PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem

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SUMMARY

The process of organ positioning has been addressed, using the pin-formed 1 (pin1) mutant as a tool. PIN1 is a transmembrane protein involved in auxin transport in Arabidopsis. Loss of function severely affects organ initiation, and pin1 mutants are characterised by an inflorescence meristem that does not initiate any flowers, resulting in the formation of a naked inflorescence stem. This phenotype, combined with the proposed role of PIN1 in hormone transport, makes the mutant an ideal tool to study organ formation and phyllotaxis, and here we present a detailed analysis of the molecular modifications at the shoot apex caused by the mutation. We show that meristem structure and function are not severely affected in the mutant. Major alterations, however, are observed at the periphery of the pin1 meristem, where organ initiation should occur. Although two very early markers of organ initiation, LEAFY and AINTEGUMENTA, are expressed at the periphery of the mutant meristem, the cells are not recruited into distinct primordia. Instead a ring-like domain expressing those primordium specific genes is observed around the meristem. This ring-like domain also expresses a boundary marker, CUP-SHAPED COTYLEDON 2, involved in organ separation, showing that the zone at the meristem periphery has a hybrid identity. This implies that PIN1 is not only involved in organ outgrowth, but that it is also necessary for organ separation and positioning. A model is presented in which PIN1 and the local distribution of auxin control phyllotaxis.

INTRODUCTION

The entire shoot system of the plant is produced by shoot apical meristems (SAMs), located at the apex of the main stem and branches. Superficially, the dome shaped SAMs appear to be composed of homogeneous populations of randomly proliferating cells. Molecular and cellular analyses, however, have shown that SAMs are highly compartmentalised structures, organised into functionally distinct zones. In higher plants, a group of slowly dividing cells at the meristem summit serves as a source of stem cells assuring self maintenance of the SAM (Lehnard and Laux, 1999). This domain, the central zone (CZ), is surrounded by the peripheral zone (PZ) where the organs are initiated and subtended by the ribzone (RZ) which generates the internal tissues of the stem. Each zone is characterised by specific gene expression patterns, reflecting their particular role in the SAM (for a review see Bowman and Eshed, 2000).

Meristems are stable structures in spite of the very dynamic nature of their constituent cells, which continuously divide, grow and differentiate while they transit from one zone to the other. The coordination of cellular behaviour in this stable system is thought to rely on an elaborate signalling network. Although the precise structure of this network is unknown, several components have been identified. A relatively well characterised example is the CLAVATA (CLV) pathway in Arabidopsis. CLV3, a putative signalling peptide produced in the central zone, is required for the activation of a complex containing the receptor kinase CLV1 located in the inner parts of the SAM (Clark et al., 1997; Fletcher et al., 1999). Together the CLV genes negatively regulate the size of the CZ (Jeong et al., 1998). This involves a repression of the activity of WUSCHEL (WUS), a homeodomain transcription factor specifying the stem fate of the CZ cells. Interestingly one of the components of the pathway, CLV3, is under the transcriptional control of WUS in the CZ, implying that the stem cell population is regulated by a feedback loop between WUS and the CLV genes (Schoof et al., 2000).

