SHI/STY* on leaf outgrowth, we counted the numbers of fused rosette leaves in 14 DAG Col, Ws, and the *shi/sty* quintuple mutant seedlings. Under control conditions, all three genotypes had nearly zero fused leaves (Figure 16). At 1 μM NPA, Col showed a mean slight increase in fused leaf counts, but only the quintuple mutant showed significantly more fused leaves than on control media (Figure 16). At 10 μM NPA, both Col and the quintuple mutant

had significantly higher numbers of fused leaves per rosette compared to untreated plants (Figure 16). However, there was no significant difference between Col and quintuple mutant leaf counts at this concentration. Interestingly, the Ws ecotype showed near-zero fused leaf counts at all NPA concentrations (Figure 16). Our fused leaf counts suggest that the NPA-dampening effects on leaf outgrowth is not amplified by components of the Ws ecotype. However, leaf fusion is hypothesized to be a consequence of increased cell recruitment during leaf blade outgrowth and expansion (Reinhardt et al., 2000), which is one key facet of leaf development (Donnelly et al., 1999; Dengler & Tsukaya, 2001). The hyposensitivity of Ws ecotype wildtype leaf outgrowth to high NPA (Figure 15) could potentially be, therefore, due to a lack of cell recruitment during leaf primordium initiation. Meanwhile, the apparent lack of hypersensitivity and hyposensitivity of leaf fusion in the *shi/sty* quintuple mutant to chemical PAT inhibition reinforces our hypothesis that *SHI/STY* alone are not heavily influential on leaf primordium initiation and leaf outgrowth.

To explore the possibility of auxin transport inhibition having unique consequences on *shi/sty* leaf venation patterning, we observed the venation patterns of 14 DAG Col, Ws, and quintuple mutant first leaves after growth on control or NPA-containing media. Growth of Col seedlings on media supplemented with moderate or high concentrations of NPA results in leaf blades with increased overall vein numbers and widths, and noticeably more pronounced marginal vascular zones (Mattsson et al., 1999). We have found similar results for our Col, Ws, and quintuple mutant leaves (Supplemental Figure 5). Our casual observations indicate that there are no obvious differences in vein numbers or thicknesses between the wildtypes and quintuple mutant at any concentration of NPA (Supplemental Figure 5), suggesting that leaf vein patterning of *shi/sty* is neither hypersensitive nor hyposensitive to auxin transport inhibition.

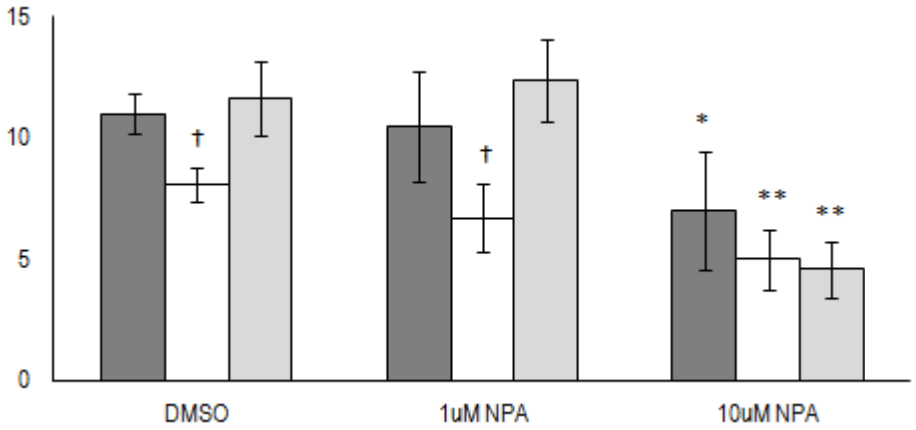


Figure 15. Rosette leaf counts at 14 DAG, after growth on media containing NPA.

Mean ± standard deviation is shown for each genotype and treatment. Dark grey bars: Col; white bars; Ws; light grey bars: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Crosses (†) mark where Ws has significantly fewer leaves than Col and the quintuple mutant under control conditions and on media containing 1 µM NPA. Significantly fewer leaves in Col seedlings grown on 10 µM NPA compared to Col under control conditions are marked by a single asterisk (*). Double asterisks (**) indicate that Ws and the quintuple mutant have significantly fewer leaves on 10 µM NPA than Ws and the quintuple mutant under control conditions, respectively. These values are also significantly smaller than Col on 10 µM NPA, but the quintuple mutant does not differ significantly from Ws on 10 µM NPA (Student's t-tests, n: 7-14, α = 0.01).

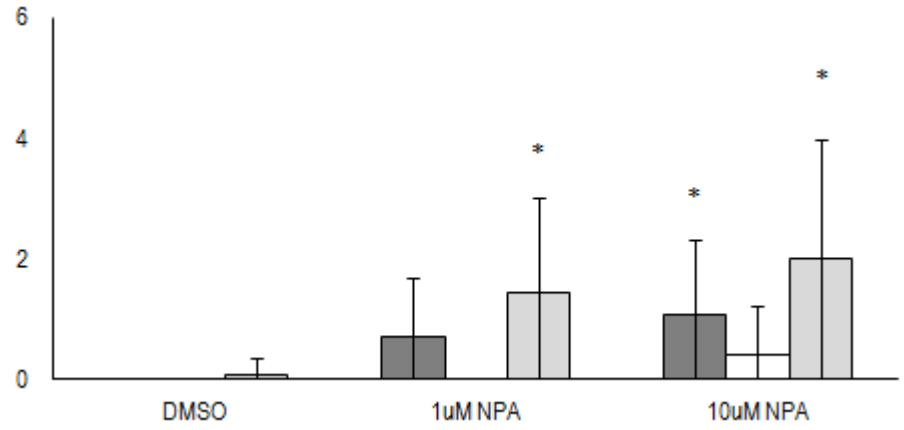


Figure 16. Fused rosette leaf counts at 14 DAG, after growth on media containing NPA.

Mean ± standard deviation is shown for each genotype and treatment. Dark grey bars: Col; white bars; Ws; light grey bars: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Single asterisks (*) mark significantly higher counts of fused leaves for Col (at 10 µM NPA) and the quintuple mutant (at 1 and 10 µM NPA) compared to their respective genotypes grown under control conditions (Student's t-tests, n: 7-14, α = 0.01).

2.4. Discussion

The *SHI/STY* gene family members have been previously implicated primarily in the development of the flower gynoecium (Kuusk et al., 2002, 2006; Sohlberg et al., 2006). Here we show evidence, based on expression patterns and mutant phenotypes, that these genes also have functions in embryo and leaf development.

2.4.1. SHI/STY *genes are expressed in cotyledon and leaf primordia*

The four promoter-reporter gene constructs analyzed here showed overlapping spatial expression patterns during embryo and rosette leaf development with *SHI, STY1* and *STY 2* in incipient and developing primordia, transiting into narrow domains of expression at the apex of maturing primordia. These genes differ primarily in their temporal expression, which is particularly noticeable with *SHI* expressed in early globular embryos, *STY1* coming on in late globular embryos, and *STY2* in triangular embryos; *SRS5* is not expressed until late in cotyledon development (Figure 4aa). Although we have no evidence for it, we speculate on two potential explanations for the serial pattern of temporal gene expression. First, it is possible that *SHI*, *STY1*, *STY2*, and *SRS5* are all induced by one or several closely related accumulating factors but display different affinities or thresholds for activation, resulting in a temporal sequence of activation. Recent sequence analyses of nine active *SHI/STY* genes suggest that *SHI*, *STY1*, *STY2*, *SRS5*, *LRP1*, and other family member genes contain conserved GCC box-like elements near their transcriptional start sites (Eklund et al., 2011), which could be ethylene response factor (ERF)-binding regulatory elements (Ohme-Takagi and Shinshi, 1990; Shinshi et al., 1995; Hao et al., 1998; Fujimoto et al., 2000). Mutation in the GCC box of *STY1::GUS* resulted in decreased levels of *STY1* expression in hypocotyls, cotyledons, leaf primordia, and gynoecia, suggesting the importance of this sequence element to normal *SHI/STY* expression (Eklund et al., 2011). Also, experiments suggest that the activation of *STY1* is mediated by DORNRÖSCHEN (DRN), DRN-LIKE (DRNL), and PUCHI from the APETALA2 (AP2)/ERF family of the putative transcription factors (Nakano et al., 2006), suggesting the AP2/ERF family may redundantly regulate *STY1* in plant development (Eklund et al., 2011). It may be possible, then, that several AP2/ERFs somehow contribute to or are affected by the

accumulating factor(s) needed for succedent expression of *SHI*, *STY1*, *STY2*, and *SRS5* during embryo and leaf development. Indeed, *DRNL* is expressed at sites of incipient cotyledon formation (Chandler et al., 2011a), and *DRN* and *DRNL* are expressed at sites of incipient floral primordia formation (Chandler et al., 2011b). Phenotypic analyses also suggest that these AP2/ERFs contribute to the normal development of these organs (Chandler et al., 2011a, 2011b). Alternatively, it could be possible that *SHI/STY* members act on each other in a series of transcriptional activation events, beginning with *SHI* and ending with *SRS5*. There is evidence that *SHI/STY* genes encode transcription factors (Sohlberg et al., 2006; Ståldal et al., 2008, 2012; Eklund et al., 2010) but so far no studies have explored the possibility of protein-DNA/RNA or protein-protein interactions between individual SHI/STY family members.

Both of the above hypotheses can be tested with molecular techniques identifying promoter–transcription factor interactions. The expression of the *SHI/STY* genes overlaps surprisingly well with the expression conferred by natural and synthetic auxin response elements, suggesting that auxin may be an accumulating activating factor. For example, DR5 (Ulmasov et al., 1997) is expressed in incipient and developing cotyledons (Benková et al., 2003) and leaf primordia, and later on at the apices of cotyledon and leaf primordia and developing hydathodes (Aloni et al., 2003; Mattsson et al, 2003). We also show here that *SHI/STY* gene expression is altered upon auxin transport inhibitor induced alterations of auxin distribution, and that *SHI/STY* gene expression domains expand and become stronger upon auxin treatment of leaf primordia. There are several arguments, however, against an unqualified auxin regulation of the *SHI/STY* genes. First, the *SHI/STY* genes are not expressed at all sites where DR5 confers expression, for example in the early embryo hypophyseal cell (Benková et al., 2003) and preprocambium and procambium of leaf primordia (Mattsson et al., 2003). Also, there is no evidence for an auxin induction of *STY1* upon exposure of whole seedlings to IAA treatments (Eklund et al., 2011). There remains a possibility, however, that *SHI/STY* gene expression depends, at least in part and indirectly, on auxin signaling. *SHI/STY* gene expression patterns coincide not only with auxin response maxima, but also with the expression of PIN1, an auxin efflux carrier implicated in generating auxin maxima at sites of cotyledon and leaf formation (Benková et al., 2003; Reinhardt et al., 2003; Scarpella et al., 2006, Wenzel et al., 2007). Based on these

overlaps of expression, we hypothesize that *SHI/STY* genes may partake in a positive feedback mechanism in which PIN1 auxin transport-generated auxin maxima lead indirectly to activation of *SHI/STY* genes that in turn activate YUCCA genes, resulting in the eventual formation of relatively stable auxin biosynthesis maxima at the cotyledon and leaf apex and hydathodes. The descriptions of the *SHI/STY* gene expression domains help narrowing the list of both potential upstream and downstream candidates of regulation to test this hypothesis. Given that DRN and DRNL affect apical embryogenesis and floral primordium initiation (Chandler et al., 2011a, 2011b), and that the activities of these AP2/ERFs are related to auxin signalling potentially via the auxin response factor MONOPTEROS (MP; Cole et al., 2009; Chandler et al., 2011a, 2011b), it would be interesting to explore if and how AP2/ERFs fit into our proposed positive feedback loop in cotyledon and rosette leaf vein development.

2.4.2. SHI/STY *genes act non-cell-autonomously in venation patterning*

We show here that *shi/sty* mutants of different combinations display a series of defects in cotyledon and leaf venation patterns. Without going into phenotypic details, there are several general conclusions that can be drawn. First, defects can be found in both the pattern (i.e. the overall distribution of veins) and in the continuity of veins (e.g. secondary vein defects), indicating that *SHI/STY* genes act on both the formation of veins as well as the subsequent differentiation of veins. Second, as both cotyledon and rosette leaf venation patterns are defective, the assessed *SHI/STY* genes act during both embryonic and post-embryonic development, in line with their expression patterns. Third, leaf venation defects are typically stronger in double, triple, and quintuple mutant combinations, indicating a dosage effect of their activity on vein patterning. This is in line with their largely overlapping spatial expression patterns. Since there are four additional active members in this gene family (Fridborg et al., 2001; Kuusk et al., 2002, 2006), it is also possible that more extensive mutant combinations would result in stronger phenotypes. Fourth, since the expression of the assessed *SHI/STY* genes is primarily in the apices and margins of cotyledon and leaf primordia, and the phenotypic defects occur in internal vascular tissues, it appears that the *SHI/STY* genes somehow act non-cell autonomously in the process of leaf vein patterning and differentiation.

Since it is known that some transcription factors can act non-cell autonomously via transport through plasmodesmata (reviewed in Xu & Jackson, 2012) it is possible that the SHI/STY transcription factors act in a similar fashion. In light of the known activation of *YUC4*, encoding an enzyme in auxin biosynthesis (Cheng et al., 2006), by STY1 (Sohlberg et al., 2006; Eklund et al., 2010), a more likely explanation is that SHI/STY proteins act cell-autonomously in the activation of *YUC* genes, triggering auxin production that in turn acts non-cell autonomously in vein development via PAT. In support of this hypothesis, *shi*/*sty* mutants have reduced auxin levels (Sohlberg et al., 2006; Ståldal et al., 2008). *STY1* expression (Figure 5g-l) partially coincides with that of *YUC4*, which is expressed at both the basal and apical regions of young rosette leaves, then later in the hydathodes and mature vascular tissues (Cheng et al., 2006). *YUC1* is expressed at the basal regions as well as at the margins only in the young leaf, while *YUC2* is expressed in the mature apical region, mature vascular tissue, and hydathodes (Cheng et al., 2006). These domains of expression also overlap with those of the *SHI*/*STY* genes that we have analysed (Figure 5). We therefore hypothesize that several *SHI*/*STY* genes act in a cell-autonomous manner as positive regulators of several *YUC* genes at the edges of young leaves.

2.4.3. shi/sty *mutant vein defects may be reconciled with reduced auxin levels*

Given that *shi/sty* mutants have reduced levels of free IAA (Sohlberg et al., 2006; Ståldal et al., 2008) the reductions of the venation system seen in the same mutants may be caused by reduced levels of inductive auxin. At first glance, the distal peg phenotype appears to be a gain-of-function phenotype. However, as our cotyledon analyses suggest that the distal peg is primarily a symptom of a basal displacement of the entire leaf vascular system (Figure 10), it is plausible that this defect is a result of a decrease in available auxin. It is hypothesized that a midvein forms when the leaf primordium first grow outwards by PIN1 proteins concentrating auxin flow towards the centre of the apical end of the primordium then towards the leaf base in a discrete cell file, which differentiates into procambium (Reinhardt et al., 2003; Scarpella et al., 2006; Smith et al, 2006; Wenzel et al., 2007). Secondary vein formation coincides with two separate events of auxin transport (i.e. LLD and ULD formation) from leaf margin auxin "sources" and the extension of PIN1 expression domains in the centre of the leaf lamina

to fuse with the procambial midvein auxin “sink” (Scarpella et al., 2006; Wenzel et al., 2007). Since *SHI/STY* members are expressed primarily at leaf primordia apical zones and margins, *shi/sty* mutant leaves may experience insufficient local auxin biosynthesis in these domains. A lack of free auxin flowing from the primordium apical end could affect the concentration of PIN1 expression into discrete cell files during midvein formation, resulting in fewer differentiated procambial cells at the most apical region of the leaf lamina. Similarly, decreased auxin at the apical and marginal regions could coerce secondary vein ULD fusion with the midvein at a more basal region, as relatively more auxin would be located in the midvein procambium. This would lead to the appearance of a distal peg in the vasculature.

The bifurcated/skewed midveins of *shi/sty* mutants could potentially be attributed to decreased available auxin as well. If there is a reduction of apical auxin biosynthesis due to decreased SHI/STY function, then lateral or marginal auxin sources regulated by other gene families may exert stronger influences on midvein formation during early primordium development. This could play a role in the split of the midvein. Those other gene families could include the NGATHA (NGA) transcription factors which are known to participate in lateral organ development, possibly in part through the regulation of *YUC2* and *YUC4* in the apical domain of the gynoecium (Alvarez et al., 2009; Trigueros et al., 2009). *NGA4*, *NGA1*, and *NGA2* are expressed at the leaf margins and may be activated by STY1 which, interestingly, may lead to the NGA activation of other *SHI/STY* members (Alvarez et al., 2009; Trigueros et al., 2009). If SHI/STY and NGA factors act cooperatively to promote auxin-mediated style development, then perhaps the families act similarly in leaf development.

2.5. Materials and Methods

2.5.1. Plant material and growth

Arabidopsis thaliana seeds containing the auxin-responsive promoter-GUS fusions STY1::GUS, STY2::GUS, SHI::GUS, and SRS5::GUS were generated as previously described (Fridborg et al., 2001; Kuusk et al., 2002, 2006). Seeds homozygous for *sty1-1*, *sty2-1*, *shi-3* and *srs5-1* were propagated from stocks of our previous work (Fridborg et al., 2001; Kuusk et al., 2002, 2006). Seeds carrying an insertion in *LRP1* (*lrp1*) were kindly donated by Nina Fedoroff, Biology Department and Huck Institutes of Life Sciences, Pennsylvania State University, University Park, PA 16302, USA. *sty1-1* and *sty2-1* are in the Columbia (Col.) ecotype background; *shi-3* is in the Wassilewskija (Ws) background; *lrp1* is in the Nossen background, and; *srs5-1* is in the Landberg *erectra* (Ler) background. All promoter-GUS fusions are in the Col. background except for SHI::GUS, which is in the Ler background. Double, triple, and quintuple homozygous mutants were generated by cross-fertilization as previously described (Kuusk et al., 2002, 2006).

