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REVIEW PAPER

Keeping it all together: auxin–actin crosstalk in plant development

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Abstract

Polar auxin transport and the action of the actin cytoskeleton are tightly interconnected, which is documented by the finding that auxin transporters reach their final destination by active movement of secretory vesicles along F-actin tracks. Moreover, auxin transporter polarity and flexibility is thought to depend on transporter cycling that requires endocytosis and exocytosis of vesicles. In this context, we have reviewed the current literature on an involvement of the actin cytoskeleton in polar auxin transport and identify known similarities and differences in its structure, function and dynamics in comparison to non-plant organisms. By describing how auxin modulates actin expression and actin organization and how actin and its stability affects auxin-transporter endocytosis and recycling, we discuss the current knowledge on regulatory auxin-actin feedback loops. We focus on known effects of auxin and of auxin transport inhibitors on the stability and organization of actin and examine the functionality of auxin and/or auxin transport inhibitor-binding proteins with respect to their suitability to integrate auxin/auxin transport inhibitor action. Finally, we indicate current difficulties in the interpretation of organ, time and concentration-dependent auxin/auxin transport inhibitor treatments and formulate simple future experimental guidelines.

Key words: Actin, auxin, auxin transport inhibitor, PIN cycling, ABCB, TWD1 ABP1.

Introduction

A series of auxin maxima and minima are produced in different regions of both shoots and roots by a plant-specific process, called polar auxin transport (PAT: for details see Grones and Friml, 2015; Vanneste and Friml, 2009). These gradients form the basis for most processes of plant development and performance, such as tropic growth, lateral root development and planar root hair polarity (Boutte *et al.*, 2007; Vanneste and Friml, 2009; Geisler *et al.*, 2014; Grones and Friml, 2015).

PAT has been widely detected in plant phyla, from Charophyta (a group of freshwater green algae) to land plants including bryophytes (Fujita *et al.*, 2008; Boot *et al.*, 2012). However, most recent PAT studies have been performed in angiosperms and predominantly in the model plant, *Arabidopsis thaliana*. PAT occurs in a cell-to-cell manner, requires energy, is relatively slow (5–20 mm/h), specific for active free auxins and is gravity independent (Vieten *et al.*, 2007). Auxin is initially transported from the shoot apex downwards to the base (basipetally), and a small part of the auxin can be redistributed laterally in the stem. In the root, the auxin stream continues to the root tip (acropetally) and part of the auxin in the tip is redirected back upwards through the root epidermis into the root elongation zone, where it can be recycled back to the main auxin stream (Michniewicz *et al.*, 2007).

Based on the known transport data, in the 1970s a chemiosmotic model was proposed to better explain the mechanism

of PAT (Rubery and Sheldrake, 1974; Raven, 1975). As a weak acid (pK_a 4.75), about 15% of IAA is protonated (IAAH) in the apoplast, where the pH is ~5.5, maintained by the PM H^+-ATPase. IAAH can enter the cell by passive lipophilic diffusion across the PM. However, almost all the IAAH will be deprotonated (IAA^-) as a result of the more alkaline cytosolic environment (pH 7.0). IAA^- cannot diffuse across the PM and is, as such, trapped inside the cell. Thus it is obvious that PAT can be efficiently controlled only at the export and not at the import level (Geisler, 2014).

This classic chemiosmotic model was strengthened by the identification of PIN-carrier proteins, AUXIN-RESISTANT1/LIKE AUX1 (AUX1/LAX) uptake permeases and ABCB transporters (reviewed in: Benjamins and Scheres, 2008; Petrasek and Friml, 2009; Zazimalova *et al.*, 2010; Peer *et al.*, 2011; Grones and Friml, 2015). Recently, some other classes of proteins were also reported to catalyse the transport of auxin, such as PIN-LIKE PROTEINS (PILS), members of the ABC transporter G family (ABCG) and NRT1.1 (Krouk *et al.*, 2010; Ruzicka *et al.*, 2010; Barbez *et al.*, 2012). However, their properties as auxin transporters are less well documented and their individual roles in PAT are less understood.

Polar auxin transport and the action of the actin cytoskeleton are tightly interconnected. From a wider perspective, this might sound like a trivial statement because, in general, plant transporters reach their final destination tracks by myosin-mediated movement of secretory vesicles along F-actin tracks (Ivakov and Persson, 2013). Moreover, transporter polarity and flexibility is dependent on transporter cycling, requiring endocytosis and exocytosis of vesicles (Kleine-Vehn and Friml, 2008). The tight connections between these processes was also underlined by the finding that the *Arabidopsis* interactome reveals a strong enrichment of a few network communities, with a high degree of shared proteins among the transmembrane-transport and trafficking communities suggesting a functional overlap (Arabidopsis Interactome Mapping, 2011).

In this respect we have reviewed the current literature on an involvement of the actin cytoskeleton in polar auxin transport and highlight differences from non-plant organisms. We discuss known effects of auxins and of auxin transport inhibitors on the stability and organization of actin and examine the suitability of auxin and auxin transport inhibitor-binding proteins to integrate auxin/auxin transport inhibitor action. Finally, we indicate current difficulties in the interpretation of auxin and auxin transport inhibitor treatments and formulate simple future experimental guidelines.

The plant actin cytoskeleton: similarities to and differences from non-plant organisms

Structure and function

Filamentous actin (F-actin) is built of globular actin subunits (G-actin) of ~42 kDa forming polymeric structures of 7–9 nm in diameter, resembling a double-stranded string of pearls with a helical twist (Staiger and Blanchoin, 2006; Thomas and Staiger, 2014). In all eukaryotic cells, actin constantly changes between polymeric and monomeric forms depending on the type, differentiation and physiological status of the cell (Karpova *et al.*, 1995; Gibbon *et al.*, 1999; Staiger, 2000; Snowman *et al.*, 2002). For example, budding yeast, Saccharomyces cerevisiae, has an extremely small pool of globular subunits (0.2 µM) because it assembles almost all of its actin into filaments (2 µM; Pollard *et al.*, 2000). In contrast, plant pollen has as much as 200 µM total actin of which only 5–10% is in its filamentous form (Gibbon *et al.*, 1999; Snowman *et al.*, 2002).

