The plasma membrane recycling pathway and cell polarity in plants: studies on PIN proteins

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Summary

The PIN-FORMED (PIN) proteins are plasma-membrane-associated facilitators of auxin transport. They are often targeted to one side of the cell only through subcellular mechanisms that remain largely unknown. Here, we have studied the potential roles of the cytoskeleton and endomembrane system in the localisation of PIN proteins. Immunocytochemistry and image analysis on root cells from Arabidopsis thaliana and maize showed that 10-30% of the intracellular PIN proteins mapped to the Golgi network, but never to prevacuolar compartments. The remaining 70-90% were associated with yet to be identified structures. The maintenance of PIN proteins at the plasma membrane depends on a BFA-sensitive machinery, but not on microtubules and actin filaments.

The polar localisation of PIN proteins at the plasma-membrane was not reflected by any asymmetric distribution of cytoplasmic organelles. In addition, PIN proteins were inserted in a symmetrical manner at both sides of the cell plate during cytokinesis. Together, the data indicate that the localisation of PIN proteins is a post-mitotic event, which depends on local characteristics of the plasma membrane and its direct environment. In this context, we present evidence that microtubule arrays might define essential positional information for PIN localisation. This information seems to require the presence of an intact cell wall.

Key words: Plant cell polarity, PIN proteins, Endomembrane, Cytoskeleton, Immunocytochemistry, Confocal microscopy

Introduction

A widely accepted model proposes that gradients of the hormone auxin are the basis of differential cell behaviour during pattern formation in higher plants (Benková et al., 2003; Friml et al., 2003; Reinhardt et al., 2003). Auxin distribution throughout the whole plant is controlled by at least two families of plasma-membrane-associated proteins, called AUX/LAX and PIN-FORMED (PIN). These so-called transport facilitators, regulate auxin fluxes in and out of the cells (Gälweiler et al., 1998; Swarup et al., 2001). Since these proteins are often localised on one side of the cell only, it has been proposed that they can generate auxin fluxes through tissues, thus creating differences in hormone concentrations. This has been particularly well established for the PIN proteins (Gälweiler et al., 1998), which are facilitators of auxin efflux. In vascular tissues, for example, these proteins occur on the apical or basal pole of cells in the same cell file. In this way, they supposedly create upwards or downwards directed auxin fluxes. Although PIN proteins have been extensively studied (Benková et al., 2003; Friml et al., 2003; Geldner et al., 2001; Geldner et al., 2003a), the cellular mechanism of their polarised positioning within the cells is not understood (Geldner et al., 2003a; Steinmann et al., 1999; Willemsen et al., 2003). Recently, it was shown that a protein kinase called PINOID (PID) plays an important role in this process (Benjamins, 2004; Friml et al., 2004). Although the precise targets of this enzyme are not known, there is evidence that it interacts with cytoskeletal proteins (Benjamins, 2004). In this context it is important to note that an important role has been proposed for the actin cytoskeleton in recycling of some of the carriers to specific sides of the cells (Geldner et al., 2001). On the whole, however, the cellular basis of PIN localisation remains poorly understood.

Here, we have used immunocytochemistry and PIN::GFP fusions to explore the cellular processes involved in PIN localisation. Our results suggest that the establishment and maintenance of polarity in plant cells is a post-mitotic event, which might involve endocytic and exocytic processes. In this context, we provide evidence that the Golgi apparatus functions as a junction between the exocytic and endocytic pathways for PIN trafficking. Actin might be involved in this, either by modulating the movement of both Golgi stacks and PIN-labelled compartments or by mediating the transport of Golgi-derived products to the cell surface, but not by directing endocytic recruitment. In addition, we show that microtubules play an indirect role in PIN localisation, as their prolonged disruption leads to a reorganisation of growth axis and cell polarity in plant cells. They therefore appear essential for the pluricellular pattern of PIN proteins.

Results

AtPIN labelled PIN proteins in root cells

Western blot analysis

The purified AtPIN antiserum labelled one major band of approximately 68-70 kDa (Fig. 1), on western blots of whole-
protein extracts of Arabidopsis thaliana roots and protoplasts. This corresponds to the molecular weight of AtPIN1 protein. In addition, another lighter band was observed close to the major 68-70 kDa band. This might correspond to closely related PIN proteins. Indeed, a BLAST analysis revealed that several PIN proteins have sequences that are close to the p74 peptide used for raising the antibody. The antibody also labelled a major band of about the same size in extracts from maize roots (Fig. 1A). An additional minor band appeared as a weak signal, and might be due to protein degradation.