All seeds were sterilized in sealed gas chambers as described by Clough & Bent (2001). The seeds were plated on plant agar media [0.5 × Murashige and Skoog salts with vitamins, 1.5%(w/v) sucrose, 0.5g/L 2-(N-morpholino)-ethane sulfonic acid (MES) buffer (Duchefa Biochemie), adjusted to pH 5.7 using KOH, 0.8%(w/v) plant agar (Duchefa Biochemie)], then placed at 5°C for 2d for stratification. Seedlings were plated at a density of no more than 20 per petri dish. For those experiments investigating the effects of auxin transport inhibition, stock solutions of 1-N-naphthylphtalamic acid (NPA) in DMSO were added to the media. The plates were then germinated at 22°C under white fluorescent light (170-180 μE m^{-2} s^{-1}), for 16h light and 8h darkness at 50% RH. For those experiments exploring the effects of exogenous auxin on gene expression, seedlings at 4 DAG were transplanted to liquid media [0.5 × Murashige and Skoog salts with vitamins, 1.5%(w/v) sucrose, 0.5g/L 2-(N-morpholino)-ethane sulfonic acid (MES) buffer (Duchefa Biochemie), adjusted to pH 5.7 using KOH] containing DMSO or 2,4-dichlorophenyoxy acetic acid (2,4-D), then placed on an illuminated shaker for 16 h at room temperature before GUS analysis. At 14 DAG, some seedlings were transplanted

to soil under the same growth conditions to allow rapid and complete maturation of rosette leaves and siliques.

Spatial and temporal gene expression patterns were observed in the first or second leaf once per day from emergence of the primordia to leaf maturity, for promoter-GUS plants. The same was done for later-formed leaves to determine if gene expression patterns are the same for all true rosette leaves. Leaf primordia for the first, second, and later-formed rosette leaves were dissected from representative seedlings in sterile water each day from the moment they were visible (3 DAG for the first leaf) until the later-formed leaves were mature (14 DAG). For the study of mature leaf venation patterns, wildtype and mutant cotyledons were dissected at 14 DAG and first/second and third leaves at 21 DAG, all in sterile water.

2.5.2. Venation pattern analysis

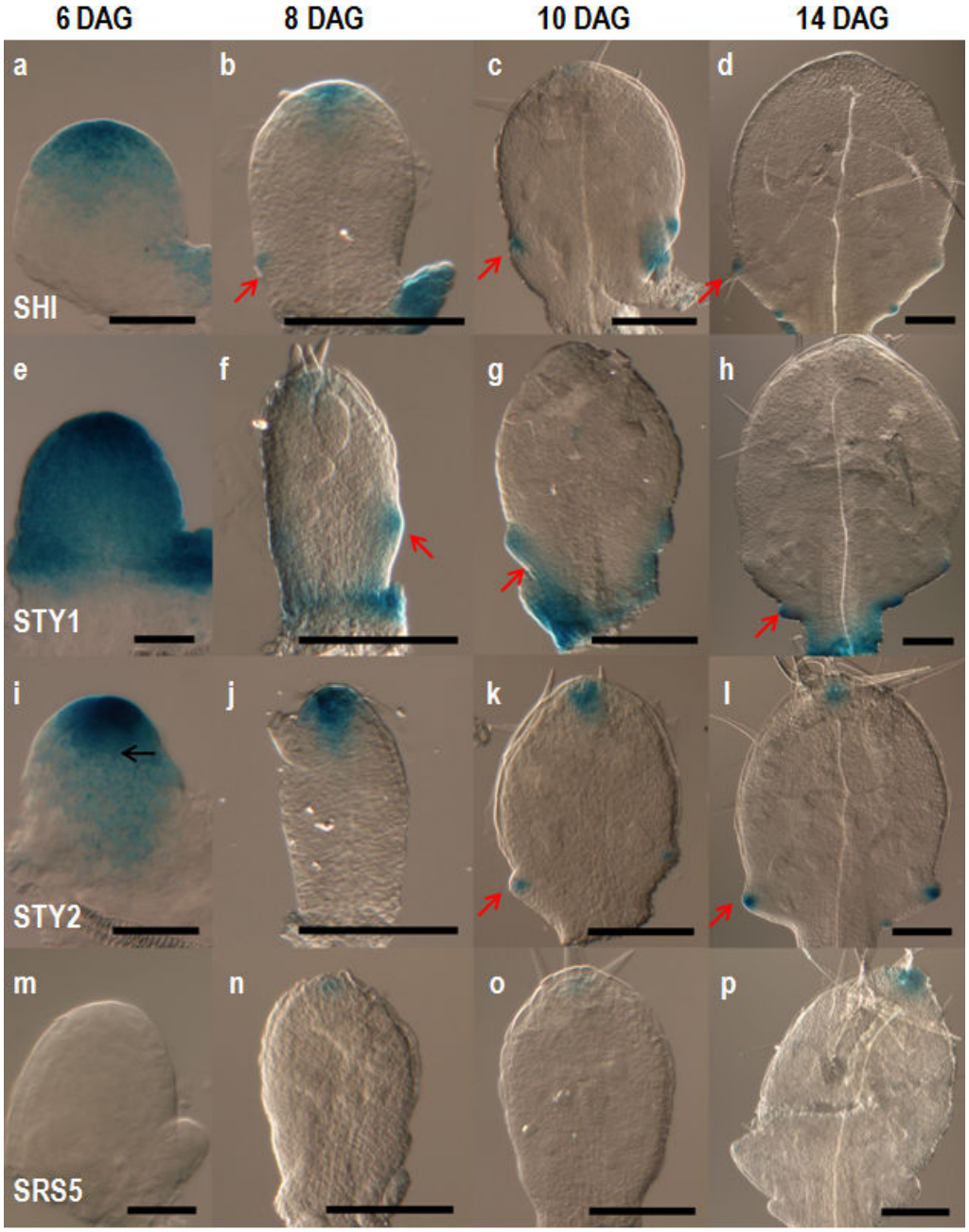
First/second leaves, third leaves and cotyledons were fixed at 4°C overnight in 100% ethanol:acetic acid (6:1, v/v). They were then washed once in 100% ethanol and again in 70% (v/v) ethanol, followed by clearing in 50% (w/v) chloral hydrate at 65°C for 1h. After being rinsed twice in water, all leaves were cleared again in 85% (w/v) lactic acid at room temperature for at least three days. All leaves were mounted in lactic acid and observed using dark field microscopy. All leaf sizes were measured and all venation features were counted using ImageJ. Mean values and standard errors were calculated and compared by ANOVA and Student's t-tests, respectively, using JMP 8.0.2 (SAS Institute Inc.). Graphs were created using Microsoft Excel.

2.5.3. GUS assay and analysis

Leaf primordia and embryos were incubated in GUS substrate solution: 50mM sodium phosphate, pH 7, 5mM K3/K4 FeCN, 0.1% (w/v) Triton X-100, and 0.67mM, 2mM or 4mM 5-bromo-4-chloro-3-indolyl-beta-GlcUA. Air was evacuated from samples, followed by incubation at 37°C for 1 to 24 h, depending on colour development. Younger leaf primordia experienced a longer staining period in order to observe the faint initiation of expression. The samples were then rinsed of excess substrate by keeping them in sterilized water at room temperature for 1h, then fixed at 4°C overnight in 100% ethanol:acetic acid (6:1, v/v) or 2% formamide. Following that, they were washed twice

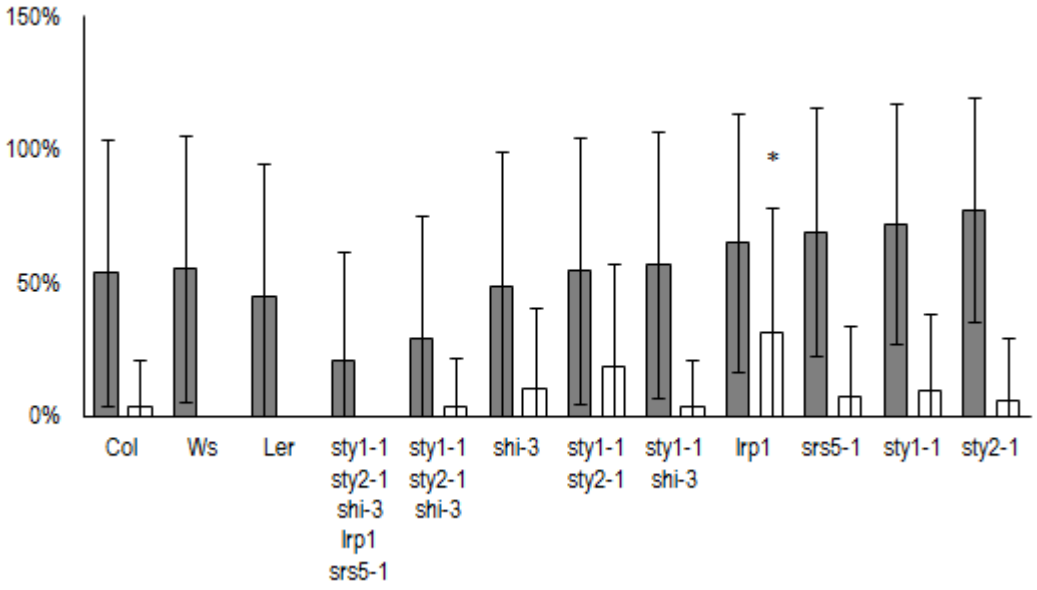
in 100% ethanol then dissected in 70% (v/v) ethanol. For analysis, leaf primordia and embryos were mounted in clearing solution (chloral hydrate:glycerol:water, 9:1:3 [w/w/v]) and observed using differential interference contrast settings on a Nikon Eclipse E600 microscope. Photographs were taken using a Canon EOS 5D Mark II digital camera, then assembled using Adobe Photoshop.

2.6. Supplemental Materials



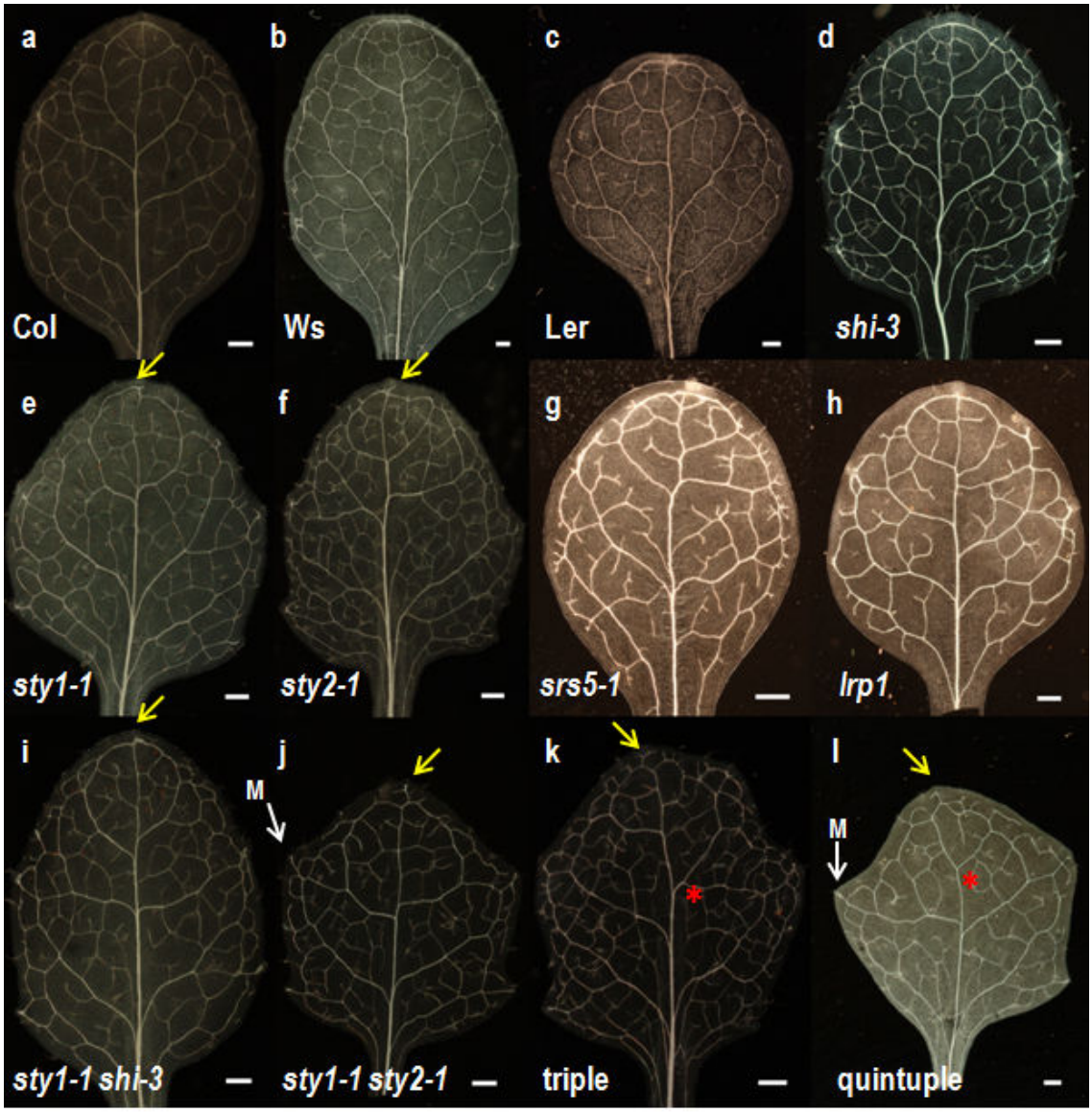
Supplemental Figure 1 **SHI/STY-promoter::GUS *expression in developing* Arabidopsis *third rosette leaves***

All frames are planar median views. Rows from the left to the right (e.g. a-d) contain representative samples for each marker line at different stages in leaf development, from youngest to oldest. Black arrows indicate expression at areas of incipient midvein formation; red arrows mark expression at leaf margins. DAG: days after germination. Scale bars = 20 µm at 6 DAG; 100 µm in all other columns.



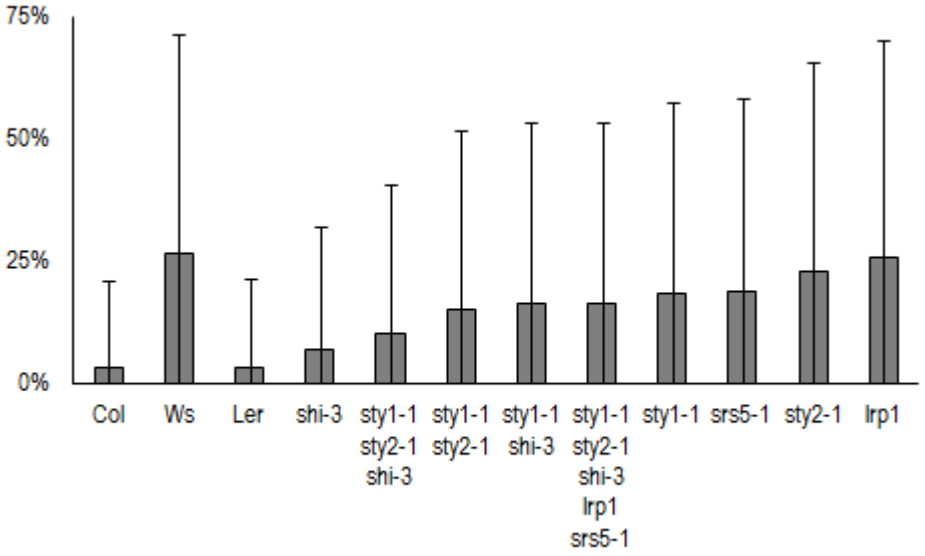
Supplemental Figure 2. Frequencies of secondary lower-loop domain (LLD) defects in* shi/sty *mutant* Arabidopsis *cotyledons and first/second rosette leaves

Means with error bars show ± standard deviation are shown. Grey bars: cotyledons; white bars: first/second rosette leaves. The single asterisk (*) indicates a significant difference between the *lrp1* mutant rosette leaf and the three ecotypes (Student's t-tests, n: 31-73 for cotyledons, n: 27-35 for rosette leaves, α = 0.01).



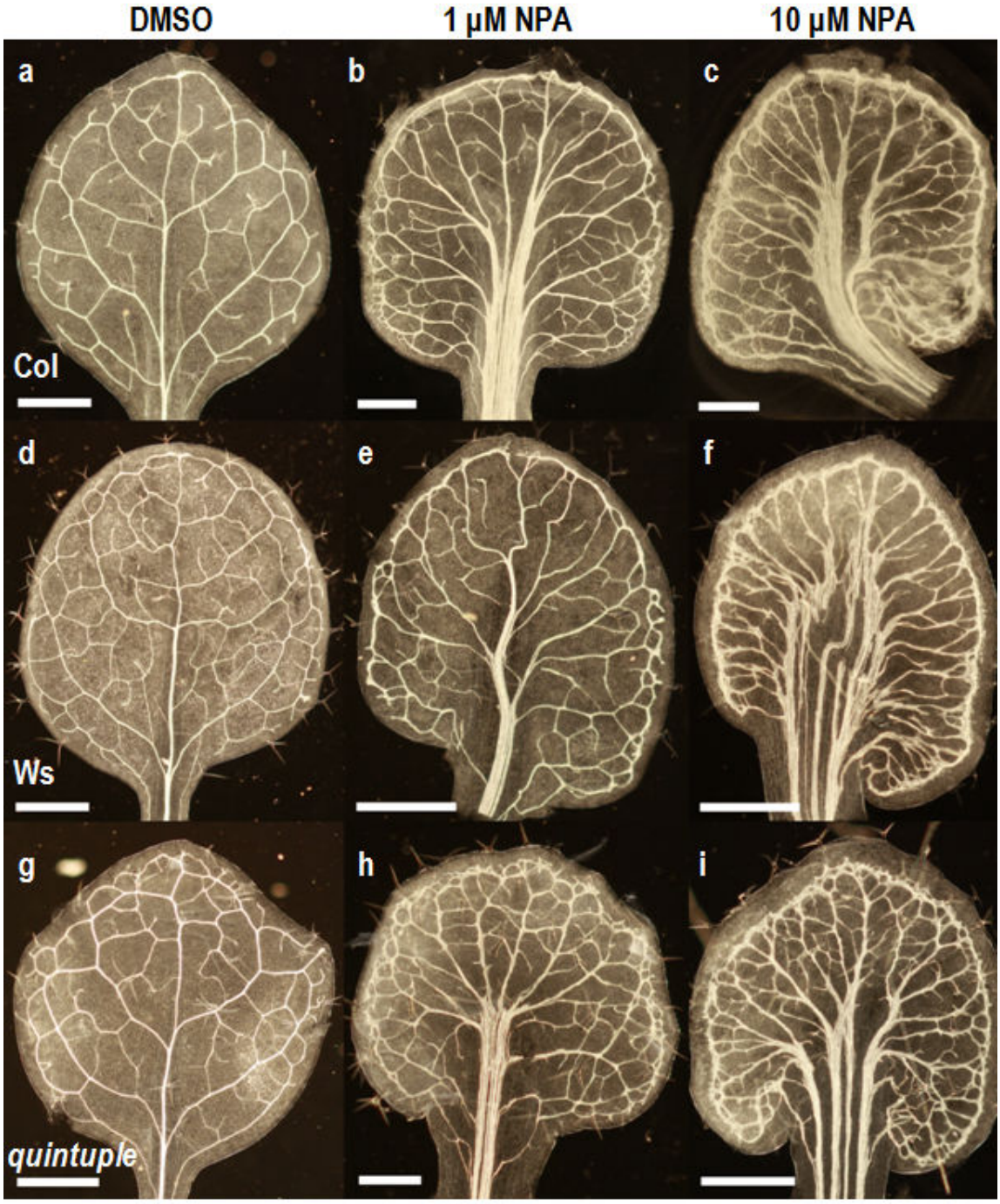
Supplemental Figure 3. Venation patterns of* shi/sty *mutant* Arabidopsis *third rosette leaves at 21 DAG

Each frame contains a representative higher order leaf of each genotype. Yellow arrows mark distal pegs of vascular systems. Red asterisks mark bifurcations in midveins. M: marginal free end. Triple: *sty1-1 sty2-1 shi-3*. Quintuple: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Scale bars = 500 μm.