In plants, actin is a highly conserved protein encoded by a large gene family. Different actin genes and their encoded protein isovariants are specialized to perform a subset of the many essential functions of auxin in different organs and tissues (Meagher *et al.*, 1999). *Arabidopsis thaliana* has eight actin genes that are grouped into two ancient classes, vegetative and reproductive actins (Kandasamy *et al.*, 2009). The vegetative class has two distinct subclasses of actin isovariant: *ACT2* and *ACT8* (subclass 1) encode for actins that vary only by a single amino acid. *ACT7* (subclass 2) encodes for a protein differing from ACT2 and ACT8 by 7%, and from the closest reproductive actin ACT11 by 4%. However, genetic analysis indicates that the two subclasses of vegetative actins have different function in multicellular development. Lack of the most abundant *ACT2* gives rise to stunted and bulbous root hairs, but normal branching of leaf trichomes (Gilliland *et al.*, 2002; Ringli *et al.*, 2002; Kandasamy *et al.*, 2009). In contrast, the other strongly expressed actin, ACT7, was suggested to affect seed germination, root growth and trichome branching, but not root hair development (Kandasamy *et al.*, 2001, 2009; Gilliland *et al.*, 2003). In a very recent study, however, ACT2 and especially ACT7 were shown to have an impact on planar root hair polarity (Kiefer *et al.*, 2015). By analysing the phenotypes of double mutants expressing only a single vegetative actin, it was suggested that the two subclasses of vegetative actin exhibit unique functional properties. For example, compared to single mutants, the act2-1 act7-4 double mutant shows additional developmental and morphological defects, such as abnormal cell division, cell expansion and extreme dwarfism (Kandasamy *et al.*, 2009). In addition, it was demonstrated that the regulation of actin genes is differential because overexpression of a single vegetative actin isovariant from multiple actin regulatory sequences could restore, or at least partially restore, the development of actin double mutants (Kandasamy *et al.*, 2009).

Proteomic analysis of single mutants indicated that expression of vegetative *Arabidopsis* actins is subclass-specific and redundant (Kandasamy *et al.*, 2009). Although *act2-1* plants have a reduced level (55–60%) of total subclass 1 actin (ACT2 and ACT8) compared with the wild type, they have the same or even a slightly higher level of total actin in shoot and root tissues compared with wild-type plants. The reason for the higher total actin detected in *act2-1* is due to an up-regulation of expression of the *ACT7* isovariant (Kandasamy *et al.*, 2009).

Interestingly, *ACT7* was the only *Arabidopsis* actin isoform to respond strongly to auxin, other hormone treatments,

the light regime and wounding, and may be thus the primary actin gene responding to external stimuli. While it was reported recently that the *act2-1* mutant has disturbed BR signalling and an enhanced auxin response (Lanza *et al.*, 2012), a redundant function of ACT7 should be considered because of the up-regulation of *ACT7* in the *act2-1* mutant.

Finally, a recent study provided evidence that the interaction of ACT7 and interacting actin-interacting protein 1 (AIP1-2) is required for correct polar positioning of ROP proteins during establishment of planar polarity (Kiefer *et al.*, 2015). Genetic analyses suggest that *ACT7* represents a main actin isoform required for planar polarity of root hair positioning.

Actin-binding proteins

The assembly and reorganization of actin filaments is provided by the action of actin- binding proteins (ABPs) (Pollard and Cooper, 2009; Blanchoin *et al.*, 2014). In plants, biochemical and genetic approaches have allowed identification of several ABPs thought to regulate actin organization and dynamics *in vitro* and *in vivo* (Higaki *et al.*, 2007; Ren and Xiang, 2007; Thomas *et al.*, 2009a; Blanchoin *et al.*, 2010; Thomas, 2012; Henty-Ridilla *et al.*, 2013).

ABP function in plants has been recently summarized in several excellent reviews (Hussey *et al.*, 2006; Staiger and Blanchoin, 2006; Thomas *et al.*, 2009b; Henty-Ridilla *et al.*, 2013; Li *et al.*, 2015). In short, ABPs reveal distinct but overlapping effects on actin organization and polymerization: monomer-binding proteins, such as profiling, ACTIN-DEPOLYMERIZING FACTOR (ADF), control the size and activity of the actin pool (Staiger *et al.*, 2009). Severing proteins, such as ADF/cofilin, villin and some formins, create filament breaks and thus generate new ends for actin assembly. Capping proteins, including villin and ACTIN-INTERACTING PROTEIN 1 (AIP1), bind with high affinity to filament ends and regulate actin turnover. Homologues of the ACTIN RELATED PROTEIN2/3 (ARP2/3) complex and formins are actin modulators and promote actin elongation, nucleation and cross-linking (Staiger *et al.*, 2010). In summary, it appears that F-actin can be stabilized against disassembly by the action of multifunctional ABPs.

Work on ABPs has been impeded by their existence as large gene families and overlapping functionality. Multiple isovariants do coexist in different tissues and cells during different plant development stages. Their function is apparently also affected by subcellular signalling because ABPs have been suggested to act as sensors and transducers of signalling cascades, in which their activities are often regulated by second messengers, such as calcium and phosphoinositides (Li *et al.*, 2015). In addition, some ABPs are able to bind to actin filaments as well as other molecules, as suggested by the interaction between profilin and PIP2 and AIP1-2 to ACT7 (Kiefer *et al.*, 2015). Therefore further detailed studies of *in vivo* roles of ABPs in regulating actin organization and dynamics are needed for a deeper understanding of the connection between actin and auxin-regulated plant cell growth and morphology.