Meristems produce organs in well defined phyllotactic patterns. Organ initiation involves the isolation of a subset of cells from the meristem, followed or accompanied by the specification of identity and the outgrowth of the incipient primordium. The control of organ identity has been well characterised (e.g. Ng and Yanofsky, 2000), in contrast to the mechanisms determining organ positioning and outgrowth. So far, two genes in Arabidopsis, CUP-SHAPED COTYLEDON (CUC)1 and CUC2 are clearly associated with the establishment of organ boundaries at the SAM (Aida et al., 1997; Aida et al., 1999; Ishida et al., 2000). However, their exact role is not known and the precise sequence of events leading to the initial selection of the primordium cells remains obscure. Likewise, very few genes involved in the control of
outgrowth have been identified, and so far only the potential transcriptional regulator AINTEGUMENTA (ANT) has been directly associated with the control of cell proliferation during organ formation (Krizek, 1999; Mizukami and Fisher, 2000). Pharmacological and microsurgical experiments have also indicated the importance of signalling in the control of phyllotaxis. According to the so-called field theories, the incipient and young growing primordium laterally inhibits the initiation of a new one in its vicinity (review: Lyndon, 1998). In the same manner the formation of primordia inside the meristem would be inhibited by a field surrounding the CZ. Although the signals involved have not been identified, plant hormones have been associated with this process. In particular auxin appears to be an important factor in organ initiation and positioning. Work on PIN-FORMED 1 (PIN1), a putative auxin carrier (Gälweiler et al., 1998), has shown that local differences in auxin levels are important in pattern formation during development and provided us with the first elements of a signalling network controlling phyllotaxis in plants. Basipetal auxin transport in the inflorescence of pin1 mutants has been shown to be reduced by up to 90% and correlated with an almost complete incapacity to produce flower primordia (Okada et al., 1991; Bennett et al., 1995). This dramatic phenotype can be phenocopied when plants are grown on a medium containing an inhibitor of auxin transport such as naphthylphthalamic acid (NPA). In addition, the localised application of auxin on the naked inflorescence apex of NPA-treated plants and pin1 mutants complements the defect in flower formation (Reinhardt et al., 2000). This suggests that polar auxin transport and local differences in concentration interfere with pattern formation at the shoot apex as was also shown for the root meristem (Sabatini et al., 1999) and embryo development (Liu et al., 1993; Hadfi et al., 1998). In accordance, mutations in two other genes associated with auxin action, PINOID and MONOPTEROS, result in a pin-like phenotype (Hardtke and Berleth, 1998; Bennett et al., 1995; Prezemek et al., 1996; Christensen et al., 2000).

The proposed role of PIN1 in signalling and the phenotype caused by its loss of function make the corresponding mutant an excellent tool to address the mechanisms controlling primordium initiation. We have therefore analysed the apex of the pin1 mutant. Our studies have focused on the inflorescence meristem for two reasons. First, the mutation has its most dramatic effects at the level of flower initiation. Second, flower initiation has been well characterised and the precise expression patterns of regulators of floral morphogenesis have been described in detail. Our results indicate that PIN1 not only affects organ outgrowth, but that the gene is also involved in the establishment of organ boundaries.

MATERIALS AND METHODS

Growth conditions and genetics

Plants were grown in vitro as described earlier by Santoni et al. (1994). Two-week-old plants were transferred to the greenhouse for further analyses. The pin1-6 and chv3 (here called chv3-EAI22) mutants used in this study had both been identified in a T-DNA mutagenesis screen carried out in our laboratory (Wassilewskaya ecotype (WS); Bechtold et al., 1993; Bouchez et al., 1993).

Allelism was confirmed by complementation tests using the following mutant strains: pin1-1 (Enkheim ecotype; En), obtained from the ABRC, Ohio State University, US. chv3-1 (Landsberg erecta background; Ler) was a gift from Dr S. Clarke, University of Michigan, USA. Since the majority of our mutants are in the WS ecotype, we chose the WS lines carrying the pin1 and chv3 alleles for further analysis. The transgenic line containing LEAFY::GUS was a gift from Dr D. Weigel, The Salk Institute, USA.

Double mutants for pin1-6 and chv3-EAI22 were identified in the F2 generation as plants with a new phenotype. Frequencies of these plants are compatible with a segregation ratio of 1:16 confirming that they correspond to double mutants.

Histological analysis and GUS staining

Histological sections of plastic (Historesin, Leica, France) and embedded material were prepared using standard procedures (Traas et al., 1995) and observed in a Nikon FXA microscope. To facilitate the visualisation of cellular arrangements, the sections were stained using non-specific staining with Toluidine Blue. GUS activity was detected using standard procedures (e.g. Jefferson et al., 1987). Briefly, plants were fixed in acetone (80% in water, –20°C) for one hour. After a rinse in water the plants were stained at 37°C in a solution containing 1 mg/ml of substrate (5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid; Duchefa, Haarlem, The Netherlands), 1 μl/ml Triton X-100, 0.5 mM ferrocyanoure, 0.5 mM ferricyanure and 10 mM EDTA in 10 mM phosphate buffer (pH 7.5).