Supplemental Figure 4. Frequencies of secondary upper-loop domain (ULD) defects in* shi/sty *mutant* Arabidopsis *rosette leaves

Means with errors bars showing ± standard deviation are shown. No significant differences were found between any mutants and the three ecotypes (Student's t-tests, n: 27-35, α = 0.01).



Supplemental Figure 5. Venation patterns of* Arabidopsis *first/second rosette leaves at 14 DAG after growth on media containing NPA.

Rows from the left to the right (e.g. a-c) contain representative samples for each genotype at different concentrations of 1-N-naphthylphtalamic acid (NPA). DMSO: control conditions. Quintuple: *sty1-1 sty2-1 shi-3 lrp1 srs5-1*. Scale bars = 500 µm.

3. The dynamic expression patterns of the auxin biosynthesis genes, TAA1 and YUCCAs, in *Arabidopsis thaliana* leaf development

Tammy Baylis[1], Eva Sundberg[2], Jim Mattsson[1]

[1] Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., V5A 1S6, Canada

[2] Department of Plant Biology and Forest Genetics, Uppsala BioCenter, Linnean Centre of Plant Biology in Uppsala, Swedish University of Agricultural Sciences, PO-Box 7080, SE-750 07 Uppsala, Sweden

3.1. Abstract

While well known that polar auxin transport plays a key role in leaf vein patterning, the contributions of local auxin biosynthesis have not been identified. The recent identification of a two-step pathway of the main auxin, indole-3-acetic acid (IAA), involves enzymes in two gene families: TAA1/TAR and YUC. Here we have examined the genetics of local auxin biosynthesis in *Arabidopsis thaliana* leaves. Some members of *TAA1/TAR* and *YUC* are expressed in distinct, dynamic patterns in developing leaf primordia. *TAA1*, previously shown to be expressed at mature leaf margins, is expressed only at the apical domains of young primordia. *YUC1* is expressed at the basal domains as well as the margins, at the flanks of developing hydathodes. *YUC2* is expressed in older leaf primordia and in differentiated vascular tissues. In older leaf primordia, *YUC4* expression emerges at the apical tip and hydathodes. None of these genes are expressed in developing leaf veins, yet previous analyses have shown leaf vein mutant phenotypes. We have identified *TAA1* and *YUC4* as coexpression partners, while corresponding coexpression partners for *YUC1* and *YUC2* are missing. Expression these four genes occurs after PAT-derived auxin maxima formation, suggesting an indirect role of *TAA1/TAR* and *YUC* genes in leaf vein patterning.

3.2. Introduction

Plant leaves have evolved to develop complex venation systems to collect and transport products of photosynthesis and also to distribute water throughout the leaf tissues. In the model organism *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*), the developmental stages of post-embryonic leaf venation system development and their close ties with young leaf (primordium) outgrowth have been characterized (Donelly et al., 1999). Through these steps, ground meristem cells first differentiate into a precursor known as procambium, which then matures into xylem and phloem cells. The fully-developed *Arabidopsis* leaf venation system consists of one midvein and a leaf size-dependent number of secondary, tertiary, and quaternary veins (Donelly et al., 1999).

The canalization of signal flow hypothesis presents a mechanism by which vascular patterning likely occurs (Sachs, 1981). This model suggests that some mobile signal acts as a developmental trigger by first diffusing through growing leaves toward the roots, then promoting the increase of polar transport through only certain queues of cells while draining auxin from adjacent cells through a positive feedback mechanism (Sachs, 1981). Substantial physiological and genetic evidence suggests that auxin (indole-3-acetic acid; IAA) is the transportable signal in question in leaf vein patterning (Mattsson et al., 1999, 2003; Rolland-Lagan & Prusinkiewicz, 2005; Scarpella et al., 2006; Wenzel et al., 2007). The membrane-bound auxin efflux protein PIN-FORMED1 (PIN1; Gälweiler et al., 1998) plays an important role in polar auxin transport (PAT) during leaf vein patterning, on account of vascular hypertrophy in *pin1* mutants (Mattsson et al., 1999) and PIN1 localization studies (Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). There is also evidence that the expression of *PIN1* as well as that of other genes is influenced by concentrated auxin flow, which presages sites of procambial cell stage acquisition in leaves (Aloni et al., 2003; Mattsson et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007).

Unlike the role of PAT, a role of local auxin biosynthesis in leaf vein patterning is not well-established. Two gene families have been attributed to what has recently been uncovered as the main, two-step pathway of IAA biosynthesis in *Arabidopsis* (Mashiguchi et al., 2011; Won et al., 2011). TRYPTOPHAN AMINOTRANSFERASE OF

ARABIDOPSIS 1 (TAA1; also known by its *weak ethylene insensitive* allele, *wei8*) and its two TAA1-related *Arabidopsis* homologs (TAR1 and TAR2) catalyze the first step of converting tryptophan (Trp) to indole-3-pyruvic acid (IPA; Yamada et al., 2009; Mashiguchi et al., 2011; Won et al., 2011). Based on mutant analyses, TAA1/TAR members have roles in shade response, ethylene interactions, embryogenesis, and flower development, and leaf vascular patterning (Stepanova et al., 2008; Tao et al., 2008). Double *wei8-1 tar2-1* and *wei8-1 tar2-2* mutants show leaves with fewer secondary and tertiary veins than the wildtype (Stepanova et al., 2008). Some members of the *YUCCA* (*YUC*) gene family facilitate the rate-limiting conversion of IPA to IAA (Mashiguchi et al., 2011; Won et al., 2011). 11 *YUC* members are known to encode flavin monooxygenases (Zhao et al., 2001) with some of the members having overlapping functions in embryogenesis, seedling development, flower development, and vascular patterning (Cheng et al., 2006, 2007). *yuc1 yuc4*, *yuc1 yuc2 yuc4*, and *yuc1 yuc4 yuc6* mutant leaves contain fewer secondary and tertiary veins than the wildtype (Cheng et al., 2006). The *yuc1 yuc2 yuc4 yuc6* quadruple mutant has an even more severe phenotype with secondary veins ending in dead ends rather than forming continuous vein loops (Cheng et al., 2006).

The spatial expression patterns of *TAA1* and some *YUC* members have been identified in leaves; specifically, both families collectively span the leaf margins (including the hydathodes), leaf base (basal domain), and apical leaf tip (apical domain; Cheng et al., 2006, 2008; Tao et al., 2008). This suggests that TAA1 and YUCs act locally in auxin biosynthesis during leaf development. However, the details of this remain elusive, particularly when evidence suggests that local auxin biosynthesis influences developmental processes in a non-cell-autonomous manner (reviewed in Zhao, 2010). During the final stages of preparation of this thesis, a study by Wang et al. (2011) was published describing a detailed analysis of *YUC* expression patterns in developing rosette leaves to explore leaf margin development, which partially overlaps with our findings. Nevertheless, the timing and relationships between molecular events in auxin transport and auxin biosynthesis during development—including that of leaf venation systems—are not currently known. Is it the formation of auxin biosynthesis maxima by TAA1 and YUCs that leads to auxin transport by PIN1 in defining paths of

leaf vein formation? Or, does local auxin transport initiate local auxin biosynthesis to support continuous vein development?

In this paper, we wanted to better characterize the roles of TAA1 and YUCs in leaf vascular development. Our preliminary approach has involved the high-resolution spatial and temporal mapping of *TAA1* and *YUC* expression during *Arabidopsis* leaf development. So far this has provided us with potentially more detail on the spatio-temporal pattern of auxin biosynthesis. Compared to the previously documented timing and domains of auxin transport and auxin maxima (Aloni et al., 2003; Mattsson et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007), we hypothesize that local auxin transport precedes local auxin biosynthesis in leaf vascular development.

3.3. Results

3.3.1. Auxin biosynthesis genes are expressed in dynamic, overlapping patterns beginning midway through rosette leaf development

We mapped the expression patterns of *TAA1* and the three *YUC* family members, *YUC1*, *YUC2*, and *YUC4* in developing rosette leaves. We used auxin-responsive promoter-GUS constructs that were generated as previously described (Stepanova et al., 2008; Tao et al., 2008; Cheng et al., 2006) and kindly donated by Yunde Zhao (Division of Biological Sciences, University of California San Diego, La Jolla CA 92093, USA) and Joanne Chory (Plant Molecular and Cellular Biology Laboratory, Salk Institute for Biological Studies, San Diego, La Jolla CA 92037, USA).

TAA1 was expressed after the emergence of the leaf primordium but before the appearance of the procambial midvein (Figure 17a-f, Figure 18a-d). As previously reported, we have confirmed that *TAA1* is expressed predominantly at the margins of older leaves (Tao et al., 2008). We have also shown that this auxin biosynthesis gene is expressed during leaf development, when expression is limited to only a small number of cells at the apical domain. As the leaf expands, this domain of *TAA1* expression also appears to expand from the apical tip downwards, but only along the leaf margins. This pattern of expression persists at leaf maturity (Figure 17e, f). There is also *TAA1*

expression at the forming hydathodes when these portions of the leaf are more prominent, as with more serrated third rosette leaves (Figure 18d). Hydathodes are zones of fluid secretion in the mature leaf, which are likely areas of high free auxin levels at some point during leaf growth (Aloni, 2001; Aloni et al., 2003).

YUC1 expression first appeared approximately at the same time as *TAA1* in the leaf primordium, lasting through until leaf maturity (Figure 17, Figure 18). However, *YUC1* expression was observed along the basal half of the primordium and rarely in the apical area (Figure 17g-l, Figure 18e-h), similar to previous findings in older leaves (Cheng et al., 2006). This suggests that YUC1 plays a role in basal leaf primordium growth and vascular development, which is particularly important given evidence of cell divisions occurring at the basal primordium zone until leaf maturity, even when cell division at the apical domain slows (reviewed in Efroni et al., 2010). The localization of *YUC1* expression included the basal portions of the leaf margins during hydathode outgrowth, and remained constant from first appearance until leaf maturity. In the case of more serrated leaves, we have found *YUC1* expression was also localized to a small number of cells flanking the hydathodes, which was not documented for any other gene in our analysis (see red arrows, YUC1 at 14 DAG, Figure 18h). The number of hydathodes in a leaf varies with leaf order, thus first and second leaves sometimes did not have hydathodes or the punctate expression pattern of*YUC1* at the margin.

The expression of *YUC2* was not observed until after the first appearances of *TAA1* and *YUC1*, nearing leaf maturity and the first appearances of procambial cells (Figure 17m-r, Figure 18i-l). Consistent with Cheng et al. (2006) and Wang et al. (2011), we first detected *YUC2* expression at the apical end of the leaf as well as in the midvein procambium (Figure 17q-r, Figure 18j-l). With leaf expansion, *YUC2* expression remained in these domains, even when the midvein had visibly differentiated into vascular elements. *YUC2* is also expressed albeit very faintly in the mature hydathodes (Figure 17r, Figure 18l).

YUC4 expression began after the start of *TAA1* and *YUC1* expression, but before the first signs of *YUC2* expression (Figure 17s-x, Figure 18m-p). Starting approximately during procambial differentiation of the midvein, *YUC4* expression was observed at the

apical end of the primordium. Closer to leaf maturity, expression still occurred at the apical end and also at the hydathodes (Figure 17x, Figure 18p).

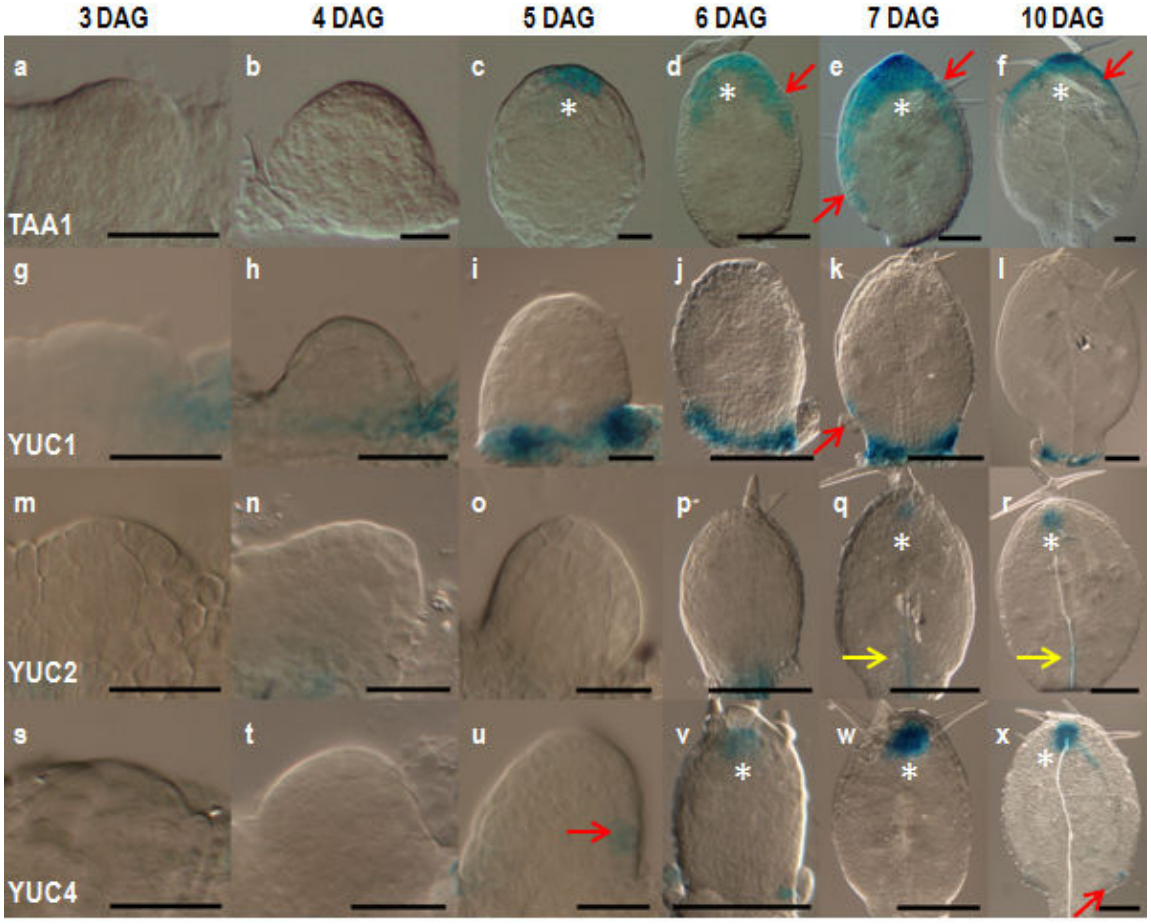


Figure 17. **TAA1-** ***and*** **YUC-promoter::GUS** ***expression in developing*** **Arabidopsis** ***first/second rosette leaves***

All frames are planar median views except for 3 and 4 DAG, which are lateral median views. Rows from the left to the right (e.g. a-f) contain representative samples for each marker line at different stages in leaf development, from youngest to oldest. White asterisks indicate expression at the leaf apical tip. Red arrows mark expression at leaf margins. Yellow arrows mark expression in procambial or vascular tissues. DAG: days after germination. Scale bars = 20 µm at 3-5 DAG; 100 µm at 6-10 DAG.

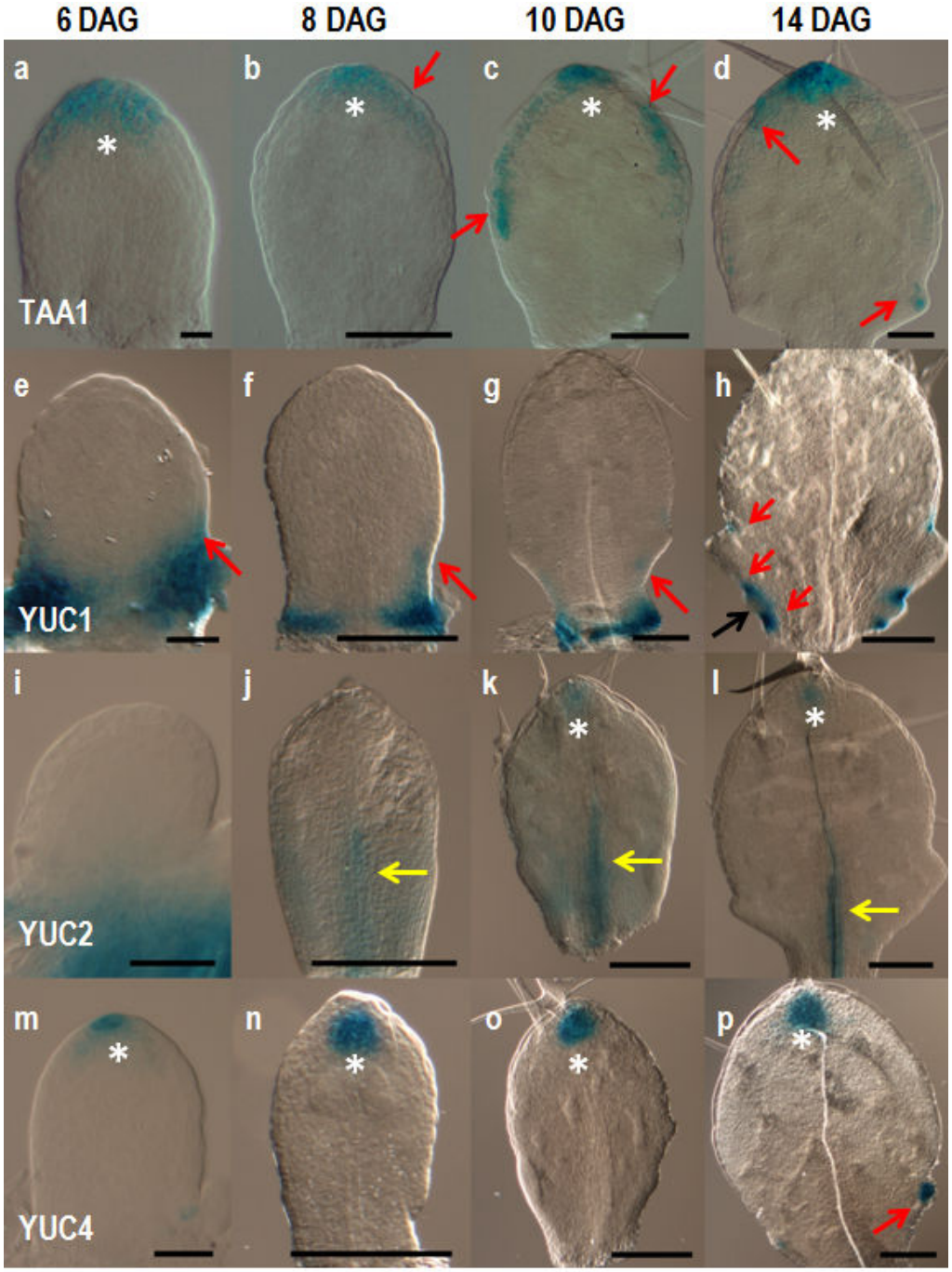


Figure 18. **TAA1-** ***and*** **YUC-promoter::GUS** ***expression in developing*** **Arabidopsis** ***third rosette leaves***

All frames are planar median views except at 6 DAG, which are lateral median views. Rows from the left to the right (e.g. a-d) contain representative samples for each marker line at different stages in leaf development, from youngest to oldest. White asterisks indicate expression at the leaf apical tip. Red arrows mark expression at leaf margins. Yellow arrows mark expression in procambial or vascular tissues. DAG: days after germination. Scale bars = 20 μm at 6 DAG; 100 μm at 8-14 DAG.