**Immunostaining of root tips with AtPIN antiserum**

The AtPIN antibody detected polar localised proteins in various tissues (Fig. 2). In whole-mount preparations of Arabidopsis roots expressing AtPIN1::GFP (Fig. 2A-C), the antibody labelled cells within and around the central cylinder and the endodermis. In most of the stained cells, the antibodies recognised the basal cell surface, giving rise to a characteristic ladder-like pattern (Fig. 2A). Although the AtPIN antibody clearly colocalised with AtPIN1::GFP protein, it also stained additional cell lines in a polar manner within the roots (see red pattern in the merged picture Fig. 2C). In stem tissues of pin-formed1 mutants of Arabidopsis that do not express AtPIN1 (Gälweiler et al., 1998), the antibody still labelled polar localised protein(s) (Fig. 2D). Together, the results confirmed that the antibody stained at least one other PIN protein.

The characteristic polar pattern of PIN distribution described in Arabidopsis roots was also observed on longitudinal sections and squashes of Zea mays roots (Fig. 2E, Fig. 3A) and Medicago sativa (not shown). In addition, intracellular organelles (1 μm in size) were labelled in these same cells (Fig. 3A-E). In conclusion, the AtPIN antibody appears to recognise a highly conserved epitope present in polar localised, PIN-like proteins in at least three species.

**Association of PIN proteins within the endomembrane system in interphase cells**

A potential colocalisation of AtPIN-labelled organelles with markers for the endomembrane system was investigated. For this purpose, we used squashes of maize roots. Moreover, we used the drug brefeldin A (BFA), which has an inhibitory effect on the polar localisation of AtPIN1 (Geldner et al., 2001) and shows a distinctive effect on the functional organisation of endomembrane compartments (Satia-Jeunemaitre et al., 1996a).

**AtPIN-labelled structures versus the ER-Golgi complex**

Typical JIM84-labelled, μm-sized Golgi stacks were homogeneously dispersed throughout the cytoplasm of all maize root cells (Fig. 3B) (Satia-Jeunemaitre and Hawes, 1992). The JIM84 antibody also labelled the plasma membrane (Satia-Jeunemaitre and Hawes, 1992) in many, but not all root cells (Fig. 3B) (see also Horsley et al., 1993 and figures and comments within) (Couchy et al., 1998; Couchy et al., 2003). Double immunostaining showed a partial association of AtPIN-labelled organelles and JIM84-labelled Golgi stacks (Fig. 3A-C). The colocalisation was variable, but on average about 20% of the AtPIN-labelled structures colocalised with JIM84 (Fig. 3D). These colocalisation events were randomly distributed within the cells.

Double immunostaining with AtPIN-antibody and antibodies against calreticulin, an endoplasmic reticulum (ER) marker (Kluge et al., 2004), showed that there was no colabelling of the two antibodies in differentiating cells (not shown). Together, these results show that the Golgi network is actively involved in trafficking of the PIN proteins. However, the topology of the labelled compartments did not reflect any polarity.

**AtPIN-labelled structures versus the prevacuolar marker m-Rab**

To characterise the remaining 70-90% of the PIN-labelled structures that were not associated with the Golgi stacks, we next investigated whether there was any colocalisation between AtPIN-labelled membranes and pre-vacuolar compartments (PVCs). PVCs are randomly distributed within cells (Bolte et al., 2004a). They possibly function in the regulation of trafficking events between the Golgi stacks and lytic vacuoles (Bolte et al., 2004a; Kotzer et al., 2004). PVCs are essentially defined by the presence of specific vacuolar sorting receptors (VSRs) involved in protein sorting to vacuoles and might represent a junction compartment between endocytic and secretory pathways (Tse et al., 2004; Geldner, 2004). In control root cells, the m-Rab5 vacuolar polyclonal antibody (a PVC marker) (Bolte et al., 2004a), stained punctate structures dispersed through the cytoplasm (Fig. 3F). In the merged picture (Fig. 3G) of an AtPIN-stained cell (Fig. 3E) and m-Rab5-stained cells (Fig. 3F), no colocalisation of the stained structures was ever observed. These results were confirmed with other markers for PVCs such as Pep12 or Bp80 (data not shown).

In conclusion, our data show that PVCs are not involved in trafficking of the PIN proteins. They indicate that the majority of AtPIN-labelled organelles is associated with subcellular compartments that have yet to be identified, but which could correspond to endosomal membranes (Geldner, 2004). In addition, we provide evidence that about 20% of the intracellular PIN labelling is associated with the Golgi apparatus.
The effect of BFA on AtPIN- and JIM84-labelled structures

We next analysed the effect of BFA on AtPIN-labelled compartments in maize root cells. As seen on longitudinal sections (Fig. 2F), polar plasma membrane staining with AtPIN was weaker after a 90-minute treatment with BFA (Fig. 2, compare F with E) and almost disappeared after a 150-minute treatment (Fig. 2G) (see also Baluska et al., 2002 and Fig. 3H within). This BFA effect on polar staining was concomitant with the formation of two to four fluorescent areas within the cell (Fig. 2F,G), reminiscent of ‘BFA compartments’ (Satiat-Jeunemaitre and Hawes, 1992; Satiat-Jeunemaitre and Hawes 1993) (see also Baluska et al., 2002 and Fig. 3H within). These BFA-induced modifications of plasma membrane staining were also clearly seen on isolated root cells (Fig. 3H,K). At the subcellular level, a third additional BFA effect was observed because there was a clear decrease, or even loss, in the number of the μm-sized intracellular immunolabelled objects (Fig. 3H).