Actin dynamics and imaging

The combination of advanced fluorescent microscopy approaches and new fluorescent protein reporters of the actin cytoskeleton have provided us with a much better view of actin dynamics in some living plant cells, such as those of the epidermis and pollen tubes (Thomas, 2012). Total internal reflection fluorescence microscopy (TIRFM) and its variant, variable-angle epifluorescent microscopy (VAEM), have been used successfully to study the functionalities of ABPs (Michelot *et al.*, 2005; Khurana *et al.*, 2010; Zhang *et al.*, 2010; Henty *et al.*, 2011; Hoffmann *et al.*, 2014) and to visualize actin filament dynamics *in planta* (Staiger *et al.*, 2009). VAEM, adjusting the illuminating light to an angle greater than the critical angle for reflection (Konopka and Bednarek, 2008), proved to be especially suitable for the generation of high contrast images of the cortical actin cytoskeleton in plant cells (Fujimoto *et al.*, 2007; Konopka and Bednarek, 2008). Most VAEM-based studies focus on epidermal cells of etiolated hypocotyls because of the correlation of cytoskeleton organization/dynamics with cell expansion/morphogenesis in this cell type. Moreover, in these cells VAEM imaging is facilitated by fairly thin cell walls and broad flat surfaces (Li *et al.*, 2015). In addition, etiolated hypocotyls are also a good model to study the effects of dynamics on cell elongation because it allows imaging of actin with a minimal interference by PAT, which is widely dependent on light (Jensen *et al.*, 1998).

Attempts to image the actin cytoskeleton by direct tagging of actin with GFP variants were unsuccessful. Therefore, domains or motifs from ABPs have been employed to construct indirect reporters of F-actin for living cell imaging (Li *et al.*, 2015). Currently, the second actin-binding domain from *Arabidopsis* Fimbrin1 (fABD2) and a 17 amino acid actin-binding motif derived from yeast Abp140 (Lifeact) are being used extensively as F-actin reporters *in vivo* (Sheahan *et al.*, 2004; Voigt *et al.*, 2005; Pegurier *et al.*, 2010; Era *et al.*, 2013). Another actin cytoskeleton reporter, mouse-talin, is now less used because of its apparent effect on bundling and stabilizing of actin filaments (Nick, 2010).

Quantification of single actin filament dynamics in cortical tissues surprisingly revealed a much more rapid growth and severing activity in comparison to animal cells (Staiger *et al.*, 2009). The rate of assembly at the barbed-end is about 2 μm s^{-1} or 720 subunits per second (Henty-Ridilla *et al.*, 2013). Such an assembly rate would allow filaments to grow from one side to the other across the cell in ~10 s (Li *et al.*, 2015). Once filaments reach a maximum length, severing will start in the older region of filaments with an average frequency of 0.011–0.026 breaks/μm s^{-1} (Li *et al.*, 2015). From filament initiation to complete disappearance of all served fragments, an individual plant filament has a short lifetime (~15–30 s; Li *et al.*, 2015). This kind of fast filament turnover has been described as stochastic dynamics (Staiger *et al.*, 2009; Li *et al.*, 2015). In addition, actin filaments are also constantly remodelled by filament buckling or 'waving'. Both processes were reduced by the putative myosin inhibitor, BDM, suggesting an involvement of myosins in this remodelling process (Staiger *et al.*, 2009).

In addition to the dynamics of single filaments, actin bundles are also generated by 'zippering' together of two or more individual actin filaments. The bundles can be disassembled by 'peeling off' filaments as well as by severing activity (Zheng *et al.*, 2013; Hoffmann *et al.*, 2014). It was shown that actin bundling in plants is also very dynamic and that different cell types have a variable bundling frequency (Li *et al.*, 2015). For example, measurement of bundling frequencies in hypocotyl epidermal cells was found at a rate of 6.9×10^{-5} events/μm^2 s^{-1} (Hoffmann *et al.*, 2014), while, in *Arabidopsis* pollen, bundling is observed at a frequency of 2.3×10^{-5} events/ μm^2 s^{-1} (Zheng *et al.*, 2013). However, the *in vivo* function of actin bundling dynamics has not been fully elucidated.

Lessons from actin drugs

Actin-specific drugs are used widely to study the relationship between actin organization, dynamics and functions *in vitro* and *in vivo.* Latrunculins, marine natural products, were the first found to bind to actin and disrupt its organization (Spector *et al.*, 1989). Latrunculins inhibit the polymerization of monomeric actin by forming a high-affinity complex with monomeric actin, thus promoting depolymerization of filamentous actin (F-actin). *In vivo* imaging revealed that latrunculin B treatment reduces the elongation rate at filament ends in a dose-dependent manner (Staiger *et al.*, 2009).

Jasplakinolide is a cyclic peptide with a 15-carbon ring containing three amino acid residues (Spector *et al.*, 1999). It was isolated from marine sponges and is known as a potent inducer of actin polymerization and/or stabilization. Jasplakinolide binds to F-actin competitively with phalloidin, a bicyclic heptapeptide from the mushroom *Amanita phalloides* (Cooper, 1987). Fluorescent derivatives of phalloidin, such as Alexa Fluor 488, are commercially available and very useful for localizing and quantifying actin filament parameters in living and fixed cells.

Cytochalasins, a group of fungal metabolites, bind to the barbed end of actin filaments leading to the inhibition of actin polymerization and depolymerization and inhibit the growth of actin filaments. For example, cytochalasin D was used to study the interaction between the actin cytoskeleton and NPA-binding proteins in extracts of zucchini hypocotyls (Butler *et al.*, 1998a; Muday, 2000). Those experiments indicated that PAT and its regulation by NPA are affected by specific drug treatments of the actin cytoskeleton (for further details and the effect of actin drugs on auxin transporter vesicle trafficking, see under heading 'Regulatory auxin-actin feedback loops').

However, a surprising finding was that both cytochalasin D and latrunculin B treatments shown to disrupt actin polymerization and thus reduce PAT (Butler *et al.*, 1998a) were ineffective in inhibiting root gravitropism (Staves *et al.*, 1997; Blancaflor and Masson, 2003; Hou *et al.*, 2004). Even more remarkable, latrunculin B promoted root gravitropism, which is accompanied by a persistant lateral auxin gradient (Hou *et al.*, 2004). Although these data were most evident by using a clinostat, they argue for the model that actin cytoskeleton is not essential for gravity perception (Hou *et al.*, 2004).

Regulatory auxin-actin feedback loops

As pointed out above, the directionality and flexibility of auxin transport relies on subcellular dynamics of specific influx and efflux transporters that own the particular property of polar targeting and endocytotic recycling (Blakeslee *et al.*, 2005; Kleine-Vehn and Friml, 2008; Titapiwatanakun and Murphy, 2009). Multiple results from genetic analysis and pharmacological studies suggest that asymmetric cellular targeting of auxin transporters and their constitutive recycling is dependent on actin filaments (Muday, 2000; Geldner *et al.*, 2001; Muday and Murphy, 2002; Boutte *et al.*, 2006; Kleine-Vehn *et al.*, 2006; Titapiwatanakun *et al.*, 2009; Cho and Cho, 2012).

