GENETIC REGULATION OF VASCULAR DEVELOPMENT IN *ARABIDOPSIS THALIANA*

by

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Bachelor of Science, Shandong University, 2003

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
Department of Biological Sciences

SIMON FRASER UNIVERSITY

Summer 2008

APPROVAL

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Degree: Master of Science

Title of Thesis:

Genetic regulation of vascular development in *Arabidopsis thaliana*

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ABSTRACT

Vascular tissues distribute water and nutrients throughout the plant. To achieve this, cells of the vascular system are elongated and interconnected and undergo specialized differentiation. Manipulation of auxin distribution has shown that this hormone plays a pivotal role in vascular patterning and differentiation, although the molecular genetic basis of this is largely missing. Here we show that the expression of PIN1, an auxin efflux carrier, is gradually narrowed down to the sites of leaf vein formation and that PIN1 is a potential component of a positive feedback loop involving auxin and the transcription factor MP. In a separate study, we assessed whether auxin-induced vascular overgrowth could be used to identify genes expressed in vascular tissues based on increased mRNA abundance as assessed by microarray analysis. We found strong support for this hypothesis as we analyzed the expression of 40 up-regulated genes in detail and initiated a phenotypic study.

Keywords: *Arabidopsis thaliana*, auxin, *MONOPTEROS*, *AtPIN-FORMED1*, microarray, *AtEXLB1*

DEDICATION

I dedicate this work to my husband, my parents and my parents-in-law. Thank you for all the love and support. Please keep supporting me for my next adventure.

ACKNOWLEDGEMENTS

I thank Dr. Mattsson for taking me as his graduate student and supervising me. I thank Drs. Plant and Bisgrove for serving as my committee members.

I thank Afsaneh, Allie and Dua'a for having them in my lab. We spent a lot of time working together. They have been very nice and friendly to me, and we made some good memories along my research life. From my first day in the lab Afsaneh was there, we spent several years working together and it was a pleasure. Chatting with Allie when we worked side by side at the bench was very fun and lightened my day. Having a female Master's student from a foreign culture in our lab like Dua'a made me feel not so alone. Her personality and example in dealing with cultural conflicts gave me much encouragement.

I thank Daniel and Agnes for being my friends in grad school. We had many fun times having lunches and picnics together.

I thank Samuel for his emotional support. He supported me and helped go through a year when I had a hard time dealing with sickness, stress and being homesick. He taught me there are other things to do and other goals to achieve than studying day and night to be a top student.

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CHAPTER 1
INTRODUCTION

1.1 Function and structure of plant vascular tissues

Plant vascular tissues distribute water, sugars, minerals and signalling molecules throughout plants and also provide structural support for upright growth (Shininger, 1979). The evolution of a vascular system was a key aspect of plant adaptation from aquatic to terrestrial life, as it allowed a separation of water uptake from the ground and assimilation of photons from sunlight (Taiz and Zeiger, 1991). Vascular tissues are unique among plant tissues in that their constituent cells must span across long distances and must be interconnected for proper function. These traits are present from the very beginning of vascular differentiation as cells selected for vascular differentiation elongate and interconnect along the same axis (Fukuda, 2004). These procambial or provascular cells differentiate into the two main constituents of vascular tissues, the vessel elements and tracheids forming the water and mineral-transporting xylem and sieve elements forming the sugar transporting phloem. Although xylem and phloem have different functions and usually carry out transport in opposite directions, they are usually found together as two poles of the same vascular bundle. The structural anatomy of the constituent transporting cells is closely related to their specialized functions and will be reviewed below.

1.1.1 Function and structure of xylem

Xylem is responsible for the conduction of water and dissolved minerals from the roots to the leaves. This function is conducted by two types of tracheary elements: tracheids and vessel elements, which are the unique cell types of xylem tissues. Tracheids are elongated, spindle-shaped cells with thick lignified secondary cell walls and bordered pits. A special characteristic of vessel elements is openings in the end walls. Vessel elements connect to each other to form a tube, called a vessel. In their final phase of differentiation, tracheids and vessel elements undergo programmed cell death (PCD) (Gray, 2004). The PCD in effect results in long, reinforced hollow tubes or capillaries, which provide low-resistance pathway for water movement throughout plants. The hollow and narrow tubes, mediate water transport through a combination of capillary forces and strong tension generated by water evaporation, primarily from leaves (Canny, 1998).The tracheary elements do not collapse from the tension as they are re-inforced with thick secondary walls. In addition to tracheary elements, xylem tissue includes parenchyma and fiber cells. Parenchyma cells remain alive at maturity, may still divide and have thin primary cell walls. Fiber cells are dead at maturity and have thick secondary cell walls providing physical support and protection to the plant (Carlquist and Schneider, 2002). Elongated and interconnected xylem and fiber cells provide the long-distance water conduction and the mechanical support needed in a terrestrial habitat. Secondary wall thickenings are particularly important in trees where vascular cells make up the supportive wood.

1.1.2 Function and structure of phloem

Phloem tissues are generally located on the outer side of vascular tissues, with unique cell types: sieve elements and companion cells. Sieve elements function in the conduction of sucrose and other organic substances throughout the plant. They are alive at maturity, have no nucleus, ribosomes, or vacuole, and they connect to each other to form a tube with sieve area pores at end walls. In angiosperms, individual sieve tube elements are joined together into an open channel sieve tube that allows transport between cells, while in gymnosperms, pores in sieve areas remain covered with plasma membranes. Companion cells are alive at maturity, providing metabolic support to associated sieve elements. The sieve tube elements and their companion cells are connected cytoplasmically via numerous plasmodesmata. Other cell types of phloem tissue include parenchyma cells and fiber cells. (Taiz and Zeiger, 1991). Translocation in the phloem is the movement of sugars, mainly sucrose, and other organic materials from sources to sinks. Sources are areas of photosynthate supply, usually mature leaves, and sinks are areas of metabolism or storage of photosynthate, such as roots, immature leaves and fruits (Taiz and Zeiger, 1991). Sugars might enter the phloem from sources, called phloem loading, through the symplast route via the plasmodesmata, or through the apoplastic route (Taiz and Zeiger, 1991; Barker et al., 2000). In the latter case, sieve element loading involves a sucrose-H^+ symporter, which facilitate the H^+-coupled uptake of sucrose that uses the energy generated by the proton pump. Several plant sucrose transporters (SUTs, also named SUCs) have been cloned and localized in the phloem (Stadler et al., 1995; Truernit and Sauer, 1995; Kuhn et al., 1997; Barker et al., 2000; Weise et al., 2000). One of them, AtSUC2, is identified as the main phloem-loading transporter in Arabidopsis (Sauer and Stolz, 1994). Transport of sugars out of the sieve

elements of sink tissues, called phloem unloading, can occur via symplastic (through cytoplasm) or apoplastic (through cell) pathways and requires metabolic energy (Patrick, 1997).

1.2 Hormonal regulation of vascular development

The first insights into the regulation of vascular development came from studies on the effects of plant hormones on vascular development. The plant hormone auxin plays a particularly prominent role in vascular development. Here I will first describe the role of auxin in vascular patterning and differentiation, followed by a description of the role of other hormones in vascular differentiation.

1.2.1 The roles of auxin in vascular development

Several important observations regarding vascular differentiation in plants were made 100 years ago. Simon (1908) found that (1) plants possessed ability to regenerate vascular bundles after injury, (2) that this regeneration occurred by transformation of parenchymatic cells into vascular cells, and (3) that this transformation was polar, always proceeding in the apical to basal direction. These observations have been built upon since. The removal of young leaf primordia inhibits the differentiation of the subtending leaf traces and also regeneration by transformation (Esau, 1965) suggesting that leaf primordia produce an inductive agent required for differentiation of the subtending leaf trace. In parallel, several studies showed that young leaves contain considerable quantities of the plant hormone auxin and that auxin moves in the apical to basal direction in stem segments (reviewed in Lomax et al., 1995). Taken together, these results suggest that the stimulatory agent might be synonymous with auxin and that auxin

is transported from young leaves in the apical to basal direction induce the differentiation of vascular bundles and also the regeneration of severed bundles. Jacobs (Jacobs, 1952) provided experimental support for this hypothesis in a series of experiments, the most important showing that the stimulatory effect of leaf primordia on vascular regeneration could be replaced with topical application of auxin at the site of the excised primordia. Auxin application basal to the severed bundle had no effect on vascular regeneration, providing additional support that auxin moves to the site of injury by a polar transport process.

Polar auxin transport is an energy-requiring process that moves auxin from cell to cell in a net apical to basal direction. In addition to vascular differentiation, the process of polar auxin transport plays a role in a number of developmental processes including tropic responses and apical dominance. Polar auxin transport has been studied extensively with various biochemical methodologies, largely outside the context of vascular differentiation. Chemical inhibitors of polar auxin transport as well as the use of radio-labelled auxin have been particularly useful in the biochemical dissection of this process (Lomax et al., 1995). Two seminal publications provided a theory on how this might work (Rubery and Sheldrake, 1974; Raven, 1975). The chemiosmotic theory states that indole acetic acid (IAA), the most prevalent natural auxin, can enter cells via either of two paths depending on the form of IAA. The charged ionic (IAA-) form can enter via transport through an influx carrier. The uncharged protonated form (IAAH) can enter cells via simple diffusion across the plasma membrane. IAA is kept in the cytoplasm by an ion trapping mechanism. At the pH of the cytoplasm (pH 7), IAA exist primarily in the charged form, and can only move out of cells via efflux carriers. The net apical to basal

flow of IAA was hypothesized to come from efflux carriers being localized primarily in the plasma membrane at the basal end of cells, and potentially also a polar localization of influx carriers at the apical end of cells (Raven, 1975). Efflux and influx, both passive and active, have electrochemical and osmotic requirements, providing the name of this theory. This theory remains intact and the molecular basis of it has largely been confirmed. Genes encoding auxin influx and efflux carriers have been identified and subcellular localization of the corresponding proteins contributing to influx and efflux transport (see genetics of patterning below).

Tsvi Sachs, a specialist on biological patterning in plants, has studied the process of intrinsic polarity in plant tissues and its role in vascular regeneration and also the formation of vascular bundles between existing vascular bundles and sites of applied auxin (Sachs, 1981, 1991). One of the key problems that he approached was the fact that topical application of auxin leads not to formation of massive vascularisation close to the application point as one would expect, but to the formation of discrete vascular bundles from the auxin source to auxin sinks in form of existing vasculature. Based on his findings and the conceptual framework of the chemiosmotic theory, Sachs proposed the canalization of auxin flow hypothesis (Sachs, 1981, 1991). According to this model, cells with slightly higher concentrations of auxin become specialized for polar transport of auxin. Auxin is delivered through those conducting cells in its apical-basal direction of flow, which induce those cells to become more conductive, thereby stabilizing auxin routes into preferred auxin canals. The most conductive canals drain auxin from surrounding tissue restricting the canalizing zone to a narrow cell file. This continuous

auxin polar transport through cells finally results in the activation of genes triggering differentiation of strands of procambial cells and, subsequently, vascular strands.

Besides the canalization of auxin flow hypothesis, researchers also proposed the diffusion-reaction hypothesis, a mathematically derived model for reticulated vein patterns. (Koch and Meinhardt, 1994). This model involves two components: an activator and an inhibitor. The activator is part of a positive feedback loop and acts non-cell autonomously as it is capable of moving to adjacent sites. The inhibitor is a predicted to be a low-molecular weight molecule which therefore can act over a long-range as an inhibitor that restricts the lateral spread of the activator. Meinhardt demonstrated mathematically that these two components can possibly generate vascular patterns that are close to normal leaf vein patterns (Meinhardt, 1984). So far, the activator and inhibitor have not been identified as two specific molecules that exist in plants (Scheres, 2000). However this model has been suggested as a better fit with the discontinuous vascular network of some vein patterning mutants, such as cotyledon vascular pattern mutants *cvp1, cvp2*, and *scarface* (*sfc/van3*), since the diffusion-reaction hypothesis can explain the formation of discontinuous veins, whereas the canalization of auxin flow hypothesis can not (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). However, a recent publication found that one of these mutants, *van3*, actually formed continuous procambial strands, and that the discontinuity occurred only at the subsequent differentiation of those cells into mature vascular cells (Scarpella et al., 2006).

1.2.2 The role of other hormones in vascular differentiation

The plant hormones, auxin, cyokinins, ethylene, gibberellins and brassinosteroids, all play roles in regulating plant development. It has been well established that auxin has

essential roles in regulating vascular differentiation (Dalessandro, 1973; DeGroote and Larson, 1984; Gersani and Sachs, 1984; Meicenlaeimer and Larson, 1985), whereas roles of other types of hormones in regulating vascular differentiation are as follows.

The cytokinins, discovered in the search for plant cells division stimuli, are involved in the regulation of vascular differentiation, and many other cellular processes. Cytokinins are found at highest concentrations in young, rapidly dividing cells of the shoot and root apical meristems. Vascular differentiation is induced by root apices, as the sources of cytokinin and other stimuli (Fosket and Torrey, 1969; Dalessandro, 1973). Cytokinin has essential roles in promoting procambial cell differentiation (Aloni, 1987; Fukuda, 2004). Mutation in the *WOODEN LEG* (*WOL/CRE1*) gene leads to differentiation of procambial cells into protoxylem and is therefore believed to promote procambial cell division and thereby suppress premature differentiation into xylem (Scheres et al., 1995; Inoue et al., 2001). It has been found to encode a cytokinin receptor (Mahonen et al., 2000; Inoue et al., 2001).

Ethylene is a gaseous hormone, known to regulate cellular processes associated with organ senescence, such as fruit ripening, and some other cellular processes. Mechanical pressure has been found to be important for secondary vascular tissue development (Brown and Sax, 1962).External pressures induce the synthesis of ethylene (Yang and Hoffman, 1984), which has roles in promoting wood formation (Nelson and Hillis, 1978; Savidge et al., 1983) and in xylem differentiation (Roberts, 1975; Roberts and Miller, 1982).

Cambium activity is regulated by the presence of both auxin and gibberellic acid (Wareing et al., 1964). It has been demonstrated in *Coleus* that the combined application

of exogenous IAA and gibberellic acid has the same affect as leaves in differentiation of primary phloem fibers (Aloni, 1979). Formation of fiber can be induced by a combination of auxin and gibberellic acid, but application of only one type of hormone is not sufficient.

Plant steroid hormones, the brassinosteroids, have been found to have roles in vascular development (Fukuda, 2004), in addition to their important roles in cell elongation and differentiation. Mutants, with deficient brassinosteroids, have defects in vascular differentiation, forming more phloem tissues and less xylem tissues (Szekeres et al., 1996; Choe et al., 1999). Recently identified brassinosteroids receptors have been proposed to facilitate brassinosteroids signalling in the procambium for the up-regulation of xylem proliferation and down-regulation of phloem proliferation. (Cano-Delgado et al., 2004)

1.3 Genetic studies of vascular development in *Arabidopsis thaliana*

The early work on vascular development was performed primarily on plants in which it was relatively easy to injure and observe individual vascular bundles, such as *Pisum* spp. and *Coleus* spp. Genetic analysis of plant development is done, however, on other species, including *Arabidopsis thaliana,* maize and *Zinnia* spp. Of these species, Arabidopsis is by far the most studied species with respect to vascular development. Therefore, we provide here a brief introductory description of Arabidopsis as a model species, before going on to the analysis of vascular development in this species.

1.3.1 *Arabidopsis thaliana*, model plant for genetic studies

Arabidopsis thaliana is an important model organism for plant research, being the equivalent to *C. elegans*, *Drosophila*, or the mouse for studies in animal kingdom. It is a small flowering dicotyledonous species, belonging to the mustard family. The English common names for Arabidopsis are thale cress, wall cress and mouse-ear cress. In history, Arabidopsis is named after Johannes Thal, who discovered this species in the Harz Mountains in the sixteenth century (TAIR, 2008). Though Arabidopsis has no agricultural value, it is one of the most well characterized and significant plant organisms in the laboratory. Arabidopsis was initially chosen to be a model plant for genetic study because of several key advantages. It has a relatively small genome of approximately 114.5 Mb to 125 Mb (Megabase pairs) compared to other plant species. It has a rapid life cycle of about 6-8 weeks from germination to mature seed. It can be transformed by *Agrobacterium tumefaciens*, which is easier and faster to manipulate than in any other plant. (TAIR, 2008) The sequencing of the Arabidopsis genome, completed in 2000, provides strategies to explore gene functions. Understanding of gene function in Arabidopsis provides a framework of strategies for studying other plant organisms and insights into gene functions of other plant species, because genes that are discovered in Arabidopsis normally exist in other flowering plants and carry out similar functions. Understanding of Arabidopsis helps people gain greater insight into the genetic basis of plant biology and may provide economic value in the future.

1.3.2 Genetics of vascular patterning

Understanding the genetic basis of vascular development has been extensively explored by developmental biologists. Rapid progress has been made in identifying genes

that regulate vascular development in Arabidopsis. In most cases, genes that are important for vascular development have been revealed by screening for mutants with altered vascular development. Previous studies have identified some of the important genes, while roles of most genes that regulate the pathway of vascular differentiation and patterning remain unclear.

Recent genetic analyses have begun to unravel the molecular mechanisms underlying vascular pattern formation within vascular bundles or veins. Mutant studies on *phan* (Waites and Hudson, 1995; Waites et al., 1998), *phb-1d* (McConnell and Barton, 1998; McConnell et al., 2001) and *avb1* (Zhong et al., 1999) have revealed that the vascular tissue organization is controlled by positional information, not clonal history of tissue cells. The common vascular organization within a vascular bundle is a parallel arrangement of xylem and phloem, as in Arabidopsis. *phb-1d* and *avb1* mutants disrupt positional information for xylem placement, a circle of xylem tissues are formed around phloem, while *phan* mutants disrupt phloem positional information, forming a circle of phloem tissue around xylem. Mutation of the *AVB1* gene, encoding a homeodomain-leucin zipper protein, disrupts vascular pattern within a vascular bundle. In addition, it also disrupts the ring-like organization of vascular bundles in stems. Taken together, the defects in vascular patterning on both levels are controlled by positional information.

Recent mutant studies on the transcription factors of class III homeodomain-leucine zipper (HD-ZIPIII) family genes, *FIBERLESS (REV), PHABULOSA/ATHB14 (PHB), PHAVOLUTA/ATHB9 (PHV), CORONA/ATHB15 (CAN)* and *ATHB8*, in Arabidopsis reveal that HD-ZIPIII is essential for correct patterning within vascular bundles (Emery et al., 2003). KANADI transcription factors also play important roles in

the radial patterning of the vascular bundle by negatively regulating HD-ZIPIII gene expression (Bowman, 2004). The *kan1 kan2 kan3* mutant has a similar phenotype to the *rev-d, phb-d* or *phv-d* gain-of-function mutants (Emery et al., 2003).

Regardless of the specific arrangement of the vascular tissues, they present a variety of patterns (Turner and Sieburth, 2002). There are different forms of vascular patterns: in primary stems and roots, vascular bundles can be organized as a single ring; and in all broad flat organs, such as leaf vein patterns, vascular bundles form reticulate patterns (Turner and Sieburth, 2002). While the vascular strands in stems and roots along the main axis is based primarily on an extension of its initial pattern, venation in leaves is created *de novo* during leaf formation (Foster, 1952; Esau, 1965). Leaf vein development is therefore an attractive subject for researchers to understand the dynamics underlying vascular pattern formation, which is included in this study (see Chapter 2). Nomenclature for leaf vascular strands is distinguished by hierarchical degrees (Hickey, 1979). In dicotyledonous leaves, secondary veins (2°) branch from the usually single midvein (primary vein, 1°). Tertiary (3°) veins branch from secondary veins. In Arabidopsis, the species of this study, vein development occurs in three stages, starting with the appearance of procambial cells in the centre of early bulge-shaped primordia, forming a procambial midvein (Mattsson et al., 1999). While the primary vein is formed acropetally, secondary procambial veins proceed starting from the leaf apex while lamina is forming. Tertiary and quaternary veins first form close to the leaf apex and then develop basipetally during secondary vein formation (Mattsson et al., 1999).

A number of mutants affecting vascular patterning have been isolated. Many of them fit the profile of being defective in both vascular patterning and vascular

differentiation, and it is possible that in at least some of these mutants, the defective pattern comes from a patchy pattern of differentiation, resulting in a defective vascular pattern, primarily in leaf venation.One mutant that sheds light on this connection is the *monopteros (mp)* mutant (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998) Seedlings of mp mutants lack hypocotyls and roots, and cotyledons and leaves have altered venation patterns, in which distal portions of secondary veins are missing, as are tertiary veins (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998). The lack of hypocotyl and root has been attributed to defective embryonic axis formation (Berleth and Jurgens, 1993). Similarly, the vascular defects in leaves of seedlings have also been attributed to defects in axialization and interconnection of vascular cells (Przemeck et al., 1996). *mp* mutants display both reduced polar auxin transport capacity as meassured by transport of radiolabelled auxin in stem segments (Przemeck et al., 1996) and reduced sensitivity to external application of auxin (Mattsson et al., 2003). The *MP* gene encodes an auxin response factor (ARF) which is a transcription factor (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998).

As described above, various manipulations of auxin sources and auxin transport have revealed that polar auxin plays a central role in vascular patterning. One of the strongest mutant defects linking auxin and auxin transport to vascular patterning has been observed in mutants of the *PIN-FORMED 1* gene. Rosette leaves of *pin1* mutants show an enhanced vascularisation with a wider midvein, larger number of secondary veins and enhanced vascularisation at the leaf margin (Mattsson et al., 1999; more detail of PIN protein will be discussed below). Other mutants defective in polar auxin transport, such as *auxin resistant 1* (*aux1*), defective in an auxin influx carrier (Bennett et al., 1996), and

mutants of P-glycoprotein (PGP) auxin transporters (Geisler et al., 2005) show no obvious defects in vascular patterning, but are implicated based on their expression patterns. Similarly, loss-of-function mutants of *PINOID* gene, which encodes protein kinase involved in the regulation of subcellular localization of the auxin efflux carrier PIN1 (Friml et al., 2004), also shows no obvious vascular defects (Bennett et al., 1995). The absence of strong phenotypes is widely attributed to genetic redundancy among gene family members, and there is for example evidence for that with respect to PIN proteins (Vieten et al., 2005). The *yabby* (Siegfried et al., 1999) and *argonaute1* (Bohmert et al., 1998) mutants exhibit altered venation. The YABBY protein plays a role in the regulation of auxin homeostasis, while the ARGONAUTE protein plays a role in the processing of micro RNAs. Many other mutants have been discovered, mainly based on their altered vein pattern in leaves. These mutations cause discontinuous, random, or reduced numbers of veins, along with defects in other aspects of plant development, for example, *cvp1, cvp2*, and *sfc/van3* (mentioned previously) mutants have discontinuous veins with reduced numbers. (Koizumi et al., 2000) The distal end of newly forming veins of *FORKED 1* mutants do not connect to previously formed veins. (Steynen and Schultz, 2003). Further examination of these mutants and genes will contribute to understanding vascular pattern formation mechanism at the molecular level.

1.3.3 Genetics of vascular differentiation

The initiation of vascular differentiation starts already during embryogenesis with the formation of procambial cells. These cells differentiate into mature vascular cells soon after germination. Thereafter the formation of new procambial cells is tightly associated with the formation of leaves from meristems. In addition to the venation

formed in leaves, the midvein, going through the blade and petiole, contribute with a vascular bundle to the stem as well (the leaf trace). In addition to this primary pattern of vascular tissues, secondary vascular meristematic tissues are formed between vascular bundles of the stem or from the pericycle in the root. These meristematic tissues, referred to as vascular cambium, form secondary vascular tissues, most commonly referred to as wood in woody plants (Esau, 1965).

During xylem and phloem differentiation from the procambium, the tracheary elements and sieve elements are interconnected to form tubes by control of polar auxin transport and other additional hormones. In recent studies, the details of this process have started to be elucidated from studying xylogen (Motose et al., 2004). The molecular identification of xylogen classifies it as a proteoglycan protein that has properties both of an arabinogalactan protein and of a lipid-transfer protein. It is located in the apical side of undifferentiated xylem cells. In tissue culture, young tracheary elements secrete xylogen, which induces neighbouring cells to transdifferentiate into trachery elements, suggesting that the function of xylogen is to mediate tracheary element differentiation to form continuous strands of xylem (Motose et al., 2004).

The secondary wall of tracheary element contributes the most in composition of the wood in trees. The formation of secondary walls during tracheary element differentiation involves the deposition of cellulose to form characteristically patterned wall thickenings. The secondary walls consist of a higher content of cellulose than primary walls, as well as lignin, which normally is not present in primary walls. Some studies on vascular differentiation have focused on the biosynthesis of these compounds. Many of the enzymes involved in cellulose and lignin biosynthesis have been described

and their corresponding genes are now the focus of study in a number of laboratories (Mellerowicz et al., 2001). A decrease in lignin content is desired in the pulp and paper industry as this compound has to be removed in the manufacturing of paper. Recently, a reduced lignin content was accomplished by significantly reducing the expression of a lignin biosynthetic gene in poplar (Hu et al., 1999). Differential screening for vascular-specific genes has been performed primarily by exploring the capacity of *Zinnia* mesophyll cells to undergo synchronous trans-differentiation into vessel elements upon exposure to auxin. These studies have elucidated a number of expected as well as unexpected genes (Yoshimura et al., 1996). This system has shortcomings, as it is restricted to vessel elements that undergo only limited elongation.

Until recently, the literature contained few descriptions of vascular mutants. Over the past few years, however, a number of mutants with vascular defects have been isolated, primarily in Arabidopsis. Many of these vasculature mutants have pleiotropic phenotypes. They are thought to be defective in auxin signaling rather than specification and differentiation of particular cell types (Berleth et al., 2000) and will not be reviewed here. Turner and Somerville (1997) screened mutagenized populations of Arabidopsis for irregular xylem tissue by microscopic examination of stem sections. They identified several independent loci that had defective xylem, and many of these genes have now been cloned. The *irregular xylem 1* and *3 (irx1, irx3)* encode cellulose synthases that are required for secondary cell wall synthesis (Taylor et al., 1999; Taylor et al., 2000), whereas the *irx4* gene encodes a cinnamoyl-CoA reductase, which is involved in lignin biosynthesis (Jones et al., 2001). As the name indicates, the *interfascicular fiber-less mutant (ifl1)* lacks interfascicular fibers in the inflorescence stem (Zhong et al., 1999).

Closer scrutiny has revealed that this deficiency is restricted to the basal portion of the inflorescence and that *ifl1* is defective in polar auxin transport. These observations suggest that the primary function of *IFL* may be in auxin transport rather than fiber differentiation (Zhong et al., 1999).

1.4 The aims of this study

1.4.1 The role of *AtPIN FORMED1* in vein formation

As mentioned previously, based on the chemiosmotic hypothesis, plants use specialized influx and efflux carrier molecules to polarly transport indole-3-acetic acid (IAA) from cell to cell. In recent years, genes that encode proteins that are believed to be components involved in polar auxin transport have been identified. One of these genes, *AtPIN FORMED1* (PIN1), is believed to encode an auxin efflux carrier (Galweiler et al., 1998), and its molecular identification revealed a family of related genes are encoded in the Arabidopsis genome (Muday and Murphy, 2002). The identification of PIN1 and additional molecules that function in the transport pathway, provides tools for identifying cells that conduct auxin transport and an indication of direction of auxin flow (Wisniewska et al., 2006).

During the globular embryo stage, PIN1 protein is expressed throughout the embryo. In heart-staged embryos, subcellular localization of PIN1 protein in the elongated procambial cells starts to be established (Galweiler et al., 1998). This expression pattern suggests that the PIN1 protein is expressed in prepocambial cells that will undergo vascular differentiation. Mattsson et al (1999) showed that *pin1* mutant rosette leaves have vein pattern defects that are similar to leaves that grow in low levels

of polar auxin transport inhibitors. It suggests defects in *pin1* partially block polar auxin transport.

The identification of carrier molecules with subcellular localizations that indicate direction of auxin flow allows, for the first time, the visualization of the canalization process as it occurs. AtPIN1 labelled with a fluorescent-protein marker has been studied during the developmental process of embryogenesis (Vieten et al., 2005), lateral root initiation (Benkova et al., 2003), flower primordial development (Heisler et al., 2005), and so on. To the date I started my study, *PIN1*-mediated leaf vein formation had not been studied (Scarpella et al., 2006). My hypothesis is that PIN1 expression in leaf primordia predicts the site of procambial vein selection in leaves. In addition, the subellular localization of PIN1 may reveal the dynamics of auxin flow during this process. To this effect, pPIN1::PIN1::GFP (PIN1-GFP) created by Eva Benkova (2003), was used in my study as a marker to visualize PIN1 distribution during vein formation in leaf primodia, mainly focusing on the formation of the primary and secondary veins in the first leaf of Arabidopsis seedlings as the development of those leaves is relatively synchronous and easy to access soon after seed germination.

1.4.2 Using microarray analysis to identify vascular differentiation-associated genes

Auxin transport inhibitors have been used in studies which revealed that the polarity of auxin movement provides a key developmental signal during embryogenesis (Liu et al., 1993), lateral root initiation (Casimiro et al., 2001), leaf vascular patterning (Mattsson et al., 1999), and root gravitropism (Parry et al., 2001). One of several chemicals, NPA (1-N-naphthylphtalamic acid) inhibits polar auxin transport by

interfering with auxin efflux (Lomax et al., 1995). Plants grown on medium with inhibitor produce increased amount of vascular strands, and cells within those strands are improperly aligned (Rubery, 1990). This study showed that plants exposed to 1-40 μM of auxin transport inhibitors such as NPA during development, developed organs with internal vascular hypertrophy. In theory, this observation offers an approach to identify genes with a role in vascular differentiation, as their transcripts should be more prevalent in tissues that are developing NPA-induced vascular hypertrophy.

DNA oligonucleotide microarray analysis was utilized by Drs. Wenzel and Mattsson before my involvement in this study to capture changes in expression levels of all genes in the Arabidopsis genome in response to NPA. It is a novel technology producing high throughput results with gene expression levels for thousands of genes simultaneously, thereby monitoring the whole genome on a single chip (Stoughton, 2005). Several studies have used microarrays to identify genes likely to be expressed in plant vascular tissues, such as in Arabidopsis stems (Zhao et al., 2000; Oh et al., 2003; Ko et al., 2004; Ehlting et al., 2005; Zhao et al., 2005) in the developing Arabidopsis root (Birnbaum et al., 2003), in cultured *Zinnia elegans* mesophyll cells transdifferentiating into xylem cells (Allona et al., 1998; Taku et al., 2002), in wood-forming tissues of several tree species (Allona et al., 1998; Sterky et al., 1998; Hertzberg et al., 2001; Lorenz and Dean, 2002; Kirst et al., 2003; Yang et al., 2003), and in maize (Nakazono et al., 2003).

The genetic basis of internal vascular differentiation and function is relatively poorly understood, partly due to the difficulty of screening for mutants defective in internal vascular tissues. My hypothesis is that a microarray analysis of differential gene

expression in RNA samples from plants developing in the absence or presence of NPA will identify large number of genes that may relate to vascular differentiation. This hypothesis is based on the assumption that genes that are involved in vascular differentiation should have increased expression in response to an auxin transport inhibitor-induced vascular hypertrophy. To test the hypothesis that genes may have potential functions in vascular differentiation if the expression profile of promoter-marker gene fusions is in vascular tissues, phenotypic study on the potential vascular-associated genes identified from a previous study was performed.