3.3.2. Coexpression pairs of TAA1/TAR *and* YUC *members could be required for local, two-step auxin biosynthesis*

The main auxin biosynthesis pathway is a two-step process requiring the aminotransferase activities of TAA1/TAR enzymes to produce IPA and the monooxygenase activities of YUC enzymes to produce IAA (Mashiguchi et al., 2011; Won et al., 2011). Thus, we assume that the coexpression of at least one member of each *TAA1/TAR* and *YUC* gene families in a domain of cells at a given time point is required for local IAA biosynthesis in that domain. We have found that *TAA1* and *YUC4* are expressed in the same apical position during late stages of leaf development (see asterisks, Figure 17; Figure 18). Therefore, we consider *TAA1* and *YUC4* to be a "coexpression pair" in local auxin biosynthesis during leaf development. The late appearance of *YUC2* expression at leaf maturation (Figure 17q-r, Figure 18k-l) has led us to consider *YUC2* as not a major coexpression partner of *TAA1* during vein patterning. As such, coexpression partners for *YUC1* and *YUC2* encoding tryptophan aminotransferases have not yet been identified based on our criteria for congruous spatial and temporal expression. In other words, we have characterized the expression of two *YUC* genes whose encoding enzymatic activities may locally convert the intermediate IPA to IAA, but the source of IPA is not currently known.

3.4. Discussion

Auxin is central to many plant processes, yet little is known about the spatio-temporal distribution of auxin biosynthesis (reviewed in Chandler, 2009; Zhao et al., 2010). Here, we provide preliminary evidence for two gene families of auxin biosynthesis having tightly regulated expression patterns and possibly very specific functions in leaf vascular development.

3.4.1. Partial overlaps of auxin biosynthesis gene expression reinforce the partial functional redundancy of YUC *family members while presenting new questions for local two-step pathways*

Evidence suggests that members of the *YUC* gene family are, in part, functionally redundant in *Arabidopsis* (Zhao et al., 2001; Cheng et al, 2006, 2007). Gain-of-function studies suggest YUC1 facilitates auxin biosynthesis (Zhao et al., 2001) while knock-out mutations in multiple *YUC* loci are needed to observe auxin-deficient developmental defects (Cheng et al., 2006, 2007). Meanwhile, each member may act in a position-specific manner, as suggested by distinct and overlapping gene expression patterns of *YUC1*, *YUC2*, and *YUC4* (Cheng et al., 2006; our data). As well, the rescue of defective plant stature and fertility in multiple *yuc* mutants is possible with the *YUC* promoter-guided expression of a bacterial auxin biosynthesis gene, but not with exogenous auxin application (Cheng et al., 2006, 2007). Our results emphasize these findings at a higher resolution of time and space, suggesting that distinct roles in leaf vascular development may also be subdivided amongst different *YUC* gene family members. Indeed, different *yuc* mutant combinations show different leaf vascular phenotypes, ranging from mild double mutant vein patterning defects to quadruple mutants missing tertiary veins and having incomplete secondary veins (Cheng et al., 2006). We hypothesize that YUC1, YUC2, and YUC4 locally convert IPA to IAA at the developing leaf apical tips, margins, and basal zones, which could collectively influence vascular patterning in leaves via PAT (see below). This would require the strict control of the different, dynamic *YUC* expression patterns we have observed, possibly by transcription factors encoded by genes such as the *SHORT INTERNODES/STYLISH* family.

The widespread expression of *TAA1* at the developing leaf margins coincides only partially with the expression of the *YUC* members studied. We have named *TAA1* and *YUC4* a coexpression pair whose gene products are required for two-step auxin biosynthesis at the apical ends of young leaf primordia. Within this position, we hypothesize that TAA1 locally converts Trp to IPA, which is locally converted to IAA. This begs the question: what are the coexpression partners of *YUC1* and *YUC2*? How is auxin synthesis compartmentalized in developing leaves? Given the functional redundancy of TAA1/TAR members in several developmental processes and the hallmarks of reduced auxin in double *wei8/tar* mutants (Stepanova et al., 2008), perhaps other TAA1/TAR members coordinate with these two YUC members in local IAA production. *TAR1* and *TAR2*, whose expression in 3-day-old seedlings has been documented albeit at a low level of resolution (Stepanova et al., 2008), could potentially be the coexpression partners of *YUC1* and *YUC2*. A detailed analysis of the spatio-temporal expression patterns of *TAR1* and *TAR2* in leaf development may support this hypothesis if these genes are expressed at the same time as *YUC1* and *YUC2* in the basal domain and maturing vasculature, respectively. Other so far unidentified factors could also contribute to local pools of IPA. For example, an alternative IPA auxin biosynthesis pathway may exist in plants involving bacteria-like IPA decarboxylases (Spaepen et al., 2007; Yamada et al., 2009).

Another possibility accounting for missing coexpression partners of *YUC1* and *YUC2* is that the intermediate IPA is a mobile particle. For instance, after conversion from Trp by TAA1 at the apical leaf margin, perhaps IPA is polarly transported towards the leaf base to be oxygenated by YUC1. At this time, however, there is no evidence of polar IPA transport occurring in plants or evidence of any sort of carriers that would facilitate such as process. Alternatively, perhaps IPA diffuses passively through leaf primordia cells. Considering how widespread *TAA1* expression is during leaf development (Figure 17a-f, Figure 18a-d), perhaps ample levels of IPA are produced at the apical leaf primordia margins. The passive intercellular diffusion of this IPA into the primordium centre and base could then trigger local IAA production by YUC1 and YUC2 in a rate-limiting step. The limited distances by which IPA can diffuse—if it can passively move intercellularly at all—still requires much exploration.

3.4.2. *Local auxin biosynthesis may be preceded by local auxin transport in early stages of leaf vein patterning*

Auxin is hypothesized to act non-cell-autonomously, i.e. by being synthesized in some positions in the plant then transported, followed by affecting target cells (reviewed in Zhao, 2010). Leaf patterning is perturbed when PAT is inhibited either chemically (Mattsson et al., 1999; Sieburth, 1999) or genetically (i.e. *pin1* mutants; Okada et al., 1991). The auxin biosynthesis genes we have characterized are expressed at the apical domains and margins of leaf primordia, and though *YUC2* is expressed in vascular tissues, this is not detectable until the bundles are mature. In other words, *TAA1* and the *YUC* genes we have studied are not initially expressed by the cells that are tagged to differentiate into vascular bundles. Nevertheless, multiple *yuc* or *wei8/tar* mutant combinations show leaf vascular phenotypes (Cheng et al., 2006, 2007; Stepanova et al., 2008). We therefore hypothesize that TAA1 and YUCs synthesize auxin locally then non-cell-autonomously contribute to leaf vein patterning through PAT.

Interestingly, the timing of apical expression of *TAA1* and the *YUCs* we have studied is late during leaf outgrowth and midvein patterning, commencing when the leaf primordium is already relatively large (Figure 17c, i, o, u; Figure 18a, e, i, m). Leaf vein patterning and procambium differentiation commences when leaf primordia are small. When a leaf primordium is initiated and first begins to bulge, the first procambial midvein cells can already be identified (Mattsson et al., 1999, 2003, unpublished). The procambial midvein forms from the primordium base, extending towards the apical end, even before the cylindrical leaf primordium has formed a partially flattened, bilateral structure (Donelly et al., 1999; Mattsson et al., 2003). Our evidence suggests that *TAA1* and *YUC1*, the genes whose expression we detected the earliest during primordia development, were expressed during the start of the shift in leaf form from cylindrical to planar (Figure 17c, i; Figure 18a, e). In line with this, the *yuc1 yuc2 yuc4 yuc6* mutant leaves observed by Cheng et al. (2006) were lacking in secondary and tertiary veins, but their fully developed, continuous midveins remained. Expanding on our hypothesis, we propose that TAA1 and YUCs contribute to the patterning of the later-formed secondary, tertiary, and quaternary leaf veins. We hypothesize that patterning of the midvein is influenced more by PAT-derived auxin maxima than by local biosynthesis-initiated auxin maxima. PIN1 proteins are expressed during leaf primordium initiation and the earliest

stages of outgrowth (Reinhardt et al., 2003), simultaneously with PIN1 expression during the earliest stages of midvein patterning (Donelly et al., 1999; Scarpella et al., 2006; Wenzel et al., 2007). *pin1* mutants show hypertrophic midvein formation, thought to be a consequence of poor auxin drainage and apical signal accumulation (Mattsson et al., 2003).

How exactly do TAA1 and YUCs influence higher-order vein formation? How do auxin biosynthesis and auxin transport coordinate in differentiating ground meristem cells into secondary, tertiary, and quaternary veins? The potential synergy of PAT and auxin biosynthesis in leaf development is evident in *yuc1 yuc4 pin1* triple mutants (Cheng et al., 2007), and *yuc1 yuc4*, *wei8-1 tar2-1* or *wei8-1 tar2-2* double mutants grown on NPA, which all fail to form leaves entirely (Won et al., 2011). In older leaf primordia, the lateral margins are hypothesized to first become primary sites of free auxin accumulation by PAT, then somehow auxin biosynthesis is triggered to make the leaf margins auxin sources (Aloni, 2001; Aloni et al., 2003; Cheng et al., 2006). *PIN1* expression could be important in this proposed phenomenon, as the subcellular localizations of PIN proteins at the leaf margins suggest paths of PAT meet at marginal convergence points (Scarpella et al., 2006). Convergence points are associated with the patterning of the first secondary veins marked by cell files of *PIN1* expression (Scarpella et al., 2006; Wenzel et al., 2007) while it is hypothesized that auxin biosynthesis at the margins influences the formation of later-formed, more basal secondary veins of rosette leaves (Cheng et al., 2006; Sawchuk et al., 2007) and perhaps the smaller tertiary and quaternary veins as well. Spatio-temporal analyses of leaf and flower primordia have shown that *PIN1* expression domains and subcellular PIN1 orientations are very dynamic, correlating with shifts in *DR5::GUS* expression (DR5, a marker of auxin response; Ulmasov et al., 1997) during primordia development or in response to exogenous auxin application (Heisler et al., 2005; Scarpella et al., 2006). *PIN1* expression, therefore, depends on auxin distribution, which depends on auxin transport—a positive feedback loop potentially at play in shoot development (Heisler et al., 2005; Scarpella et al., 2006). Based on these and our findings, we hypothesize that PAT precedes auxin biosynthesis in the patterning of secondary and tertiary leaf veins. Epidermal PIN1 proteins first transport auxin to the very young leaf primordium apex, then the localizations of these auxin carriers shift to restricted zones at the primordium

base and margins upon the first signs of secondary vein patterning (Scarpella et al., 2006; Wenzel et al., 2007). *PIN1* expression domain restriction, reinforced by positive feedback, would result in auxin accumulation. Then, we hypothesize that the onset of auxin biosynthesis by TAA1, YUC1, and YUC4 at the margins would contribute to local auxin pools, forming auxin maxima. These PAT-derived, biosynthesis-supported auxin maxima act as auxin sources, triggering auxin flow by PIN1 towards midvein procambium sinks in the patterning of secondary veins. Decreased auxin in the margins would limit auxin transport from source maxima and disrupt secondary vein differentiation, as seen in *yuc1 yuc4* mutants with decreased secondary vein numbers (Cheng et al., 2006). We also hypothesize that YUC2 and YUC6 could play supportive, compensatory roles in this process since these *YUC2* is expressed relatively late during leaf development and *YUC6* is not expressed in wildtype leaves (Cheng et al., 2006) but *yuc1 yuc2 yuc4 yuc6* mutant venation is more defective than in *yuc1 yuc4* mutants (Cheng et al., 2006).

3.5. Materials and Methods

3.5.1. Plant material and growth

Columbia (Col) ecotype *Arabidopsis thaliana* seeds containing the auxin-responsive promoter-GUS fusions TAA1::GUS were kindly donated by Joanne Chory, Plant Molecular and Cellular Biology Laboratory, Salk Institute for Biological Studies, San Diego, La Jolla CA 92037, USA. YUC1::GUS, YUC2::GUS, and YUC4::GUS lines were kindly donated by Yunde Zhao, Division of Biological Sciences, University of California San Diego, La Jolla CA 92093, USA.

Seeds were sterilized in sealed gas chambers as described by Clough & Bent (2001). The seeds were plated, at a density of no more than 20 per petri dish, on plant agar media [0.5 × Murashige and Skoog salts with vitamins, 1.5%(w/v) sucrose, 0.5g/L 2-(N-morpholino)-ethane sulfonic acid (MES) buffer (Duchefa Biochemie), adjusted to pH 5.7 using KOH, 0.8%(w/v) plant agar (Duchefa Biochemie)], then placed at 5°C for 2d for stratification. Seeds were then germinated at 22°C under white fluorescent light (170-180 μE m^{-2} s^{-1}), for 16h light and 8h darkness at 50% RH. Leaf primordia for the

first, second, and later-formed rosette leaves were dissected from representative seedlings in sterile water each day from the moment they were visible (3 DAG for the first leaf) until the later-formed leaves were mature (14 DAG).

3.5.2. GUS assay and analysis

Dissected leaf primordia were incubated at GUS substrate solution: 50mM sodium phosphate, pH 7, 5mM K3/K4 FeCN, 0.1% (w/v) Triton X-100, and 0.67mM, 2mM or 4mM 5-bromo-4-chloro-3-indolyl-beta-GlcUA. The samples then underwent air evacuation, then incubation at 37°C for 1.0h to 24.0h depending on colour development. Longer staining periods were needed for younger primordia in order to observe the faint initiation of expression of auxin biosynthesis genes, for example. After, samples were rinsed of excess substrate by immersion in sterilized water at room temperature for 1h, then samples were fixed at 4°C overnight in 100% ethanol:acetic acid (6:1, v/v). They were then washed twice in 100% ethanol then dissected in 70% (v/v) ethanol. For GUS expression analysis, leaf primordia were mounted in clearing solution (chloral hydrate:glycerol:water, 9:1:3 [w/w/v]), heat-fixed, and observed using differential interference contrast (DIC) settings on a Nikon Eclipse E600 microscope. Photographs were taken using a Canon EOS 5D Mark II digital camera, then assembled using Adobe Photoshop.

4. Discussion of Research

The primary focus of my thesis was to explore the roles of auxin biosynthesis in *Arabidopsis* leaf vein patterning. I have mapped the spatial and temporal expression patterns of members from three relevant families of genes: *SHI/STY*, which includes factors involved in the transcriptional regulation of genes encoding auxin biosynthesis enzymes, and; *TAA1* and the *YUCs*, an aminotransferase and several flavin monooxygenases, respectively, that together convert Trp to IAA. I have also analysed the venation phenotypes of *shi/sty* mutants and changes in *SHI/STY* expression in response to altered auxin transport and exogenous auxin application. Although these studies encompass a limited number of genes, the timing of expression events as well as phenotypes suggest that auxin homeostasis may play a role in vein patterning, and may act primarily in the subsequent differentiation of veins. Regulation of auxin biosynthesis is also likely to contribute indirectly to vein patterning, by providing auxin for auxin transport-dependent auxin maxima formation, and subsequent vein patterning.

4.1. Evidence for spatio-temporal coexpression of *SHI/STY*, *YUC*, and *TAA1* genes

The genes I have studied display expression patterns that are spatially and temporally distinct in leaf primordia, with each gene's transcription covering discrete domains of cells (Figure 5; Supplemental Figure 1; Figure 17; Figure 18). Some of these domains overlap with one another, identifying coexpression domains indicative of potential functional units. Taken together, results from Chapters 2 and 3 show that *SHI*, *STY1*, *STY2, YUC4*, *TAA1*, and DR5 expression largely coincides at the apical tips of rosette leaf primordia (Figure 5a-c, g-k, m-r; Figure 12 a, d, g, m; Figure 17c-f, v-x). Domains of *STY1*, *YUC1*, and *TAA1* expression are also shared at mid and basal margins of the primordia, including sections flanking the hydathodes in mature leaves (Figure 5i-l; Supplemental Figure 1f-h; Figure 17e, k; Figure 18d, h). The developing

hydathodes express *SHI*, *STY1*, *STY2*, *TAA1*, *YUC4*, and DR5 (Figure 5e-f, j-l, r; Supplemental Figure 1b-d, f-h, k-l; Figure 13a; Figure 17e, x; Figure 18d, p). Since there is evidence for direct, transcriptional regulation of *YUC4* by STY1 (Sohlberg et al., 2006; Eklund et al., 2010, 2011), the expression of *STY1* is positively correlated with seedling auxin levels (Sohlberg et al., 2006; Ståldal et al., 2008), and recent evidence suggests that YUC and TAA1 proteins act together to synthesize IAA (Mashiguchi et al., 2011; Won et al., 2011), I hypothesize that these three groups of genes may form functional units in the regulation of auxin homeostasis at the apical and hydathode regions of developing leaf primordia.

It must be noted that the expression patterns of *SHI/STY*, the auxin biosynthesis genes, and DR5 are not purely synchronous. The general trend in my data appears to be that the transcription of *SHI/STY* members I have analysed precedes the expression of *TAA1* and the *YUCs* by at least two days (Figure 5; Supplemental Figure 1; Figure 17; Figure 18) while previous studies suggest that DR5 is expressed from the first emergence of the leaf primordium until leaf maturity (Mattsson et al., 2003). For example, the overlap in expression of *STY1* and *YUC4* in leaf primordia is limited to a brief period at the apical tip of the leaf, approximately during early midvein differentiation (Figure 5j, k; Figure 17v, w). I therefore present two alternative though not necessarily mutually exclusive hypotheses as follows.