The impact of the actin cytoskeleton on PAT, however, is still controversial (Boutte *et al.*, 2006; Rahman *et al.*, 2007; Titapiwatanakun *et al.*, 2009). In the following we will therefore review findings on how auxin modulates actin expression and actin organization/dynamics and how actin and actin stability affects auxin transporter endocytosis and recycling by regulating vesicular trafficking.

Auxin modulates actin expression, organization and dynamics

Plants perform auxin signalling through at least two distinct pathways. One is provided by the transcriptional AUX/IAA-$SCF^{TIR1/AFB}$ receptor, which senses high cellular auxin in the nucleus (Mockaitis and Estelle, 2008; Grones and Friml, 2015). Another, more rapid auxin signalling pathway seems to be conducted by a portion of the non-transcriptional AUXIN-BINDING PROTEIN1 (ABP1) receptor situated on the apoplastic PM surface of cells (Robert *et al.*, 2010; Grones and Friml, 2015). As elaborated in the following, multiple lines of evidence indicates that auxin can modulate actin expression, actin organization and actin dynamics and that the underlying signalling is transduced through both pathways, although obviously on transcriptional and post-transcriptional levels, respectively. Moreover, recent evidence supports both receptors' ability to co-contribute to auxin-mediated transcriptional auxin responses, including control of actin organization and dynamics (Tromas *et al.*, 2013; Grones and Friml, 2015; for details, see below). However, as a very recent report sheds some serious doubt on the role of ABP1 in auxin signalling and auxin-mediated plant development (Gao *et al.*, 2015), we prefer to restrict our discussion on ABP1 and ABP1-$SCF^{TIR1/AFB}$ crosstalk to a minimum.

Auxin induces ACTIN expression

The role of auxin during the regulation of plant morphogenesis is tightly linked with the differential regulation of actin genes (Kandasamy *et al.*, 2001). One of the three vegetative actins in *Arabidopsis*, *ACT7,* was variably expressed in different tissues and organs (McDowell *et al.*, 1996). The highest levels of *ACT7* mRNA were found in rapidly expanding vegetative organs (McDowell *et al.*, 1996), known to contain high levels of auxin originating form local auxin synthesis or PAT. By promoter-GUS analyses, it was shown that *ACT7*

is the only one of the eight actin genes that is strongly active in the hypocotyl and that *ACT7* is the only actin gene to respond strongly to auxin and other environmental stimuli (McDowell *et al.*, 1996). This is probably due to the fact that the *ACT7* promoter sequence contains a remarkable number of motifs with sequence similarity to putative phytohormone response elements (McDowell *et al.*, 1996). In cultured tissues of *Arabidopsis*, homozygous *act7-1* mutant plants were shown to produce callus tissues in response to exogenous auxin, the mutant callus contained at least 2- to 3-fold lower levels of ACT7 protein than the wild type (Kandasamy *et al.*, 2001). A null mutant in *ACT2*, another vegetative actin gene, did not significantly affect callus formation (Kandasamy *et al.*, 2001). This indicates that auxin induces *ACT7* but not *ACT2* and that this isovariant is essential in the process of auxin-induced cell proliferation and callus formation (Kandasamy *et al.*, 2001).

Auxin induces reorganization of the actin cytoskeleton

Studies on cytoplasmic streaming, a process provided by organelle-associated myosin XI moving along actin bundles, broadened our understanding of how auxin influences actin organization. Application of IAA accelerates cytoplasmic streaming at low concentration and inhibits it at high concentration in several plant cell types (Sweeney and Thimann, 1942; Kelso and Turner, 1955; Ayling *et al.*, 1994). Measurements of cytoplasmic calcium concentrations indicated that the application of auxin was followed by a small and slow increase in calcium in some of the treated cells (Ayling *et al.*, 1994). Furthermore, in root hair cells of *Hydrocharis*, the orientation of actin filaments was disturbed by application of high concentration of the synthetic auxin, NAA, resulting in an inhibition of cytoplasmic streaming (Tominaga *et al.*, 1998). Therefore, it was hypothesized that cytoplasmic streaming is inhibited by NAA via acidification of the cytoplasm. Acidification of the cytoplasm by different acids could mimic the inhibited cytoplasmic streaming and disturbed actin organization (Tominaga *et al.*, 1998). Interestingly, recently a link between the velocity of cytoplasmic streaming and plant size was provided: expression of high- and low-speed chimeric myosin XI-2 resulted in larger and smaller plant sizes (Tominaga *et al.*, 2013).

In rice coleoptiles, mouse talin-YFP (mTalin-YFP) imaging revealed that the process of auxin-promoted elongation is accompanied by a change of actin bundling from thick bundles to fine strands (Holweg *et al.*, 2004). IAA and NAA (but not 2,4-D) could rescue the bundled configuration of actin induced by the overexpression of mTalin not only in intact rice coleoptiles (10–50 µM, 0–60 min; Nick *et al.*, 2009), but also in tobacco BY-2 cell lines (2 µM; Maisch and Nick, 2007). Based on these results, a model was provided where auxin triggers in the shoot the reorganization of F-actin bundles into finer filaments leading to a more efficient transport of auxin-signalling or transport components toward the cell pole (Maisch and Nick, 2007). Despite the fact that this study uses mTalin as actin probe, our own data employing the actin reporter, fABD2-GFP, in *Arabidopsis* support these findings (Zhu and Geisler, unpublished).

In *Arabidopsis*, it was reported that IAA and NAA inhibit root growth primarily through a reduction of the growth zone rather than the maximal rate of elongation and that these auxins do not reduce the rate of cell production (Rahman *et al.*, 2007). In contrast, 2,4-D inhibits root growth primarily by reducing the rate of cell production (Rahman *et al.*, 2007). Correspondingly, IAA (30 nM) and NAA (100 nM) tend to increase actin bundling, while 2,4-D (30 nM) removes actin in roots after a 2-day treatment (see Fig. 1), as imaged in living cells or by chemical fixation and immunocytochemistry (Rahman *et al.*, 2007). However, it was shown that a high concentration of auxin (50 µM, 1 h) unbundled actin filaments in roots in a manner similar to brassinosteroids (Lanza *et al.*, 2012). In contrast, 10 µM of IAA enhances actin bundling in the root of rice after a 6 h treatment (Li *et al.*, 2014).