In situ hybridisation

In situ hybridisations were performed as described earlier (Laufs et al., 1998). Briefly plants were fixed in 4% formaldehyde (freshly made from paraformaldehyde) in PBS under vacuum for 2× 20 minutes, and left in fixative overnight. After fixation, plants were washed, dehydrated and embedded in paraffin wax essentially as described by Jackson (1991). Paraffin sections (8-10 μm thick) were cut with a disposable metal knife and attached to precoated glass slides (DAKO). Antisense probes were synthesised using digoxigenin (DIG)-UTP (Boehringer Mannheim) according to the manufacturer’s instructions. Immunodetection of the DIG-labelled probes was performed using an anti-DIG antibody coupled to alkaline phosphatase as described by the manufacturer (Boehringer Mannheim). Sections were counterstained with calceinolur white (0.01% in water) before viewing in a FXA Nikon microphot fluorescence microscope.

The following probes were used: a full length cDNA of SHOOTMERISTEMLESS (STM) (a gift from Dr K. Barton, University of Wisconsin-Madison, USA), cDNAs fragments of LFY, AP1, AP3 (a gift from Dr F. Parcy, Gil-s-Yvette, France), CUC2 (a gift from Dr M. Tasaka, Nara, Japan), ANT (a gift from Dr M. Pastuglia, INRA, France) and PIN1 (a gift from Dr K. Palme, MPI, Koln, Germany).

Confocal microscopy analyses and scanning electron microscopy

Shoot apical meristems were fixed in 4% formaldehyde in phosphate-buffered saline and stained with propidium iodide as described earlier (Laufs et al., 1998). Longitudinal sections were made using a Leica TCS confocal microscope. Projections of the L1 layer of pin and wild-type inflorescence meristems were analysed using Optimas image analysis software. Meristems were divided into concentric zones. First a central circle, four cells wide, was drawn on the summit of the meristem, which approximately corresponds to the size of the CZ (Laufs et al., 1998). Subsequently concentric zones, 2 cells wide, were drawn around the centre at intervals. The mitotic index was directly determined in the surface layer (L1) of each zone. Cell size was estimated by measuring the distance between the nuclei in the L1 layer. Per meristem, 50 measurements were made.

Meristem structure was studied using low-temperature scanning electron microscopy as described by Traas et al. (1995).

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RESULTS

Organ initiation is strongly affected in the pin1-6 mutant

The recessive allele pin1-6 in the WS background, isolated in our laboratory, produced a very similar phenotype to previously described strong pin1 alleles (Fig. 1; Okada et al., 1991; Bennett et al., 1995). The phenotype became visible during embryogenesis as the position of the cotyledons was usually perturbed (Fig. 1B). This often led to the formation of completely or partially fused cotyledons. The wild-type plants initiated 4-6 leaves (5.1±0.3, n: 167) during the vegetative phase. The number of leaves initiated by the mutant was variable (1-5) with an average of 1.7±0.5 (n: 127), which represents a significant reduction compared to the wild type (Table 1). Leaves were also often fused and were abnormal in shape and size. Most strikingly, the inflorescence stem produced by the pin1-6 mutant was naked (Fig. 1D and E). Next to the summit of the pin stem small bulges could be observed, but these flattened out during further elongation. Occasionally a flower was formed, in particular at the more slowly growing apex of older plants. Flowers, like the other organs, were usually fused and/or had abnormal architectures. Occasionally, fasciation of the mutant inflorescence stem was observed. This confirmed earlier observations (Okada et al., 1991; Bennett et al., 1995; Gälweiler et al., 1999) that pin1 mutants are severely impaired in organ initiation and also in lateral organ separation during both the vegetative and the floral stages.

The overall organisation of the meristem is not affected in pin1 mutants

The defects in lateral organ formation in pin1-6 suggested a specific role of the PIN1 gene in the control of primordia formation per se. However, this role could also be indirect, and the defects in organ formation could be due to other abnormalities, such as meristem patterning and self-maintenance. To discriminate between these two possibilities, we first studied the structure of the meristem in the pin1-6 mutant. Conventional staining of plastic embedded sections or optical sectioning of the inflorescence apex in the confocal microscope, showed a layered meristematic zone and a clearly recognisable rib zone (Fig. 2 and data not shown). Similar observations were also made on vegetative meristems (not shown), which indicated that a normal organisation of the SAM is maintained throughout development in pin1 mutants.

We then studied the expression of meristem-specific genes in the pin1-6 mutant (Fig. 2C-F). STM is a marker for meristematic cells as the gene is expressed specifically in the meristem. Initially it is excluded from the flower primordia, but later it is expressed in the zones that have not been allocated to the flower organ primordia (Fig. 2C; Long et al., 1996).