BFA is known to block exocytosis but not endocytosis (for a review see Satiat-Jeunemaitre et al., 1996a). Therefore, the observed AtPIN staining of BFA-like compartments might come from two sources: an accumulation of Golgi-derived PIN proteins on their way to the plasma membrane (because of a block of exocytosis) and/or an accumulation of PIN proteins derived from the plasma membrane (through an internalisation process) (see also Geldner et al., 2001). Regarding the observed decrease of plasma membrane labelling, it might either be due to protein degradation (simply reflecting the half-life of PIN proteins) or blocked recycling processes.

We next performed double labelling with JIM84 and AtPIN antibody on roots treated with BFA. This showed that PIN proteins (Fig. 3H,K) are localised in the same BFA compartments as the JIM84 labelled Golgi membranes (Fig. 3). Within these compartments, however, JIM84-labelling only partially overlapped with PIN labelling. This often led to a ring-like pattern of double-stained membranes around a domain only stained by AtPIN antibody (Fig. 3J). This specific organisation within the BFA compartment suggests that the two labelled populations have a distinct molecular signature which is sufficient to keep them separated. Incidentally, the fact that the JIM84 plasma membrane-labelling remained in many BFA-treated cells suggests that, the JIM84-labelled BFA structures are predominantly made of Golgi- rather than plasma membrane-derived membranes (see also Satiat-Jeunemaitre and Hawes 1992; Satiat-Jeunemaitre and Hawes, 1994; Steele-King et al., 1999).

After BFA treatment AtPIN-labelled membranes colocalised neither with ER markers (not shown) nor with PVC markers (Fig. 3K-M). mRab-labelled structures were often bigger than those in control cells, but the basic 3D pattern was rarely altered by BFA (Fig. 3L). This was also found for other PVC markers such as PEP12 and BP80 (data not shown), or trans-Golgi-network markers (Geldner et al., 2003a).

Analysis of AtPIN labelling in dividing cells

In interphase cells, we never observed any asymmetry in the distribution of intracellular...
PIN compartments, which could be related to the polar insertion of the proteins at the cell surface. We next investigated whether the polar localisation could be traced back to a mitotic event. In particular, we tested the hypothesis that the proteins were asymmetrically inserted into the phragmoplast.

The cell plate was strongly labelled by AtPIN (Fig. 4C,D), suggesting that the insertion of PIN proteins within the plasma membrane takes place as the new plasma membrane is formed (see also Geldner et al., 2001 and fig. 2F within). A triple staining of dividing cells with DNA specific probes, microtubule antibodies and AtPIN confirmed that, the insertion of proteins began during anaphase together with the progression of the phragmoplast (Fig. 4A-D). At higher magnification, no asymmetry in membrane labelling was ever detected at this level, suggesting that proteins were targeted to both sides of the cell plate. Intracellular objects, resembling those in interphase cells gathered preferentially around the phragmoplast. This spatial distribution was symmetrical on each side of the cell plate (Fig. 4D). This symmetry of PIN-labelled structures around the cell plate was also clearly seen in BFA-treated cells (Fig. 4E), where BFA-induced aggregates were distributed in a symmetric manner on each side of the cell plate.

As a whole, these observations suggest that, the asymmetrical localisation of PIN proteins is not linked to positional information within the endomembrane system and occurs after cell division. It, therefore, depends on local properties of the cortical cytoplasm or the plasma membrane.

Involvement of the actin network in polar distribution of PIN proteins
The cytoskeleton might be involved in the formation of local cytoplasmic subdomains. In particular, actin appears to be involved in the recycling of PIN proteins (Geldner et al., 2001). We further extended these observations by analysing the detailed effects of actin depolymerisation on the localisation of PIN proteins. Here, we took advantage of the better resolution provided by isolated cells of maize roots.