In addition, it is well known that the tip growth of pollen tubes correlates with the presence of a highly dynamic complex, the cortical actin fringe (Su *et al.*, 2012). Disturbance of intracellular auxin homeostasis, for example through overexpression of *PIN8*, enhances pollen tube elongation by an unknown mechanism (Ding *et al.*, 2012). *pin8* and *pin5* loss-of-function mutants have morphologically defective pollen grains with reduced pollen germination rates; the latter process depends on the turnover of actin organization (Dal Bosco *et al.*, 2012a; Ding *et al.*, 2012; Cao *et al.*, 2013). Interestingly, the *pin5 pin8* double mutant showed a rescue of pollen morphology defects compared to single mutants (Dal Bosco *et al.*, 2012b), suggesting a compensatory role of both carriers during ER auxin transport. It is therefore possible that the change of intercellular auxin levels, maintained by auxin transporters, affects pollen morphology and germination and the polar growth of pollen tubes by modulating actin dynamics.

Together, these findings suggest that auxin modulates actin organization and dynamics by an unclear but highly variable mechanism. However, the limited numbers of often-conflicting results derived from different model plants make it a challenge to understand the physical and functional relation between auxin and the actin cytoskeleton.

Auxin activates the expression of ACTIN-BINDING PROTEINS

Although the role of exogenous auxin in inducing actin re-organization is clearly demonstrated by some reports, the underlying mechanism is still not properly understood. Actin-binding proteins might work as a mediator of auxin action because of their multiple functions in regulation actin organization and dynamics.

Tobacco BY-2 cells are a good cellular model for studying auxin-dependent patterning (Maisch and Nick, 2007). The synchronized cell division in BY-2 cell files is auxin dependent and relies on intact F-actin organization (Maisch and Nick, 2007). In *Nicotiana tabacum* it was found that ACTIN-DEPOLYMERIZING FACTOR 2 (ADF2) is an important player in auxin-induced actin reorganization (Durst *et al.*, 2013). Overexpression of *NtADF2* leads to alteration of synchronized cell division in BY-2 cell files by depolymerizing or fragmenting the cortical actin meshwork. It was also found that

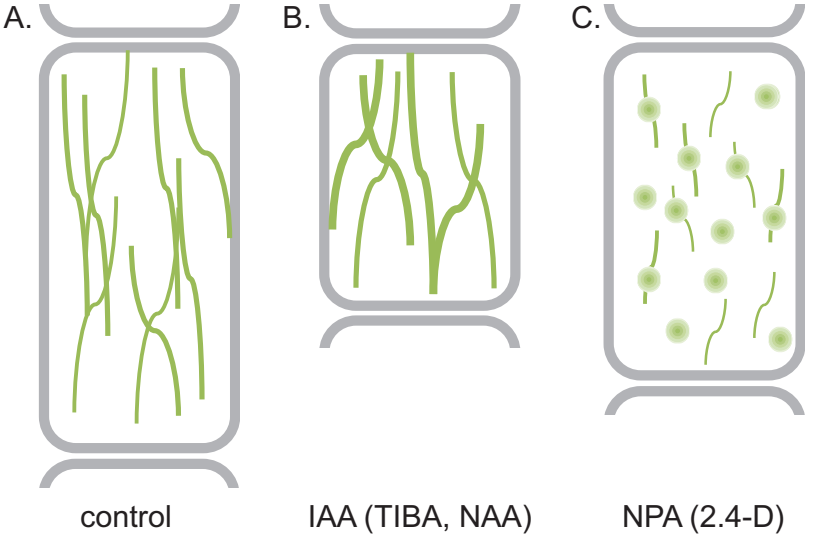

Fig. 1. Effect of auxins and auxin transport inhibitors on actin stability and cell growth in the root elongation zone. IAA (like NAA and TIBA) reduces cell length drastically, which is accompanied with actin filament (green) bundling (B) compared to the solvent control (A). In contrast, NPA (like 2.4-D) decreases cell elongation moderately but leads to partial depolymerization of actin filaments showing often punctuated structures (C). Modified from Rahman *et al.* (2007). (This figure is available in colour at *JXB* online.)

the altered pattern of cell division could be rescued by expressing *PIP2*, an ADF regulator, or by treatment with phalloidin, an actin filament stabilizer, respectively (Durst *et al.*, 2013). Those data indicate that ADF2 could control dynamic cortical actin filaments and play an important role in auxin-dependent cell division synchrony in BY2 (Durst *et al.*, 2013).

In mammalian cells, FYVE domain-containing proteins were identified as one class of formin interactors (reviewed by Cvrckova, 2013). Two of the members, FAB1A and FAB1B, were experimentally characterized as type II phosphatidylinositol 3-phosphate-5-kinases (PIKfyve), a family which functions in regulation of endocytosis and actin dynamics in metazoan cells (Shisheva, 2008). FAB1A/1B proteins participate in endomembrane homeostasis and are possibly involved in auxin transporter recycling (Hirano and Sato, 2011; Hirano *et al.*, 2011). Although these effects may be due to various regulatory effects of PIP2 produced by PIKfyve, it is possible that formins, especially those type II members that bind to PIP2, are involved in auxin signalling on actin.

