THE ROLES OF AUXIN SIGNALING AND TRANSPORT IN LEAF INITIATION AND VASCULAR PATTERN FORMATION IN ARABIDOPSIS THALIANA.

by

Mathias M. Schuetz
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Name: Mathias M. Schuetz
Degree: Doctor of Philosophy
Title of Thesis: Roles of Auxin Signaling and Transport in Leaf Initiation and Vascular Pattern Formation in Arabidopsis thaliana.
Examining Committee:
Chair: Dr. Leah Bendell-Young
Professor, Simon Fraser University

Dr. Jim Mattsson, Senior Supervisor
Associate Professor, Simon Fraser University

Dr. Carl Lowenberger, Supervisor
Associate Professor, Simon Fraser University

Dr. Aine Plant, Supervisor
Associate Professor, Simon Fraser University

Dr. Sherryl Bisgrove, Internal Examiner
Assistant Professor, University of British Columbia

Dr. Geoffrey Wasteneys, External Examiner
Professor, University of British Columbia

Date Defended/Approved: June 26, 2009
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ABSTRACT

The plant hormone auxin has been implicated in many diverse processes in plant development including the formation of leaves from the shoot apical meristem and subsequent leaf vascular tissue patterning. Both processes of leaf initiation and leaf vascular patterning share many commonalities including: 1. They appear to be formed by a gradual limitation process that may be synonymous with canalization of auxin transport. 2. Genetic evidence from Arabidopsis thaliana, links both processes to the MONOPTEROS (MP) gene, involved in auxin signaling and the PIN-FORMED1 (PIN1) gene, involved in auxin transport.

To elucidate the potential functions of MP and PIN1 in vascular patterning, we assessed detailed MP and PIN1 expression dynamics during vascular tissue formation in A. thaliana leaf primordia. Taken together, the results presented provide novel molecular support for the canalization of auxin flow hypothesis. Since PIN1 expression appears to be regulated by MP function, it seemed possible that MP affects leaf formation exclusively as a positive regulator of auxin transport. To explore this notion further, we analyze a novel, completely leafless phenotype in mp pin1 double mutants. Subsequent analysis indicates multiple MP dependent pathways are involved in leaf initiation. Shoot meristem function, including central stem cell zone maintenance is severely disrupted in mp pin1 double mutants. Mutant analysis of characterized genes involved in central zone maintenance in mp mutant backgrounds indicate that MP functions as a negative regulator of central zone size in parallel to established mechanisms. While it is clear that MP is an important player in both processes of leaf initiation and vascular patterning, loss of function mutations in MP have a relatively limited phenotypic impact. By analyzing the temporal and spatial expression profiles of genes closely related to MP we identified the ARF3 and ARF7 genes, as candidates for putative overlapping function with MP. Subsequent mutant
analysis demonstrate that ARF3 and ARF7 play novel functional roles in leaf initiation in *mp* loss of function mutants.

The results presented in this thesis contribute to our general understanding of auxin mediated developmental processes through analysis of leaf initiation and vascular pattern formation.

**Keywords:** *Arabidopsis thaliana*, Auxin, *MONOPTEROS (MP)*, Auxin Response Factor (ARF), *PINFORMED1 (PIN1)*, Vascular tissue, Shoot Apical Meristem (SAM), whole mount in situ.
DEDICATION

To my childhood paradise Klais and my aunt Ursula

"Live as if you were to die tomorrow. Learn as if you were to live forever."  Mahatma Gandhi
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1: INTRODUCTION

1.1 Auxin Signaling

Chemical messengers were hypothesized to be regulators of plant growth and development far before any substances were isolated and identified as phytohormones (Taiz and Zeiger, 2002). Charles Darwin described a directional transport of a mobile substance necessary for grass coleoptile phototropism which was moving from the coleoptile tip toward the base in 1880. 55 years later this substance was identified as the auxin IAA (Indole-3-acetic acid), a small readily diffusible organic molecule (Taiz and Zeiger, 2002). The term auxin includes other structurally different substances, which elicit a variety of growth responses. Synthetic auxins such as 2,4-Dichlorophenoxyacetic acid (2,4-D) elicit unsustainable growth in high concentrations and have been commercially exploited for use as herbicides (Taiz and Zeiger, 2002). The most abundant naturally occurring auxin in plants is IAA, which regulates auxin-dependent processes including leaf/flower initiation, shoot apical dominance, lateral root formation, and fruit development (Srivastava, 2002; Taiz and Zeiger, 2002). Auxin accumulation in specific groups of cells in the developing plant embryo leads to generative processes resulting in the generation of the embryo proper at the two cell stage, the root apical meristem at the globular stage and cotyledons
at the triangular stage of embryogenesis. Auxin is also a potent regulator of plant cell elongation (Taiz and Zeiger, 2002). High auxin concentrations in plant root cells retards cell elongation whereas it stimulates cell elongation and growth in shoot tissues. Oriented plant growth in response to environmental signals like light (phototropism) or gravity (gravitropism), are based on differential auxin accumulation within shoot and root tissues. For example, in phototropic responses, auxin accumulates on the shaded side of the shoot stem in response to a unilateral light source. The resulting local increase in cell elongation and growth results in differential stem elongation leading to the formation of a curve toward the light source. Gravitropic responses of roots are similar to phototropic responses in the shoot except auxin accumulation on the bottom side of a horizontally orientated root result in retardation of cell elongation. This leads to differential growth between the bottom and top portions of a horizontal orientated root leading to the formation of a downward bend. Formation of plant vascular systems composed of xylem and phloem tissues are dependent on auxin signaling and transport. The process of vascular tissue regeneration around severed vascular strands is auxin-dependent and has recently been correlated with basipetal auxin transport (Jacobs, 1952; Sauer et al., 2006). Identification of genes disrupted in mutants displaying defects in vascular tissue formation or patterning in Arabidopsis thaliana support a role of auxin biosynthesis, signaling or transport in vascular tissue formation (Turner, 2002; Fukuda, 2004; Cheng et al., 2006).
Various genetic screens in *A. thaliana* have identified many of the components of an auxin signal transduction pathway. Auxin signaling appears to be dependent on ubiquitin-proteosome regulation of protein degradation (Mockaitis and Estelle, 2008). The process of using the protein ubiquitin (Ub) to selectively target other proteins for degradation is mediated by three enzyme complexes called E1 (Ub-activating), E2 (Ub-conjugating) and E3 (Ub-ligase) in both plants and animals. (Fig. 1-1) (Hershko and Ciechanover, 1998; Thrower et al., 2000). Several mutants, which display reduced auxin responses and defects in auxin mediated developmental processes in *A. thaliana* encode components of the ubiquitin dependent protein degradation system. This points to protein degradation as a crucial component of auxin signaling. The SCF (Skp1-Cul1-Fbox) E3 ligase complex is the point of substrate specificity and this type of E3 ligase represents the most abundant class of E3’s in plants (Fig. 1-1) (Moon et al., 2004; Smalle and Vierstra, 2004; Mockaitis and Estelle, 2008). A crucial component of the SFC E3 ligase complex is the CUL1 scaffolding protein (Hellmann et al., 2003). Loss of function cul1 mutants display severe embryogenic phenotypes and germinate without a hypocotyl or root and display defects in cotyledon formation (Hobbie et al., 2000; Hellmann et al., 2003). Further mutant screens in *A. thaliana* identified additional components involved in auxin signaling including proteins putatively targeted for degradation by the SCF E3 ligase. The majority of auxin signaling mutants isolated fall into the Auxin Response Factor (ARF) and AUX/IAA gene families. Subsequent analysis of
these genes has lead to significant advances in the understanding of auxin signal transduction.

The MONOPTEROS (MP) gene was isolated in a screen for mutants displaying defects in embryogenesis and was found to encode one member of a family of 23 transcription factors termed Auxin Response Factors (Berleth and Jurgens, 1993; Ulmasov et al., 1997; Guilfoyle et al., 1998; Hardtke and Berleth, 1998). mp loss of function mutants display similar phenotypes as cul1 mutants and highlight a similar resistance to auxin application (Przemeck et al., 1996; Hobbie et al., 2000; Mattsson et al., 2003). Only four ARF genes have documented single mutant phenotypes, of which mp phenotypes are the most severe, leading to the notion that there might be a high degree of functional redundancy among ARF genes (Guilfoyle and Hagen, 2007). ARF proteins generally share an N-terminal to C-terminal protein motif organization. An N-terminal B3 DNA binding motif is followed by a middle region functioning as a transcriptional activator or repressor domain which is subsequently followed by two C-terminal protein dimerization motifs (Fig. 1-1) (Ulmasov et al., 1997; Guilfoyle et al., 1998; Tiwari et al., 2003). All ARF genes have DNA binding, activation / repression, and dimerization domains except for ETTIN/ARF3, 13 and 17 which lack the C-terminal dimerization domain and ARF23 which has a stop codon near the middle of the DNA binding domain and thus may represent a pseudogene (Okushima et al., 2005; Guilfoyle and Hagen, 2007). ARF proteins specifically bind to TGTCTC DNA sequences called auxin response elements (AuxREs). Promoters of primary auxin response genes are enriched in AuxREs.
and thereby implicate ARF proteins as key regulators of auxin responsive gene transcription (Ulmasov et al., 1999a; 1999b; Guilfoyle and Hagen, 2007). \textit{ARF5 - ARF8} and \textit{ARF19} contain transcriptional activation domains which are enriched with glutamine and serine amino acids whereas the remaining ARFs contain transcriptional repression domains which are enriched with serine, proline, and glycine amino acids (Guilfoyle and Hagen, 2007). ARF transcriptional activator versus repressor function was determined by assaying ARF transcriptional activity in plant protoplasts transfected with yeast GAL4 DNA binding domains fused to ARF middle regions and interaction with constitutive or minimal promoter:reporter genes containing corresponding GAL4 DNA binding motifs (Ulmasov et al., 1999a; 1999b; Tiwari et al., 2003; Hardtke et al., 2004; Wang et al., 2005; Wilmoth et al., 2005). The results from these experiments using plant protoplasts systems have largely been supported by later studies that examined ARF function in more specific developmental contexts (Okushima et al., 2005; Weijers et al., 2005; Wilmoth et al., 2005). The ARF C-terminal dimerization domain has been shown to facilitate ARF homo and heterodimer formation but importantly also facilitates the binding of members of the AUX/IAA gene family of transcriptional inhibitor proteins (Fig. 1-1) (Kim et al., 1997; Ulmasov et al., 1997; Ulmasov et al., 1999a; 1999b). Analysis of truncated ARF proteins missing the C-terminal dimerization motif established that these domains are not required for ARF DNA binding or transcriptional activation / repression activity since truncated ARFs are still able to bind auxin response elements and function to activate or repress gene transcription (Tiwari et al., 2003; Wang et al., 2005).
A key regulator in ARF function is the binding of AUX/IAA repressor proteins to ARF proteins via a homologous C-terminal dimerization domain. Only a subset of ARF proteins interact significantly with each other by forming ARF homo or heterodimers in yeast two hybrid experiments leading to the conclusion that ARF interactions are highly selective (Ulmasov et al., 1997; Hardtke et al., 2004). In contrast, ARF AUX/IAA protein interactions are not selective and it appears that any AUX/IAA protein can bind to any ARF proteins sharing a C-terminal dimerization motif as long as they are co-expressed (Kim et al., 1997; Tiwari et al., 2004; Weijers et al., 2005). Therefore ARF proteins that lack the C-terminal dimerization motif (i.e. ETTIN/ARF3, 13 and 17) are unlikely to be regulated by AUX/IAA protein binding. Interestingly, ETTIN/ARF3 is one of the four ARF genes that has an obvious loss of function phenotype thereby indicating a non-redundant functional role of ETTIN/ARF3 mediated gene repression (Sessions et al., 1997). The majority of AUX/IAA genes share a common organization and 24 canonical proteins are found in the A. thaliana genome (Liscum and Reed, 2002; Leyser, 2006). The N-terminal domain 1 acts as a transcriptional repression domain, domain 2 facilitates binding of ubiquitin and hence determines protein stability, while the two most C-terminal domains facilitate AUX/IAA homo and heterodimer as well as ARF AUX/IAA heterodimer formation (Fig. 1-1) (Ulmasov et al., 1997; Tiwari et al., 2004). Several AUX/IAA mutants have been isolated in A. thaliana all of which turned out to be dominant or semi dominant gain of function mutations in a highly conserved sequence in domain 2 referred to as a degron (reviewed in (Mockaitis and Estelle, 2008)).
When the reporter protein luciferase is fused to domain 2 from AUX/IAA proteins, the chimeric protein is degraded in response to auxin in plant systems, thereby illustrating the significance of this domain in the regulation of AUX/IAA protein stability (Tiwari et al., 2001). The BODENLOS (BDL) gene was identified based on phenotypes strikingly similar to those observed for cul1 and mp mutants and was shown to be a dominant mutation in domain 2 of the AUX/IAA12 gene (Hamann et al., 1999; Hamann et al., 2002; Liscum and Reed, 2002). Ten other dominant AUX/IAA mutants were isolated and all mutations were mapped to the GWPPV sequence contained in domain 2 (reviewed in Mockaitis and Estelle (2008)). All of the dominant AUX/IAA mutants displayed various auxin related phenotypes including defects in auxin mediated gene activation, thereby demonstrating a functional role in auxin signal transduction. Although loss of function mutants of AUX/IAA genes have been isolated in reverse genetic studies, no phenotypes have been reported and thus AUX/IAA genes appear to function redundantly with each other. This notion is supported by the observation that various AUX/IAA proteins bind promiscuously to any ARF proteins containing C-terminal dimerization domains (Kim et al., 1997; Weijers et al., 2005). 

With the identification of TIR1, an F-box protein part of the SCF E3 ligase complex as an auxin receptor, the mechanism of how auxin promotes the degradation of AUX/IAA proteins became clear (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). TIR1 directly binds the naturally occurring auxin IAA and also synthetic auxins including NAA (1-naphthaleneacetic acid) and 2,4-D
(2,4-dichlorophenoxyacetic acid) all of which have been observed to function as elicitors of AUX/IAA protein degradation (Gray et al., 2001; Tiwari et al., 2001; Zenser et al., 2001; Tian et al., 2003; Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). The auxin binding site of the TIR1 protein is the same site used for TIR1 interaction with AUX/IAA proteins leading to their subsequent ubiquitination and degradation (Fig. 1-1) (Tan et al., 2007). Auxin does not cause a conformational change in the TIR1 auxin receptor, but acts as ‘molecular glue’ by binding to a hydrophobic cavity on the surface of the TIR1 protein thereby facilitating AUX/IAA binding (Tan et al., 2007). These results were obtained from a series of crystallographic studies that also highlighted the significance of the highly conserved AUX/IAA domain 2 GWPPV motif which represents the hallmark of the domain 2 degron (Tan et al., 2007). This amino acid motif is crucial and sufficient for the interaction of AUX/IAA proteins with the auxin bound TIR1 protein (Tan et al., 2007). TIR1 functions as one of a small family of auxin receptors, which have been named auxin signaling F-box proteins 1 through 5 (AFB1-5) (Dharmasiri et al., 2005b; Mockaitis and Estelle, 2008). Single mutants of TIR1 or any of the other AFB proteins do not result in severe phenotypes suggesting functional redundancy. Severe developmental phenotypes similar to what is observed in mp, cul1 and bdl loss of function mutants are observed in tir1 afb2 afb3 triple mutants (Dharmasiri et al., 2005b). These phenotypes together with biochemical evidence illustrating reduced IAA binding in tir1 afb mutant plant extracts points to a family of auxin receptors in plants (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b). The resulting auxin signal transduction model
includes TIR1 auxin perception, which mediates the ubiquitination of AUX/IAA repressor proteins (Fig. 1-1). The subsequent degradation of AUX/IAA proteins bound to ARF transcription factors results in the de-repression of auxin responsive gene transcription by facilitating ARF homo and heterodimer formation (Fig. 1-1). Additional components to this model are continually being identified and will be added over time. Recent findings implicate the TOPLESS (TPL) protein as an inhibitor of ARF dependent gene transcription (Szemenyei et al., 2008). The TPL protein functions as a transcriptional repressor and binds to domain 1 of the AUX/IAA protein BDL/IAA12, a protein known to interact with the ARF transcription factor MP. This observation leads to the possibility that BDL/IAA12 carries out its function in repressing MP transcriptional activity by facilitating TPL-MP interaction, a statement that is supported by the observation that tpl loss of function results in partial rescue of the dominant bdl mutant phenotype in tpl bdl double mutants (Mockaitis and Estelle, 2008; Szemenyei et al., 2008). A growing body of evidence suggests that post-transcriptional regulation of various ARF transcripts is mediated by various small silencing RNAs (reviewed in Guilfoyle and Hagen (2007); Ghildiyal and Zamore (2009)). Mutations that interfere with small silencing RNA production or binding to ARF mRNA’s result in various developmental defects ranging from defective root cap or flower formation, to defective leaf vascular patterning (reviewed in Guilfoyle and Hagen (2007)). Interestingly, two of the three ARF genes, ARF3 and ARF17, which lack the C-terminal dimerization motifs and are therefore insensitive to known AUX/IAA mediated regulatory mechanisms, are developmentally
regulated by small interfering RNA’s (Mallory et al., 2005; Sorin et al., 2005; Adenot et al., 2006; Garcia et al., 2006). These studies emphasize the importance of incorporating other genes and factors, which manipulate components of auxin signaling into the existing TIR1-AUX/IAA-ARF framework.

A TIR1 independent mechanism of auxin perception also appears to exist in plants, which involves the AUXIN BINDING PROTEIN-1 (ABP1) gene. Auxin has been demonstrated to bind to ABP1 protein isolated from various plant species including Zea mays, Nicotiana tabacum, Oryza sativa and Arabidopsis thaliana and acts to promote auxin-dependent processes such as cell expansion (Ray et al., 1977; Shimomura et al., 1986; Jones and Venis, 1989; Brown and Jones, 1994; Jones et al., 1998; Kim et al., 1998; Chen et al., 2001; Braun et al., 2008). ABP1 expression levels differ depending on tissue type and at the subcellular level it is primarily localized to the endoplasmic reticulum (ER) (Inohara et al., 1989; Jones and Venis, 1989). The discrepancy between the documented ER localization of ABP1 and experimental evidence demonstrating that ABP1-mediated auxin perception and action occurs at the plasma membrane became clear with detailed analysis of subcellular ABP1 localization using electron microscopic immunocytochemistry (Jones and Herman, 1993). Jones and Herman confirmed that ABP1 is primarily localized to the ER but is also present at the plasma membrane and within the cell wall. Based on the ABP1 protein localization in the Golgi, ABP1 is thought to be transported to the cell periphery through the cells secretory system (Jones and Herman, 1993). Homozygous abp1 loss of function in A. thaliana result in embryo lethality
demonstrating that ABP1 is essential for plant development (Chen et al., 2001). The lethality of abp1 mutant embryos hampered further studies of the role of ABP1 in post-embryonic developmental processes until an inducible system of ABP1 silencing was developed in A. thaliana (Braun et al., 2008). Braun and colleagues demonstrated that ABP1 is required for progression of the cell cycle, cell expansion, cell plate formation and expression of members of the early auxin response AUX/IAA gene family during post-embryonic growth and development (David et al., 2007; Braun et al., 2008). The observation that ABP1 function influences the expression of several members of AUX/IAA genes provides a convergence point for TIR1 mediated and ABP1 mediated auxin signaling.

1.2 Polar Auxin Transport
Sites of auxin production are not necessarily correlated with auxin mediated physiological and developmental processes. This leads to a disconnect between auxin synthesis and sites of auxin perception and action. Auxin transport therefore becomes an important aspect of auxin signaling. Auxin is primarily produced in young shoot organs and is transported basipetally toward the root in a process termed polar auxin transport (PAT). The chemiosmotic hypothesis describes how the auxin IAA becomes preferentially retained inside plant cells due to differences in pH between the cytoplasm and cell wall (Fig. 1-2) (Rubery and Sheldrake, 1973; Goldsmith et al., 1981; Taiz and Zeiger, 2002). The differences in pH is mainly achieved by action of ATPase proton pumps which transport H\(^+\) ions out of the cytoplasm and into the surrounding apoplast (Taiz and Zeiger, 2002). In the acidic cell wall environment, IAA\(^-\) becomes protonated
and this uncharged IAAH can diffuse through the hydrophobic centre of the plasma membrane into the cytoplasm. The neutral pH in the cytoplasm results in the preferential deprotonation of IAAH into the negatively charged IAA\(^{-}\), which is unable to diffuse out of the cell. Auxin efflux proteins can then directionally export the cytoplasmic IAA\(^{-}\) out the cell. PAT is at the heart of the canalization of auxin transport hypothesis which describes a positive feedback loop regulating auxin transport (Sachs, 1981, 1991). The hypothesis predicts that; (1) auxin moves out of cells primarily via plasma membrane-bound efflux carriers, (2) that efflux carriers are found primarily in the basal ends of cells and (3) that asymmetric distribution of efflux carriers result in a directional transport of auxin from the shoot toward the root.

This model has been linked to various patterning processes in plants including the formation of leaf vascular patterns and phyllotaxis (Sachs, 1981; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Heisler and Jonsson, 2007; Wenzel et al., 2007; Bayer et al., 2009). The canalization of auxin process leads to preferential auxin accumulation in a subset of plant cells leading to their specialization for auxin transport (Fig. 1-3 A, B). The subsequent formation of preferred avenues of auxin transport efficiently depletes surrounding areas of auxin. The canalization of auxin hypothesis is supported by numerous studies including recent molecular evidence implicating the auxin transport protein PIN1 and the ARF transcription factor MP to the establishment of a positive feedback loop during the earliest stages of leaf vascular development (Scarpella et al., 2006; Wenzel et al., 2007). High auxin concentrations have
been observed in auxin transporting provascular cells using auxin responsive reporter gene constructs like ProDR5::GUS and is consistent with recent computational modelling of auxin transport (Fig. 1-3B) (Mattsson et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007; Bayer et al., 2009). The increased auxin concentration in these preferred avenues of auxin flow is thought to trigger a developmental program which leads to their differentiation into vascular cells (Jacobs, 1952; Lyndon, 1990; Aloni, 1995). These results are consistent with ectopic auxin application experiments, where distinct strands of vascular tissue are formed in response to local application of auxin (Fig. 1-3 D) (Sachs, 1981). The formation of discrete strands of vascular cells connecting the local auxin source with existing vascular tissues is consistent with a canalization of auxin process (Fig. 1-3D). Further evidence is provided by experiments where basipetal auxin transport is interrupted by severing vascular strands. Auxin transport is redirected around the wound leading to the transdifferentiation of parenchymatic cells into vascular cell types, which then reconnect to existing vascular tissues (Jacobs, 1952; Sauer et al., 2006).

Three main gene families have been implicated in auxin transport in A. thaliana; the PIN FORMED (PIN) and ATP-binding cassette (ABC) transport families of auxin efflux proteins which function to transport auxin out of cells and the AUX family of auxin influx proteins which transport auxin into cells (Fig. 1-2) (reviewed in Blakeslee (2005)). The PIN-FORMED1 (PIN1) gene is the founding member of a family of 8 auxin efflux proteins which are localized to the plasma membranes of cells (Galweiler et al., 1998; Palme and Galweiler, 1999). pin1
loss of function mutants were first identified based on defects in the formation of flowers from the reproductive meristem leading to the formation of pin-shaped inflorescences devoid of flowers or leaves, a phenotype similar to plants exposed to chemical inhibitors of auxin transport (Okada et al., 1991). Similar phenotypes were found in monopteros (mp) and pinoid (pid) mutants implicating these proteins as regulators of auxin transport (Bennett, 1995; Przemeck et al., 1996). Studies using functional ProPIN::PIN::GFP fusion transgenes and PIN1 antibodies have shown that the asymmetric subcellular localization of PIN proteins accurately predict the direction of auxin flow through cells (Galweiler et al., 1998; Benkova et al., 2003; Friml et al., 2003; Reinhardt et al., 2003; Wisniewska et al., 2006). Functional analysis of PIN1 through PIN4 and PIN7 proteins have confirmed that these proteins act as auxin efflux carriers when tested in plant or in heterologous mammalian or yeast systems (Petrasek et al., 2006; Wisniewska et al., 2006). Subcellular PIN localization experiments and subsequent analysis of auxin accumulation have lead to many insights into how auxin transport and signaling are integrated in the processes of embryogenesis, lateral organ initiation, leaf vascular patterning, tropic responses, lateral root initiation and primary root patterning (reviewed in Benjamins and Scheres (2008)).

Regulators of PIN expression and subcellular PIN distribution have also been identified in A. thaliana. The PINOID (PID) gene is a serine-threonine kinase, which directly phosphorylates PIN1 proteins resulting in asymmetric PIN1 protein targeting to the cell membrane (Friml et al., 2004). pid loss of function
mutants display flower initiation defects identical to *pin1* loss of function mutants thereby demonstrating the significance of asymmetric PIN1 distribution to auxin transport. Asymmetric targeting of PIN1 is regulated by a *PINOID*-dependent binary switch mechanism where low or high PID expression levels result in either apical or basal targeting of *PIN1* proteins respectively (Friml et al., 2004). However, the phosphorylation activity of the PID protein kinase and D6 protein kinases (D6PK) is antagonized by the activity of the protein phosphatase 2A (*PP2A*) in *A. thaliana* (Fig. 1-2) (Michniewicz et al., 2007; Zourelidou et al., 2009). These studies demonstrate that the PID, D6PK’s, and PP2A proteins act antagonistically on the reversible phosphorylation of PIN proteins, thereby regulating the asymmetric localization of PIN proteins in plant cells (Michniewicz et al., 2007; Zourelidou et al., 2009). Auxin flow can therefore be determined by changing the phosphorylation state and subsequent polar targeting of PIN proteins (Fig. 1-2). Directional auxin transport also requires endosomal cycling, which appears to be the main mechanism of PIN trafficking to the plasma membrane (Fig. 1-2). Several genes implicated in vesicle transport are important regulators of PIN intracellular trafficking including ADP ribosylation factors which are small GTP binding proteins. These include *GNOM* and *BEN1*, encoding ADP ribosylation GTP/GDP exchange factors (ARF-GEF), and *SCARFACE/VAN3* an ADP ribosylation factor GTPase activating protein (ARF-GAP) (Steinmann et al., 1999; Geldner et al., 2003; Koizumi et al., 2005; Sieburth et al., 2006; Tanaka et al., 2009). The mechanisms regulating PIN polar targeting to cell membranes are not fully understood but new components are continually being identified. The
identification of defects in PIN1 localization in mutants of the transcriptional scaffolding protein ENHANCER OF PINOID (ENP) indicate complex transcriptional interactions are also involved in controlling PIN1 localization (Furutani et al., 2007).

Auxin efflux out of cells is also performed by type B members of the ATP-binding cassette (ABCB) transport proteins in plants (reviewed in Bandyopadhyay (2007)). Loss of function mutants of this gene family have reduced auxin transport capacity and/or show defects in auxin mediated development and growth, especially in double or triple mutants (Multani et al., 2003; Noh et al., 2003; Bandyopadhyay et al., 2007; Mravec et al., 2008; Titapiwatanakun et al., 2009). The phenotypes observed in mutant analysis in A. thaliana are supported by experiments demonstrating that ABCB1, 4 and 19 transport auxin out of plant cells and also out of non-plant heterologous host cells (Geisler et al., 2005; Terasaka et al., 2005; Blakeslee et al., 2007; Cho et al., 2007; Lewis et al., 2007; Wu et al., 2007). Although ABCB proteins appear to be able to transport auxin they are generally not as asymmetrically localized to cell membranes compared to PIN proteins (Fig. 1-2)(Mravec et al., 2008). The relationship between ABCB and PIN proteins is not entirely clear although recent results have demonstrated that when ABCB19 co-localizes with PIN1 in the plasma membrane it acts to stabilize PIN1 localization in membrane microdomains (Titapiwatanakun et al., 2009). This finding is supported by previous studies which have shown that interaction between ABCB and PIN proteins is functionally relevant for auxin transport when they are expressed in
non-plant heterologous systems. Co-expression of ABCB and PIN protein results in enhanced substrate specificity, increased rate of efflux and reduced sensitivity to auxin transport inhibitors (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Mravec et al., 2008). These results are consistent with a model in which ABCB proteins and PIN proteins are able to facilitate auxin efflux independently, but function together in promoting auxin flow in developmental processes such as embryogenesis, lateral organ formation and tropic responses (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Mravec et al., 2008).