### Table 1. Number of leaves produced during the rosette stage

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Range</th>
<th>Mean ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (WS)</td>
<td>167</td>
<td>4-6</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td>pin1</td>
<td>127</td>
<td>1-5</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>clv3</td>
<td>132</td>
<td>6-9</td>
<td>7.0±1.1</td>
</tr>
<tr>
<td>pin1 clv3</td>
<td>48</td>
<td>0-3</td>
<td>1.2±0.7</td>
</tr>
</tbody>
</table>

n, numbers of plants analysed.

![Fig. 1.](image1.png) **Fig. 1.** Morphology of wild-type and pin1-6 plants. 10-day old wild-type (A) and pin1-6 (B) seedlings. Leaf production is reduced in pin1-6 seedlings and fusion at the base of the cotyledon petioles can be observed. Wild-type (C) and pin1 (D) inflorescence stem. Note the complete absence of cauline leaves, lateral branches and flowers on the pin1-6 stem. (E) Electron micrograph of a pin1 inflorescence apex. Scale bar, 200 μm.

![Fig. 2.](image2.png) **Fig. 2.** Structure of the meristem and expression of meristem-specific genes in the inflorescence of wild-type and pin1-6 inflorescence apices. Inflorescence apices of (A,C,E) wild-type and (B,D,F) pin1-6 plants. (A,B) Histological section of meristem stained with Toluidine Blue. An organisation into different layers (L1, L2 and L3) can be observed in both wild-type and pin1-6 meristems. Below the L3 layer the typical cell files of the rib meristem can be distinguished in pin1-6 apices. (C,D) STM expression pattern. (E,F) WUS expression pattern. IM, inflorescence meristem; FM, floral meristem. Scale bars, 100 μm.
was similarly expressed in pin1-6 in a small population of cells at the summit of the shoot apex, confirming the existence of a meristematic zone (Fig. 2D). This zone appeared slightly wider than in the wild type, which could be related to the slight increase in cell size observed in the mutant meristem (see below). Indeed, this meristematic zone expressing STM was 12-16 cells wide (counted on median sections of 12 meristems), which is comparable to wild-type values under our conditions (12-15 cells). WUS was used to study further the organisation of the meristem. WUS is a marker for a subpopulation of central zone cells and was used to study the CZ. In wild-type inflorescence meristems, WUS is only expressed in a few cells at what is supposed to be the base of the CZ (Mayer et al., 1998). The pattern of WUS was indistinguishable in wild-type and in pin1-6 mutants (Fig. 2E,F), suggesting that the CZ is of normal size in the mutant.

To determine whether the mechanisms controlling meristem maintenance were still functional in pin1-6 we subsequently crossed the mutant with clv3-EAI22 in the WS background. clv3 mutants have an enlarged meristem resulting from the enlargement of the CZ. Pin1/clv3 double mutants all showed an enlarged meristem and extensive fasciation as observed in clv3 single mutants. This implied that the regulation of meristem size in the pin1 single mutant still required a functional CLV3. Interestingly, pin1-6 was epistatic over clv3-EAI22 as far as organ numbers were concerned. Clv3 mutants produce extra leaves (Table 1) and flowers (Fig. 3; Fletcher et al., 1999) supposedly because more meristematic cells are available in the mutant. However, pin1/clv3 double mutants did not show a dramatic increase in the number of leaves (Table 1) or flowers (Fig. 3). This was particularly evident in the inflorescence meristem: even though the number of meristematic cells potentially available for organ initiation had dramatically increased we rarely observed any production of flower primordia. This again confirmed that PIN1 is essential for organ production.

In conclusion, pin1 mutants essentially have a normally sized and structured meristem, requiring CLV3. Thus, although a major function of the SAM, organ production, is defective, a functional PIN1 gene is not necessary for the establishment of an organized meristem able to maintain itself. Together, these results further point to a major role for PIN1 in organ initiation at the meristem periphery.