An exciting example of auxin-formin regulation was recently found in rice: Li *et al.* (2014) reported that RICE MORPHOLOGY DETERMINANT (RMD) link actin reorganization and auxin signalling. *RMD* encodes for type II formin-like protein consisting of two highly conserved formin-homology domains, FH1 and FH2, and an N-terminal PTEN-like domain, which mediates the localization of RMD to the chloroplast envelope (Yang *et al.*, 2011; Zhang *et al.*, 2011). Biochemical *in vitro* analyses indicated that RMD can nucleate actin assembly from free or profiling-bound monomeric actin, and can cap the barbed end of actin filaments (Yang *et al.*, 2011; Zhang *et al.*, 2011). The FH2 domain of RMD bundles actin filaments directly and stabilizes actin filaments but also binds to and bundles microtubules *in vitro* (Yang *et al.*, 2011; Zhang *et al.*, 2011). It was reported that *rmd* mutants have aberrant microfilament and microtubule networks, which results in abnormal cell elongation and altered plant morphology, such as wavy inflorescence in adult plants, dwarfism and enhanced gravitropic responses (Yang *et al.*, 2011; Zhang *et al.*, 2011). The putative role of RMD in auxin signalling was confirmed by the finding that *rmd* mutants have a reduced sensitivity to IAA treatments and a shorter elongation zone due to reduced cell length rather than cell number (Li *et al.*, 2014). This phenotype resembles *Arabidopsis ACTIN* mutant roots (Gilliland *et al.*, 2003; Kandasamy *et al.*, 2009) and wild-type *Arabidopsis* roots treated with IAA (30 nM) or NAA (100 nM) (Rahman *et al.*, 2007). The reduced IAA sensitivity of root growth corresponds to the finding that IAA (10 µM, 6 h) induced F-actin bundling in root cells, which is absent in *rmd* mutants, indicating that RMD is essential for the auxin-mediated reorganization of F-actin arrays (Li *et al.*, 2014). Moreover, the expression of *RMD* can be promoted by additional IAA in a way similar to *ACT7* (Gilliland *et al.*, 2003; Li *et al.*, 2014). The signalling pathway was strengthened by the finding of two functionally redundant AUXIN RESPONSIVE FACTORS (ARFs), OsARF23 and OsARF, which directly regulate *RMD* expression and RMD-dependent actin reorganization (Li *et al.*, 2014). In summary, the work in rice established the presence of a nuclear TIR1/AFB-dependent auxin-signalling pathway that controls F-actin arrays, which is required in turn for auxin transporter recycling. However, there are still some open questions: for example, while the role of NtADF2 in synchrony of cell division was shown, it was not indicated how NtADF2 participates in this process of cell division itself. In addition, the RMD localized to the chloroplast-envelope supports its putative role in the association of chloroplasts with the cytoskeleton (Zhang *et al.*, 2011), but not the regulation of longitudinal bundling of F-actin (Li *et al.*, 2014). And finally, the effects of RMD on microtubules should also be properly considered in the response to the auxin signalling, since microtubules participate in maintaining polarized PINs on the PM (Boutte *et al.*, 2006).

In the future, single-cell systems will be highly useful for studying auxin-modulated actin cytoskeleton behaviour. Those systems, like root hairs, pollen tubes, leaf trichomes or leaf pavement cells, make it easier to quantify actin reorganization and dynamics. Also, the study of auxin signalling in single-cell systems seems more reliable because of the relative simple PAT pattern compared to more complex tissues or organs.

Auxin modulates actin dynamics through the ABP1-ROP-RIC pathway

In recent years, studies on plant small GTPases have elucidated a rapid regulatory pathway from auxin to cortical cytoskeletal dynamics, which is thought to be mediated by the membrane-associated auxin receptor, ABP1 (Ren and Lin, 2015). By binding to ABP1 on the cell surface, auxin promotes the activity of plant ROP (Rho guanidine triphosphate hydrolases of plants), a family of Rho-like GTPases, and their associated RICs (ROP-interactive CRIB motif-containing proteins), which control actin assembly by targeting the downstream Arp2/3 complex (Ren and Lin, 2015). In *Arabidopsis*, the ABP1-ROP-RIC signalling pathway was reported to be non-transcriptional and self-organized not only in leaf pavement but also in root cells (Basu *et al.*, 2008; Xu *et al.*, 2010; Chen *et al.*, 2012; Lin *et al.*, 2012; Nagawa *et al.*, 2012).

In leaf pavement cells, auxin treatment was shown to determine the formation of a jigsaw puzzle-like shape by activating the two exclusive ROP2-RIC4 and ROP6-RIC1 pathways (Xu *et al.*, 2010). Auxin-activated ROP2-RIC4 interaction inhibits the clathrin-dependent endocytosis of PIN1 by inducing cortical fine F-actin polymerization (Nagawa *et al.*, 2012). The RIC4-dependent assembly of cortical F-actin contributes to the lobe outgrowth of pavement cells. Meanwhile, in the adjacent cell, auxin-activated ROP6 promotes RIC1-dependent microtubule ordering for outgrowth suppression (Xu *et al.*, 2010).

In roots, ROP-RIC-mediated actin stabilization results in the inhibition of PIN2 endocytosis, and these results are consistent with data for pavement cells (Chen *et al.*, 2012; Lin *et al.*, 2012). This is further supported by the finding that treatment with the actin depolymerization drug, latrunculin B, induces more PIN2 internalization, but that stabilization of actin via ROP activity reduces PIN2 internalization (Lin *et al.*, 2012).

An important progress was the identification of SPK1, belonging to the DHR2-Dock family of ROP guanine nucleotide exchange factors that function upstream in activating ROP6 (Lin *et al.*, 2012). Before this finding, it was already reported that SPK1 controls auxin-dependent cell morphogenesis through the ARP2/3 complex (Szymanski, 2005; Basu *et al.*, 2008; Xu *et al.*, 2014). However, it is still unclear how SPK1 is activated or how the auxin signal is passed from ABP1 to SPK1. A recent paper reported that ABP1 interacts with the PM-localized, receptor-like kinases of the TMK1 family in the regulation of ROP proteins and thus influences downstream, non-transcriptional cytoskeletal organization (Chen and Yang, 2014; Xu *et al.*, 2014).

ABP1-mediated auxin activation of the ROP-RIC-actin pathways is seen as rapid auxin signal transduction processes that have been thought to function primarily as pre-transcriptional events independent of AUX/IAA-SCF$^{TIR1/AFB}$ signalling. In pavement cells, it was found that the activation of ROP by ABP1 occurs within minutes, and that auxin activates ROP2 and ROP6 in a dosage-dependent manner (Xu *et al.*, 2014). However, the time frame from auxin application to the observed actin reconfiguration via the ROP-RIC-actin signalling pathway still needs to be determined.