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CHAPTER 2
ASSESSMENT OF PIN1 EXPRESSION IN LEAF VENATION PATTERNING

Originally published in *The Plant Journal* 2007 Feb;49(3):387-98, under the title "Dynamics of *MONOPTEROS* and PIN-FORMED1 expression during leaf vein pattern formation in *Arabidopsis thaliana*" under the authorship of C. Wenzel, M. Schuetz, Q. Hester and J. Mattsson, with the gracious permission of The Plant Journal/Blackwell Synergy it is replicated here. Figure 1 and related description in the main text are altered from the original paper to preserve this information for M. Schuetz's thesis.

Dynamics of PIN-FORMED1 expression experiments were performed by joint effort between Dr. Wenzel and Ms. Hester (see figure 2, 3, 4, 9), including sample preparation and confocal laser scanning microscopy of PIN1. Dynamics of MONOPTEROS expression experiments were performed by Mr. Schuetz. Experiments on auxin induction of MP expression were performed by Drs. Mattsson and Wenzel as well as Mr. Schuetz (see figure 1, 5, 6, 7, 8 and 9). Figure 1 has been replaced by a simplified figure produced by Dr. Mattsson and the corresponding text has been shortened accordingly.

2.1 Summary

Genetic evidence links the Arabidopsis *MONOPTEROS (MP)* and *PIN-FORMED1 (PIN1)* genes to the patterning of leaf veins. To elucidate their potential functions and interactions in this process, we have assessed the dynamics of *MP* and PIN1 expression during vascular patterning in Arabidopsis leaf primordia. Both genes undergo a dynamic process of gradual refinement of expression into 1-2 cell wide files before overt vascular differentiation. The subcellular distribution of PIN1 is also gradually refined from a non-polar distribution in isodiametric cells to strongly polarized in elongated procambial cells and provides an indication of overall directions of auxin

flow. We found evidence that *MP* expression can be activated by auxin exposure and that PIN1 as well as DR5::GUS* expression is defective in *mp* mutant leaves. Taken together the results suggest a feedback regulatory loop that involves auxin, MP and PIN1 and provide novel experimental support for the canalization of auxin flow hypothesis.

2.2 Introduction

Vascular tissues differ from most other plant tissues in that cells need to be precisely connected in order for the tissue to carry out its functions. Vascular organization is particularly conspicuous in the highly ordered pattern of veins in leaves. *Arabidopsis thaliana* leaves develop a hierarchical reticulate venation pattern first with the formation of a central primary vein, followed by successive basipetal addition of secondary veins and finally higher order veins (Mattsson et al., 1999; Mattsson et al., 2003).

Numerous experiments suggest that auxin has an essential role in vascular patterning. Local auxin application induces vascular strand formation (Sachs, 1981) and high levels have been detected in preprocambial cells (Uggla et al., 1996; Mattsson et al., 2003) suggesting that auxin may act as a positional signal controlling vascular development. Pharmacological inhibition of auxin transport has a dramatic effect on vascular pattern formation (Mattsson et al., 1999; Sieburth, 1999), and mutations in auxin signaling-related *MONOPTEROS (MP), BODENLOS,* and *AUXIN-RESISTANT6* disrupt vascular continuity (Berleth and Jurgens, 1993; Przemeck et al., 1996; Hamann et al., 1999; Hobbie et al., 2000). The incomplete vascular continuity in the *mp* mutant and

* The construct is a vector where seven synthetic auxin response elements have been fused to generate a strong promoter reporter of cellular auxin content (DR5), fused to the uidA gene encoding the β-glucuronidase enzyme as a reporter gene.

expression of the auxin response factor *MP* in vascular tissues suggests an early function of *MP* in the establishment of vascular patterns (Hardtke and Berleth, 1998). Mutants in *MP* are defective in the auxin induction of several genes that may affect procambial development (Mattsson *et al.*, 2003).

Auxin is actively transported within the plant (reviewed in Sachs, 1981; Aloni, 1995; Friml, 2003; Geisler et al., 2005). AUXIN1 (AUX1) and similar proteins are auxin influx carriers and the PIN-FORMED (PIN) family are auxin efflux carrier proteins that become polarly localized within cells and facilitate directional auxin flow (Bennett et al., 1996; Galweiler et al., 1998). Several ABC proteins are also involved in auxin transport, possibly acting independently of PINs (Noh et al., 2001; Muday and Murphy, 2002; Geisler et al., 2005). Polar localization of PIN carrier complexes is mediated by vesicle trafficking (Steinmann et al., 1999; Muday and DeLong, 2001; Geisler et al., 2005), and by polarity and cell fate determinants ((Friml et al., 2004; Treml et al., 2005; Wisniewska et al., 2006). Auxin seems to regulate PIN transcription, cellular trafficking and localization (Leyser, 2005; Paciorek et al., 2005; Vieten et al., 2005; Wisniewska et al., 2006).

Organ and vascular formation in plants may rely on auxin carrier proteins and other upstream factors driving formation of local auxin maxima (Benkova et al., 2003 Friml *et al.*, 2003; Wisniewska et al., 2006). In the shoot apical meristem, the sub-cellular localization of AUX1 and PIN1 proteins suggests that auxin flows in the epidermis towards a point where an auxin maximum is formed, followed by an internalization of flow, triggering the formation of leaf primordia and the midvein (Benkova *et al.*, 2003; Reinhardt *et al.*, 2003). PIN1 is further implicated in vascular formation since it becomes

polarly localized in vascular cells to transport auxin from the stem towards the root (Galweiler *et al.*, 1998; Vieten *et al.*, 2005), and *pin1* mutants show increased vascularization similar to the effects seen with polar auxin transport inhibition (Galweiler et al., 1998; Mattsson et al., 1999; Reinhardt et al., 2003; Vieten et al., 2005).

Two models have been proposed describing the selection of cells for vascular strand formation. The auxin canalization hypothesis proposes a positive feed-back mechanism whereby an initial broad region of auxin is actively channeled into a file of cells that eventually undergo vascular differentiation (Sachs, 1981; Mattsson et al., 1999; Rolland-Lagan and Prusinkiewicz, 2005). Support for this model comes from experiments showing that chemical or genetic inhibition of auxin transport can lead to thicker veins, presumably since auxin cannot drain properly and thus forms broader canals of cells with high auxin concentrations (Mattsson et al., 1999; Rolland-Lagan and Prusinkiewicz, 2005). A reaction-diffusion model based on the interaction of two or more diffusing substances has also been used to describe vascular patterning (Nelson and Dengler, 1997). Local fluxes of an activator such as auxin may trigger a positive feedback loop leading to cells with high activator concentration to undergo vascular differentiation. Koizumi *et al.* (2000) suggest that the *van* mutants, which show discontinuous but generally normal vein patterning, support the reaction-diffusion model since the canalization model assumes a continuous generation of strands. However, two studies using computer modeling (Rolland-Lagan and Prusinkiewicz, 2005) or showing localization of PIN1 in the *van3* mutant (Scarpella *et al.*, 2006) provide support for the existence of a canalization mechanism in mutants with discontinuous vein strands.

Both *MP* and *PIN1* are implicated in leaf vein formation since the corresponding *mp* and *pin1* mutants have vein defects. In this study we explore the roles of *MP* and *PIN1* in Arabidopsis leaf vein patterning by assessing their expression during this process. In the final stages of preparation of this manuscript a study was published by Scarpella *et al.* (2006) describing PIN1 localization in developing Arabidopsis leaf primordia that overlaps in part with this study. These results will be further discussed in context to our own results (see below).

2.3 Results

In this study, we are assessing the spatio-temporal expression patterns of two components involved in leaf vascular development in *Arabidopsis thaliana - MONOPTEROS (MP)* and PIN-FORMED 1 (PIN1) and their potential interaction with auxin. Hereafter, we refer to '*MP* expression' as the level of *MP* mRNA transcript indicated by *in situ* RNA hybridization, and 'PIN1-GFP expression or localization' as indicated by the fusion protein PIN1-GFP in *pPIN1::PIN1-GFP* transformed lines. Vascular terminology are as described in (Mattsson et al., 2003) and (Hickey, 1979), and also shown in Figure S1. Terminology regarding positions within organs and individual cells are as defined in Friml *et al.* (2006). First rosette leaf primordia stages are given in days after germination (DAG).

2.3.1 Dynamics of *MONOPTEROS* (*MP*) expression

In this study, we show how *MP* expression is progressively restricted from broad regions to preprocambial cells. In 2 DAG leaf primordia, *MP* is initially expressed at elevated levels in the incipient primary vein in first leaf primordia (Figure 1a). By 3

DAG, *MP* expression indicates the incipient secondary veins along the margin before the formation of elongated procambial cells of secondary veins (Figure 1b). For all subsequent basipetally formed secondary veins, *MP* expression is first low in a large region between the margin and existing secondary preprocambial strand, before a distinct strand appears. *MP* expression is also gradually restricted during the formation of tertiary veins (Figure 1c). In summary, *MP* expression appears to go through a process of gradual refinement from regions of low levels of expression in many cells into single files of cells with strong expression before procambial differentiation occurs.

2.3.2 Dynamics of PIN1-GFP expression

PIN1-GFP expression overlaps considerably with *MP* expression and also becomes restricted to preprocambial strands. In the first leaves, by 2 DAG a 2-3 cell wide region of PIN1-GFP expressing cells depicts the primary vein connecting to the hypocotyl vasculature (Figure 2a), with expression narrowing to a 1-3 cell wide primary vein in older leaf primordia (Figure 2b-d). A similar progressive delimitation of the primary vein strand is observed for later forming leaves. In all rosette leaves, the first indication of secondary preprocambial veins based on PIN1-GFP expression appears as a cluster of PIN1-GFP expressing cells in the basal adaxial region (asterisks in Figure 2b,f,k and arrows in 2e). This region of PIN1-GFP-expressing cells is gradually restricted to form a continuous 1-2 cell wide secondary strand (Figure 2f-h and 2k-m). Secondary vein formation often occurs in two stages, with the strand forming between the epidermis and midvein prior to connecting to a more distal region of the midvein (Figure 2b-d) or more distal secondary strand (Figure 2g,h). This biphasic connection always occurs for the second to fourth secondary veins in later formed rosette leaves (Figure 2i-k), but is

usually more simultaneous for the fourth and fifth pairs of secondary veins in the first leaves and the fifth and subsequently formed secondary veins in later rosette leaves (data not shown). PIN1-GFP expression in developing higher order veins appears as either outgrowth from existing veins, or connection of isolated or clustered PIN1-GFP-expressing cells to a lower order vein (Figure S2a,b). PIN1-GFP expression in all vein types is progressively lost in a basipetal direction as the more distal veins differentiate (e.g. Figure 3e).

2.3.3 PIN1-GFP expression extends from the epidermis into ground tissue

PIN1-GFP expression suggests a role of the epidermis in directing auxin into developing vascular regions. In slightly bulging primordia, PIN1-GFP fusion proteins are localized to the apical end of all epidermal cells leading towards the primordia apex, which overlies the developing primary vein (Figure 3a,b, 4s). About the time of emergence of the first pair of secondary veins, PIN1-GFP is predominantly lost from the abaxial epidermis and becomes progressively more restricted to basal adaxial and marginal epidermal cells (Figure 3c,d, S2c). Thereafter, PIN1-GFP is always localized to the basal adaxial and marginal epidermal cells that are adjacent to the newly forming secondary veins (Figure 2e, 3d,e, S2c), connecting the epidermis and developing veins (e.g. Figure 4t, S2e) until the procambial strand is clearly delimited (Figure 2g,h and 2l,m). In later formed rosette leaves, this epidermal region includes areas just proximal and distal to a serration (Figure 3e), respectively involved in the formation of secondary (arrowheads in Figure 2k-m) and tertiary marginal (Figure S2b) veins. Some of the later formed rosette leaves (<5%) also maintain abaxial PIN1-GFP expression along much of

the length of the primordia up until the formation of the fourth or fifth secondary veins (Figure S2d).

2.3.4 PIN1-GFP polarization during procambial development

All vein classes show polar PIN1-GFP localization once the narrow procambial strands are clearly formed. Our results, however, indicate that PIN1-GFP proteins are initially diffusely distributed throughout the cytoplasm and/or on the plasma membrane. In young primordia that show PIN1-GFP polarity on the apical ends of epidermal cells, the procambial midvein cells often appear to have PIN1-GFP expression throughout the cytoplasm (Figure 4a), which later localizes in the basal end of cells (Figure 4b,c). Developing secondary preprocambial cells often show diffuse PIN1-GFP cytoplasmic expression (e.g. arrows in Figure 4d), which later becomes polarized (Figure 4e). The most obvious examples of early non-polarized PIN1-GFP expression are observed in later forming rosette leaves that have broad regions of cells with diffuse PIN1-GFP expression (Figure 4j,o). In these regions, PIN1-GFP gradually becomes more polarly localized in fewer cells, eventually forming a secondary procambial strand with highly polarized PIN1-GFP localization (Figure 4k-n). Epidermal cells adjacent to developing secondary preprocambial cells are often observed with PIN1-GFP expression throughout their cytoplasm, which is eventually more localized to the membrane as the secondary procambial strands develop or as a leaf serration expands (Figure 4o-r,t).

Based on PIN1-GFP protein localization we can predict the direction of auxin flow. Auxin flow in the primary vein is predominantly in the basal direction (Figure 4b,c). Auxin flow is predominantly bi-directional along secondary strands, with the distal cells having apical (Figure 4f) or basal PIN1-GFP polarity, and the more basal cells

always having basal polarity where they connect to the midvein (Figure 4h), with one or more cells within the strand having bi-directional PIN1-GFP localization (Figure 4g). Less frequently, PIN1-GFP occurs only on basal ends of procambial cells, resulting in a basal direction of auxin flow along a given secondary strand (Figure 4i). In young leaf primordia PIN1-GFP becomes localized to the apical ends of epidermal cells, presumably resulting in auxin flow to the apex before it is internalized through the primary procambial strand region (Figure 3a,b, 4s). PIN1-GFP can also become polarly localized in epidermal cells adjacent to developing secondary strands, indicating epidermal auxin flow towards a serration apex and then internally into the developing secondary strand (Figure 4q,r,t).

2.3.5 *MP* expression is induced by auxin and similar to auxin response patterns

Previous experiments to assess auxin induction of *MP* and other auxin response factors (ARFs) transcription have been negative (Ulmasov *et al.*, 1999). Instead, the auxin response mediated by ARFs is believed to come from auxin-induced degradation of Aux/IAA proteins that otherwise dimerize with and inhibit the DNA binding of ARFs (Leyser, 2002). As shown in Figure 1, the expression of *MP* is highly regulated. If it is not regulated at least in part by auxin, one has to consider mechanisms other than canalization of auxin flow for the patterning of *MP* expression. To assess whether *MP* expression can be induced by auxin on a local level, we exposed 3 or 4 day old seedlings grown in liquid medium to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) for 16 hr followed by whole-mount *in situ* hybridization. We used the strong auxin-response marker DR5::GUS (Ulmasov *et al.*, 1997) as a control to identify suitable conditions for auxin exposure. In 3 day old seedlings, exposure to 1 μM 2,4-D resulted in

a patchy and weak activation of DR5:GUS expression, whereas exposure to 10 μM 2,4-D resulted in a strong response throughout the lamina (Figure 5b,c). Exposure to 1 μM 2,4-D enhanced preprocambial *MP* expression and also resulted in ectopic expression in ground cells between preprocambial veins predominantly in the basal part of leaf primordia regions where new secondary veins are forming (Figure 5e compared to 5d). Exposure to 10 μM 2,4-D, resulted in strong *MP* expression throughout the lamina except for a 2-3 cell-wide region proximal to the margin (Figure 5f). Cells in this region appear to lack not only the competence to express *MP* in response to auxin, but also to respond to accumulation of endogenous auxin by vascular differentiation (Mattsson *et al.*, 1999; Mattsson *et al.*, 2003). That cellular competence may play a role in the response of *MP* to auxin is further supported by exposure of 4 day old leaf primordia to auxin (Figure S5), where the response is limited almost entirely to the actively growing basal part of the primordia. The response of *MP* in primordia of this age to exogenous auxin is similar to the auxin response reported for *pPIN1::PIN1-GFP* to topical application of auxin (Scarpella *et al.*, 2006). We can reproduce a similar induction of *PIN1* mRNA transcript by auxin exposure in liquid medium and detection by *in situ* hybridization (Figure S5), providing further evidence of a similar response of *MP* and *PIN1* to auxin.

To assess whether *MP* expression responds to changes in the distribution of endogenous auxin, we exposed seedlings to auxin transport inhibitors rather than auxin. This kind of treatment is known to cause major alterations in auxin distribution (Mattsson *et al.*, 2003) and correlated changes in venation patterns (Mattsson et *al.*, 1999; Sieburth, 1999). In young leaf primordia of seedlings germinated in the presence of 10 μM NPA, *MP* is expressed primarily in a region 2-3 cells proximal and parallel to the leaf margin,

with low levels of *MP* expression throughout the central lamina (Figure 6g,h). In more advanced primordia, there can also be preprocambial strands of *MP*-expressing cells extending from the margin towards the petiole (Figure 6i). This pattern of expression in response to NPA treatment is almost identical to that observed with the auxin-sensitive DR5::GUS reporter (Mattsson et al., 2003).

Since *MP* encodes an auxin response factor, it is in theory possible that it regulates its own expression. To assess this possibility, we analyzed the expression of *MP* in primordia of *mp* mutants that produce a defective *MP* transcript. We found *MP* expression in young primordia indicative of preprocambial primary and the first two pairs of secondary veins, which is comparable to the expression in wildtype primordia of equal size (compare Figure 6a,b with 6d,e). Thus it appears that the *MP* gene product does not feed back significantly on its own expression during preprocambial strand formation. *MP* expression in *mp* mutants is much reduced in later stages (Figure 6f), possibly due to the lack of preprocambial cells at this stage, and a limited number of cells undergoing procambial differentiation. In summary, we have found experimental support that *MP* expression can be altered by manipulation of auxin distribution, and that early *MP* expression does not depend on activity of the MP protein.

2.3.6 PIN1-GFP expression is regulated by MP and altered by auxin transport inhibition

Since the *MP* gene encodes a transcriptional regulator, it is possible that PIN1 depends on MP for normal expression. To assess this possibility we introduced the *pPIN1::PIN1-GFP* marker into the *mp* mutant backround. In contrast to the initially normal *MP* expression in *mp* mutants, PIN1-GFP expression in *mp* mutants is much

reduced, both quantitatively and qualitatively (Figure 7d-f). The expression of PIN1-GFP in *mp* mutant primordia is evident in developing primary veins and incomplete secondary veins that terminate in a diffuse PIN1-GFP expressing region (compare Figure 7d-f and S3f-i with 7a-c). In *mp* mutants, PIN1-GFP appears to be localized in apical ends of epidermal cells in young emerging leaf primordia (Figure S3e). In older *mp* primordia, PIN1-GFP expression becomes predominantly restricted to basal adaxial regions (Figure S3f) with little or no epidermal PIN1-GFP polarity (Figure S3g-i). Similar to *MP,* in NPA-treated tissue PIN1-GFP has diffuse lamina expression 2-3 cells proximal to the margin in young primordia and marginal expression with strands leading towards the petiole in older primordia, although *MP* expression is higher near the margin of young primordia (compare Figures 7g-i with 6g-i). NPA treatment predominantly inhibits the development of sub-cellular PIN1-GFP localization in most internal cells (Figure S4) and in the epidermis (Figure S3a-d), although PIN1-GFP expression does become restricted to the basal adaxial and marginal epidermis in older primordia (Figure S3b).

2.3.7 *mp* mutants are defective in the organization of auxin maxima

The canalization of auxin flow hypothesis predicts a feedback regulatory loop where auxin transport alters the pattern of auxin distribution (Sachs, 1981). If *MP* is part of such a regulatory loop, one may expect alterations in the auxin response pattern in *mp* mutant leaves. The auxin response pattern of DR5::GUS expression in wild type primordia can be briefly summarized as having maxima at the apex and lateral serrations, in addition to preprocambial and procambial expression (Mattsson *et al.*, 2003; Figure 8a-c). Most *mp* primordia have an abnormally large and diffuse apical maximum, usually no lateral maxima, diffuse preprocambial and rarely occurring procambial DR5::GUS

expression (Figure 8d-f). In wildtype primordia, NPA treatment predominantly shifts DR5::GUS expression to the primordia margin (Mattsson *et al.*, 2003; Figure 8g-i). A similar distribution of DR5::GUS expression is seen in older primordia of *mp* and *pin1* mutants, although the latter can often also form two or more apical maxima of DR5::GUS activity (Figure S6). In summary, *mp* mutants appear to fail to delimit and organize vascular and marginal auxin maxima, while *pin1* mutants can organize extra auxin maxima.

2.4 Discussion

The expression of *MONOPTEROS (MP)* and *PIN-FORMED1 (PIN1)* during venation patterning of leaves is of particular interest since mutants in both genes have vascular defects and the gene products are involved in auxin signaling and transport. Here we propose a feedback model that involves *MP, PIN1* and auxin in the formation of leaf veins. A part of our study, PIN1-GFP expression in developing leaf primordia, overlaps with a recent publication (Scarpella et al., 2006). Points where we provide additional information will be discussed below.

2.4.1 A model for canalization of auxin flow that involves MP and PIN1 activity

Presently, two models are favored for the explanation of the patterning of veins in leaves; the canalization of auxin flow hypothesis (Sachs, 1981), and the diffusion-reaction hypothesis (reviewed by Koch and Meinhardt, 1994). Our assessment is potentially biased towards the first hypothesis in that we have actively sought to understand the role of auxin-related components in vein formation. This hypothesis predicts a positive feed-back mechanism whereby cells respond to auxin by expressing

auxin efflux carriers, which eventually become polarly localized to produce discrete files of auxin-transporting cells that undergo vein differentiation (Sachs, 1981).

Although the gradual refinement of expression that we observe for both *MP* and PIN1-GFP is consistent with both models, our observations support the following positive feedback cellular response pathway driving auxin canalization, as summarized in Figure 9a. (1) In response to increased auxin levels, the *MP* gene becomes transcriptionally activated (Figure 5 and S5). To our knowledge, this is the first experimental support for an auxin-induced expression of a gene encoding an auxin response factor, although we can not rule out indirect induction at this point. (2) The activity of the MP protein is modulated by inhibitory Aux/IAA proteins, which can be targeted for auxin-induced degradation (Ulmasov et al., 1999; Leyser, 2002). (3) The *MP* and PIN1-GFP expression patterns in developing leaf primordia overlap considerably and undergo a similar gradual restriction of expression (Figures 1 and 2). We have shown that PIN1-GFP protein is reduced quantitatively and qualitatively in the *mp* mutant (Figure 7), suggesting that the expression of the *PIN1* gene is partially regulated by the MP protein, possibly by direct interaction of MP protein with the *PIN1* promoter. There is evidence that *PIN1* is at least in part activated by auxin (Paciorek *et al.*, 2005; Scarpella *et al.*, 2006; Figure S5) and this response may be mediated by MP. (4) We have shown that the subcellular localization of PIN1-GFP develops gradually from no obvious polarity in isodiametric cells to strong polarity that indicates auxin flow along a source-sink vector in elongated and interconnected procambial cells (Figure 4). PIN1 polarity is presumably regulated by mechanisms that involve vesicle trafficking and regulatory proteins such as PINOID (e.g. Friml *et al.*, 2004; Steinmann *et al.*, 1999). (5) The resulting gradual

concentration of auxin into certain cells would lead to stronger activation of MP, possibly both on transcriptional and posttranscriptional levels, and as a consequence a stronger expression of the *PIN1* gene. (6) Cells that are drained of auxin would react by decreasing expression of *MP* and PIN1. While the above scenario provides a cellular framework for a positive feedback loop, the relatively minor venation defects in mature leaves of both *pin1* and *mp* mutants suggests that other regulatory and carrier proteins are likely involved.

2.4.2 A module for major vein formation

Based on the patterns of *MP* mRNA transcript and PIN1-GFP fusion protein expression and subcellular localization, we propose a module for secondary vein formation as an extension of what has been proposed for leaf primordia and midvein formation based on polar auxin transport (Reinhardt *et al.*, 2003). A similar model has been proposed by Scarpella *et al.*, (2006) based on PIN1-GFP expression, and the main difference between the models is that we include the positive feedback regulatory loop described above. The module includes several steps beginning with the expression of PIN1 in the adaxial and marginal epidermis and *MP* and PIN1 expression in the basal adaxial ground tissues of leaf primordia. PIN1 epidermal expression may not be driven by auxin alone as there is no corresponding *MP* and DR5::GUS expression in the epidermis. Growth initially occurs throughout the primordia and gradually becomes restricted to basal, actively growing regions in older primordia (Kang and Dengler, 2002), which are competent to respond to auxin with respect to *PIN1* and *MP* regulation (Figure 5e and S5). The auxin produced by cells in actively growing ground tissue and epidermis triggers the canalization of auxin flow mechanism, beginning with wide

regions of cells with low levels of MP and non-polarized PIN1 proteins and ending in narrow files of cells with high levels of MP and highly polarized PIN1 proteins. The source-sink relationship between actively growing regions (sources) and pre-existing veins (sinks) triggers first the formation of the midvein, then in turn the progressive formation of secondary veins in the growing basal regions of the primordia. Secondary veins often appear to emanate from a primary source at the leaf margin where PIN1 is particularly strongly expressed. From there, elongated clouds of *MP* and PIN1 expressing cells connect to the midvein (sink) and a pre-existing secondary vein (sink). These clouds of expression are gradually resolved into strands in parallel with the gradual polar localization of PIN1. Figure 9b summarizes this module for reiterative secondary vein formation based on *MP* and PIN1 expression.

Based on our results for subcellular PIN1-GFP localization, an entry point of auxin appears in conjunction with the formation of each secondary vein, through which auxin moves from the epidermis into the ground tissues. The mechanism behind the formation of these entry points is unknown. We have noticed though that in *mp* mutant background, the level of PIN1-GFP expression and degree of epidermal polarization is much reduced. This is surprising as *MP* is not expressed in the epidermis, and one has to consider indirect effects of MP activity to explain this apparent contradiction. The strong ground tissue expression of PIN1-GFP normally seen near these points is also weak or absent in the *mp* mutants. One possibility is that the internal canalization driven by the MP/PIN1 feedback loop is required to form a highly focused entry point in the epidermis. This scenario can be likened to pulling the plug in a bath tub (sink), resulting in a strong and focused vortex on the surface (epidermis) bringing surface particles (auxin) to the

vortex, before they are engulfed. In *mp* mutants, a weakened vortex forms that results in partial secondary veins close to the sink (midvein), but is not focused enough at the surface to result in the formation of distal parts of secondary veins, a typical phenotype of young *mp* mutant leaves. Thus MP and PIN1 appear to be important components in auxin canalization affecting leaf vein formation. Additional components are likely to be added with time.

2.5 Experimental procedures

2.5.1 Plant material and growth

Arabidopsis thaliana Columbia-0 containing a *pPIN1::PIN1-GFP* fusion construct (Stock no. CS9362) was obtained from the Arabidopsis Biological Resource Centre, Ohio State University, USA. Col-0 plants containing the auxin-responsive promoter DR5::GUS construct were obtained from Tom Guilfoyle (University of Missouri, Columbia; Ulmasov *et al.*, 1997). Sterilized seeds were plated in 9cm Petri dishes containing solid *Arabidopsis thaliana* salts media (Lincoln et al., 1994). For the auxin transport inhibitor treatments, seedlings were plated on media containing 0.1, 1 or 10 μM N-(1-naphthyl)phthalamic acid (NPA; TCI, Tokyo, Japan). Seeds were stratified at 4°C for at least two days, and grown in a short-day chamber (8h light/16h dark) at approximately 20°C and 50 μEinsteins light intensity.

For the auxin induction treatment, approximately 50 DR5::GUS seeds were grown in 9cm Petri dishes containing 10 ml of sterile ATS media on a rotary shaker for 3 or 4 days at 20°C. At these time points 0.1% (w/v) pluronic-F68 surfactant (Sigma) and 2,4-dichlorophenoxyacetic acid (Sigma) to final concentrations of 0, 1 or 10 μM were

added to the media and seedlings grown for about 16h. The seedlings were then GUS stained or prepared for *in situ* hybridization to detect DR5::GUS or *MP* expression. Observations were made on at least 10 primordia for each treatment.

2.5.2 PIN1-GFP and DR5::GUS localization

Expression was observed in leaf primordia extracted on sequential days. Results are based on observations of at least 20 leaves taken from each developmental stage. Individual leaves or leaf primordia were extracted from seedlings and mounted in a 30% aqueous glycerol solution. In order to avoid disruption of inherent PIN1-GFP localization, we mostly base polarity observations on tissues that were examined within about 15 min of mounting in water. In some cases determination of the polarization of PIN1-GFP localization in procambial cells was facilitated with induction of plasmolysis by mounting the extracted leaves in either a 2M NaCl or 100 μM aqueous propidium iodide (Sigma) solution. A Zeiss LSM 410 confocal laser scanning microscope was used to image PIN1-GFP localization. GFP was imaged using a 488 nm excitation filter and 500-530 nm emission filter combination. Background red autofluorescence was detected using a 568 nm excitation filter and an LP 580 emission filter set. DR5::GUS assays were performed as described in Mattsson *et al.* (2003). DIC images were taken on a Nikon Eclipse E600 microscope using a digital camera. Adobe Photoshop software was used to merge images with slightly different focal planes.

2.5.3 Whole-mount *in situ* hybridization

Whole mount *in situ* hybridization was carried out as described in (Zachgo *et al.*, 2000) with several changes. In our hands, a fixation time of least 4 hours and generally

overnight, had to be used to obtain expected expression patterns of several published genes, including *MP*. In addition, background signal was decreased by agitation in a fresh solution containing 0.1 M triethanolamine (pH 8) and 0.5% (v/v) acetic anhydride for 15 min, followed by two washes in 1x PBT solution prior to hybridization for two days at 60°C. *MP* sense and antisense probes were prepared as described in Hardtke and Berleth (1998).

2.6 Acknowledgements

We thank Afsaneh Haghighi-Kia for technical assistance, Steven Chatfield, Jiri Friml and the Arabidopsis Biological Resource Centre (Ohio State University, Columbia) for providing the *pPIN1::PIN1-GFP* line. The work was supported by a National Science and Engineering Research Council of Canada grant to J.M.

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2.8 Figures*

Figure 1 Expression of MP in leaf primordia.

Whole-mount *in situ* hybridizations of *MP* mRNA transcript in developing leaf primordia. (a) lateral view of the first two leaf primordia 2 days after germination (DAG) showing MP expression in the incipient primary veins. (b) Planar view of 3 DAG primordia showing the emergence of MP expression before the formation of elongated procambial cells of secondary veins. (c) Base of 5 DAG primordia. MP expression predicting sites of procambial tertiary veins between procambial secondary veins, also expressing MP.

* Supplemental material is available at http://www3.interscience.wiley.com/journal/118488445/suppinfo (also see appendix 1)

Figure 2 Expression of PIN1-GFP in leaf primordia.

First (a-h) or later formed (i-m) rosette leaves in *pPIN1::PIN1-GFP* transformed plants. Images show formation of the primary (a,b) and first (c,d) or second (e-h) secondary veins in the first leaves, and the second or third secondary veins in later rosette leaves (i-m). All are planar median views except (a, b) which are lateral median views. Scale bars are 20 μm. Arrows point at various aspects of PIN1-GFP expression (see text).