In addition to diffusion, auxin also enters cells via membrane bound auxin influx proteins (Fig. 1-2). *AUXIN RESISTANT1 (AUX1)* is the founding member of a small gene family of putative auxin influx carriers that includes three other AUX1-like (LAX) proteins in *A. thaliana* (Bennett et al., 1996). *AUX1* functions as an auxin influx protein without the need for additional plant specific cofactors since *AUX1* expression in non-plant cells demonstrated auxin uptake (Yang et al., 2006; Carrier et al., 2008). Similar experiments have not been performed on the remaining three *A. thaliana* LAX proteins although the miss-expression of an AUX1-like protein (*PaLAX1*) from wild cherry (*Prunus avium*) in *Nicotiana tabacum* and *Arabidopsis thaliana* cells demonstrated significantly increased internal auxin concentrations within those cells (Hoyerova et al., 2008). *AUX1* is asymmetrically localized in cell membranes and distinct regulators of *AUX1* polar localization have been identified (Dharmasiri et al., 2006). In the shoot apical meristem (SAM) of *A. thaliana* plants, *PIN1* and *AUX1* localization partially overlap, and suggests that the proteins appear to work together in the
redistribution of auxin to future primordia initiation sites (Reinhardt et al., 2003). In a model of regulation of phyllotaxy by polar auxin transport, *AUX1* functions to maintain high auxin concentrations in the L1 layer of the meristem by moving apoplastic auxin into the L1 layer. Auxin is subsequently directionally transported toward incipient primordia by action of *PIN1* proteins (Reinhardt et al., 2003; Reinhardt, 2005). In *A. thaliana*, observable *aux1* loss of function phenotypes are restricted to gravitropic defects in the root, where AUX1 functions to asymmetrically redistribute auxin in concert with PIN proteins in response to gravity (Bennett et al., 1996; Swarup et al., 2005). The absence of an *aux1* mutant phenotype in the formation of lateral organs from the SAM has recently been reconciled by generation of *aux1, lax1, lax2, lax3* quadruple mutants. These mutants display phyllotactic defects thereby supporting a functional role of auxin influx in the process of leaf / flower initiation (Bainbridge et al., 2008).

### 1.3 Shoot apical meristem organization

The success of land plants can be attributed to a large extent to their ability to continuously produce new lateral organs including roots, flowers and leaves. In the plant shoot system, flowers and leaves are produced from existing growth zones termed meristems. The shoot apical meristem (SAM) is a flat to dome shaped structure consisting of two clonally distinct outer cell layers (L1 and L2), which cover a population of cells in the interior (L3) (Fig. 1-4) (Esau, 1965). The cells comprising the SAM can be divided into three zones based on differences in cell behaviour and cell fate. The most central region of the SAM, termed the central zone (CZ), consists of stem cells that divide at relatively low frequency in
comparison to cells in the flanking peripheral zone (PZ) (Reddy et al., 2004; Reddy and Meyerowitz, 2005). Lateral organ formation occurs at regular intervals in the peripheral zone. Below the CZ and PZ cells of the SAM lies the rib zone (RZ) from which the majority of the pith cells of the stem are derived (Fig. 1-4) (Esau, 1965). The CZ provides and maintains a source of stem cells for the PZ which continually loses cells to differentiating organs, thereby facilitating the indeterminate growth of plant shoots (Esau, 1965). The size of the central zone of the SAM in A. thaliana is regulated by a well characterized negative feedback loop involving the homeobox transcription factor WUSCHEL (WUS) and the CLAVATA1, 2 and 3 (CLV) genes (Fig. 1-4) (Schoof et al., 2000). The WUS gene is expressed below the CZ of the meristem and positively regulates the expression of the CLV3 gene and specifies CZ stem cell fate (Mayer et al., 1998; Schoof et al., 2000). CLV3 encodes a small, secreted hydroxyproline-containing polypeptide signaling molecule which diffuses from the CZ cells until it is sequestered by the heterodimeric CLV1 / CLV2 receptor protein which is expressed in the area surrounding and above WUS expression (Clark et al., 1993; Kayes and Clark, 1998; Brand et al., 2000; Trotochaud et al., 2000; Lenhard and Laux, 2003). The subsequent binding of the membrane bound CLV1/2 receptor in turn activates a signaling cascade leading to the negative regulation of WUS transcription (Schoof et al., 2000). The exact mechanism of how CLV1/2 mediated signaling results in WUS repression are unknown, although two protein phosphatases (POLTERGEIST and POLTERGEIST-LIKE1) and the chromatin remodelling factor SPLAYED have been implicated in this
process (Kwon et al., 2005; Song et al., 2006; Sablowski, 2007). Other genes, including the class III homeodomain leucine zipper transcription factors, _CORONA_, _PHABULOSA_ and _PHAVOLUTA_, as well as the GATA-3-like transcription factor _HANABA TARANU_, have also been implicated in the specification of stem cells in the SAM but appear to play lesser, complementary roles to _WUS_ function (Zhao et al., 2004; Green et al., 2005; Prigge et al., 2005). By using the _WUS_ and _CLV_ gene regulatory loop, a stable population of central zone stem cells in plant SAMs is maintained.

Surrounding the CZ of the meristem is the PZ of rapidly dividing and differentiating cells where lateral organ formation occurs. Small groups of 4 to 10 peripheral zone cells are specified for lateral organ cell fate, and lateral organs develop as a result of a subsequent increase in cell division and expansion rates (Bossinger and Smyth, 1996; Reddy et al., 2004). The pattern of lateral organ initiation, termed phyllotaxy, is usually invariant within plant species and consists of a highly regular and repetitive pattern of initiation events. The lateral organ primordia differentiate into leaves until the SAM switches to a reproductive mode when flowers are formed instead. In 2003, a model where polar auxin transport (PAT) mediates phyllotaxy was presented, (Reinhardt et al., 2003). This model involves auxin transport to specific areas of the meristem resulting in local auxin maxima formation and lateral organ initiation. The developing lateral organs subsequently act as an auxin sinks, functioning to locally remove auxin from the surrounding area of the meristem. The collective action of existing lateral organs thereby limits auxin maxima formation in nearby areas of the meristem.
Therefore auxin maxima and subsequent lateral organ initiation only occur at a maximum distance from existing primordia resulting in a regular pattern of initiation events. This model of PAT regulated phyllotaxy is largely based on imaging of the auxin transport proteins \textit{PIN1} and \textit{AUX1} in the SAM which suggests that the proteins work together in the redistribution of auxin to future primordia initiation sites (Reinhardt et al., 2003). This model is supported by subsequent studies indicating formation of auxin maxima at sites of incipient lateral organ formation (Heisler et al., 2005). Some modifications of this PAT driven model for phyllotaxy have been suggested based on computational approaches and some molecular evidence pointing to high auxin concentrations in the CZ cells of the meristem (de Reuille et al., 2006). Although the vast majority of computational models and molecular evidence do not include auxin accumulation in CZ cells, the underlying model of regulation of phyllotaxy by PAT is accepted in all cases (de Reuille et al., 2006; Jonsson et al., 2006; Smith et al., 2006; Heisler and Jonsson, 2007; Bayer et al., 2009). The ability of plants to directionally transport auxin to various locations in the meristem and subsequently respond to local auxin maxima formation is consistent with experimental and genetic evidence linking the efflux (PIN), influx (AUX/LAX) transport proteins and ARF transcription factors to play important roles in these processes (Galweiler et al., 1998; Hardtke and Berleth, 1998; Benjamins et al., 2001; Reinhardt et al., 2003; Bainbridge et al., 2008).

The formation of the SAM occurs during embryogenesis and the \textit{SHOOTMERISTEMLESS (STM)} gene is a crucial component for the
specification of the meristem. \textit{STM} is a member of the KNOTTED class of homeodomain transcription factors (KNOX genes) (Long et al., 1996). Loss of function \textit{stm} mutants do not undergo SAM formation during embryogenesis and upon germination do not undergo any postembryonic growth (Long et al., 1996). Analysis of partial loss of function alleles of \textit{STM}, which are able to form postembryonic adventitious shoot meristems, showed that \textit{STM} function is also crucial for postembryonic SAM maintenance and function (Clark et al., 1996; Endrizzi et al., 1996; Hake et al., 2004). Analysis of other KNOX genes in \textit{A. thaliana} including \textit{BREVIPEDICELLUS (BP)} and \textit{KNAT6} have demonstrated that \textit{STM} is the most important member of this gene family in specifying the formation of the SAM during embryogenesis. The \textit{BP}, \textit{KNAT6} and to a lesser extent \textit{KNAT2} genes have been implicated in playing roles in post-embryogenic meristem maintenance and function (Belles-Boix et al., 2006; Hay et al., 2006). These genes may carry out this function, in part by regulating cytokinin biosynthesis since the KNOX gene STM positively regulates the cytokinin biosynthesis gene \textit{ISOPENTENYL TRANSFERASE 7 (IPT7)} in \textit{A. thaliana} (Jasinski et al., 2005; Yanai et al., 2005).

Cytokinin is a plant hormone which stimulates cell division and plays many important developmental roles in diverse aspects of plant development (reviewed in To and Kieber (2008)). Three cytokinin receptors have been identified in \textit{A. thaliana}, and although single mutant phenotypes show no defects in SAM morphology or function, triple mutants display a dramatic reduction in meristem size and leaf initiation (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al.,
STM could therefore function in part by maintaining SAM cell fate by increasing local cytokinin production in the meristem. Two lines of additional experimental evidence support this statement. First, *stm* mutants are partially rescued when cytokinin production is coupled to the *STM* promoter, demonstrating the relevance of *STM* activated cytokinin production in SAM formation (Yanai et al., 2005; Shani et al., 2006). Secondly, partial loss of function *stm* phenotypes become more severe in double mutants with the cytokinin receptor mutant *WOODEN LEG (wol)* (Jasinski et al., 2005; Shani et al., 2006). *STM* is a crucial regulator of SAM function throughout the life of the plant but other components of meristem function (discussed below) have also been identified (Fig. 1-4) (Mayer et al., 1998; Laux et al., 2004). The relevance of hormone signaling and SAM function have been reinforced by a study that identified the gene targets for the *WUS* transcription factor. Type-A Arabidopsis Response Regulators (ARR’s) have been implicated in negatively regulating cytokinin signaling, where constitutive expression of these genes leads to cytokinin insensitivity and loss of function mutants leads to cytokinin hypersensitivity respectively (Hwang and Sheen, 2001; Kiba et al., 2003; To et al., 2004). Leibfried and co-workers propose that *WUS* signals the overlying cells to maintain stem cell identity, in part through direct regulation of cytokinin-inducible type-A ARR’s (Leibfried et al., 2005). These results are further supported by experimental evidence presented by Leibfried et al (2005) which show that the expression of a mutant *ARR7* protein, which mimics the active phosphorylated state, resulted in defects in meristem function similar to what is
observed in wus mutants. Thus it appears that WUS-mediated stem cell specification is at least partially achieved through inhibiting inhibitors of cytokinin signaling in the CZ of SAMs, resulting in increased ability of CZ stem cells to respond to cytokinin.

Lateral organ founder cell specification is marked by expression of the AINTEGUMENTA (ANT) and ASYMMETRIC LEAVES 1 and 2 (AS1, AS2) genes (Fig. 1-4) (Long and Barton, 1998; Byrne et al., 2000; Vernoux et al., 2000; Iwakawa et al., 2002). ANT and AS1 expression occurs on the flanks of the meristem before any morphological changes associated with lateral organ formation is observed. (Long and Barton, 1998; Byrne et al., 2000; Vernoux et al., 2000; Schuetz et al., 2008). The ANT protein appears to stimulate cell proliferation since loss of function mutants have reduced cell numbers in all shoot organs and gain of function mutants display correspondingly larger numbers of cells in larger shoot organs (Mizukami and Fischer, 2000). AS1 and AS2 interact genetically and co-regulate the repression of the meristem genes BP and KNAT2 in lateral organs (Fig. 1-4) (Byrne et al., 2000; Byrne et al., 2002). The repression of BP and KNAT2 in lateral organs is complemented by the repression of AS1 and AS2 expression in the meristem by STM (Fig. 1-4) (Byrne et al., 2000; Byrne et al., 2002). In the absence of AS1 or AS2 function, BP and KNAT2 become expressed in the lateral organs leading to abnormal leaf and flower development (Byrne et al., 2000; Byrne et al., 2002). The meristem defects observed in partial loss of function stm alleles is thought to be partially the result of expanded AS1 and AS2 expression in the meristem. This notion is supported by similar
phenotypes of weak *stm* and *as1* or *as2* mutants and by experiments that illustrate that *stm* mutant phenotypes are partially rescued in *as1* mutant backgrounds (Byrne et al., 2000; Byrne et al., 2002). These results point to a model where a mutual repression of factors promoting meristem identity and factors promoting lateral organ identity are crucial to SAM function.

The population of cells in the region between initiating lateral organs and the meristem and between lateral organs, expresses genes involved in boundary formation. This boundary region consists of cells which have lower mitotic indexes and hence undergo cell divisions less frequently as judged from analyzing cell proliferation patterns in the *A. thaliana* floral meristems and monitoring several cell cycle control genes (Breuil-Broyer et al., 2004; Aida and Tasaka, 2006). Furthermore, detailed analysis of the surface cells of the shoot apex revealed that boundary cells preferentially expand in one plane leading to elongated cell files arranged in parallel along the boundary axis (Kwiatkowska and Dumais, 2003; Kwiatkowska, 2004). Experiments performed to analyze diffusion of fluorescent probes small enough to allow passage through plasmodesmata have shown that the boundary region acts as a barrier to diffusion of small molecules and acts to symplastically isolate the developing primordia from the meristem (Rinne and van der Schoot, 1998; Aida and Tasaka, 2006). Examples of developmentally important transcription factors including the KNOX proteins *STM* and *BP* have been shown to move throughout different layers of the meristem and this movement is functionally required to rescue strong *stm* loss of function mutants (Kim et al., 2003). Thus, boundary regions
may play important roles in excluding mobile *STM* and *BP* proteins from developing lateral organs. The *A. thaliana* CUP-SHAPED COTYLEDON 1, 2 and 3 (*CUC1*, 2, and 3) genes are members of the NAC family of transcription factors and play redundant roles in establishing organ separation boundaries (Aida et al., 1997; Ishida et al., 2000; Takada et al., 2001; Vroemen et al., 2003). *CUC1*, 2 and 3 are expressed in boundary regions between developing lateral organs and meristems as early as the globular stage of embryogenesis and continue to be expressed in boundary regions of vegetative and reproductive meristems during postembryonic growth and development (Fig. 1-4) (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). Computational predictions of putative miRNA targets identified several members of NAC gene family including *CUC1* and *CUC2* as putative targets of miR164 (Rhoades et al., 2002). Experiments including ectopic expression of miR164 lead to developmental defects similar to *cuc1cuc2* double mutants, therefore confirming miR164 as an important regulator of *CUC1* and *CUC2* expression (Laufs et al., 2004; Mallory et al., 2004). Analysis of plants constitutively expressing *CUC1*, *CUC2* or *CUC3* genes revealed that overexpression of CUC genes leads to the formation of lobed cotyledons (Takada et al., 2001). This phenotype was interpreted as caused by ectopic growth suppression which is consistent with the observed low mitotic indices of CUC-expressing boundary cells (Takada et al., 2001). The formation of leaf serrations and phyllotaxy may be regulated by a balance between CUC mediated growth repression and miR164 regulation of *CUC1* / *CUC2* expression (Nikovics et al., 2006; Peaucelle et al., 2007). Interestingly, in mutants defective in auxin
signaling or transport where auxin mediated shoot patterning is disrupted, CUC gene expression is similarly altered (Vernoux et al., 2000; Aida et al., 2002; Furutani et al., 2004). Furthermore, PIN1-dependent auxin maxima formation at sites of incipient lateral organ primordia has been suggested to directly or indirectly exclude CUC2 expression from developing lateral organs (Vernoux et al., 2000; Aida and Tasaka, 2006). Other studies have shown that CUC1 and CUC2 are targets for STM-mediated transcriptional repression from embryonic SAMs (Aida et al., 1999; Takada et al., 2001; Aida and Tasaka, 2006). These results allowed the formulation of a model where STM would exclude CUC expression in the meristem while auxin maxima formation at sites of primordia initiation would repress CUC genes and hence restrict CUC expression to the boundary region between the meristem and the developing primordia (Aida and Tasaka, 2006). However, results including observations that STM expression at least partially depends on CUC function are inconsistent with this model and point to a more complex mechanism specifying boundary formation (Aida et al., 1999; Aida and Tasaka, 2006).

Although auxin’s role in initiating lateral organ development from the SAM is an important aspect of meristem function, evidence presented in Chapter 2 and 3 of this thesis provide support for additional, yet undefined roles of auxin in meristem patterning and maintenance. The discovery of abnormally sized SAMs defective in leaf initiation as a consequence of simultaneous interference with auxin signaling and auxin transport may provide an entry point in the analysis of
auxin’s role in regulating SAM patterning including possible crosstalk with other plant hormones.

1.4 Vascular patterning

The plant vascular system is comprised of xylem and phloem tissue types which are specialized for long distance transport of solutes throughout the plant (Esau, 1965; Turner, 2002). Xylem primarily functions in transporting water and dissolved minerals from the root to the shoot region and phloem primarily functions in transporting photosynthetic products (i.e. sugars) from the shoot to the root and other sinks. The formation of the vascular system in plants is a highly regulative and coordinated process involving specification of different cell types along different symmetrical planes (Esau, 1965; Turner, 2002). For example, the xylem tissues in plant leaves is always located on the adaxial side of the leaf with phloem tissues on the abaxial side while both tissue types are coordinately arranged throughout the leaf proximo-distal and centro-lateral axes (Fig. 1-5 A, B).

The process of leaf initiation from the SAM and the process of leaf vascular pattern formation share many commonalities. They appear to be formed by a gradual limitation process that may be synonymous with canalization of auxin transport. This is further supported by both processes depending on the function of similar genes, including the MP and PIN1 genes, which display loss of function phenotypes in both leaf / flower initiation and leaf vascular pattern formation (Przemeck et al., 1996; Galweiler et al., 1998; Hardtke and Berleth, 1998; Mattsson et al., 1999). Another link between vascular development and
leaf initiation is observed in as1 and as2 mutants. These genes play important roles in meristem patterning and leaf founder cell specification but also have defects in leaf vascular tissue patterning (Byrne et al., 2000; Byrne et al., 2002; Turner, 2002).

Auxin has been implicated in various aspects of vascular tissue development and pattern formation in various plant species (Jacobs, 1952; Klee, 1987; Aloni, 1995; Aloni et al., 2003). Genes responsible for defective leaf vascular tissue formation or patterning in mutants isolated in A. thaliana have been identified and shown to act in an auxin-related pathway. These genes include the auxin response factor MP, the auxin efflux protein PIN1, AXR6/CUL1 which is a crucial component of the SFC E3 ligase, and SCF/VAN3 and GNOM both of which are involved in the intracellular trafficking of auxin efflux proteins (reviewed in Turner (2002); Benjamins and Scheres (2008)).

Mature xylem cells have thick secondary cell walls which are easily observed in the leaf and therefore the majority of mutants displaying vascular patterning defects have been identified in screens that focused on this tissue type (Turner, 2002). Xylem development occurs from the initial specification of isodiametrically shaped mesophyll cells soon after leaf initiation and are referred to as pre-procambial cells (Fig. 1-5C). Pre-procambial cells are the earliest indication of future vascular cell fate and can be identified based on expression of PIN1 and MP genes or reporter genes driven by the synthetic auxin responsive promoter DR5 (Mattsson et al., 2003; Scarpella et al., 2006; Wenzel et al., 2007). PIN1-mediated auxin flow through undifferentiated leaf mesophyll
cells appears to predict the formation of pre-procambial cells (Scarpella et al., 2006; Wenzel et al., 2007). For example, the specification of the leaf midvein is formed from the redistribution of auxin accumulated in the L1 layer of the SAM. PIN1 expression dynamics in the SAM has been shown to predict the initiation of lateral organs by forming auxin convergence points in the L1 layer of the SAM (Fig. 1-3 Bi) (Reinhardt et al., 2003; Heisler et al., 2005; Schuetz et al., 2008). The auxin accumulated in those cells is subsequently redirected basipetally by PIN1 leading to the formation of a discrete channel of auxin transporting cells (Fig. 1-3 B, Ci). Upon leaf initiation from the meristem, PIN1 expression continues to be refined in this file of cells located directly in the center of the developing primordium (Fig. 1-3 Ci). The specification of secondary vein loops is achieved by a similar mechanism where formation of auxin convergence points occurs in the epidermal layer of the leaf primordium (Fig. 1-3 Ciii) (Scarpella et al., 2006; Wenzel et al., 2007). Subsequent PIN1-mediated redistribution of auxin leads to the formation of channels of auxin flow for both the lower and upper regions of the loop connecting to the midvein (Fig. 1-3 C iv-vii) (Scarpella et al., 2006; Wenzel et al., 2007). The following formation of elongated and interconnected procambial cells from pre-procambial cells is associated with the activation of other genes including \textit{AtHB8} and \textit{TED3} (Fig. 1-5 C) (Baima, 1995; Igarashi et al., 1998; Turner, 2002). Procambial cells give rise to either xylem or phloem tissue types and are specified by action of distinct transcription factors (Baima et al., 2001; Bonke et al., 2003). Xylem forming procambial cells undergo a process of patterned secondary cell wall deposition as development continues.
that first appears in annular or spiral thickenings in protoxylem, followed by more complex reticulate patterns in metaxylem (Fig. 1-5 D) (Esau, 1965; Turner, 2002). The radial orientation of xylem and phloem in various plant structures is regulated by members of the \textit{A. thaliana} HD-ZIP III, YABBY and KANADI class of transcription factors, which also are involved in the specification of other abaxial and adaxial cell fates in leaves (reviewed in Fukuda (2004)).

Several genes including members of the plant specific NAC-domain transcription factors have been shown to developmentally regulate the formation of proto and metaxylem cells (Kubo et al., 2005). Misexpression of the \textit{VASCULAR-RELATED NAC-DOMAIN6 (VND6)} or \textit{VND7} genes in various different cell types in \textit{A. thaliana} or \textit{Populus tremuloides} result in the transdifferentiation of even highly specialized cell types including trichomes and guard cells into metaxylem-like cells (Kubo et al., 2005). Mature metaxylem cells subsequently undergo apoptosis resulting in the formation of a series of interconnected hollow tracheary elements (TE). Xylem tissue is primarily composed of tracheary elements, which are the water conducting component of xylem tissue and xylem parenchyma and fiber cells. Xylem parenchyma cells are slightly elongated cells with uniform secondary cell walls that do not undergo apoptosis at maturity (Esau, 1965; Turner, 2002). These specialized parenchyma cells have been implicated in TE secondary cell wall lignification, a process that results in the strengthening of secondary cell walls (Wegner and Raschke, 1994; Turner, 2002; Ros Barcelo, 2005). Xylem fibers have uniformly
thick secondary cell walls and are thought to provide mechanical support (Zhong et al., 1997; Zhong and Ye, 1999; Turner, 2002).

The canalization of auxin flow hypothesis builds a framework of how auxin signaling and auxin transport are integrated to result in the patterning of leaf vascular tissues. In this hypothesis, cells that are exposed to auxin become specialized for auxin transport, which results in the formation of preferred channels of auxin flow. These auxin channels drain any local accumulation of auxin and eventually differentiate into vascular cells thus forming the connected vascular system. Mutations in the \textit{SCARFACE/VAN3} gene resulted in the formation of interrupted vascular islands resulting in a fragmented leaf vascular pattern (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). These observations appeared to be inconsistent with a canalization model of vascular cell fate specification. However, detailed analysis of \textit{ProPIN1:PIN1:GFP} expression dynamics in developing \textit{scarface/van3} mutant leaves demonstrated that \textit{PIN1} mediated auxin flow is initially continuous in the mutants and the defect in vascular differentiation appears to be a result of a gradual decay of the continuous auxin transport route (Scarpella et al., 2006). These results are consistent with the observation that the fragmented vascular islands observed in \textit{scarface/van3} mutants do not occur in random patterns and is best described as a \textit{WT}-like pattern of fragmented vascular islands (Deyholos et al., 2000; Koizumi et al., 2000). Therefore the primary defect in \textit{scarface/van3} mutants is the maintenance rather than the initial establishment of a continuous vascular pattern (Scarpella et al., 2006). Proteins that potentially act as facilitators of canalization
of auxin flow include the ARF family of transcription factors, PIN family of auxin efflux carriers and AUX family of auxin influx carriers (Reviewed in Rolland-Lagan (2008)). The results presented in chapter 4 of this thesis suggest a feedback regulatory loop that involves auxin, MP and PIN1, and provides novel experimental support for the canalization of auxin flow hypothesis (Wenzel et al., 2007). While it is clear that MP is an important player in leaf vascular pattern formation and lateral organ initiation, loss of function mp mutants are still able to undergo leaf initiation and form reduced leaf vascular systems indicating possible genetic redundancy. In chapter 5, the six ARFs phylogenetically most similar to MP were analyzed for putative roles in leaf initiation and vascular pattern formation. Based on partially overlapping expression patterns and similar auxin inducible transcriptional expression profiles compared to MP in developing leaf primorida, ARF3 was identified as playing a potential role in leaf initiation and vascular pattern formation. Other ARF genes analyzed in this study highlight ARF7 and ARF19 transcriptional expression coinciding with TE differentiation.
1.5 Figures

Figure 1-1 TIR-AUX/IAA-ARF model of auxin signaling.