The PIN1 gene is upregulated at very early stages of primordia formation

We next studied the expression patterns of the PIN1 gene in the shoot apex by in situ hybridisation. We confirmed earlier observations that the gene is strongly expressed in the vascular tissues of the inflorescence stem and in the organ primordia at the shoot apex (Fig. 4 and data not shown; Gälweiler et al., 1998; Christensen et al., 2000). In addition the upregulation of PIN1 mRNA was observed at very early stages of organogenesis, even before the incipient primordium started to grow out (Fig. 4A). Throughout the meristem the level of labelling was low, although above background level. PIN1 was also strongly expressed in all primordia during the different stages of flower development (Fig. 4B). Thus the PIN1 gene is not only expressed in vascular tissues, but is also upregulated at the shoot apex in the incipient and very young primordia, suggesting a role for the protein in the early stages of organ initiation.

Expression of meristem and floral identity genes in pin1 mutants

Taken together, the previous results strongly indicated a role

![Fig. 3](image-url) Phenotype of 3-week-old pin1-6 and clv3-EAI22 plants and double mutants. (A) Wild-type inflorescence. (B) clv3 produces more flowers than wild type, while (C) pin1 single mutants and (D) pin1/clv3 double mutants have a naked inflorescence. (E-H) Scanning electron microscopy. (E) Typical organisation of a wild-type meristem. (F) clv3 has an enlarged meristem and an increased number of organ primordia. (G) Primordia formation is completely blocked in pin1 single mutants and in (H) pin1/clv3 double mutants, but a fasciation typical of clv3 is also observed in the double mutant. Below the apex of the young double mutant stem, trichomes are visible just as observed in wild-type at a similar stage. A to D are at the same magnification. Scale bars in panels E to H, 200 μM.

![Fig. 4](image-url) PIN1 expression in wild-type inflorescences. PIN1 mRNA was detected using in situ hybridisation. (A) In the inflorescence meristem (IM), a strong expression is detected before the emergence of the primordia (primordium anlage: arrowhead) and persists in the centre of floral meristems (stage 1 and 2; FM). PIN1 mRNA is also detected in the vascular bundles (VB, arrow). (B) In this transverse section of an inflorescence apex, PIN1 expression is detected in a flower anlage (1) and in flower primordia (2–6). The primordia are numbered from youngest to oldest. Scale bars, 100 μM.
for PIN1 at the periphery of the meristem, at the level of primordium initiation. To investigate this role further, we first asked how far organ initiation in the inflorescence meristem proceeds in the mutant. Initially, the expression patterns of two very early markers of primordia, LEAFY (LFY) and ANT (Elliott et al., 1996; Weigel et al., 1992) were examined. In wild-type meristems, LFY and ANT mRNA accumulate in the incipient primordium before it starts to grow out, at about the same stage when PIN1 expression is increased, LFY and ANT were expressed at the periphery of all pin1-6 apices tested (Fig. 5A,B,E,F). However, while the level of ANT was comparable to wild type, the level of LFY was frequently lower, requiring longer staining of the in situ preparations. A strong expression of LFY was only observed in the very few primordia that did grow out (data not shown). This indicates that the early development of primordia was perturbed in pin1-6 mutants during or just after the activation of LFY. Identical results were obtained when LFY expression was visualised in the pin1-1 null allele (data not shown). To define whether LFY was able to activate downstream targets in the pin1-6 mutant, the expression patterns of the floral homeotic genes APETALA (AP)1 and AP3 were also examined. Although AP1 is a direct target of the LFY transcription factor and is activated early during stage 1 of floral development (Mandel et al., 1992; Gustafson-Brown et al., 1994; Parcy et al., 1998; Wagner et al., 1999), the gene was not switched on in pin1-6 mutants. When there was some outgrowth of a primordium in pin1-6, AP1 expression could only be observed (Fig. 6B). The same results were obtained for AP3, which is activated later on in wild-type, during early stage 3 of floral development (Fig. 6C,D; Jack et al., 1992). This shows that the expression of specific floral genes is never observed in pin1 except when the rare flower primordium further develops.

In conclusion, the activation of the differentiation program of primordia is partially defective in pin1 mutants and leads to an early block in floral primordia development after or at the level of the induction of LFY and ANT.

Organ separation is defective in pin1 mutants

Since cells at the periphery of the meristem of pin1 mutants adopt an organ identity despite the absence of primordia outgrowth, we next investigated whether they were located in discrete primordia in the classical phyllotactic pattern usually observed. For this purpose, we analysed serial longitudinal sections of the pin1-6 mutant probed for LFY and ANT mRNA.