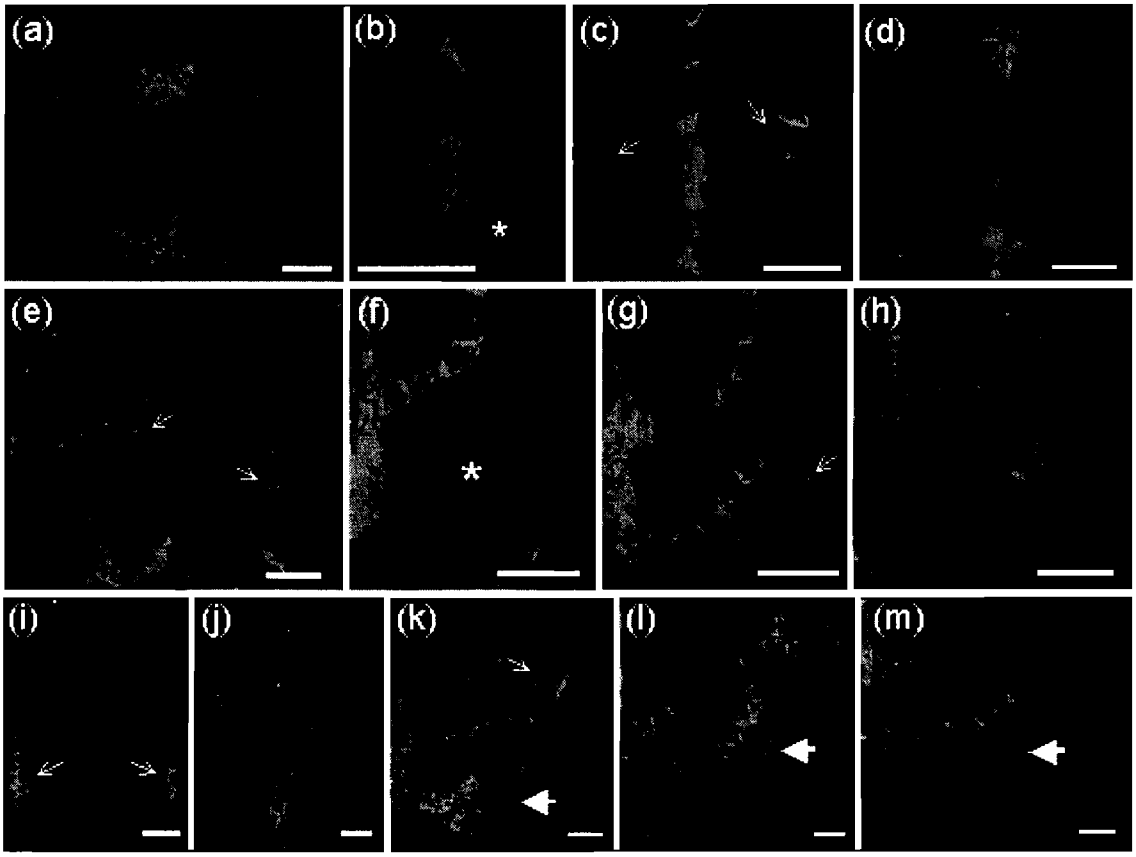


Figure 3 Expression of PIN1-GFP in epidermal tissues of primordia.

First two (a-d) or later forming (e) rosette leaves. All show images taken through the median section (lower) or adaxial epidermal surface (upper) of the same primordia. Arrows point at subcellular localization (a,b) or high expression region (c) of PIN1-GFP. Scale bars are 20 μm.

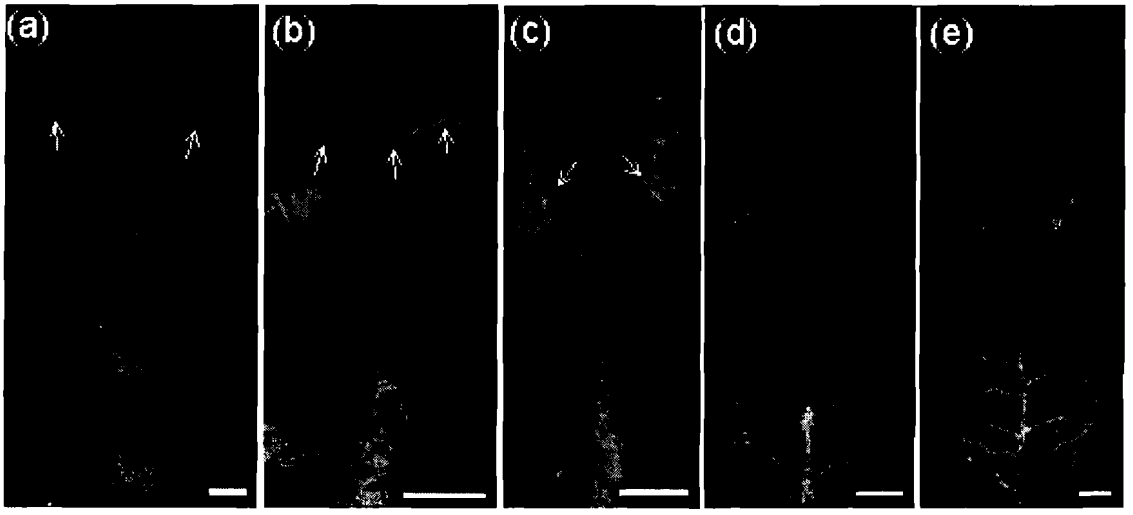


Figure 4 Subcellular localization of PIN1-GFP.

All are planar median views except o-q which show epidermal (upper) and median (lower) sections. Arrows point at subcellular localization of PIN1-GFP and also indicate presumed direction of auxin transport except for d and l which indicate preprocambial strand location. Large arrowhead in r shows 'hotspot' of non-polarized PIN1-GFP in the epidermis adjacent to the next site of preprocambial strand development. Scale bars are 10 μm.

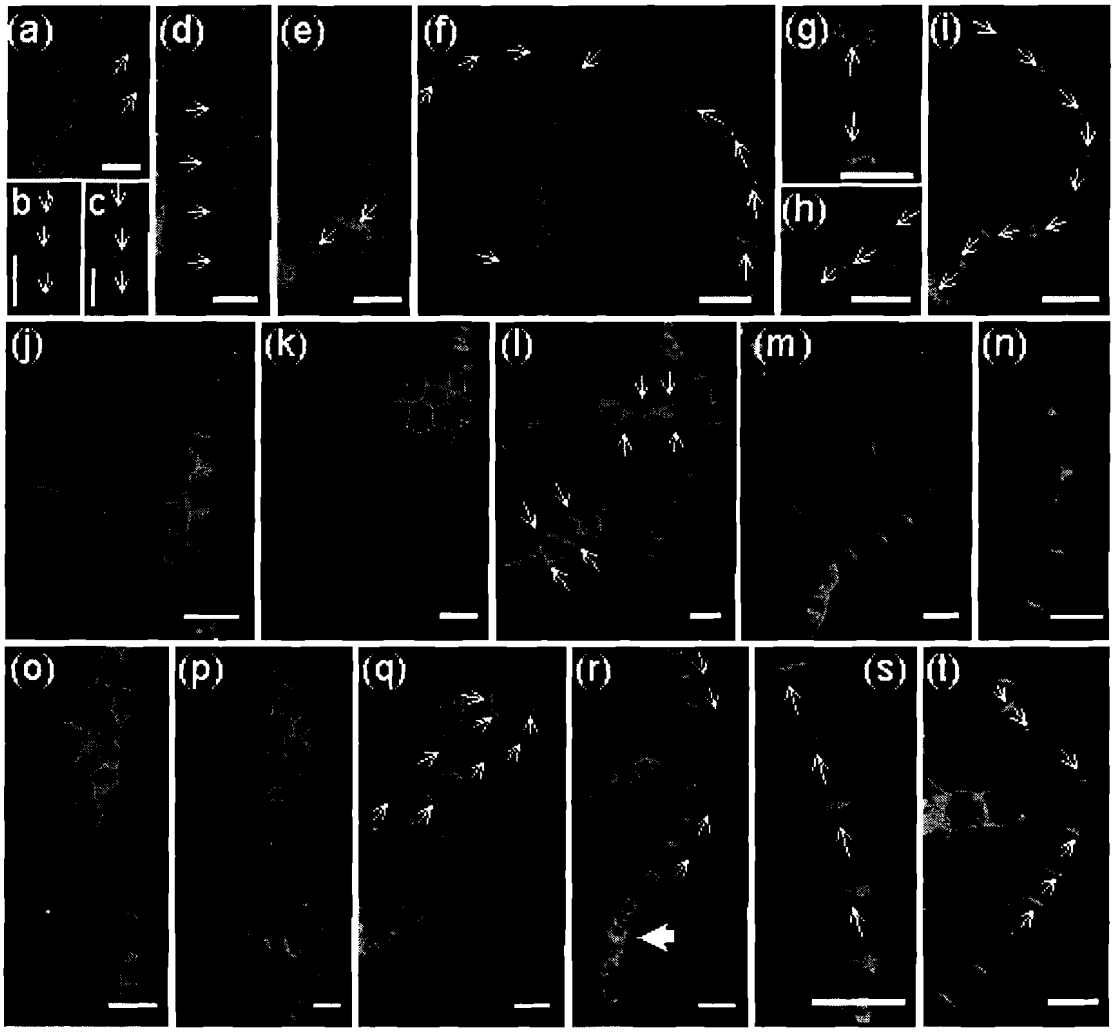


Figure 5 Auxin induction of MP expression.

Expression of DR5::GUS (a-c) and *in situ* hybridizations to *MP* mRNA transcript (d-f) in leaf primordia after 3 DAG seedlings had been exposed to 0 (a,d), 1 (b,e) or 10 (c,f) μM of exogenous 2,4-D. Scale bars are 20 μm.

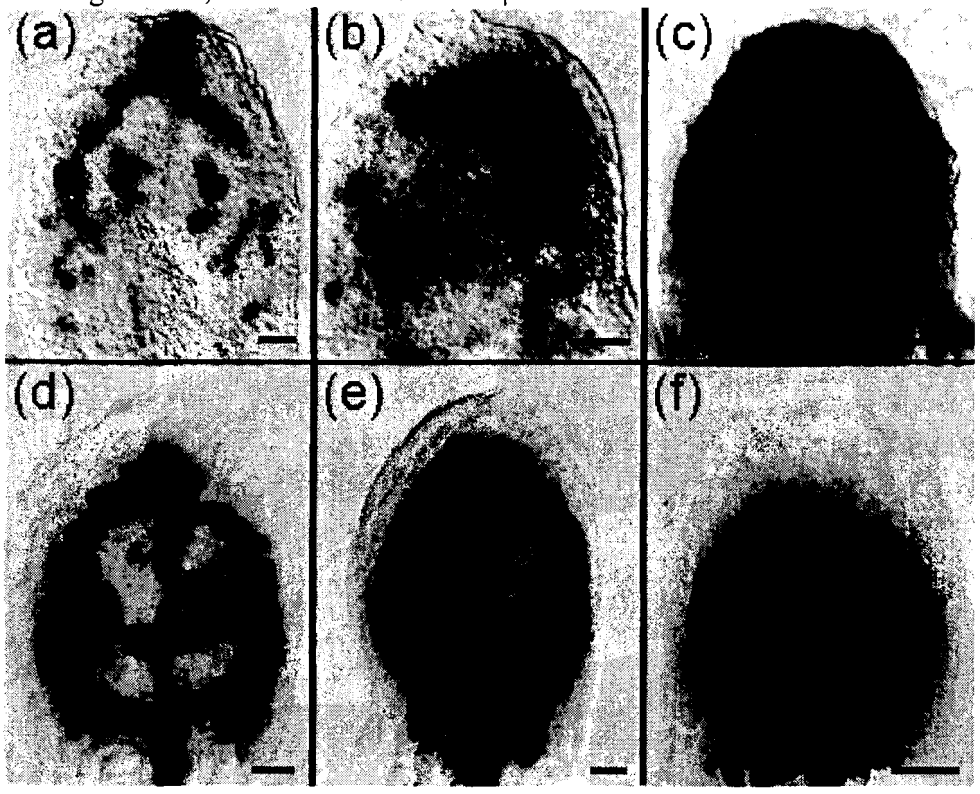


Figure 6 MP expression in primordia from mp mutant and NPA-grown plants.

Whole mount *in situ* hybridizations showing *MP* mRNA transcript in wild-type (a-c) compared to similar stages in the *mp* mutant (d-f) and in response to auxin transport inhibition with 10 μM NPA (g-i). All are planar median views. Scale bars are 50 μm.

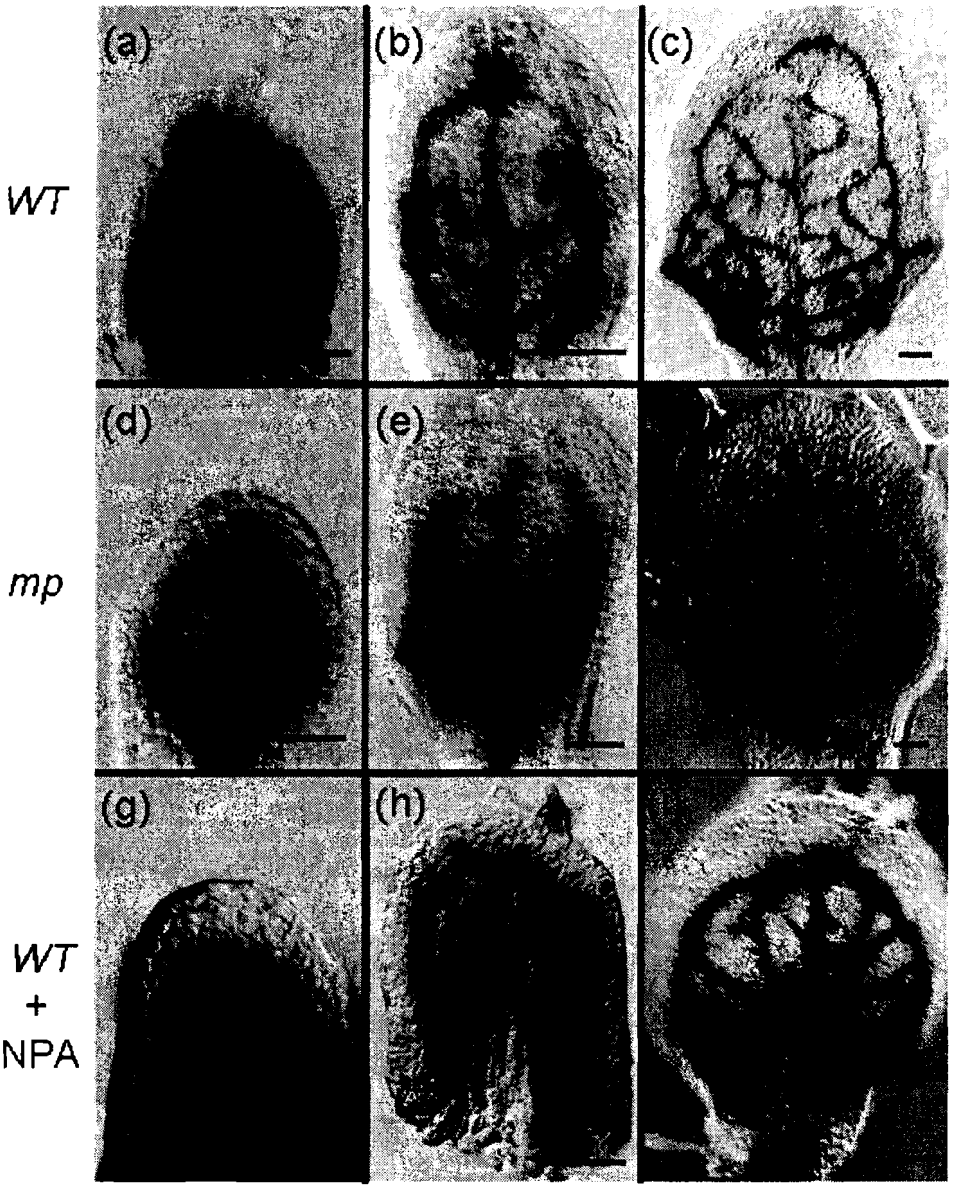


Figure 7 PIN1-GFP expression in primordia from mp mutant and NPA-grown plants.

Control plants (a-c) compared to similar developmental stages in the *mp* mutant (d-f) and in response to auxin transport inhibition with 1 μM NPA (g-k). All are planar median views. Scale bars are 20 μm.

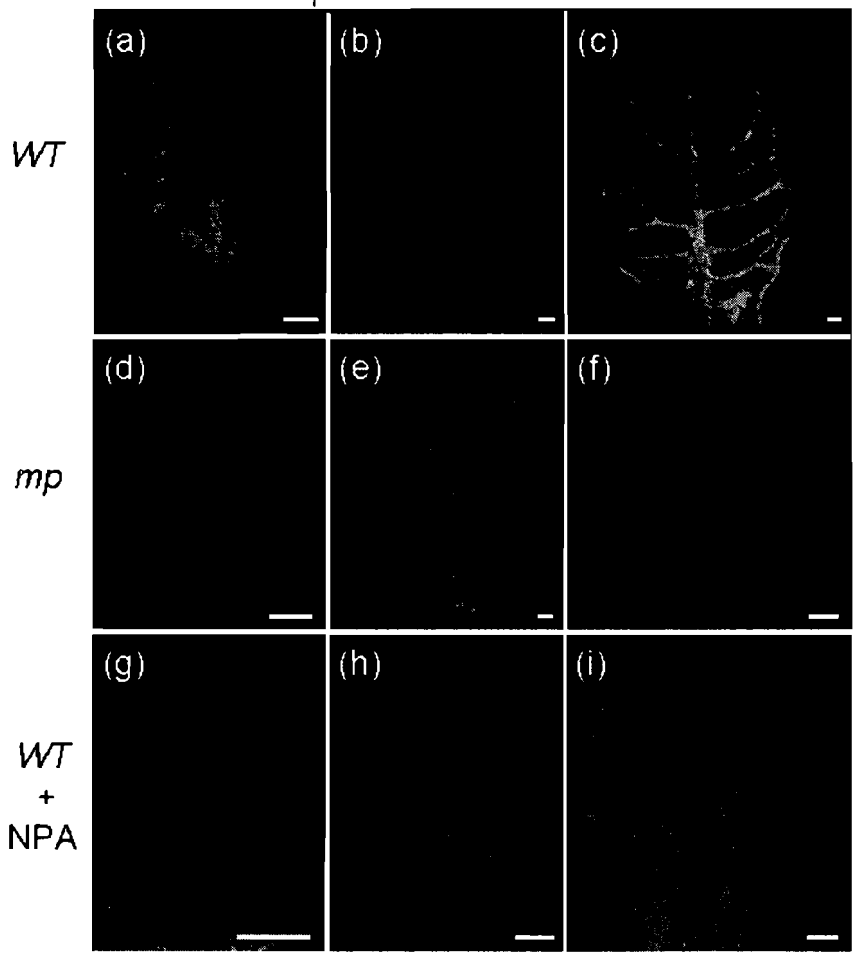


Figure 8 Auxin-response patterns in primordia of mp mutant and NPA-grown plants.

DR5::GUS expression expression in wild-type (a-c) compared to *mp* (d-f), and in response to polar auxin transport inhibition with 10 μM NPA (g-i). All are planar median views. Scale bars are 50 μm.

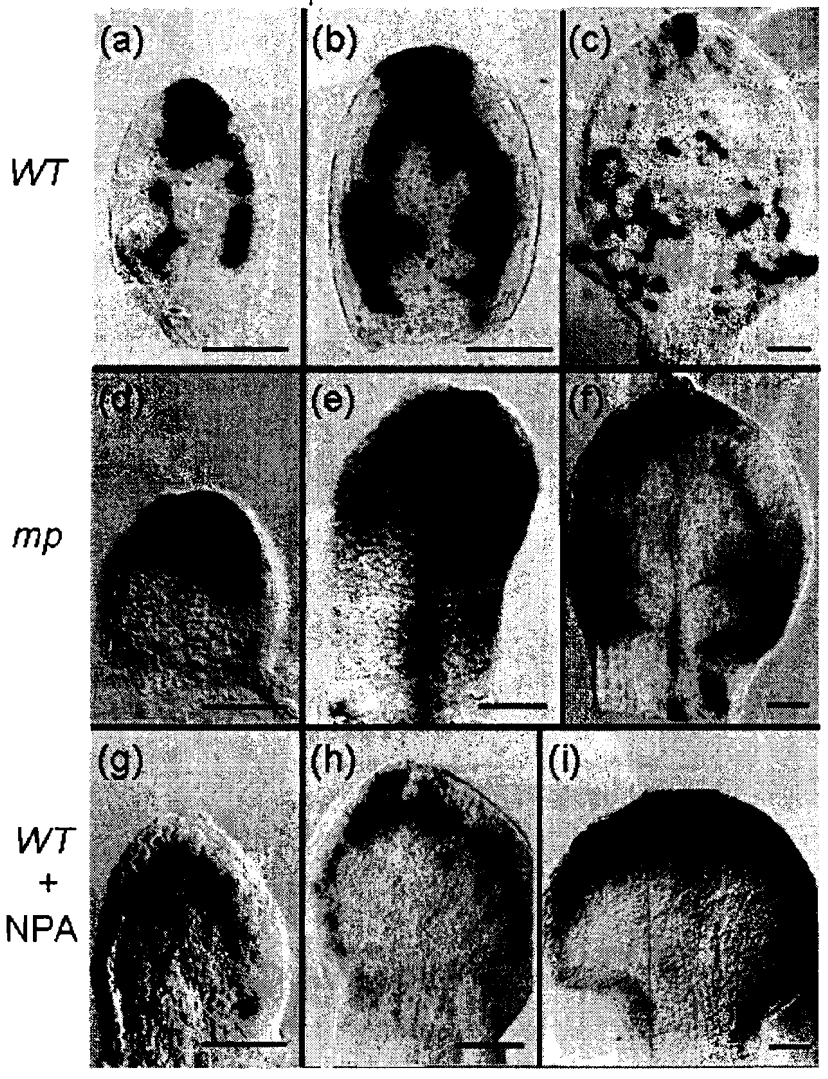
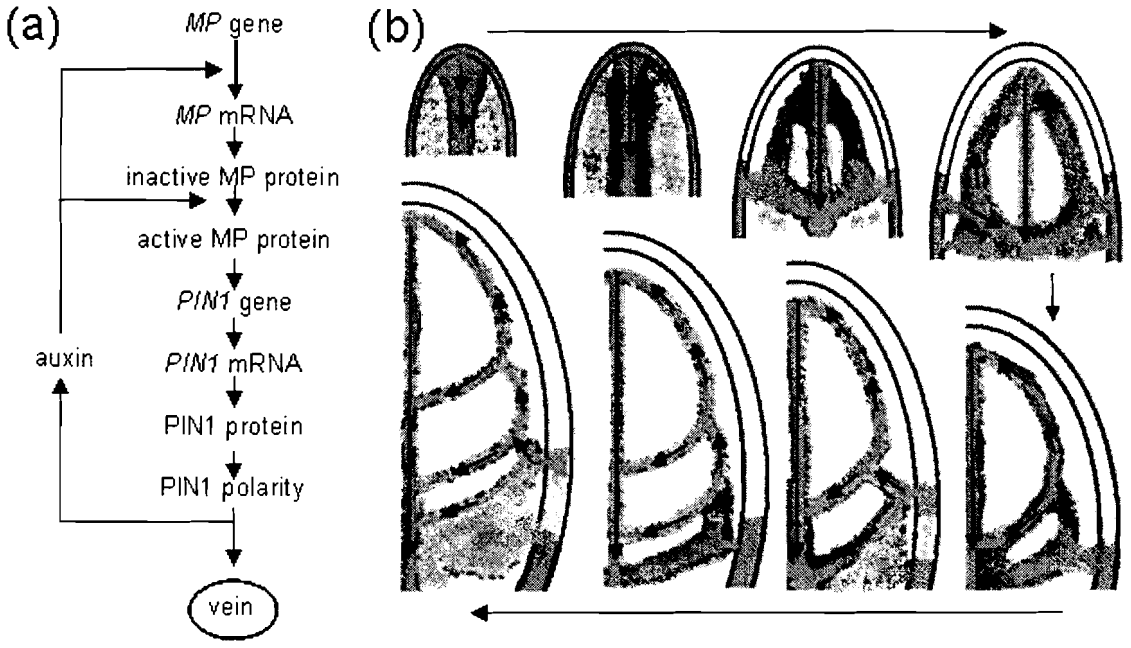


Figure 9 Proposed regulatory loop and module for major vein formation.

Models on (a) a cellular feedback regulatory loop that leads to vein formation and (b) a module for major vein patterning based on overlapping and gradually refined patterns of *MP* (blue) and PIN1 (green) expression. Arrows indicate direction of auxin transport based on subcellular localization of PIN1 proteins. PIN1 expression at the margins indicates expression in adaxial and marginal cells.



CHAPTER 3
IDENTIFICATION OF GENES EXPRESSED IN VASCULAR TISSUES

Originally published in Plant & Cell Physiology 2008 March 49(3):457-468, under the title of "Identification of genes expressed in vascular tissues using NPA-induced vascular overgrowth in Arabidopsis" under the authorship of C. Wenzel, Q. Hester and J. Mattsson, with the gracious permission of Plant & Cell Physiology/Oxford Journals it is replicated here.

Microarray and northern blot analyses to identify genes expressed in vascular tissues were performed by Dr. Mattsson and Dr. Wenzel (see figure 10 and 11). Gene expression patterns study by constructing promoter::GUS fusions were performed by joint effort between Dr. Wenzel and Ms. Hester (see figure 12 to 17), within which Ms Hester constructed approximately 20 genes into promoter::GUS fusions and transformed to Arabidopsis (see appendix 3). Free hand sectioning of leaf and inflorescence stems were mainly performed by Ms. Hester. Photograph of tissue section samples was mainly performed by Dr. Wenzel. Arabidopsis planting, seed isolation, GUS staining, hormones and sucrose treatments were performed by Dr. Wenzel, Ms. Hester, a lab technician and an undergraduate student.

3.1 Summary

The genetic basis of vascular differentiation and function is relatively poorly understood, partly due to the difficulty of screening for mutants defective in internal vascular tissues. Here we present an approach based on a predicted increase in vascular-related gene expression in response to an auxin transport inhibitor-induced vascular overgrowth. We used microarray analyses to identify 336 genes that were up-regulated two-fold or more in shoot tissues of *Arabidopsis thaliana* showing vascular overgrowth. Promoter-marker gene fusions revealed that 38 out of 40 genes with four-fold or more

up-regulation in vascular overgrowth tissues had vascular-related expression in transgenic Arabidopsis plants. Obtained expression patterns included cambial tissues and differentiating xylem, phloem and fibers. A total of 15 genes were found to have vascular-specific expression patterns in the leaves and/or inflorescence stems. This study provides empirical evidence of the efficiency of the approach and describes for the first time the *in situ* expression patterns of the majority of the assessed genes.

3.2 Introduction

The plant vascular system plays a fundamental role in the transport of water, nutrients and other compounds throughout the plant. It also provides physical support for upright growth, which is perhaps most obvious in tree trunks consisting almost exclusively of supportive vascular tissues. Despite the considerable economic and ecological importance of trees and the potential for breeding, the underlying genetic basis of vascular differentiation is only beginning to be unraveled (Ye, 2002; Fukuda, 2004).

Previous studies have identified genes with potential functions in vascular differentiation. In *Arabidopsis thaliana*, vascular gene expression profiling has been done along the developmental gradient of maturation in the inflorescence stem (Ehlting et al., 2005), and also following induction of secondary vascular growth by either repeated removal of inflorescence stems (Zhao et al., 2000, 2005; Oh et al., 2003) or by weight load of the stem (Ko et al., 2004). Several studies compiled numerous microarray databases to identify genes in Arabidopsis involved in cellulose biosynthesis (Brown et al., 2005; Persson et al., 2005) or secondary xylem development (Ko et al., 2006). Stage-specific profiling of vascular gene expression has also been done in the developing Arabidopsis root (Birnbaum et al., 2003), in cultured Arabidopsis or *Zinnia* cells

differentiating into xylem cells (Demura et al., 2002; Kubo et al., 2005), in wood-forming tissues of several tree species (Allona et al., 1998; Sterky et al., 1998; Hertzberg et al., 2001; Lorenz and Dean, 2002; Kirst et al., 2003; Yang et al., 2003; Andersson-Gunnerås et al., 2006), and in maize (Nakazono et al., 2003).

Auxin plays a key role in vascular strand formation and differentiation (Berleth and Sachs, 2001; Fukuda, 1997). Auxin accumulates in preprocambial cells (Mattsson et al., 2003; Uggla et al., 1996) presumably via the gradual active canalization of auxin into narrow vascular strands (Sachs, 1981; Scarpella et al., 2006; Wenzel et al., 2007). Partial inhibition of auxin transport using pharmacological inhibitors leads to extensive vascular overgrowth or hypertrophy, presumably due to the formation of broader auxin canals during vascular strand formation (Mattsson et al., 1999; Sieburth, 1999).

Here we have used auxin transport inhibitor-induced vascular overgrowth to identify genes expressed in vascular tissues, as their transcripts should be more prevalent in tissues developing vascular overgrowth. Using microarray analysis we identified a large number of genes whose expression was up-regulated in shoots developing vascular overgrowth and assessed expression of 40 genes that showed four-fold or more up-regulation. Our results suggest that this approach is highly efficient at identifying genes with expression in vascular tissues.

3.3 Results

3.3.1 38 genes had vascular-related expression

Two-week-old plants grown on NPA-containing media provided suitable material for this study as most rosette leaves were in advanced stages of vascular differentiation

leading to vascular overgrowth (Fig. 10B). Microarray analyses of purified RNA samples identified 336 genes that showed at least two-fold up-regulation in NPA-grown tissues (available at NASCArrays), and we focused on 40 genes showing at least four-fold up-regulation. These 40 genes fall into several functional categories based on predicted gene annotations (http://www.arabidopsis.org) including DNA/RNA binding, signaling, stress and metabolism (Fig. 11). Several genes were associated with cell wall modification, lignin biosynthesis, and membrane dynamics (Fig. 11). Northern blot hybridizations of independent RNA samples confirmed that 6 randomly selected genes showed up-regulation with NPA treatment (Fig. 10C), validating the differential expression observed in microarray experiments for these 6 genes. Presumably this finding is representative of the remaining 34 genes in this study. Analyses of promoter-GUS fusions showed that 38 out of the 40 genes were expressed in vascular-related tissues, 15 of which had vascular-specific expression in the leaves and/or inflorescence stems (Fig. 10D and 11). These results provide empirical evidence that microarray analyses comparing control and vascular overgrowth tissues can be used to identify genes expressed in vascular-related tissues.

3.3.2 Leaf expression patterns could be separated into distinct classes

Since the genes under study were identified based on their elevated expression in NPA-grown leaf rosettes, we first compared expression in leaves of control and NPA-grown plants. A comparison of the leaf expression patterns revealed that they could be categorized into eight different classes of expression in leaves (Fig. 12). Leaf expression classes 1-4 all showed gene expression predominantly in veins. Class 1 was characterized by an expression in veins of both control and NPA-grown plants. Classes 2 and 3 showed

little or no vein expression in control leaves and more extensive vein expression in NPA-grown leaves, with class 3 also showing hydathode expression in control leaves. Class 4 was predominantly expressed in marginal vascular tissues. Classes 5 and 6 also had vascular or vascular-associated expression, but this was often associated with ground and/or epidermal expression. Class 6 was further distinguished by discontinuous expression in vascular or vascular-associated cells. Class 7 included expression in midvein-associated cells. Class 8 had non-vascular or no expression in leaves but did have expression in vascular tissues of stems. Taken together, the expression analyses in leaves demonstrated that the majority of genes had expression in leaf veins (Fig. 11). Regardless of the classification, expression was always more extensive in NPA-grown leaves, thereby providing additional independent support for the up-regulation observed in microarray experiments.