Auxin Response Factors (ARFs) bind to TGTCTC auxin response elements (Pink) via a B3-type DNA binding domain (Blue). ARF function is inhibited by Aux/IAA proteins, which contain a potent repressor domain (orange) that also facilitates binding of transcriptional co-repressor proteins like TPL. Aux/IAA proteins bind to ARFs via two conserved C-terminal dimerization domains (Red, and Green). In the presence of high auxin concentrations, auxin binds the TIR1 auxin receptor, which is a component of the SCF^{TIR1} E3 protein ligase complex. The auxin-bound TIR1 protein facilitates interaction between domain 2 of Aux/IAA proteins and the E3 protein ligase complex resulting in the ubiquitination of the Aux/IAA protein. The poly-ubiquitination of Aux/IAA proteins is achieved via concerted action of ubiquitin activating (E1), ubiquitin carrier (E2) and ubiquitin protein ligase (E3). Degradation of poly-ubiquitinated Aux/IAA proteins via the 26S proteosome results in de-repression of ARF function. This allows for ARF Homo or Heterodimer formation and subsequent ARF dependent activation of target gene transcription. (Reviewed in (Mockaitis and Estelle, 2008)). Adapted from (Berleth et al., 2004)
Figure 1-1 TIR-AUX/IAA-ARF model of auxin signaling.
Polar auxin transport (PAT) is achieved by dynamic asymmetric localization of PIN auxin efflux proteins (Blue rectangles) and AUX auxin influx proteins (Red squares) to the plasma membrane. This is achieved via continual cycling of these proteins between endosomal compartments and the plasma membrane. The cycling of PIN proteins from the endosomes to the plasma membrane requires the function of the ADP-ribosylation factor GTPase guanine nucleotide exchange factor (ARF-GEF) GNOM and ADP-ribosylation factor GTPase activating protein (ARF-GAP) SCF/VAN3. The subsequent apical or basal targeting of PIN proteins is achieved via the antagonistic action of the PID protein kinase and protein phosphatase 2A (PP2A). These proteins determine the phosphorylation state of PIN proteins resulting in either apical or basal targeting. The asymmetric localization of the AUX1 protein is also facilitated by continuous cycling between endosomal compartments and the plasma membrane but is not GNOM or SCF/VAN3 dependent and instead requires the action of the endoplasmic reticulum accessory protein AXR4. The chemiosmotic hypothesis for PAT describes that IAA, the major form of auxin, preferentially accumulates in the cytoplasm due to the differences in pH between the cytoplasm and cell wall. In the relatively acidic cell wall environment, IAA becomes protonated and this uncharged IAAH can diffuse through the hydrophobic centre of the plasma membrane into the cytoplasm. The more neutral pH in the cytoplasm results in the preferential deprotonation of IAAH into the negatively charged IAA-, which is unable to diffuse out of the cell. The cytosolic IAA then becomes a substrate for PIN and B type ATP-binding cassette (ABCB) efflux proteins. This illustration does not reflect the localization of transport proteins at any one given time.
Figure 1-2 Auxin transport.
Figure 1-3 Canalization of Auxin Transport

A. Computational output of auxin accumulation (blue) and PIN1 (red) expression dynamics in a rectangular grid of cells during leaf midvein formation. The length and width of arrows indicate the magnitude and dominant direction of auxin flux with increasing time (i-iv). B. Simulation model of midvein formation of cellular meristem template. PIN1 (red) and auxin accumulation (green) shown at increasing time points (i-iii). C. Model for PIN1 mediated auxin flow during midvein (i-ii) and first secondary loop (iii-vii) formation. Auxin is transported to the tip of the developing leaf primordia and accumulates in convergence points (dark blue), where it is subsequently redistributed and transported basipetally by PIN1 (red arrows) through the centre of the developing leaf primordia (i). The resulting auxin flow specifies procambial cells (orange) in the future leaf midvein (ii). Auxin accumulation on epidermal convergence points (dark blue) and subsequent PIN1 mediated redistribution and refinement of auxin flow leading to connection with the midvein (iii). PIN1 mediated auxin transport from epidermal convergence point and surrounding area leading to the formation of the upper region of the loop, leading to the specification of a complete loop of procambial cells (orange) (iv-vii). D. Diffusion vs Canalization mechanism for formation of vascular tissues from ectopic auxin application (*). Formation of discrete strands of vascular tissue connecting ectopic auxin source (*) to existing vasculature support a canalization of auxin mechanism vs diffusion. (A-B from (Bayer et al., 2009) and C adapted from (Scarpella et al., 2006, Wenzel et al., 2007))
Figure 1-3 Canalization of Auxin Transport
Figure 1-4 Gene interactions in meristem patterning and maintenance

The shoot apical meristem is divided into three main growth zones. The central zone (stem cell source), peripheral zone (organogenesis), and rib zone (source of stem pith cells). The closely related KNOX genes STM, BP and KNAT2 are important regulators of meristem maintenance and are expressed throughout the meristem. AS1 and ANT positively regulate leaf founder cell specification in the peripheral zone. Boundaries between lateral organ primordia and the meristem are marked by expression of the CUC1, CUC2 and CUC3 genes. Mutually antagonistic interactions between AS1 and the KNOX genes STM, BP and KNAT2 result in exclusion of lateral organ founder cell fates in the meristem centre and meristem cell fates in developing lateral organs. The central zone of stem cells is regulated by a negative feedback loop involving the WUS transcription factor, CLV3 signaling peptide and CLV1/2 heterodimeric receptor protein.
Figure 1-4 Gene interactions in meristem patterning and maintenance.
Figure 1-5 Overview of auxin mediated vascular tissue patterning and development

A. Vein classification (Hickey, 1979). (1\textsuperscript{st}) Primary mid vein in Red, (2\textsuperscript{nd}) Secondary veins in pink, (3\textsuperscript{rd}) Tertiary veins in green, (4\textsuperscript{th}) Quaternary veins in blue, and (5\textsuperscript{th}) Quinary veins in yellow. B. Dark field image of mature \textit{WT A. thaliana} fourth leaf illustrating relevant axes. C. Whole mount in situ hybridization of \textit{WT A. thaliana} leaf with \textit{MP} antisense probes. Isodiametric mesophyll cell outlined in brown, isodiametric pre-procambial cell outlined in light blue, and elongated procambial cell outlined in green. D. Xylem cells in successively more mature stages of xylogenesis from left to right. Secondary cell wall deposition in annular rings, followed by helix pattern, and subsequent tighter helix patterns, until a reticulate pattern of mature xylem vessel is formed (not shown).
Figure 1-5 Overview of auxin mediated vascular tissue patterning and development.
1.6 References


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2: MULTIPLE MONOPTEROS-DEPENDENT PATHWAYS ARE INVOLVED IN LEAF INITIATION.

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Statement of contribution:

M. Schuetz performed all experimental work, in part as follow up experiments first performed by J. Mattsson. All the authors contributed to the writing of the manuscript.

2.1 Abstract

Initiation of leaves at the flanks of the shoot apical meristem occurs at sites of auxin accumulation and pronounced expression of auxin-inducible PIN genes, suggesting a feedback loop to progressively focus auxin in concrete spots. Since PIN expression is regulated by Auxin Response Factor (ARF) activity, including MONOPTEROS (MP), it appeared possible that MP affects leaf formation as a positive regulator of PIN genes and auxin transport. Here we analyze a novel, completely leafless phenotype arising from simultaneous interference with both auxin signaling and auxin transport. We show that mp pin1 double mutants, as well as mp mutants treated with auxin-efflux inhibitors, display synergistic abnormalities, not seen in wild type regardless of how strongly auxin transport was reduced. The synergism of abnormalities indicates that the role of MP in
shoot meristem organization is not limited to auxin transport regulation. In mp mutant background, auxin transport inhibition completely abolishes leaf formation. Instead of forming leaves, the abnormal shoot meristems dramatically increase in size harbouring correspondingly enlarged expression domains of CLAVATA3 and SHOOTMERISTEMLESS, molecular markers for the central stem cell zone and the complete meristem respectively. The observed synergism under conditions of auxin efflux inhibition was further supported by an unrestricted PIN1 expression in mp meristems, as compared to a partial restriction in wildtype meristems. Auxin transport-inhibited mp meristems also lacked detectable auxin maxima. We conclude that MP promotes the focusing of auxin and leaf initiation in part through pathways not affected by auxin efflux inhibitors.

2.2 Introduction

Plants continuously produce lateral organs, primarily leaves and flowers, at the flanks of shoot apical meristems (SAMs). Considerable advances have been made over the past ten years on the understanding of the genetic basis of meristem maintenance, proliferation and lateral organ formation (see recent reviews by (Williams and Fletcher, 2005; Carraro et al., 2006; Shani et al., 2006; Tucker and Laux, 2007)). At the center of the meristem, a central zone (CZ) of generally slower dividing cells provides stem cells for the more rapidly dividing surrounding peripheral zone (PZ) and underlying rib zone (RZ) (Reddy et al., 2004). Together, the CZ, PZ and RZ make up the shoot apical meristem. The
meristem provides cells for lateral organ, i.e. leaf and flower formation and underlying pith formation. The size of the central zone is regulated by a feedback loop involving the *CLAVATA* genes and the *WUSCHEL (WUS)* gene (Fletcher et al., 1999; Schoof et al., 2000; Clark, 2001). The meristem is specified and maintained by the *SHOOTMERISTEMLESS (STM)* gene (Long et al., 1996; Muday and DeLong, 2001; Kumaran et al., 2002) along with other members of the same gene family, primarily *BREVIPEDICELLUS (BP)/KNAT1* and *KNAT2* (Chuck et al., 1996; Ori et al., 2000; Muday and DeLong, 2001; Byrne et al., 2002). *STM* appears to carry out this function at least in part by preventing the expression of *ASYMMETRIC LEAVES1 (AS1)* in the meristem (Byrne et al., 2000; Long and Barton, 2000). *AS1* in turn promotes lateral organ formation at the flanks of the peripheral zone by down-regulating *BP/KNAT1* and *KNAT2* at the sites of lateral organ formation (Byrne et al., 2000; Byrne et al., 2002). Lateral organ formation also depends on the *AINTEGUMENTA (ANT)* gene which promotes cell proliferation in these structures (Mizukami and Fischer, 2000). Both *ANT* and *AS1* have been used as early molecular markers for the formation of lateral organs (Long and Barton, 1998; Byrne et al., 2000; Vernoux et al., 2000). Although some of the interactions of meristem organizing genes have been documented, clear evidence of how primordia-specific genes become expressed at the sites of lateral organ formation remain elusive. The separation of the emerging lateral organs is promoted by several genes, most notably the *CUP-SHAPED COTYLEDON* genes, adding another level of regulation involved in
lateral organ formation (Aida et al., 1997; Aida et al., 1999; Hibara et al., 2003; Vroemen et al., 2003; Koyama et al., 2007).

It has long been known that the formation of lateral organs can be influenced by the plant hormone auxin (Reinhardt et al., 2000 and references therein). Application of auxin as well as auxin efflux inhibitors results in a range of phenotypes from altered numbers and positions of flowers and leaves to a complete block of flower formation from reproductive SAMs (Wardlaw, 1949; Meicenheimer, 1981; Okada et al., 1991; Mattsson et al., 1999). Recent advances suggest that auxin accumulation is required for lateral organ initiation and that auxin is transported to these sites by membrane bound efflux transport proteins that asymmetrically localize to apical or basal ends of cells (Benkova et al., 2003; Reinhardt et al., 2003; Friml et al., 2004; Heisler et al., 2005; Petrasek et al., 2006). A key component in this process is PIN-FORMED1 (PIN1), a member of the PIN family of membrane bound auxin efflux proteins (Okada et al., 1991; Galweiler et al., 1998). Loss of function mutations in the PIN1 gene result in reduced auxin transport, and defective cotyledon and flower formation (Okada et al., 1991). Petrasek et al (2006) have recently shown that PIN auxin efflux proteins are sufficient to facilitate auxin efflux in yeast cells, suggesting that directionality of auxin flow can be regulated by the subcellular localization of PIN proteins. The PINOID (PID) gene, encoding a protein-serine/threonine kinase, acts as a positive regulator of polar auxin transport by regulating the sub-cellular localization of PIN1 (Bennett, 1995; Benjamins et al., 2001; Friml et al., 2004; Lee and Cho, 2006). Loss of function pid mutants display defects in lateral organ
formation similar to *pin1* mutants consistent with its role in regulating *PIN1* mediated auxin efflux.

Auxin transport is promoted by the activity of the *MONOPTEROS (MP)* gene (Wenzel et al., 2007), which belongs to the Auxin Response Factor (ARF) family of transcription factors (Guilfoyle et al., 1998; Hardtke and Berleth, 1998). Members of this family are post-translationally activated in response to auxin via auxin-mediated degradation of members of the AUX/IAA family of nuclear repressor proteins that bind to ARFs and inhibit ARF dimerization and subsequent target gene transcription (Kim et al., 1997; Ulmasov et al., 1997; Leyser and Berleth, 1999; Ulmasov et al., 1999; Dharmasiri and Estelle, 2002; Liscum and Reed, 2002). Not only mutations in *PIN1* and *PID* but also in the *MP* gene interfere with lateral organ formation on inflorescence meristems (Przemeck et al., 1996). Local auxin application can restore flower formation on the flanks of *pin1* and *pid*, but not *mp* mutant inflorescences (Reinhardt et al., 2000; Reinhardt et al., 2003), suggesting that in *mp* mutants not the local supply of auxin, but auxin sensitivity is diminished. Similarly, cotyledon response assays show that *mp* mutants are more resistant to the effects of exogenous auxin treatments than the strong auxin resistant mutant allele *axr1-12* demonstrating that *mp* mutants are severely defective in auxin signaling (Mattsson et al., 2003).

Recent reports show that ARFs, including *MP*, may regulate the expression of *PIN* genes (Sauer et al., 2006; Wenzel et al., 2007). To test if *MP* exerts its effect on lateral organ formation exclusively as a regulator of *PIN* genes and
auxin transport, we created *mp pin1* double mutants and also grew *mp* mutants on media supplemented with auxin efflux inhibitors. Here we show that *mp pin1* double mutants, as well as *mp* mutants treated with auxin efflux inhibitors, display strong synergistic abnormalities. These mutants fail to develop any lateral organs and the SAM develops into a leafless dome. The appearance of a synergistic defect indicates that the role of *MP* in shoot meristem organization is not limited to the regulation of auxin transport and the novel meristem phenotype implicates auxin transport and signaling in the regulation of meristem size.

### 2.3 Materials and Methods

#### Plant material and growth

The *mp*\textsuperscript{G12, G33, Tu399}, *pid3* and *pin1-1* mutant alleles used for double mutant and single mutant analysis have been described previously in (Berleth, 1993; Hardtke and Berleth, 1998) (Christensen et al., 2000; Benjamins et al., 2001) (Okada et al., 1991). All *MP* alleles used in this study are characterized as strong alleles and no differences were observed between different alleles and subsequent treatments or double mutant generation. The 35S::*MP* line was generated as described in Hardtke et al (2004) and over-expression of *MP* transcripts was confirmed by qPCR using a Rotor-Gene\textsuperscript{Tm} 3000 real time quantitative thermocycler (Corbett Life Sciences, San Francisco, CA) and the Platinum SYBER Green qPCR SuperMix (Invitrogen), with the primers MP-RT-F (CGATTGGATCCCTGAGAT) and MP-RT-R (ACCCATTCACTTCCAGCAG) ((Hardtke et al., 2004); data not shown). The Pro\textsubscript{DRS}::*GUS* (Ulmasov et al., 1997),
ProPIN1:PIN1:GFP (Benkova et al., 2003), ProSTM:GUS (Kirch et al., 2003), ProCLV3:GFP:ER (Lenhard and Laux, 2003), transgenes were crossed into the mp mutant background. Attempts to introgress a ProDR5::GFP construct obtained from ABRC into mp mutant background failed, possibly as a consequence of repulsion due to linkage. Surface-sterilized seeds were grown on ATS medium (Lincoln et al., 1990) and exposed to NPA as described (Mattsson et al., 1999). For quantification of CLV3 and STM expression domains in meristems, images were taken and subsequently analyzed using ImageJ v1.37 software (NIH). mp seedlings germinate approximately 1 day after WT most likely due to lack of hypocotyl and root. Comparable developmental stages were chosen for each data set defined by the WT, for example; The 3 DAG stage is defined as 3 DAG WT plants and 4 DAG mp plants, with similar sizes of leaf primordia.

In situ hybridization, Histology and GUS assays

All gene fragments were amplified from cDNA generated from total RNA extracted from 14 day old WT seedlings using Trizol reagent (Invitrogen) and subsequently reverse transcribed using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) and cloned into pBluescript II Sk(-) (Stratagene). The ANT and AS1 fragments were generated as described (Long and Barton, 1998); (Byrne et al., 2000). Whole mount in situ hybridization procedure was as described (Zachgo et al., 2000) with some modifications including overnight fixation and 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. For histological analysis, plant material was fixed and sectioned as described in (Ruzin, 1999). Localization of $b$-glucuronidase activity was carried out as described in (Mattsson et al., 2003).

**Microscopy**

A Zeiss LSM 410 was used to image $Pro_{PIN1}:PIN1:GFP$ and $Pro_{CLV3}:GFP:ER$ 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. DIC images were taken on a Nikon Eclipse 600 microscope using a Canon D30 digital camera and tissue clearing and preparation were performed as described in (Mattsson et al., 1999). Samples for Scanning electron micrographs were fixed o/n in 2.5% gualeraldehyde in .05 M Cacodylate buffer, dehydrated in a graded ethanol series before being critically point dried and mounted on stubs. Samples were then coated with gold-palladium in a SEM Prep2 sputter coater (Nanotech), and imaged using a Hitachi S-2600N VP-SEM.

**2.4 Results**

*mp pin1 double mutants fail to form leaves*

The shoot meristems of both *pin1* and *mp* single mutants produce a functional rosette of leaves from the vegetative SAM but are highly defective in the analogous process of flower formation from the reproductive SAM (Okada et al.,
to assess whether MP function in shoot organization acts exclusively through the regulation of auxin transport, we generated mp pin1 double mutants. Analysis of progeny from a cross between heterozygous mp and pin1 plants, resulted in the identification of a fraction of mp-like plants which had formed a leafless dome from the SAM (Fig. 2-1 C,D). The segregation ratio of this novel phenotype was not significantly different from an expected theoretical value based on chi-square analysis (p = 0.75; Table 2-1), supporting the notion that the individuals were double mutants. The domes had a smooth surface and lacked differentiated epidermal, trichome and stomata cells (Fig. 2-1 D). After 2-3 weeks of culture in short day conditions, the majority of the putative double-mutants had developed additional leafless dome structures arising from the base of the initial dome (Fig. 2-1 E). Such domes were never observed in single mp or pin1 mutant populations. The appearance of a novel phenotype in the absence of both gene activities leads us to conclude that MP and PIN1 act, at least in part, in separate pathways (see discussion).

Phenotypes of mp pin1 double mutant plants ranged from highly fasciated domes (Fig. 2-1 F) to single or multiple dome formation and in the vast majority of all plants, leaf formation was absent. After 3-4 weeks of culture, many of the domes had formed one or more filament like projections from its surface. A large number of these projections were formed after prolonged culture (Fig. 2-1 G,H). We interpreted these as inflorescences as they sometimes produced pistil-like or petal-like structures at their apices (Fig. 2-1 I, Fig. 3-2 E). We found further evidence that MP acts on another pathway distinct from the regulation of PIN1 by
the evaluation of *mp pid* and *pin1 pid* double mutants. The *PID* gene is known to be required for subcellular localization of PIN1 in plant cells transporting auxin (Friml et al., 2004) and may thus be thought to act in the same pathway as *PIN1*. Consistent with this interpretation, the *mp pid* double mutants produced phenotypes that were indistinguishable from the *mp pin1* phenotype (Fig. 2-1 J; Table 2-1). Further, as previously reported (Furutani et al., 2004), the *pin1 pid* double mutants were characterized by a variable degree of wide or fused leaves, but did not produce the leafless dome phenotype observed in *mp pin1* or *mp pid* double mutants. (Fig. 2-1 K). The fact that the *pin pid* double mutant displays defects that are qualitatively similar to those of both single mutants is consistent with *PIN1* and *PID* acting in the same pathway, in line with molecular evidence (Friml et al., 2004). In summary, *mp pin1* and *mp pid* double mutants produced an identical, novel synergistic phenotype, suggesting that *MP* function in shoot meristem organization goes beyond the regulation of auxin transport processes (see discussion).

**Reduction of auxin transport does not abolish lateral organ formation**

The phenotypes from the *mp pin1* and *mp pid* double mutants suggests that in *mp* mutant background, leaf initiation becomes extremely sensitive to reduction of auxin transport. To assess this possibility, we grew *mp* seedlings on medium supplemented with the polar auxin efflux inhibitor naphthylphtalamic acid (NPA). The observed defects very much resembled the phenotype of *mp pin1* and *mp pid* double mutants (Fig. 2-1 M). In addition a large part of the heterogeneity
observed in double mutants was lost at NPA concentrations at or above 10 µM NPA, suggesting that the heterogeneity was due to a comparatively weaker reduction in auxin transport in pin1 or pid mutants. Similar phenotypes were obtained with other, chemically distinct auxin efflux inhibitors, i.e. 9-hydroxyfluorene-9-carboxylic acid (HFCA) and 2,3,5-triiodobenzoic acid (TIBA) (Fig. 2-1 N,O) when applied to mp mutants.

Since auxin transport is reduced in mp mutants (Przemeck et al., 1996), we next asked whether the leafless dome phenotype could simply be a consequence of particularly weak auxin transport. To this end, we grew wild-type (WT) plants and mp mutants in the presence of increasing NPA concentrations. As shown in Figure 2-2 A, leaf formation in wild type, but also in pin1 and pid shoots could not be abolished by any concentration of NPA, not even at 100 µM NPA an eventually lethal concentration. Upon exposure to NPA WT, pin1 and pid3 mutants developed leaf fusions or tubular leaves but never formed leafless domes (Fig. 2-1 L; Fig. 2-2 A). In WT plants, 0.1 µM and 1 µM NPA had no significant effect on the numbers of leaves produced by 21 days after germination (DAG) (Fig. 2-2 B). In contrast, in mp mutants NPA concentrations as low as 0.1 µM resulted in a dramatic decrease in leaf initiation (Fig. 2-2 B) and at concentrations of 1 µM NPA and higher, the majority of mp mutants developed leafless domes. The novel leafless domes continued to grow, demonstrating that their inability to produce leaves was not the expression of a general growth defect. We conclude that MP, in addition to promoting auxin transport, must
stimulate another activity that leads to the actual formation and growth of leaf primordia (see discussion).

Plants with ectopic expression of MP display similar NPA hypersensitivity

The above results suggest that a loss of MP function is required for the formation of the leafless dome phenotype in the presence of NPA. To test if altered spatio-temporal expression of MP may also interfere with leaf formation under conditions of PAT inhibition we grew plants miss-expressing MP from the constitutive CAMV 35S promoter on medium supplemented with NPA. Growth of Pro35S:MP plants in the presence of NPA did result in the frequent formation of leafless domes (Fig. 2-2; Fig. 2-1 P). The response of Pro35S:MP plants to NPA was intermediate between WT and mp mutants, as leaf formation was abolished at 10 µM NPA in the majority of plants. Therefore, not only the expression of MP per se but also its restriction to distinct domains is critical for the outgrowth of leaf primordia under conditions of reduced PAT.

The shoot apical meristem enlarges during leafless dome formation

The strict requirement of defects in MP activity for the formation of leafless domes lead us to have a more careful look at the mp meristem and its ability to form leaves in the absence of PAT inhibition. We found various defects in phyllotaxy and growth of mp primordia compared to WT (Fig. 2-3 A-C, Table 2-2), suggesting that the mp meristem is already labile in this process. We also observed an immediate response of mp meristems to NPA. In the presence of 10
µM NPA, WT meristems initiated a normal first pair of leaf primordia, while mp meristems did not form any visible leaf primordia (Fig. 2-3 D-E). Instead, in mp mutants the cells immediately surrounding the meristem appeared to elongate forming a ring of elongated cells around the meristem (Fig. 2-3 G). Subsequently the meristem region began to enlarge to initiate the formation of the leafless dome (Fig. 2-3 E,F). The ring of cells initially expressed the leaf founder cell marker AS1 (Fig 2-3 F) but expression of AS1 and growth of these cells ceased by 6 DAG (Fig. 2-7). After approximately 4-6 DAG all subsequent growth came from the meristem (Fig. 2-3 G-I) and the resulting leafless dome structure is derived entirely from this region.

To determine the extent and organization of the meristem domain in leafless domes, we assessed the expression conferred by the SHOOTMERISTEMLESS (STM) gene promoter in these structures. Figure 2-4 A-D shows a comparison of ProSTM::GUS meristem expression in WT and mp plants grown in the presence or absence of 10 µM NPA after 7 days of growth. While the size of the meristem in 7 day old WT, mp plants and WT plants grown in the presence of NPA appeared comparable, the ProSTM::GUS expression domain was more curved and visibly wider in NPA-grown mp plants (Fig. 2-4 D). After 21 days of growth, a distinct leafless dome structure had developed in NPA-grown mp plants. The ProSTM::GUS expression was localized at the apex of these structures (Fig. 2-4 F,G), and although highly variable in size appeared both wider and deeper than corresponding expression domain in WT plants grown in parallel on the same media (Fig. 2-4 E). In NPA-grown mp plants, the leafless
domes also expressed ProSTM::GUS in thin strands along the apical-basal axis (arrows in Fig. 2-4 F). Upon closer inspection, these strands appeared to consist of elongated and narrow cells typical of procambial strands (Fig. 2-4 H). Similar procambial expression of STM has previously been reported in the pith meristem of WT plants (Long et al., 1996). The procambial strands, however, are frequently interrupted and never differentiate into vascular tissues (Fig 2-4 I, data not shown).

To further explore the enlarged SAMs in NPA-grown mp plants, we quantified the area of expression of the central zone marker, CLAVATA3 (CLV3) and the meristem marker STM at 10 and 21 days after germination using ProCLV3::GFP and ProSTM::GUS respectively (Fig. 2-5; representative imaged areas showed in Fig. 2-8). After 10 days, the average areas of CLV3 and STM expression were significantly larger in NPA-grown mp mutants. Although highly variable, the area of CLV3 expression was on average 4.1 times and the STM expression 2.0 times as large in NPA-grown mp plants as compared to NPA-grown WT plants thereby illustrating that leafless domes have enlarged central zones and meristem identity respectively (Fig. 2-5 B,E). After 21 days of growth, the differences had increased further with 5.5 times larger area of CLV3 expression and 4.2 times larger area of STM expression in NPA-grown mp plants as compared to NPA-grown WT plants (Fig. 2-5 C,F). The expansion of ProCLV3::GFP observed in NPA-grown mp mutants was not observed in the leafless reproductive meristems of the strong loss of function pin1-1 mutant and hence does not appear to be general response of the meristem in the absence of
lateral organs such as leaves or flowers (Fig. 2-9)(Vernoux et al., 2000). In summary, the leafless domes appear to have the organization of an enlarged shoot apex, comprising an apical meristem, and a basal radially organized stem region, but the central zone as well as the entire meristem region are enlarged and the basal region shows limited internal and external cellular differentiation.

**Leafless domes fail to focus PIN1 expression and auxin**

Previous studies have reported that PIN1 expression is upregulated at sites of flower primordia formation in the reproductive SAM (Heisler et al., 2005). We used a ProPIN1:PIN1:GFP marker to visualize PIN1 expression in vegetative SAMs defective in mp and/or auxin transport functions. Our analysis showed that PIN1 expression was most pronounced in discrete epidermal spots on the surface of vegetative WT SAMs and internal procambial midveins of young primordia (Fig. 2-6 A), in agreement with previous findings from the reproductive SAM. In mp meristems, PIN1 expression domains were more diffuse, occurred in defective phyllotactic patterns, and expression appeared spuriously in cells that are normally not involved in primordia formation (Fig. 2-6 B). PIN1 expression in NPA-grown WT seedlings was very weak or absent in the central zone area of the meristem thereby forming a ring of high expression in the peripheral zone possibly predicting the future formation of a tubular leaf (Fig. 2-6 C). Remarkably, in NPA-grown mp plants, PIN1 expression was not even restricted to the peripheral zone and instead expression was evenly distributed throughout the entire surface of young domes, including the central zone and
more basal parts of the leafless dome (Fig. 2-6 D). To assess if the lack of \textit{PIN1} focus formation in NPA-grown \textit{mp} plants is accompanied by a lack of auxin maxima formation, we analyzed the expression of the auxin responsive \textit{Pro\textsubscript{DR5}:GUS} marker. In \textit{WT} seedlings, \textit{Pro\textsubscript{DR5}:GUS} is expressed initially at the apices of emerging leaf primordia, and also internally in leaf primordia in conjunction with the formation of procambial tissues but \textit{Pro\textsubscript{DR5}:GUS} expression is not found in the central and peripheral zones of the SAM (Fig. 2-6 E) (Mattsson et al., 2003). In \textit{mp} seedlings, the \textit{Pro\textsubscript{DR5}:GUS} expression in leaf primordia apices was always more diffuse than in \textit{WT} seedlings (Fig. 2-6 F). \textit{WT} plants responded to NPA with a considerable delay in leaf primordia formation and when leaf primordia emerged, the \textit{Pro\textsubscript{DR5}:GUS} expression was found at the margins of the circular or close to circular leaf primordia (Fig. 2-6 G). At no point did we observe localized \textit{Pro\textsubscript{DR5}:GUS} expression at the flanks of NPA-grown \textit{mp} meristems (Fig. 2-6 H). In summary, the leafless dome meristems of NPA-grown \textit{mp} mutants show defects in the focusing of \textit{PIN1} expression and do not form local auxin-response maxima as judged by \textit{Pro\textsubscript{DR5}:GUS}.

**Leaf founder cell markers are expressed in leafless dome meristems**

The synergistic phenotype in \textit{mp pin1} double mutants suggests that \textit{MP} acts not only through regulation of polar auxin transport in the process of leaf formation, but may separately promote the growth of leaf primordia. Potential target genes could be involved in leaf founder cell fate specification or associated with subsequent organ outgrowth. The \textit{AINTEGUMENTA (ANT)} and \textit{ASYMMETRIC
LEAVES 1 (AS1) genes are expressed in the leaf founder cell population and subsequently during outgrowth of leaf primordia (Elliott et al., 1996; Long and Barton, 1998; Byrne et al., 2000). We used the expression of these genes to assess if leaf founder cell populations are established at the flanks of the meristem in leafless domes. In WT plants, we found that the expression of these markers preceded the formation of leaf primordia and that they were expressed in outgrowing primordia (Fig 2-6 I,M), in agreement with published results. The expression of ANT and AS1 in mp mutants appears identical to WT expression patterns (Fig. 2-6 L,P) except for the defects in phyllotaxy already described (Fig. 2-3 B,C). In response to NPA, WT plants expressed ANT and AS1 in a circular domain in the meristem (Fig. 2-6 K,O) consistent with the subsequent formation of a tubular leaf. We observed a similar ring-shaped expression of ANT and AS1 near the apex of leafless domes in NPA-grown mp plants (Fig. 2-6 L,P). Thus, leaf founder cell populations appear to be specified in the peripheral zones of WT and mp plants treated with NPA but this specification is not sufficient for leaf formation in the latter. The failure to form leaves in leafless domes appears to be due to a defect in outgrowth of leaf primordia. In WT plants, early leaf initiation can be detected by a switch from anticlinal to periclinal cell divisions in the L2 layer (Medford et al., 1992). We screened longitudinal medial sections of more than 15 leafless domes without finding any indications of periclinal divisions in the L2 layer. Instead we observed smooth surfaces of the peripheral zone, and a pattern of cell walls in the L2 layer that indicated strict anticlinal cell division planes (Fig. 2-6 Q,R).
In summary, we conclude that the defect in leaf primordia formation in NPA-grown $mp$ plants does not involve a block in the formation of leaf founder cells, but appears to involve a block of subsequent periclinal divisions in the process of leaf outgrowth, which appears to depend on $MP$ activity.