Expanding upon my hypothesis above, STY1 and other SHI/STY members may only activate specific auxin biosynthesis gene expression at certain points in time in specific primordium domains, in a tightly regulated manner. Expression of *TAA1* and *YUCs* very late in leaf primordia development—when *SHI/STY* members are not expressed—could indeed be regulated by other factors. Those factors that regulate *TAA1* have not yet been characterized, but *YUC2* and *YUC4* are known to also be regulated by factors such as LEAFY COTYLEDON2 (LEC2) and the NGATHAs (NGAs). While their roles in rosette leaf vein patterning have not yet been identified, *LEC2* and some *NGA* members are expressed in young leaves and their mutations are associated with altered cotyledon or gynoecium morphology—types of shoot organs (Stone et al., 2001, 2008; Alvarez et al., 2009; Trigueros et al., 2009). As well, the spatio-temporal expression patterns of other *YUCs* (YUC5, 8, 9, 10, 11) and *TAR1/2* have not yet been analyzed in developing leaves, and as I have already mentioned in Chapter 3 these may

potentially overlap more adequately with *SHI/STY* expression. The limited coexpression of genes shown by my results could merely be a glimpse of the many possible functional units of auxin homeostasis involved in leaf vein development.

Another possible explanation for my observations is that SHI/STY transcripts or proteins may have to accumulate to a threshold level before they can overcome repression by currently unknown factors, such as repressor proteins and/or small RNAs (reviewed in Voinnet, 2009), in order to cell-autonomously activate *TAA1* and *YUC* expression. Or, perhaps SHI/STY transcripts or proteins could act non-cell-autonomously (concepts reviewed in Xu & Jackson, 2012). It has already been suggested that STY1 may be mobile at the transcript or protein level in the maintenance of SAMs (Eklund et al., 2010) while transcription of this transcription factor occurs at points of incipient cotyledon formation (Figure 4i, j; Kuusk et al., 2002). If this is the case, it is possible that some SHI/STY family members can act non-cell-autonomously in certain scenarios in the positive regulation of auxin biosynthesis genes. Specifically, *SHI/STY* genes could be translated or merely transcribed at the apical leaf primordium tip, transported intercellularly, then bound to activate the expression of *YUCs* and maybe *TAA1* at the primordium margins. While this mechanism is a possibility, my results favour my initial hypothesis as I explain in the next section.

I must mention that these hypotheses are mainly based on the spatio-temporal expression patterns I have analysed in *Arabidopsis* rosette leaves. I have found similar expression patterns of each *SHI/STY* gene member studied in both cotyledonary and rosette leaf primordia (Figure 4; Figure 5), suggesting that this gene family influences vein development embryonically and post-embryonically. The corresponding mutant cotyledon phenotypes of the *YUC* and *TAA1/TAR* gene family members whose expression patterns I have investigated during rosette leaf development (*YUC1*, *2*, *4* and *TAA1*) have not yet been studied. However, the spatio-temporal expression patterns have been analysed for *YUC1* and *4* during embryogenesis (Cheng et al., 2007) which do appear to be similar to the results I have obtained for *YUC1* and *4* expression during rosette leaf development (Figure 17g-l, s-x; Figure 18e-h, m-p). It is quite likely, therefore, that expression overlaps of *SHI/STYs*, *YUCs*, and *TAA1/TARs* are the same in cotyledon and rosette leaf primordia and that my hypotheses in this chapter apply to both embryonic and post-embryonic leaf development.

4.2. *SHI/STY* genes act non-cell-autonomously in vein development

The *SHI/STY* genes are expressed primarily at the apices of leaf primordia as well as in developing hydathodes. Yet, when mutant combinations in these genes are generated, we see defects in the differentiation of internal veins—some distance away from the domains of expression. This discrepancy in expression and phenotype strongly suggests non-cell autonomous functions of the *SHI/STY* gene products. Although there are instances of known cell-to-cell movement of transcription factors in meristems and young leaf primordia (reviewed in Xu & Jackson, 2012), a more plausible scenario in this context is that the SHI/STY genes act cell autonomously in the regulation of *YUC* genes, which in turn act on auxin homeostasis. Auxin, of course, is well known to act non-cell-autonomously via polar auxin transport (reviewed in section 1.1.4). The reduced auxin flux in *shi/sty* mutants may then impart the aberrant differentiation of leaf veins, a process well known to depend upon auxin (reviewed in Fukuda, 2004).

My leaf venation phenotype analyses have shown that disrupted *SHI/STY* function is associated with higher frequencies of discontinuous, defective ULDs of secondary veins, but not necessarily for the LLDs. Defects in LLDs occur occasionally in wildtype leaves at frequencies that do not significantly differ from the mutants (Supplemental Figure 2), but typical wildtype leaves have the distal portions of their secondary veins in continuous files of differentiated vascular cells. *shi/sty* mutant leaves, particularly the cotyledons, frequently have these distal vein portions missing (Figure 6k-l; Figure 7; Supplemental Figure 4). This could be explained by observations of these ULDs frequently forming after LLDs during wildtype leaf development, with both patterning events associated with PIN1-influenced PAT (Figure 3b, c; Scarpella et al., 2006). ULDs may therefore depend more on the auxin synthesis-supported maintenance of auxin maxima, in which SHI/STY genes may play a role, rather than earlier polar auxin transport-driven auxin maxima formation (see section 3.4.2 and below). In addition, the strength of auxin flow may be more limited during the formation and differentiation of ULDs in general compared to LLDs because: (1) earlier-forming, developing LLDs are presumed to already act as competing auxin sinks, depleting auxin from surrounding cells (Scarpella et al., 2006), and; (2) developing ULDs connect with the apical portion of the midvein (Scarpella et al., 2006) distant from high auxin given the

predicted general trend of apical to basal flow of auxin in leaf primordia (Reinhardt et al., 2003). There is also evidence that a narrow window of competence exists for PIN1 expression activation in maturing leaves (Scarpella et al., 2006) while ground meristem tissues differentiate and cell divisions decrease in a basipetal (apical-to-basal) wave nearing leaf maturity (Donnelly et al., 1999). Taking this into consideration, decreased *SHI/STY* function may make developing leaves particularly sensitive to disruption in ULD development, compared to LLD development. A decrease in the positive regulation of *YUC* expression by SHI/STY could result in a decrease in auxin biosynthesis at the primordium apical and marginal zones, where members of both gene families are expressed (Figure 5; Figure 17), leaving less auxin to be polarly transported and focused near the apical tip to form the ULDs of secondary vein loops. Similar speculation could be made for the higher numbers of free-ending veins found in *shi/sty* leaves (Table 1, Table 2), i.e. decreased locals auxin levels would not be sufficient for the differentiation of closed tertiary vein loops.

The distal peg phenotype of *shi/sty* mutant leaves could also be attributed to a decrease in local auxin biosynthesis (Figure 6e, f, i-l; Figure 9; Figure 10; Figure 11e, f, i-l). Midvein formation is associated with the concentration of PIN1 expression in a file of cells at the centre of the young leaf primordium, which presumably focuses PAT and promotes procambium differentiation (Figure 3a; Reinhardt et al., 2003; Scarpella et al., 2006; Smith et al, 2006; Wenzel et al., 2007). Then, as I mentioned in the previous paragraph, the secondary veins likely form with the LLDs depending on auxin transport-driven auxin maxima at the margins and ULDs relying on maxima maintained by auxin biosynthesis (Figure 3b, c). This hypothesized, normal shift in relative contribution to local auxin levels at the primordium margins can be correlated with the hypothesized transition of auxin production from the apical tip to the leaf blade margins (Figure 3a, b, c; Aloni et al., 2003). If SHI/STY members normally and cell-autonomously activate the expression of *YUC* or other auxin homeostasis genes at the apical ends of leaf primordia, then defective SHI/STY activity in *shi/sty* mutants would also decrease local auxin levels at primordium apices. Insufficient PAT from this position could affect the concentration of PIN1 expression into discrete cell files during midvein formation, resulting in fewer differentiated procambial cells at the apical leaf lamina which we see in the basally displaced vascular systems of *shi/sty* cotyledons that have distal pegs

(Figure 10). This decrease in local auxin as well as that in the leaf margins could cause secondary vein ULD fusion with the midvein at a more basal region, since relatively more auxin would be situated along the midvein procambium, giving the appearance of a distal peg at vascular maturity. In *sty1-1 shi-3* mutants, the presence of distal pegs that are more likely apical extensions could be an intermediate phenotype where perturbed auxin homeostasis still causes the aforementioned shift in the point of secondary vein-midvein fusion. Local auxin levels in relation to the elongated *sty1-1 shi-3* midvein could be investigated further by analysis of DR5 expression in a *sty1-1 shi-3* background.

Decreased local auxin biosynthesis at the apical ends of leaf primordia may explain the bifurcated/skewed midvein phenotype seen in *shi/sty* mutants (Figure 6i-l; Figure 8; Figure 11h, k, l). Midvein development is hypothesized to result from the concentrated, basipetal flow of auxin from a convergence point at the apical tip along a narrow file of ground meristem cells (Figure 3a; Sachs, 1981; Mattsson et al., 1999, 2003; Reinhardt et al., 2003; Scarpella et al., 2006). Two midvein portions that fork from the primordium centre could result from relatively stronger influences on midvein formation by auxin at more lateral positions than the central apical end. This could mean that the apical auxin convergence point, like marginal convergence points, correlate with auxin maxima initiated by auxin transport but maintained by auxin biosynthesis. Genes expressed at the centre of the apical end early in leaf development—*SHI*, *STY1*, *STY2*, and *TAA1*—could be in part responsible for the local generation of auxin at the centre apical domain. Without the proper function of these genes' products in *shi/sty* mutants, perhaps other gene families contribute to relatively higher rates of auxin biosynthesis at the leaf primordium margins. As I stated in the previous section, other genes such as *LEC2*, the *NGAs*, and *TAR1* and *2* may be good candidates for genes encoding other functional units in leaf vein development. There are likely other so far unidentified but relevant genes expressed at the leaf margins as well.

The leaf venation systems of quintuple *shi/sty* mutant leaves are abnormal, showing distal pegs, more free vein ends than the wildtype, and occasional ULD defects and bifurcated/skewed midveins (Figure 11l). Interestingly, vein development in these mutants appeared to be neither hypersensitive nor hyposensitive to chemical inhibition of PAT by NPA (Supplemental Figure 5). In agreement with my previous interpretations, I hypothesize that the apparent non-differential response of *shi/sty* quintuple mutant leaf

venation to chemical PAT inhibition is due to functional redundancy of the SHI/STY family with other regulators of auxin homeostasis. SHI/STY members are not the only potential transcriptional regulators of *YUC* expression (see 1.1.2; Figure 2). The regulation of a rate-limiting step in auxin biosynthesis is likely complex and redundant (Zhao et al., 2001, 2002; Cheng et al., 2006, 2007) and YUCs may not be the only enzymes that facilitate the biosynthesis of auxin in the leaf. Continuing genetic screens and functional studies are needed to uncover what is likely numerous regulatory and auxin homeostasis pathways in leaf vein development.

4.3. *SHI/STY* genes may act to transform polar auxin transport-driven auxin maxima into auxin biosynthesis-supported auxin maxima

The genes I have analysed here in the context of leaf vein development encode components involved in auxin homeostasis. Integration of my results with the spatio-temporal expression patterns of the auxin efflux carrier PIN1 (Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007) suggests a distinct sequence of auxin transport and auxin biosynthesis events occurs during leaf primordia development and contributes to leaf vein development (Figure 19), to which I have already alluded in this chapter. PIN1 is subcellularly localized throughout leaf primordium development suggesting directional PAT at the sites of vein formation (Figure 3; Figure 19a; Scarpella et al., 2006; Wenzel et al., 2007). The auxin response factor MP is expressed at similar sites, suggesting a feedback loop of auxin perception and auxin transport takes place (Figure 19a; Wenzel et al., 2007). As the leaf primordium bulges out adjacent to the SAM, the apical converge point—that corresponds to an auxin maximum—emerges as marked by PIN1 localization suggesting two channels of PAT at the epidermis collide and deflect towards the leaf centre and base (Figure 3; Figure 19a; Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). While the leaf primordium grows into a cylindrical shape, *PIN1* expression at the primordium centre concentrates to a narrow streak of ground meristem cells, suggesting canalization of auxin flow (Figure 3; Figure 19a; Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). At this early stage in development, these central cells expressing *PIN1* may begin to differentiate acropetally into the procambial midvein, according to observations of cell divisions

through planes along the axis of the incipient midvein (Mattsson et al., 1999, 2003, unpublished). It is only at this point that, with the exception of *STY2*, *SHI/STY* gene family members, *TAA1*, and *YUC4* begin to be expressed at the apical convergence point (Figure 19a). This leads me to hypothesize that patterning of the midvein relies primarily on the transport-driven auxin maximum at the leaf apical tip. Since they are expressed relatively late, the auxin biosynthesis genes I have studied could contribute to the apical auxin maximum, but by maintaining local auxin levels to uphold signaling strength (Figure 19c). Based also on our knowledge of the timing of the appearance of the procambial midvein (Donelly et al., 1999; Mattsson et al., 1999, 2003, unpublished; Kang & Dengler, 2002; Sawchuk et al., 2007), I hypothesize that *SHI*, *STY1*, *SRS5*, *TAA1*, and *YUC4* primarily influence later stages of vein patterning and vein differentiation in leaf vein development (Figure 19c).

Before the growth of the leaf primordium into a semi-flat, bilateral structure, *PIN1* expression is detected at distinct positions at the leaf margins (Figure 3b; Figure 19a, b; Scarpella et al., 2006; Wenzel et al., 2007). These correlate to new convergence points and auxin maxima, as well as the differentiation of secondary procambium (Scarpella et al., 2006; Wenzel et al., 2007). As with what appears to be a delayed onset of auxin biosynthesis at the apical auxin maxima, only *STY1* and *TAA1* may be expressed early at the marginal auxin maxima, while the expression of other *SHI/STY* gene members, *TAA1*, and *YUC1* and *4* appear later as the hydathodes emerge (Figure 19a, b). These expression patterns and the sensitivity to defects of the ULDs of *shi/sty* mutant leaf secondary veins (Figure 6k, l; Figure 7; Supplemental Figure 3) lead me to hypothesize that auxin biosynthesis is required for the maintenance of auxin transport-driven maxima at the margins, as I mentioned previously (see 4.2). The patterning of the secondary veins could be initiated by auxin transport, but auxin homeostasis may be required for incipient vein pattern maintenance and procambium differentiation (Figure 19c).

The specific mechanisms by which *SHI/STY* family and auxin biosynthesis genes contribute to auxin maxima maintenance and leaf vein development could potentially be predicted by identifying their expression patterns and functions. As I have already noted in this section, previous studies have suggested that polar auxin transport facilitated by MP and PIN1 presages sites of leaf midvein formation (see also section 1.2.1). My research has shown that *STY2*, in the rosette leaves only, is also expressed along the

future midvein early in rosette leaf development (Figure 5m-o), suggesting STY2 is involved with MP, PIN1, and auxin in midvein development (Figure 19a). However, my results indicate that *sty2-1* single mutants display very mild leaf venation defects and severe bifurcated/skewed midvein phenotypes are seen only in triple and quintuple *shi/sty* mutants (Figure 8k-m). I hypothesize, then, that there may be a role for *STY2* in midvein formation, but a functionally redundant one. The downstream targets of STY2 have not been yet been identified, but it is likely that STY2 may function to regulate auxin biosynthesis like STY1 based on genetic analyses and sequence similarity (Kuusk et al., 2002, 2006; Sohlberg et al., 2006). STY2 could, therefore, positively regulate local auxin biosynthesis at the incipient midvein during procambium differentiation. A potential purpose of this could be to increase local free IAA levels at the incipient midvein, increase MP activity, increase polar PIN1 expression to subsequently increase auxin canalization, perhaps as part of some sort of buffering mechanism against abnormal auxin fluxes (dashed line, Figure 19c). In this case, we would expect the localization of PIN1 proteins in multiple *shi/sty* mutants containing non-functional *STY2* alleles to be abnormal and unfocused. Since even quintuple *shi/sty* mutants show mild venation defects, more drastic alterations to PIN1 expression could potentially be achieved by dominant negative repressor constructs that knockdown multiple *SHI/STY* genes at once such as the 35S::STY1-SRDX fusion used by Eklund et al. (2011).

There is an interesting overlap in the expression patterns of *STY1*, *YUC1*, *TAA1*, and *PIN1* at the leaf primordium margins. When the primordium has expanded to a point when the midvein differentiation just begins, *STY1* and *YUC1* are both expressed at the leaf base and basal leaf margins (Figure 19a, b). Previous studies on *Arabidopsis* rosette leaves have shown that the expression of *PIN1* occurs in similar margin domains with similar timing (Figure 19b; Scarpella et al., 2006; Wenzel et al., 2007). Mutant phenotype analysis (Cheng et al., 2006) and my expression pattern studies (Figure 17; Figure 18) suggest that YUC1 does not play a large role in midvein patterning, whereas evidence suggests that PIN1 does (Mattsson et al., 1999; Reinhardt et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). However, these experiments along with my data have led me to hypothesize that STY1, YUC1, and PIN1 may more likely coordinate in the patterning of leaf secondary vein loops. In cells flanking the hydathodes as they develop at the basal leaf margin (Figure 19b), YUC1 may be transcriptionally and cell-

autonomously activated at least in part by STY1. Increased levels of YUC1 could increase the rate-limiting conversion of IPA—provided by TAA1 activity in a nearby, slightly more apical leaf margin position (Figure 19b)—to free IAA. Since auxin activates the transcription of *PIN1* via MP signalling (Wenzel et al., 2007), the generation of auxin biosynthesis maxima at the basal half of the leaf margins would increase local *PIN1* expression, for which evidence has been found (Scarpella et al., 2006; Wenzel et al., 2007). As well, other studies suggest that free auxin levels come to maxima at the developing hydathodes (Aloni et al., 2003; Mattsson et al., 2003). I hypothesized in Chapter Two that a cell-autonomous positive feedback loop may occur at auxin maxima, where *PIN1* may indirectly activate *SHI/STY* expression which in turn activates *YUC* expression and auxin biosynthesis (see section 2.4.1). Considering also evidence from Chapter Three and previous work with PIN1, I hypothesize that auxin homeostasis contributes to secondary leaf vein development (Figure 19c). Increased auxin biosynthesis by TAA1 and YUC1 at the leaf margins, regulated by STY1, could activate and refine PIN1 expression in order to maintain marginal auxin maxima, the canalization of auxin transport, and the differentiation of narrow cell files into vascular bundles (Figure 19c). This mechanism likely occurs in a functionally redundant manner with other TAA1/TAR, YUC, and SHI/STY gene family members, given the dose-response dependence of secondary vein formation on TAA1/TAR (Stepanova et al., 2008), YUC (Cheng et al., 2006) and SHI/STY (Chapter 2) family activities. It would be interesting to analyze the leaf venation of plants with *pin1* and non-functional alleles from all three auxin biosynthesis gene families I have studied here.