3.3.3 Most genes were also expressed in stem vascular tissues

Most genes were also expressed in the inflorescence stems, usually in more than one vascular cell type (Fig. 11 and 13). Some genes were expressed predominantly in the phloem (At4g00670, DNA-binding remorin 2; At3g16360, phosphorelay mediator; At1g07430, protein phosphatase 2C), cambium (At2g39330, myrosinase-binding protein), or xylem (At2g37870 and At3g53980, lipid-transfer proteins; At5g37970, methyltransferase) (Fig. 11 and 13). Three genes were expressed predominantly in developing xylem and interfascicular fibers of inflorescence stems: At2g46680, HD-Zip transcription factor; At1g57590, pectinacetylesterase; and At5g42180, peroxidase (Fig. 11, 13H and 14). Fourteen genes had vascular-specific expression in inflorescence stems,

partially overlapping with the eight genes having vascular-specific leaf expression (Fig. 11).

3.3.4 The majority of genes were expressed in later phases of vascular differentiation

Our original selection of material was intended to identify genes involved in various stages of vascular differentiation (Fig. 10A,B). A comparison between differentiation and expression was most easily done in cross sections of inflorescence stems. None of the assessed genes were expressed in the most immature regions of inflorescence stems where procambial tissues could be discerned, but were expressed in slightly older tissues and continued throughout development (Fig. 14 and S1). Genes having expression in immature, differentiating xylem maintained expression until cell death (Fig. 15). A similar analysis in leaf primordia confirmed that these genes were expressed in late phases of vein differentiation (Fig. S2). In summary, the temporal gene expression patterns indicate a potential role of many genes in the differentiation of vascular cells, especially in xylem and fiber cells that are functional only after death. We also found genes that are likely to be involved in vascular function, especially those genes that were continually expressed in living, differentiated phloem and vascular-associated cells.

3.3.5 Genes implicated in defense were also expressed in vascular tissues

We found five potential defense-related genes (Fig. 11), three of which had homology to myrosinase-binding proteins (MBPs; At2g39330, At1g52000, At1g52040). Myrosinases hydrolyse glucosinolates forming toxic defense compounds (Rask et al., 2000), and have also been implicated in auxin homeostasis whereby indole glucosinolates

are converted to indole acetonitrile, an intermediate in indole-3-acetic acid biosynthesis (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). MBPs are required for myrosinase complex formation (Eriksson et al., 2002), but their role in defense or auxin homeostasis is unknown. In Arabidopsis, myrosinases are known to accumulate in specialized idioblast (myrosin) cells scattered along vascular bundles in leaves (Andréasson et al., 2001) and are spatially separated from glucosinolates that accumulate in vascular-associated sulfur-rich S-cells. Here we show that the three putative MBPs identified in this study were expressed in vascular or vascular-associated cells (Fig. 16B-D), and not in idioblast cells where we found that an acid phosphatase (At3g17790) was expressed (Fig. 16A). Thus these MBPs were not co-localized with idioblast myrosinases, but their close proximity may enable association upon wounding, as previously suggested for myrosinases and glucosinolates (Rask et al., 2000). One of these MBP genes (At1g52000) also showed an intriguing expression in the basal regions of leaves where new secondary veins form (Fig. 16C), the functional significance of which remains to be determined.

3.3.6 Expression of anthocyanin-related genes

Two genes, *TRANSPARENT TESTA 19* (*TT19*, At5g17220), encoding a glutathione transferase, and At4g14090, encoding an anthocyanidin glucosyltransferase, are both known to be involved in anthocyanin metabolism, the former of which affects pigment production in seed coats and vegetative tissues (Kitamura et al., 2004). We found that the promoter marker gene fusions of these two genes conferred vascular-associated expression. *TT19* was expressed in cells associated with the midvein of developing leaves (Fig. 16E), and the glucosyltransferase was expressed in phloem cells

in leaves (Fig. 16F). For *TT19*, NPA treatment induced additional sporadic expression in ground and epidermal tissues throughout the leaf lamina (Fig. 16E). Both genes also had vascular expression in the inflorescence stems (Fig. 11). Previous findings link both flavonoids and also these two genes to functions in the regulation of auxin transport, a process known to affect the patterning and differentiation of vascular tissues (see discussion).

3.3.7 Several genes responded to exogenous hormones and/or sucrose levels

Several hormones have been implicated in vascular patterning and differentiation (reviewed by Fukuda, 2004). Here we tested regulation of the 40 identified genes to exogenous hormone treatments. We identified 12 genes that responded to hormones with an increased level of expression – 4 to auxin, 4 to GA, 1 each to BA and SA, and 9 to ABA (Fig. 11 and 17). Several genes responded to more than one hormone suggesting that their expression may be dependent on multiple endogenous hormones. A number of genes regulating the storage, mobilization and translocation of carbohydrates are themselves regulated by availability of carbohydrates as part of feedback mechanisms (Lalonde et al., 2004; Rook et al., 2006). We therefore also assessed whether gene expression of the identified genes responded to different sucrose levels, 0.2% and 1% (w/v). Eight genes were up-regulated in response to elevated sucrose levels, with over half involved in metabolism and 6 of the genes having phloem-associated expression (Fig. 11 and S3). Two genes responded with even stronger expression to 3% sucrose - At5g59320 (lipid transfer protein 3) and At3g17790 (acid phosphatase) (Fig. S3).

3.4 Discussion

3.4.1 A novel approach identified genes expressed in vascular tissues

Herein we describe a novel approach to the identification of genes potentially involved in vascular differentiation or vascular cell functions. We assessed differential gene expression in shoots that were developing strong vascular overgrowth in response to growth in the presence of an auxin transport inhibitor (NPA) with the intention of identifying up-regulated transcripts of proteins involved in vascular differentiation or vascular transport functions. The vascular overgrowth that can be induced by growing Arabidopsis plants on medium supplemented with auxin transport inhibitors can be substantial (Mattsson et al., 1999; Sieburth, 1999; Fig. 10B). The quantitative differences are generally larger than those observed between wild type plants and available mutants with reduced or enhanced venation and should thus be a more suitable material for differential gene expression studies. Nevertheless, there are several potential artifacts induced with this method. First, NPA is known to have toxic side effects, ranging from inhibition of growth (Mattsson et al., 1999) to potentially also affecting vesicle trafficking other than that involved in auxin transport (Geldner et al., 2001). Second, NPA is likely to cause changes in auxin distribution and homeostasis (Mattsson et al., 2003; Yoshida et al., 2005) that affect the expression of genes unrelated to vascular differentiation or function. Third, little is known about the effects of NPA on cell types other than conspicuous vascular cells. Fourth, NPA-induced defects in vascular continuity may result in osmotic and nutrient stress.

Thus, to assess the applicability of this approach, we sampled young rosettes of Arabidopsis plants grown in the absence or presence of 10 μM NPA. These samples

contained a wide range of developmental stages of leaves and may therefore capture genes involved in vascular patterning, differentiation and function. A first surprise came from the relatively few genes (336) that were up-regulated more than two-fold in NPA-grown shoots, suggesting that the long-term impact of NPA on global gene expression was limited. The annotations of these 336 genes provided a first indication of the validity of the screen as it revealed genes with known functions in vascular patterning such as *PIN-FORMED1* (Scarpella et al., 2006; Wenzel et al., 2007), and *ATHB15* (Zhou et al., 2007), and genes with functions in vascular differentiation such as *IRREGULAR XYLEM 5/CELLULOSE SYNTHASE 4* (Brown et al., 2005) and several genes in lignin synthesis (original data available at NASCArrays). Further support for the validity of the screen came from the considerable overlap between our list of genes and other screens for genes with potential functions in vascular differentiation (Table SI). Three of these previous screens (i.e. Ehlting et al., 2005; Kubo et al., 2005; Zhao et al., 2005) each also identified 25-60% of the 40 genes that were four-fold or more up-regulated in our screen (Table SI).

To assess in an unbiased manner whether the identified genes were indeed expressed in vascular tissues, we created promoter-marker gene fusions of genes that were four-fold or more up-regulated, regardless of the annotations and potential functions associated with them. This labour-intensive approach was not taken by any of the published screens for genes with potential functions in vascular differentiation. The evaluation of the expression of these 40 constructs in transgenic Arabidopsis plants provided empirical evidence of the validity of the approach, as 38 out of the 40 tested promoter fragments (i.e. 95%) generated vascular-related expression patterns in the leaves and/or inflorescence stems. In leaves, 27 were expressed in vascular tissues and 7

were expressed in either midvein- or vascular-associated cells (Fig. 11). Four of the 38 genes had non-vascular expression in leaves, but all four had vascular expression in inflorescence stems, suggesting that their initial differential expression may come from vascular overgrowth in the rosette stem included in the material sampled for microarray analysis. Furthermore, 30 of the 38 genes (79%) also had vascular expression in inflorescence stems (Fig. 11). These results provide convincing evidence that our approach selected for genes having vascular-related expression with considerable efficiency.

In line with the original material selection, focusing primarily on leaves with veins in advanced stages of differentiation or fully mature veins, we found that the majority of analyzed genes were expressed in later phases of vascular differentiation and in mature vascular tissues. Thus, based on the expression profiles, it appears that we have successfully identified candidate genes for roles in later stages of vascular differentiation as well as genes involved in vascular function. It should be possible to selectively harvest material to enrich for transcripts involved in early phases of vascular differentiation or to more specifically target vascular function. Likewise, the screening approach taken here could probably also be applied to other species, in particular those species where mutant screening is not a viable option.

3.4.2 Expression patterns indicate potential gene functions

Based on expression, the genes most likely involved in vascular-specific processes are the eight genes with vascular-specific expression in leaves, and the partially overlapping set of 14 genes with vascular-specific expression in the inflorescence stems (Fig. 11). This study adds to previous literature on these genes (Table SI) by revealing for

the first time their *in situ* vascular expression patterns. All tested promoter fragments generated expression in more than one vascular cell type suggesting cellular functions such as differentiation that transcend the involved cell types. Here we highlight a few of the identified genes with potentially novel roles in vascular differentiation or function.

Six genes with predominantly xylem or fiber expression may be of particular interest with regards to secondary xylem differentiation. Three genes were expressed almost exclusively in differentiating interfascicular fibers: a peroxidase (At5g42180), a pectinacetylesterase (At1g57590), and a homeodomain-leucine zipper-type transcription factor ATHB7, (At2g46680) (Fig. 11 and 14). To our knowledge, this is the first study to implicate the pectinacetylesterase in fiber development. Several previous microarray studies have also identified the peroxidase gene (At5g42180) as a potential candidate in vascular differentiation without revealing its expression *in situ* (Table SI). A recent study found a cis sequence in the promoter region of the peroxidase gene At5g42180 with homology to the 11 bp TERE element, a sequence that confers xylem-specific expression (Pyo et al., 2007). However, this putative cis-element did not confer any vascular expression. Interestingly, the approximately 2 kb promoter fragment of this gene used in our study does reveal xylem and fiber-specific expression (Fig. 14A-C), suggesting that there are other cis elements in this promoter that confer this type of expression. The ATHB7 gene is known to be induced by drought stress and ABA treatment (Söderman et al., 1994; Hjellström et al., 2003). We have confirmed the ABA induction of this gene by promoter-marker gene fusion and also added a novel aspect to its expression, a strong expression in differentiating interfascicular fibers and vessel elements. Since these cell types undergo programmed cell death (PCD), and ABA has been implicated in the

suppression of PCD (Bethke and Jones, 2001; Steffens and Sauter, 2005), one may speculate that *ATHB7* is involved in the suppression of PCD to allow a timely maturation of these cells. Two lipid transfer proteins (At2g37870 and At3g53980) and a methyltransferase (At5g37970) had predominantly xylem expression (Fig. 11 and 12). Although the function of these genes is unknown, related genes have demonstrated functions in vessel element differentiation (Motose et al. 2004; Carland et al. 2002).

We found several genes that were expressed predominantly in phloem of both leaves and inflorescences - a putative two-component phosphorelay mediator (At3g16360), a protein phosphatase 2C (At1g07430), a DNA-binding remorin protein (At4g00670), and a polyadenyl-binding protein mediator (At4g14270). None of these genes has previously been known to have phloem expression. Remorins have been found to be expressed in vascular tissues of tomato (Bariola et al., 2004), but their functions in this tissue are unknown.

Among the 40 genes with four-fold or more upregulation were two genes that have been previously linked to auxin transport. At4g14090, an anthocyanidin glucosyltransferase, and *TRANSPARENT TESTA 19*, a glutathione transferase (At5g17220) are involved in the biosynthesis and vacuolar sequestration respectively of flavonoids (Kitamura et al., 2004; Tohge et al., 2005), which are known to inhibit polar auxin transport (Lazar and Goodman, 2006). The vascular-associated expression of *TT19* and the phloem expression of the anthocyanidin glucosyltransferase described for the first time here provide additional support for functions in auxin transport, as vascular tissues are well known to carry out polarized auxin transport (Berleth and Sachs, 2001).

3.5 Material and methods

3.5.1 Microarray and northern blot analyses

Seeds of *Arabidopsis thaliana* ecotype Col-0 were sterilized with chlorine gas and plated onto sterile ATS media (Lincoln et al., 1990) supplemented with either N-(1-Naphthyl) phthalamic acid (NPA; Sigma, St. Louis, MO) dissolved in DMSO to a final concentration of 10 μM, or an equivalent volume of DMSO for control material. Seeds were stratified for at least 2 days at 4°C and then grown at ca 20°C with 16h illumination for 2 weeks. Shoot tissues were immediately immersed in liquid nitrogen after excision, stored at -80°C, and total RNA purified according to Chang et al. (1993). Purified RNA was prepared for microarray analysis with Affymetrix Arabidopsis ATH1 Genome chips (performed by the Center for Applied Genomics, The Hospital for Sick Children, Toronto, Canada) and differential gene expression profiles analyzed using DNA-Chip Analyzer (d-Chip) Version 1.3 software (Li and Wong, 2003). Microarray data have been donated to the Nottingham Arabidopsis Stock Centre's microarray database, NASCArrays and are available under reference number 428. For northern blot analyses of select up-regulated genes, total RNA extracted from 2 week old plants was separated by electrophoresis and blotted onto Hybond N membrane (Sambrook and Russell, 2001). PCR products were used for random priming DNA synthesis including ^{32}P-dCTP and hybridized in Church buffer (Sambrook and Russell, 2001). Table SII lists the primers used for northern blots.

3.5.2 Promoter-marker gene fusion analyses

Promoter-marker gene fusions were used to assess tissue specific gene expression, although we acknowledge that the 5' sequence fragments used may not contain all cis-

elements. Promoter-marker gene fusions were made for 40 genes showing at least four-fold up-regulation with NPA treatment with exception for promoter fragments that we failed to clone by TOPO cloning, especially in the 4-5 fold range. Duplicate promoter segments were cloned by PCR using one primer designed against or near the 5' UTR and up to 2 kb upstream of the ORF, following Finnzyme's Phusion high-fidelity DNA polymerase protocol. Table SIII lists primers used for PCR products. PCR products were cloned into the pKGWFS7 GATEWAY destination vector (Karimi et al. 2002) and Arabidopsis Col-0 ecotype plants were transformed with these constructs by floral dip (Clough and Bent, 1998). Kanamycin resistance was used to select for T2 and T3 lines containing a single TDNA insert.

For assessment of expression, histochemical GUS analyses were done on the first leaves of 5-14 day old plants or in older rosette leaves taken from 2-3 week old plants grown on media with or without NPA as above. 3-5 mm long segments were taken along the length of inflorescence stems in 3-4 wk old plants grown either in sterile media as above or in soil (20°C, 16hr/8hr light/dark). GUS staining and mounting in 8:2:1 chloral hydrate:glycerol:water was performed as in Mattsson et al. (1999). For determination of cell type expression, inflorescence stems, petioles and some leaf blades were hand-sectioned prior to mounting. Images were taken using a Nikon Eclipse E600 microscope and a Canon EOS D30 digital camera.

Gene responses to various hormones or sucrose concentrations were tested. 10-20 seeds of selected promoter::GUS T2 or T3 lines were grown in 10 ml liquid ATS medium for 7 or 14 days at ca 20°C under constant light conditions and agitation. At these time points, 0.1% w/v of the surfactant pluronic F-68 (Sigma; St. Louis, MO) was added to

each along with one of the following to a final concentration of 10 μM: 2,4 – dichlorophenoxyacetic acid (2,4-D; Sigma), abscisic acid (ABA; Sigma), benzyl adenine (BA; Sigma), epibrassinolide (BR; Sigma), gibberellic acid (GA, ICN Biomedicals Inc., Aurora, Ohio), salicylic acid (SA; Sigma). After ca 15 hr, the seedlings were rinsed in water and then assayed for GUS activity. Transformed promoter::GUS lines were also grown for 7 or 14 days in liquid ATS medium containing low (0.2% w/v) or high (3.0% w/v) sucrose concentrations compared to the control ATS medium (1% w/v sucrose) and then GUS stained as above.

3.5.3 Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org (also see appendix 2).

3.6 Funding

National Science and Engineering Research Council funding to Jim Mattsson.

3.7 Acknowledgments

We thank Afsaneh Haghighi-Kia, Micky Yip and Mathias Schuetz for technical assistance. We also thank the reviewers for valuable comments

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3.9 Figures

Figure 10 Genes up-regulated in NPA-grown shoots were expressed in vascular tissues.

(A) Dark-field image of control plant shoot. (B) NPA-treated shoot tissue showing vascular hypertrophy (arrows). (C) Northern blot hybridizations comparing control (-) and NPA (+) treatments showed up-regulation in either shoot or inflorescence tissues for genes: (1) At2g37870; (2) At2g42540; (3) At2g18050; (4) At5g37970; (5) At3g16360; (6) At3g02480. Northern blot hybridizations confirmed microarray results except that At5g37970 (4) was not detected in shoot tissues although it was upregulated in inflorescence stems. (D) Promoter-GUS analyses confirmed that most of the 40 selected genes were expressed in vascular tissues, fifteen of which had vascular-specific expression in either the leaf (1 gene), inflorescence (7 genes) or both (7 genes) (see Fig. 11). Bars = 1 mm.

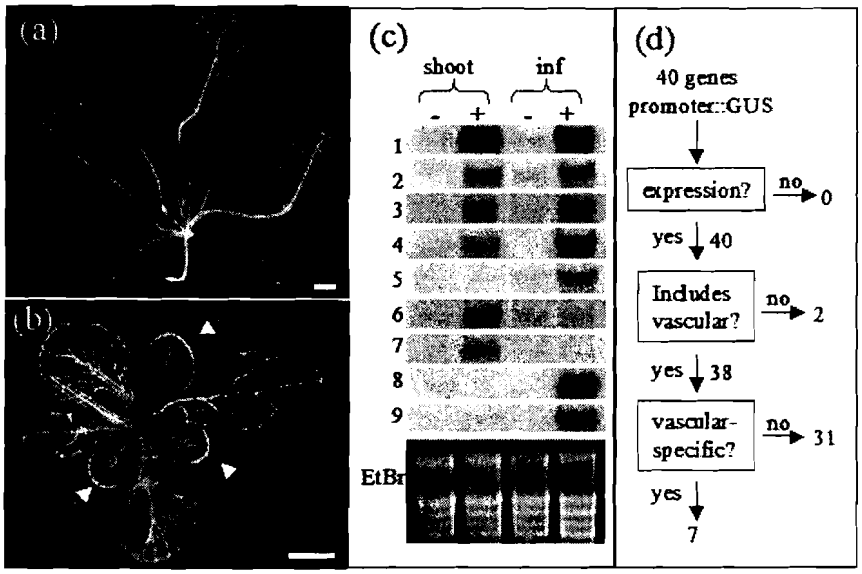


Figure 11 Annotation and summary of expression analysis for identified genes.

Cell-specific and overall leaf vascular patterning for 38 genes from the shoot screen. Outlined cell types indicate predominant expression pattern. Many genes also had some expression in 'other' non-vascular cell types (hatched pattern = sporadic/variable expression; closed pattern = strong expression). For cell types: L = leaf; I = inflorescence; V = vascular-associated; H = hydathode; M = midvein-associated; T = trichomes. *V-specific = vascular specific expression in the leaf (L) or inflorescence (I). Some genes also had sporadic or stage-specific leaf (i) midvein-associated expression (At5g06510, At3g02480, At3g08860, At1g10970, At1g27030), (ii) non-vascular expression (At5g42180, At2g39330), or (iii) patchy vascular expression (At4g17030, At4g00670). All stress treatments show up-regulation of expression. For stress treatments: s = shoot; r = root; g = ground; v = vascular; e = epidermis; SUC = sucrose. For example, svg: ABA = up-regulated expression in shoot vascular & ground tissue with ABA treatment.

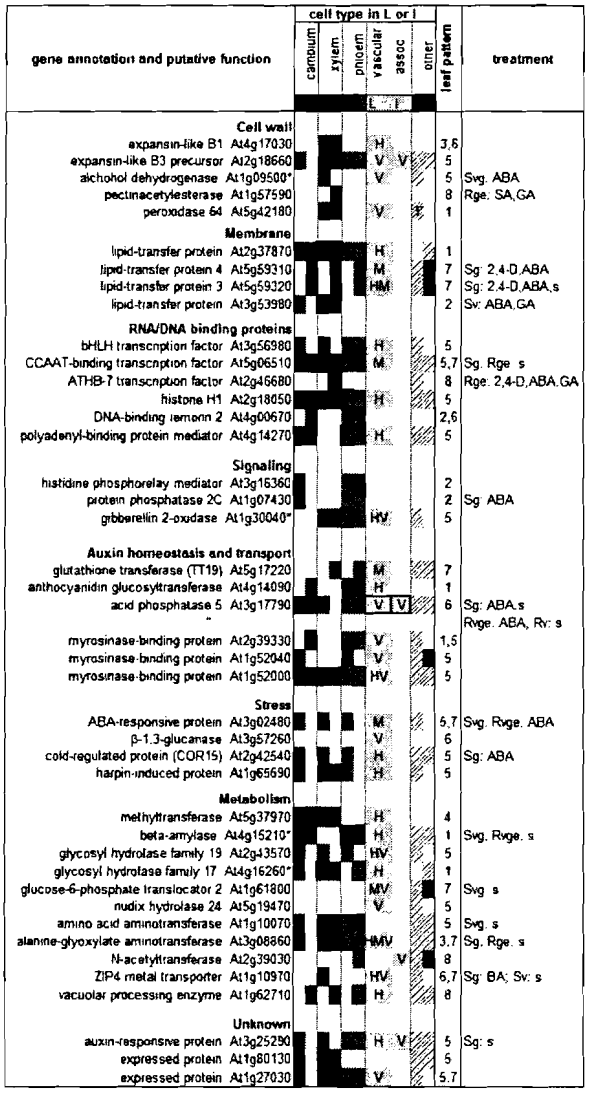


Figure 12 Eight different gene expression categories in leaves.

Leaf gene expression patterns 1-8 in control (-) and NPA-treated (+) leaves for genes: (1) At2g37870, lipid-transfer protein; (2) At3g16360, phosphorelay mediator; (3) At3g08860, amino-transferase; (4) At5g37970, methyltransferase; (5) At2g42540, cold-responsive protein; (6) At3g17790, acid phosphatase type 5; (7) At5g17220, glutathione transferase (TT19); (8) At1g62710, VPE. See text for descriptions of expression categories. Leaves were often cut to facilitate GUS staining. Bars = 500 μm.

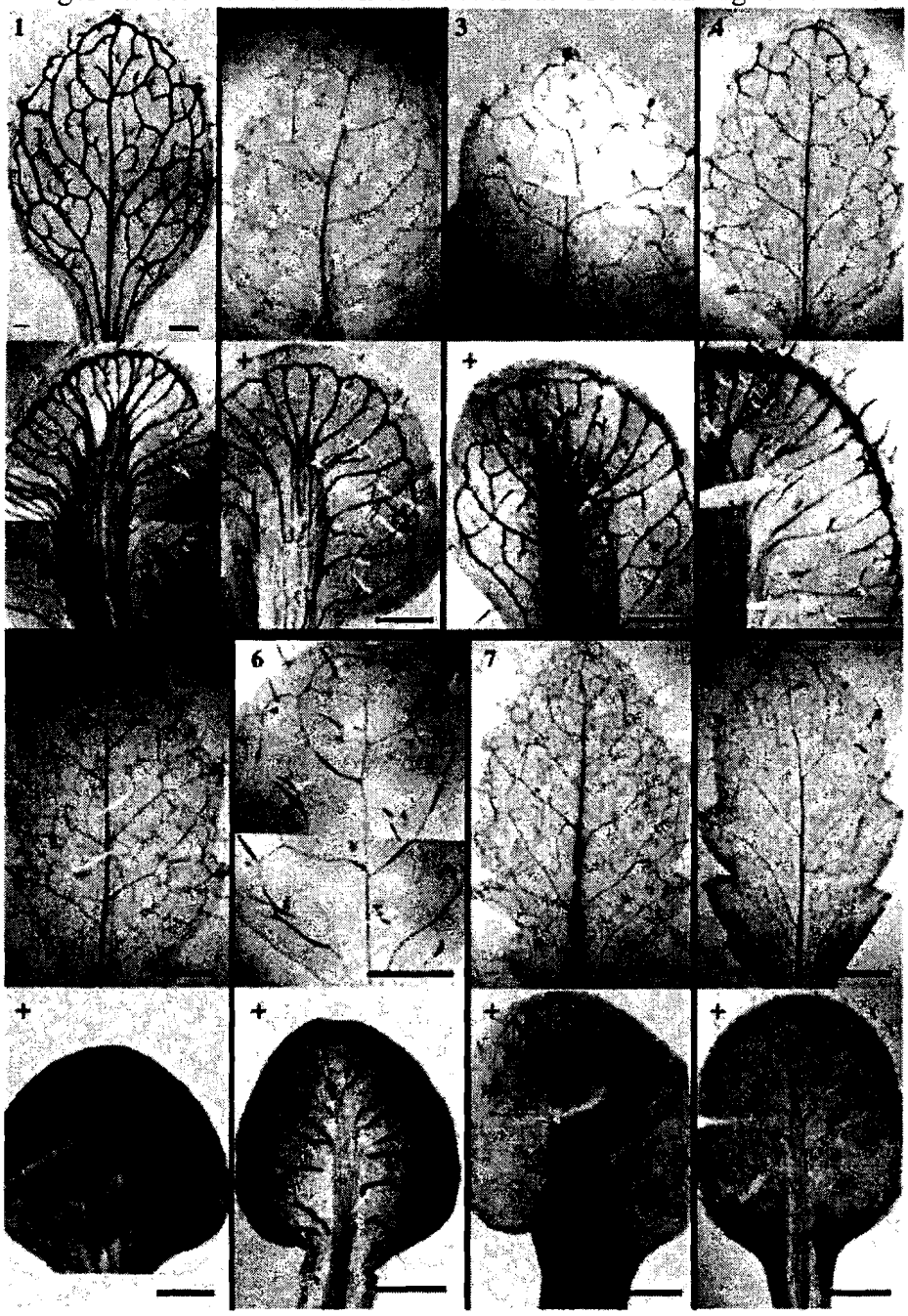


Figure 13 The diversity of expression patterns in inflorescence vascular bundles.

Transverse sections showing expression patterns in inflorescence stems for genes: (A) At4g00670, DNA-binding remorin 2; (B) At2g39330, myrosinase-binding protein; (C) At3g17790, acid phosphatase type 5; (D) At5g06510, CCAAT-binding transcription factor; (E) At1g10070, aminotransferase; (F) At1g27030, expressed protein; (G) At2g18660, expansin-like B3 precursor; (H) At5g42180, peroxidase 64. At1g27030 and At2g18660 also had sporadic phloem expression (Fig. 11). p = phloem; c = cambium; emx = early metaxylem; mx = metaxylem; vp = vascular parenchyma; if = interfascicular fibres. Bars = 10μm.

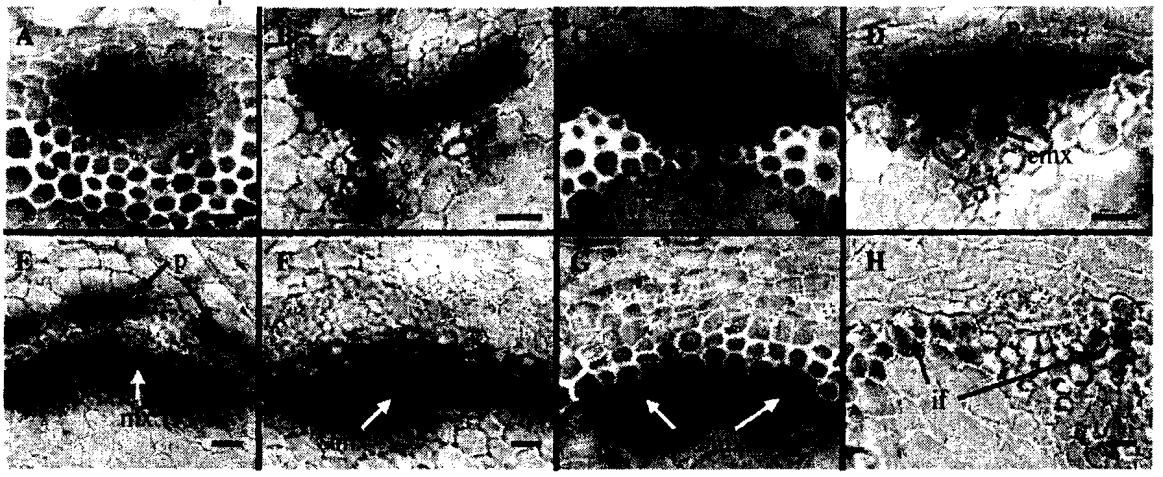


Figure 14 Three genes were expressed in differentiating fibers.

Transverse sections showing gene expression patterns in developing xylem and interfascicular fibers of genes: (A-C) At5g42180, peroxidase 64; (D-F) At1g57590, pectinacetylesterase; (G-I) At2g46680, ATHB7 transcription factor. For each gene, a developmental series was taken from an immature region near the inflorescence tip (A,D,G), an older region showing expression in interfascicular fibers (B,E,H) and a mature region with most expression in the more recently formed fibers (arrows in C,F,I). Bars = 10 μm.

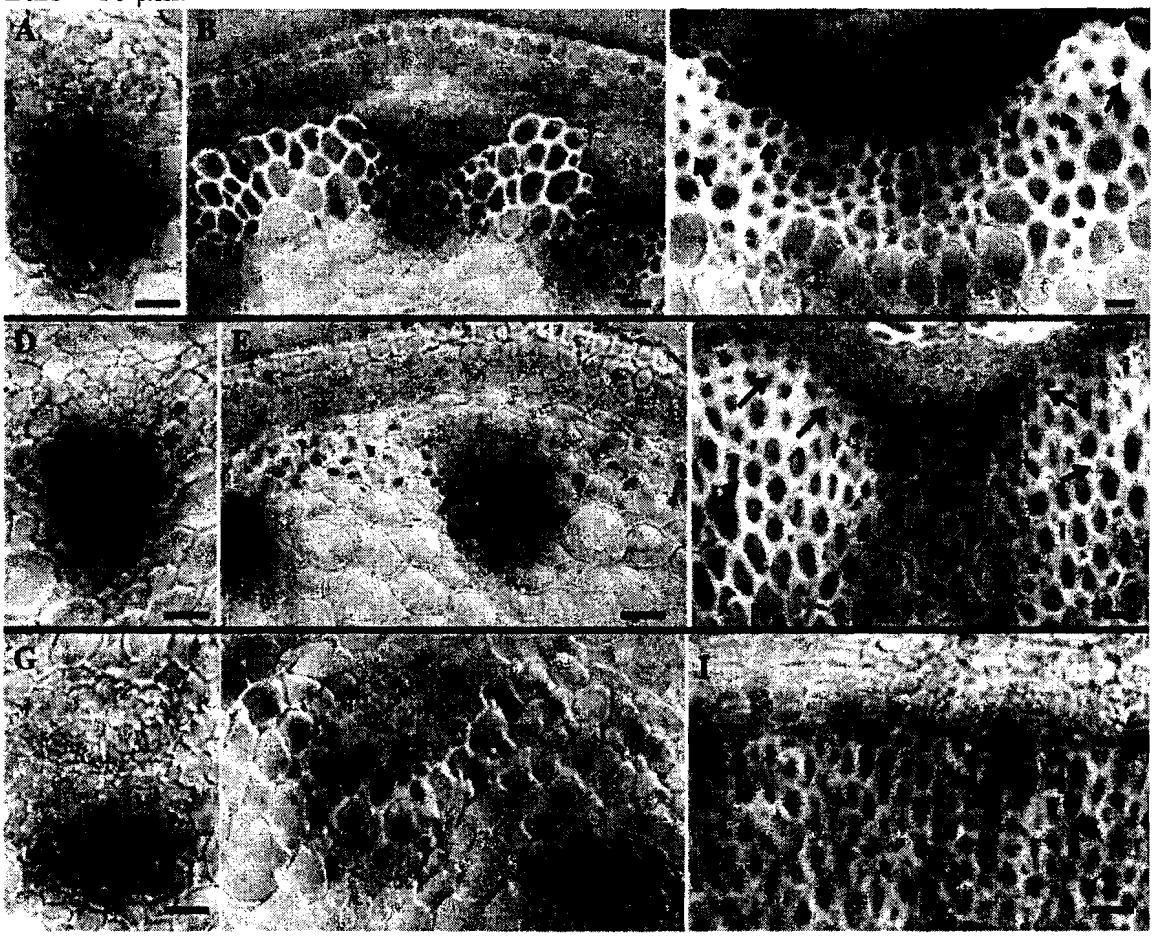


Figure 15 Genes were expressed during vascular differentiation.