2.5 Discussion

Several lines of evidence have indicated that $PIN$ gene expression is auxin (Heisler et al., 2005; Vieten et al., 2005; Scarpella et al., 2006; Vieten et al., 2007) and ARF (Sauer et al., 2006; Wenzel et al., 2007) dependent suggesting that $MP$ functions in leaf initiation by mediating the $PIN$ gene expression. In this case, however, one would expect that loss of $MP$ function should not matter in plants severely compromised in auxin transport. Here we observed that $mp$ mutants of various allele strengths are hypersensitive to NPA treatment and display synergistic defects in double mutants with $pin1$. These findings provide strong evidence for an involvement of $MP$ in a process beyond the control of auxin transport. Importantly, the synergistic defects cannot be mimicked by applying increased concentration of NPA to $WT$ or $pin1$ plants, further supporting that $MP$ regulates further, hitherto unexplored processes to promote leaf initiation. As one of those processes, we propose that $MP$ has a role in promoting the actual outgrowth of leaves and flowers. Notably, it has also been suggested that activating ARFs, including MP, could bind to the promoters of auxin-regulated leaf specification genes thereby promoting leaf formation in the peripheral zone of the meristem while interaction with other ARFs limit this action.
in the central zone of the meristem (Leyser, 2006). Given this scenario, ARFs like MP would therefore be implicated in also having functions in conferring differential properties to zones in the SAM.

Reinhardt et al. (2003) have formulated a model in which leaf primordia form at sites of elevated epidermal auxin concentration. Pre-existing primordia are thought to influence the position of new primordia by depleting the vicinity of auxin through auxin transport. Thus, new leaf primordia would only form at sites far enough away from existing primordia to allow new auxin maxima to form. This mechanism would not only explain the dependence of leaf formation on auxin maxima, but also the phyllotactic pattern of leaves and how it is influenced by the position of pre-existing primordia. Mathematical modelling of leaf initiation based on these findings postulated a positive feedback loop concentrating auxin into concrete spots on the surface of the SAM, because of positive influence of auxin on the amount and orientation of PIN1 efflux carriers in neighboring cells (de Reuille et al., 2006; Jonsson et al., 2006; Smith et al., 2006). These models also proposed that no further specified mechanisms restrict the self-regulated auxin-focusing process to the peripheral zone of the SAM. Mutants in the PIN1 gene as well as NPA treated plants fail to focus auxin through convergent PIN1 polarity (Heisler et al., 2005) and do not form flowers from the inflorescence meristem (Okada et al., 1991; Vernoux et al., 2000). This is evidenced by the fact that in NPA treated plants, the concentration and polarization of PIN1-GFP towards individual spots is much reduced (Heisler et al., 2005). These interpretations are also consistent with the fact that flowers can be formed in both pin1 mutant and
NPA treated inflorescence meristems upon local application of auxin. Apparently, the local application bypasses the need for auxin-transport driven focusing of auxin towards flower initiation sites.

\textit{MP} is another likely component of the postulated mechanism since \textit{mp} mutants also fail to form flowers from the inflorescence meristem and have reduced auxin transport capacity (Przemeck et al., 1996). Further, \textit{MP} encodes an auxin response factor (Ulmasov et al., 1997; Hardtke and Berleth, 1998), which might be involved in the auxin-dependent regulation of PIN expression (Sauer et al., 2006; Wenzel et al., 2007). No flowers can be induced by local auxin application on the flanks of \textit{mp} inflorescence meristems (Reinhardt et al., 2003), suggesting that it is not only auxin transport and auxin accumulation that is defective in \textit{mp} mutants, but also a failure to trigger lateral organ outgrowth even when auxin is locally provided (Reinhardt et al., 2003). Thus published auxin application experiments already hint to a role of \textit{MP} in controlling auxin responses in lateral organ outgrowth.

The inhibition of auxin transport in \textit{mp} mutant backgrounds generates an unprecedented type of abnormal SAM development, which not only completely obstructs the formation of lateral organs but also vastly expands the shoot apex. Marker gene expression indicates that the enlarged apical dome is composed of expanded \textit{STM} and \textit{CLV3} expressing domains surrounded by a wide circular peripheral zone, marked by \textit{ANT} and \textit{AS1}. Although no leaf primordia are formed under these conditions, there seems to be some dispersed growth as the \textit{ANT} and \textit{AS1} expression domains are extremely wide.
Under conditions of normal auxin transport, ARFs acting redundantly to
*mp* appear to be sufficient for triggering organ formation from the vegetative, yet
not from the reproductive SAM, as *mp* mutants produce leaves. Conversely,
inhibition of auxin transport seems to allow for sufficient auxin focusing in the
epidermis to trigger vegetative leaf initiation as long as *MP* is functional.
However, poorly defined leaf initiation points seem to be insufficient to trigger
organ outgrowth through redundantly acting ARFs when *MP* is not functional.
While failed leaf initiation may thus be explainable as the superimposition of
defects in two interdependent steps, the reasons for the enlargement of the
central zone seem to reflect other, unknown levels of control. It has been
proposed that the restriction of leaf-initiating auxin focusing to the peripheral
zone reflects auxin sensitivity zones due to the specific expression domains of
competing ARFs (Leyser, 2006). In this interpretation it is plausible that the
removal of an important ARF may destabilize the zoning sufficiently to promote
cell proliferation also in the central zone. In this context it is remarkable that we
observed equally strong PIN1-GFP expression in the peripheral and central
zones uniquely in NPA exposed *mp* mutants. Formally, it is also possible that
the expansion of the central zone could be a necessary consequence of
defective lateral organ formation. Several levels of mutually antagonistic gene
activities have been implicated in the control of stem cell pool size of the shoot
meristem (reviewed in; (Clark, 2001; Williams and Fletcher, 2005; Carraro et al.,
2006; Tucker and Laux, 2007) in which some negative regulators originate from
the peripheral zone. As there are no other leafless genotypes available, we
cannot genetically separate leaflessness from SAM expansion. However, it should be noted that in the inflorescences of *pin1* mutants devoid of lateral flowers, the size of the meristem and its constituent zones have been described as normal (Vernoux et al., 2000; Fig.2-9), arguing against a mechanism where signals negatively regulating shoot meristem size are derived from concrete flower or leaf primordia.

The sizes of SAMs vary considerably across the plant kingdom (Steeves and Sussex, 1989) and the influences of new regulators on SAM size are continuously being revealed (Chaudhury et al., 1993; Clark et al., 1993, 1995; Running et al., 2004; Green et al., 2005; Chiu et al., 2007). The discovery of highly abnormally sized SAMs as a consequence of simultaneous interference with auxin transport and ARF function may provide an entry point in the genetic analysis of auxin’s role in this process.
2.6 Figures

Figure 2-1 Development of leafless domes from *mp* meristems. (A) WT rosette of leaves at 14 days after germination (DAG) compared to *mp* at 21 DAG (B). (C,D) Photograph and SEM of *mp pin1* double mutant at 40 DAG. *mp pin1* double mutants at 60 DAG (E-F) and 75 DAG (G,H). Multiple leafless domes (E), and example of extreme fasciation leading to leafless flattened structures (F). Examples of filamentous projections (G,H) sometimes ending in pistil-like structures (I). *mp pid* double mutant at 50 DAG (J). Single fused leaf and no cotyledons in a *pid pin1* double mutant at 14 DAG (K). A tubular 3rd leaf in a 21 DAG WT seedling treated with 10µM NPA (L). *mp* grown on medium with 10µM NPA at 50 DAG (M), 40 µM HFCA at 35 DAG (N), and 40 µM TIBA at 35 DAG (O). Leafless dome formation in Pro\textsubscript{35S:MP} plant grown on medium with 10 µM NPA for 40 DAG (P). Scale bars: 1µm in A,B; 500 µm in C,E-H,J-P; 100 µm in D; 50µm in I.
Figure 2-2 Frequency of leafless dome formation in response to NPA.
(A) WT Columbia, WT Enkheim, pin1(Enk) segregating population, pid segregating population, Pro35S:MP and mp plants were grown on a series of media containing 0-100 µM NPA. All genotypes were scored at 35 DAG and leafless dome formation was judged by presence of leafless dome structure. Between 42 and 178 plants were scored for each genotype and treatment. (B) WT Col (dark bars) and mp (light bars) plants were grown on media as in (A) and were scored for number of leaf primordia visible under a dissecting microscope at 21 DAG. ** illustrates significant difference between NPA-grown WT and mp mutants within the respective NPA treatment as determined by student’s t test analysis, p < 0.05. Error bars indicate standard deviation.
Figure 2-3 Phyllotactic defects in the *mp* meristem and the initiation of leafless domes.

The first leaf primordia in WT seedlings (A), and *mp* mutants (B,C). *mp* mutants with two opposite cotyledons were used for analysis to preclude any effects of cotyledon placement on subsequent leaf primordia formation. WT (D) and *mp* mutants (E-I) grown on medium with 10µM NPA. The cells in the peripheral region of the NPA-grown *mp* SAM elongate to form a collar of cells (arrows in E-I) which have leaf cell fate as judged by *in situ* hybridization with an AS1 anti-sense probe (F). Central region of meristem indicated by arrow heads in G-I. All samples are 2 DAG except (H) 4 DAG, (I) 9 DAG. Scale bars: 50µM in A-I
Figure 2-4 Expression of STM in leafless domes and procambial defects.
Expression of ProSTM::GUS in: WT (A), mp (B), WT grown on medium with 10μM NPA (C,E), and mp grown on medium with 10μM NPA (D,F-I). Arrows in (F,I) indicate expression of ProSTM::GUS in elongated procambial cell types orientated along the longitudinal axis. Longitudinal medial sections of leafless domes shows procambial strands (H,I) that are frequently interrupted (I). (A-D) 7 DAG, (E-I) 21 DAG, Size bars 50μm (A-G) and 10μm (H,I).
**Figure 2-5 Quantification of central zone and meristem areas.**

WT or *mp* plants were grown in absence or presence of 10 μM NPA and the areas based on *ProCLV3:GFP:ER* expression domains at 10 (A) and 21 DAG (B), and *ProSTM:GUS* expression domains at 10 (C) and 21 DAG (D). Y-axis show measured areas in 10^2 μm^2. Bars represent average of measured areas from 6-13 meristems, error bars are standard deviations. ** illustrates significant difference between NPA-grown *mp* mutants compared to all other genotypes and treatments, as determined by student’s t test analysis, p < 05. Representative images of measured areas are shown in Fig. S2.
Figure 2-6 Marker analysis of leafless dome meristems
Material grown on medium supplemented with 10µM NPA indicated as "+ NPA". ProPIN1::PIN1::GFP expression in WT (A), mp (B), WT + NPA (C), and mp NPA (D). Order of leaf primordia, present and incipient, is indicated in WT (A) and mp (B). Aberrant expression is indicated by I. ProDR5::GUS expression in WT (E), mp (F), WT + NPA (G), mp+NPA (H) at 21 DAG. ANT anti-sense probe at 3 DAG in WT (I), mp (J) and 21 DAG WT+NPA (K) and mp+NPA (L). AS1 anti-sense probe at 3 DAG in WT (M), mp (N) and 21 DAG WT+NPA (O) and mp+ NPA (P). Apex of 21 DAG leafless dome (Q) and higher magnification of marked area in (R). Arrows in (R) indicate cell walls produced by anticlinal cell divisions. All size bars are 50µm except for (H) which is 100µm, and (R), which is 25 µm.
Figure 2-7 Developmental series of AS1 and STM expression in leafless domes.
Whole mount in situ hybridization illustrating the switch of AS1 expression which is initially expressed in a collar of cells surrounding the meristem at 3 and 4 DAG to simultaneous expression in the meristem at 6 DAG and is exclusively expressed in the enlarging meristem from 9 DAG onwards. STM is expressed in the meristem region of developing domes throughout all time points. (A,B) 3 DAG, (C,D) 4 DAG, (E,F) 6 DAG, (G,H) 9 DAG, (I,J) 21 DAG, scale bars are 50μm.
Figure 2-8 Quantification of central zone and meristem areas.
Material grown on medium supplemented with 10μM NPA indicated as "+ NPA". Representative images of *Pro*\textsubscript{CLV3}:GFP:ER and *Pro*\textsubscript{STM}:GUS expression at 3, 10 and 21 DAG in WT, mp, WT +NPA, and mp+NPA meristems. Size bars are 50μm.
Figure 2-9 Central zone sizes are comparable in WT and pin1-1 inflorescence tips.

Pro\textsubscript{CLV3}:GFP:ER expression in 4 week old (A) WT and (B) pin1-1 mutant reproductive Shoot Apical Meristems. Size bars are 30\mu m.
Figure 2-10 *MP* and *DR5* expression dynamics in WT meristems under control and 10 NPA treatments

*Pro<sub>DR5::GUS</sub>* (A,B) and MP whole mount in situ (C,D) expression dynamics in 4 week old WT meristems (A,C) WT DMSO, (B,D) WT 10µM NPA, (A,B) Size bars are 50µm.
2.7 Tables

Table S1. Segregation of double mutants

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<th>genotype, parental plants</th>
<th>phenotype, offspring</th>
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<td></td>
<td>WT</td>
</tr>
<tr>
<td>mp/+pin1/+</td>
<td>425 (61)</td>
</tr>
<tr>
<td>mp/+pid/+</td>
<td>247 (57)</td>
</tr>
<tr>
<td>pin/+pid/+</td>
<td>468 (57)</td>
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</table>

Table 2-1 Segregation of double mutants

Segregation of mp pin1, mp pid and pid pin1 double mutants stemming from doubly heterozygous parental plants. Double mutant populations were identified first by screening for mp seedling phenotypes and pin1 inflorescence phenotypes. In double mutant populations, seedlings with mp phenotypes were transferred to new petridishes and kept in short day conditions for observation of possible segregation of mp single and double mutant phenotypes in the vegetative meristem. Plants were scored for parental phenotypes (mp, pin1, pid), and for phenotypes different from parental phenotypes (novel; see figure 1 for description). Absolute numbers are followed by percentage of total number, given in brackets. Expected number is based on a 9:3:3:1 segregation of two unlinked loci. Statistical analysis of segregation ratios using Chi-square analysis resulted in no statistical difference between expected and observed numbers of individuals with novel phenotypes in the segregating population; mp pin1 double mutant p = 0.75, mp pid double mutants p = 0.69, pin1 pid double mutants p = 0.88.
Table 2-2 Phyllotactic defects in *mp* dicotyledoneous seedlings

The categories indicate conformation of the first leaf primordia in dicotyledoneous 7 DAG WT and mp mutant seedlings as absolute numbers and their percentage contribution in parentheses.

<table>
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<th>WT dicot</th>
<th>mp dicot</th>
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<tr>
<td>double opposite</td>
<td>50 (100)</td>
<td>16 (30)</td>
</tr>
<tr>
<td>one reduced</td>
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<td>10 (18)</td>
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<tr>
<td>single</td>
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<tr>
<td>total</td>
<td>50 (100)</td>
<td>54 (100)</td>
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2.8 References


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3: **WUSCHEL IS NECESSARY FOR LEAF FORMATION IN MONOPTEROS MUTANTS**

Statement of contribution:

M. Schuetz performed all experimental work and wrote the manuscript together with J. Mattsson.

### 3.1 Abstract

We have previously found that a combination of mutation in *MONOPTEROS* (*MP*), an auxin response factor, and *PIN-FORMED 1* (*PIN1*), an auxin efflux carrier, results in plants with oversized leafless shoots. This phenotype is associated with an enlarged central zone of the meristem. Therefore, it is possible that the failure of *mp pin1* mutants to produce leaves is due to the enlargement of the central zone. To test this hypothesis, we generated double mutants of *mp* with *clavata 1* (*clv1*), a mutant defective in restricting central zone size, and *wuschel* (*wus*), a mutant defective in central zone maintenance. The *mp clv1* mutants were able to form leaves and showed additive phenotypes. We therefore conclude that the loss of leaf initiation in *mp pin1* mutants is not a result of an oversized central zone domain. On the other hand, *mp wus* mutants generated oversized leafless shoots somewhat similar to *mp pin* mutants. The overall smaller size of leafless shoots produced in *mp wus* mutants compared to
mp pin1 mutants correlated with defective formation of a central zone of stem cells. We hypothesize that WUS normally acts to suppress auxin signaling in the central zone. In the absence of this suppression, together with defective MP-mediated auxin signaling, auxin is insufficiently focused in the peripheral zone leading to a block in leaf initiation.

3.2 Introduction

The shoot apical meristem (SAM) is the region of the plant from which the postembryonic shoot system is derived. The indeterminate nature of plant growth is facilitated by plant meristems, which must balance the number of cells lost to differentiating organs while maintaining a population of stem cells. The SAMs of angiosperms share a common organization where the meristem is composed of two clonally distinct outer layers (L1 and L2) with predominantly anticlinal divisions supported by an underlying third layer (L3) displaying more randomly orientated cell divisions (Esau, 1965). The meristem is divided into a Central Zone (CZ) of generally slowly dividing cells on the apical most region of the meristem, which is surrounded by a Peripheral Zone (PZ) of more rapidly dividing cells (Esau, 1965; Reddy et al., 2004). The maintenance of the CZ of stem cells has been characterized in Arabidopsis thaliana and is regulated by a negative feedback loop involving the WUSCHEL (WUS) and CLAVATA (CLV) genes (Schoof et al., 2000). The WUS gene is a homeodomain-containing transcription factor, which is essential in specifying and maintaining CZ stem cell
fate (Mayer et al., 1998). The SAM is specified during embryogenesis and expression of genes including *SHOOTMERISTEMLESS (STM)* is unaffected at the time of germination in *wus* mutants (Mayer et al., 1998). In *wus* mutants, the loss of cells from the meristem to differentiating leaves and lack of stem cell renewal leads to the termination of the SAM after formation of one to several leaves (Laux et al., 1996; Mayer et al., 1998). However, post embryonic growth continues in *wus* plants from axial meristems after SAM termination (Laux et al., 1996).

Three genes have been identified that negatively regulate *WUS* expression thereby limiting the size of the CZ. The *clavata* mutants share a common phenotype in having enlarged CZ encompassing increased numbers of stem cells resulting in larger SAMs (Clark et al., 1993, 1995; Kayes and Clark, 1998). *WUS* is expressed in a domain of cells directly below the CZ of the meristem and functions to specify stem cell fate including activation of the *CLV3* gene (Mayer et al., 1998; Schoof et al., 2000). *CLV3* encodes a small secreted signaling peptide and is a marker for CZ identity (Fletcher et al., 1999). *CLV3* is sequestered by the *CLV1/2* heterodimeric receptor protein, which is expressed below the peripheral zone in the area surrounding the *WUS* expression domain (Fletcher et al., 1999; Rojo et al., 2002; Lenhard and Laux, 2003). The membrane bound *CLV1/2* receptor protein is composed of *CLV1*, encoding a receptor like protein kinase of which *CLV2* is a crucial component required for *CLV3* binding (Clark et al., 1997; Jeong et al., 1999; Trotochaud et al., 2000). The *CLV3* bound *CLV1/2* receptor leads to subsequent signal transduction,
resulting in the suppression of \( WUS \) transcription. (Schoof et al., 2000; Lenhard and Laux, 2003).

Recent evidence suggests that \( WUS \) signals the overlying cells to maintain stem cell identity, in part through negative regulation of cytokinin-inducible type-A response regulators (ARR) in \( A. \) thaliana (Leibfried et al., 2005). Cytokinins are a class of plant hormones which were discovered for their effects in promoting plant cell division and have been shown to be important regulators of shoot development (reviewed in To and Kieber (2008)). Type-A ARR’s have been implicated in negatively regulating cytokinin signaling, where overexpression of these genes leads to cytokinin insensitivity and loss of function mutants lead to cytokinin hypersensitivity (Hwang and Sheen, 2001; Kiba et al., 2003; To et al., 2004; Leibfried et al., 2005). In the model presented by Leibfried et al (2005), \( WUS \) binds to the promoters of multiple type-A ARR genes and functions to repress their transcription in the central zone thereby enhancing the ability of central zone cells to respond to cytokinin.

Three cytokinin receptors have been identified in \( A. \) thaliana of which multiple mutants display stronger phenotypes (reviewed in To and Kieber (2008)). Triple cytokinin receptor mutants display various phenotypes including smaller meristems and a reduction in leaf initiation rate indicating that cytokinin perception plays a role in these processes (Nishimura et al., 2004; Shani et al., 2006). This evidence is supported by studies that limit the amount of active cytokinin in plants through misexpression of cytokinin degrading enzymes (Werner et al., 2001; Werner et al., 2003; Shani et al., 2006). Plants with reduced
cytokinin levels display similar defects as observed in the cytokinin receptor triple mutants. Interestingly, genes that are necessary for meristem function like STM and BREVIPEDICELLUS (BP), have been implicated in positively regulating cytokinin synthesis (Jasinski et al., 2005; Yanai et al., 2005). These genes may therefore carry out their function in part by maintaining relatively high cytokinin levels throughout the life of the meristem (Jasinski et al., 2005; Yanai et al., 2005).

Loss of function mutations in genes involved in auxin transport or signaling have been identified and display various defects in the initiation of lateral organs, ie, flowers or leaves from SAMs (Okada et al., 1991; Galweiler et al., 1998; Hardtke and Berleth, 1998; Christensen et al., 2000). The PINFORMED1 (PIN1) gene is a member of a small family of membrane-bound auxin efflux proteins that has been shown to mediate auxin transport out of cells (Galweiler et al., 1998; Petrasek et al., 2006). PIN-mediated auxin transport appears to be achieved by the asymmetric localization of PIN proteins to one side of cells leading to directional auxin flow. With the use of the subcellular localization of various PIN::GFP fusion proteins, significant advances in the understanding of how developmental processes are coupled to polar auxin transport (PAT) have been made (Benkova et al., 2003; Friml et al., 2003; Vieten et al., 2007). Examination of PIN1-mediated auxin transport dynamics in the shoot apical meristem revealed that auxin is acropetally transported to incipient lateral organ primordia mainly in the L1 layer of the meristem (Reinhardt et al., 2003). Once a threshold level of auxin is reached, auxin transport is reversed and proceeds basipetaly
through the central region of the initiated leaf or flower (Reinhardt et al., 2003; Heisler et al., 2005). The subcellular distribution of PIN1 proteins is regulated by the PINOID (PID) protein kinase and pid loss of function mutants display identical defects in flower initiation as observed in pin1 mutants (Bennett, 1995; Christensen et al., 2000; Benjamins et al., 2001). Local auxin application experiments on WT plants and mutants severely compromised in auxin transport have shown that auxin is sufficient to initiate lateral organs from SAMs (Reinhardt et al., 2000; Reinhardt et al., 2003). MONOPTEROS (MP) is a member of the Auxin Response Factors (ARFs) gene family of transcription factors that play key roles in auxin responsive gene regulation (Hardtke and Berleth, 1998; Mattsson et al., 2003; Hardtke et al., 2004). mp mutants were more resistant to auxin-mediated adventitious root formation than the well-characterized auxin resistant1 (axr1) mutant, demonstrating that the mp mutant is severely compromised in auxin perception (Mattsson et al., 2003). mp mutants display flower initiation defects similar to pin1 mutants (Okada et al., 1991; Przemeck et al., 1996). However, local auxin application is not able to rescue flower initiation in mp reproductive meristems in contrast to what is observed in pin1 mutants (Reinhardt et al., 2003). These studies have demonstrated that auxin signal transduction is essential in mediating developmental processes like flower and root formation while defects in auxin transport can be rescued by local auxin application.

The synergistic leafless dome phenotype resulting from simultaneous interference of auxin signaling and auxin transport in PAT-inhibited mp mutants
suggests that MP acts not only through regulation of auxin transport in the initiation of lateral organs from SAMs (Schuetz et al., 2008). The meristematic region residing on the apex of these leafless domes was enlarged as judged by increased STM and CLV3 expression domains (Schuetz et al., 2008). In addition to the leafless phenotype and enlarged meristematic domains of PAT-inhibited mp plants, extreme fasciation of the meristem region was observed. Fasciated structures formed after prolonged culture and resulted in the formation of multiple filamentous-like projections (Schuetz et al., 2008). Observations of petal-like and pistil-like structures forming at the tips of the filamentous projections identifies these structures as small inflorescences (Schuetz et al., 2008). Although expression of the leaf founder cell markers ASYMMETRIC LEAVES 1 (AS1) and AINTEGUMENTA (ANT) was observed in the peripheral regions of the enlarged meristematic zone of PAT inhibited mp plants, leaf outgrowth did not occur (Schuetz et al., 2008). In this context, it seems possible that the expanded meristem domain could have allowed for leaf founder cell fate to be specified while those cells, at least in part, maintained CZ cell fate. It has been demonstrated that silencing of CLV3 expression results in expansion of the CZ in part by the re-specification of PZ cells to adopt CZ fate (Reddy and Meyerowitz, 2005). Although the re-specification of PZ zone cells into CZ cells resulted in the enlargement of the meristem, it appeared to have limited impact on flower formation (Reddy and Meyerowitz, 2005). This result argues against PZ re-specification leading to defects in lateral organ initiation. In order to explore this
question further, *mp clv1* and *mp wus* double mutants were generated, thereby genetically expanding and eliminating the CZ in *mp* mutants respectively.

### 3.3 Materials and Methods

**Plant material and growth**

The *mp*<sup>G12, G33</sup>, *wus*-1 and *clv1*-1 mutant alleles used for double mutant and single mutant analysis have previously been described (Clark et al., 1993; Hardtke and Berleth, 1998; Mayer et al., 1998). Plants harbouring the *Pro<sub>CLV3</sub>:GFP:ER* (Lenhard and Laux, 2003) or *Pro<sub>CLV3</sub>:GUS* (Brand et al., 2002) transgenes were crossed with *mp* heterozygous plants and *mp* mutants expressing these markers were isolated in subsequent generations. Surface-sterilized seeds were grown on ATS medium (Lincoln et al., 1990) and exposed to NPA as described in (Mattsson et al., 1999).

To generate *mp clv1* double mutants *clv1* homozygous pollen was used to pollinate heterozygous *mp* plants and the resulting F1 generation was allowed to self fertilize. *clv1* homozygous plants were isolated from the resulting F2 generation and their offspring populations were screened for *mp* mutants displaying lack of hypocotyl and root phenotypes. The identified *mp* plants would have the *mp clv1* double mutant genotype and were analyzed in parallel to *mp* single mutants.

Heterozygous *wus* plants were crossed with heterozygous *mp* plants since homozygous mutation in either plants are infertile. The resulting F1 generation was allowed to self fertilize and the resulting F2 generation would include *mp*
wus double heterozygous plants. Individual F2 plants that segregated for both mp and wus were used for further analysis.

**In situ hybridization, Histology and GUS assays**

STM and CLV3 gene fragments were amplified from cDNA generated from total RNA extracted from 14 day old WT seedlings using Trizol reagent (Invitrogen) and subsequently reverse transcribed using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) and cloned into pBluescript II Sk(-) (Stratagene). Primers used for amplification were STM-F (TCCTTCCTCTTGTGCTCCTG), STM-R (CAACATCAAACGCTTTGTTCA) and CLV3-F (TTCCTTCATGATGCTTCTGG) and CLV3-R (CCAAACGAAACAGATTGCAC). Whole mount in situ hybridization procedure was performed as described (Zachgo et al., 2000) with the following modifications. Fixation of plant tissue was performed overnight instead of 30 min. After the proteinase K treatment and post fixation step, plant tissue was incubated in a fresh solution containing 0.1 M triethanolamine (pH 8) and 0.5% (v/v) acetic anhydride for 15 min. The triethanolamine treatment was followed by two washes in 1x PBT solution prior to hybridization for two days at 60°C. Localization of β-glucuronidase activity was carried out as described in Mattsson et al., (2003)

**Microscopy**

A Zeiss LSM 410 was used to image Pro\(_{CLV3}:GFP:ER\) 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. DIC images were taken on a Nikon Eclipse 600 microscope using a Canon D30 digital camera. Samples for Scanning electron micrographs were fixed o/n in 2.5% glutaraldehyde in 50 mM cacodylate buffer, dehydrated in a graded ethanol series before being critically point dried and mounted on stubs. Samples were then coated with gold-palladium in a SEM Prep2 sputter coater (Nanotech), and imaged using a Hitachi S-2600N VP-SEM.