Latrunculin has no effect on polar staining but affects both AtPIN- and JIM84-labelled structures
Short latrunculin treatments of up to 2 hours had no obvious...
Polar distribution of PIN proteins

LatrunculinB inhibits some but not all effects of BFA on PIN-labelled structures

To further test the involvement of actin on PIN trafficking, we analysed the effects of latrunculin pre-treatment on the BFA-induced reorganisation of PIN proteins in maize roots. This showed that the BFA effects were partially dominant over the latrunculin effects. First, 92% of the PIN-labelled cells lacking actin filaments still lost their plasma membrane staining after subsequent treatment with BFA (Fig. 4J), suggesting that the BFA-induced protein retrieval from the plasma membrane was actin independent. Second, the intracellular AtPIN-labelled compartments significantly increased in size under BFA treatment, even in the absence of actin filaments (Fig. 4, compare J with G). Similar observations were made for JIM84 epitopes under the same experimental conditions (Fig. 4, compare I with F) and, again, the two populations merged in the same aggregates (Fig. 4K). These results indicate that both PIN-labelled compartments and Golgi stacks were modified by BFA in an actin-independent manner. Third, the formation of the two or three massive BFA compartments did not occur anymore in the absence of actin filaments (compare Fig. 4J with Fig. 3H), suggesting that the final coalescence of BFA-induced Golgi and PIN-labelled compartment is actin dependent.

These observations are summarised in Fig. 5, concordant with our earlier hypothesis that the formation of BFA compartments results from a sequence of distinct events (see Satiat-Jeunemaitre et al., 1996b). Some of these are actin dependent (aggregation of Golgi units and PIN-labelled structures in two to four fluorescent areas), whereas others are actin independent (modification of compartment morphology). These two effects probably concern two distinct molecular machineries. In all cases, our latrunculin- and latrunculin-BFA-based experiments show that actin is not involved in BFA-induced retrieval of PIN proteins from plasma membrane. In addition, the data do not indicate that actin is involved in initiating specific local asymmetry and polarity within the cell cortex.

Involvement of microtubules in the polar distribution of AtPIN-stained proteins

Microtubules are usually present in highly organised cortical

Fig. 4. AtPIN and cytoskeleton in isolated Zea mays root cells. (A-D) Co-immunostaining of AtPIN with microtubules. AtPIN-Cy3–tubulin-FITC double labelling of cells in (A) interphase, (B) metaphase, (C) late anaphase, (D) telophase. Chromosomes are stained with Hoechst 33342 (blue). PIN proteins (red) are inserted within the newly formed plasma membrane at the cell plate. Notice the presence of PIN-labelled intracellular structures on each side of the cell plate. (E) A cell plate in a cell treated with BFA. Notice the strong labelling of the plasma membrane and the symmetrical repartition of BFA-aggregates on each side of the cell plate. (F-H) Effect of latrunculin-B on the colocalisation of AtPIN and the Golgi marker JIM84. (F) JIM84–FITC (green). Golgi stacks tend to form small aggregates in absence of actin whereas the plasma membrane remains labelled. (G) AtPIN-CY3 (red). A strong polar labelling of the plasma membrane at the cell base is still observed in absence of actin. However, intracellular compartments form labelled aggregates. (H) Merged image. AtPIN and JIM84 labelled membranes are often juxtaposed in these latrunculin-B-induced aggregates, PIN proteins being generally in the core of these aggregates. (I-K) Effect of BFA on latrunculin-B-induced aggregates. Maize root cells were immersed in latrunculin-B solution to which BFA was added after 150 minutes. (I) JIM84–FITC (green). Increase of the initial latrunculin-induced aggregates (compare with F). (J) AtPIN-CY3 (red). BFA induces the disappearance of polar staining (compare with G) and the latrunculin-B-induced aggregates become thicker. (K) Merged picture, showing that AtPIN- and JIM84-labelled membranes colocalise in these fluorescent structures. Bars, 8 μm.
arrays. They could, therefore, provide a basis for the polar localisation of PIN proteins. Previous analyses on Arabidopsis roots have shown that microtubules might be involved in a cytokinetic trafficking pathway of PIN proteins, but were not required for the maintenance of polarity of PIN proteins (Geldner et al., 2001). Nevertheless, these experiments were based on short-term treatments with oryzalin and did not question the long-term effects of microtubule disruption on polar organisation at cell and tissue level. We, therefore, performed two additional experiments. First, we investigated the distribution of PIN proteins in a mutant with a disorganised interphase microtubule arrays. Second, we looked at the short- and long-term effects a complete absence of microtubules might have.

AtPIN labelling in ton mutants

The ton mutants are characterised by a the lack of pre-prophase bands and the presence of disorganised cortical microtubule arrays (Fig. 6A), the division planes being randomly organised within the tissue (Traas et al., 1995; Camilleri et al., 2002). Cells expressing the weak allele ton2.1 (Fig. 6B), PIN proteins, were still asymmetrically distributed, although the pattern along cell files was less regular. In root cells expressing the strong allele ton2.2 (Fig. 6C), labelling of subdomains of the plasma membrane was still observed but organisation of the polarity at the tissue level was lost. These observations suggest that, the presence of ordered cortical arrays of microtubules is not required for the establishment of polarity at the cell level but in their absence, cells lose the ability to target the PIN protein in a coherent way. Therefore, microtubules appear to be essential for the tissue patterning of PIN proteins.