Another question concerns whether the rapid auxin signalling mechanism at the cell surface is essential for cell-growth required actin remodelling. The dynamics of cortical actin are important for membrane-related signalling responses, including endocytotic processes. But, apparently this is not the whole story of actin dynamics in plant cells and it does not mean that cortical actin regulation is independent of auxin-induced transcriptional events. However, by studying the rapid auxin-induced cell expansion and gene expression in a multiple SCF$^{TIR1/AFB}$ loss-of-function mutant it was shown that gene expression, mediated by SCF$^{TIR1/AFB}$ receptors, most likely does not play a major role in triggering the very rapid phase of cell elongation (Schenck *et al.*, 2010).

In this respect, it is of interest that a recent study indicated that ABP1 functions as a negative regulator of the SCF$^{TIR1/AFB}$ pathway (Tromas *et al.*, 2013). ABP1 is thought to counteract the phenotypes caused by loss-of-*TIR1* function. In summary these results indicate that ABP1 at the cell surface might influence the sensitivity of nuclear SCF$^{TIR1/AFB}$ and therefore that both receptors are able to co-contribute to auxin-mediated transcriptional auxin responses (Tromas *et al.*, 2013; Grones and Friml, 2015), such as actin organization and dynamics.

Auxin transport inhibitors bind to and inhibit auxin exporters

Our knowledge on the mechanisms of PAT was widened by the use of synthetic auxin transport inhibitors (ATIs), such as 1-N-naphtylphtalamic acid (NPA), a non-competitive auxin-efflux inhibitor (Butler *et al.*, 1998a; Cox and Muday, 1994). At low concentrations (1–5 µM), NPA efficiently inhibits the basal polar auxin flow required for plant development, while at high (>50 µM) concentrations, NPA seems to have an effect on trafficking components (Geldner *et al.*, 2001; Gil *et al.*, 2001; Peer *et al.*, 2009).

Before the identifications of auxin transporters, most studies on auxin transport focused on the identification of NPA binding-proteins (NBPs). However, the exact number and the identity of the NBP is surprisingly still controversial (Luschnig, 2001). Using biochemical *in vitro* assays, NBPs were reported to be PM-associated and NPA-binding activity was localized to the cytoplasmic face of the membrane (Cox and Muday, 1994).

Auxin exporters, ABCB1 and ABCB19, and the FKBP42, TWISTED DWARF1 (TWD1; originally misannotated as cyclophilin5), have been identified together with M1 aminopeptidase (APM1) in so-called 'high' affinity fractions of an

NPA chromatography (see Fig. 2), where NPA was immobilized via a spacer allowing efficient binding (Murphy *et al.*, 2002; Geisler *et al.*, 2003). Obviously, affinities of immobilized NPA might differ from unbound NPA. Moreover, these kinds of experiments do not directly indicate that both pulled-down components, ABCBs and TWD1, are both high-affinity components because TWD1 and ABCB1/19 were found to interact (Geisler *et al.*, 2003; Bouchard *et al.*, 2006). However, independently NPA-binding to ABCBs and TWD1 was verified biochemically (Geisler *et al.*, 2005; Rojas-Pierce *et al.*, 2007; Nagashima *et al.*, 2008). In agreement, micromolar NPA concentrations cause inhibition of auxin efflux catalysed by ABCB1 and ABCB19 (Geisler *et al.*, 2005; Bouchard *et al.*, 2006; Bailly *et al.*, 2008), while similar NPA concentrations obviously have no significant direct effect on the activity of members of the PIN family (Rojas-Pierce *et al.*, 2007; Kim *et al.*, 2010). Moreover, all trials to demonstrate NPA-binding to PINs have failed so far (Geisler, 2014).

APM1 is so far the only NBP for which NPA binding affinities have been estimated. Affinities 3–50 µM (Peer *et al.*, 2009) qualify APM1 as a medium-affinity NBP. Interestingly, co-expression of ABCB1 with its PM chaperon, TWD1, in yeast reduced NPA binding to TWD1 levels (Bailly *et al.*, 2008), suggesting that NPA-binding affinities are interdependent. This was supported by the finding that ABCBs were found to be ER delocalized and degraded in *twd1* (Wu *et al.*, 2010; Wang *et al.*, 2013). Further sustenance came from the result that interaction of ABCB1 with TWD1 is disrupted by nM NPA concentrations resulting in loss-of ABCB1 activation (Bailly *et al.*, 2008). Individual binding affinities for ABCBs and TWD1 have not been determined. However, identification of two different NPA-binding affinities on plasma-membrane located TWD1 and ABCBs are in agreement with initial proposals of the transporter itself and the NPA-binding regulatory subunit (Michalke *et al.*, 1992).