3.4 Results

In order to evaluate the effect of CZ size on leaf initiation in PAT-inhibited mp mutants, we first carried out a detailed examination of the expansion of CLV3 expression domains in mp mutants grown in the presence of the PAT inhibitor NPA (1-naphthylphthalamic acid). Thereafter, we manipulated the size of the CZ in mp mutants by generating mp clv1 and mp wus double mutants.

The expanded CZ in NPA-treated mp plants gives rise to numerous distinct CLV3 expressing centres

The expansion of CLV3-expressing cells in the meristematic tip of NPA-grown mp leafless domes is highly variable but does not appear to be significantly different at 10 or 21 DAG (Schuetz et al., 2008). Domains of maximum expression of CLV3, as judged by visualizing ProCLV3::GUS and ProCLV3::GFP, occurs during the vegetative phase of plant development before the initiation of pin-shaped outgrowths (Fig. 3-1 A and C). The switch to reproductive development appears to lead to a process of CZ refocusing. This phenomenon
is can be observed by the larger domains of CLV3 expression in newly formed leafless domes compared to older domes which appear to have switched to reproductive phase of development (Fig. 3-1 A, arrowhead and arrow respectively). During this process, large CLV3 expressing centres break up and give rise to numerous CLV3 expressing centres (Fig. 3-1 B, C, D). These smaller and newly formed CZs are correlated with the formation of pin-shaped projections, each expressing a single CLV3 expressing centre at the apex (Fig. 3-1 B, and E, F). Since the CLV3 expression domains on these projections are smaller than the large CLV3 expressing centre from which they arose, it suggests that additional factors act to restrict CZ size in reproductive SAMs.

**mp clv1 double mutants show additive phenotypes**

*mp* and *mp clv1* mutants were indistinguishable during vegetative development (Fig. 3-2 A and B). To assess inflorescence development in *mp* and *mp clv1* mutants, mutants that had formed adventitious roots were transferred to soil. Although no obvious differences were observed between *mp* and *mp clv1* mutants during vegetative development, fasciated inflorescence meristems were observed in *mp clv1* double mutants compared to *mp* mutants during reproductive development (data not shown). The double *mp clv1* mutant phenotype, with a seedling phenotype of *mp* mutants and a fasciated inflorescence typical of *clv1* mutants is additive, indicating that the two genes do not act in the same pathway.
We observed more extreme meristem fasciation in NPA-treated \textit{mp clv1} double mutants compared to NPA-treated \textit{mp} mutants (Fig. 3-2 C and D). Furthermore, the meristem fasciation observed in NPA-treated \textit{mp clv1} double mutants appeared to form at least several weeks earlier compared to NPA-treated \textit{mp} mutants (Fig. 3-2 C and D). Individual pin-shaped projections were phenotypically similar between \textit{mp} and \textit{mp clv1} mutants, but pistil-like structures formed from these projections appeared to be wider in NPA-treated \textit{mp clv1} mutants (Fig 3-2 E and F). We also interpret this phenotype as additive because \textit{mp} loss of function and auxin transport inhibition are known to result in fasciated inflorescences (Przemeck et al., 1996, Okada et al., 1991) and the phenotype seen here shows only quantitative rather than qualitative changes when compared to any single mutant effects.

\textbf{Putative \textit{mp wus} double mutants form leafless shoots}

In order to observe the effect of eliminating stem cell maintenance in \textit{mp} mutants, \textit{mp wus} double mutants were generated. \textit{mp} plants are easily identified due to lack of hypocotyl and root while \textit{wus} mutant plants can be identified by delayed leaf or aberrant leaf initiation compared to \textit{WT} (Fig. 3-3 A-D). The segregation ratio of offspring populations from \textit{mp wus} double heterozygous plants was close to what is expected for the segregation of two independent recessive loci (Table 3-1). No obvious phenotypic differences were observed in the \textit{mp}-like seedlings from \textit{mp wus} double heterozygous parental plants compared to single \textit{mp} mutants at 10 DAG (Table 3-1) (data not shown).
However, at 40 DAG the population of *mp*-like plants from *mp wus* double heterozygous parental plants could be separated into two categories based on morphological differences. All *mp* single mutant plants form a relatively normal rosette of leaves at 40 DAG compared to a portion of *mp*-like plants isolated from *mp wus* double heterozygous plants, which displayed various novel defects (Fig 3-3 B, E-H). The phenotype of putative *mp wus* double mutants is variable but includes a block in leaf formation and subsequent formation of a leafless dome structure somewhat similar to what is observed in NPA-treated *mp* plants (Fig. 3-3 F and H). Other putative *mp wus* double mutant phenotypes include formation of fewer leaves compared to *mp* single mutants, and leaf initiation occurring from enlarged apical regions (Fig. 3-3 E and G). The number of *mp*-like plants that germinated but did not undergo further development also increased from less than 1% in *mp* single mutants to approximately 10 % in *mp*-like plants from *mp wus* double heterozygous parental plants (data not shown). When *mp*-like plants with novel morphological defects and *mp*-like plants that do not undergo any further postembryonic growth are added together, this population of putative *mp wus* double mutants does not differ significantly from the expected proportion of double mutants based on chi square analysis (Table 3-1, p=0.67).

Leafless shoots of putative *mp wus* double mutants have lost meristem identity.

The unexpected formation of a leafless dome-like structure in putative *mp wus* double mutants was interesting since similar phenotypes are described in PAT-inhibited *mp* plants (Schuetz et al., 2008). However, significant differences
between the two structures were observed. The leafless domes from putative mp wus double mutants were smaller in overall size, never fasciated and did not form any pin-like projections compared to those observed in NPA-treated mp (Fig. 3-3 E-H). Apical regions of NPA-treated mp leafless domes exhibit expanded expression domains of STM and CLV3 (Schuetz et al., 2008). STM expression has been observed in wus mutant SAMs but is absent in terminated meristems of older wus seedlings (Mayer et al., 1998). In order to evaluate if STM or CLV3 expression is a prerequisite to formation of a leafless dome structure, expression of these genes was evaluated in putative mp wus plants. STM transcripts were detected in SAMs of mp seedlings in contrast to no expression in the leafless domes of putative mp wus double mutants (Fig. 3-4 A-B and E-F). However, STM expression was observed in the axial region between the cotyledon base and the base of the leafless dome (Fig. 3-4 F arrow). Expression of CLV3 transcripts in mp mutants was restricted to a small number of cells in the SAM (Fig. 3-4 C and D). No expression of CLV3 was detected in the leafless structures from putative mp wus double mutants (Fig. 3-4 H). The differences in CLV3 expression between mp and putative mp wus double mutants are in line with previous studies that have shown that WUS is necessary for CLV3 expression (Schoof et al., 2000). The lack of CLV3 expression in putative mp wus double mutant plants can be taken as evidence that these plants are indeed mp wus double mutants. We also conclude that the absence of both STM and CLV3 expression from putative mp wus leafless
domes shows that the activity of these genes is strictly not required for the formation of a leafless dome-like phenotype.

3.5 Discussion

Central zone size

The expanded CLV3-expressing central zones in NPA-treated mp leafless shoot meristems appear to undergo a process of refocusing during the vegetative to reproductive phase change. This notion is supported by the formation of numerous independent CLV3 expressing centres from large dispersed CLV3 expression domains (Fig. 3-1 C and D). These smaller CLV3 expressing centres subsequently give rise to numerous individual pin-shaped structures (Fig. 3-1 E and F). Previous studies using microinjection experiments of fluorescent dyes and localization of plasmodesmata in SAM cells have revealed that CZ and PZ cells are segregated into distinct symplasmic fields (Rinne and van der Schoot, 1998; Ormenese et al., 2000; Ormenese et al., 2002). Extensive reorganization of these symplasmic fields has been observed during the vegetative to reproductive meristem phase change in various plant species, indicating significant differences between vegetative and reproductive SAMs. (Rinne and van der Schoot, 1998; Ormenese et al., 2000; Ormenese et al., 2002). The meristem identity proteins STM and BP, have been shown to carry out their function in part by trafficking throughout the SAM (Lucas et al., 1995; Kim et al., 2002; Kim et al., 2003). STM protein trafficking in the meristem is particularly relevant to CLV3 expression since STM has been implicated in positively
regulating CLV3 expression in parallel with WUS (Brand et al., 2002). The reorganization of symplasmic connections between meristem cells could contribute to the observed redistribution of CLV3 expressing cells during the vegetative to reproductive phase change observed in NPA-treated mp mutant meristems.

The expansion of CLV3 expression domains previously observed in NPA-treated mp mutants suggested that the leafless shoot phenotype may be related to the expansion of the CZ (Schuetz et al., 2008). However, in this study we show that the combination of mp with the CZ-expanded mutant clv1 did not result in a leafless shoot phenotype, thereby rejecting this hypothesis. In addition, the additive effects observed in mp clv1 mutants and NPA-treated mp clv1 mutants suggests that MP and PAT activities are unrelated to CLV1 mediated signaling (Fig. 3-2 C, D). Since CZ size in mp mutant SAMs are comparable to WT during vegetative growth and MP is not expressed in the CZ, we hypothesize that MP indirectly restricts CZ cell fate. An obvious source of signals that could restrict CZ size, are differentiating cells in the peripheral zone of the meristem. However, we have previously shown that leaf founder cell fate is specified in peripheral zones of NPA-treated mp leafless shoots with enlarged CZs (Schuetz et al., 2008). These data suggest that concrete leaf primordia, rather than dispersed peripheral regions would be the sources for putative signals negatively regulating shoot meristem size.
Leaf initiation.

Various studies have demonstrated that the formation of local auxin maxima is required for the initiation of lateral organs from the shoot meristem (Reinhardt et al., 2000; Reinhardt et al., 2003; Heisler et al., 2005; de Reuille et al., 2006; Smith et al., 2006; Schuetz et al., 2008). The ARF transcription factor MP and the PIN1 auxin efflux protein appear to be key components mediating auxin maxima formation and lateral organ initiation (Reinhardt et al., 2000; Heisler et al., 2005; Wenzel et al., 2007; Schuetz et al., 2008). Recent evidence suggests that PIN1 expression is largely dependent on MP function (Wenzel et al., 2007) and that PIN1 transcription is regulated by auxin (Paciorek et al., 2005; Scarpella et al., 2006; Wenzel et al., 2007). However, while both mp and pin1 mutants show defective flower formation, both mutants are able to undergo the analogous process of leaf formation during vegetative development (Okada et al., 1991; Przemeck et al., 1996). We have previously shown that mp pin1 double mutants cease to form leaves almost directly after germination, and instead form an enlarged dome-shaped structure (Schuetz et al 2008). This phenotype can be copied by growing mp mutants on medium supplemented with auxin efflux inhibitors (Schuetz et al., 2008). There are several important conclusions from these observations. First, MP and PIN1 have important roles also in the process of leaf formation. Second, the synergistic phenotype observed in mp pin1 mutants is best explained by MP playing additional roles to promote leaf initiation, independent of PIN1 and polar auxin transport. This interpretation is consistent with the function of MP as a transcription factor, presumably regulating
the expression of multiple target genes. If MP and PIN1 act in part independently from each other in leaf initiation, it is not difficult to envision that other genetic components active in the meristem could act independently from MP.

Putative mp wus double mutant phenotypes show a reduction in leaf initiation that appears to subsequently result in the formation of enlarged apical shoot regions. The synergistic leafless phenotype of putative mp wus double mutants is not present in any of the single mutants and suggests that both MP and WUS act independently to promote leaf initiation. The phenotypes of putative mp wus double mutants are reminiscent of mp pin1 and NPA-treated mp mutant phenotypes (Schuetz et al., 2008). These mutants display a complete block in leaf initiation correlated with the subsequent formation of a large leafless shoot (Schuetz et al., 2008). Leafless shoots of putative mp wus double mutants are generally much smaller in size compared to mp pin1 or NPA-treated mp leafless shoots. This difference in size could be the result of defective stem cell maintenance in mp wus mutants that limits the supply of cells for incorporation into the meristem region. While MP is tied to leaf initiation both by function and expression, the WUS gene has not been linked directly to leaf initiation, nor is it expressed at sites of leaf initiation. It therefore appears likely that WUS indirectly promotes this process. Below we will provide some hypotheses on how WUS might mediate such a function.

We have previously shown that the auxin inducible PIN1 gene, normally confined to the peripheral zone, is also expressed in the central zone of NPA-treated mp mutants (Schuetz et al., 2008). It is possible that the reduced auxin
response and transport in these plants results in a failure to delimit auxin to the peripheral zone, presumably via defective canalization of auxin flow. A similar scenario could occur if the central zone normally has a reduced sensitivity to auxin due to the action of WUS, and that the perception of auxin in the CZ is enhanced in wus mutants. The combination of wus and mp could then result in a failure to create local auxin maxima required for leaf initiation in the peripheral zone. There is precedent for a reduced auxin perception in the CZ. Expression of reporter genes driven by the auxin responsive promoter DR5 show no auxin response in the CZ of vegetative A. thaliana meristems, suggesting low levels of auxin in this region (Mattsson et al., 2003; Heisler et al., 2005; de Reuille et al., 2006; Smith et al., 2006; Schuetz et al., 2008). There is recent evidence, however, that the cells of the central zone contain considerable amounts of auxin but are unable to respond to it (de Reuille et al., 2006). Similarly, topic application of auxin to the apex of leafless auxin transport-inhibited stems has no effect on the apex itself, but results in the formation of leaves from the peripheral region, suggesting that the apex is insensitive to auxin (Reinhardt et al., 2000; Reinhardt et al., 2003).

Local auxin transport to incipient lateral organ initiation sites results in high auxin/cytokinin ratios compared to the CZ of meristems that exhibit low auxin/cytokinin ratios (Shani et al., 2006). However, cytokinin is necessary for leaf initiation to occur as demonstrated by strong leaf initiation defects observed in cytokinin receptor mutants and in plants engineered to degrade active cytokinin (Werner et al., 2001; Werner et al., 2003; Nishimura et al., 2004; Shani
et al., 2006). WUS has been implicated in enhancing cytokinin signaling in the CZ and STM activity has been linked to promote cytokinin biosynthesis in the SAM (Jasinski et al., 2005; Leibfried et al., 2005; Yanai et al., 2005). Since STM expression is not observed in putative mp wus leafless shoots (Fig. 3-4 F) and WUS function is compromised, putative mp wus SAMs likely have low levels of active cytokinin and are less sensitive to cytokinin signaling. mp mutants have a reduced capacity for auxin signaling and transport, but vegetative meristems are able to undergo leaf initiation albeit with defective initiation patterns (Przemeck et al., 1996; Mattsson et al., 2003; Schuetz et al., 2008). If this is correct, it appears that both cytokinin and auxin can act as positive regulators of leaf initiation.
3.6 Figures

Figure 3-1 Localization of CLV3 expression in fasciated NPA-grown mp leafless domes.

Expression of Pro\textsubscript{CLV3}:\textit{GFP} (A inset, C-D,F) or Pro\textsubscript{CLV3}:\textit{GUS} (A,B) in the apex of \textit{mp} + NPA plants is reduced in developmentally more mature domes undergoing a switch to reproductive development (A,B). Expanded CLV3 expression domains give rise to numerous smaller independent CLV3 expressing centres associated with the formation of pin shaped projections from apical region of the initial mp + NPA leafless dome (C-F). \textit{mp} +10 \textmu M NPA at approximately 21 DAG (A inset, C and D) 35 DAG (B, E and F). All size bars are 100\textmu m except for A (inset), and D which are 50\textmu m.
Figure 3-2 Meristem stability in *mp* single and *mp clv1* double mutants treated with and without NPA.

*mp* single mutants (A) and *mp clv1* double mutants grown under control conditions (B) are indistinguishable during vegetative growth. *mp clv1* double mutants grown in the presence of NPA (D) display more severe meristem fasciation defects, which are generally observed earlier compared to *mp* single mutants grown under the same conditions (C). Pin-like projections formed from both, *mp* (E) and *mp clv1* (F) double mutants often terminate with pistil like cells. A-B, 21 DAG, size bars 1mm; C-D, 35 DAG, size bars 500µm; E-F, approx 50 DAG, size bars 100 µm.
Figure 3-3 Phenotypic analysis of putative *mp wus* double mutants compared to *mp* and *wus* single mutants.

*WT* (A) and *mp* (B) form a functional rosette of leaves at 21 DAG compared to aberrant placement and reduced number of leaf initiation events observed in *wus* mutants at 21 DAG (C-D). Novel phenotypes of putative 42 DAG *mp wus* double mutants, highlighting enlargement of the apical meristem region associated with loss or defective leaf initiation events (E-F). Size bars are 5mm in A-D and 500µm in E-H.
Figure 3-4 Expression of the meristem identity and maintenance markers in *mp* and putative *mp wus* double mutants.

*STM* transcripts are localized to the SAM in *mp* mutants (A, B) compared to more restricted expression domain observed in the cells between the enlarged leafless dome structure and the base of the cotyledon in putative *mp wus* double mutants (arrow in F). *CLV3* transcripts are localized to a subset of cells in the CZ of the SAM in *mp* mutants (C, D). *CLV3* transcripts are not detectable in putative *mp wus* double mutants (H). Representative images of whole mount in situ hybridization experiments in A-D, F, H. Putative *mp wus* double mutants before and after in situ hybridization in E-F and G-H respectively. 14 DAG *mp* plants (A-D) and 42 DAG putative *mp wus* double mutants (E-H) Size bars are all 100 µm except for B and D which are 50 µm.
3.7 Tables

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Table 3-1 Segregation of putative *mp wus* double mutants.

Segregation of *mp wus* double mutants stemming from doubly heterozygous parental plants. Double mutant populations were identified first by screening for *mp* seedling phenotypes followed by screening for *wus* phenotypes. Five double heterozygous plants were used for further analysis. In double mutant populations, seedlings with *mp* phenotypes were transferred to new petri dishes and kept in short day conditions for observation of possible segregation of *mp* single and double mutant phenotypes in the vegetative meristem. Absolute numbers are followed by expected percentages based on a 9:3:3:1 segregation of two unlinked loci followed by observed percentages. Statistical analysis of segregation ratios using Chi-square analysis resulted in no statistical difference between expected and observed numbers of individuals with novel phenotypes in the segregating population p = 0.67.
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4: DYNAMICS OF MONOPTEROS AND PIN-FORMED1 EXPRESSION DURING LEAF VEIN PATTERN FORMATION IN ARABIDOPSIS THALIANA.

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Statement of contribution:
M. Schuetz performed all experimental work relating to MP and PIN1 in situ hybridizations, crossing and imaging PrPIN1::PIN1::GFP and PrDR5::GUS in mp mutant backgrounds. Imaging of PrPIN1::PIN1::GFP in various stages of WT leaf development was also performed but was not included in the final publication. All the authors contributed to the writing of the manuscript.

4.1 Abstract

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.

### 4.2 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 (Kang and Dengler, 2002; Mattsson *et al*., 1999).

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 (Mattsson *et al*., 2003; Uggla *et al*., 1996) 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, AUXIN-RESISTANT6* disrupt vascular continuity (Hamann *et al*., 1999; Hobbie *et al*., 2000; Przemeck *et al*., 1996). The
incomplete vascular continuity in the mp mutant and 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 Aloni, 1995). 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; Boutté et al., 2006; Friml, 2003; Petrášek et al., 2006; Wiśniewska et al., 2006). Several ABC proteins are also involved in auxin transport, possibly acting independently of PINs (Murphy et al., 2002; Noh et al., 2001; Petrášek et al., 2006). Polar localization of PIN carrier complexes is mediated by vesicle trafficking (Steinmann et al., 1999), and by polarity and cell fate determinants (Friml et al., 2004; Treml et al., 2005; Xu et al., 2006). Auxin seems to regulate PIN transcription, cellular trafficking and localization (Blilou et al., 2005; Leyser, 2005; Paciorek et al., 2005; Vieten et al., 2005).

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; Xu 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 predominantly localized at the poles in vascular cells to transport auxin from the stem toward 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 (Mattsson et al., 1999).

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 channelled into a file of cells that eventually undergo vascular differentiation (Sachs, 1981). 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; Sieburth, 1999). A reaction-diffusion model based on the interaction of two or more diffusing substances has also been used to describe vascular patterning (Koch and Meinhardt, 1994). Local fluxes of an activator such as auxin may trigger a positive feedback loop leading to cells with high activator concentration that undergo vascular differentiation. Koizimi 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 modelling (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).

4.3 Materials and Methods

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). Sterilized seeds were plated in 9 cm petri dishes containing solid Arabidopsis thaliana salts media (ATS; Lincoln et al., 1990). For the auxin transport inhibitor treatments, seedlings were plated on media containing 0.1, 1 or 10 µM 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 9 cm 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.

**PIN1 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 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). Adobe Photoshop software was used to merge images with slightly different focal planes. DIC images were taken on a Nikon Eclipse E600 microscope using a digital camera.

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).

4.4 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) transcript and PIN-FORMED 1 (PIN1) protein and their potential interaction with auxin. Vascular terminology are as described in Mattsson et al. (2003) and Hickey (1979). 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).
Dynamics of *MONOPTEROS (MP)* expression

In this study, we show how *MP* expression is progressively restricted from broad regions to pre-procambial cells. In 2 DAG leaf primordia, *MP* is initially expressed internally throughout the lamina excluding the margin in first leaf primordia (Fig. 4-1 A,B) or at elevated levels in the incipient primary vein (Fig. 4-1 C). By 3 DAG, *MP* expression indicates the incipient secondary veins along the margin (arrows in Fig. 4-1 D), which eventually sharpen to form 1-2 cell-wide secondary veins (Fig. 4-1 E,F). For all subsequent basipetally formed secondary veins, *MP* expression is first low in a larger region between the margin and existing secondary preprocambial strand (arrows in Fig. 4-1 G,I,J), before a distinct strand appears (Fig. 4-1 H,K). The same process occurs in the proximity of a leaf serration (arrows in Fig. 4-1 J,K). *MP* expression is also gradually restricted during the formation of tertiary veins (arrowheads in Fig. 4-1 I,J).

In the subsequent leaves, the larger final size is accompanied by a more complex venation and *MP* expression pattern. *MP* is expressed at the site of incipient leaf primordia becoming confined to an internal domain of expression (arrows in Fig. 4-1 L,M). *MP* expression is initially strong and widespread (Fig. 4-1 N), and can remain high and diffuse simultaneously in the first two secondary preprocambial veins (Fig. 4-1 O). Secondary veins in later leaves can appear in a two-step process with strong and focused *MP* expression in preprocambial veins connecting the midvein with the margin prior to connecting with the more distal preprocambial veins (arrow in Fig. 4-1 O). Figure 1p shows how the level of *MP* expression is highest in preprocambial veins without overt cellular differentiation.
(arrow), intermediate in procambial veins with elongated cells (arrowhead), and low or absent in fully differentiated veins (asterisk). 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.

**Dynamics of PIN1-GFP expression**

PIN1 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 expressing cells depicts the primary vein connecting to the hypocotyl vasculature (Fig. 4-2 A), with expression narrowing to a 1-3 cell wide primary vein in older leaf primordia (Fig. 4-2 B-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 expression appears as a cluster of PIN1 expressing cells in the basal adaxial region (asterisks in Fig. 4-2 B,F,K and arrows in E). This region of PIN1-expressing cells is gradually restricted to form a continuous 1-2 cell wide secondary strand (Fig. 4-2 F-H and K-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 (Fig. 4-2 B-D) or more distal secondary strand (Fig. 4-2 G,H). This biphasic connection always occurs for the second to fourth secondary veins in later formed rosette leaves (Fig. 4-2 I-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 expression in developing higher order veins appears as either outgrowth from existing veins, or connection of isolated or clustered PIN1-expressing cells to a lower order vein. PIN1 expression in all vein types is progressively lost in a basipetal direction as the more distal veins differentiate (e.g. Fig. 4-3 E).

**PIN1 expression extends from the epidermis into ground tissue**
PIN1 expression suggests a role of the epidermis in directing auxin into developing vascular regions. In slightly bulging primordia, PIN1 proteins are localized to the apical end of all epidermal cells leading towards the primordia apex, which overlies the developing primary vein (Fig. 4-3 A,B, 4-4 S). About the time of emergence of the first pair of secondary veins, PIN1 is predominantly lost from the abaxial epidermis and becomes progressively more restricted to basal adaxial and marginal epidermal cells (Fig. 4-3 C,D). Thereafter, PIN1 is always localized to the basal adaxial and marginal epidermal cells that are adjacent to the newly forming secondary veins (Fig. 4-2 E, 4-3 D,E), connecting the epidermis and developing veins (e.g. Fig. 4-4 T) until the procambial strand is clearly delimited (Fig. 4-2 G,H and L,M). In later formed rosette leaves, this epidermal region includes areas just proximal and distal to a serration (Fig. 4-3 E), respectively involved in the formation of secondary (arrowheads in Fig. 4-2 K-M) and tertiary marginal veins. Some of the later formed rosette leaves (<5%)
also maintain abaxial PIN1 expression along much of the length of the primordia up until the formation of the fourth or fifth secondary veins.

**PIN1 polarization during procambial development**

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

Based on PIN1 protein localization we can predict the direction of auxin flow. Auxin flow in the primary vein is predominantly in the basal direction (Fig. 4-
4 B,C). Auxin flow is predominantly bi-directional along secondary strands, with the distal cells having apical (Fig. 4-4 F) or basal PIN1 polarity, and the more basal cells always having basal polarity where they connect to the midvein (Fig. 4-4 H), with one or more cells within the strand having bi-directional PIN1 localization (Fig. 4-4 G). Less frequently, PIN1 occurs only on basal ends of procambial cells, resulting in a basal direction of auxin flow along a given secondary strand (Fig. 4-4 I). In young leaf primordia, PIN1 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 (Fig. 4-3 A,B, 4-4 S). PIN1 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 (Fig. 4-4 Q,R,T).

**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 4-1, 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 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 (Fig. 4-5 B,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 (Fig. 4-5 E compared to D). 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 (Fig. 4-5 F). 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 (Fig. 4-10), where the response is limited almost entirely to the actively growing basal part of 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 using liquid exposure and detection by in situ hybridization (Fig. 4-10), 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 (Fig. 4-6 G,H). In more advanced primordia, there can also be preprocambial strands of MP-expressing cells extending from the margin towards the petiole (Fig. 4-6 I). 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; Figure 4-8 G-I).

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 Fig. 4-6 A,B with D,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 (Fig. 4-6 F), 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.

**PIN1 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 background. In contrast to the initially normal MP expression in mp mutants, PIN1 expression in mp mutants is much reduced, both quantitatively and qualitatively (Fig. 4-7 D-F). The expression of PIN1 in mp mutant primordia is evident in developing primary veins and incomplete secondary veins that terminate in a diffuse PIN1 expressing region (compare Fig. 4-7 D-F with A-C). In mp mutants, PIN1 appears to be localized in apical ends of epidermal cells in young emerging leaf primordia. In older mp primordia, PIN1 expression becomes predominantly restricted to basal adaxial regions with little or no epidermal PIN1 polarity. Similar to MP, in NPA-treated tissue PIN1 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 Fig. 4-7 G-I with 4-6 G-I). NPA treatment predominantly inhibits the development of sub-cellular PIN1 localization in most internal cells and in the epidermis, although PIN1 expression does become restricted to the basal adaxial epidermis in older primordia.
**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; Fig. 4-8a-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 (Fig. 4-8d-f). In wildtype primordia, NPA treatment predominantly shifts DR5::GUS expression to the primordia margin (Mattsson et al., 2003; Fig. 4-8 G-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 (Fig. 4-11). In summary, mp mutants appear to fail to delimit and organize vascular and marginal auxin maxima, while pin1 mutants can organize extra auxin maxima.

### 4.5 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 expression in developing leaf primordia, overlaps with a recent publication (Scarpella et al., 2006). Points where we provide additional information will be discussed below.