Effect of microtubule depolymerisation on AtPIN-labelling pattern

Having established that ordered microtubules are required for organised PIN patterns at the tissue level, we next investigated the short- and long-term effects of a complete depolymerisation of the microtubules.

On short treatment of maize roots with high concentrations of oryzalin, microtubules rapidly depolymerised, whereas polar AtPIN-labelling remained unchanged (Fig. 6D-F). These observations confirm that, in interphase cells, microtubules are not involved in the establishment or short-term maintenance of cell polarity.

After longer treatments (8-42 hours), growth of maize or Arabidopsis plantlets continued but showed radially swollen root tips (not shown). The changes in root morphology were associated with laterally swollen cells. Whereas PIN proteins were still present in the plasma membranes, the typical baso-apical polarity of the labelling was hardly recognisable on longitudinal root sections (Fig. 6, compare H-J with G). Sub-domains of the plasma membrane were still labelled, but cell polarity had changed. Analysis of both transverse (Fig. 6K) and longitudinal sections (Fig. 6H-J) revealed that the PIN proteins were not redistributed randomly, but were relocalised towards the centre of the central cylinder of the roots. The phenomenon of PIN relocation was also observed in maize roots (Fig. 6L). These results suggest that microtubules were indirectly involved in the control of the final cellular location of PIN.

PIN1 labelling in BY-2 cells and protoplasts

To study the behaviour of PIN proteins in a simple system, we constructed a BY-2 cell line expressing AtPIN1::GFP. Growing

**Fig. 5.** Diagram summarizing the BFA and latrunculin-B (LAT) effects on AtPIN- and JIM84 labelled compartments in maize root cells. (1) In control cells, PIN- and JIM84-labelled compartments are dispersed throughout the cytoplasm; strong AtPIN polar staining of the basal membrane. G, Golgi stacks labelled with JIM84. E, endosome-like structures labelled with AtPIN. (2) Under latrunculin-B treatment, both populations tend to mix and form small aggregates, suggesting a similar actin dependency for their distribution. However, AtPIN polar staining is still observed, suggesting that actin is not involved in the maintenance of cell polarity. (3) Treatment with brefeldin A results in the formation of typical BFA compartments, trapping both Golgi stacks and endosome-like membranes. These observations are the sum of two distinct dynamic events: first, the gathering of compartments in cytoplasmic subdomains, a phenomenon that is actin dependent and, second, a vesiculisation of compartments that is actin independent – as shown for Golgi membranes (Satiat-Jeunemaitre et al., 1992). (4) In latrunculin pre-treated cells, BFA still targets the BFA-sensitive compartments in an actin-independent process because the polar labelling of the membrane is lost and the small, induced latrunculin aggregates increase significantly in size, probably due to an actin-independent deconstruction process as described in (3).
BY-2 cells exhibit helical arrays of microtubules within the cells that become randomly organised when cell walls are removed (Couchy et al., 2003). AtPIN1::GFP was localised only to the transverse cell surfaces of the BY-2 cell ribbon, and labelled intracellular structures (Fig. 7A). In dividing cells, the cell plate was symmetrically labelled by AtPIN1::GFP. Interestingly, this symmetry was maintained after mitosis (Fig. 7B), outlining the transverse cell surfaces of the BY-2 cell ribbons. The fate of this transverse staining was followed when microtubules were disorganised during protoplast formation (Fig. 7C-H) or by treatment with oryzalin (Fig. 7I-L). Fig. 7 shows a redistribution of the AtPIN1 proteins over the plasma membrane during protoplast formation (Fig. 7C-H). Preferential staining of membrane subdomains between two cells is still observed as long as the cells stay in close contact, even if these cells lose their original shape. The cell plate was still stained for AtPIN1 (Fig. 7D). When wall digestion finally separated the two cells, AtPIN1 staining of plasma membrane subdomains was still visible for a few minutes (Fig. 7E), but rapidly exhibited a perfect regular staining of the whole plasma membrane (Fig. 7G). When BY-2 cells were treated with oryzalin, cell shape was progressively modified (Fig. 7I-L). Cells enlarged laterally, finally producing aggregates of round cells (Fig. 7J-L). Differential labelling with AtPIN was still observed as long as the initial cell-cell contacts were maintained (Fig. 7K,L).

Together, these results – obtained on plants and cell suspensions – suggest that microtubules act indirectly on PIN localisation. They might do so by interfering with the establishment of cell-cell interactions, which seems to play an important role in the final cellular distribution of PIN proteins.

