Multiple *MONOPTEROS*-dependent pathways are involved in leaf initiation

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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 harboring 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.
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 less frequently dividing cells provides cells for the more frequently dividing cells in the 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 which polarly 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
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.

**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., 1991; Przemeck et al., 1996) (Fig. 1A,B). 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. 1C,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 S1), supporting the notion that the individuals were double mutants. The domes had a smooth surface and lacked differentiated epidermal, trichome and stomata cells (Fig. 1D). 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. 1E). 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. 1F) 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. 1G,H). We interpreted these as inflorescences as they sometimes produced pistil-like or petal-like structures at their apices (Fig. 1I, data not shown). 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. 1J; Table S1). 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. 1K). 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 later 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. 1M). 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. 1N,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 2A, 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. 1L; Fig. 2A). 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. 2B). In contrast, in *mp* mutants NPA concentrations as low as 0.1 μM resulted in a dramatic decrease in leaf initiation (Fig. 2B) 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. Since MP is expressed specifically in leaf anlagen and primordia at the flanks of the meristem (Hardtke and Berleth, 1998; Wenzel et al., 2007), we tested if altered expression of MP would suffice to interfere with leaf formation under conditions of PAT inhibition. To this effect, 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; Fig. 1P). 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 appears 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. 3A-C), 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. 3D-E). Instead, in mp mutants the cells immediately surrounding the meristem appeared to elongate forming a ring of elongated cells around the meristem (Fig. 3G). Subsequently the meristem region began to enlarge to initiate the formation of the leafless dome (Fig. 3E,F). The ring of cells initially expressed the leaf founder cell marker AS1 (Fig 3F) but expression of AS1
and growth of these cells ceased by 6 DAG (Fig. S1). After approximately 4-6 DAG all subsequent growth came from the meristem (Fig. 3G-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 4A-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. 4D). 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. 4F,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. 4E). In NPA-grown mp plants, the leafless domes also expressed ProSTM:GUS in thin strands along the apical-basal axis (arrows in Fig. 4F). Upon closer inspection, these strands appeared to consist of elongated and narrow cells typical of procambial strands (Fig. 4H). 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 4I, 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. 5; representative imaged areas showed in Fig. S2). 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. 5B,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. 5C,F). 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 SAM’s and internal procambial midveins of young primordia (Fig. 6A), 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. 6B). *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. 6C). 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. 6D). To assess if the lack of *PIN1* focus formation in NPA-grown *mp* plants is accompanied by a lack of auxin maxima formation, we analyzed the expression of the auxin responsive *ProDR5:GUS* marker. In *WT* seedlings, *ProDR5: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 *ProDR5:GUS* expression is not found in the central and peripheral zones of the SAM (Fig. 6E) (Mattsson et al., 2003). In *mp* seedlings, the *ProDR5:GUS* expression in leaf primordia apices was always more diffuse than in *WT*
seedlings (Fig. 6F). WT plants responded to NPA with a considerable delay in leaf primordia formation and when leaf primordia emerged, the ProDR5:GUS expression was found at the margins of the circular or close to circular leaf primordia (Fig. 6G). At no point did we observe localized ProDR5:GUS expression at the flanks of NPA-grown mp meristems (Fig. 6H). In summary, the leafless dome meristems of NPA-grown mp mutants show defects in the focusing of PIN1 expression and do not form local auxin-response maxima as judged by ProDR5:GUS.

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

The synergistic phenotype in mp pin1 double mutants suggests that 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 AINTEGUMENTA (ANT) and 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 6 I,M), in agreement with published results. The expression of ANT and AS1 in mp mutants appears identical to WT expression patterns (Fig. 6L,P) except for the defects in phyllotaxy already described (Fig. 3B,C). In response to NPA, WT plants expressed ANT and AS1 in a circular domain (Fig. 6K,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. 6L,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 later. 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. 6Q,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.

**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 dependent (Sauer et al., 2006; Wenzel et al., 2007) 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 ARF’s limit this action in the central zone of the meristem (Leyser, 2006). Given this scenario, ARF’s
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 phylloptic pattern of leaves and how it is influenced by the position of pre-existing primordia. Mathematical modeling 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 (Jonsson et al., 2006; Smith et al., 2006). 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.

MP is another likely component of the postulated mechanism since mp mutants also fail to form flowers from the inflorescence meristem and have reduced auxin transport capacity (Przemeck et al., 1996). Further, MP encodes an auxin response factor (Ulmasov et al., 1997; Hardtke and Berleth, 1998), which might be involved in the auxin-independent 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 mp inflorescence meristems (Reinhardt et al., 2003), suggesting that it is not only auxin transport and auxin accumulation that is defective in 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 *MP* in controlling auxin responses in lateral organ outgrowth.

The inhibition of auxin transport in *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 *STM* and *CLV3* expressing domains surrounded by a wide circular peripheral zone, marked by *ANT* and *ASI*. Although no leaf primordia are formed under these conditions, there seems to be some dispersed growth as the *ANT* and *ASI* 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), 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.

MATERIALS AND METHODS

Plant material and growth
The $\text{mp}^{G12, G33, Tu399}$, 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-GeneTm 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 (CGATTGGATCCGTTGAGAT) and MP-RT-R (ACCCCATTCAGTTTCACCAG) (Hardtke et al., 2004); data not shown). The $\text{Pro}_{\text{DR5}}$::GUS (Ulmasov et al., 1997), $\text{Propin1}$::PIN1::GFP (Benkova et al., 2003), $\text{Pro}_{\text{STM}}$::GUS (Kirch et al., 2003), $\text{Pro}_{\text{CLV3}}$::GFP::ER (Lenhard and Laux, 2003), transgenes were crossed into the mp mutant background. Attempts to introgress a $\text{Pro}_{\text{DR5}}$::GFP construct 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 ASI 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 β-glucuronidase activity was carried out as described in (Mattsson et al., 2003).

**Microscopy**

A Zeiss LSM 410 was used to image ProPIN1:PIN1:GFP and ProCLV3: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% gluteraldehyde 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.

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FIGURE LEGENDS

Fig. 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 cotelydons 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 Pro35S:MP plant grown on medium
with 10 μM NPA for 40 DAG (P). Scale bars: 1mm in A,B; 500μm in C,E-H,J-P; 100 μm in D; 50μm in I.

Figure 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 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 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 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 < 0.05. Representative images of measured areas are shown in Fig. S2.

Figure 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 LEGENDS, SUPPLEMENT

Table S1. 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.

Figure S1. Time series of AS1 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 exclusive expression in the enlarging meristem from 9 DAG onwards. (A) 3 DAG, (B) 4 DAG, (C) 6 DAG, (D) 9 DAG, (E) 21 DAG, a = apex of meristem, scale bars are 50μm.

Figure S2. Quantification of central zone and meristem areas.
Material grown on medium supplemented with 10μM NPA indicated as "+ NPA". Representative images of Pro<sub>CLV3</sub>:GFP:ER and Pro<sub>STM</sub>:GUS expression at 3, 10 and 21 DAG in WT, mp, WT+NPA, and mp+NPA meristems. Size bars are 50μm.