**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 is consistent with both models, our observations support the following positive feedback cellular response pathway driving auxin canalization, as summarized in Figure 4-9 A. (1) In response to increased auxin levels, the MP gene becomes transcriptionally activated (Fig. 4-5 and 4-10). 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) MP and PIN1 expression patterns in developing leaf primordia overlap considerably and undergo a similar gradual restriction of expression (Figures 4-1 and 4-2). We have shown that PIN1 protein is reduced quantitatively and qualitatively in the *mp* mutant (Fig. 4-7), 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; Fig. 4-10) and this response may be mediated by MP. (5) We have shown that the subcellular localization of PIN1 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 (Fig. 4-4). PIN1 polarity is presumably affected by mechanisms that involve vesicle trafficking and regulatory proteins such as PINOID (e.g. Friml *et al.*, 2004; Steinmann *et al.*, 1999). (6) 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. 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.
A module for major vein formation

Based on the patterns of the 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 (Fig. 4-5 E and Fig 4-10). The auxin brought in by epidermal expression of PIN1 and/or produced by cells in actively growing regions triggers the canalization of auxin flow mechanism, involving the feedback regulatory loop described above in ground cells. 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 polarization of PIN1. Figure 4-9 (b) summarizes this module for reiterative secondary vein formation based on $MP$ and PIN1 expression.

Based on 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 the $mp$ mutant background, the level of PIN1 expression and the 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 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 close to 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, and additional components are likely to
be added with time.
4.6 Figures

Figure 4-1 Whole-mount in situ hybridizations of MP mRNA transcripts. First two developing primordia (a-k) or later forming (l-p) rosette leaves. All are planar median views of the leaf primordia, except (a,l,m) which are lateral median views. Scale bars are 20 µm (a-f,l,m) or 40 µm (g-k,n-p). Arrows point at emerging veins (see text).
Figure 4-2 Expression of PIN1-GFP in leaf primordia.

First (a-h) or later formed (i-m) rosette leaves. 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 expression (see text).
Figure 4-3 Expression of PIN1-GFP in epidermal tissues of developing 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. Scale bars are 20 µm.
Figure 4-4 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 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 in the epidermis adjacent to the next site of preprocambial strand development. Scale bars are 10 µm.
Figure 4-5 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 4-6 *MP* expression in primorida 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 4-7 Expression of PIN1::GFP in primorida 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 4-8 Auxin response patterns in primordia of *mp* mutant and NPA-grown plants.

DR5::GUS expression in wildtype (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 4-9 Canalization of auxin model specifying leaf vascular patterns.

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.
Figure 4-10. Auxin induction of MP and PIN1 in more mature leaf primordia.

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 situ were done as for MP, with the PIN1 probe made from a cDNA sequence (using primers TTTGTGTGAGCTCAAGTGC and CTGCGTGTGTTTGGCTTA ) 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 4-11 *PromDR5::GUS* expression in *mp* and *pin1* mutants.

Planar median views of *PromDR5::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).
4.7 References


Statement of contribution:

M. Schuetz performed all experimental work and wrote the manuscript together with J. Mattsson.

5.1 Abstract

Genetic evidence links the Auxin Response Factor (ARF) gene MONOPTEROS (MP) to both lateral organ initiation and leaf vascular patterning in Arabidopsis thaliana. Loss of MP function leads to severe defects in flower initiation but relatively mild defects in leaf initiation. mp mutants also develop leaves with a simplified leaf veination pattern. Since MP is a member of a gene family it is possible that other closely related ARF genes play partially redundant roles with MP in these processes. Here we analyzed expression patterns of closely related ARF genes throughout various stages of leaf development to identify potential candidates for overlapping function with MP. We found that ARF3 and ARF7 expression patterns overlapped in part with MP throughout leaf development and displayed a similar local accumulation of transcripts in response to ectopic auxin application. Subsequent analysis of mp arf3 and mp arf7 double mutants confirmed a role of ARF3 and ARF7 acting redundantly with MP in the process of leaf initiation. Results of this study provide a framework for ARF-mediated
establishment of differential gene expression in response to local auxin gradients leading to leaf and vein formation.

5.2 Introduction

The phytohormone auxin has been implicated in a diverse set of plant developmental processes ranging from pattern formation in embryogenesis to regulating environmental responses like gravitropism. Studies exploring how auxin elicits such diverse responses in the model plant Arabidopsis thaliana, have resulted in the identification of important components of auxin signal transduction. A key component of the auxin signaling system are Auxin Response Factors (ARFs), a family of transcription factors that activate or repress target gene transcription in response to auxin. MONOPTEROS/ARF5 is an important member of this 23 member ARF gene family (Hardtke and Berleth, 1998; Guilfoyle and Hagen, 2007). mp mutants display embryonic phenotypes including lack of root, hypocotyl and variably fused cotyledons (Berleth and Jurgens, 1993). Postembryonic mp development is aided by the formation of adventitious root systems that can be induced by growing bisected apical regions in the presence of Indole butyric acid (IBA) (Przemeck et al., 1996). Postembryonic mp defects include the formation of reduced and incomplete leaf vascular patterns and defects in flower initiation (Przemeck et al., 1996). Of the 23 annotated ARFs in the A. thaliana genome, five have been shown to act as transcriptional activators when assayed in plant protoplast transfection assays while the remaining ARFs appear to act as transcriptional repressors (Ulmasov et al., 1999; Tiwari et al., 2003; Hardtke et al., 2004; Wang et al., 2005; Guilfoyle
and Hagen, 2007). All ARFs acting as transcriptional activators, *MP/ARF5, ARF6, ARF7, ARF8* and *ARF19* are phylogenetically related based on protein sequence similarity and form one of the three main clades in the ARF phylogenetic tree (Liscum and Reed, 2002; Guilfoyle and Hagen, 2007). This clade of ARF genes also includes the transcriptional repressors, *ARF3* and *ARF4* (Liscum and Reed, 2002; Teale et al., 2006; Guilfoyle and Hagen, 2007). It has been observed that double mutants of phylogenetically closely related ARFs result in enhancement of loss of function phenotypes compared to single mutants, which points to possible genetic redundancy among closely related ARFs (Guilfoyle and Hagen, 2007). Furthermore, *ARF7* has previously been implicated in playing a partial redundant role with *MP* in cotyledon initiation, and has been shown to strongly interact with *MP* in yeast two hybrid experiments (Hardtke et al., 2004).

Numerous studies have demonstrated that ARF function is dependent on the auxin-mediated degradation of AUX/IAA repressor proteins (reviewed in Mockaitis and Estelle (2008)). AUX/IAA proteins can bind to ARF proteins via a shared C-terminal dimerization domain and thus inhibit ARF function. In the presence of auxin, AUX/IAA proteins are targeted for degradation resulting in the de-repression of ARF activity. The perception of auxin is mediated via the *TIR1* auxin receptor, which is associated with a SCF (Skp1-Cul1-F-box) ubiquitin E3 ligase enzyme complex. The SCF E3 ligase, ubiquitinates AUX/IAA proteins and targets them for degradation via the 25S proteosome thus de-repressing ARF function (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007).
Plants are therefore able to activate or repress auxin responsive gene transcription by manipulating auxin concentrations in various plant tissues.

Auxin is directionally transported throughout plant tissues via a family of auxin efflux (PIN) and influx (AUX/LAX) proteins. These proteins asymmetrically localize to ends of cells thereby determining the direction of auxin flow (Friml et al., 2003; Reinhardt et al., 2003; Petrasek et al., 2006; Wisniewska et al., 2006; Yang et al., 2006; Carrier et al., 2008). Loss of function in the PIN1 gene results in mild vascular hypertrophy in leaf and inflorescence stems, and loss of flower initiation similar to what is observed in WT plants treated with chemicals functioning as polar auxin transport (PAT) inhibitors (Okada et al., 1991; Galweiler et al., 1998; Mattsson et al., 1999). Several studies have suggested a link between ARF activity and expression or localization of members of the PIN family of auxin efflux proteins (Krogan, 2006; Sauer et al., 2006; Wenzel et al., 2007). This notion is consistent with the observation of similar flower initiation defects observed in both mp and pin1 mutants. PIN1 has been shown to be an important regulator of leaf vascular pattern formation and functions by facilitating auxin flow through leaf mesophyll cells in discrete cell files (Scarpella et al., 2006; Wenzel et al., 2007). The high auxin concentrations in these auxin transporting mesophyll cells is thought to lead to their differentiation into vascular tissue types. The vascular hypertrophy observed in pin1 mutants and WT plants treated with PAT inhibitors is thought to be the result of auxin accumulation in response to reduced auxin removal from the leaf (Mattsson et al., 1999; Sieburth, 1999; Mattsson et al., 2003).
Auxin signaling and auxin transport may be components of a positive feedback loop described in the canalization of auxin hypothesis first proposed by Sachs (1981). This hypothesis outlines a process where cells, which initially have low auxin transport capacity, become more specialized and subsequently have greater auxin transporting capacity than neighbouring cells. This positive feedback leads to the formation of preferential avenues of auxin flow that efficiently drains the surrounding area of auxin. The canalization of auxin flow hypothesis is supported by several lines of experimental evidence which has lead to a model where MP mediated auxin signaling feeds into PIN1-mediated auxin transport in leaf vascular pattern formation (Wenzel et al., 2007). In this model, increased auxin concentrations in preferred auxin transport routes leads to the degradation of AUX/IAA proteins resulting in the activation of MP. MP functions to promote the expression of PIN1 in these cells, thereby increasing their auxin transport capability. In addition to the post-translational activation of MP in response to auxin, MP appears to be transcriptionally upregulated in the presence of auxin (Wenzel et al., 2007). Since PIN1 expression is also upregulated in response to auxin (Heisler et al., 2005; Paciorek et al., 2005; Scarpella et al., 2006; Vieten et al., 2007; Wenzel et al., 2007) and PIN1 expression depends on MP function (Wenzel et al., 2007), there is evidence for a basic molecular positive feedback loop in the process of vein formation.

The processes of leaf initiation and leaf vascular patterning appear to be similar, in so far as they depend on the activity of the same genes including MP and PIN1 (Wenzel et al., 2007; Schuetz et al., 2008). Schuetz et al., 2008
demonstrated that leaf initiation in mp mutants is sensitive to reduction of PAT either genetically through pin1 or pid loss of function mutations or through application of chemical PAT inhibitors. Concentrations as low as 0.1 µM of the PAT inhibitor NPA (1-naphthylphthalamic acid) resulted in a drastic reduction of leaf initiation in mp mutants and leaf initiation was abolished at concentrations of 1 µM NPA and higher (Schuetz et al., 2008). In the absence of PAT inhibition it is possible that mp mutants are able to undergo leaf initiation and sporadic vascular tissue formation due to the action of partially redundant ARF gene(s). In order to evaluate putative overlapping function, expression profiles of candidate ARF genes were examined for overlap with MP expression domains throughout leaf development. ARF3 and ARF7 expression profiles were the most similar to those observed for MP, and putative functional roles were assessed in mp arf3 and mp arf7 double mutants. The results presented suggest that ARF3 and ARF7 play a redundant role with MP in promoting leaf initiation.

5.3 Materials and Methods

Plant material and growth

The mpG12, G33, BS1354, arf4-2, arf3-1, arf7-1, arf7/nph4-1, arf19-1, arf8-2, arf6-1 mutant alleles used for double mutant and single mutant analysis have been described previously in (Hardtke and Berleth, 1998; Harper et al., 2000; Okushima et al., 2005). Col-0 plants containing the auxin-responsive PromDR5::GUS construct were obtained from Tom Guilfoyle (University of Missouri, Columbia). Surface sterilized seeds were plated on solid Arabidopsis
thaliana salts media (ATS) (Lincoln et al., 1990). For the auxin transport inhibitor treatments, seedlings were plated on media containing 0.1, 1 or 10 µM 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.

In situ hybridization and RT QPCR

ARF4 and ARF19 gene fragments were amplified from cDNA using the primers ARF19_Probe_F (CGCGCTCTCATCTTTTAACC), ARF19_Probe_R (CCTCCA CCATTCATGATTC), ARF4_Probe_F (AGGTTCGATCACCCTC) and ARF4 Probe R (TGCCCTTCTGTTTTCCCATC). cDNA was generated from total RNA extracted from 14 day old WT seedlings using Trizol reagent (Invitrogen) and subsequently reverse transcribed using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) and cloned into pBluescript II Sk(-) (Stratagene). Plasmids containing cloned ARF3 (U09387), ARF6 (H7D8), ARF7 (E11H5), and ARF8 (M34A11) cDNAs were obtained from Arabidopsis Biological Resource Centre (ABRC) with the indicated stock number. All plasmids were sequenced to verify the cDNA sequence and determine insert orientation. DIG-labelled RNA nucleotides (Roche Applied Science) were incorporated into single stranded RNA probes by using either T3 or T7 RNA polymerase (Fermentas) from linearized plasmid DNA. MP probes were generated as described in Hartdke et al (1998). Whole mount in situ hybridization procedure was performed as described (Zachgo et al., 2000) with some modifications including overnight fixation and
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. Localization of β-glucuronidase activity was carried out as described in Mattsson et al (2003).

RT QPCR was performed using a Rotor-Gene\textsuperscript{TM} 3000 real time quantitative thermocycler (Corbett Life Sciences, San Francisco, CA) and the PERFECTA SYBR SUPERMIX (Quanta BioSciences, Gaithersburg, MD) with the primers listed in Table 5-1. WT seedlings were germinated on solid ATS media and were transferred to liquid ATS media supplemented with or without 2,4-D or IAA (Sigma, St. Louis, MO) to a final concentration of 10 µM, and 0.1% (w/v) pluronic-F68 surfactant (Sigma, St. Louis, MO) at approximately 3 DAG. Seedlings were subsequently incubated on a rotary shaker for approximately 16 hours under normal growth conditions. cDNA was generated as described for preparation of cloned ARF4 and ARF19 cDNA's. Relative gene abundance was determined using the \textDelta\DeltaCT method as described in Livak and Schmittgen (2001). Relative gene abundance was normalized to the ORNITHINE TRANSCARBAMILASE (OTC) gene (Cnops et al., 2004; Hay et al., 2006). DIC images were taken on a Nikon Eclipse 600 microscope using a Canon D30 digital camera.
5.4 Results

Phenotypic analysis of ARF mutants

*mp* mutants display a drastic reduction in leaf initiation events compared to *WT* in response to treatment with the PAT inhibitor NPA (Fig. 2-2) (Schuetz et al., 2008). Mutations in other closely related ARF genes have not been reported to display leaf initiation defects when grown under standard conditions. However, we hypothesized that these mutants could be more sensitive to leaf initiation under conditions of reduced PAT similar to what is observed in *mp* mutants. ARF proteins which share significant homology to *MP* in phylogenetic analysis are *ARF3, ARF4, ARF6, ARF7, ARF8* and *ARF19* (Liscum and Reed, 2002). Previously characterized loss of function T-DNA insertion lines of candidate ARF genes (Okushima et al., 2005) were grown on media supplemented with 0.1, 1, and 10 µM NPA and were analyzed for leaf initiation defects. The site of T-DNA insertion into the coding region of the tested ARF genes is illustrated in Figure 5-1. With the exception of *mp*, the ARF mutants did not respond differently compared to *WT* in response to NPA and often resulted in tubular leaf formation identical to what is observed in *WT* plants (Fig. 5-2; data not shown). Furthermore, no differences in leaf vascular pattern formation under standard conditions and in the presence of NPA were observed in *arf3, 4, 6, 7, 8* and *19* mutants compared to *WT* plants (data not shown). We therefore conclude that in the presence of a functional *MP* protein, the process of leaf initiation and
vascular pattern formation is not affected by single mutations of other closely related ARF genes, even under conditions of reduced PAT.

**Differential expression of ARF genes during leaf development**

The sequence of events leading to the formation of mature xylem vascular tissues starts with isodiametrically shaped mesophyll cells called pre-procambial cells. Pre-procambial cells elongate giving rise to procambial cells. Procambial cells are arranged in narrow cell files forming continuous strands that are easily observed in developing leaves (Fig. 1-5) (Esau, 1965). Xylem-forming procambial cells undergo a process of xylogensis, where a thick secondary cell wall is deposited, eventually forming a mature xylem vessel (Esau, 1965).

In descriptions for expression analysis of ARF genes during leaf development we will use the following terms for leaf developmental stages. Stage 1 of leaf development is defined by MP expression in pre-procambial and procambial cells of the midvein (Fig. 5-4 C). Stage 2 leaf primordia are defined as the stage where MP expression has defined the first pair of loop-shaped secondary veins (Fig. 5-4 E). Stage 3 primordia are defined as the stage where MP expression has formed two pairs of secondary loops (Fig. 5-5 M). Stage 4 leaves are defined by midveins lacking MP expression in procambial cells just prior to undergoing xylogensis (Fig. 5-3 G). Stage 5 leaves have midveins and/or first secondary vascular loops undergoing xylogensis (Fig. 5-3 N). Stage 6 leaves are nearly mature with complete reticulate veins patterns (Fig. 5-3 O).
In order to assess to what degree the expression profiles of candidate ARFs overlap with those of MP during the process of leaf development, transcriptional profiles of ARF 3, 4, 6, 7, 8 and 19 were assessed using whole mount in situ hybridization. The experiments revealed highly dynamic temporal and spatial expression patterns of ARF3, 4, 6, 7, 8 and 19. Representative images of expression dynamics during stage 4, stage 5 and stage 6 of leaf development for ARF3, 4, 6, 7, 8 and 19 along with MP are presented in Figure 5-3. All ARF genes tested displayed a strong expression maximum at the tip of nearly mature leaves similar to what is observed in plants expressing the auxin sensitive PromDR5:GUS transgene (Aloni et al., 2003; Mattsson et al., 2003) (Fig. 5-3). ARF4, ARF6 and ARF8 transcripts were not detected prior to stage 4 leaf development (data not shown) and ARF6 and ARF8 displayed weak expression in the petiole region of stage 5 and 6 leaves (Fig. 5-3). Expression of ARF6 was observed in the trichomes of stage 6 leaves, which was unique among all the ARFs tested (arrow in Fig. 5-3 U). ARF7 and ARF19 were both expressed in vascular cells undergoing xylogenesis in stage 5 leaves (Fig. 5-3 K-L and N-O; Fig. 5-4 L and M). ARF19 expression was restricted to cells that were undergoing xylogenesis and was much more pronounced in these differentiating vascular cells compared to ARF7 (Fig. 5-3 M-N and J-K; Fig. 5-4 L and M). ARF7 expression was also observed in the elongated procambial cells (arrow in Fig. 5-4 M) and hydathode regions of stage 4 leaves (arrow in Fig. 5-3 J-K). ARF3 was expressed in procambial cells throughout different developmental stages and as vascular development continued in stage 5 and 6 leaves its
expression faded and was absent from mature veins (Fig. 5-3 D-F). *MP* expression has been characterized as being present in pre-procambial and procambial cell types in developing leaf vascular systems and is one of the earliest markers of future vascular cell fate (Fig. 4-1 and 4-2) (Wenzel et al., 2007). *ARF3* spatio and temporal expression domains considerably overlapped with *MP* expression domains throughout different stages of leaf development (Fig. 5-3 D-F and G-I).

A closer examination of *ARF3* expression dynamics in the earliest stages of leaf development (stage 1) revealed that *ARF3* is expressed on the flanks of the meristem and in the isodiametrically shaped pre-procambial cells of the future midvein region of leaf primordia (Fig. 5-4 A, F). *MP* and *PIN1* expression is also observed pre-procambial cells during comparable stages of development (Fig. 5-4 C and I). Although *ARF3* expression is similar to the expression domains of *MP* and *PIN1* during this developmental stage, there are significant differences. *MP* and *PIN1* start to be expressed at nearly the same time and only differ in *MP* having broader expression domains in the developing midvein and lamina compared to *PIN1* (Wenzel et al., 2007) (Fig. 5-4 C-E and I-K). *ARF3* expression domains appear more similar to those observed for *PIN1* in the developing midvein of stage 1 to 1.5 primordia, where both genes are expressed in a 1-2 cell wide file of cells. (Fig. 5-4 F-H and I-K). It is only during the initial specification of the midvein that pre-procambial *ARF3* expression is observed, during later stages of development *ARF3* expression appears to become restricted to procambial cells (Fig. 5-4 F-H). During the specification of the first vascular loop
in stage 2 leaf primordia, ARF3 expression is observed in the procambial cells at the apical region of leaf primordia (Fig. 5-4 H). During comparable stages of development, MP and PIN1 expression is continuous throughout the entire loop domain including both procambial and pre-procambial cell types (Fig. 5-4 E, H, K) (Wenzel et al., 2007). In later stages of leaf development (stage 4 and 5), ARF3 expression occurs in procambial cells of primary, secondary and tertiary veins but is absent in higher order veins. (Fig. 5-3 D-E). MP expression appears more extensive and precedes ARF3 expression in tertiary and quaternary veins of stage 4 and 5 leaves (Fig. 5-3 G-H to D-E). In summary, ARF3 is initially expressed together with MP and PIN1 in pre-procambial cells of the developing midvein, but ARF3 expression becomes restricted to procambial cells during subsequent stages of vascular tissue formation.

**ARF transcriptional induction in response to auxin application.**

Although auxin mediated degradation of AUX/IAA genes has been implicated as the main mechanism governing ARF dependent gene regulation, we previously showed that steady state levels of MP transcripts increase in response to auxin application in developing leaf primordia (Fig. 4-5) (Wenzel et al., 2007). The transcriptional activation of the MP gene in response to auxin suggests an additional, more direct role of auxin mediating ARF gene activity. In order to evaluate putative transcriptional auxin induction of ARFs most closely related to MP, 3 DAG (days after germination) seedlings were exposed to the synthetic auxin 2,4-D (2,4-dichlorophenoxy acetic acid) for 16 hours. The response to
auxin was documented using whole mount in situ hybridization experiments as well as RT Q-PCR. Plants harbouring the auxin responsive ProDR5::GUS transgene were used as a control of auxin induced gene transcription for whole mount in situ hybridization experiments. Upregulation of the GUS reporter gene was observed in auxin-treated young leaf primordia and root tips (Fig. 5-5 A-D). A response indicating an upregulation of ARF transcription in response to auxin was observed for ARF3, MP/ARF5 and possibly also ARF19 in both young leaf primordia and primary root tips compared to control (Fig. 5-5 I-L, M-P, U-X). In young leaf primordia (stage 1 to 3), both MP and ARF3 exhibited wider expression domains especially in the more central and basal regions of leaf primordia treated with auxin (arrows in Fig. 5-5 I-J and M-N; Fig. 4-10). Expansion of MP and ARF3 expression domains were also observed in root tips treated with auxin where ectopic gene transcription was observed extending distally from the root tip to more mature regions of the root (arrows in Fig. 5-5 K-L and O-P). ARF19 transcription appears to be developmentally restricted to more mature vascular tissues in developing leaves, which are not observed prior to stage 4 leaf primordia (Fig. 5-3 M-O). However, weak ARF19 expression was consistently observed throughout auxin treated stage 2 and 3 leaf primordia, and was never observed in the absence of auxin application (Fig. 5-5 U-V).

RT Q-PCR experiments were performed to provide independent support of results from in situ hybridization experiments, and seedlings were grown under identical conditions. Two auxin treatments were included for RT Q-PCR experiments, the naturally occurring auxin, IAA (indole-3-acetic acid) and
synthetic auxin, 2,4-D. The characterized early auxin response gene Aux/IAA1 was used to test the effectiveness of auxin application. The Aux/IAA1 gene showed an 11 fold increase in transcript abundance in response to treatment with 10 µM IAA compared with control (Fig. 5-6). In response to treatment with 10 µM 2,4-D, an average 29 fold increase was observed compared with control (Fig. 5-6). In contrast to data obtained using whole mount in situ hybridization, ARF3 was not significantly upregulated in response to auxin as evaluated by RT Q-PCR (Fig. 5-6). However, the remaining data from both whole mount in situ and RT Q-PCR were in agreement for the remainder of the ARF genes tested. Several attempts at amplifying a gene-specific fragment suitable for RT Q-PCR for ARF6 failed and therefore was not included in this analysis. Relative MP transcript abundance increased 1.9 fold in response to IAA treatments and 3.3 fold in response to 2,4-D (Fig. 5-6). Relative ARF19 transcript abundance increased 2.3 fold in response to IAA treatment and 4.1 fold in response to 2,4-D treatment (Fig. 5-6). Q-PCR results for both MP and ARF19 were statistically significant after students t-test analysis at the p < 0.05 level of significance. With the exception of ARF3, the results obtained from RT Q-PCR experiments were consistent with what was documented from whole mount in situ hybridization experiments.

**ARF3 expression is partially dependent on MP function.**

ARF3 and MP are both expressed on the flanks of the meristem thereby predicting the site of future leaf initiation (Fig 5-4 A, B). Their expression also
overlaps in procambial cells in developing leaf vascular systems and both appear to elicit a transcriptional response following auxin application, at least on the local level. Since MP expression preceded ARF3 expression in secondary, tertiary and quaternary veins, it seemed possible that ARF3 could be dependent on MP function. To test this hypothesis, ARF3 expression domains were evaluated in developing mp mutant leaves. Detection of non-functional MP transcripts in mp mutants has shown that MP expression is not severely affected during the formation of the primary midvein and subsequent formation of the first two pairs of secondary veins (Fig. 4-6) (Wenzel et al., 2007). However, MP expression is dramatically reduced and occurs in sporadic regions associated with procambial cell fate in more mature mp mutant leaves (Fig. 5-7 C and E; Fig. 4-6) (Wenzel et al., 2007). ARF3 expression was detected in young stage 2 mp leaf primordia (Fig. 5-7 B). However, ARF3 expression was not detectable during later stages of mp leaf development (Fig. 5-7 D and F). The absence of ARF3 expression in mp mutants supports the notion that MP dependent signaling positively influences ARF3 expression. ARF7 and ARF19 transcripts were not detectable in young (stage2) mp leaf primordia, which is consistent with what is observed in WT (data not shown). However, both ARF7 and ARF19 expression was associated with vascular cell types of mature mp mutant leaves (Fig. 5-7 G and H). ARF7 and ARF19 expression therefore do not appear to directly depend on MP function but do depend on MP-mediated processes promoting vascular patterning and vascular cell specification.
**mp arf7 and mp arf3 double mutants display defects in leaf initiation**

Of the six ARF genes analyzed in this study three genes showed some similarites to MP expression dynamics in developing leaf primordia. ARF3 and ARF7 were the only ARF genes expressed in procambial cells during vascular patterning, of which ARF3 had the most significant overlap with MP. ARF19 showed a similar transcriptional upregulation in response to auxin as MP but was never expressed in procambial cells. In order to ascertain any functional overlap between MP and the three candidate ARF genes, double mutants were generated. Generation of mp arf19 double mutants failed probably due to their very close physical distance on chromosome one (approximately 200kb). mp arf3 and mp arf7 double mutants were generated and assayed for enhanced defects in either leaf initiation and / or vascular pattern formation.

*mp arf7* double mutants have already been characterized as showing enhanced defects in cotyledon formation and cotyledon vascular pattern formation compared to *mp* single mutants (Hardtke et al., 2004). Hartke et al (2004) demonstrated an overlapping role of ARF7 with MP during embryogenesis but limited data was presented on any postembryonic effects. *mp arf7* double mutants show severe defects in post-embryonic leaf initiation and form an enlarged leafless shoot structure (Fig. 5-8 C-D). This enlarged leafless shoot is reminiscent of what is observed in *mp pin1*, *mp pid* and *mp* mutants treated with PAT inhibitors (Fig. 2-1) (Schuetz et al., 2008). *mp arf7* double mutants continue
to grow and the size of the enlarged meristem region continues to increase along
with axillary leafless meristems forming from axial regions identical to what is
observed in mp pin1 mutants (Fig. 5-8 D)(Schuetz et al., 2008) The abolishment
of leaf initiation in mp arf7 double mutants confirms a functional role for ARF7 in
postembryonic leaf initiation.

mp arf3 double mutants initially showed no morphological differences from
mp single mutant seedlings. Compared to mp single mutants, mp arf3 double
mutant meristems appear to undergo fewer leaf initiation events and eventually
arrest leaf initiation entirely (Fig. 5-8 E-F). In contrast to the enlarged leafless
shoots formed in mp pin1 and mp arf7 double mutants, mp arf3 double mutant
meristems remain relatively normal in size and do not form enlarged dome-
shaped structures. Formation of axillary leafless domes is not observed in mp
arf3 double mutants (Fig. 5-8 E-F). The results documented from double mutant
analysis show that ARF7 and ARF3 function redundantly with MP in promoting
leaf initiation from vegetative meristems.