**Discussion**

**PIN protein trafficking involves the Golgi to plasma membrane pathway**

Previous studies have shown that PIN proteins were distributed in a polar manner at the plasma membrane of *Arabidopsis* (Gälweiler et al., 1998; Geldner et al., 2001) but little attention was paid to intracellularly labelled organelles. Here, we show that PIN proteins are differentially associated with these organelles. The major proportion of PIN-labelled organelles (80%) was associated with punctate structures distinct from ER domains, Golgi stacks and prevacuolar compartments. By extrapolation of what has been suggested in *Arabidopsis* root cells (Geldner et al., 2001), the major part of these PIN-labelled organelles might be recycling or endocytic compartments. Endosomes in plant cells may be described as punctate structures covering heterogeneous populations of...
compartments stained by FM4-64 – a definition to be handled with care (see Bolte et al., 2004b and discussion within). These structures are similar in size but are indeed distinct from the Golgi (Robert et al., 2005; Geldner, 2004). They might represent an heterogenous population of organelles, including PVCs (Tse et al., 2004), and might specifically bear proteins like GNOM, an ARF-GEF protein involved in PIN1 protein recycling (Geldner et al., 2003a; Geldner et al., 2003b), or KOR1, an endo-1,4-β-glucanase involved in cellulose synthesis (Robert et al., 2005). The morphological and functional features of the endosome population harbouring PIN proteins still need to be clarified.

We now also provide evidence, that a significant proportion of the PIN proteins is associated with the Golgi apparatus. This association suggests that, a level of regulation for PIN trafficking takes place at the Golgi, operating either as an exocytic and/or endocytic compartment (Tanchak et al., 1984; Bolte et al., 2004b; Hawes and Satiat-Jeunemaitre, 2005). These active PIN trafficking pathways between the Golgi stacks and plasma membrane do neither overlap with the vacuolar secretory pathway nor with the putative ‘late’ endocytic pathways (Tse et al., 2004) because no PVCs were PIN-labelled.

The question remains as to when and how PIN proteins are polarly distributed within the cell. An obvious moment when PIN can be polarly distributed is cytokinesis. In such a scenario, PIN would be only deposited on one side of the phragmoplast. It has been previously shown that, in Arabidopsis root cells, PIN proteins are inserted in the newly forming cell plate between daughter cells (Geldner et al., 2001). Our results in maize root cells confirm this feature. In addition, we show that a symmetric vesicle distribution on each side of the cell plate occurs, suggesting that PIN proteins are continuously inserted on both sides of the forming cell plate. Therefore, the basal membrane to be targeted ultimately by PIN is defined after mitosis, an hypothesis already suggested by Geldner et al. (Geldner et al., 2004). Nevertheless, the fact that the phragmoplast systematically accumulates PIN proteins shows that cell division has to be considered as a level of regulation. Interestingly, the post-mitotic definition of the targeted membrane seems to be correlated to the organisation of cells in tissues: in BY-2 cells organised in a ribbon-like structure, this asymmetry does not appear on the transverse walls. It has been proposed that specific endocytic trafficking processes explain the maintenance of specific subdomains within the cells (Geldner, 2004; Grebe, 2004), although the occurrence of such differential turnover needs further experimental evidence.

![Fig. 7. Redistribution of AtPIN1::GFP in BY-2 protoplasts and oryzalin-treated BY-2 cells.](image)

(A) Stably transformed BY-2 cells exhibit a polar pattern of AtPIN1::GFP because the radial surface (but not their longitudinal membranes) was clearly fluorescent. (B) High-magnification of the transverse membranes shows that the two sides of the cell-cell contact are indeed labelled. (C-H) Progressive relocation of AtPIN1::GFP-labelling during the process of making protoplasts. (C) Bottom cell becomes rounder and labelling begins to extend to the whole plasma membrane. Notice the more intense labelling when cells are still in contact (top cells). (D) The arrow marks the cell followed over time and shown in the two following sequences (E) and (G). (E) When the protoplast finally detaches from its neighbour, differential staining is still displayed for a few minutes on the plasma membrane (arrowhead). (F) Corresponding DIC image of (E). (G) Same protoplast at a later stage, AtPIN1::GFP labelling of plasma membrane is still conserved but it has extended to the whole surface. (H) Corresponding DIC image. (I-L) Progressive relocation of AtPIN1::GFP under oryzalin treatment. (I) After 10 minutes, cells begin to round up, labelling is still observed on cell plate and transverse cell surface. (J-L) Labelling at the cell surface is still observed as long as there is some cell-cell contact, regardless of the loss of the ribbon form of BY-2 cells (arrowheads). Bars, 16 μm.