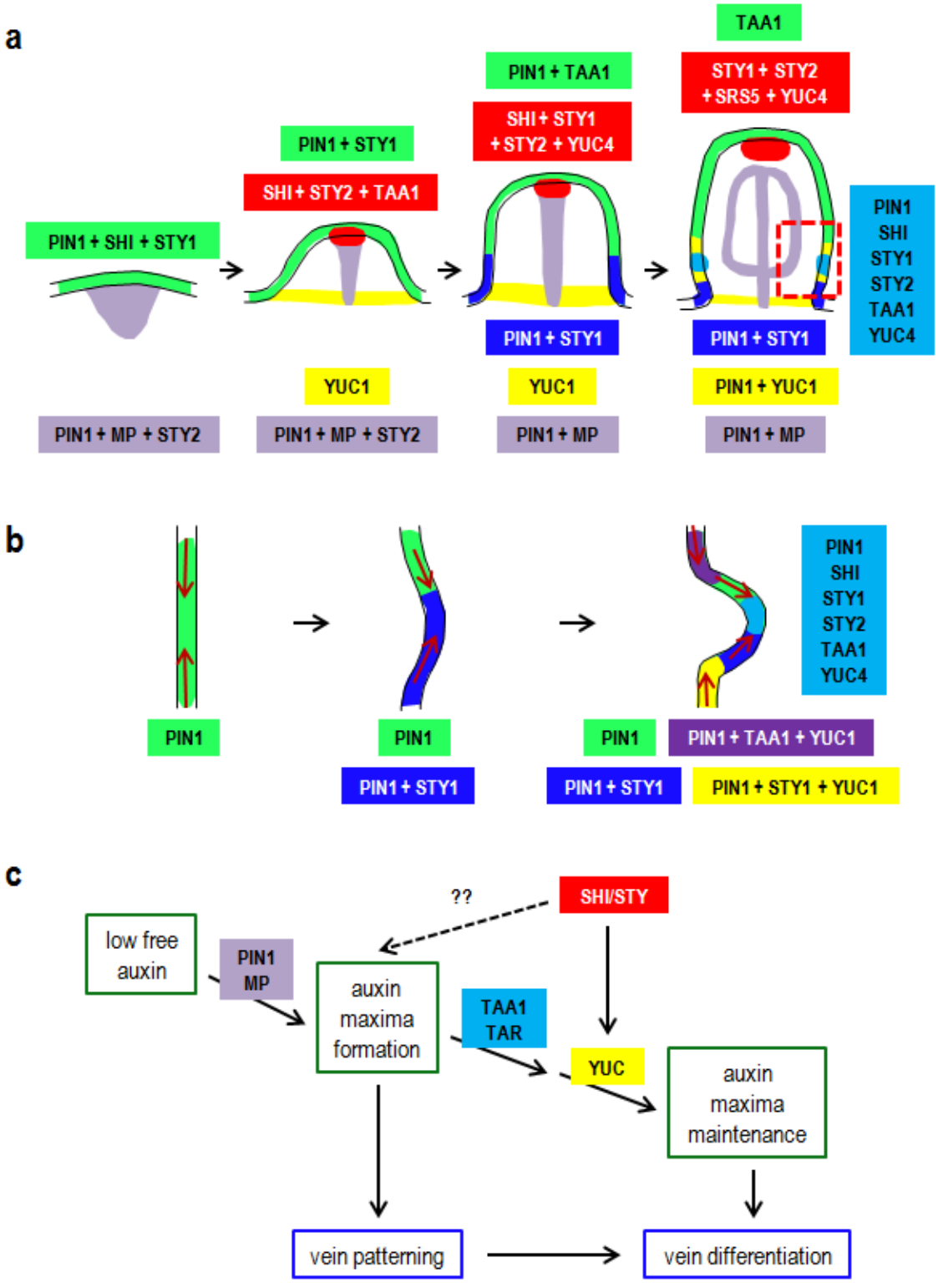


Figure 19. ***PIN1, SHI/STY, and auxin biosynthesis gene expression in a module for leaf vein development***

(Figure 19, continued from previous page.) The spatio-temporal gene expression patterns and related mutant phenotypes of the listed genes suggest they have roles in leaf vein development. Groupings of genes that are expressed in the same positions are described in coloured text boxes above and below the pictures of developmental stages at which expression overlap occurs, corresponding to domains of expression of the same colour. For example, in the first frame of (a), *PIN1*, *MP*, and *STY2* (violet) are all expressed in the incipient midvein (also in violet) during an early stage of leaf primordium outgrowth.

a: *SHI*, *STY1*, and *STY2* expression collectively overlaps with that of *PIN1* and *MP* during an early stage of leaf outgrowth, contributing somehow to an apical auxin maximum and midvein development. At the next stage, *STY2* is still expressed at the incipient midvein while *TAA1* expression begins at the apex and *YUC1* expression is visible at the basal domain. As the leaf primordium expands and starts to take on a semi-flat appearance, *TAA1*and other *SHI/STY*s and *YUCs* are expressed at various apical, lateral, and basal portions of the margins—onsets that may be associated with the shift from PAT-driven auxin maxima to biosynthesis-maintained auxin maxima (see c).

b: A close-up of the red dash-outlined box in (a) is shown starting at an earlier stage, with red arrows symbolizing the direction of PAT. Evidence suggests that PIN1 initiates auxin convergence points at the margins (Scarpella et al., 2006; Wenzel et al., 2007). When the leaf hydathode begins to emerge, *STY1* is expressed in the basal leaf margins. This is followed by *YUC1* expression at the basal leaf margins, *TAA1* expression at the more apical leaf margins, and the expression of other auxin biosynthesis-related genes at the hydathode bulge that may influence secondary vein ULD formation.

c: Proposed cascade of gene activity (coloured boxes) and developmental events (hollow boxes) in leaf vein development. Low free auxin levels in cells adjacent to the SAM are canalized via MP and PIN1 to form auxin maxima in leaf primordia that influence vein patterning. SHI/STY members may regulate YUCs that maintain these auxin maxima through auxin homeostasis to influence the differentiation of patterned veins. SHI/STY members may also somehow influence auxin maxima formation (dashed line), for example during midvein development.

4.4. Limitations in the use of transcriptional fusion constructs in gene expression analyses leave possible alternatives to the model for *SHI/STY* members in leaf vein development

My evidence so far favours a hypothesis for a cell-autonomous role for SHI/STY family members in *YUC* gene activation and a related non-cell-autonomous role for auxin in leaf vein development. However, the techniques I have used in my research do not necessarily show the whole picture of molecular mechanisms involved in *SHI/STY* expression and function. The four GUS marker lines I have used to analyse *SHI*, *STY1*, *STY2*, and *SRS5* expression are transcriptional marker lines, each having a construct of 2 kb genomic upstream *cis* regulatory sequence fused to the open reading frame of the *uidA* reporter gene (Fridborg et al., 2001; Kuusk et al., 2002, 2006). While these constructs could contain the regulatory sequences required by *SHI/STY* genes for normal expression, such as their conserved GCC box-like elements (Eklund et al., 2011), they do not include whatever embedded and downstream elements may exist in each member sequence. The spatio-temporal expression patterns shown by these GUS marker lines may or may not, therefore, accurately represent the expression of their corresponding endogenous genes.

An inconsistency has been previously established between an *LRP1::GUS* marker line expression and expression analysis by reverse transcription-PCR (RT-PCR) and microarray, where the first method suggests *LRP1* expression is lateral root primordium-specific while the latter two suggest it is expressed also in floral organs (Smith & Fedoroff, 1995; Schmid et al., 2005; Kuusk et al., 2006). The inadequacy of this *LRP1::GUS* line as a marker of *LRP1* expression is indicative of a need for supplementary experiments in my expression analyses of the other *SHI/STY* gene members. *in situ* hybridization analyses, for example, can mark tissue domains of gene transcription as per endogenous gene expression. It could also be valuable to compare the expression patterns of transcriptional marker lines with translational marker lines, in case SHI/STY transcripts are localized differently from SHI/STY proteins. If there happens to be a large discrepancy between my promoter-GUS expression data and future *in situ* and translational fusion analyses, then alternative hypotheses to what I have postulated in section 4.2 should be considered. Perhaps, for example, SHI/STY

proteins are transported from source cells to effected cells, with SHI/STY proteins themselves acting as non-cell-autonomous signals in leaf vein development.

My lack of data for the spatio-temporal expression of *LRP1* during leaf development at this time brings additional questions. Out of all the single *shi/sty* mutant rosette leaves I have analysed, *lrp1* appears to have the most decreased overall vein density compared to the wildtypes (Figure 11; Table 2). As well, while the *sty1-1 sty2-1 shi-3* triple mutant shows a slight increase in vein density, the *sty1-1 sty2-1 shi-3 lrp1 srs5-1* quintuple mutant have fewer venation markers suggesting that LRP1 and the other four SHI/STY family members could potentially have opposing effects (Table 2). Of the five *SHI/STY* genes I have studied, *LRP1* shares the least sequence homology with the others and is placed in a separate clade with *SRS8* and *SHI/STY* homologs of various other plant species (Kuusk et al., 2006). As well, with no high-resolution expression data for the leaves currently available, one must ponder the expression pattern and functions of *LRP1.* Could this *SHI/STY* member have a cell-autonomous function at incipient vein sites, in contrast to the potential non-cell-autonomous functions of the others? Or, could my phenotypic analyses be misleading should there be unique genetic factors in the Nossen ecotype background (from which the *lrp1* mutant was developed) that contribute to development? Additional studies of *LRP1* could contribute to a better understanding of the dynamic roles of the *SHI/STY* gene family in leaf vein development.

5. Future Perspectives and Conclusion

My research has provided me with several hypotheses on the roles of auxin homeostasis in leaf vein patterning. My data has provided evidence suggesting three families of genes having dynamic, functionally redundant roles in vein development. However, questions remain unanswered and additional data is still needed to support my hypotheses.

Previous publications and my evidence suggest that the *SHI/STY* gene family regulates auxin homeostasis while SHI/STY activity is activated by auxin (Genevestigator; Hruz et al., 2008; Eklund et al., 2011). However, the exact mechanisms by which either of these occurs is not entirely clear. It is only known that STY1 directly and positively regulates the expression of *YUC4* (Eklund et al., 2010) and possibly *YUC8* (Eklund et al., 2011), while the molecular aspects of *STY2*, *SHI*, *SRS5*, and *LRP* have not yet been explored. Even so, the spatial and temporal specification by which STY1 activates *YUC4* has yet to be determined. A next step in this study, therefore, would be to investigate the relationships between individual *SHI/STY* and *YUC* gene family members in specific tissues and time points. One approach for which I have preliminary results is the analysis of *YUC* expression patterns in *shi/sty* mutant backgrounds. Interestingly, my promoter-reporter approach so far suggests that *YUC1* and *YUC4* expression patterns are unchanged in the leaf primordia of *sty1-1*, *shi-3*, and *sty1-1 shi-3* backgrounds compared to the wildtype (data not shown). There does seem to be a decrease in *YUC2* and *YUC4* expression with *STY1* or *SHI* loss-of-function, but this is only the case for mature leaves and not young primordia (data not shown); it would be more interesting to see perturbations in expression early on in leaf development when auxin-dependent leaf vein patterning occurs. One would expect to see at least some alteration in early leaf primordium *YUC* expression when defects in leaf vein patterning, such as the distal peg, are observed in single and double *shi/sty* mutants. On the other hand, since the *SHI/STY* gene family members are at least partially redundant in their functions in organ development (Kuusk et al., 2002; 2006),

the regulation of auxin biosynthesis genes could also be quite complex, potentially involving multiple upstream regulators. It would be interesting to observe *YUC* and *TAA1/TAR* expression patterns in leaf primordia of higher order *shi/sty* mutants and mutants of other known *YUC* regulators.

The main hypothesis I have made in this thesis is that SHI/STY members are cell-autonomous, positive regulators of local auxin biosynthesis genes in developing leaf primordia, especially at the leaf margins. This implies that local auxin biosynthesis-maintained maxima are important auxin sources and regulators of the source-to-sink transport of auxin flow in secondary vein development (Figure 3b, c). Are the distinct, dynamic expression patterns of *SHI/STY*, *YUC*, and *TAA1/TAR* members really part of the complex allocation of roles alongside PIN1 in leaf vein development? Future studies on this hypothesis could involve more expression pattern analyses. I have mentioned that *YUC* expression does not appear to change in single and double *shi/sty* background compared to the wildtype (data not shown) but analyses have yet to be done in multiple *shi/sty* mutants. It may also be valuable to observe *PIN1* expression in higher order *shi/sty* × *yuc* mutants, as well as to analyze the resulting venation patterns of these mutants. The potential appearance of reduced or altered *PIN1* expression and venation defects at the leaf primordium margin would suggest that local auxin levels are decreased and normal auxin maxima are required for normal secondary vein patterning.

In summary, I have found preliminary evidence of roles for auxin homeostasis in *Arabidopsis* leaf vein patterning. Four *SHI/STY* gene family members and genes involved in auxin biosynthesis, *YUCs* and *TAA1*, have dynamic and distinct spatial and temporal expression patterns in and leaf development. Venation defects are found in single and higher order *shi/sty* mutants. Perturbations are also seen in *SHI/STY* gene expression upon auxin transport inhibition and exogenous auxin application, whereas *shi/sty* mutant leaves are not particularly sensitive or resistant to auxin transport inhibition. Ergo I have made several hypotheses in which auxin transport is crucial in leaf vein patterning while local auxin biosynthesis plays a secondary but important part. My work will hopefully contribute to molecular studies in auxin and vascular development in the near future.

References

Aida M, Vernoux T, Furutani M, Traas J, Tasaka M (2002) Roles of *PIN-FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo. Development 129:3965-3974

Aloni R (1979) Role of auxin and gibberellins in differentiation of primary phloem fibers. Plant Physiol 63:609-614

Aloni R (1982) Role of cytokinin in differentiation of secondary xylem fibers. Plant Physiol 70:1631-1633

Aloni R (2001) Foliar and axial aspects of vascular differentiation: hypotheses and evidence. J Plant Growth Regul 20:22-34

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

Alvarez JP, Goldshmidt A, Efroni I, Bowman JL, Eshed Y (2009) The *NGATHA* distal organ development genes are essential for style specification in *Arabidopsis*. Plant Cell 21:1373-1393

Badescu GO, Napier RM (2006) Receptors for auxin: will it all end in TIRs? Trends Plant Sci 11:217-223

Baima S, Nobili F, Sessa G, Lucchetti S, Ruberti I, Morelli G (1995) The expression of the *Athb-8* homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*. Development 121:4171-4182

Baima S, Possenti M, Matteucci A, Wisman E, Altamura MM, Ruberti I, Morelli G (2001) The Arabidopsis ATHB-8 HD-zip protein acts as a differentiation-promoting transcription factor of the vascular meristems. Plant Physiol 126:643-655

Bainbridge K, Guyomarc'h S, Bayer E, Swarup R, Bennett M, Mandel T, Kuhlemeier C (2008) Auxin influx carriers stabilize phyllotactic patterning. Genes Dev 22:810-823

Bandyopadhyay A, Blakeslee JJ, Lee OR, Mravec J, Sauer M, Titapiwatanakun B, Makam SN, Bouchard R, Geisler M, Martinoia E, Friml J, Peer WA, Murphy AS (2006) Interactions of PIN and PGP auxin transport mechanisms. Biochem Soc Trans 35:137-141

Barkoulas M, Galinha C, Grigg SP, Tsiantis M (2007) From genes to shape: regulatory interactions in leaf development. Curr Opin Plant Biol 10:660-666

Bartel B, Fink GR (1995) ILR1, an amidohydrolase that releases active indole-3-acetic acid from conjugates. Science 268:1745-1748

Benjamins R, Quint A, Weijers D, Hooykaas P, Offringa R (2001) The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. Development 128:4057-4067

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol 59:443-65

Benková E, Ivanchenko MG, Friml J, Shishkova S, Dubrovsky JG (2010) A morphogenetic trigger: is there an emerging concept in plant developmental biology? Trends Plant Sci 14:189-193

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115:591-602

Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schulz B, Feldmann KA (1996) *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. Science 273:948-950

Bennett SRM, Alvarez J, Bossinger G, Smyth DR (1995) Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. Plant J 8:505-520

Berleth T, Jürgens G (1993) The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. Development 118:575-587

Berleth T, Mattsson J, Hardtke CS (2000) Vascular continuity and auxin signals. Trends Plant Sci 5:387-393

Bishopp A, Benková E, Helariutta Y (2011) Sending mixed messages: auxin-cytokinin crosstalk in roots. Curr Opin Plant Biol 14:10-16

Blakeslee JJ, Bandyopadhyay A, Lee OR, Mravec J, Titapiwatanakun B, Sauer M, Makam SN, Cheng Y, Bouchard R, Adamec J, Geisler M, Nagashima A, Sakai T, Martinoia E, Friml J, Peer WA, Murphy AS (2007) Interactions among PIN-FORMED and P-glycoprotein auxin transporters in *Arabidopsis*. Plant Cell 19:131-147

Blein T, Hasson A, Laufs P (2010) Leaf development: what it needs to be complex. Curr Opin Plant Biol 13:75-82

Bowman JL (2004) Class III HD-zip gene regulation, the golden fleece of ARGONAUTE activity? BioEssays 26:938-942

Caño-Delgado A, Lee J, Demura T (2010) Regulatory mechanisms for specification and patterning of plant vascular tissues. Annu Rev Cell Dev Biol 26:605-637

Caño-Delgado A, Yin Y, Yu C, Vafeados D, Mora-García S, Cheng J, Nam KH, Li J, Chory J (2004) BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. Development 131:5341-5351

Carlsbecker A, Helariutta Y (2005) Phloem and xylem specification: pieces of the puzzle emerge. Curr Opin Plant Biol 8:512-517

Casson SA, Chilley PM, Topping JF, Evans IM, Souter MA, Lindsey K (2002) The *POLARIS* gene of Arabidopsis encodes a predicted peptide required for correct root growth and leaf vascular patterning. Plant Cell 14:1705-1721

Ceserani T, Trofka A, Gandotra N, Nelson T (2009) VH1/BRL2 receptor-like kinase interacts with vascular-specific adaptor proteins VIT and VIK to influence leaf venation. Plant J 57:1000-1014

Chandler JW (2009) Local auxin production: a small contribution to a big field. BioEssays 31:60-70.