5.5 Discussion

Redundant function of ARF genes

Auxin signaling and transport have been established as important components
regulating leaf initiation and vascular tissue formation. Loss of function in the
ARF gene MP leads to strong defects in flower initiation but relatively less
penetrant defects in the analogous process of leaf initiation. Thus the mp mutant
phenotype is somewhat contradictory; since mp mutants fail to form one type of
lateral organs – flowers – they are perfectly able to form another type – leaves. It should be pointed out though that mp mutants show defects in cotyledon number and placement, (Mayer et al., 1991; Berleth and Jurgens, 1993) and defects in leaf phyllotaxy (Schuetz et al., 2008). mp mutants also show defects in leaf vein formation where distinct parts of secondary veins and much of tertiary veins are missing (Przemeck et al., 1996). While it is possible that mp function is largely restricted to flower formation and the formation of distal portions and higher order veins, the expression patterns of the mp gene tells a different story. MP is expressed at sites of leaf and flower initiation and at all sites of leaf vein formation including midvein and proximal portions of secondary veins. In light of these observations, it appears more likely that MP is a component of a signaling mechanism required for both leaf and flower initiation and formation of all orders of leaf veins. If so, the reduced auxin signaling in mp mutants may result in an obvious phenotype primarily at sites of reduced auxin input, or at sites that require enhanced auxin signaling. The range and extent of mp mutant phenotypes could be explained by the action of other closely related ARFs playing a partially redundant role with MP in these processes. This notion is consistent with double mutant analysis of phylogenetically closely related ARF genes that have been shown to play partially redundant roles (Nagpal et al., 2005; Pekker et al., 2005; Wilmoth et al., 2005; Guilfoyle and Hagen, 2007). Furthermore, overlapping expression patterns among phylogenetically related genes have been shown to predict similar functional roles (Dharmasiri et al., 2005; Cheng et al., 2006, 2007). By analyzing the expression profiles of the six
ARF genes phylogenetically most similar to MP (Liscum and Reed, 2002), we identified ARF3 and ARF7 as having overlapping expression profiles with MP in developing leaf vascular systems (Fig. 5-3 and 5-4). However, ARF3 expression was most similar to MP and was co-expressed in pre-procambial cells of the developing midvein of stage 1 leaf primordia and in procambial cells of more mature leaves (Fig. 5-3 and 5-4).

**Overlapping expression of MP and ARF3**

We previously implicated MP function in regulating leaf vascular tissue formation by positively regulating PIN1-mediated auxin transport (Wenzel et al., 2007). In this study, induction of MP transcription was observed in response to ectopic auxin treatment in young leaf primordia, which are confirmed in this study (Fig. 5-5 and 5-6) (Wenzel et al., 2007). These data add the component of auxin-mediated ARF gene transcription to the established model of post-translational activation of ARF protein function (reviewed in (Mockaitis and Estelle, 2008)). Auxin responsive gene transcription could be a unique feature of MP but may also be shared by other ARF genes potentially playing similar functional roles. ARF3 exhibited a local, transcriptional increase in response to auxin application, similar to what is observed for MP (Fig. 5-5). Although this auxin mediated ARF3 transcriptional response was not significant when averaged over entire seedlings, the qualitatively similar ARF3 and MP expression profiles in young leaf primordia do suggest that ARF3 gene transcription is responsive to auxin at this developmental stage.
Detailed observation of auxin accumulation and transport during leaf initiation and midvein formation has lead to the notion that both processes are regulated by the same mechanism (Mattsson et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Heisler and Jonsson, 2007; Wenzel et al., 2007; Rolland-Lagan, 2008; Bayer et al., 2009). These observations are consistent with a recent computational model that has resolved how auxin accumulation can simultaneously occur together with high rates of auxin flux on the flanks of the meristem and in leaf vascular systems (Bayer et al., 2009). ARF3 and MP are co-expressed not only in procambial cells during leaf development but also on the flanks of the meristem at sites of presumptive leaf initiation prior to any morphological changes are observed (Fig. 5-4 A, B). Since ARF3 and MP gene transcription also appears to be induced by ectopic auxin application, one can postulate that both genes are activated at sites of leaf primordia formation in response to local auxin accumulation. After leaf initiation, MP and ARF3 continue to be co-expressed during the specification of the leaf midvein and in procambial cells of higher order veins. Although the MP and ARF3 genes are co-expressed during the process of leaf initiation and leaf vascular tissue specification, the MP and ARF3 proteins have been shown to have opposite functions. MP has been shown to act as a transcriptional activator whereas ARF3 has been shown to act as a transcriptional repressor protein (Ulmasov et al., 1999; Tiwari et al., 2003). Several ARF proteins have been shown to interact in yeast two hybrid assays but any direct interaction between MP and ARF3 proteins is unlikely since the ARF3 gene lacks the C-terminal
dimerization domain required for ARF-ARF interactions to occur (Ulmasov et al., 1999; Tiwari et al., 2003; Hardtke et al., 2004). ARF3 proteins are also unlikely to be post-translationally regulated by Aux/IAA genes in the absence of a C-terminal dimerization domain (Ulmasov et al., 1999; Tiwari et al., 2003; Hardtke et al., 2004; Weijers et al., 2005). Therefore, auxin-mediated ARF3 transcriptional induction becomes an important regulator of ARF3 function. Interestingly, ARF3 expression is not observed in more mature mp mutant leaves, suggesting that ARF3 expression is at least partially dependent on MP function (Fig. 5-7 D-F). However, this observation may be the result of an indirect effect since ARF3 expression may depend on well-defined local auxin gradients that are absent or severely reduced in mp mutants (Przemeck et al., 1996; Wenzel et al., 2007). This notion is consistent with previous studies implicating ARF3 in translating auxin gradients into developmental relevant pattern formation in Arabidopsis gynoecium development (Sessions et al., 1997; Nemhauser et al., 2000).

**Double mutant analysis**

MP has already been established as a crucial regulator of leaf initiation and vascular tissue formation but no similar defects are apparent in arf3 mutants. However, ectopic ARF3 expression has been shown to result in epinastic leaf shape and simplified leaf vascular patterns (Hunter et al., 2006). Since a functional MP gene could mask any obvious defects in arf3 mutants we generated mp arf3 double mutants. mp arf3 double mutants initiated several
leaves similar to what is observed in mp single mutants by 7-10 DAG and were indistinguishable during this developmental stage (data not shown). However, leaf initiation in mp arf3 double mutants was abolished during later stages of vegetative development leading to the formation of a leafless meristem by 21 DAG (Fig. 5-8 E-F). This phenotype was never observed in mp single mutants and thus reflects a novel mp arf3 double mutant phenotype. The leaf initiation defects observed in mp arf3 double mutants are consistent with ARF3 and MP expression at sites of incipient leaf formation in the meristem and demonstrates a novel role for ARF3 promoting leaf initiation in the absence of MP function.

Previous studies have shown that there is considerable overlap between ARF7 and MP expression during embryogenesis and that ARF7 partially acts redundantly with MP during cotyledon formation (Hardtke et al., 2004). Hardtke et al (2004) also provided evidence of ARF7 acting redundantly with MP during post-embryogenic development of tertiary and quaternary leaf veins (Hardtke et al., 2004). Unlike ARF3, the ARF7 and MP proteins have been documented to strongly interact with each other in yeast two hybrid studies and act as transcriptional activators in plant protoplast systems (Ulmasov et al., 1999; Tiwari et al., 2003; Hardtke et al., 2004). The post-embryogenic defects previously described were based on analysis of strong loss of function arf7 mutants crossed with plants constitutively expressing a transgenic MP antisense construct (35S::MPAS) (Hardtke et al., 2004). The 35S::MPAS plants did not fully recapitulate the phenotypes of the weakest mp mutant alleles suggesting that MP function was just slightly reduced (Berleth and Jurgens, 1993; Przemeck et al.,
We hypothesized that using strong \textit{mp} loss of function mutants instead of 35S::\textit{MPAS} constructs in \textit{arf7} mutant backgrounds could reveal additional roles of \textit{ARF7} in post-embryogenic growth and development. To explore this possibility, \textit{mp arf7} double mutants were generated from the strong loss of function \textit{mp} \textsuperscript{G12} and \textit{arf7} \textsuperscript{nph4-1} alleles. Analysis of postembryonic phenotypes was limited since the vast majority of \textit{mp} \textsuperscript{G12} \textit{arf7} \textsuperscript{nph4-1} double mutants did not undergo leaf formation after germination (Fig. 5-8 C and D). Instead of forming leaves, an enlarged leafless shoot formed that was phenotypically similar to \textit{mp pin1}, \textit{mp pid} or \textit{mp} mutants treated with auxin efflux inhibitors (Schuetz et al., 2008). Significant overlap between \textit{MP} and \textit{ARF7} expression domains throughout leaf development was observed in procambial cells and not in meristems at sites of leaf primordia formation (Fig. 5-3 J-L, data not shown). Expansion of \textit{ARF7} expression domains to include leaf initiation sites in peripheral zones of SAMs was also not observed in \textit{mp} mutants (data not shown). This provides a paradox of how \textit{ARF7} promotes leaf initiation when \textit{ARF7} expression is absent at sites where leaf initiation occurs. The absence of \textit{ARF7} expression in \textit{WT} or in \textit{mp} mutant meristems suggests that \textit{ARF7} promotes leaf initiation by an undefined non-cell autonomous mechanism.

This study identifies \textit{ARF3} as a marker for pre-procambial cell fate and leaf initiation. \textit{ARF3} is thought to function in interpreting local auxin gradients resulting in the repression of target genes thereby promoting cell differentiation. In this scenario \textit{ARF} could repress genes involved in maintenance of pluripotent cell fates while \textit{MP} functions to promote cell differentiation. The formation of
enlarged leafless shoots in mp arf7 double mutants compared to relatively normal sized leafless shoots in mp arf3 double mutants appears to be correlated with fewer leaf initiation events. This observation supports the impression that oversized leafless shoot meristems increase in size partially due to lack of cell removal from dispersed peripheral growth zones (Schuetz et al., 2008). Future analysis of mp arf3 and mp arf7 mutants will provide significant insights into how auxin signaling regulates leaf initiation and its subsequent effect on meristem growth.
Figure 5-1 Sites of molecular lesions of ARF mutants used in this study

arf4-2, arf3-1, arf7-1, arf19-1, arf8-2 and arf6-1 alleles are confirmed T-DNA insertion lines. Location of molecular lesion in the each ARF gene is indicated by arrowheads. mp/arf5 alleles BS1354 and G33 are comprised of a single nucleotide substitution and deletion respectively (Hardtke and Berleth, 1998). Both mp/arf5 mutations result in the formation of a premature stop codon.
Figure 5-2 Leaf initiation defects in NPA treated ARF mutants

WT rosette of leaves grown under control conditions (A) or in the presence of 10μM NPA (B). arf4-2, arf3-1, arf7-1, arf19-1, arf8-2 and arf6-1 mutants did not respond differently to NPA treatment compared to WT (data not shown). arf3-1 mutant grown under control conditions (C) and in the presence of 10μM NPA (D) is presented as a representative of all ARF mutants tested. mp mutants undergo leaf initiation events forming a rosette of leaves under control conditions (E) but leaf initiation is abolished if mp mutants are grown in the presence of NPA (F). All images of plants at 21 days after germination (DAG), all size bars are 500 μm.
**Figure 5-3 Differential expression of ARF genes during leaf development.**

Increasing developmental age of leaves from left to right (Approximately, Stage 4, Stage 5 and Stage 6) for each ARF gene analyzed. Phylogeny of ARF genes is indicated and is based on protein similarity as described in Liscum and Reed (2002). ARF4 (A-C), ARF3 (D-F), ARF5/MP (G-I), ARF7 (J-L), ARF19 (M-O), ARF8 (P-R) and ARF6 (S-U). Blue/purple stain indicates presence of respective ARF mRNA signal as visualized using whole mount in situ hybridizations. Arrows in J, K indicate procambial ARF7 expression and arrow in M indicate ARF19 expression in differentiating xylem cells. All size bars are 50 µm except C,F,I,J,O,R, and U which are 500 µm.
Figure 5-3 Differential expression of ARF genes during leaf development.
Fig. 5-4 Comparison of ARF5/MP, ARF3 and PIN1 expression in the earliest stages of leaf primordia development

ARF3 (A) and MP (B) expression is observed on the flanks of the SAM prior to leaf initiation. Stage 1 (C,F,I), 1.5 (D,G,H) and 2 (E,H,K) leaf primordia illustrating expression of ARF3 (F-H), ARF5/MP (C-D) and PIN1 (I-K) respectively. Differences observed in expression of ARF19 (L) and ARF7 (M) in stage 5 leaves initially described in Figure 5-3, show that ARF19 expression is primarily observed in vascular cells undergoing xylogenesis (arrowheads in L). ARF7 expression is observed in procambial cells (arrow in M) before and during xylogenesis (arrowhead in M). Size bars are 20 µm in C-K and 50 µm in A-B, L-M.
Figure 5-4 Comparison of ARF5/MP, ARF3 and PIN1 expression in earliest stages of leaf primordia development.
Figure 5-5. Auxin responsive ARF gene transcription in leaf primordia and primary roots.

All plant material is from 3 DAG (Stage 3) WT plants except for A-D which are plants harbouring the PromDR5::GUS reporter gene. Whole mount in situ hybridization were performed on control and auxin treated seedlings as indicated. Representative images of each treatment and ARF in situ are presented. Arrows in I-P highlight auxin mediated ectopic expression of ARF3 and ARF5/MP respectively, in both leaf primordia and primary roots. All images are the same magnification and size bar in A is 50 µm.
Figure 5-5 Auxin responsive ARF gene transcription in leaf primordia and primary roots.
Experiments were conducted in 2 biological replicates, resulting in two control and four auxin treatments (two IAA and two 2,4-D). The data from the biological replicates were pooled and subjected to students T-test analysis. Statistically significant differences between control and auxin treatments are indicated. Several attempts at amplifying a gene specific fragment suitable for Q-PCR for ARF6 failed and therefore was not included in this analysis. p-values for statistically significant auxin mediated induction of the ARF5/MP gene are; DMSO-IAA p=.02, DMSO-2,4D p=.002 and for ARF19 are; DMSO-IAA p= .026, DMSO-2,4D p= .006. Error bars are standard deviations.
Figure 5-7  Analysis of ARF3, ARF7 and ARF19 expression in mp mutants.

Expression of ARF5/MP (A,C,D), ARF3 (B,D,F), ARF7 (G) and ARF19 (H) in mp mutant leaves at 3 DAG (A,B), 7 DAG (C,D) and 14 DAG (E-H). ARF3 is initially expressed in very young mp mutant leaf primordia (B) but is absent in later stages of development (D,F). Procambial cells still form in mp mutants and express mutant mp transcripts. Scale bars are 20 µm in A,B and 50 µm in C-H.
**Figure 5-8 Leaf initiation defects in mp arf7 and mp arf3 double mutants.**

*mp* mutant (A,B), *mp arf7* double mutant (C,D) and *mp arf3* double mutant (E, F) at 21 DAG. Emerging leaf primordia are highlighted by white arrows in *mp* mutants (B) and the approximate centre of the meristem is highlighted by a red asterix (B, D, F). Size bars are 500 µm in A, C, E and 100 µm in B, D and F.
<table>
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<tr>
<th>qPCR primers</th>
<th>Sequence</th>
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<tr>
<td>ARF3 RT F</td>
<td>TCATCACCTCTTCCGTTCTT</td>
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<tr>
<td>ARF3 RT R</td>
<td>TTCCTTGGCGAATGATGATGA</td>
</tr>
<tr>
<td>ARF4 RT F</td>
<td>TCCTGAAAAAGGATGGAGGAGGA</td>
</tr>
<tr>
<td>ARF4 RT R</td>
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</tr>
<tr>
<td>MP RT F</td>
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<tr>
<td>MP RT R</td>
<td>CCTTACGCATCCCACAAAAT</td>
</tr>
<tr>
<td>ARF7 RT F</td>
<td>AACTTTGCGCGGTGACCAGT</td>
</tr>
<tr>
<td>ARF7 RT R</td>
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<tr>
<td>ARF19 RT F</td>
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</tr>
<tr>
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<tr>
<td>IAA1 RT R</td>
<td>CAGGAGAGAGGAGCAGATTCTT</td>
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</table>

**Table 5-1** Primer sequences used for RT Q-PCR.
5.7 References


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6: THESIS SUMMARY

Auxin is an important regulator of plant growth and development. The results presented in this thesis contribute to the understanding of how auxin regulates the processes of leaf initiation and leaf vascular tissue patterning. Hundreds of genes are differentially regulated by auxin as demonstrated by microarray experiments (Pufky et al., 2003; Okushima et al., 2005a; Vanneste et al., 2005). Nearly 90% of these auxin-induced or repressed genes contain the TGTCTC ARF DNA binding motif within the first 2kb of their 5’ promoter region, supporting a putative role of ARF proteins in their regulation (Okushima et al., 2005). Differential gene expression is impaired in ARF mutants in response to auxin and this defect is more pronounced in double ARF mutants (Okushima et al., 2005b; Okushima et al., 2005a). These results are in line with the observation that ARF single mutant phenotypes are more severe in double mutants of closely related ARF genes (Hardtke et al., 2004; Ellis et al., 2005; Nagpal et al., 2005; Okushima et al., 2005a). Auxin elicits diverse responses in different plant tissues and this may be mediated in part by tissue specific expression of auxin signaling components (Leyser, 2006). Analysis of ARF gene expression has revealed overlapping and non-redundant expression patterns of ARF1-8, 12, 16 and 19 throughout different plant organs including meristems and leaves (reviewed in Guilfoyle and Hagen, 2007). These diverse ARF expression patterns are
complemented by similarly diverse expression patterns of AUX/IAA genes, which have been shown to be important regulators of ARF function (reviewed in Mockaitis and Estelle (2008)). The resulting ‘auxin code’ could determine how auxin drives various patterning and growth responses throughout the plant (Leyser, 2006).

The roles of auxin transport and signaling in leaf initiation

The MONOPTEROS and PIN-FORMED 1 genes have been shown to be important components of auxin signal transduction and auxin transport respectively (Galweiler et al., 1998; Hardtke and Berleth, 1998). Interestingly, previous studies have demonstrated that mp and pin1 mutants display similar defects in flower initiation from reproductive shoot apical meristems and both have reduced capacities for polar auxin transport (PAT) (Okada et al., 1991; Przemeck et al., 1996). These studies suggest a mechanistic link between MP-mediated auxin signaling and PIN1-mediated auxin transport. To test whether MP exerts its effect on lateral organ formation exclusively as a regulator of PIN-mediated auxin transport, we created mp pin1 double mutants and also grew mp mutants on medium supplemented with auxin efflux inhibitors.

In chapter 2 of this thesis we described a novel leafless phenotype in mp pin1 and mp mutants treated with various PAT inhibitors including NPA (1-naphthylphthalamic acid). Instead of forming leaves these mutants generated a large dome-like structure, which encompassed enlarged expression domains for molecular markers for the central zone and entire meristem zone. Leaf founder
cell markers were expressed in dispersed peripheral regions surrounding the enlarged meristem domain of NPA-treated mp leafless domes. These results indicate that leaf founder cell specification was not defective in these mutants but that they are defective in subsequent leaf outgrowth. The synergistic phenotype was never observed in either mp or pin1 single mutants and indicates that MP promotes leaf initiation in a process not limited to regulation of PAT (Fig. 6-1). The data also suggested that MP-mediated processes restrict shoot apical meristem size, particularly the central zone (Fig. 6-1).

The enlarged expression of the central stem cell zone maker gene CLAVATA3 in NPA-treated mp leafless shoots could explain the leaf initiation defects observed. To test this hypothesis, we generated double mutants of mp with clvata 1 (clv1), a mutant defective in restricting central zone size, and wuschel (wus), a mutant defective in central zone maintenance. The results presented in chapter 3 indicate that MP restricts central zone size independently of the established negative feedback loop involving the WUSCHEL (WUS) and CLAVATA genes (Fig. 6-1). Elimination of WUS function in mp wus double mutants results in various defects in leaf initiation which is associated with formation of leafless shoots somewhat similar to what is observed in mp pin1 mutants. We hypothesized that WUS normally acts to suppress auxin signaling in the central zone of the meristem and/or that WUS-mediated cytokinin signaling could be involved in the leaf initiation defects observed in mp wus double mutants (Fig. 6-1).
The roles of auxin transport and signaling in leaf vascular tissue formation.

In chapter 4 of this thesis we proposed a feedback regulatory loop that involves auxin, MP and PIN1 and provided novel experimental support for the canalization-of-auxin-flow hypothesis. We observed that MP transcription is induced in response to ectopic auxin application and that PIN1::GFP expression is severely reduced in mp mutant leaves. The reduced PIN1::GFP expression in mp mutants suggested that PIN1 expression is dependent on MP function. Defects in auxin accumulation as visualized by the PromDR5::GUS transgene, are also observed in mp and pin1 mutant leaves, which confirms a role for these proteins in auxin distribution. The data presented provide some of the first molecular support for the canalization of auxin flow hypothesis originally proposed by Sachs (1981).

In chapter 5 we continued to evaluate the roles of auxin signaling in leaf vascular tissue formation by aiming to identify ARF genes that could function redundantly with MP. By evaluating the expression patterns of the six ARF genes phylogenetically most related to MP, we identified the ETTIN/ARF3, NPH4/ARF7 and ARF19 genes as playing potential roles in leaf vascular tissue formation. Expression patterns of ETTIN/ARF3 were most similar to MP throughout leaf development and both genes were expressed in pre-procambial cells of the developing midvein and procambial cells of secondary, tertiary and quaternary leaf veins. NPH4/ARF7 was expressed in procambial cells before and after xylogenesis. ARF19 expression appeared to coincide with the initiation of xylogenesis in procambial cells and continued to be expressed in developing
protoxylem cells. Furthermore, ectopic auxin application experiments demonstrate that MP/ARF5, ETTIN/ARF3 and ARF19 transcription appear to be responsive to auxin. These results reveal a specific expression network of four different ARF genes in leaf vascular tissues over time (Fig. 6-2). The ARF expression network documented in this study support the idea of an ‘auxin code’ functioning to interpret local auxin gradients into developmentally relevant patterning or differentiation processes. Although defects in vascular pattern formation are only observed in mp mutants, a functional MP gene appears to mask any obvious defects in ettin/arf3 or nph4/arf7 mutants. Analysis of mp ettin/arf3 and mp nph4/arf7 double mutants revealed novel roles for both ETTIN/ARF3 and NPH4/ARF7 in leaf initiation. Subsequent analysis of leaf vascular tissue patterning and differentiation in these double mutants could result in the uncovering of novel functional roles for ETTIN/ARF3 and NPH4/ARF7 in vascular tissue formation.
The MP and PIN1 proteins function to promote auxin transport to sites of incipient leaf formation on the flanks of the SAM. MP plays additional roles in promoting leaf initiation independent of PIN-mediated auxin transport. WUS promotes the expression of the CVL3 gene and maintains central zone cell fate. Central zone size is regulated by a negative feedback loop involving the CLV3 and CLV1/2 genes. Activation of the CLV1/2 receptor by the CLV3 ligand leads to the repression of WUS expression. WUS also appears to promote leaf initiation through an unknown mechanism and MP-dependent leaf initiation results in restriction of central zone size independently of CLV1/2. Auxin accumulation is highlighted in blue.
Figure 6-2 Auxin signaling and transport in leaf vascular tissue formation.

The ARF transcription factor MP/ARF5 and auxin transport protein PIN1 positively regulate auxin transport and accumulation in pre-procambial cells. Factors involved in procambial cell differentiation like *AtHB8* (Baima et al., 2001) have also been implicated as being positively regulated by MP *(Mattsson et al., 2003; Krogan, 2006)*. Expression of other ARF genes including *ETTIN/ARF3*, *NPH4/ARF7* and *ARF19* are observed during specific stages of vascular cell formation as indicated (pre-procambial, procambial, and procambial cells undergoing xylogenesis). Auxin appears to be a key regulator in promoting the expression of *MP/ARF5*, *ETTIN/ARF3* and *ARF19* indicated in blue.
6.1 References


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APPENDICES

Induction of xylem and fiber differentiation in *Populus tremuloides*

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Statement of contribution:

M. Schuetz was involved in the initial planning of the study and performed all RT QPCR experiments including indentifying candidate genes from publically available microarray and EST databases. All authors contributed to the writing of the manuscript.

1. Abstract

Vascular tissues are of particular importance to terrestrial plants as they allow long-distance transport within the plant and also provide support for upright growth. Nowhere are these traits more obvious than in tree species. Here we have evaluated the role of auxin transport in the differentiation of primary and secondary vascular tissues in a tree species, trembling aspen (*Populus tremuloides* Michx). We found that a partial inhibition of auxin transport resulted in increased width and numbers of veins in leaves. A similar vascular overgrowth was observed during early secondary vascular differentiation of stems. This stem overgrowth consisted almost entirely of early differentiation of metaxylem and fibers. We hypothesize that the early differentiation of metaxylem and fibers
result from inhibitor-induced accumulation of auxin in stems and that the differentiation of these tissues require higher levels of auxin exposure than protoxylem. The controlled conditions used in this study also provide a framework for reverse genetics approaches to identify genes involved in vascular differentiation based on elevated expression in tissues developing vascular overgrowth.
2. Introduction

The vascular system of plants distributes water, nutrients and photoassimilates throughout the plant, as well as providing structural support for upright growth in a terrestrial environment (Esau, 1965; Nelson and Dengler, 1997). Woody trees provide extreme examples of these traits. Despite the economic and environmental importance of woody species, the developmental regulation of vascular patterning, differentiation and wood formation is relatively poorly understood. Considerable inroads to the genetic basis of vascular patterning and differentiation have been made in the model species Arabidopsis thaliana and Zinnia elegans (reviewed by Fukuda, 2004; Sieburth and Deyholos, 2006). A large part of this information relates to the role of auxin in vascular development. Jacobs (1952) reported that polar auxin transport (PAT) of auxin can induce the formation of new vascular strands in basal tissues, a finding corroborated by many subsequent experiments including alterations of endogenous auxin levels in transgenic plants (Klee, 1987). Auxin influx and efflux carrier proteins are now known to play key roles in PAT (Lomax et al. 1995; Friml 2003).

Sachs (1989) proposed the canalization of auxin flow hypothesis, suggesting that polar auxin transport arises by an autocatalytic mechanism in which initially diffusely distributed auxin is actively canalized into distinct strands of cells as some cells become better transporters of auxin than others. Genetic and chemical manipulations of PAT provide evidence for the auxin canalization hypothesis since partial PAT inhibition causes the formation of more and wider veins in A. thaliana (Mattsson et al., 1999; Sieburth, 1999). Visualization of the
auxin efflux carrier protein PINFORMED1 (PIN1) shows the gradual focusing of expression into discrete procambial strands, providing further evidence for the canalization of auxin (Scarpella et al. 2006; Wenzel et al. 2007). PIN1 appears to be part of a feedback regulatory loop that includes MONOPTEROS (MP), an auxin response factor, and the transcriptional and post-transcriptional regulation of MP by auxin (Wenzel et al., 2007). The remarkably dynamic patterns of auxin carrier protein distribution have provided insight into the direction of auxin flow and its role in the formation of distinct auxin maxima that appear to be required for the formation of virtually every organ in plants (Benkova et al., 2003), as well as for vascular strand formation (Mattsson et al. 2003).

While the molecular mechanisms behind vascular patterning and differentiation are beginning to be unraveled in the annual plant A. thaliana, the same cannot be said for the formation of wood in trees. For a number of reasons, trees are generally intractable for genetic analysis by mutant screens and map-based gene cloning. Novel genetic resources such as the sequenced genome of *Populus trichocarpa* (Tuskan et al., 2006) and extensive microarrays combined with relatively rapid growth and easy genetic transformation makes this species highly suitable for various reverse genetics approaches. However, the lack of mutants restricts the application of technologies such as microarrays in tree species as they rely on differential gene expression between for example control plants and plants defective in vascular differentiation. Hence, in this study we have evaluated the response of the vascular system of *Populus tremuloides* to PAT inhibitor-induced reduction of auxin transport with emphasis on identifying
differences that could be used in screens to identify genes with potential functions in vascular differentiation and wood formation of a tree species. Our observations confirm findings from similar experiments in *A. thaliana*, and also provide novel findings on PAT inhibitor-induced early formation of metaxylem and fibers, the primary constituents of wood.