![Fig. 8. Position of the p74 peptide chosen for raising antibodies AtPIN against AtPIN1.](image)

The p74 peptide is made of 16 aa (bold in grey box) placed in the α glycine-rich domain (asterisks), in the intracellular loops placed between two transmembrane domains (sequence shaded grey).
Effects of BFA outline different sites of BFA action to regulate PIN

The striking effect of BFA on PIN labelling (i.e. the disappearance of polar PIN labelling, concomitantly with the appearance of BFA compartments), has been interpreted as evidence of endocytic PIN being blocked to recycle back to the plasma membrane (Geldner et al., 2001; Geldner et al., 2003a; Baluska et al., 2002), which would be actin dependent. However, global BFA effects are probably the sum of distinct BFA effects (Merigout et al., 2002), rendering their interpretation difficult. Our results modulate the interpretation of BFA data because they show that PIN retrieval from the plasma membrane under BFA treatment is independent of actin. They also show that PIN proteins are trapped, together with JIM84-labelled epitopes, in BFA-induced structures – PIN proteins being often in the core of these compartments. The accumulation of PIN proteins in BFA compartments might result from both the retrieval of PIN from plasma membrane (which in this case is an actin-independent process), a reorganisation of PIN-labelled Golgi membranes (which is only partly actin dependent, see Results) and a BFA-induced block of PIN proteins en route to the plasma membrane (an actin-dependent process). In contrast to PIN proteins, the accumulation of JIM84-labelled epitopes in BFA-compartments mostly derived from Golgi membranes because plasma membrane staining is still in place in most of the BFA-treated cells. Such sub-compartmentation between PIN and Golgi markers in these BFA-induced compartments has already been described for other probes of the endomembrane system (Geldner et al., 2003a; Grebe et al., 2003). It probably results from slight differences in the molecular signature of Golgi stacks and endosome-like compartments, leading to a structural hierarchy within the endomembrane system. It is tempting to consider that the original molecular gradients between Golgi and plasma membrane present in control cells, are actually mirrored in the organisation of BFA compartments, as suggested by earlier electron microscopy studies (Satiat-Jeunemaitre and Hawes, 1993; Satiat-Jeunemaitre et al., 1996a). These differences in molecular signatures might be due to the lipid component (known to be differentially affected by BFA) (Mérigout et al., 2002), or to proteins such as distinct ARF-GEFs with different sensitivities to BFA (Peyroche et al., 1999). The ARF-GEF protein Gnom has been reported to be a key BFA-sensitive marker for plant endosome-like structures (Geldner et al., 2003a), but not for the Golgi stacks. To discover the rules governing the formation of a BFA compartment could identify sorting mechanisms along the secretory pathway.

Microtubules are required for developmental information underlying PIN insertion

Our results do not indicate any direct role of actin filaments or microtubules in the maintenance of cell polarity, because PIN localisation is maintained during treatments of up to several hours with latrunculin and oryzalin. This does not mean that microtubules in the maintenance of cell polarity, because PIN is known to be involved in exocytosis processes (Picton and Steer, 1981) and, as discussed above, in the movement of PIN-labelled vesicles; therefore actin must somehow interact with the mechanism that controls the insertion of the proteins in specific domains. The position of certain (endo-)membrane associated proteins also depends on the presence of microtubules, as shown recently for the KOR1 protein involved in cell wall synthesis (Robert et al., 2005). Although we did not find such a direct role of the microtubules in PIN labelling, our results clearly indicate an indirect one.

The defects in polar PIN pattern observed in ton-mutant cells coincide with severe defects in cell-to-cell alignment, suggesting that ordered arrays of microtubules are necessary for cells to coordinate their polarity with respect to each other. After long-term oryzalin treatment, PIN proteins are redistributed on lateral plasma membranes specifically on the inner side of the cells towards the central cylinder showing that, in absence of microtubules, the cells have lost information on the position of the main polar axis.

This shows that the microtubule network (or its associated proteins) might play an indirect role in the final location of PIN, by determining somehow essential positional information within tissues. Hereby, cell-cell contacts might be particularly important as illustrated by our observations of BY-2 cells expressing AtPIN1::GFP, which show that polar PIN labelling was lost as soon as the cell-cell contacts were lost. Therefore, future work aimed at understanding the mechanism of PIN localisation should also be focused on the extracellular matrix.

Materials and Methods

Plant material

Arabidopsis thaliana cells and BY-2 cells in suspension were used as previously described (Bolte et al., 2004a). Maize caryopses (Zea mays, LG20.80, Limagrain, France) were grown as described in Satiat-Jeunemaitre and Hawes (Satiat-Jeunemaitre and Hawes, 1992). Arabidopsis seeds (ecotype Columbia) were grown for 7 days in continuous light (24 hours) at 23°C, on a solid Murashige and Skoog medium. Ton, pinoid and pin-formed1 mutants were grown as previously described (Traas et al., 1995; Christensen et al., 2000; Vernoux et al., 2000). Seeds of stably transformed plantlets of Arabidopsis expressing an AtPIN1::GFP construct under its own promoter were kindly provided by J. Friml (Benková et al., 2003).