GUS expression in differentiating xylem from transverse sections (TS, upper images) and longitudinal sections (LS, lower images) in similar regions of inflorescence stems. The stem center is at the right side for TS images. (A) At1g57590 (pectinacetylesterase) expression in differentiating protoxylem (px) without secondary wall thickenings. (B) At5g42180 (peroxidase) expression in px elements having secondary wall thickenings, early metaxylem (emx) and more mature metaxylem elements (mx) with scalariform wall thickenings, with loss of expression in mature protoxylem (right side). Bars = 10 μm.

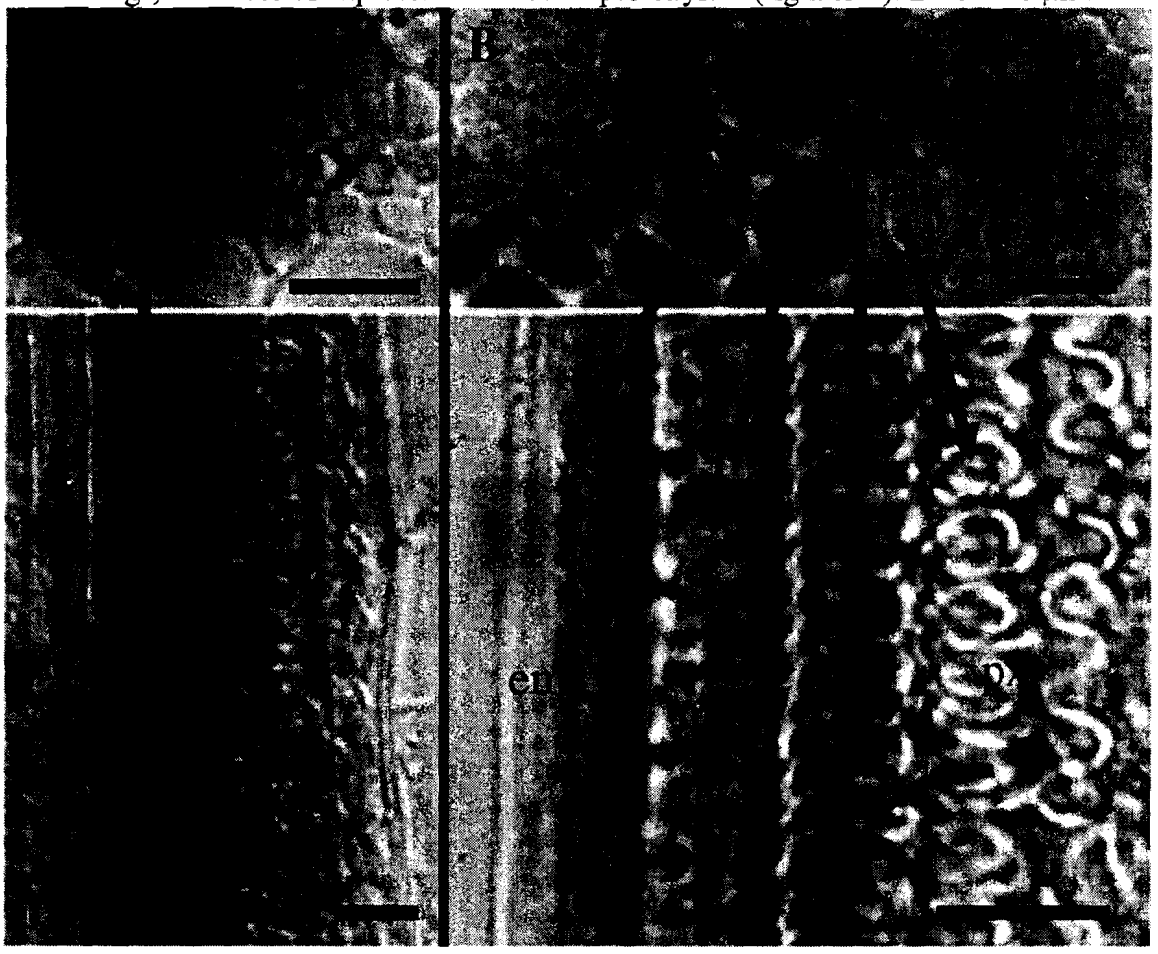


Figure 16 Defense and anthocyanin-related genes were expressed in vascular-associated cells.

Expression patterns in control (-) or NPA-treated (+) plants for genes: (A) At3g17790 (acid phosphatase) in phloem-associated idioblasts; (B) At2g39330 (myrosinase-binding protein, MBP) and (C) At1g52000 (MBP) in bundle sheath and phloem cells; (D) At1g52040 (MBP) in phloem; (E) At5g17220 (glutathione transferase) in midvein-associated cells (in or near the epidermis); and (F) At4g14090 (anthocyanidin glucosyltransferase) in phloem cells. Top and middle images show whole mounts of 7-10 day old leaves (Bars = 100 μm). Lower image shows high magnification in or near leaf veins (Bars = 10 μm). Note that At5g17220 has no expression in the vascular tissues (lower image of E).

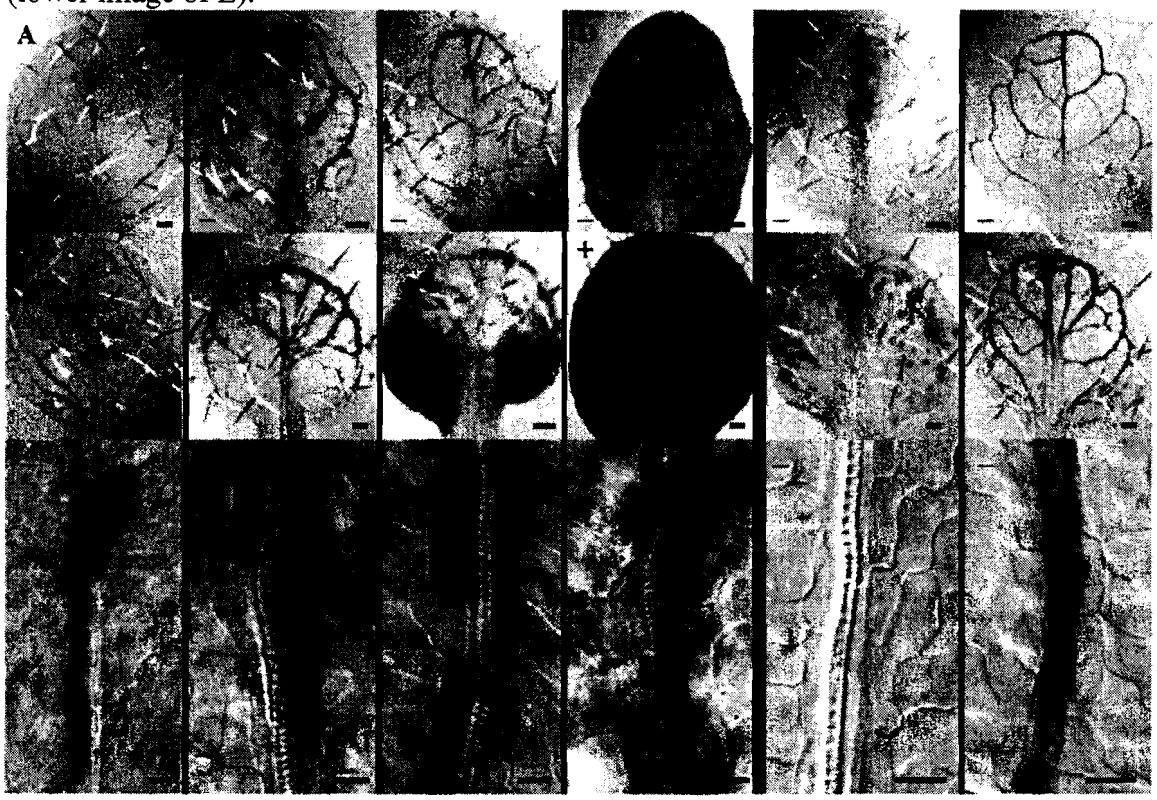
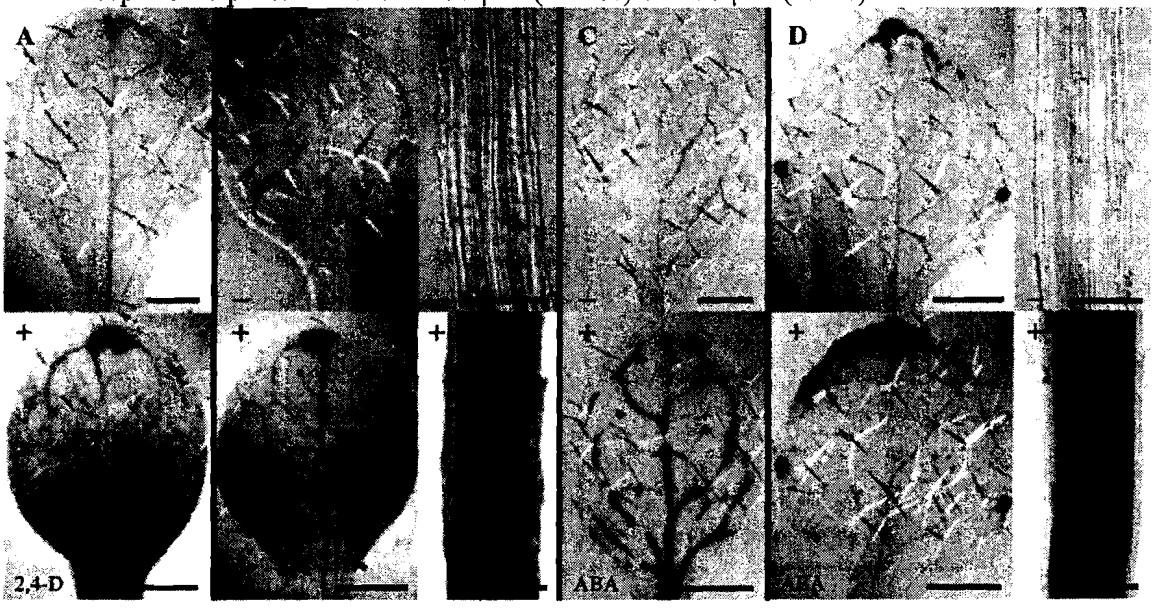


Figure 17 Exogenous hormone treatment affected gene expression.

Control (-) and treated (+) leaves and roots (right side of B and D) with 2,4-D (A), GA (B) or ABA (C,D) for genes: (A) At3g25290, auxin-responsive protein; (B) At3g16360, histidine phosphorelay mediator; (C) At3g53980, lipid transfer protein; (D) At3g02480, ABA-responsive protein. Bars = 500 μm (leaves) or 100 μm (roots).



CHAPTER 4
THE *ARABIDOPSIS THALIANA EXPANSIN-LIKE B1* GENE HAS A POTENTIAL FUNCTION IN ROOT GROWTH REGULATION

The research presented in this manuscript was performed by Qian Hester, under the supervision of Dr. Jim Mattsson. Unpublished pictures of more than three weeks old rosette leaves of promoter::GUS lines were taken by Dr. Carol Wenzel during a previous project described in chapter 3.

4.1 Summary

While many expansin proteins have been linked to a function in cell expansion during various processes of plant development no function has to date been assigned to any member of a related protein family, the expansin-like proteins. Here we present a functional analysis of the *Arabidopsis thaliana EXPANSIN-LIKE B1* (*AtEXLB1*) based on assessments of its spatio-temporal expression pattern and a morphometric analysis of a mutant defective in this gene. The analysis of an *AtEXLB1*promoter-marker gene construct in transgenic Arabidopsis plants showed that *AtEXLB1* was primarily expressed in multiple tissues of the root elongation zone and in maturing root vascular tissues. Mutants in the *AtEXLB1* gene displayed reduced growth of primary roots, and reduced length of root vessel elements. Taken together, the expression and phenotypic study suggest that *AtEXLB1* acts as positive regulator of root growth and vessel element elongation and provides for the first time phenotypic evidence for a function of an expansin-like gene.

4.2 Introduction

Plant vascular tissues transport photoassimilates, water and minerals throughout plants. As such, plant vascular tissues differ from other plant tissues in that they have a systemic rather than local function. To acquire this systemic transport function, vascular cells undergo specialized differentiation that sets them apart from other tissues. Transporting cells become extremely elongated to span as much distance as possible, and also become interconnected at their ends to allow continuous flow from cell to cell. Plant cell growth is obtained by a combination of cell hydrostatic pressure as a driving force for growth coupled with deposition of new cell wall material (Geisler et al., 2005). With limited directional control of growth, plant cells grow in all directions, referred to as isotropic growth (Wasteneys and Fujita, 2006). The highly polarized or anisotropic growth of vascular cells appears to be due to a restriction of growth perpendicular to the axis of growth by cellulose microfibrils encircling and in effect constrain the growth of the cells, coupled with a controlled diffuse expansion of the cell wall in the opposite direction (Geisler et al., 2005). The directional deposition of microfibrils in the cell wall in turn appears to be controlled by a parallel intracellular network of cortical microtubules (Geisler et al., 2005). Actin has also been implicated in the control of anisotropic growth although its function is not well understood (Geisler et al., 2005). The rate of anisotropic growth varies with different tissues and is also influenced by environmental factors. In response to unequal phototropic or gravitropic stimuli, unequal cell elongation of cells in shoots and roots can result in bending of shoot or root apices within minutes (Taiz and Zeiger, 1991). Such rapid responses have been attributed to auxin-mediated acidification of cell wall space leading to both non-enzymatic and

enzymatic hydrolysis and loosening of cell wall components, allowing anisotropic growth (Rayle and Cleland, 1992). Enzymatic cell wall loosening has primarily been attributed to expansins, a large family of proteins (McQueen-Mason et al., 1992). While the exact mechanism of expansin action is not clear, it has been suggested that they act by generating slippage between molecules of both glycans and hemicellulose that crosslink cellulose microfibrils to each other, thereby allowing turgor-driven expansion (Cosgrove et al., 2002). Expansins have been implicated in cell wall loosening at various phases of development including anisotropic epidermal, vascular and root hair elongation, as well as isotropic expansion during fruit ripening and leaf abscission (Cosgrove et al., 2002). Accordingly, the factors that induce their transcriptional activation vary depending on the gene, and include the growth regulators auxin, ethylene and abscisic acid (Cosgrove et al 2002). It should be pointed out that the expression of some of the expansin genes does not correlate with cell expansion, and that the function of many of the family members is unknown (Caderas et al., 2000; Li et al., 2002). Phylogenetic analysis of expansin genes found in sequenced plant genomes and EST populations has revealed that expansins form a superfamily (Li et al., 2002; Sampedro and Cosgrove, 2005). In *Arabidopsis thaliana*, the 36-member superfamily consists of 25 members of the α-expansin family, 5 members of the β-expansin subfamily, and a family of 4 expansin-like proteins. When integrated with expansin-like proteins from other species, the expansin-like family consists of two subfamilies; expansin-like A and B, with 3 and 1 members respectively in Arabidopsis. No biological or biochemical function has yet been established for any member of the expansin-like families. (Kende et al., 2004).

We recently reported the identification of a large number of genes expressed in vascular tissues of Arabidopsis (Wenzel et al., 2008). Among these genes were *Arabidopsis thaliana expansin-like B1* (*AtEXLB1*), the sole member of the expansin-like B sub-family in Arabidopsis. Here we have assessed the developmental function of *AtEXLB1* gene based on further analysis of spatio-temporal gene expression pattern and morphometric analysis of related growth. We found that *AtEXLB1* is primarily expressed the root elongation zone and in the vascular cylinder of the root maturation zone. The morphometric analysis of growth uncovered a corresponding phenotype in *Atexlb1* mutants as they showed reduced root growth as well as shortened vessel elements in the root maturation zone.

4.3 Results

4.3.1 The *AtEXLB1* gene is expressed primarily in the root elongation and vascular maturation zones

As part of a larger study focusing on the identification of a large group of genes and the analysis of their expression in developing leaves, we have reported in the previous project described in chapter 3 that the *AtEXLB1* promoter-marker gene construct is expressed in immature xylem of leaf hydathodes in Arabidopsis (Wenzel et al., 2008). To produce a more complete picture of where and when this gene might act, we have assessed its expression throughout the life of Arabidopsis plants. Since we previously observed that *AtEXLB1* is up-regulated in shoot tissues grown in the presence of the auxin influx inhibitor NPA (Wenzel et al., 2008), we compared expression of *AtEXLB1* in tissues unexposed and exposed to NPA to identify potential flexibility in *AtEXLB1* expression.

4.3.1.1 *AtEXLB1* gene expression in leaves and stems

There is no expression of the *AtEXLB1* promoter::GUS fusion in the first pair of rosette leaves during much of leaf development without and with NPA treatment, based on observation of 7 and 14 day old rosette leaves (data not shown). However, in more than three week old rosette leaves, promoter::GUS expression is in vascular tissues of hydathode and leaf petioles without NPA treatment and there is sporadic vascular expression with NPA treatment (Figure 18). The leaf cross sections show that *AtEXLB1* promoter::GUS is expressed in a position consistent with immature xylem cells. (Figure 18c,f). Similarly, in leaf blades, the expression appears in the focal plane of xylem tissues (Figure 18b.d), consistent with expression in immature xylem cells. There is no *AtEXLB1* promoter::GUS expression in early and late development stage sections of inflorescent stems with and without NPA treatment (data not shown). The absence of expression in stems and limited expression of *AtEXLB1* in leaf veins suggest that it has a minor, if any, role in differentiation of vascular tissues in these organs.

4.3.1.2 *AtEXLB1* gene expression in roots

In 7 day old roots, *AtEXLB1* promoter::GUS expression is strong in a diffuse region behind the root apical meristem (RAM) without and with NPA treatment (Figure 19b,d). This region corresponds roughly to the root elongation zone. The expression is not restricted to central vascular tissues, but appears to include all tissues. There is no expression in the maturation zone at this stage (corresponds roughly with formation of root hairs; Figure 19a,c). Surprisingly, in 14 day old roots, *AtEXLB1* expression is almost absent in the root tip region (Figure 19f). Instead, *AtEXLB1* is now expressed strongly in the vascular cylinder in the maturation zone behind the root elongation zone (Figure 19e).

In 14 day old roots grown in the presence of NPA, the expression remains much the same as in 7 day old roots, with strong expression in the elongation zone (Figure 19h), and no expression in the maturation zone of the root (Figure 19g). This observation suggests that inhibition of auxin transport inhibits also the transition from *AtEXLB1* expression in the elongation zone to expression in the maturation zone observed between 7 and 14 day old roots grown in the absence of NPA (see discussion). Since the root tip region usually grows continuously, the down regulation of *AtEXLB1* expression in the root elongation zone of 14 day old roots grown in the absence of NPA is surprising and requires further analysis. In summary, *AtEXLB1* promoter::GUS confers expression primarily in roots. In roots, it is initially expressed in the elongation zone of all tissues, but later become expressed in vascular tissues in the maturation zone. This expression profile is in part in line with the function of an expansin, as it correlates with cell elongation..

4.3.2 An *AtEXLB1* insertion mutant line displayed a reduced growth of roots and reduced length of root vessel elements

To assess the function of *AtEXLB1,* we carried out an analysis of a mutant defective in the *AtEXLB1* gene. While the function of expansin-like genes is unknown, we initiated a morphometric analysis based on the hypothesis that *AtEXLB1* could have a function in cell expansion, similar to expansin genes with known functions in this process. In the several T-DNA insertion Arabidopsis seed lines that are available for *AtEXLB1*, the Salk_03488 line was chosen as it had an exon insertion closest to the 5' end of the open reading frame region. According to the Salk_03488 sequence in *The Arabidopsis Information Resource (TAIR)* database, the T-DNA is early in the second exon of *AtEXLB1*. This insertion predicts in truncation of more than half of the predicted

protein, suggesting that Salk_03488 is likely to be a strong or complete loss of function mutant. We have confirmed the insertion site by PCR and identified parental plants *exlb1-3* homozygous for the insertion (Figure 20). We used lines that were identified as homozygous wildtype from the same material as a wildtype control for comparison. Although the expression analysis above indicated a potential function primarily in the root, we carried out an extensive morphogenetic analysis of the growth of hypocotyl, cotyledons, first leaves and inflorescence length (see material and methods). We found no statistically significant differences between wildtype and mutant lines, and therefore do not show these data here. However, We found differences in root growth, much in line with the recorded expression of *AtEXLB1*.

4.3.2.1 Root growth is reduced in *exlb1* mutants

Two independent sets of experiments were setup under similar conditions. In the first set of plants, 18 plants of each line were measured. In this set, *exlb1-3* plants showed obviously shorter roots compare to wild types during early stages of root development. At 4 day-after-germination (4 DAG), root length of *exlb1-3* is on average 78% of wild type roots (Figure 21a). At 8 DAG, root length of *exlb1-3* is 88% of wild type roots (Figure 21b). In the second set of plants, 10 plants of each line were measured. The second set of plants provided similar results. At 5 DAG, root length of *exlb1-3* mutants was 78% of wild type roots (Figure 21c). At 6 DAG, root length of *exlb1-3* mutants were on average 81% of wild type roots (Figure 21d), and at 10 DAG, root length of *exlb1-3* was 85% of wild type roots (Figure 21e). Student's T-test showed a statistically significant difference between the length of wild type and *exlb1-3* roots from different DAG with a probability of null hypothesis being correct at less than 0.0001. In summary, the results showed that

exlb1-3 plants have reduced root growth compare to wild type plants and strongly suggested a role of *AtEXLB1* in root growth.

4.3.2.2 Length of root vessel elements is reduced in *exlb1* mutants

Since we recorded strong expression of *AtEXLB1* in the vascular cylinder or stele of maturing roots (Figure 19e,f), we analyzed this tissue for potential defects. We focused our analysis on vessel elements as the conspicuous secondary cell wall of these cells makes them relatively easy to identify. An analysis using confocal laser scanning microscopy showed no defects in the overall anatomy of root vessel elements in the *exlb1* mutants either at 6 DAG (Figure 22) or 11 DAG (not shown). To visualize potential differences in length of vessel elements, we used differential interference microscopy (DIC) optical sectioning of cleared roots to identify the end walls of vessel elements, and direct measurement in the microscope by a staged micrometer. The results were based on about 200 mature xylem root cells of each line growing under the same condition at 6 DAG. Measurements were taken just after the point where vessel elements have reached their full length in the early root hair zone. The results showed that *exlb1-3* vessel elements are on average 20% shorter than wild type vessel elements (Figure 23). To evaluate the result that is based on a large quantity of cell observations, a Student's T-test was conducted on the data and concluded that there is a statistically significant difference between wild type and *exlb1-3* with respect to length of root vessel elements (Probability of null hypothesis < 0.0001).

4.4 Discussion

In this study we set out to asses a potential developmental function of the *EXPANSIN-LIKE B* gene from Arabidopsis. To date, no function, biochemical or developmental, has been assigned to any expansin-like gene from any plant species (Sampedro and Cosgrove, 2005). We discovered that *exlb1-3* mutants generally have about 20% shorter primary roots than corresponding wildtype plants under the tested conditions. We also discovered that *exlb1-3* have 20% shorter vessel elements in the early maturation zone of roots. The overall reduction of root growth and reduction in root vessel element length provides evidence that *AtEXLB1* normally has a positive influence on root growth and root vessel element elongation. While these phenotypes is consistent with a localized function in cell expansion typical of some expansins, this cannot however be taken as evidence of biochemical function of *AtEXLB1* as an expansin mediating cell wall loosening. This will require further experimentation for example extensometer assays where activity is tested by adding back expansin to denatured cell walls to assess if they generate cell wall extension ability (McQueen-Mason et al., 1992; Rayle and Cleland, 1992). In addition, further support can also be gained by sub-cellular localization analysis, which should reveal an extracellular localization of *AtEXLB1*. Such an experiment can be accomplished relatively easily in Arabidopsis by the construction of a translational fusion of EXLB1 with GFP or other spectral variant and high magnification confocal laser scanning microscopy of transgenic plant cells.

The observed root phenotypes correlate very well with the observed expression of *AtEXLB1* by promoter marker gene analysis. Here we saw an initial expression in what appears to be all cell types in the elongation zone. Additional microscopy, possibly of

sectioned root tips is required to assess if internal tissues express *AtEXLB1* in the elongation zone. In this zone multiple tissues undergo anisotropic expansion (Taiz and Zeiger, 1991; Geisler et al., 2005). In addition we observed a strong expression of *AtEXLB1* in the maturation zone further back from the root apex. In this position, only vascular cells undergo anisotropic growth in order to obtain their extremely long cell lengths, relative to surrounding tissues. It is not clear which of these domains of expression contributes to the observed overall reduction in root growth. It is possible that a combination of reduced anisotropic growth of all tissues in the elongation zone as well as a reduction of anisotropic vessel element growth is responsible for the reduction in root growth. This scenario requires that the reduced elongation of vessel elements somehow physically limits elongation of other tissues. Such a scenario is not unheard of, and there is evidence for similar limitations on growth to be imposed by epidermal tissues on sub-epidermal tissues in stems (reviewed by Sinha, 2000). However, the expression of *AtEXLB1* in the root stele above the elongation zone suggests either that *AtEXLB1* has multiple roles in cell elongation or does not have a function as an expansin. In the opposite direction, we have also observed that the elongation zone expression is absent in older roots. It is possible though that this is an artefact stemming from a termination or reduction of growth of the primary root. Thus it is clear that further analysis of the spatio-temporal expression of *AtEXLB1* is required to clarify these issues. Nevertheless, it appears that we have uncovered both correlative and causative evidence of *AtEXLB1* having a function in root growth and development and as such evidence for a function of the first expansin-like gene to date.

4.5 Materials and methods

4.5.1 Plant material and growth conditions

Arabidopsis thaliana T-DNA insertion Salk seed lines (*exlb1-3* = Salk_034888) were obtained from the Salk Institute Genomic Analysis Laboratory through the Arabidopsis Biological Resource Center (ABRC). The Salk seed lines had been planted to collect second generation seeds, when I started this study. Seeds of *Arabidopsis thaliana* Salk lines were surface sterilized by exposure to chlorine gas for three hours and plated onto sterile *Arabidopsis thaliana* salts (ATS) media (Lincoln et al., 1994). Seeds were vernalized for at least two days at 4°C and then grown at approximately 20°C with constant illumination. Plants were transferred to soil after two weeks and grown at approximately 18°C in a greenhouse.

4.5.2 Salk line mutant genotyping

Genomic DNA of each plant was isolated separately using one leaf of 4 week old plants or several leaves of 2 week old plants as in Edwards et al (Edwards et al., 1991). Salk line mutant genotyping PCR was performed according to Taq DNA Polymerase manufacturer (Invitrogen; www.invitrogen.com). In a 25µl system, 100ng genomic DNA, 0.2µg of each primer (LBa1+LP+RP), 25nmol $MgCl_2$, 1 unit Taq DNA Polymerase, 6.25nmol dNTP, buffer and water were used to perform PCR at an annealing temperature of 60°C for 30 second. 5µl of PCR products were used to perform gel electrophoresis. Salk T-DNA verification primer design program provided on SIGnAL website (http://signal.salk.edu/tdnaprimers.2.html) is used to design primers. This program is based on Primer3, a popular web-based free primer design program, with primer-picking

conditions suitable for Salk line genotyping PCR. LBa1 is chosen as the left border primer on T-DNA, since LBb1 (a type of the left T-DNA border primers) gave false positives in some of my experiments (data not shown; SIGnAL website http://signal.salk.edu/) Of the several T-DNA insertion Arabidopsis seed lines that are available for *AtEXLB1*, Salk_03488, which has the earliest exon insertion, was chosen for further study. According to the Salk_03488 sequence in TAIR database, T-DNA is inserted at early mid exon (the second exon) of *AtEXLB1*. This suggests that Salk_03488 is likely to be a strong or complete loss of function mutant. The right primer (RP) and left primer (LP) of *AtEXLB1* are chosen when Max N is 440, Ext5 is 440 and Ext3 is 315 and gives a PCR product size of 1319 bps. The primers that were used to do PCR are: Lba1: 5' TGGTTCACGTAGTGGGCCATC 3'; Salk_034888 RP: 5' CCACCAACGTACAAGACAAGG 3'; Salk_034888 LP: 5' GGGTTTATGCCAACCCTAATG 3'. Wild type allele is produced with primers Salk_034888 LP and Salk_034888 RP. Mutant allele is produced with primers Lba1 and Salk_034888 RP. In the second generation of seeds produced from the Salk_034888 seeds obtained from the stock center, *exlb1-3* lines are found to be *AtEXLB1* homozygous mutant plants. More than 20 individual plants from *exlb1-3* have been tested. (Figure 20)

4.5.3 Microtechniques and microscopy

Histochemical detection of GUS activity, fixation, and clearing were performed as described in the previous chapter. Samples were viewed with a Nikon Eclipse E600 microscope equipped with a Canon EOS D30 digital camera. Images were assembled using Adobe Photoshop 7.0 (Adobe Systems). For Confocal Laser Microscopy, root vascular cells were stained with safranin as described (Kitin et al., 2003). Samples were

observed with a Leica SP2 confocal microscope equipped with a helium neon laser for excitation (wavelength, 543 nm; long-pass filter, 590 nm), and either a 63×/1.32 oil or a 40×/1.25 oil objective lens for imaging. For differential interference contrast (DIC) microscopy, plant material was fixed in ethanol/acetic acid (6:1), hydrated in a series of decreasing concentrations of ethanol, and mounted in a mixture of chloralhydrate/glycerol/water (8:1:2), according to the method of Berleth and Jürgens (1993). Root cell lengths were measured using a Nikon Eclipse E600 microscope using differential interference contrast settings, with the micrometer eyepiece (Nikon, WWW.Nikon.com).

4.5.4 Morphometric analysis of T-DNA insertion mutant

For the root length measurement experiment, five or six sterilized seeds from each line were placed onto the same ATS media plate. After two days at 4°C, plates were placed vertically on a shelf at approximately 20°C with constant illumination. Plants to be used for hypocotyl length measurements were transferred to 6 x 6 x 10 cm magenta boxes with fresh ATS media and grown directly with constant illumination. To generate longer, etiolated seedlings, magenta boxes with seedlings were exposed to constant light for 12 hour then wrapped with alumina foil and kept in dark. The length of plant organs were measured with ruler or calliper.