3. Materials and methods

**Plant material and growth conditions**

*Populus tremuloides* Michx (trembling aspen) seeds were provided by the National Tree Seed Centre, Natural Resources Canada, Fredricton, NB. Seeds were surface-sterilized for 4 h with chlorine gas (3% HCl in commercial bleach) and then plated on solid ATS medium (Lincoln et al., 1990) containing a final concentration of 0, 2, 4, 8 or 16 µmol L\(^{-1}\) of the polar auxin transport (PAT) inhibitor 1-N-naphthylphtalamic acid (NPA; Chemical Service, West Chester, PA, USA) or 2-chloro-9-hydroxyfluorene-9-carboxylic acid (HFCA; Sigma, St. Louis, MO, USA). Both PAT inhibitors were dissolved in dimethylsulfoxide (DMSO) and final equivalent volumes of DMSO were added to all media. Seeds were initially plated in 9 cm Petri dishes (ca 20 seeds/plate). Seedlings displayed uneven germination rate and we selected the best performing plants for subculture after 2 weeks into 6 x 6 x 10 cm magenta boxes (9 plants/magenta). Seedlings were grown at approximately 24°C and 8 hr dark/16 hr light (120-150 µmol m\(^{-2}\) s\(^{-1}\))
with timing of days after germination (DAG) commencing at the start of incubation.

**Microscopic analyses**

The effect of PAT inhibitors on leaf venation and root, hypocotyl and stem vasculature was determined for 4-week-old seedlings exposed to the different treatments. To examine leaf venation patterns, excised first-node leaves were fixed, cleared, mounted and examined with dark-field illumination as in Mattsson et al. (1999). To assess internal anatomy of axial organs, 1-4 mm segments were taken (a) ca 2 mm proximal of the root tip, (b) from the hypocotyl just above the root/hypocotyl junction, and (c) from the mid region of the 1st, 2nd and 3rd stem internodes, where 1st is the most mature internode. These tissues were fixed overnight in FAA (5% formaldehyde, 15% acetic acid, 50% ethanol), dehydrated and embedded in paraffin as described in Ruzin (1999). 8-12 µm thick sections were cut with a Leitz Wetzlar (Walter A. Carveth Ltd.) microtome, and adhered for 1 d at 40°C to slides coated in 1% w/v gelatin. Paraffin was removed by two 15 min incubations in xylene, and the sections then mounted in Permount (Fisher Scientific, Waltham, Massachusetts, USA) and hardened for 1 d at 60°C. Microphotographs were taken with a Nikon Eclipse E600 microscope using dark-field or differential interference contrast settings and a Canon EOS D30 digital camera.
**Gene expression analysis**

We assessed the expression of two genes, *PttHB9*, a poplar homolog of *PHAVOLUTA/PHABULOSA* (Schrader et al. 2004), and *PttF5H*, a poplar homolog of the gene encoding ferulate-5-hydroxylase (Franke et al. 2000). Aspen plants were grown as above on media containing 0 or 2 µmol L\(^{-1}\) NPA for 14 or 21 DAG, at which times the entire shoots above the cotyledons were excised and immediately frozen in liquid nitrogen. Total RNA from aspen shoots was extracted using TRIZOL reagent (Invitrogen, Carlsbad, California, USA), DNasel-treated and used for cDNA synthesis using Revertaid reverse transcriptase (Fermentas, Burlington, Ontario, Canada) and oligo(dT) primers according to manufacturers’ specifications. cDNA of *PttHB9* and *PttF5H* was amplified using Platinum SYBER Green qPCR SuperMix (Invitrogen) on the Rotor-Gene\textsuperscript{TM} 3000 real-time quantitative PCR machine (Corbett Life Sciences, San Francisco, California, USA). cDNA was made from RNA collected from two independent biological experiments, and three qPCR reactions were performed on each cDNA sample using the primers for *PttHB9* (TGCTTGTGCTCAGCTTGTCT and AAAATTGGAACGCGATTGTCT) and for *PttF5H* (GACGAGGTGGACTCAATGGT and CCAAGGAATGAAATCCGAAA). Primers were designed against hybrid aspen ESTs (Genbank accessions AY919619 (*PttHB9*) and DT502577 (*PttF5H*)). The CYCLOPHILIN internal control gene primers are as described in (Brunner et al., 2004). Relative fold induction of gene expression was calculated using the 2\(^{-\Delta\Delta CT}\) method.
(Applied_Biosystems, 2001; Livak and Schmittgen, 2001) and standard deviations calculated on technical replicates.

4. Results
Previous experiments primarily with *Arabidopsis thaliana* have provided evidence that the process of polar auxin transport is a central component of the mechanism that regulates patterning as well as the extent of vascular tissue formation (Mattsson et al. 1999; Sieburth 1999). Due to the lack of appropriate genetic mutants, in this study we used a pharmacological approach to assess if auxin transport plays an equally important role in vascular development of the woody tree species *Populus tremuloides* (trembling aspen). We grew *P. tremuloides* from seed to 1-month-old plantlets in axenic cultures on medium supplemented with chemical auxin transport inhibitors. In lieu of independent mutants of various allelic strengths, we used two chemically distinct inhibitor substances and a series of concentrations to discern dosage-dependent inhibition on overall plant growth and vascular differentiation. We focus on describing anatomical and morphological tendencies from a range of inhibitor concentrations so as to provide a holistic picture of the potential role of auxin transport in vascular development of this species. Results are based on at least 3 or 10 plants for anatomical and morphological trends respectively.
Effect of auxin transport inhibitors on *P. tremuloides* shoot and root growth

After 4 weeks, *P. tremuloides* plants grown in vitro on media without auxin transport inhibitors had produced a 15-20 mm long stem with 10 or more leaves and a ramified root system (Figs. 1A, 2A). Plants grown on media supplemented with HFCA or NPA had a concentration-dependent decrease in stem length, and greatly reduced number of leaves at the highest concentrations (Fig. 1). With HFCA, stem length was reduced to about 50% (1 and 2 µmol L\(^{-1}\)), 33% (4 µmol L\(^{-1}\)), and 25% (8 and 16 µmol L\(^{-1}\)) compared to control plants (Fig. 1). Leaf number was only affected at HFCA concentrations above 4 µmol L\(^{-1}\): six-eight leaves at 4 µmol L\(^{-1}\) HFCA; only two leaves at 8 µmol L\(^{-1}\), with plants often having one or two short shoots from the cotyledon axils (arrow, Fig. 1E); and at 16 µmol L\(^{-1}\) HFCA plants usually had only one fused or tubular leaf (Fig. 1F). Growth in the presence of NPA also resulted in increasingly dramatic reduction in shoot growth and leaf number with higher inhibitor dosages (Figs. 1G-K). The root system was also reduced with both NPA and HFCA, resulting in increasingly reduced root length and lateral branch number at higher concentrations. At high concentrations of PAT inhibitors, plants had only a short primary root with no lateral branch roots (Fig. 2). Exceptions to the above tendencies were a few (<5%) seedlings that harbored either a fasciated root tip (Fig. 2L) or a large number of clustered lateral roots (Fig. 2M).
Effect of auxin transport inhibitors on P. tremuloides leaf venation

*P. tremuloides* leaves have a hierarchal venation pattern, with lower order primary midvein and secondary veins and higher order tertiary and quaternary veins. The first true leaves of control plants had a narrow primary midvein. Under our growth conditions, the first leaf had produced a lamina with an average of eight secondary veins after 4 weeks of growth (Fig. 3A). The secondary veins formed a series of prominent arches in which the distal end of a secondary vein joined the neighboring secondary vein or the primary vein. A network of tertiary and quaternary veins extended respectively from secondary or tertiary veins into the intercostal areas and also close to the leaf margin.

In the first leaves of plants grown in the presence of a series of concentrations of auxin transport inhibitors, a number of alterations in overall vein organization were observed. Here we will again first describe the effects of HFCA, followed by a short comparison to NPA. While 2 µmol L⁻¹ HFCA resulted in visibly smaller leaf lamina, it had little effect on the venation pattern (Fig. 3B). On the other hand, plants grown on 4 µmol L⁻¹ HFCA had first leaves with considerably broader lower order veins and generally more discernable secondary veins than in control leaves (Fig. 3C). These effects were even more pronounced at 8 µmol L⁻¹ HFCA and frequently accompanied by a partial fusion of the first two leaves (Fig. 3D). At 16 µmol L⁻¹ HFCA, the lamina of the first two leaves was much reduced, and the petiole extended around much or all of the stem, resulting in a first leaf that appeared tubular (Fig. 3E) or indeed was tubular in shape (Fig. 1F). Such leaves appeared to have multiple major veins
connecting to the petiole, resulting in a very high proportion of vascular tissue in the lamina (Fig. 3E). Although a similar tendency of increased venation and leaf fusion was observed for NPA, the effect of corresponding concentrations of NPA on the leaf venation was much less pronounced than the effects for HFCA (Figs. 2F-I).

Both HFCA and NPA also affected higher order venation patterns in leaves. The inhibitor treatments often made it almost impossible to separate tertiary and quaternary veins from each other, and in these cases we therefore refer to them collectively as higher order veins. We compared four regions of the leaves: the apex, the midvein approximately half way along the proximo-distal axis of the blade, the leaf margin at one of the major serrations, and the petiole. Vascular overgrowth was evident in all vein types already at the lowest concentration of 2 µmol L⁻¹ HFCA (Fig. 4). Several general tendencies could be discerned as an effect of auxin transport inhibition by HFCA. First, vascular tissues accumulated at the leaf apex (Figs. 4C-E). Second, the density of secondary veins increased and they often bifurcated and thickened towards the margin, often blurring the distinction between secondary and higher order veins (Figs 4F-O). Third, the density of higher order veins increased especially at the margin (Figs. 4K-O). Finally, the width of the midvein generally increased in plants grown in medium with 2-16 µmol L⁻¹ HFCA, and at 8 and 16 µmol L⁻¹ HFCA the blades and petioles appeared to have a very wide midvein or multiple primary veins (Figs. 4F-J and P-T). Similar tendencies were observed from
growth in medium with corresponding NPA concentrations, albeit with weaker effects than those observed from HFCA treatment (Fig. 5).

Effect of auxin transport inhibition on stem vascular tissues in P. tremulodies

Figure 6 shows a panel of cross sections through several positions along stems of 4-wk-old plants, revealing the effects of auxin transport inhibitors on the extent of vascular tissues in stems. While the effects of HFCA on leaf venation were much stronger than NPA, the effect was reversed in the stem, where NPA had a stronger effect on vascular tissues. Figure 6 therefore shows a more detailed picture of the effect of NPA on stem vascular tissues, and only a brief summary of the effects of HFCA. While 1 µmol L\(^{-1}\) NPA had no effect on the vascular tissues of the hypocotyl (Fig. 6N), the 1st internode contained approximately 2-3 times as much xylary tissues as the first internode of control plants (arrow in Fig. 6J, compare to 6I). Moreover, the first internode of plants grown in the presence of 1 or 2 µmol L\(^{-1}\) NPA contained more differentiated phloem fibers than the control plants (arrow in Fig. 6K, compare to Fig. 6I). In 1 µmol L\(^{-1}\) NPA samples, xylary and fiber overgrowth was observed also in the second and third internodes, albeit less pronounced (Figs 6B, F). The xylary and fiber overgrowth was also evident in the first and second internodes of plants grown on 2 µmol L\(^{-1}\) NPA (Figs. 6G, K). In contrast, the hypocotyl and third internode of plants grown on 2 µmol L\(^{-1}\) NPA showed a reduction of xylem tissues (Figs. 6C, O). At 4 µmol L\(^{-1}\) NPA, only a phloem fiber overgrowth was observed in the first internode (Fig. 6L), and all other internodes and the hypocotyl displayed a marked reduction of
xylem tissues (Figs. 6D, H, P). HFCA-grown material displayed similar xylary and fiber overgrowths as NPA-grown material, although the xylary overgrowth was less extensive than in NPA grown material (Figs. 6Q-T).

Medial longitudinal sections of the first internode of NPA-grown plants confirmed the induced xylem and fiber overgrowths (Fig. 7) and also provided an unexpected finding. Control samples contained conspicuous protoxylem, identified by annular or spiral secondary wall thickenings (Fig. 7A). In contrast, NPA-grown samples taken from the same position showed an approximately equal amount of protoxylem as controls, but the xylem overgrowth was almost entirely due to early formation of metaxylem cells, characterized by scalariform secondary cell wall thickenings (Fig. 7B). NPA-grown plants also had phloem fibers (Fig. 7B) that were usually absent in control material of the same age (Fig. 7A). In summary, relatively low concentrations of both NPA and HFCA resulted in early development of metaxylem as well as fibers. These effects appeared to peak at 2 µmol L⁻¹, and higher concentrations of inhibitors resulted in reduced amounts of vascular tissues.

**Expression of vascular markers in auxin transport-inhibited P. tremuloides tissues**

In theory, the observed vascular overgrowth in auxin-transport-inhibited tissues could be used for large-scale identification of differentially expressed genes, for example by microarray analysis. To carry out an initial assessment of this assumption, we analyzed the expression of two genes in control shoots versus shoots developing vascular overgrowth when grown in 2 µmol L⁻¹ NPA. The
tissues grown in 2 μmol L⁻¹ NPA included internodes showing vascular overgrowth (e.g. Figs. 6G, K), although the leaves had little overgrowth (Figs. 5A, E, I, M). We chose a potential early and a late marker of xylem differentiation: PttHB9, which is similar to the Arabidopsis HD-ZIP III family of transcription factors and is expressed in xylary procambial cells of aspen (Hertzberg et al., 2001; Schrader et al., 2004); and PttF5H, which encodes the enzyme ferulate-5-hydroxylase (F5H) involved in lignin biosynthesis (Franke et al., 2000). We hypothesized that the observed vascular overgrowth would be preceded by an up-regulation of PttHB9 and PttF5H in the NPA-grown material. Neither PttHB9 nor PttF5H were significantly up-regulated at 14 DAG (data not shown). At 21 DAG, however, the analysis of expression in two biological replicates at 21 DAG revealed a small but significant up-regulation of both genes in NPA-grown shoots (Fig. 8). It is clear though that these small differences in expression would be difficult to capitalize upon in a screen for novel genes involved in vascular differentiation. Larger differences in gene expression can probably be obtained though by optimizing sampling time and by collecting specific organs with vascular overgrowth (see discussion).

5. Discussion

Although auxin is known to be an essential growth hormone in plants, the extent of the role of polar auxin transport in vascular patterning in plants of various growth patterns and phylogenetic origins remains to be shown. The effects of auxin transport inhibition on vascular organization of the dicot plant A. thaliana
are quite spectacular with extensive modifications of leaf, stem and root vascular patterns (Mattsson et al., 1999; Sieburth, 1999). On the other hand, the effects of auxin transport inhibition on the leaf venation of Zea mays and Oryza sativa are relatively small with limited overgrowth in veins and minor changes in vein spacing (Tsiantis et al. 1999; (Scarpella et al., 2002). These varied responses may potentially be related to differences in the efficacy of inhibitors on dicot and monocot classes of flowering plants or fundamental differences in vein patterning between dicot and monocot plants as suggested by Scarpella et al. (2002). It is also possible that the strong response in A. thaliana is an exception rather than an indication of a general trend. Polar auxin transport has also been implicated in the differentiation of secondary vascular cambium in aspen trees (Uggla et al., 1996; Tuominen et al., 1997; Hertzberg et al., 2001; Schrader et al., 2004). While elegant, these studies are based on correlations since the genetic tools for experimental manipulation of auxin transport are not yet available in tree species. Here we have shown that chemical inhibitors of polar auxin transport dramatically affected early development of the woody tree species Populus tremuloides (trembling aspen).

**P. tremuloides growth and vascular development depend on auxin transport**

Similar to previous observations in A. thaliana, we observed extensive modifications of growth, lateral organ formation and vascular development in P. tremuloides in response to chemical inhibition of auxin transport. These observations suggest that auxin transport plays equally fundamental roles in the
development of this species and also provides some credence for similar roles throughout at least dicot species. Several studies have identified young leaf primordia as sources of free IAA (Davies, 1995; Ljung et al., 2001) and it has long been known that auxin is generally transported basally down the stem (Jacobs 1952). Since auxin stimulates cell division and expansion (Ullah et al. 2003; Campanoni and Nick 2005), it is not surprising that axial stem and root growth were progressively more stunted with higher inhibitor dosages that would impair auxin flow into these more basal organs (Figs. 1, 2). Similarly, the decreased growth of the primary root and frequency of lateral root formation that we observed in response to auxin transport inhibition can be explained by reduced levels of free IAA reaching the root, as auxin and polar auxin transport are essential for root growth and lateral root formation (Reed et al., 1998). Differently from observations in A. thaliana (Mattsson et al. 1999), we rarely observed fasciated root tips in P. tremuloides, possibly due to stronger inhibition of growth on root apical meristems or more stable patterning in P. tremuloides root meristems.

Auxin has long been suspected to play a pivotal role in leaf formation and phyllotaxy (Steeves and Sussex 1989). There is now evidence that auxin influx and efflux carriers canalize auxin to form localized auxin maxima that are required for formation of leaf primordia (Benkova et al. 2003; Reinhardt et al. 2000, 2003; Reinhardt 2005), as well as discrete vascular strands (Sachs 1989). We observed reduced leaf number with increasing inhibitor concentration (Fig. 1) presumably owing to disruption of auxin maxima formation, thus initiating fewer
leaf primordia with these treatments. The formation of fused or tubular leaves that we observed in *P. tremuloides* (Figs. 3D, E, I) may be explained by reduced canalization leading to wider leaf primordia formation in a competent zone, sometimes all around the meristem. Another contributing component to the wider primordia may be a reduced suppression of auxin maxima formation from existing primordia, due to reduced drainage of auxin through them (Reinhardt 2005).

**Reduced auxin transport affected vascular differentiation in *P. tremuloides***

The auxin canalization theory suggests that initially diffuse auxin distribution is gradually actively channeled into discrete canals that differentiate into vascular strands (Sachs 1989). Our results support a role for the canalization and polar transport of auxin in *P. tremuloides* vascular differentiation. Just as with *A. thaliana* (Mattsson et al. 1999; Sieburth 1999), PAT inhibitors induced vascular overgrowth in *P. tremuloides* leaves (Figs. 3-5). We found that the extent of vascular overgrowth in *P. tremuloides* leaves was correlated with vascular differentiation in the stem. At low PAT inhibitor concentrations of 1 and 2 µmol L⁻¹, both leaves and stems of *P. tremuloides* showed vascular overgrowth (Figs. 4-6). At these concentrations auxin canalization was impaired, presumably forming broad channels of auxin that still permitted sufficient auxin transport into the stem resulting in wider vascular strands throughout the plant, as is also true in *A. thaliana* (Mattsson et al. 1999, 2003). In *A. thaliana*, stronger PAT inhibition resulted in leaves having predominantly marginalized vascular tissue with little or
no venation at the base of the leaves (Mattsson et al. 1999). At PAT inhibitor dosages $\geq 4 \mu\text{mol L}^{-1}$, _P. tremuloides_ leaf vascular overgrowth increased significantly and we also observed a shift towards the margin of vascular tissues, especially with HFCA treatment (Figs. 3C-E, 4). This possibly indicates the presence of marginal auxin sources and thus marginal pooling of auxin with PAT inhibition in _P. tremuloides_ leaves, similar to what is observed in _A. thaliana_ leaves (Mattsson et al., 1999; Aloni et al., 2003; Mattsson et al., 2003) Unlike with _A. thaliana_, we did not observe the absence of vascular differentiation at the base of _P. tremuloides_ leaves with high inhibitor dosages (Figs. 3-5), but we did find decreased stem vascular differentiation (Fig. 6). At these high inhibitor dosages, more extensive leaf overgrowth likely significantly disrupted basal polar auxin transport, resulting in insufficient auxin supply for stem vascular differentiation (Fig. 6). Likewise, compared to NPA, the more extensive vascular overgrowth in HFCA-treated leaves (Figs. 4 versus 5) was correlated with less vascular differentiation in HFCA-treated stems than for NPA at all dosages tested (Fig. 6), probably because HFCA treatment more significantly impaired polar auxin transport from the leaves into the stems.

Our observations are consistent with the proposed role of auxin flows in orienting vascular cell differentiation and thereby promoting vascular continuity. Broadening zones of vascular differentiation would be the likely outcome of local auxin accumulation and lateral auxin spread under conditions of slightly impaired drainage, whereas higher inhibitor dosages causing insufficient auxin flow would prevent vascular differentiation in more basal tissues. Furthermore, since
metaxylem and fibers were induced in stems with mild PAT inhibition at a time when they were still missing in control plants (Figs. 6, 7), we propose that slightly higher amounts of auxin exposure, either by elevated concentrations or prolonged time of exposure, are needed to induce metaxylem and fiber cell fate determination and differentiation than for the induction of protoxylem (Fig. 9). A similar hypothesis has been proposed to explain the formation of phloem ahead of xylem (Aloni, 1987), but to the best of our knowledge not for protoxylem, metaxylem and fibers.

**Induced vascular overgrowth may be used to identify genes expressed in vascular tissues**

The genetic basis of vascular patterning and differentiation is currently based primarily on molecular genetic studies in the annual plants *A. thaliana* and *Zinnia elegans* (Fukuda, 2004; Sieburth and Deyholos, 2006), and studies on secondary vascular differentiation in wood formation primarily in poplar species (Uggla et al., 1996; Tuominen et al., 1997; Hertzberg et al., 2001; Schrader et al., 2004). Poplar is an attractive model for these studies because of its annotated and sequenced genome (Tuskan et al., 2006), gene transformation technology (Han et al., 2000), and extensive microarray resources. The long generation cycle and dioecious nature of poplar species make them unsuitable for rapid forward genetic studies. Progress in the understanding of vascular and wood development in poplar species is therefore likely to be gleaned from the study of potential poplar gene orthologues to genes of proved function in plants like *A. thaliana* and *Z. elegans* in combination with various reverse genetics studies. In
This study we have found evidence suggesting that two genes potentially involved in xylem differentiation are up-regulated in tissues showing induced vascular overgrowth (Fig. 8). The recorded differences in gene expression were small though, and at least based on these marker genes, not suitable for large-scale microarray analysis. Differences in gene expression could most likely be further increased with careful separation of the specific organ developing the overgrowth such as NPA-grown internodes (Fig. 6) or HFCA-grown leaves (Fig. 4). Similarly, temporal analyses of the expression of specific marker genes involved in vascular differentiation could be used to identify peaks of expression and thereby suitable times for tissue harvesting. In addition, the observed differences between control and inhibitor-grown material with respect to metaxylem and fiber formation (Figs. 7, 9) could potentially result in much larger differences in gene expression if the identified genes are truly specific to either of these cell types. In summary, we have used a pharmacological approach to uncover some of the roles of polar auxin transport in vascular patterning and differentiation in *Populus tremuloides* and provided an avenue for the identification of genes involved in primary and secondary vascular differentiation in *Populus* and potentially other tree species.
Fig. 1. Effect of PAT inhibitors on stem growth and number of leaves in *P. tremuloides*.
Dark-field images of stems of 4-week-old plants grown on medium without inhibitor (A) or medium supplemented with 1 (B), 2 (C), 4 (D), 8 (E) or 16 (F) \( \mu \text{mol L}^{-1} \) HFCA, and 1 (G), 2 (H), 4 (I), 8 (J), 16 (K) \( \mu \text{mol L}^{-1} \) NPA. Asterisks mark the positions of cotyledons. Arrow in (E) indicates site of lateral shoot, and the arrow in (F) indicates a tubular leaf. Scale bar is 1 mm.
Fig. 2. Effect of PAT inhibitors on *P. tremuloides* root growth and branching.

Dark-field images of roots of 16-d-old plants grown on medium without inhibitor (A) or medium supplemented with 1 (B), 2 (C), 4 (D), 8 (E), or 16 (F) µmol L⁻¹ HFCA, and 1 (G), 2 (H), 4 (I), 8 (J), 16 (K) µmol L⁻¹ NPA. Scale bar is 5 mm. Right part of panel shows fascinated seedling root tip (L; 2 µmol L⁻¹ HFCA) and seedling root with high density of lateral roots (M; 4 µmol L⁻¹ HFCA). Scale bar is 1 mm.
Fig. 3. Effect of PAT inhibitors on *P. tremuloides* leaf morphology and venation. Dark-field images of first node leaves of 4-wk-old plants grown on medium without inhibitor (A) or supplemented with 2 (B), 4 (C), 8 (D), 16 (E) μmol L⁻¹ HFCA, and 2 (F), 4 (G), 8 (H), 16 (I) μmol L⁻¹ NPA. Scale bars are 1 mm.
Fig. 4. Effect of HFCA on leaf venation in P. tremuloides.
Dark-field images of 4-wk-old first node leaves sampled in four different positions: apex (A-E), midvein halfway along blade (F-J), margin at site of serration (K-O), petiole (P-T). Different HFCA concentrations are indicated by columns: control without HFCA (A, F, K, P); 2 µmol L\(^{-1}\) (B, G, L, Q); 4 µmol L\(^{-1}\) (C, H, M, R); 8 µmol L\(^{-1}\) (D, I, N, S); 16 µmol L\(^{-1}\) (E, J, O, T). Scale bar is 0.2mm.
Fig. 5. Effect of NPA on leaf venation in *P. tremuloides*.
Dark-field images of 4-wk-old first node leaves sampled in four different positions: apex (A-D), midvein halfway along blade (E-H), margin at site of serration (I-L), petiole (M-P). Different NPA concentrations are indicated by columns: 2 µmol L⁻¹ (A, E, I, M); 4 µmol L⁻¹ (B, F, J, N); 8 µmol L⁻¹ (C, G, K, O); 16 µmol L⁻¹ (D, H, L, P). Controls without NPA are as in Figs. 4A, F, K, P. Scale bar is 0.2 mm.
Fig. 6. Dosage-dependent regulation of vascular tissue formation in stems. DIC images of cleared transverse paraffin sections from 4-wk-old plants. Stems were sampled in four different positions indicated in rows: 3<sup>rd</sup> internode (I<sub>3</sub>, A-D), 2<sup>nd</sup> internode (I<sub>2</sub>, E-H); 1<sup>st</sup> internode (I<sub>1</sub>, I-L); hypocotyl (H, M-P). Concentration of NPA is indicated by columns: control without NPA (A, E, I, M); 1 µmol L<sup>-1</sup> (B, F, J, N); 2 µmol L<sup>-1</sup> (C, G, K, O); 4 µmol L<sup>-1</sup> (D, H, L, P). Examples of corresponding results for HFCA illustrated by sections from 1<sup>st</sup> internode (I<sub>1</sub>) in Q-T: control (Q); 1 µmol L<sup>-1</sup> (R); 2 µmol L<sup>-1</sup> (S); 4 µmol L<sup>-1</sup> (T). Arrow in (J) points at xylary overgrowth, arrow in (K) points at phloem fiber overgrowth. Scale bar is 0.1 mm.
Fig. 7. NPA-induced metaxylem and fiber differentiation.
DIC images of cleared median longitudinal paraffin sections from first internodes of 4-wk-old plants. Control (A), 2 μmol L⁻¹ NPA (B). PX = protoxylem, MX = metaxylem. F = fibers. Scale bar is 50 μm.
Fig. 8. Illustration of vascular overgrowth on the gene expression level
Real-time quantitative PCR expression analysis for an early (*PttHB9*) and late (*PttF5H*) marker of xylem differentiation in 21 DAG shoots developing stem vascular overgrowth in response to 2 µmol L\(^{-1}\) NPA relative to control plants. Two independent biological replicates were conducted (experiment 1 and 2). Error bars represent standard deviations of three technical qPCR replicates. Student’s T tests indicated statistically significant differences in gene expression between control and NPA-grown shoots. P-values experiment 1: *PttHB9* = 0.035, *PttF5H* = 0.013. P-values experiment 2: *PttHB9* = 0.0001, *PttF5H* = 0.009.
Fig. 9. Model explaining the qualitative differences in tissue differentiation induced by a reduction in auxin transport.

The model suggests that different cell types have different threshold values (dashed lines) of auxin exposure for cell fate specification/determination/differentiation (SDD) to occur in aspen stems. Threshold values are reached by a combination of time as well as concentration of auxin. Protoxylem (PX) SDD has the lowest threshold, while metaxylem (MX) and fibers (F) have higher threshold values for SDD to occur. In control samples ([IAA]\textsuperscript{control}) auxin transport is unperturbed and the concentration of auxin in cambial tissues remains relatively low as much of it passes through en route to the root. In NPA-grown samples ([IAA] = 2 \mu mol L\textsuperscript{-1} NPA), auxin transport is perturbed and auxin accumulates closer to the source (young leaves) resulting in MX and F differentiation in the first and second internodes at the cost of vascular hypotrophy in hypocotyls.
7. References


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