Drug treatments

Treatments were performed either by immersing the roots in the treating solutions at 26°C in darkness, or on solid medium containing the drug – either in Petri dishes or in 500 ml or 1 litre Schott bottles. Brefeldin A (BFA, Sigma) stock solution was 20 mg/ml in dimethylsulfoxide (DMSO). Treatment with BFA (100 μg/ml) was for 1 hour or more (see text) as previously described (Satiat-Jeunemaitre and Hawes, 1992). Oryzalin (gift of DowElanco, Letchembe Regis, U.K.) stock solution was 288 μM in aceton; freshly prepared as previously described (Satiat-Jeunemaitre et al., 1996b). Cells were treated with 1.7 μM, 17 μM and 28 μM for 3, 8, or 42 hours. Latrunculin B (Sigma) stock solution was 10 nM in water and cells were treated with 25 μM for 1 hour.

AtPIN antibody design

A highly conserved peptide sequence (p74) of 16 amino acids and located in a glycosine-rich domain of the large intra-cytosolic loop of AtPIN1 (indicated by asterisks in the AtPIN1 sequence in Fig. 8) was used to raise a rabbit antiserum (Eurogentec, Belgium). The serum was purified on an affinity column by using the peptide, and subsequently named AtPIN-antibody.

AtPIN1::GFP fusion and BY-2 cell transformation

pBIn4 plasmid containing AtPIN1::GFP (Benková et al., 2003) was inserted into an LBA4404 Agrobacterium tumefaciens strain that contained the helper plasmid. Agrobacteria were co-cultured with BY-2 cells for 2 days at 26°C in the dark. Co-cultures were washed with 400 μg/ml cefotaxim and spread on Petri dishes holding 400 μg/ml cefotaxim and 50 μg/ml kanamycin. Resistant calli were transferred into liquid-medium culture supplemented with 50 μg/ml kanamycin.

Protein extraction and western blot analysis

Protein extracts from maize and Arabidopsis root apices, BY-2 and Arabidopsis suspension cultures, and protoplasts were blotted from a SDS-polyacrylamide gel in a Trans-Blot SD semi-dry transfer cell (Bio-Rad France, Marne la Coquette, France). Immunoblots were visualised by chemiluminescence (Lumi-Light System, Roche, Mannheim, Germany), using horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies at 1:1000 (Roche, Mannheim, Germany).

Immunofluorescence staining

Immunostaining procedures were performed on tissue sections embedded in PEG,
on partially digested tissues or cells, or on whole-mounts of Arabidopsis thaliana roots as previously described (Bolte et al., 2006; Safati-Jeunemaitre and Hawes, 2001; Geldner et al., 2003b, respectively). Single- and double-labeling was performed as previously described (Couchy et al., 2003; Bolte et al., 2004a). A rat monoclonal antibody JIM84, was used undiluted as a Golgi marker (Horsley et al., 1993). A rabbit polyclonal antibody against m-Rab1, was used as a PreVacuolar Compartment marker (Bolte et al., 2004a). A mouse monoclonal antibody against α-tubulin (clone DM1A, Interchim) was used at 1/200. Secondary antibodies were purchased either from Sigma (FITC-conjugated anti-rabbit, anti-mouse or anti-rat IgGs, used at 1:1, 1:40 or 1:40, respectively), Interchim (Cy3-conjugated anti-mouse, anti-rat, or anti-rabbit IgG used at 1:800), or Molecular Probes (Alexa Fluor 488- or Alexa Fluor 568-conjugated anti-mouse, anti-rat, anti-rabbit IgG, used at 1: 800). Nuclei were counterstained with 33342 or propidium iodide (1 μg/mL).

Confocal microscopy – image analysis and assessment of colocalisation events

Slides were observed and images were collected with an upright laser scanning confocal microscope TCS SP2 (Leica Microsystems, Mannheim, Germany). Different fluorochromes were detected sequentially frame-by-frame with the acousto-optical tunable filter system (AOTF) using 488 nm and 543 nm laser lines. The images were usually coded green (fluorescein-isothiocyanate or Alexa Fluor 488) and red (Alexa Fluor 568) giving yellow colocalisation signals in merged images. The oil objectives used were 40× NA 1.25 and 63× NA 1.30, giving a resolution of approximately 200 nm in the X7-plane and 400 nm along the Z-axis (pinhole 1 Airy unit). Images were processed using Adobe Photoshop (Adobe Systems).

Colocalisation events after double-labeling were assessed by statistical analysis using Metamorph (Roper Scientific). In this method, the distance between the centroids of two labelled objects was calculated for each apparent colocalisation event. When this distance was below the resolution limit of the objective used (63×), we considered that there was a true colocalisation of labels. We then determined the percentage of co-stained objects per optical section (n=73 optical sections).

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