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Fig. 5. Expression of primordia genes in wild-type and pin1-6 inflorescence apices. (A-C) Inflorescences probed with LFY. (A) Wild-type inflorescence. Note the very early expression of LFY in the flower anlage (arrowhead). IM, inflorescence meristem; FM, floral meristem. (B) pin1-6 inflorescence: median longitudinal section of a meristem showing LFY expression. (C) pin1-6 inflorescence: tangential longitudinal section through the flank of the same meristem as in B, showing that LFY is expressed in a ring surrounding the meristem. (D) GUS detection in pin1-6 inflorescence expressing the LFY::GUS transgene. Note the ring-like domain of expression of the transgene, which confirms the data obtained using in situ hybridisation. (E-G) Inflorescences probed with ANT. (E) Wild-type inflorescence. ANT is expressed in the IM in early primordia (not visible here) and in floral organ primordia in the FM. (F) pin1-6 inflorescence: median longitudinal section of a meristem. (G) pin1-6 inflorescence: tangential longitudinal section through the flank of the same meristem as in (F), showing that ANT is also expressed in a ring surrounding the meristem. Scale bars, 100 μm.

Fig. 6. Expression of floral homeotic genes in wild-type and pin1-6 inflorescence apices. (A,B) AP1 expression. (A) In the wild-type inflorescence AP1 is an early marker of primordium emergence. It is excluded from the inflorescence meristem (IM) and accumulates in floral meristem (FM) from early stage 1 onwards. (B) In the pin1-6 inflorescence, AP1 is normally not expressed, except when the occasional bulges (FM) develop into flower primordia. (C,D) AP3 expression. (C) In wild-type inflorescence AP3 is expressed later at stage 3 of flower development. (D) In the pin1-6 inflorescence AP3 is normally not expressed except when an occasional flower develops. Scale bars, 100 μm.
This revealed that these genes were not expressed in discrete primordia. Instead, the expression patterns defined ring-like domains encircling the meristem (Fig. 5B,C,F,G). Confirming these results, in pin1-6 mutants expressing the LFY::GUS transgene, GUS activity was detected in a ring around the meristem (Fig. 5D). This implies, that one of the earliest defects in organ initiation in pin1-6 mutants occurs at the level of organ separation. This aspect was investigated further by studying the expression pattern of CUC2, a gene involved in organ separation (Aida et al., 1997). In wild-type inflorescence apices, CUC2 expression is specifically restricted to organ boundaries (Fig. 7A; Ishida et al., 2000) and its mutation leads to partial organ fusions. In the pin1-6 mutant, we observed an enlarged CUC2-expressing domain, encircling the meristem, as observed for LFY and ANT (Fig. 7B-D), suggesting that LFY and CUC2 expression overlapped in pin1 apices. In wild-type plants CUC2 was specifically expressed at the boundary of the LFY expression domain as shown by in situ hybridisations using adjacent sections (Fig. 7E-H). Although it cannot be excluded that some cells express both markers in the wild type, the large majority of the cells in the LFY domain does not express CUC2. In contrast, probing of adjacent sections demonstrated that CUC2 expression was no longer limited to the boundary of the LFY expressing zone in pin1, but that both domains largely overlapped (Fig. 7G-H). The same results were obtained for the pin 1-1 allele (data not shown).

From the expression pattern of CUC2 we concluded that organ separation is dramatically affected in pin1 mutants and that cells at the periphery of the pin1 meristem have the characteristics of both primordium and boundary zones.

DISCUSSION

The PIN1 gene encodes a transmembrane protein that is strongly expressed in the vascular tissues of the inflorescence stem where it is located at the basal end of elongated parenchymatous cells (Galweiler et al., 1998). The predicted structure of the PIN1 protein, its homology to carrier proteins and its polar localisation in auxin transport competent cells, suggest that it might act as a catalytic auxin efflux carrier. In accordance with this hypothesis polar transport of the hormone is severely reduced in the mutant (Okada et al., 1991; Bennett et al., 1995). This most probably leads to a deficit of auxin at the periphery of the inflorescence SAM, as the phenotype can be compensated for by the local application of hormone at the apex (Reinhardt et al., 2001). In addition, inhibitors of auxin transport such as NPA, mimic the pin1 phenotype, causing the formation of naked inflorescence stems (Okada et al., 1991). It is also noteworthy, that the related PIN2 protein appears to...
have the same function in roots (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Together these data provide compelling evidence that PIN1 is involved in auxin transport, and that the perturbed distribution of the hormone in pin1 is the cause of the inflorescence phenotype.