Three sets of experiments were setup to measure hypocotyl length. In the first set of plants, we performed the experiments under constant light. 18 plants of each line were measured. At 12 DAG, hypocotyls reach a measurable length of about 5 mm. In the second set of plants, we performed the experiments in dark to acquire longer hypocotyls. 20 plants of each line were measured. In the third set of plants, we performed the

experiments under reduced light to reduce dark growth stress. 15 plants of each line were measured. Morphometric analysis of cotelydons was performed under constant light. 36 cotyledons of mutant and wildtype plants were measured. At 12 DAG, we measured cotyledon blade width, blade length and petiole length of each line.

We measured the inflorescence stem length of two-and-half-month-old plants grown under greenhouse condition of T-DNA insertion mutants and wild type, 25 plants of each line.

4.5.5 Statistical methods

Standard error was calculated for comparisons in Figure 21. The standard error is calculated from the standard deviation, which is based on the differences between the average length percentage of root (100%) and individual length percentage of roots (x %). The following equations for calculation were used:

$$\sigma \text{ (Of mutant group as \%)} = \left(\sum_{i=1}^{n} (\sigma_{xi} / \bar{x}_i)\right)*(100/n)$$

Standard error = $\sigma/\sqrt{n}$

Where: n = the number of petri dish plates in the same set; x = the plant line.

Student's T-test was used to determine if two sets of data are statistically different. This was performed using an online program (http://www.physics.csbsju.edu/stats/t-test.html) that enables entry of two sets of data for comparison.

4.6 References

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4.7 Figures

Figure 18 AtEXLB1 promoter::GUS gene expression in leaf rosettes that are more than three weeks old.

Leaves of plants grown in the absence of NPA (a-c), and grown in the presence of 10 μM NPA (d-f). Leaf blade (a,b,d,e), leaf petiole cross section (c,f). Scale bars are 0.1 mm.

- NPA + NPA

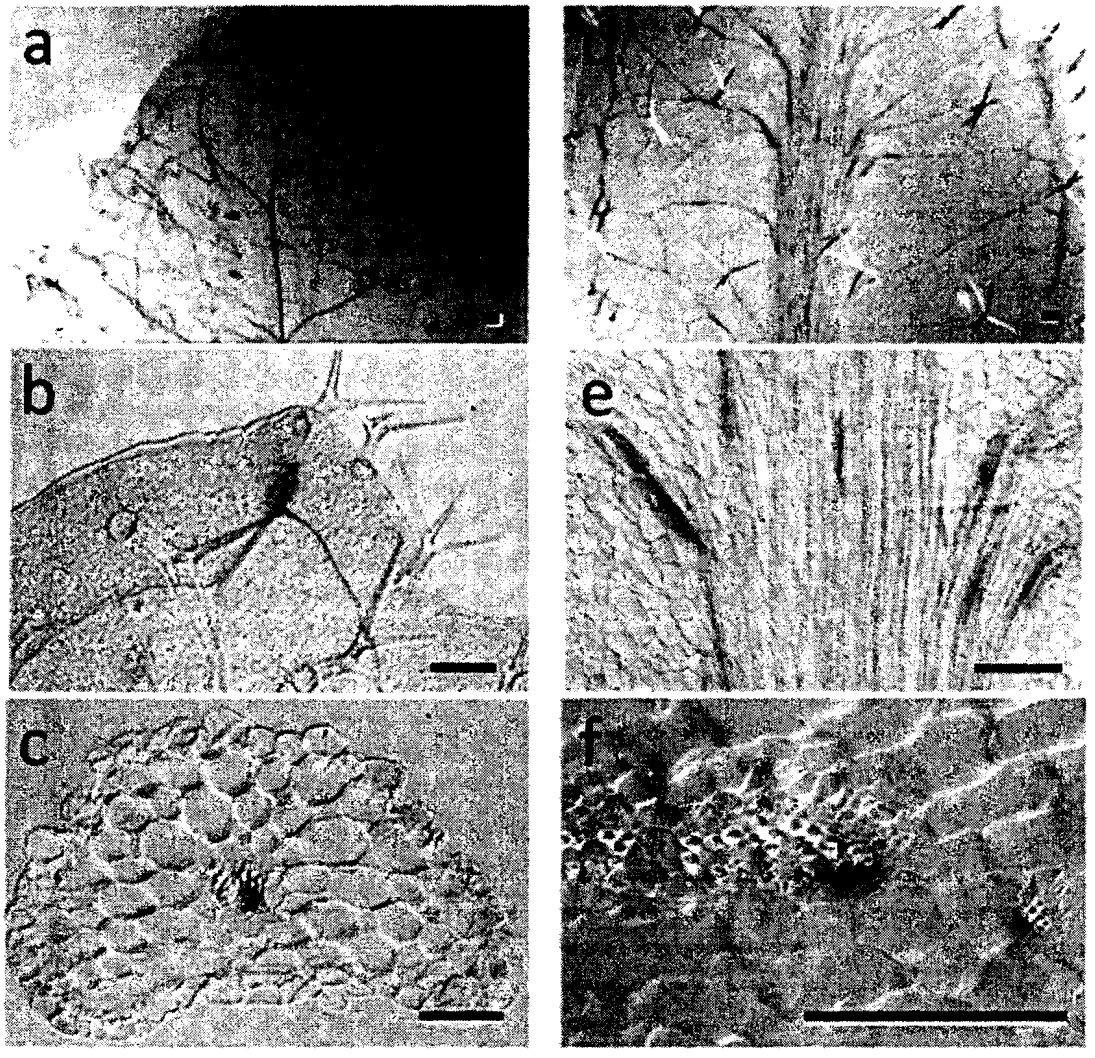


Figure 19 AtEXLB1 promoter::GUS gene expression in roots and root tips.

Roots 7 (a-d) and 14 (e-h) days after germination, without exposure to NPA (a,b,e,f) and with exposure to 10 μM NPA (c,d,g,h). Scale bars are 0.1mm.

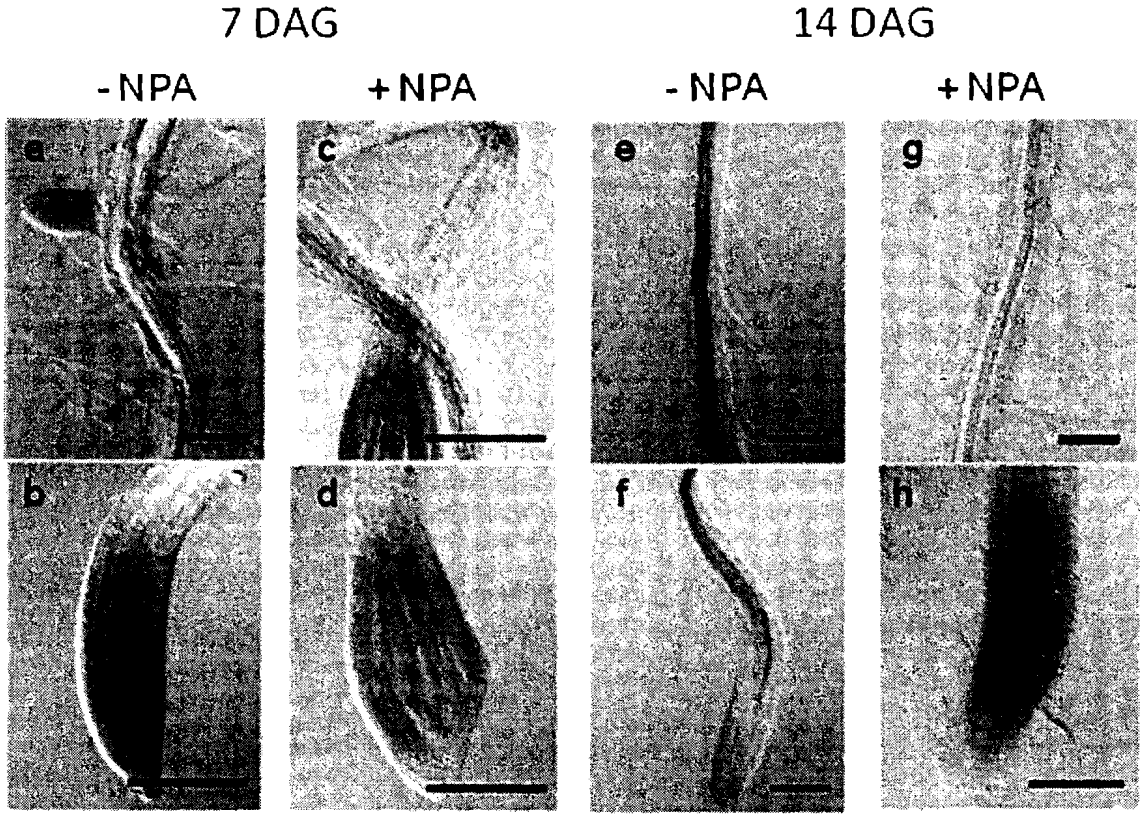


Figure 20 Identification of homozygous T-DNA insertion mutants in Salk_034888.

Lane 1 is Invitrogen 100bp ladder plus; lane 2 and 3 are PCR results of two plants from a heterozygous line; lane 4, 5 and 6 are PCR results of three plants from the homozygous line *exlb1-3*.

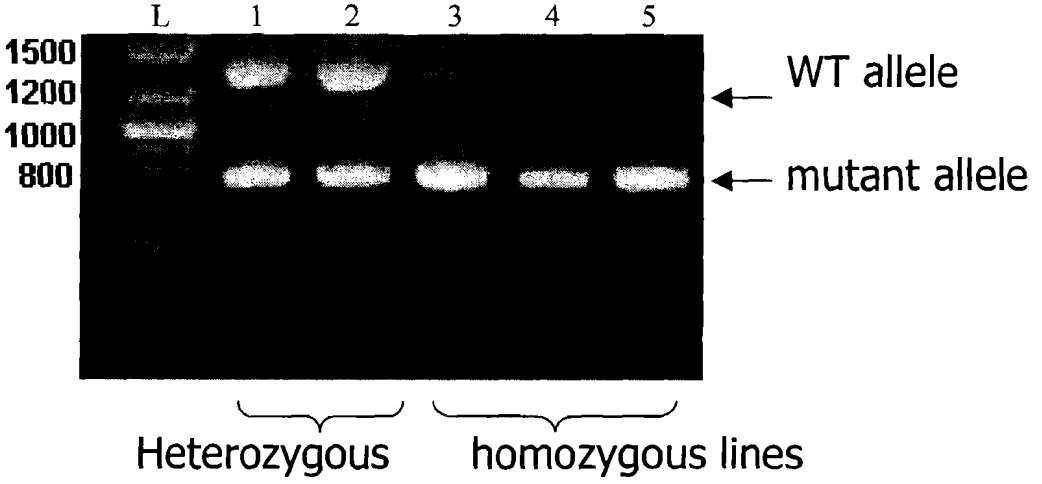


Figure 21 Root length measurements of salk line mutants and wild types.

Root length measurements of set 1 plants with 18 plants of each line at 4 DAG (a) and at 8 DAG (b). Root length measurements of set 2 plants with 10 plants of each line at 5 DAG(c), 6 DAG(d), and at 10 DAG(e). Error bars are the standard error in the sample set (see materials and methods for statistical equations). Student's T-test showed a real difference between wild type and *exlb1-3*. (Probability of null hypothesis < 0.0001)
Column 1: root length mean of wild type as control (100%).
Column 2: root length mean of *exlb1-3* mutants as a percentage of the control.

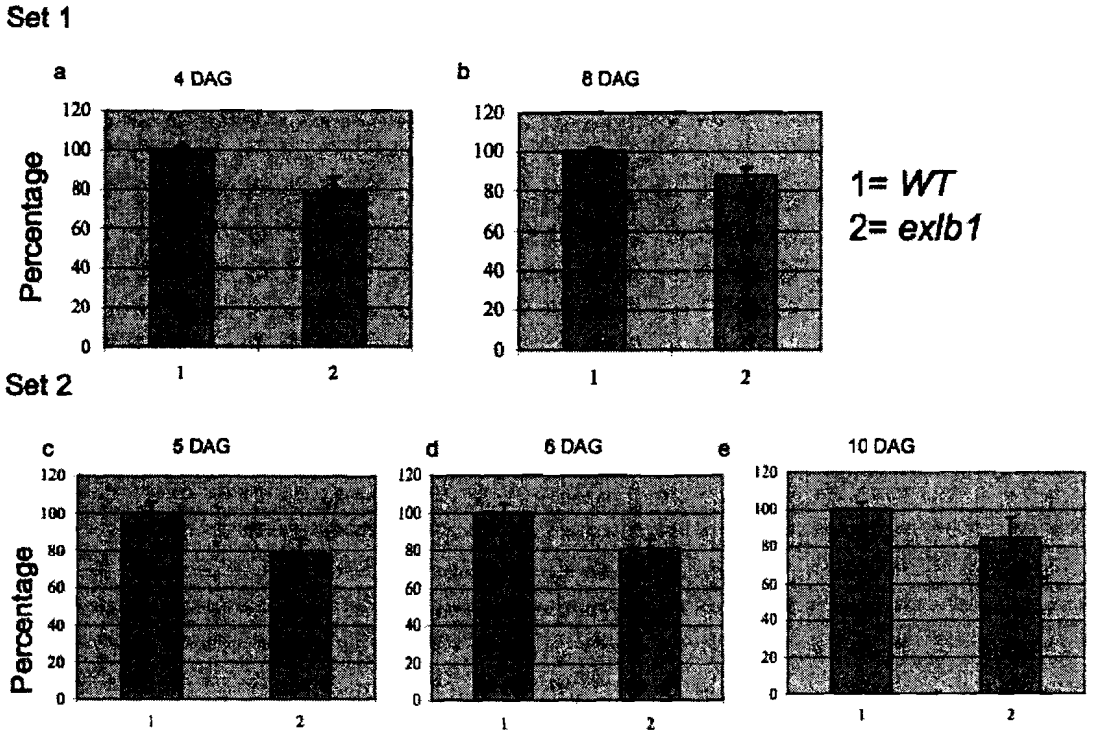


Figure 22 Vascular root cells in wild type and *exlb1-3* insertion mutant at 6 DAG.

Confocal laser scanning microscopy. The arrow points out the end wall of a metaxylem vessel element. Scale bars are 10μm.

(a) wild type (b) *exlb1-3*

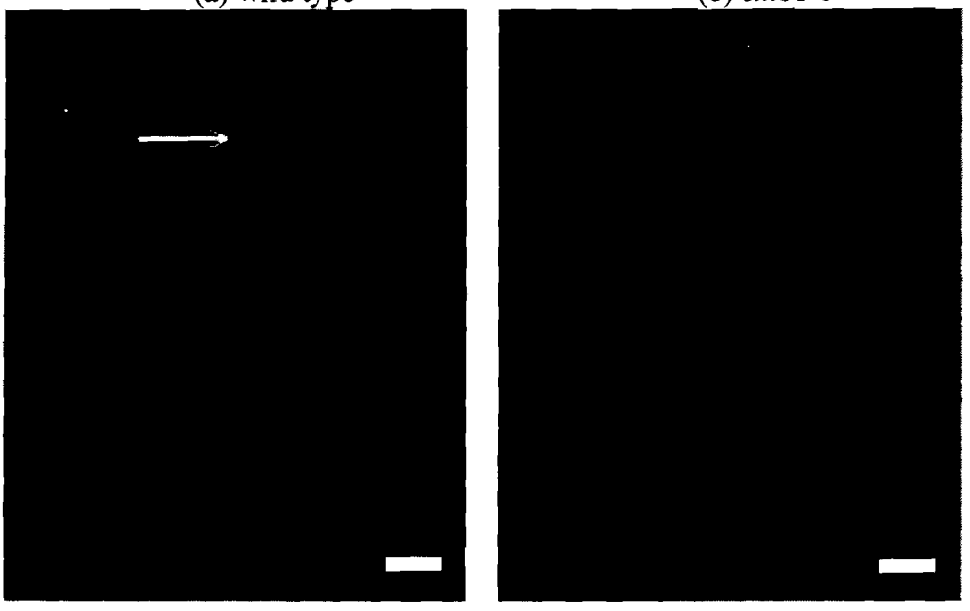
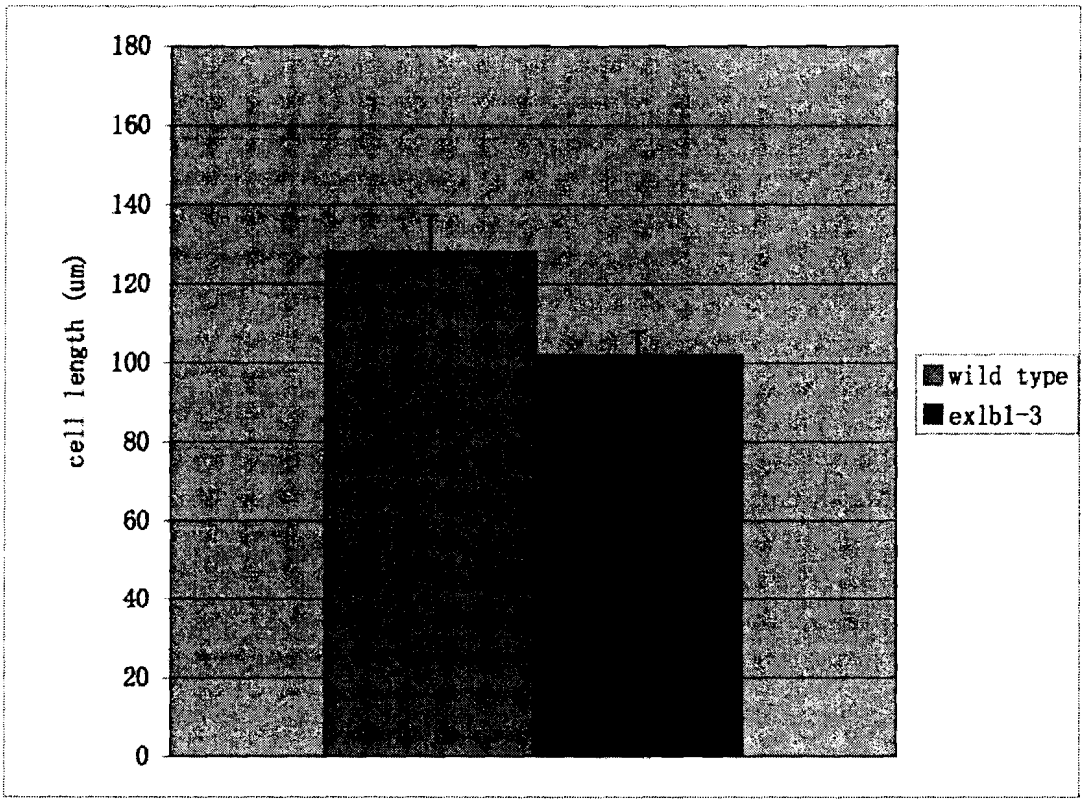


Figure 23 Length of root vessel elements in wildtype and *exlb1-3* mutants.

About 200 metaxylem root cells of each line were measured at 6 DAG. Error bars are the standard error in the sample set, which is calculated based on the differences between the average cell lengths and individual cell lengths (see materials and methods for statistical equations). Student's T-test showed a real difference between wild type and *exlb1-3*. (Probability of null hypothesis < 0.0001) Cell length is shown in μm.



CHAPTER 5
GENERAL DISCUSSION

While the structure and function of plant vascular tissues are well understood, the understanding of the genetic basis of plant development remains largely unknown. There are several reasons why such an understanding is desirable. First, and perhaps foremost, we wish to satisfy a scientific curiosity on how a plant and its functional tissues develop. For example, the issue on how the venation of a plant leaf is organized into a symmetrical pattern has been recognized for centuries as one of nature's many beautiful mysteries. Second, among plant tissues, vascular tissues hold a special position, as it is the only tissue that requires extensive inter-cellular organization for normal function. Third, plant vascular tissues hold enormous economical value as their constituent fibers are used in numerous products including clothing, paper products, and construction material.

The overall process of vascular development can be separated conceptually into the process of vascular patterning and vascular differentiation. In this thesis, I worked on the identification and characterization of genes with roles in each of these processes. With respect to the process of vascular patterning, my work focused on the characterization of the expression of the PIN1 gene, encoding an auxin efflux carrier. This work is described in chapter two. The overall hypothesis was that PIN1 plays a role in the process that selects cells for a vascular fate during vein formation, in particular the primary and secondary veins. More specifically, it was suspected that PIN1 may participate in a gradual canalization of auxin flow into limited strands of cells that

eventually are selected for a vascular fate as predicted by Sachs (see chapter 1). To this effect, I worked closely with Dr Wenzel to analyze the dynamics of PIN1 expression during vein formation. In summary, we found that (1) PIN1 localization precedes and predicts the formation of veins, (2) PIN1 localization is gradually confined to single files of cells. (3) PIN1 is gradually localized to the basal end of procambial cells. (4) PIN1 localization in epidermis and margin correlates with vein formation and suggest an overall flow of auxin in the epidermis and margin to the distal point of vein formation, where auxin flow may become internalized and participate in vein formation. Taken together, the gradual limitation of PIN1 expression across fields of cells and the development of directional subcellular localization of PIN1 provides strong molecular support for the canalization of auxin flow hypothesis as well as a positive feedback loop that includes auxin and PIN1.

The third chapter describes the by far most extensive component of my thesis work. The overall hypothesis for this project was formulated by Dr Mattsson before my Master's study and addressed the identification of genes potentially involved in vascular differentiation. The hypothesis is that genes with a function in vascular differentiation can be identified by screening for mRNAs that are more abundant in leaf primordia that are producing extra vascular tissues in response to NPA, an auxin transport inhibitor. While this hypothesis is straightforward, the work to confirm or reject the hypothesis has required an immense amount of work. Candidate genes were identified before my involvement in this study, and I have worked in close collaboration with Dr Wenzel on the assessment of whether or not identified genes are expressed in vascular tissues. To this effect, I constructed promoter-marker gene fusions of approximately 20 out of total

40 assessed genes. Each promoter fragment was amplified and cloned independently in duplex copies to avoid assessing artifact expression patterns due to PCR-induced errors, and resulting constructs were transformed through Agrobacterium-mediated transformation into Arabidopsis. Multiple transgenic lines from the duplicate constructs were assessed by segregation analysis as well as assessed by histochemical analysis of expression. The study has proven successful; as it revealed that (1) Thirty eight of the 40 genes had expression patterns that included vascular tissues, most of them previously not associated with functions in vascular tissues. (2) Fifteen of the genes showed vascular-specific expression, again most of them previously not associated with functions in vascular tissues. Thus, we could conclude that the tested strategy was highly efficient in identifying genes expressed in vascular tissues. In addition, we found highly interesting expression patterns, including genes expressed at high levels in cambium, phloem and xylem, including fibers. The identified genes provide material for many future studies of the genetic basis of vascular differentiation in Arabidopsis.

I went on to carry out a largely independent initial study of the function of two of the identified genes based on the use of T-DNA insertion mutants. The analysis of one of them, the *EXPANSIN-LIKE B1* (*EXLB1*) gene, resulted in a detectable phenotype and is described in chapter four. The other gene, a gene encoding a putative basic Helix-loop-Helix transcription factor, did not result in a detectable phenotype and is therefore described in an abbreviated form in appendix 4. In the morphometric analysis of development of the insertion mutant in the *EXLB1* gene I uncovered evidence that this gene is required for normal root growth which correlates with an expression in the root elongation zone. I also found that nearly mature metaxylem cells of *exlb1* mutant plants

were on average approximately 20% shorter than corresponding wildtype cells. Although additional analysis is required before this study can be published, the results provide preliminary evidence for a role of *EXLB1* in root growth, both at the organ and cellular level.

APPENDICES

Appendix 1

Chapter 2 additional material. Originally published as supplementary material for the article in *The Plant Journal* 2007 Feb;49(3):387-98, under the title "Dynamics of *MONOPTEROS* and PIN-FORMED1 expression during leaf vein pattern formation in *Arabidopsis thaliana*".

Figure S1. Dark-field images of mature first (a) and fifth (b) cleared leaves of wild type plants. The 1°, 2°, and 3° vein orders are shown, and for the first leaf the 1', 2' and 3' forming secondary vein pairs are indicated. Scale bars are 500 μm.

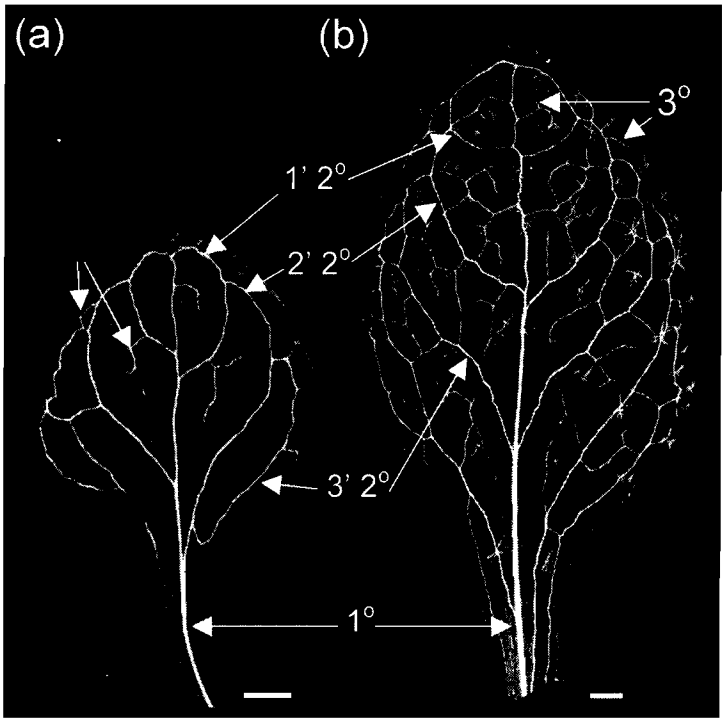


Figure S2a. PIN1-GFP expression in tertiary veins of the first leaves in *pPIN1::PIN1-GFP* plants. In 5-7 DAG primordia, tertiary and higher order veins appear to be formed through outgrowth from (a-c) or by connection of isolated PIN1-GFP expressing cells to (d) an existing lower order vein. Scale bars are 20 μm.

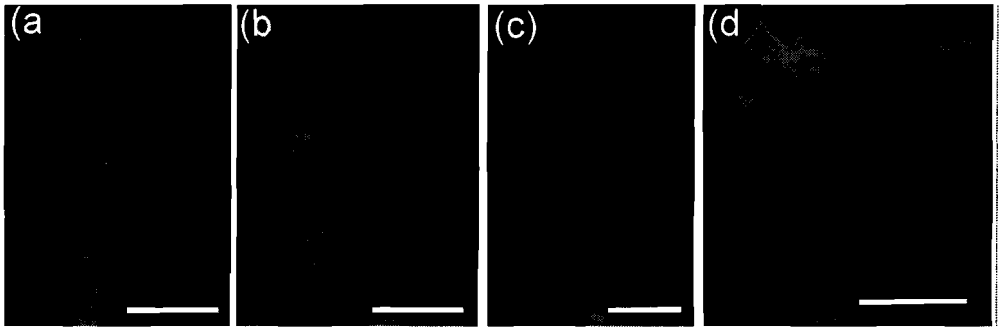


Figure S2b. PIN1-GFP expression in tertiary veins of the later formed rosette leaves in *pPIN1::PIN1-GFP* plants. PIN1-GFP expression in marginal veins suggests three possible connection mechanisms: (1) between a secondary strand epidermal bridge and the distal secondary vein (arrows in a,b); (2) via the epidermis just distal to the first serration, which can later expand to form another serration (arrowheads, c-e; arrows show vein strands); (3) directly between two secondary veins (not shown). All tertiary and higher order veins can occur via connection of isolated or clusters of PIN1-GFP expressing cells (e.g. asterisk in b), or outgrowth from existing lower order veins (e.g. arrows in a). Scale bars are 20 μm.

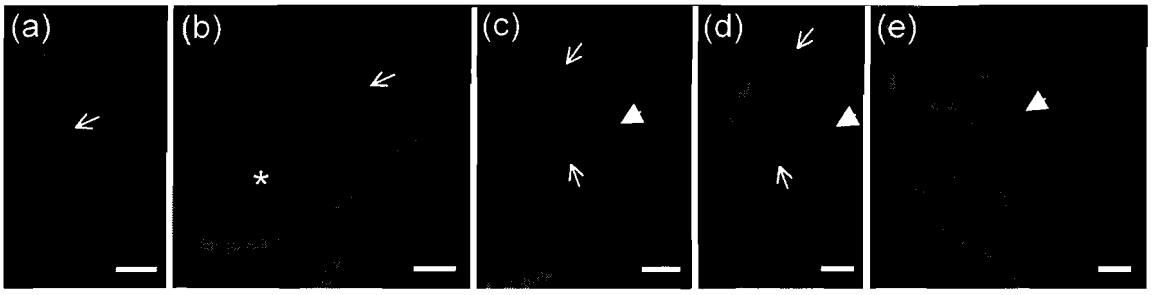


Figure S2c. PIN1-GFP expression in developing primordia of the later formed rosette leaves of *pPIN1::PIN1-GFP* plants. All show images taken through the median section (lower) or adaxial epidermal surface (upper) of the same primordia. PIN1-GFP becomes gradually confined to the basal adaxial and marginal epidermis. Scale bars are 20 μm.

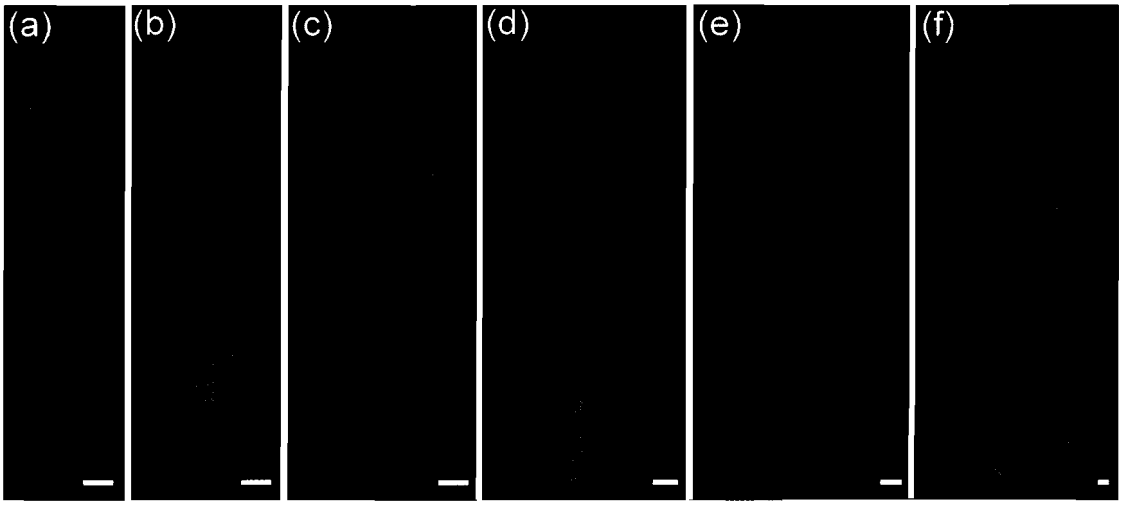


Figure S2d. In a few primordia of later formed rosette leaves of *pPIN1::PIN1-GFP* plants, PIN1-GFP expression remains in the abaxial epidermis up until formation of later secondary veins. Image positions are: median section (left in a, middle in b), abaxial epidermis (right in a,b), adaxial epidermis (left in b). Scale bars are 20 μm.