Chandler JW, Cole M, Jacobs B, Comelli P, Werr W (2011a) Genetic integration of DORNRÖSCHEN AND DORNRÖSCHEN-LIKE reveals hierarchical interactions in auxin signalling and patterning of the Arabidopsis apical embryo. Plant Mol Biol 75:223-236

Chandler JW, Jacobs B, Cole M, Comelli P, Werr W (2011b) DORNRÖSCHEN-LIKE expression marks Arabidopsis floral organ founder cells and precedes auxin response maxima. Plant Mol Biol 76:171-185

Chavrier P, Goud B (1999) The role of ARF and Rab GTPases in membrane transport. Curr Opin Cell Biol 11:466-475

Cheng Y, Dai X, Zhao Y (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev 20:1790-1799

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

Clay NK, Nelson T (2002) VH1, a provascular cell-specific receptor kinase that influences leaf cell patterns in Arabidopsis. Plant Cell 14:2707-2722

Clay NK, Nelson T (2005) Arabidopsis *thickvein* mutation affects vein thickness and organ vascularisation, and resides in a provascular cell-specific spermine synthase involved in vein definition and in polar auxin transport. Plant Physiol 138:767-777

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735-743

Clouse SD, Zurek D (1991) Molecular analysis of brassinolide action in plant growth and development. In Cutler HG, Yokota T, Adam G (ed) Brassinosteroids—Chemistry, Bioactivity, and Applications. Washington, American Chemical Society, pp 122-140

Cohen JD, Bandurski RS (1982) Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol 33:403-30

Cole M, Chandler J, Weijers D, Jacobs B, Comelli P, Werr W (2009) *DORNRÖSCHEN* is a direct target of the auxin response factor MONOPTEROS in the *Arabidopsis* embryo. Development 136:1643-1651

Davies PJ (2010) The plant hormones: their nature, occurrence, and functions. In: Davies PJ (ed) Plant hormones. 3rd ed. New York: Dordrecht, pp 1-15

Davies RT, Goetz DH, Lasswell J, Anderson MN, Bartel B (1999) *IAR3* encodes an auxin conjugate hydrolase from Arabidopsis. Plant Cell 11:365-376

Dayan J, Schwarzkopf M, Avni A, Aloni R (2010) Enhancing plant growth and fiber production by silencing GA 2-oxidase. Plant Biotech J 8:425-435

Dayan J, Voronin N, Gong F, Sun T, Hedden P, Fromm H, Aloni R (2012) Leaf-induced gibberellins signalling is essential for internode elongation, cambial activity, and fiber differentiation in tobacco stems. Plant Cell 24:66-79

Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N, Minami A, Nagata-Hiwatashi M, Nakamura K, Okamura Y, Sassa N, Suzuki S, Yazaki J, Kikuchi S, Fukuda H (2002) Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. PNAS 99:15794-15799

Dengler N, Kang J (2001) Vascular patterning and leaf shape. Curr Opin Plant Biol 4:50-56.

Dengler NG, Tsukaya H (2001) Leaf morphogenesis in dicotyledons: current issues. Int J Plant Sci 162:459-464.

Dettmer J, Elo A, Helariutta Y (2009) Hormone interactions during vascular development. Plant Mol Biol 69:347-360

Deyholos MK, Cordner G, Beebe D, Sieburth LE (2000) The *SCARFACE* gene is required for cotyledon and leaf vein patterning. Development 127:3205-3213

Dharmasiri N, Dharmasiri S, Estelle M (2005a) The F-box protein TIR1 is an auxin receptor. Nature 435:441-445

Dharmasiri N, Dharmasiri S, Jones AM, Estelle M (2003) Auxin action in a cell-free system. Curr Biol 13:1418-1422

Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens, Estelle M (2005b) Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell 9:109-119

Digby J, Wareing PF (1966) The effect of applied growth hormones on cambial division and the differentiation of the cambial derivatives. Ann Bot 30:539-548

Donelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Dev Biol 215:407-419

Donner TJ, Sherr I, Scarpella E (2009) Regulation of preprocambial cell state acquisition by auxin signalling in *Arabidopsis* leaves. Development 136:3235-3246

Dreher KA, Brown J, Saw RE, Callis J (2006) The *Arabidopsis* Aux/IAA protein family has diversified in degradation and auxin responsiveness. Plant Cell 18:699-714

Dubrovsky JG, Sauer M, Napsucialy-Mendivil S, Ivanchenko MG, Friml J, Shishkova S, Celenza J, Benková E (2008) Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. PNAS 105:8790-8794

Efroni I, Eshed Y, Lifschitz E (2010) Morphogenesis of simple and compound leaves: a critical review. Plant Cell 22:1019-1032

Eklund DM, Cierlik I, Ståldal V, Claes AR, Vestman D, Chandler J, Sundberg E (2011) Expression of Arabidopsis *SHORT INTERNODES/STYLISH* family genes in auxin biosynthesis zones of aerial organs is dependent on a GCC box-like regulatory element. Plant Physiol 157:2069-2080

Eklund DM, Ståldal V, Valsecchi I, Cierlik I, Eriksson C, Hiratsu K, Ohme-Takagi M, Sundström 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

Elo A, Immanen J, Nieminen K, Helariutta Y (2009) Stem cell function during plant vascular development. Semin Cell Dev Biol 20:1097-1106

Engstrom EM, Izhaki A, Bowman JL (2004) Promoter bashing, microRNAs, and Knox genes. New insights, regulators, and targets-of-regulation in the establishment of lateral organ polarity in Arabidopsis. Plant Physiol 135:685-694

Esau, K (1965) Plant anatomy. John Wiley, New York

Evans ML, Ishikawa H, Estelle MA (1994) Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: comparison of wild type and auxin-response mutants. Planta 194:215-222

Foster, AS (1952) Foliar venation in angiosperms from an ontogenetic standpoint. Am J Bot 39:752-766

Fridborg I, Kuusk S, Moritz T, Sundberg E (1999) The Arabidopsis dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell 11:1019-1031

Fridborg I, Kuusk S, Robertson M, Sundberg E (2001) The Arabidopsis protein SHI represses gibberellin responses in Arabidopsis and barley. Plant Physiol 127:937-948

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgen G (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature 426:147-153

Friml J, Yang X, Michniewicz M, Weijers D, Quint A, Tietz O, Benjamins R, Ouwerkerk PBF, Ljung K, Sandberg G, Hooykaas PJJ, Palme K, Offringa R (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science 306:862-865

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell 12:393-404

Fukuda H (2004) Signals that control plant vascular cell differentiation. Nat Rev Mol Cell Biol 5:379-391

Fukuda H, Komamine A (1980) Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol. 65:57-60

Furutani M, Vernoux T, Traas J, Kato T, Tasaka M, Aida M (2004) *PIN-FORMED1* and *PINOID* regulate boundary formation and cotyledon development in *Arabidopsis* embryogenesis. Development 131:5031-5030

Gälweiler L, Guan C, Müller A, Wisman E, Mendgen K, Yephremov A, Palme K (1998) Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science 282:2226-2230

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112:219-230

Geisler M, Blakeslee JJ, Bouchard R, Lee OR, Vincenzetti V, Bandyopadhyay A, Titapiwantanakun B, Peer WA, Bailly A, Richards EL, Ejendal KFK, Smith AP, Baroux C, Grossniklaus U, Müller A, Hrycyna CA, Dudler R, Murphy AS, Martinoia E (2005) Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. Plant J 44:179-194

Geldner N, Friml J, Stierhof Y, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature 413:425-428

Geldner N, Richter S, Vieten A, Marquardt S, Torres-Ruiz RA, Mayer U, Jürgens G (2004) Partial loss-of-function alleles reveal a role for *GNOM* in auxin transport-related, post-embryonic development of *Arabidopsis*. Development 131:389-400

Goldberg RB, de Paiva G, Yadegari R (1994) Plant embryogenesis: zygote to seed. Science 266:605-614

Goldsmith MHM (1977) The polar transport of auxin. Annu Rev Plant Physiol 28:439-478.

Gray WM, Kepinski S, Rouse D, Leyser O, Estelle M (2001) Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature 414:271-276

Guilfoyle TJ, Hagen G (2001) Auxin response factors. J Plant Growth Regul 20:281-291

Guilfoyle TJ, Hagen G (2007) Auxin response factors. Curr Opin Plant Biol 10:453-460

Guilfoyle TJ, Ulmasov T, Hagen G (1998) The ARF family of transcription factors and their role in plant hormone-responsive transcription. Cell Mol Life Sci 54:619-627

Hagen G, Guilfoyle TJ (1985) Rapid induction of selective transcription by auxins. Mol Cell Biol 5:1197-1203

Hamann T, Benková E, Bäurle I, Kientz M, Jürgens G (2002) The *Arabidopsis BODENLOS* gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. Genes Dev 16:1610-1615

Hamann T, Mayer U, Jürgens G (1999) The auxin-insensitive *bodenlos* mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. Development 126:1387-1395

Hao D, Ohme-Takagi M, Sarai A (1998) Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plants. J Biol Chem 273:26857-26861

Hardtke CS, Berleth T (1998) The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. EMBO J 17:1405-1411

Hardtke CS, Ckurshumova W, Vidaurre DP, Singh SA, Stamatiou G, Tiwari SB, Hagen G, Guilfoyle TJ, Berleth T (2004) Overlapping and non-redundant functions of the *Arabidopsis* auxin response factors *MONOPTEROS* and *NONPHOTOTROPIC HYPOCOTYL 4*. Development 131:1089-1100

Hardtke CS, Dorcey E, Osmont KS, Sibout R (2007) Phytohormone collaboration: zooming in on auxin-brassinosteroid interactions. Trends Cell Biol 17:485-492

Haughn GW, Somerville CR (1988). Genetic control of morphogenesis in *Arabidopsis*. Dev Genet 9:73-89

Hay A, Barkoulas M, Tsiantis M (2006) ASYMMETRIC LEAVES1 and auxin activities converge to repress *BREVIPEDICELLUS* expression and promote leaf development in *Arabidopsis*. Development 133:3955-3961

Heisler MG, Jönsson H (2006) Modeling auxin transport and plant development. J Plant Growth Regul 25:302-312

Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. Curr Biol 15:1899-1911

Hellmann H, Hobbie L, Chapman A, Dharmasiri S, Dharmasiri N, del Pozo C, Reinhardt D, Estelle M (2003) *Arabidopsis AXR6* encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. EMBO J 22:3314-3325

Hobbie L, McGovern M, Hurwitz LR, Pierro A, Liu NY, Bandyopadhyay A, Estelle M (2000) The *axr6* mutants of *Arabidopsis thaliana* define a gene involved in auxin response and early development. Development 127:23-32

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinforma 2008:ID 420747

Huang F, Zago MK, Abas L, van Marion A, Galván-Ampudia CS, Offringa R (2010) Phosphorylation of conserved PIN motifs directs *Arabidopsis* PIN1 polarity and auxin transport. Plant Cell 22:1129-1142

Ibañes M, Fàbregas N, Chory J, Caño-Delgado AI (2009) Brassinosteroid signalling and auxin transport are required to establish the periodic pattern of *Arabidopsis* shoot vascular bundles. PNAS 106:13630-13635

Ilegems M, Douet V, Meylan-Bettex M, Uyttewaal M, Brand L, Bowman JL, Stieger PA (2010) Interplay of auxin, KANADI and class III HD-ZIP transcription factors in vascular tissue formation. Development 137:975-984

Jackson RG, Lim E, Li Y, Kowalczyk M, Sandberg G, Hoggett J, Ashford DA, Bowles DJ (2001) Identification and biochemical characterization of an *Arabidopsis* indole-3-acetic acid glucosyltransferase. J Biol Chem 276:4350-4356

Jacobs WP (1970) Regeneration and differentiation of sieve tube elements. Int Rev Cytol 28:239-273

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907

Kakehi J, Kuwashiro Y, Niitsu M, Takahashi T (2008) Thermospermine is required for stem elongation in *Arabidopsis thaliana*. Plant Cell Physiol 49:1342-1349

Kang J, Dengler N (2002) Cell cycling frequency and expression of the homeobox gene *ATHB-8* during leaf vein development in *Arabidopsis*. Planta 216:212-219

Kang J, Dengler N (2004) Vein pattern development in adult leaves of *Arabidopsis thaliana*. Int J Plant Sci 165:231-242

Kepinski S, Leyser O (2004) Auxin-induced SCR^{TIR1}-Aux/IAA interaction involves stable modification of the SCF^{TIR1} complex. PNAS 101:12381-12386

Kim J, Harter K, Theologis A (1997) Protein-protein interactions among the Aux/IAA proteins. PNAS 94:11786-11791

Kim J, Jung J, Reyes JL, Kim Y, Kim S, Chung K, Kim JA, Lee M, Lee Y, Kim VN, Chua N, Park C (2005) microRNA-directed cleavage of *ATHB15* mRNA regulates vascular development in Arabidopsis inflorescence stems. Plant J 42:84-94

Klein-Vehn J, Huang F, Naramoto S, Zhang J, Michniewicz M, Offringa R, Friml J (2009) PIN auxin efflux carrier polarity is regulated by PINOID kinase-mediated recruitment into GNOM-independent trafficking in *Arabidopsis*. Plant Cell 21:3839-3849

Klein-Vehn J, Łangowski Ł, Wiśniewska J, Dhonukshe P, Brewer PB, Friml J (2008) Cellular and molecular requirements for polar PIN targeting and transcytosis in plants. Mol Plant 1:1056-1066

Koizumi K, Naramoto S, Sawa S, Yahara N, Ueda T, Nakano A, Sugiyama M, Fukuda H (2005) VAN3 ARF-GAP-mediated vesicle transport is involved in leaf vascular network formation. Development 132:1699-1711

Koizumi K, Sugiyama M, Fukuda H (2000) A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. Development 127:3197-3204

Kowalczyk M, Sandberg G (2001) Quantitative analysis of indole-3-acetic acid metabolites in Arabidopsis. Plant Physiol 127:1845-1853

Kowalczyk S, Jakubowska A, Zielińska E, Bandurski RS (2003) Bifunctional indole-3-acetyl transferase catalyses synthesis and hydrolysis of indole-3-acetyl-*myo*-inositol in immature endosperm of *Zea mays*. Physiol Plant 119:165-174

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T (2005) Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev 19:1855-1860

Kuusk S, Sohlberg JJ, Eklund DM, Sundberg E (2006) Functionally redundant *SHI* family genes regulate Arabidopsis gynoecium development in a dose-dependent manner. Plant J 47:99-111

Kuusk S, Sohlberg JJ, Long JA, Fridborg I, Sundberg E (2002) *STY1* and *STY2* promote the formation of apical tissues during *Arabidopsis* gynoecium development. Development 129:4707-4717

LaMotte CE, Jacobs WP (1963) A role of auxin in phloem regeneration in Coleus internodes. Dev Biol 8:80-98

Lau S, Ehrismann JS, Schlereth A, Takada S, Mayer U, Jürgens G (2010) Cell-cell communication in *Arabidopsis* early embryogenesis. Eur J Cell Biol 89:225-230

Lee D, Van Norman JM, Murphy C, Adhikari E, Reed JW, Sieburth LE (2012) In the absence of BYPASS1-related gene function, the *bps* signal disrupts embryogenesis by an auxin-independent mechanism. Development 139:805-815

Lee SH, Cho H (2006) PINOID positively regulates auxin efflux in *Arabidopsis* root hair cells and tobacco cells. Plant Cell 18:1604-1616

Lehmann T, Hoffmann M, Hentrich M, Pollmann S (2010) Indole-3-acetamide-dependent auxin biosynthesis: a widely distributed way of indole-3-acetic acid production? Eur J Cell Biol 89:895-905

Lewis DR, Wu G, Ljung K, Spalding EP (2009) Auxin transport into cotyledons and cotyledon growth depend similarly on the ABCB19 multidrug resistance-like transporter. Plant J 60:91-101

Li J, Yang H, Peer WA, Richter G, Blakeslee J, Bandyopadhyay A, Titapiwantakun B, Undurraga S, Khodakovskaya M, Richards EL, Krizek B, Murphy AS, Gilroy S, Gaxiola R (2005) *Arabidopsis* H^+-PPase AVP1 regulates auxin-mediated organ development. Science 310:121-125

Li L, Qin G, Tsuge T, Hou X, Ding M, Aoyama T, Oka A, Chen Z, Gu H, Zhao Y, Qu L (2008) *SPOROCYTELESS* modulates *YUCCA* expression to regulate the development of lateral organs in Arabidopsis. New Phytol 179:751-764

Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD, Sandberg G (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol Biol 49:249-272

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

Magnus V, Nigović B, Hangarter RP, Good NE (1992) N-(indol-3-ylacetyl)amino acids as sources of auxin in plant tissue culture. J Plant Growth Regul 11:19-28

Mähönen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev 14:2938-2943

Mansfield SG, Briarty LG (1991) Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can J Bot 69:461-476

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. EMBO J 18:2066-2073

Martin DN, Proebsting WM, Hedden P (1999) The SLENDER gene of pea encodes a gibberellins 2-oxidase. Plant Physiol 121:775-781

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*. PNAS 108:18512-18517

Mattsson J, Ckurshumova W, Berleth T (2003) Auxin signalling in Arabidopsis leaf vascular development. Plant Physiol 131:1327-1339

Mattsson J, Sung ZR, Berleth T (1999) Responses of plant vascular systems to auxin transport inhibition. Development 126:2979-2991

Mayer U, Büttner G, Jürgens G (1993) Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development 117:149-162

Mayer EP, Martindale SJB (1980) Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. Biochem Genet 18:1041-1053

McCready CC (1963) Movement of plant growth regulators in plants: I. Polar transport of 2,4-dichlorophenoxyacetic acid in segments from the petioles of *Phaseolus vulgaris*. New Phytol 62:3-18

McCready CC, Jacobs WP (1963) Movements of growth regulators in plants: II. Polar transport of radioactivity from indoleacetic acid (14C) and 2,4-dichlorophenoxyacetic acid (14C) in petioles of *Phaseolus vulgaris*. New Phytol 62:19–34

Meinke DW, Franzmann LH, Nickle TC, Yeung EC (1994). *Leafy cotyledon* mutants of Arabidopsis. Plant Cell 6:1049-1064.