Is PIN1 involved in meristem structure and self maintenance?

The existence of clearly defined layers and the presence of an organised rib zone shows that the organisation of the meristem proper (defined here as a zone covering the CZ, PZ and RZ) in pin1 mutants is essentially unaffected. We could not find any major alteration in meristem patterning: the size of the STM-expressing domain was comparable to wild type and a functional central zone, expressing WUS and requiring CLV3 was observed. These data suggest that meristem patterning and self-maintenance are largely independent from PIN1, despite the low but significant expression of the gene throughout the apical dome of wild-type plants. Our results, therefore, conform and further complete the findings by Reinhardt et al. (2000) who showed that the pin-like structures induced in tomato by NPA expressed LeT6, a gene encoding a homeobox protein that is normally active in the meristem. Our results on pin1 are also very similar to those reported for the pid mutant, which has an identical inflorescence phenotype. PID is a kinase which has been implied in auxin response. In pid mutants the expression patterns of UFO and CLV1 were comparable to those seen in wild-type plants which further strengthens the idea that meristem patterning does not require PIN1 activity and a strict regulation of polar auxin transport (Christensen et al., 2000).

PIN1 is required from the very early stages of flower initiation onwards

While meristem organisation itself does not require fully functional PIN1, the gene appears to play a key role during the early steps of organogenesis as was already suggested by the up regulation of the gene in young primordia. The first phase of organogenesis can be divided into different steps, including positioning of the primordium, the definition of its boundaries, the establishment of its identity and the induction of outgrowth. Although the exact sequence of events has not yet been determined, primordium formation must somehow start with the definition of organ boundaries leading to the isolation of a subset of PZ cells from the rest of the SAM. This is associated with the inactivation of meristem genes such as STM (Long et al., 1996) and the activation of a subset of primordia-specific regulatory genes.

Apparently the patterning processes that switch off STM in early primordia are not affected in pin1 mutants indicating that not only the organisation of the meristem proper but also the early determination of a primordium-specific domain where the STM gene is inactivated occurs normally in the mutant. Subsequent steps associated with the change in identity required for primordium initiation also occur in pin1 as shown by the activation of two early markers, LFY and ANT, involved in organ identity and outgrowth respectively (Weigel et al., 1992; Weigel and Nilsson, 1995; Elliott et al., 1996; Klucher et al., 1996; Krizek, 1999; Mizukami and Fischer, 2000). Although always detectable, LFY was generally weakly expressed in pin1 apices suggesting that the activation of this gene is partially deficient in the absence of fully functional PIN1. This puts the first effects of the mutation on organ initiation at the very early stage of LFY activation, before its downstream targets such as AP1 and AP3 are activated, i.e. even before the primordium starts to bulge out from the apical dome. PIN1 is therefore involved in the early regulation of LFY, perhaps indirectly, via the local accumulation of auxin. Correspondingly, we observed that PIN1 is upregulated at this stage.

The low expression levels of LFY could explain why AP1 is not activated in the pin1 apex. AP1, which is switched on at very early stages, throughout the flower primordium (Mandel et al., 1992; Gustafson-Brown et al., 1994), is an immediate transcriptional target of LFY (Percy et al., 1998; Wagner et al., 1999). It has been suggested that LFY may interact with other factors to induce AP1, as this induction is limited to certain tissues (Percy et al., 1998). In addition, lag times of up to 56 hours have been reported between the activation of LFY and the onset of AP1 induction (Simon et al., 1996; Hempel et al., 1997). It has been proposed that AP1 is only activated after a critical threshold of LFY has been reached. The low level of LFY generally observed in pin1 mutants could thus explain the absence of activation of AP1 in the mutant. Consistent with this hypothesis is the observation that when cells escape the block in pin1 mutants, and are incorporated in developing primordia we always observed a strong expression of LFY and an activation of AP1. An alternative, but not necessarily mutually exclusive, possibility is based on the hypothesis that a LFY-independent function ensures that AP1 is only activated when the primordium has reached a particular stage. PIN1 activity would either directly activate this function, and/or allow the primordium to reach this developmental stage.