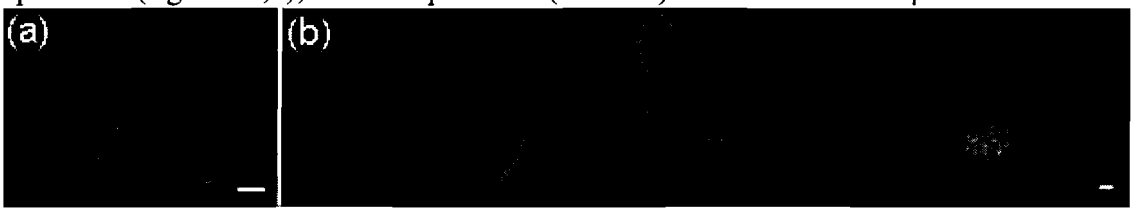


Figure S2e. Three series of images (a,b,c) progressing from the adaxial/marginal epidermal surface (1) towards a more median section (4) of *pPIN1::PIN1-GFP* plants. Arrows indicate the continuum of PIN1-GFP expressing cells from the adaxial/marginal epidermis to the preprocambial cells of a developing secondary vein. Scale bars are 20 μm.

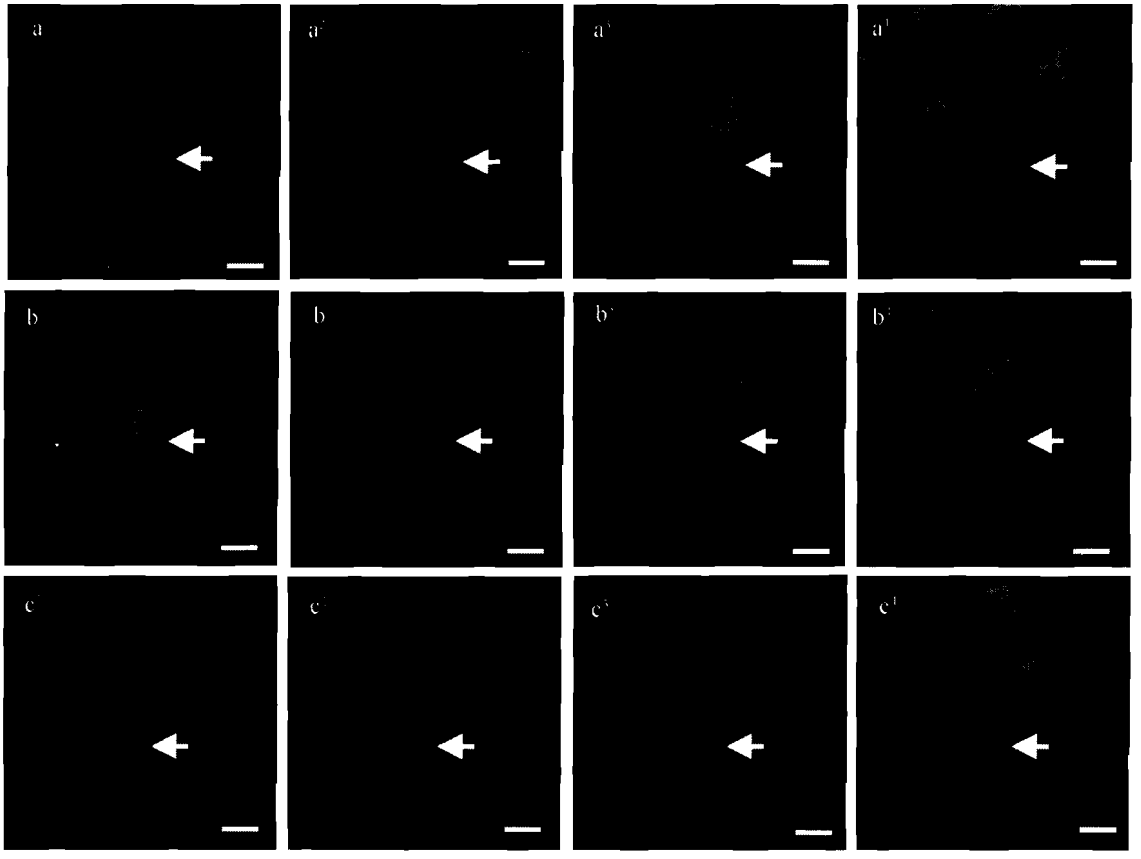


Figure S3. Epidermal PIN1-GFP expression in wild-type *pPIN1::PIN1-GFP* plants treated with 0.1-10 μM NPA (a-d) or in *mp* mutants crossed to *pPIN1::PIN1-GFP* plants (e-i). NPA treatment predominantly inhibits epidermal PIN1-GFP polarity but not epidermal expression pattern in young leaf primordia (a,b) and rarely shows marginal PIN1-GFP polarization since serrations rarely form (c,d). In *mp* plants (e-i), PIN1-GFP shows apical localization in most epidermal cells of young primordia (e) and no polarity in the basal adaxial epidermal margins of older primordia (f,g). In some *mp* mutants, leaf serrations form and these have little or no polarized PIN1-GFP localization in the serration epidermis (h,i). Scale bars are 20 μm.

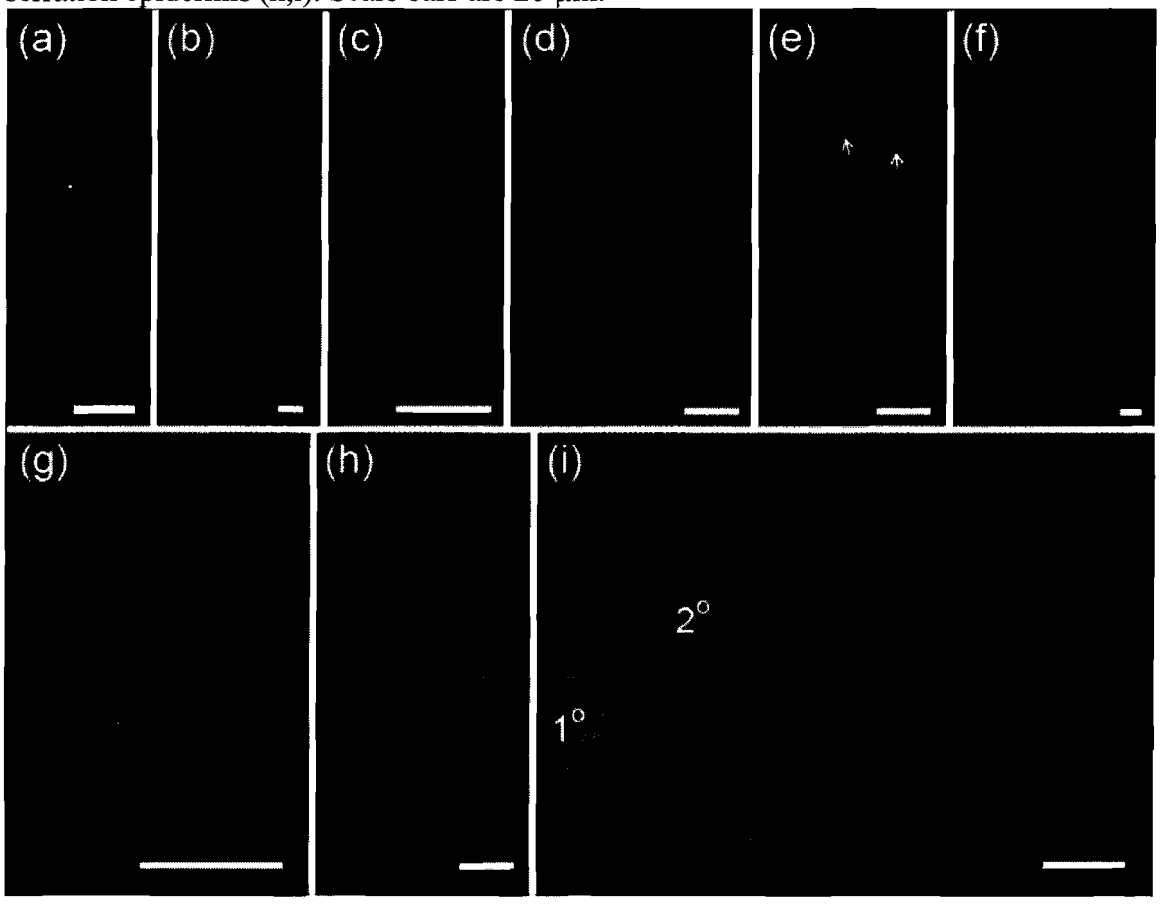


Figure S4. NPA treatment inhibits most PIN1-GFP subcellular polarization in *pPIN1::PIN1-GFP* plants. PIN1-GFP expression is initially spread throughout most internal tissues, eventually becoming more restricted to 2°-like (b) and sometimes 1°-like vascular strands (arrows in a). Scale bars are 20 μm.

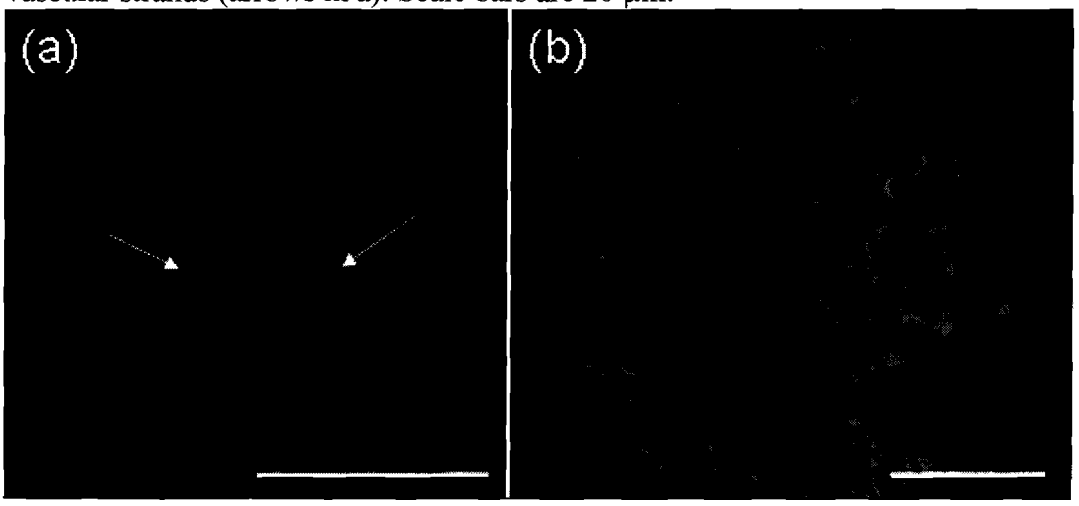


Figure S5. Planar median views of DR5::GUS (a-c), or *in situ* hybridizations of either *MP* (d-f) or *PIN1* (g-i) mRNA transcripts in wild-type plants exposed to 0 (a,d,g), 1 (b,e,h) or 10 (c,f,i) μM 2,4-D for approximately 16h after 4 days growth in liquid ATS. *PIN1 in situs* were done as for *MP*, with the *PIN1* probe made from a cDNA sequence (using primers TTTGTGTGGAGCTCAAGTGC and CTGCGTCGTTTTGTTGCTTA) inserted into pBluescript vector. With increasing 2,4-D concentration, auxin levels increase throughout the lamina (b,c), whereas *MP* (e,f) and *PIN1* (h,i) expression increased predominantly in the basal regions where new veins were forming (arrows). Scale bars are 50 μm.

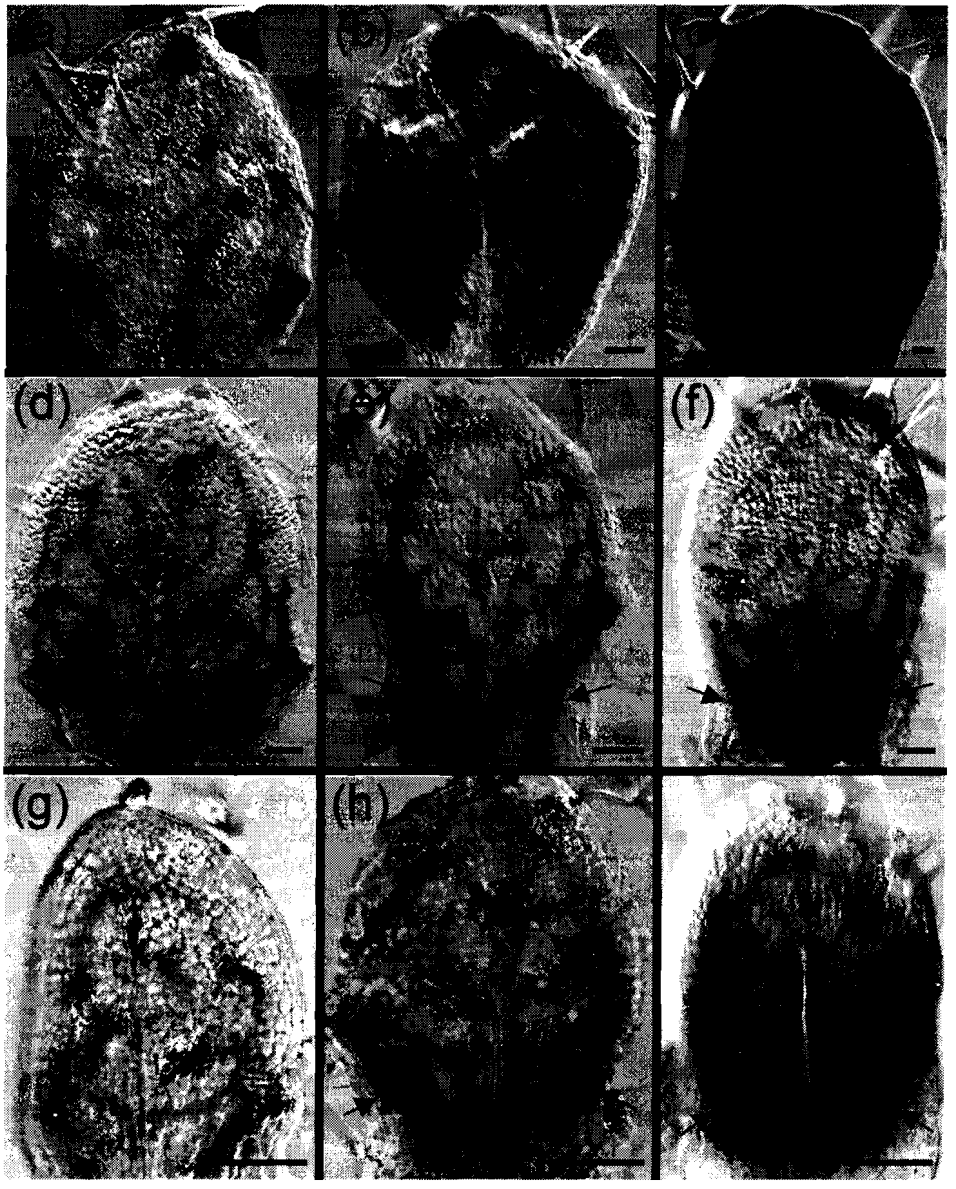


Figure S6. Planar median views of DR5::GUS expression in *mp* (a) and *pin1* (b) mutants. Auxin distribution is apparently marginalized in both mutants, with the appearance of additional apical maxima in *pin1* (arrows in b). Scale bars are 500 μm (a) or 50 μm (b).

Appendix 2

Chapter 3 addtional material. Originally published as supplementary material in Plant & Cell Physiology 2008 March 49(3):457-468, under the title of "Identification of genes expressed in vascular tissues using NPA-induced vascular overgrowth in Arabidopsis".

Supplementary Table SI. Previous literature showing potential gene function.

Gene annotation and putative function		Function[1]	Overlap[2]
Cell wall			
expansin-like B1	At4g17030		1
expansin-like B3 precursor	At2g18660		2,6,7
pectinacetylesterase	At1g57590	A	
peroxidase 64	At5g42180	B	1,2,3,4,6,7
Membrane			
lipid-transfer protein	At2g37870	C	2,3,9
lipid-transfer protein 4	At5g59310		7
lipid-transfer protein 3	At5g59320		7
lipid-transfer protein	At3g53980	C	2,3
RNA/DNA binding proteins			
bHLH transcription factor	At3g56980		2,7
CCAAT-binding transcription factor	At5g06510		7
ATHB-7 transcription factor	At2g46680	D	1,3
histone H1	At2g18050		1,4,9
DNA-binding remorin 2	At4g00670	E	2
polyadenyl-binding protein mediator	At4g14270	F	3
Signaling			
histidine phosphorelay mediator	At3g16360	G	2
protein phosphatase 2C	At1g07430	G	1,2
Defense			
β-1,3-glucanase	At3g57260		2,4
myrosinase-binding protein	At2g39330		1,2,4
myrosinase-binding protein	At1g52040		1,2
myrosinase-binding protein	At1g52000		2
HIN1-related protein	At1g65690		2
Stress			
acid phosphatase 5	At3g17790		1,3
ABA-responsive protein	At3g02480		
cold-regulated protein (COR15)	At2g42540		
Metabolism			
methyltransferase	At5g37970	H	
glycosyl hydrolase family 19	At2g43570		4,5
glucose-6-phosphate translocator 2	At1g61800		
nudix hydrolase 24	At5g19470		2
amino acid aminotransferase	At1g10070		1,2,4,7
alanine-glyoxylate aminotransferase	At3g08860		5,7
N-acetyltransferase	At2g39030		2
ZIP4 metal transporter	At1g10970		1,2,4
vacuolar processing enzyme	At1g62710		1,2
glutathione transferase (TT19)	At5g17220	I	
anthocyanidin glucosyltransferase	At4g14090	I	2
Unknown			
auxin-responsive protein	At3g25290		2,3,8,9
expressed protein	At1g80130		
expressed protein	At1g27030		2,3,4

iPossible Gene Functions:

(A) The pectinacetylesterase (At1g57590) may be involved in cell wall degradation necessary for xylem and fiber elongation.

(B) The predominantly xylem and fiber specific peroxidase (At5g42180) may be involved in lignin formation (as per Boerjan et al. 2003. Ann. Rev. Plant Biol. 54: 519-546).

(C) Two lipid transfer proteins (At2g37870 and At3g53980) with predominantly xylem expression (Table I, Fig. 2) may affect xylem differentiation similar to xylogen, a lipid transfer protein essential for continuous xylem differentiation along a vascular strand (Motose et al. 2004. Nature 429: 873-878).

(D) ATHB7, (At2g46680) is involved in stress-induced inhibition of plant growth (Hjellstrom et al. 2003. Plant Cell Env. 26: 1127–1136) and may also have a role during xylem and fiber differentiation leading to cell death, possibly along with other stress-related and metabolic genes that were up-regulated with ABA treatment (Table I).

(E) A remorin is known to be expressed in tomato vascular tissues (Bariola et al. 2004. Plant Mol. Biol. 55: 579-594), and we have identified a phloem-specific DNA-binding remorin protein (At4g00670) whose function is unknown.

(F) The predominantly phloem-specific polyadenyl-binding protein mediator (At4g14270) may have a role in post-transcriptional gene regulation, similar to other such proteins (Caponigro and Parker. 1995. Genes Dev. 9: 2421-2432).

(G) Two predominantly phloem-specific genes encode signal transduction proteins - a putative two-component phosphorelay mediator (At3g16360) and a protein phosphatase 2C (PP2C, At1g07430). Phosphorelay mediators and protein phosphatases have multiple roles in plant hormone signaling (Hwang et al. 2002. Plant Physiol. 129: 500-515; Rodriguez. 1998. Plant Mol. Biol. 38: 919-927). Some other PP2Cs are known to be expressed in vascular tissues and affect vascular development and stem cell specification (Song and Clark. 2005. Dev. Biol. 285: 272-284).

(H) Vascular continuity is affected by a sterol methyltransferase (Carland et al. 2002. Plant Cell 14: 2045-2058) and a similar role may be established for At5g37970, a methyltransferase with predominantly marginal expression of xylem in leaves (Fig. 2) where auxin and vascular tissues are known to accumulate in auxin transport inhibited leaves (Mattsson et al. 1999. Dev. 126: 2979–2991; Mattsson et al. 2003. Plant Physiol. 131: 1327-1339). Methyltransferases are involved in various activities including methylation of IAA (Zubieta et al. 2003. Plant Cell 15: 1704-1716), gibberellin (Varbanova et al. 2007. Plant Cell 19: 32-45), pectin (Bourlard et al. 1997. Plant Cell Physiol. 38: 259-267), and lignin (Zhong et al. 1998. Plant Cell 10: 2033-2045), all of which could affect cell expansion and wall properties.

(I) Anthocyanidin glucosyltransferase (At4g14090) and glutathione S-transferase (TRANSPARENT TESTA 19, At5g17220) are involved in the biosynthesis and vacuolar sequestration respectively of flavonoids (Kitamura et al. 2004. Plant J. 337: 104-114; Tohge et al., 2005. Plant J. 42: 218-235), which are known to inhibit polar auxin transport (Lazar and Goodman. 2006. P.N.A.S. 103: 472-476).

2Overlap with other genomic studies indicating a possible role in vascular tissues

(1) Ehlting, J., Mattheus, N., Aeschliman, D.S., Li, E., Hamberger, B., Cullis, I.F. Zhuang, J., Kaneda, M., Mansfield, S.D., Samuels, L., Ritland, K., Ellis, B.E., Bohlmann, J. and Douglas, C.J. (2005) Global transcript profiling of primary stems from Arabidopsis thaliana identifies candidate genes for missing links inlignin biosynthesis and transcriptional regulators of fiber differentiation. Plant J. 42: 618-640.

(2) Zhao, C., Craig, J.C., Earl Petzold, H., Dickerman, A.W. and Beers, E.P. (2005) The xylem and phloem transcriptomes from secondary tissues of the Arabidopsis root-hypocotyl. Plant Physiol. 138: 803-818.

(3) Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W. and Benfey, P.N. (2003) A gene expression map of the Arabidopsis root. Science 302: 1956-1960.

(4) Oh, S., Park, S. and Han, K.H. (2003) Transcriptional regulation of secondary growth in Arabidopsis thaliana. J. Exp. Bot. 54: 2709-2722.

(5) Ko, J.-H., Han, K.-H., Park, S. and Yang, J. (2004) Plant body weight-induced secondary growth in Arabidopsis and its transcription phenotype revealed by whole-transcriptome profiling. Plant Physiol. 135: 1069-1083.

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

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

(8) Andersson-Gunnerás, S., Mellerowicz, E.J., Love, J., Segerman, B., Ohmiya, Y., Coutinho, P.M., Nilsson, P., Henrissat, B., Moritz, T. and Sundberg, B. (2006) Biosynthesis of cellulose-enriched tension wood in Populus: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. Plant J. 45: 144-165.

(9) Hertzberg, M., Aspeborg, H., Schrader, J., Andersson, A., Erlandsson, R., Blomqvist, K., Bhalerao, R., Uhlén, M., Teeri, T.T., Lundeberg, J., Sundberg, B., Nilsson, P. and Sandberg, G. (2001) A transcriptional roadmap to wood formation. P.N.A.S. 98: 14732-14737.

(10) ** Ko, J.-H., Beers, E.P. and Han, K.-H. (2006) Global comparative transcriptome analysis identifies gene network regulating secondary xylem development in Arabidopsis thaliana. Mol. Gen. Gen. 276: 517-531.4

(11) ** Brown, D.M., Zeef, L.A.H., Ellis, J., Goodacre, R. and Turner, S.R. (2005) Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. Plant Cell 17: 2281-2295.

(12) ** Persson, S., Wei, H., Milne, J., Page, G.P. and Somerville, C.R. (2005) Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. P.N.A.S. 102: 8633-8638.

** References 10-12 had no overlap with the genes focused on in our study.

Supplemental Table SII. Primers used to generate probes for northern blot hybridization.

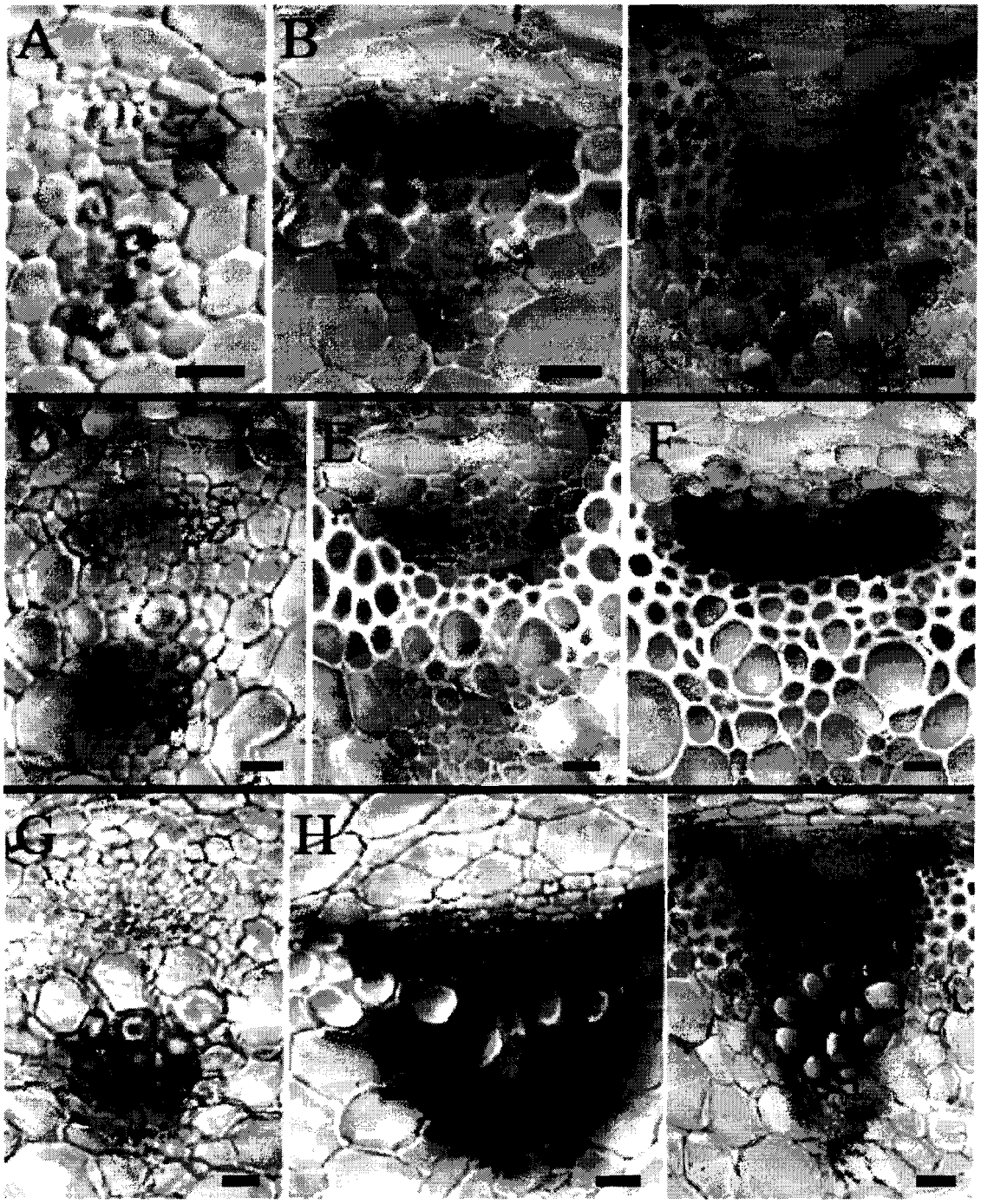
Annotation	Primer 1	Primer 2
At2g18050	aggcagcaggacaagaagaa	acccaaaagagaccacacca
At2g37870	tgcagtggctttgatgagtc	tgtaacgtccacatcgcttg
At2g42540	ttgtggcatccttagcctct	agagtcggccagaaaactca
At3g02480	acaagcaaaacgcgagctac	aatggacgcaaggaaacaac
At3g16360	gcagggatatctcgacgaac	gctcgcctggaaataatgtt
At5g37970	cgttcattggaaacgattga	gttatccccacatgcactcc

Supplemental Table SIII. Primers used for promoter::GUS fusions.