Meinhardt H (1996) Models of biological pattern formation: common mechanism in plant and animal development. Int J Dev Biol 40:123-134

Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiene I, Heisler MG, Ohno C, Zhang J, Huang F, Schwab R, Weigel D, Meyerowitz EM, Luschnig C, Offringa R, Friml J (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell 130:1044-1056

Möller B, Weijers D (2009) Auxin control of embryo patterning. Cold Spring Harb Perspect Biol 2009;1:a001545

Morris DA, Friml J, Zažímalová E (2010) The transport of auxins. In: Davies PJ (ed) Plant hormones. 3rd ed. New York: Dordrecht, pp 451-484

Motose H, Fukuda H, Sugiyama M (2001a) Involvement of local intercellular communication in the differentiation of zinnia mesophyll cells into tracheary systems. Planta 213:121-131

Motose H, Sugiyama M, Fukuda H (2001b) An arabinogalactan protein(s) is a key component of a fraction that mediates local intercellular communication involved in tracheary element differentiation of zinnia mesophyll cells. Plant Cell Physiol 42:129-137

Motose H, Sugiyama M, Fukuda H (2004) A proteoglycan mediates inductive interaction during plant vascular development. Nature 429:873-878

Moubayidin L, Mambro RD, Sabatini S (2009) Cytokinin-auxin crosstalk. Trends Plant Sci 14:557-562

Mravec J, Kubeš M, Bielach A, Gaykova V, Petrášek J, Skůpa P, Chand S, Benková E, Zažímalová E , Friml J (2008) Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development 135:3345-3354

Muñiz L, Minguet EG, Singh SK, Pesquet E, Vera-Sirera F, Moreau-Courtois CL, Carbonell J, Blázquez MA, Tuominen H (2008) ACAULIS5 controls *Arabidopsis* xylem specification through the prevention of premature cell death. Development 135:2573-2582

Nakamura A, Nakajima N, Goda H, Shimada Y, Hayashi K, Nozaki H, Asami T, Yoshida S, Fujioka S (2006) Arabidopsis *Aux/IAA* genes are involved in brassinosteroid-mediated growth responses in a manner dependent on organ type. Plant J 45:193-205

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol 140:411-432

Nakazawa M, Yabe N, Ichikawa T, Yamamoto YY, Yoshizumi T, Hasunuma K, Matsui M (2001). *DFL1*, an auxin-responsive *GH3* gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length. Plant J 25:213-221

Naramoto S, Sawa S, Koizumi K, Uemura T, Ueda T, Friml J, Nakano A, Fukuda H (2009) Phosphoinositide-dependent regulation of VAN3 ARF-GAP localization and activity essential for vascular tissue continuity in plants. Development 136:1529-1538

Nelson T, Dengler N (1997) Leaf vascular pattern formation. Plant Cell 9:1121-1135

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell 16:1365-1377

Noh B, Murphy AS, Spalding EP (2001) *Multidrug Resistance*-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell 13:2441-2454

Normanly J (1997) Auxin metabolism. Physiol Plant 100:431-442

Normanly J (2009) Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. Cold Spring Harb Perspect Biol 2010;2:a001594

Normanly J, Bartel B (1999) Redundancy as a way of life – IAA metabolism. Curr Opin Plant Biol 2:207-213

Normanly J, Cohen JD, Fink GR (1993) *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. PNAS 90:10355-10359

Northcote DH (1995) Aspects of vascular tissue differentiation in plants: parameters that may be used to monitor the process. Int J Plant Sci 156:245-256

Ohashi-Ito K, Demura T, Fukuda H (2002) Promotion of transcript accumulation of novel *Zinnia* immature xylem-specific HD-zip III homeobox genes by brassinosteroids. Plant Cell Physiol 43:1146-1153

Ohashi-Ito K, Kubo M, Demura T, Fukuda H (2005) Class III homeodomain leucine-zipper proteins regulate xylem cell differentiation. Plant Cell Physiol 46:1646-1656

Ohashi-Ito K, Oda Y, Fukuda H (2010) *Arabidopsis* VASCULAR-RELATED NAC-DOMAIN6 directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. Plant Cell 22:3461-3473

Ohme-Takagi M, Shinshi H (1990) Structure and expression of a tobacco beta-1,3-glucanase gene. Plant Mol Biol 15:941-946

Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y (1991) Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. Plant Cell 3:677-684

Östin A, Kowalczyk M, Bhalerao RP, Sandberg G (1998) Metabolism of indole-3-acetic acid in Arabidopsis. Plant Physiol 118:285-296

Palmgren MG (2001) Plant plasma membrane H^+-ATPases: powerhouses for nutrient uptake. Annu Rev Plant Physiol Plant Mol Biol 52:817-45

Park WJ, Schäfer A, Prinsen E, van Onckelen H, Kang BG, Hertel R (2001) Auxin-induced elongation of short maize coleoptiles segments is supported by 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one. Planta 213:92-100

Parry G, Calderon-Villalobos LI, Prigge M, Peret B, Dharmasiri S, Itoh H, Lechner E, Gray WM, Bennett M, Estelle M (2009) Complex regulation of the TIR1/AFB family of auxin receptors. PNAS 106:22540-22545

Parry G, Marchant A, May S, Swarup R, Swarup K, James N, Graham N, Allen T, Martucci T, Yemm A, Napier R, Manning K, King G, Bennett M (2001) Quick on the uptake: characterization of a family of plant auxin influx carriers. J Plant Growth Regul 20:217-225

Petrášek J, Malínská K, Zažímalová E (2011) Auxin transporters controlling plant development. In: Geisler M, Venema K (ed) Transporters and Pumps in Plant Signaling. New York: Dordrecht, pp 255-290

Petrášek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wiśniewska J, Tadele Z, Kuběs M, Čovanová M, Dhonukshe P, Skůpa P, Benková E, Perry L, Křeček P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zažímalová E, Friml J (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312:914-918

Pickett FB, Wilson AK, Estelle M (1990) The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol 94:1462-1466

Pollmann S, Müller A, Piotrowski M, Weiler EW (2002) Occurrence and formation of indole-3-acetamide in *Arabidopsis thaliana*. Planta 216:155-161

Przemeck GKH, Mattsson J, Hardtke CS, Sung ZR, Berleth T (1997) Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. Planta 200:229-237

Rademacher EH, Möller B, Lokerse AS, Llavata-Peris CI, van den Berg W, Weijers D (2011) A cellular expression map of the Arabidopsis *AUXIN RESPONSE FACTOR* gene family. Plant J 68:597-606

Rampey RA, LeClere S, Kowalczyk M, Ljung K, Sandberg G, Bartel B (2004) A family of auxin-conjugate hydrolases that contributes to free indole-3-acetic acid levels during Arabidopsis germination. Plant Physiol 135:978-988

Raven JA (1975) Transport of indoleacetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. New Phytol 74:163-172

Reinhardt D, Mandel T, Kuhlemeier C (2000) Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell 12:507-518

Reinhardt D, Pesce E, Stieger P, Mandel T, Baltensperger K, Bennett M, Traas J, Friml J, Kuhlemeier C (2003) Regulation of phyllotaxis by polar auxin transport. Nature 426:255-260

Rieu I, Eriksson S, Powers SJ, Gong F, Griffiths J, Woolley L, Benlloch R, Nilsson O, Thomas SG, Hedden P, Phillips AL (2008) Genetic analysis reveals that C19-GA 2-oxidation is a major gibberellins inactivation pathway in Arabidopsis. Plant Cell 20:2420-2436

Rizzardi K, Landberg K, Nilsson L, Ljung K, Sundås-Larsson A (2011) *TFL2/LHP1* is involved in auxin biosynthesis through positive regulation of *YUCCA* genes. Plant J 65:897-906

Rolland-Lagan A (2008) Vein patterning in growing leaves: axes and polarities. Curr Opin Genet Dev 18:348-353

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

Rubery PH, Sheldrake AR (1974) Carrier-mediated auxin transport. Planta 118:101-121

Sachs T (1981) The control of patterned differentiation of vascular tissues. Adv Bot Res 9:151-262

Sachs T (1989) The development of vascular networks during leaf development. Curr Top Plant Biochem Physiol 8:168-183

Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. Nature 459:1071-1078

Sarafian V, Kim Y, Poole RJ, Rea PA (1992) Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. PNAS 89:1775-1779

Sawchuk MG, Head P, Donner TJ, Scarpella E (2007) Time-lapse imaging of Arabidopsis leaf development shows dynamic patterns of procambium formation. New Phytol 176:560-571

Scarpella E, Francis P, Berleth T (2004) Stage-specific markers define early steps of procambium development in *Arabidopsis* leaves and correlate termination of vein formation with mesophyll differentiation. Development 131:3445-3455

Scarpella E, Helariutta Y (2010) Vascular pattern formation in plants. Curr Top Dev Biol 91:221-265

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

Scheres B, Di Laurenzio L, Willemsen V, Hauser M, Janmaat K, Weisbeek P, Benfey PN (1995) Mutations affecting the radial organisation of the *Arabidopsis* root display specific defects throughout the embryonic axis. Development 121:53-62

Schlereth A, Möller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jürgens G, Weijers D (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature 464:913-916

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU (2005) A gene expression map of *Arabidopsis thaliana* development. Nat Genet 37:501-506

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JA, Amasino RM (2003) Overexpression of a novel class of gibberellins 2-oxidases decreases gibberellins levels and creates dwarf plants. Plant Cell 15:151-163

Schuetz M, Berleth T, Mattsson J (2008) Multiple *MONOPTEROS*-dependent pathways are involved in leaf initiation. Plant Physiol 148:870-880

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

Shevell DE, Leu W, Gillmor CS, Xia G, Feldmann KA, Chua N (1994) *EMB30* is essential for normal cell division, cell expansion, and cell adhesion in Arabidopsis and encodes a protein that has similarity to Sec7. Cell 77:1051-1062

Shinshi H, Usami S, Ohme-Takagi M (1995) Identification of an ethylene-responsive region in the promoter of a tobacco class I chitinase gene. Plant Mol Biol 27:923-932

Sieburth LE (1999) Auxin is required for leaf vein pattern in Arabidopsis. Plant Physiol 121:1179-1190

Sieburth LE, Muday GK, King EJ, Benton G, Kim S, Metcalf KE, Meyers L, Seamen E, Van Norman JM (2006) *SCARFACE* encodes an ARF-GAP that is required for normal auxin efflux and vein patterning in *Arabidopsis*. Plant Cell 18:1396-1411

Smith DL, Fedoroff NV (1995) *LRP1*, a gene expressed in lateral and adventitious root primordia of Arabidopsis. *Plant Cell* 7:735-745

Smith RS, Guyomarc'h S, Mandel T, Reinhardt D, Kuhlemeier C, Prusinkiewicz P (2006) A plausible model of phyllotaxis. PNAS 103:1301-1306

Sohlberg JJ, Myrenås M, Kuusk S, Lagercrantz U, Kowalczyk M, Sandberg G, Sundberg E (2006) *STY1* regulates auxin homeostasis and affects apical-basal patterning of the Arabidopsis gynoecium. Plant J 47:112-123

Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism-plant signalling. FEMS Microbiol Rev 31:425-448

Ståldal V, Cierlik I, Chen S, Landberg K, Baylis T, Myrenås M, Sundström JF, Eklund DM, Ljung K, Sundberg E (2012) The *Arabidopsis thaliana* transcriptional activator STYLISH1 regulates genes affecting stamen development, cell expansion, and timing of flowering. Plant Mol Biol 78:545-559

Ståldal V, Sohlberg JJ, Eklund DM, Ljung K, Sundberg E (2008) Auxin can act independently of *CRC*, *LUG*, *SEU*, *SPT*, and *STY1* in style development but not in apical-basal patterning of the *Arabidopsis* gynoecium. New Phytol 180:798-808

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

Steynen QJ, Schultz EA (2003) The FORKED genes are essential for distal vein meeting in *Arabidopsis*. Development 130:4695-4708

Stone SL, Braybrook SA, Paula SL, Kwong LW, Meuser J, Pelletier J, Hsieh T, Fischer RL, Goldberg RB, Harada JJ (2008) *Arabidopsis* LEAFY COTYLEDON2 induces maturation traits and auxin activity: implications for somatic embryogenesis. PNAS 105:3151-3156

Stone SL, Kwong LW, Yee KM, Pelletier J, Peiniec L, Fischer RL, Goldberg RB, Harada JJ (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. PNAS 98:11806-11811

Swarup K, Benková E, Swarup R, Casimiro I, Péret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S, Levesque MP, Carrier D, James N, Calvo V, Ljung K, Kramer E, Roberts R, Graham N, Marillonnet S, Patel K, Jones JDG, Taylor CG, Schachtman DP, May S, Sandberg G, Benfey P, Friml J, Kerr I, Beeckman T, Laplaze L, Bennett MJ (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol 10:946-954

Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. Genes Dev 15:2648-2653

Swarup R, Kramer EM, Perry P, Knox K, Leyser HMO, Haseloff J, Beemster GTS, Bhalerao R, Bennett MJ (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nat Cell Biol 7:1057-1065

Sze H, Li X, Palmgren MG (1999) Energization of plant cell membranes by H^+-pumping ATPases: regulation and biosynthesis. Plant Cell 11:677-689

Szekeres M, Németh K, Koncz-Kálmán Z, Mathur J, Kauschmann A, Altmann T, Rédei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell 85:171-182

Szemenyei H, Hannon M, Long JA (2008) TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. Science 319:1384-1386

Tam YY, Epstein E, Normanly J (2000) Characterization of auxin conjugates in Arabidopsis. Low steady-state levels of indole-3-acetyl-aspartate, indole-3-acetyl-glutamate, and indole-3-acetyl-glucose. Plant Physiol 123:589-595

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

Tanaka H, Dhonukshe P, Brewer PB, Friml J (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell Mol Life Sci 63:2738-2754

Tao Y, Ferrer J, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman ME, Ivans LJ, Cheng Y, Lim J, Zhao Y, Ballaré CL, Sandberg G, Noel JP, Chory J (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell 133:164-176

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellins 2-oxidases, multifunctional enzymes involved in gibberellins deactivation. PNAS 96:4698-4703

Titapiwatanuakun B, Blakeslee JJ, Bandyopadhyay A, Yang H, Mravec J, Sauer M, Cheng Y, Adamec J, Nagashima A, Geisler M, Sakai T, Friml J, Peer WA, Murphy AS (2009) ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis. Plant J 57:27-44

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

Trigueros M, Navarrete-Gómez M, Sato S, Christensen SK, Pelaz S, Weigel D, Yanofsky MF, Ferrándiz C (2009) The *NGATHA* genes direct style development in the *Arabidopsis* gynoecium. Plant Cell 21:1394-1409

Tsugeki R, Ditengou FA, Palme K, Okada K (2010) *NO VEIN* facilitates auxin-mediated development in Arabidopsis. Plant Signal Behav 5:1249-1251

Tsugeki R, Ditengou FA, Sumi Y, Teale W, Palme K, Okada K (2009) NO VEIN mediates auxin-dependent specification and patterning in the *Arabidopsis* embryo, shoot, and root. Plant Cell 21:3133-3151

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

Van Norman JM, Frederick RL, Sieburth LE (2004) BYPASS1 negatively regulates a root-derived signal that controls plant architecture. Curr Biol 14:1739-1746

Van Norman JM, Sieburth LE (2007) Dissecting the biosynthetic pathway for the *bypass1* root-derived signal. Plant J 49:619-628

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136:669-687

Wang W, Xu B, Wang H, Li J, Huang H, Xu L (2011) *YUCCA* genes are expressed in response to leaf adaxial-abaxial juxtaposition and are required for leaf margin development. Plant Physiol 157:1805-1819

Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM (1992) *LEAFY* controls floral meristem identity in Arabidopsis. Cell 69:843-859

Weijers D, Benková E, Jäger KE, Schlereth A, Hamann T, Kientz M, Wilmoth JC, Reed JW, Jürgens G (2005a) Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators. EMBO J 24:1874-1885

Weijers D, Sauer M, Meurette O, Friml J, Ljung K, Sandberg G, Hooykaas P, Offringa R (2005b) Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in *Arabidopsis*. Plant Cell 17:2517-2526

Went FW (1974) Reflections and speculations. Annu Rev Plant Physiol 25:1-26

Wenzel CL, Hester Q, Mattsson J (2008) Identification of genes expressed in vascular tissues using NPA-induced vascular overgrowth in *Arabidopsis*. Plant Cell Physiol 49:457-468

Wenzel CL, Schuetz M, Yu Q, Mattsson J (2007) Dynamics of *MONOPTEROS* and PIN-FORMED1 expression during leaf vein pattern formation in *Arabidopsis thaliana*. Plant J 49:387-398

West MAL, Harada JJ (1993) Embryogenesis in higher plants: an overview. Plant Cell 5:1361-1369

Wisniewska J, Xu J, Seifertova D, Brewer PB, Ruzicka K, Blilou I, Rouquie D, Benková E, Scheres B, Friml J (2006) Polar PIN localization directs auxin flow in plants. Science 312:883

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*. PNAS 108:18518-18523

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot 95:707-735

Xu XM, Jackson D (2012) Cell-to-cell movement of homeodomain transcription factors: yesterday, today, and tomorrow. In: Kragler F, Hülskamp M (ed) Advances in Plant Biology, Volume 3. New York: Springer, pp 87-98

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol 42:1017-1023

Yamada M, Greenham K, Prigge MJ, Jensen PJ, Estelle M (2009) The *TRANSPORT INHIBITOR RESPONSE2* gene is required for auxin synthesis and diverse aspects of plant development. Plant Physiol 151:168-179Yamaguchi M, Goué N, Igarashi H, Ohtani M, Nakano Y, Mortimer JC, Nishikubo N, Kubo M, Katayama Y, Kakegawa K, Dupree P, Demura T (2010) VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system. Plant Physiol 153:906-914

Yamaguchi M, Kubo M, Fukuda H, Demura T (2008) VASCULAR-RELATED NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. Plant J 55:652-664

Yamaguchi M, Mitsuda N, Ohtani M, Ohme-Takagi M, Kato K, Demura T (2011) VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. Plant J 66:579-590

Yamamoto R, Demura T, Fukuda H (1997) Brassinosteroids induce entry into the final stage of tracheary element differentiation in cultured *Zinnia* cells. Plant Cell Physiol 38:980-983

Yamamoto R, Fujioka S, Demura T, Takatsuto S, Yoshida S, Fukuda H (2001) Brassinosteroid levels increase drastically prior to morphogenesis of tracheary elements. Plant Phsiol 125:556-563

Yamamoto M, Yamamoto KT (1998) Differential effects of 1-naphthaleneacetic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin-resistant mutant of Arabidopsis, *aux1*. Plant Cell Physiol 39:660-664

Yang H, Murphy AS (2009) Functional expression and characterization of Arabidopsis ABCB, AUX1 and PIN auxin tranporters in *Schizosaccharomyces pombe*. Plant J 59:179-191

Zhang J, Nodzyński T, Pěnčík A, Rolčík J, Friml J (2010) PIN phosphorylation is sufficient to mediate PIN polarity and direct auxin transport. PNAS 107:918-922

Zhao Y (2008) The role of local biosynthesis of auxin and cytokinin in plant development. Curr Opin Plant Biol 11:16-22

Zhao Y (2010) Auxin biosynthesis and its role in plant development. Annu Rev Plant Biol 61:49-64

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

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL (2002) Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev 16:3100-3112

Zhong R, Ye Z (2007) Regulation of *HD-ZIP III* genes by microRNA 165. Plant Signal Behav 2:351-353.