PIN1 regulates primordia separation and outgrowth

Besides the arrest in primordium outgrowth, pin1 presents another, even earlier defect in organogenesis. The cells expressing LFY and ANT are not grouped in discrete primordia in the well-defined phyllotactic patterns observed in wild-type apices. Instead, they are apparently recruited in a single domain encircling the apex, as demonstrated by the ring-like expression domain of LFY and ANT observed in pin1 mutants. This could imply that the mutant simply forms one ring like ‘anlage’ which then fails to grow out. The situation is more complex however, as the cells expressing primordium markers also express CUC2. From the observation of fused organs in single and double mutants and from the expression pattern of CUC2, which is restricted to organ boundaries, it has been concluded that this gene, together with at least one other factor, CUC1, probably controls the initial definition of organ boundaries (Aida et al., 1997, 1999; Ishida et al., 2000). The inflorescence meristem of pin1 is thus surrounded by a zone where the cells have acquired both primordium and boundary characteristics. PIN1 is therefore necessary to separate both identities and since it is strongly upregulated in the primordia themselves, we propose that auxin accumulation by PIN1 represses CUC2 expression in the incipient primordia, leading to the specific expression of CUC2 in the boundaries.

The failure to repress CUC2 in specific domains at the meristem periphery is also relevant for the defects in outgrowth observed in the mutant. As mentioned earlier, cucl/cuc2 mutants show organ fusions throughout development (Aida et al., 1997). One of the interpretations of this phenotype is that
these putative transcription factors negatively regulate growth between the primordia. In such a scenario, the absence of primordium outgrowth in pin1 would be due to an inhibitory effect of CUC2 throughout the organogenic zone. This inhibition could involve downstream targets of ANT or other regulators controlling organ outgrowth. It should be noted however, that the mitotic index at the periphery of the pin1 meristem is not reduced, indicating that the expression of CUC2 is not necessarily correlated with a decrease in cell proliferation. Indeed, if we suppose that the length of M-phase is not modified in the mutant, the unchanged mitotic index and increased cell size in the pin1-6 meristem suggests even an increase in overall growth rates. The mode of action of the gene is therefore more complex than a simple inhibition of cell cycle activity.

Our results show that PIN1 is required for the extinction of CUC2 in zones where primordia genes are expressed i.e. zones where PIN1 itself is upregulated and where CUC2 is downregulated. However, since the expression of primordium genes and CUC2 overlap at the periphery of the mutant meristem, PIN1 is also required for the downregulation of primordia genes in the boundary zones. This could be an indirect effect of the stimulation of primordium outgrowth. Indeed, a large body of evidence suggests that developing primordia inhibit the formation of new primordia in their vicinity (for reviews see Lyndon, 1998). According to these so-called field theories, primordia could do so either by producing a diffusible inhibitor, or, more simply, by depleting an activator from their environment. Combined with an inhibitory activity, a diffusable inhibitor, or, more simply, by depleting an activator from their environment. Combined with an inhibitory activity.

From our results an outline of the regulatory network controlling phyllotaxis can be drawn (Fig. 8). We propose that the PIN1 gene, via the local accumulation of auxin, participates in the production of organs in a defined phyllotactic pattern. In our model this auxin accumulation stimulates the primordium program and represses certain boundary characteristics such as CUC2 expression. The primordium itself then represses organ initiation in its vicinity leading to the establishment of a zone with boundary identity. Further work is needed to elucidate the precise role of known players and to identify new factors, such as those involved in the lateral inhibition which prevent the expression of primordium genes in boundary areas. It will also be crucial to develop methods permitting the precise determination of auxin concentrations at the shoot apex.

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**Conclusion: a framework for phyllotaxis**

From our results an outline of the regulatory network controlling phyllotaxis can be drawn (Fig. 8). We propose that the PIN1 gene, via the local accumulation of auxin, participates in the production of organs in a defined phyllotactic pattern. In our model this auxin accumulation stimulates the primordium program and represses certain boundary characteristics such as CUC2 expression. The primordium itself then represses organ initiation in its vicinity leading to the establishment of a zone with boundary identity. Further work is needed to elucidate the precise role of known players and to identify new factors, such as those involved in the lateral inhibition which prevent the expression of primordium genes in boundary areas. It will also be crucial to develop methods permitting the precise determination of auxin concentrations at the shoot apex.

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