Annotation	Primer 1	Primer 2
At1g07430	cacctcaaaagtaaatgattttgcagactt	aactcgatcgccgcctctactaaatat
At1g10070	caccagaagttttctgcactggaactg	agagcctctgttttaattctcttga
At1g10970	cacctccccatcttacaaagttaccg	aacaagagtttattcttcttcttcctc
At1g27030	caccagaaccagcttgttttgttcc	gagaaatgagccgtttgctt
At1g52000	caccaaccccacctgcatcataaattc	ccgttgaattccgtttgata
At1g52040	caccaatctttattaaaaacttttccccaca	tgaatgcataatactaaagaaaactcg
At1g57590	caccccaagggaaagagacaaagag	ggatgttttagacttgatcacatacc
At1g61800	caccccattgtgtaaacgatgaacatcataa	tatgatctcgaggtgggtttt
At1g62710	caccaacaaatcaatttcttcttctaaattc	ggcagcgagaaacaaaagag
At1g65690	caccagaccacaaattctttgattagaaa	tttggaaaatcttgatgacacc
At1g80130	caccttttaatttaatgtttatgaccgaca	tgatccactcggttaccaaa
At2g18050	caccatgcagaactccattgtat	tgttgctttgcttgtcttgtc
At2g18660	caccggttaaaaatggtcatgtgatgag	ttaacttgtttgtgaaataacgaagc
At2g37870	caccaaaagtgttctgaaacaaa	tccttcttgttttgttttgttga
At2g39030	caccgtgtgattcctcgtcatatttgc	ttctgttacttgtttttgaagatagtg
At2g39330	caccacgaagattgtcaaatttttacgg	ctaaattatccaaatccaaataaagtg
At2g42540	caccccttcggaacaacaacaagagtt	ccagtgagaacagctcctga
At2g43570	caccatttataccgcaccccaaagaa	tgtttttgtgtctgtgatgcag
At2g46680	caccttgcgatatactagttaaccacgac	ctcatcggaatttttcctcaga
At3g02480	caccttcgttgaaaaataaacgagattg	ttctctcgttctggttttgct
At3g08860	caccttgtacaatacaagggttcattgtt	tttcaccgacgttaaaacgttac
At3g16360	caccatatttgttattagaggggag	cgacgtacgaagttatattacgaaaa
At3g17790	caccttgttacaagaaataagatttccttcc	ttgatcaaataagcctcacacaa
At3g25290	caccccaaagtatacggatattgtgttttcg	tgttggatggcttgtagtaacc
At3g53980	caccagatttcacatgggcaggtt	tcttggagagattttaaatgaaccaa
At3g56980	caccccctggtcttcaatcagcacaa	ttgcttactaaggacaagtattgtaga
At3g57260	caccaagactttactgttaacgtgttggt	tttcttgatttttctatgattctgtga
At4g00670	caccgtgaaattactccatagtggggact	gattgagaatgagtttcaggcttt
At4g14090	caccctgaaaaccaaccgaaaagc	tttagaatgttttgaagatgttataagtttt
At4g14270	caccccgaacaacatttttgtgtctctg	tttcacctcaaactcacactgaa
At4g17030	caccctctgcatttcaaacttagg	ttttttcttaacaaatgataggaattga
At5g06510	caccctcaacttaattttaatcgaatgtttg	gcttatgttccaaaagtccaatg
At5g17220	caccaaaatgattaacggatatgtgtttga	cttgtctatcaaactggtttataaatg
At5g19470	caccctgatgattgtaagtaaaacctcactc	tgtggaggagacacaatgct
At5g37970	caccggacacgtgttttatgtttaaaaagtt	tttagttgcttagaagagaggaagaa
At5g42180	cacctattggattagactagtttcgtaatgg	tgagtgaaaagaaatgtatttagaatg
At5g59310	caccatttgtatgaattttggttaagtcatt	tgtgtttgctcttctcttttgg
At5g59320	caccgatgtcttagagagcttgg	tgggagatgatgatgggttt

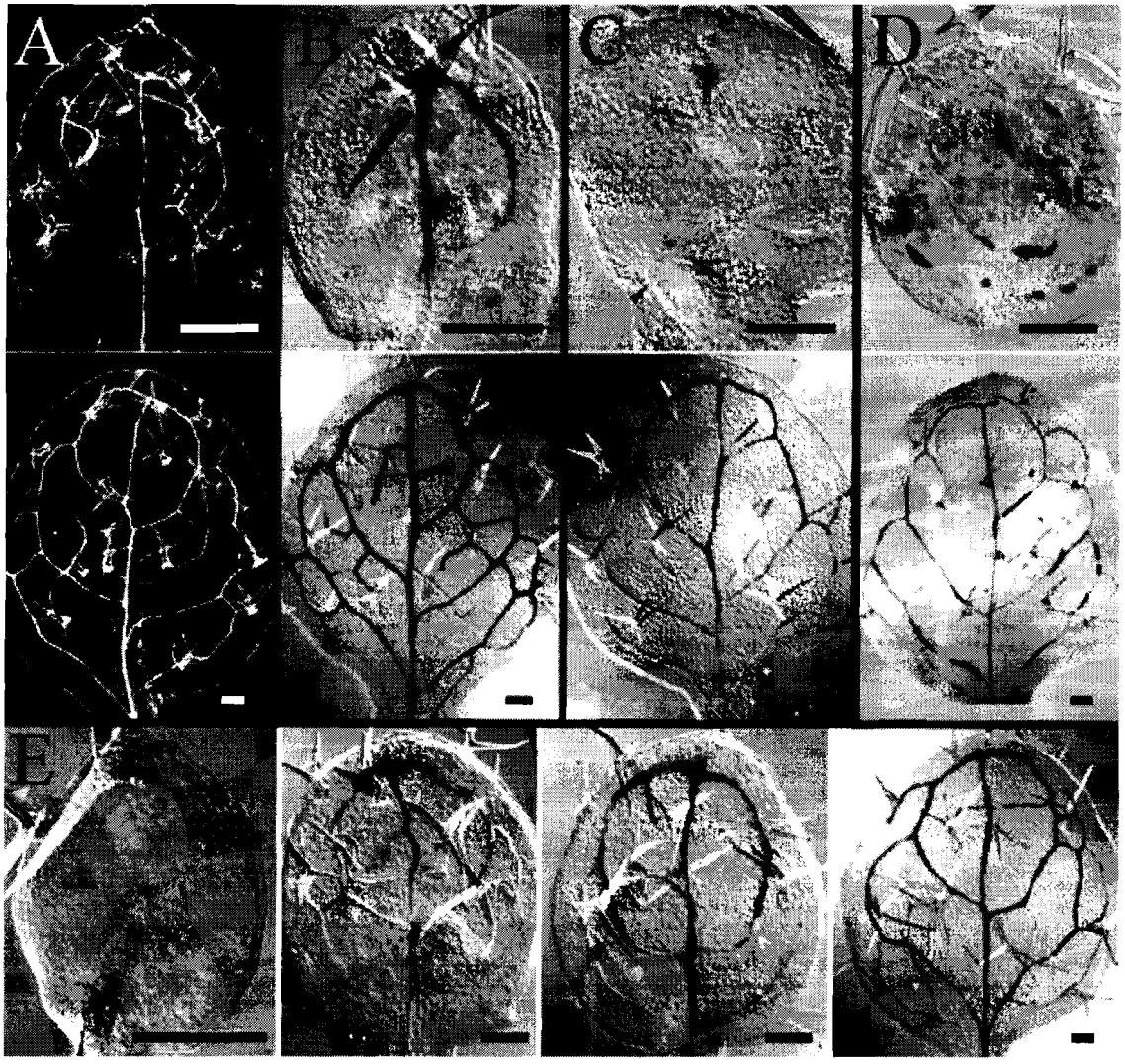
Supplemental Figure S1. Progressive development of expression in inflorescence vascular bundles.

Transverse sections of inflorescence stems showing gene expression in progressively more mature tissues (left to right) predominantly in the cambium (A-C; At2g39330, myrosinase-binding protein), phloem (D-F; At4g00670, DNA-binding protein) and xylem (G-I; At2g37870, lipid-transfer protein). Bars = 10 μm.

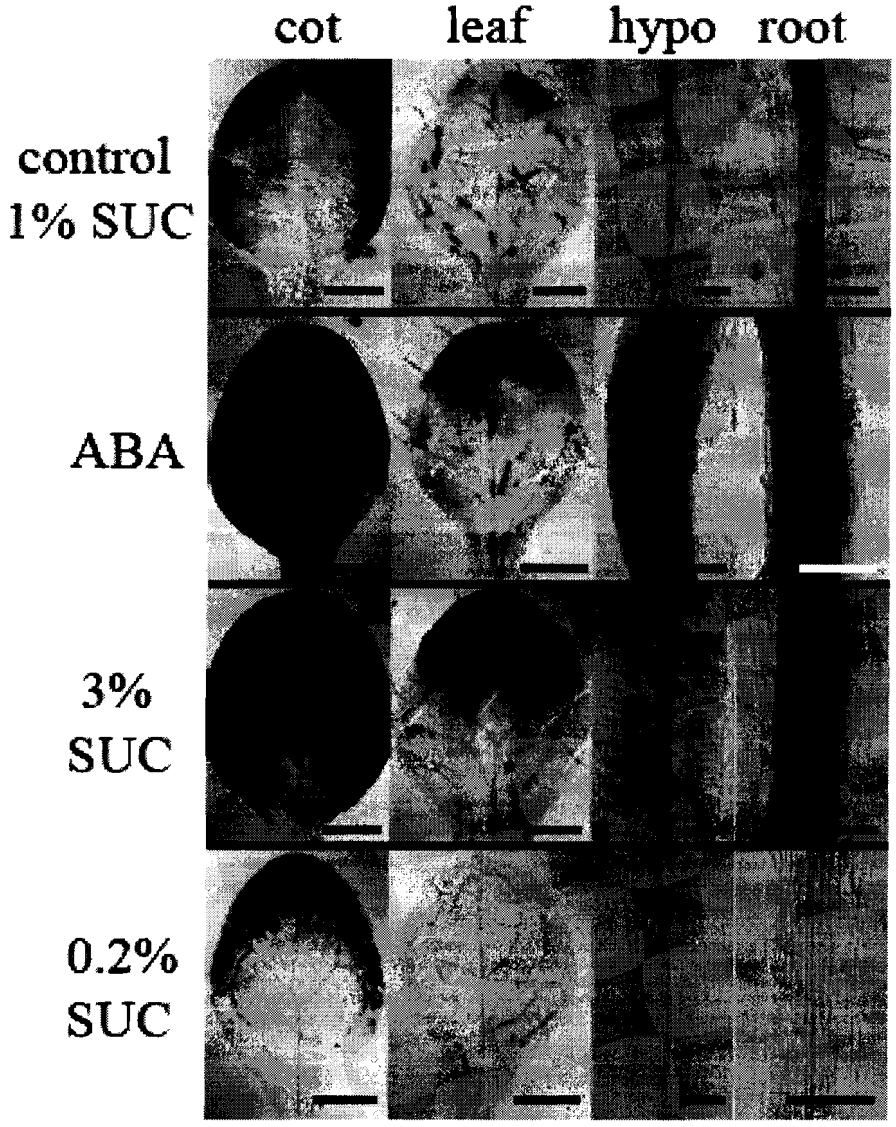


Supplemental Figure S2. The majority of genes were expressed late in leaf development.

For most genes little or no expression was seen in the 5-7 day old first two leaf primordia, with expression progressing basipetally as vein maturation occurred, to be evident in most differentiated veins by 14 days after germination. A-D show darkfield (A) and GUS expression (B-D) in either 7 (top) or 11-14 (bottom) day old leaves for genes: (A) wild type; (B) At4g14090, anthocyanidin glucosyltransferase; (C) At2g37870, lipid transfer protein; (D) At3g17790, acid phosphatase type 5. (E) Progressive basipetal gene expression (left to right) of At2g37870 (lipid transfer protein) in primary, secondary and tertiary veins of 5-9 day old leaves. Bars = 500 μm.



Supplemental Figure S3. ABA and sucrose levels affected At3g17790 gene expression. At3g17790 (acid phosphatase) gene expression increased with exposure to ABA and increasing sucrose concentration. cot = cotyledon, hypo = hypocotyls, SUC = sucrose. Bars = 500 μm (cotyledon and leaf) or 100 μm (hypocotyl and root).



Appendix 3

Chapter 3 unpublished data. Summary of unpublished pictures (specimens prepared by Ms. Qain Hester, pictures taken by Dr. Carol Wenzel) of more than three weeks old rosette leaves and inflorescence GUS expression of promoter::GUS lines that were primarily constructed by Ms. Qian Hester.

Figure S3a. Promoter::GUS lines that are constructed by Ms. Qian Hester and Dr. Wenzel.

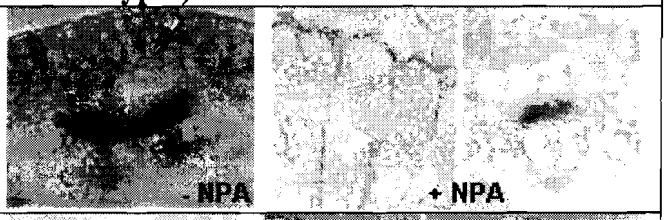
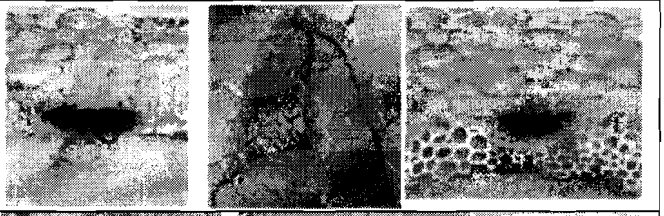
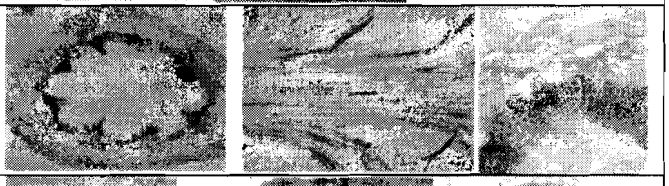
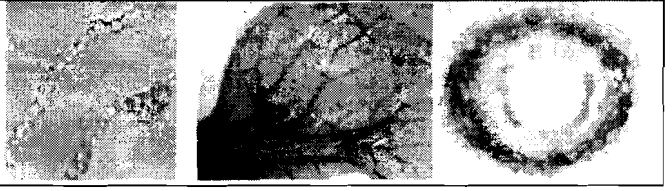
Gene	**Putative function**	**GUS expression (left image(s)-NPA; right two images +NPA, using wide margin to separate the two types)**
At2g39330	Putative myrosinase-binding protein	
At3g56980	bHLH protein	
At4g00670	DNA binding remorin	
At4g17030	Expansin-like protein, *AtEXLB1*	
At1g61800	glucose-6-phosphate/phosphate-translocator precursor, putative	

Figure S3b. Promoter::GUS lines that are constructed by Ms. Qian Hester.

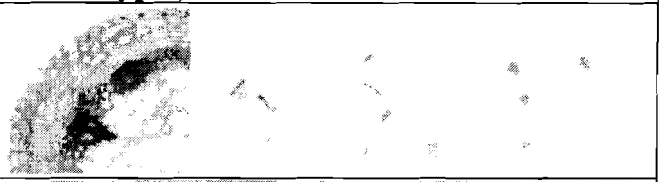
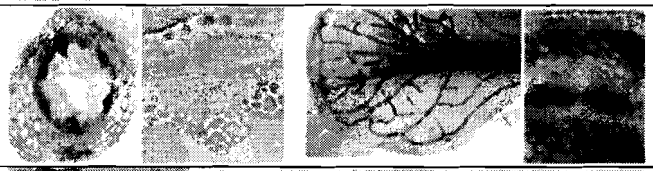
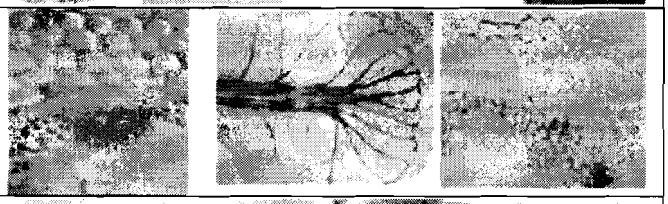
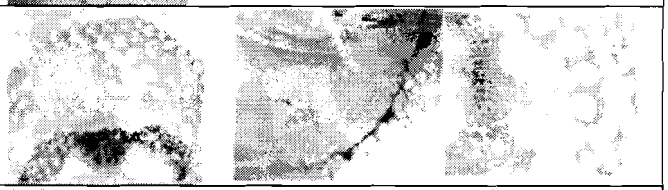
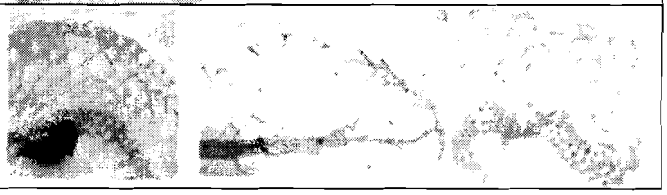
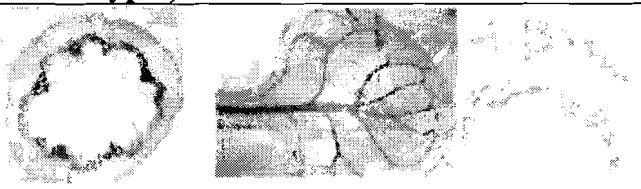
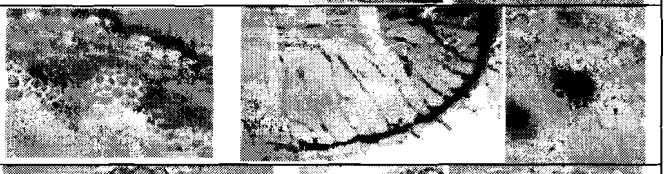
Gene	Putative function	GUS expression (left image(s)-NPA; right two images +NPA, using wide margin to separate the two types)
At3g57260	Glycosyl hydrolase family 17	
At3g08860	Putative aminotransferase	
At5g42180	peroxidase, putative	
At3g44990	xyloglucan endotransglycosylase, putative	
At1g64360	expressed protein	

Figure S3c. Promoter::GUS lines that are constructed by Ms. Qian Hester.

Gene	Putative function	GUS expression (left image(s)-NPA; right two images +NPA, using wide margin to separate the two types)
At2g42540	cold-regulated protein cor15a precursor	
At3g45140	lipoxygenase AtLOX2	
At4g16260	glycosyl hydrolase family 17	
At5g37970	Methyltransferase -related	
At1g80130	expressed protein	

Appendix 4

A gene expression and function analysis of the *Arabidopsis thaliana BASIC-HELIX-LOOP-HELIX 039* (*AtBHLH039*) with no significant mutant phenotype. The experiments followed the same procedure as those used for the analysis of the *AtEXLB1* gene.

Figure S4.1. *AtBHLH039* promoter::GUS gene expression in 7 (a, b) and 14 (c, d) days old rosette leaves. Scale bars are 0.1mm.

(a) – NPA (b) + NPA (c) – NPA (d) +NPA

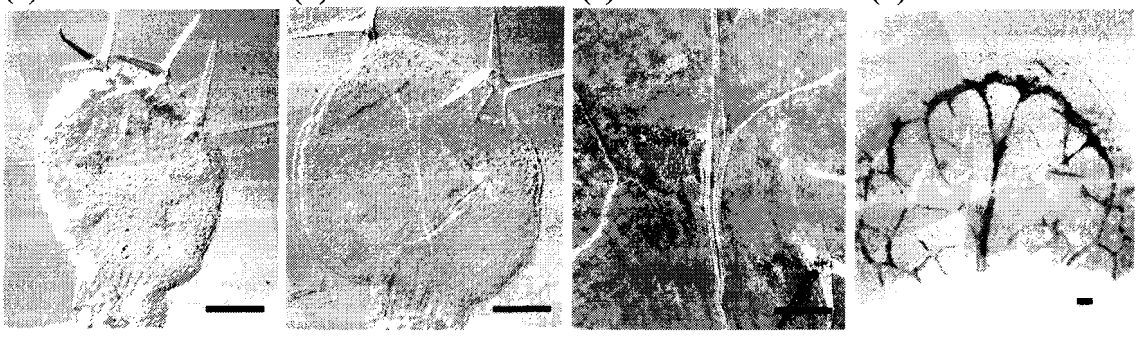


Figure S4.2. *AtBHLH039* promoter::GUS gene expression in 7 (a, b) and 14 (c, d) day old roots (top) and root tips (bottom). Scale bars are 0.1mm.

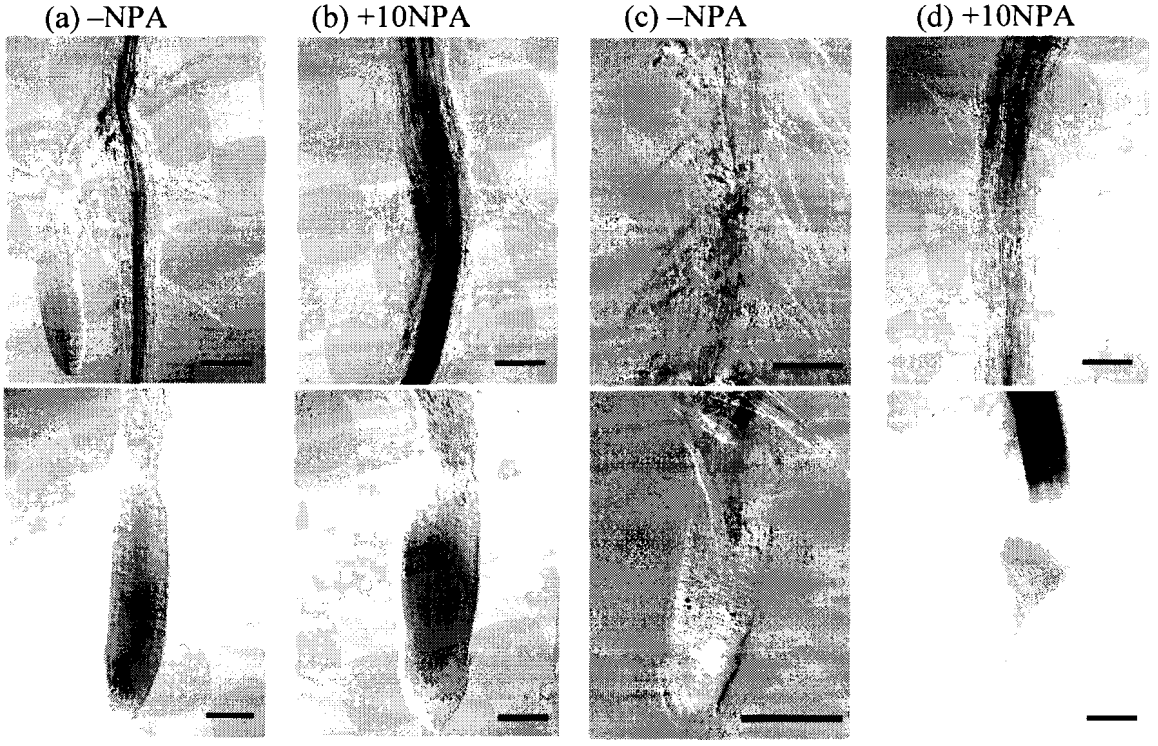


Figure S4.3. *AtBHLH039* promoter::GUS gene expression in more than three weeks old rosette leaves and leaf blade cross sections (b, bottom). Scale bars are 0.1mm.

(a) –NPA (b) +10NPA

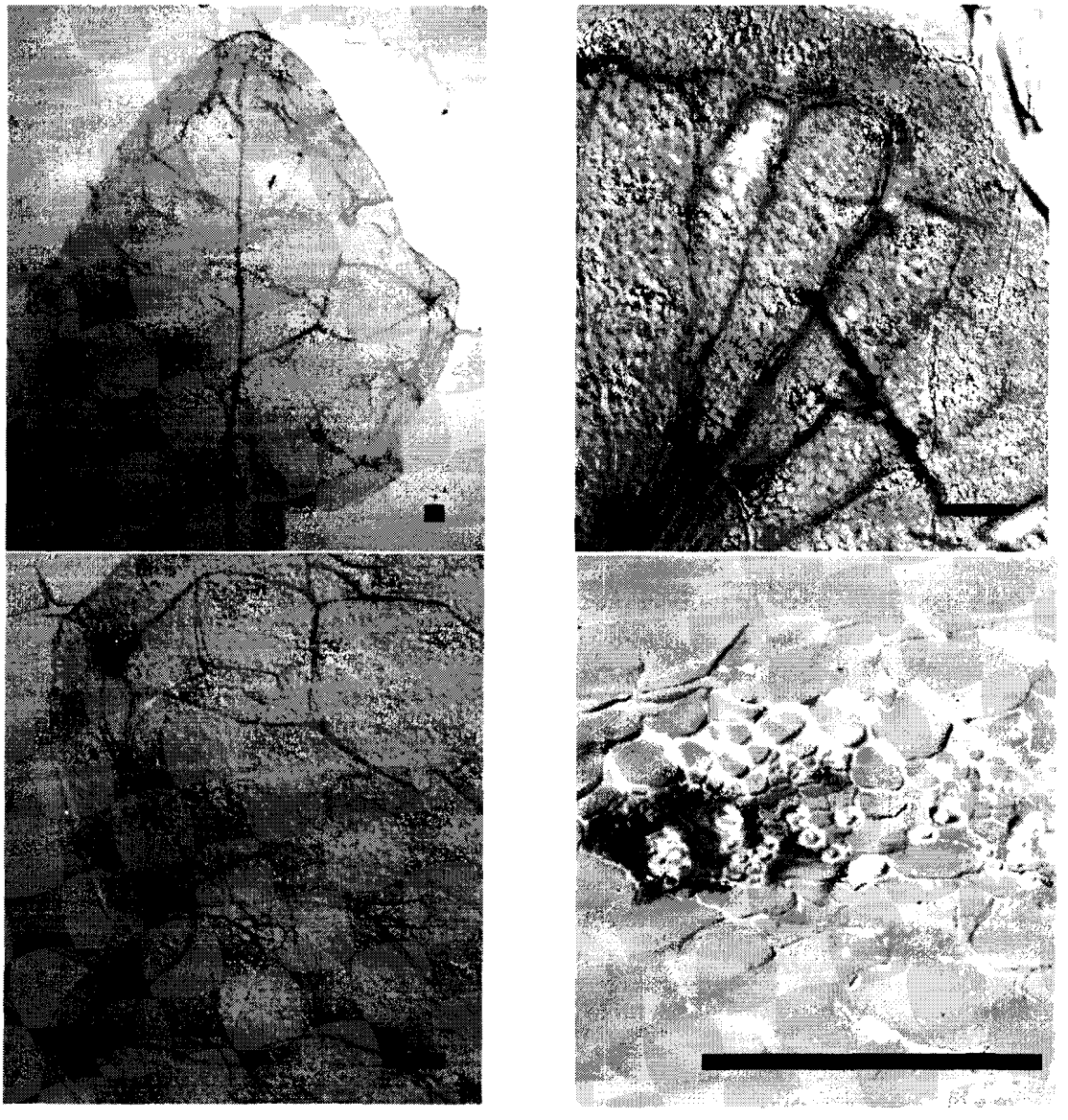


Figure S4.4. *AtBHLH039* promoter::GUS gene expression in inflorescent stems. Scale bars are 0.1mm (top) or 50μm (bottom).

(a) –NPA (b) +10NPA

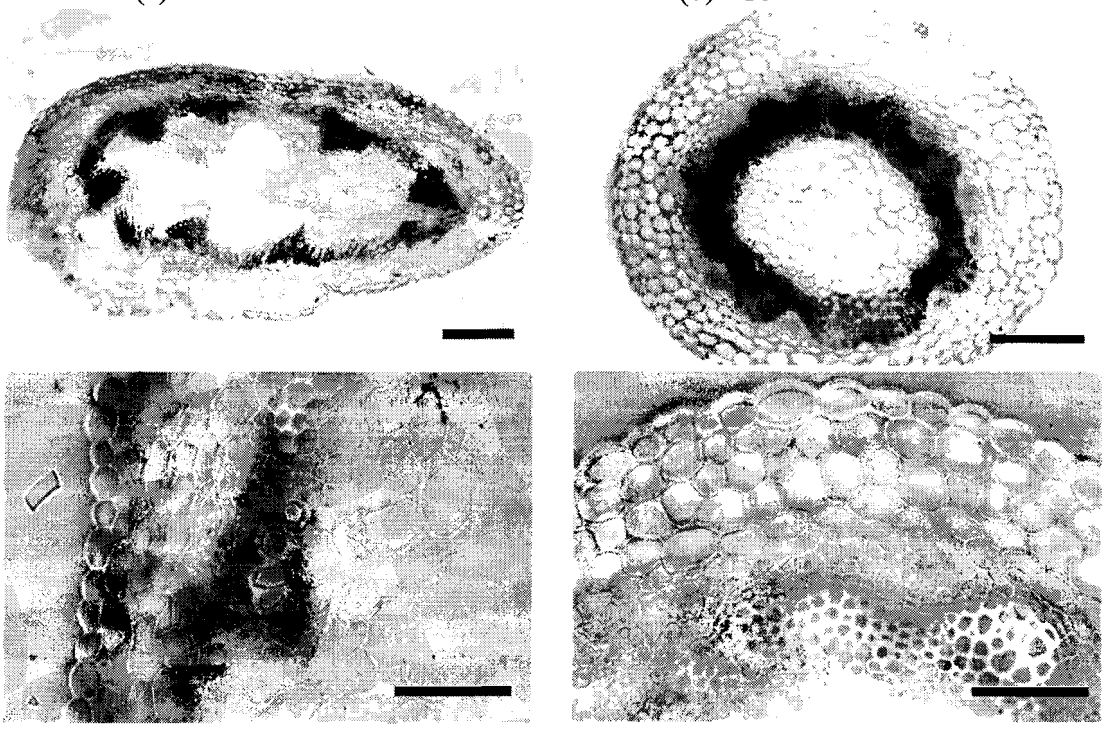


Figure S4.5. Identification of homozygous T-DNA insertion mutants of *AtBHLH039* in Salk_025676.
Lane 1 is Invitrogen 100bp ladder plus; lane 2 is λHindIII ladders; lane 3 and 4 are PCR results of two plants from the homozygous line *bhlh039-5*; lane 5 is PCR result of a plant from an *AtBHLH039* heterozygous T-DNA insertion line.

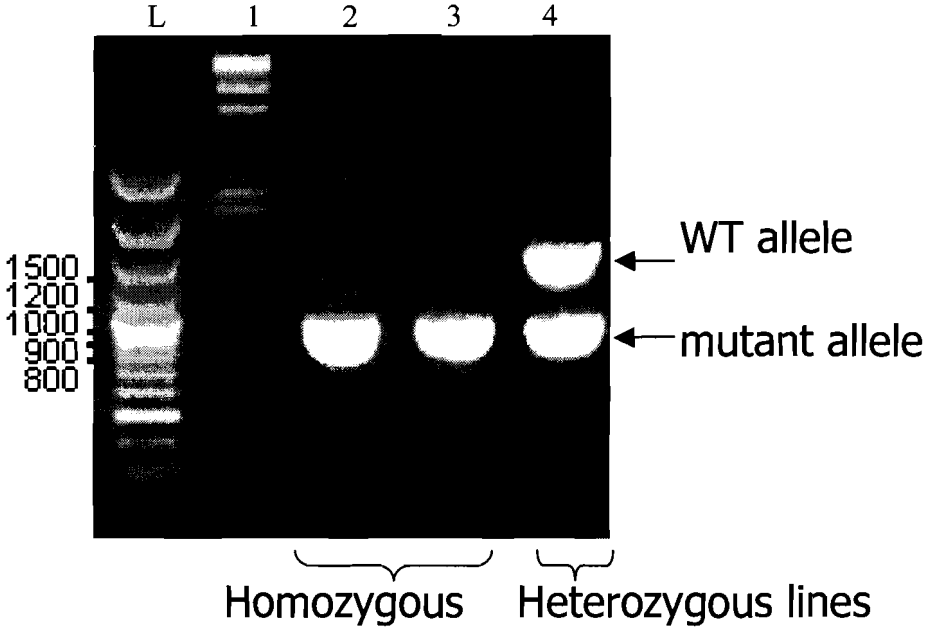


Figure S4.6. Vascular root cells in wild type and *bhlh039-5* T-DNA insertion mutant at 6 DAG under Confocol Laser Scanning Microscopy. The arrow points out the end wall of a metaxylem vessel element. Scale bars are 10μm.

(a) wild type (b) *bhlh039-5*

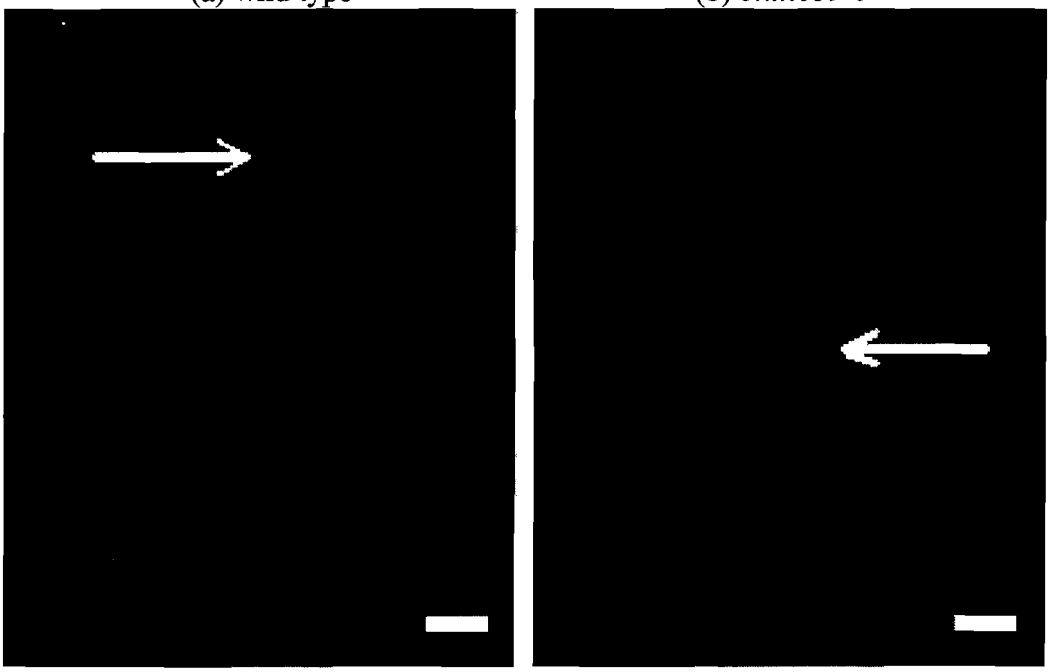


Figure S4.7. Plant length measurements of salk line mutants *bhlh039-5* and wild types. Root length measurements of 18 plants at 4 DAG (a) and at 8 DAG (b). Hypocotyl length measurements of 18 plants at 7 DAG (c). Cotyledon blade width measurements of 18 plants at 12 DAG (d). Error bars are the standard error in the sample set (see materials and methods in Chapter 4 for statistical equations).
Column 1: mean of wild type as control (100%).
Column 2: mean of *bhlh039-5* mutants as a percentage of the control.