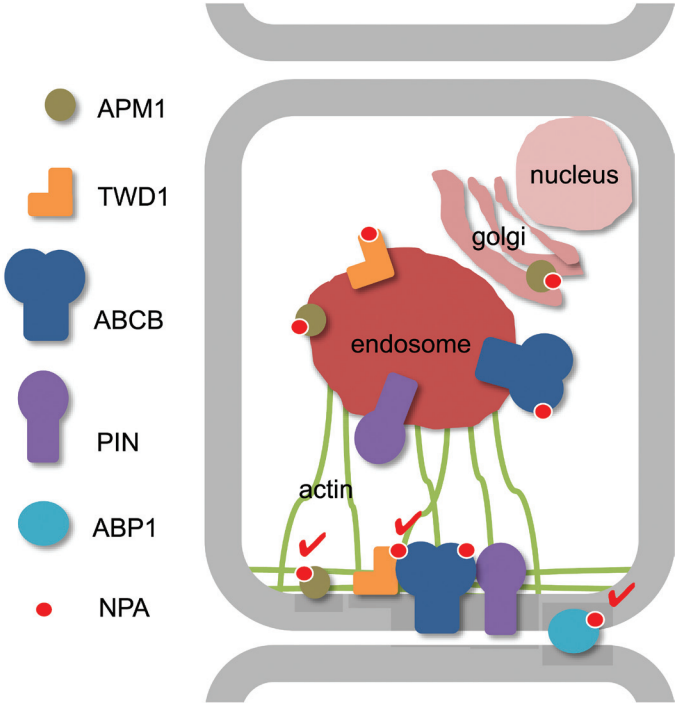

Fig. 2. Location and roles of verified NPA targets. Vesicle-dependent transport of components of the auxin efflux complex, such as the FKBP42/TWISTED DWARF1 (TWD1)/UCU2 and members of the PIN and ABCB-type auxin efflux transporters, is provided by actin filaments (green). Locations of experimentally verified NPA-binding proteins, such as TWD1, ABCBs, APM1 and ABP1, are indicated; note that NPA sensitivities of PINs and TIR3/BIG await conformation and might be of indirect nature. Proven or very likely direct regulatory involvements of NPA targets, such as for APM1 (Peer *et al.*, 2009), TWD1 (Wu *et al.*, 2010; Wang *et al.*, 2013) and ABP1 (Robert *et al.*, 2010) in transporter targeting are marked by ticks. (This figure is available in colour at *JXB* online.)

Auxin transport inihibitor binding activity is associated with actin stability and reorganization of the actin cytoskeleton

Although NPA-binding activity can be released from the plasma membrane by detergent treatment, the yield of the material released is very low compared to the amount of the activity in the ultracentrifugation pellet of detergent-extracted membrane (Butler *et al.*, 1998b; Muday *et al.*, 2000). In addition, both the amount of NPA-binding activity and pelletable actin decreased with the treatment of cytochalasin D, but increased by the treatment of phalloidin, which can fragment F-actin and stabilize F-actin, respectively (Butler *et al.*, 1998b). In contrast, the microtubule stabilizer, taxol, resulted in an increase in pelletable tubulin but caused no detectable change in NPA-binding activity (Butler *et al.*, 1998b). Furthermore, the interaction between NBP and purified actin filaments was demonstrated by selective elution of NPA-binding activity from the F-actin column (Cande *et al.*, 1973; Hu *et al.*, 2000). Together, those reports indicate the association of NBPs with the actin cytoskeleton.

It was hypothesized that the interaction of the NPA-binding protein with the actin cytoskeleton may be necessary for either the movement of auxin across the membrane or for the polar localization of the efflux carrier complex (Muday, 2000). This hypothesis was sustained by the finding that cytochalasin treatments reduced auxin transport both in corn coleoptiles (Cande *et al.*, 1973; Hu *et al.*, 2000) and zucchini hypocotyls (Butler *et al.*, 1998b). Importantly, auxin transport in zucchini hypocotyls treated by cytochalasin D is less sensitive to NPA (Butler *et al.*, 1998b), which is in line with the finding that actin bundling (induced by overexpression of mouse talin) enhances the sensitivity toward NPA (Maisch and Nick, 2007; Higaki *et al.*, 2010a, b).

In contrast to the relatively convincing effects of actin drugs on NPA-binding activity, the effects of NPA on actin reorganization remain largely exclusive. In the brown alga, *Fucus distichus*, it was reported that NPA (50 µM, 1 h) reduced the polar localization of actin patches in polarized embryos in response to gravity and light (Sun *et al.*, 2004). However, the effect of NPA was thought to be indirect because the same effect was observed with IAA treatment (50 µM, 1 h; Sun *et al.*, 2013). On the other hand, in *Arabidopsis* it was described that a 2-day treatment with NPA (10 µM) and 2,4-D (30 nM) inhibits root growth primarily by reducing cell production rate, while IAA (30 nM), NAA (100 nM) and TIBA (40 µM) inhibit root growth primarily through reducing the length of cells in growth zone

(Rahman *et al.*, 2007). Further observations implied that the different effects of those compounds on the root growth might be attributed to their different actions on the actin cytoskeleton. By actin imaging using fABD2-GFP in living cells or anti-actin antibodies in fixed cells, it was shown that NPA (10 μM) as well as 2,4-D (30 nM) reduced filamentous actin and generated punctuated structures in epidermal cells, while IAA (30 nM), NAA (100 nM) and TIBA (40 μM) tended to bundle actin filaments (Rahman *et al.*, 2007) (see Fig. 1). Furthermore, neither the auxin-efflux mutant *pin2/eir1-1* nor the auxin-influx mutant *aux1-7* could resemble the effects of NPA and 2,4-D on actin organization, suggesting that their effects are independent of PIN or AUX1-mediated auxin transport (Rahman *et al.*, 2007). 2,4-D is thought to act differently from IAA in plants (Rahman *et al.*, 2006), and very recently it was reported that 2,4-D might affect the actin cytoskeleton by inducing oxidative and S-nitrosylated modifications on the actin, and disturbing the polymerization of actin (Rodriguez-Serrano *et al.*, 2014). Although the effect of NPA on actin is resembled by 2,4-D in the *Arabidopsis* root (Fig. 1), the mechanism behind NPA action is still unclear. Dhonukshe *et al.* (2008) claimed that NPA does not affect actin stability by short-term treatment, which is in line with data using cultured tobacco cells (Petrasek *et al.*, 2003). However, these findings are in conflict with data showing that 200 μM NPA reduced PIN1 cycling to a similar magnitude caused by 25 μM TIBA (Geldner *et al.*, 2001). This concentration is about two orders of magnitude higher than the concentration of NPA required to saturate inhibition of auxin efflux. Moreover, they are close to the NPA solubility limits of around 280 μM (Peer *et al.*, 2009); therefore, the relevance of these findings (Geldner *et al.*, 2001) was criticized (Petrasek *et al.*, 2003).

In roots, NPA removes filamentous actin and generates punctuated actin (Fig. 1). This action might be indirect by changing cellular IAA concentrations. Although it is still not clear whether cellular auxin levels in epidermal cells of the root elongation zone are enhanced, several evidences support it. For example, it was reported that low concentrations of NPA could inhibit shootward auxin fluxes in roots (Rashotte *et al.*, 2000), suggesting higher auxin concentrations in epidermal cells of the elongation zone. In addition, the effect of NPA treatment (10 μM, 2 d) in reducing filamentous actin (Rahman *et al.*, 2007) can be somehow copied by high concentrations of IAA (50 μM, 1 h), which results in shorter actin strands (Lanza *et al.*, 2012). In contrast, in epidermal cells of etiolated hypocotyls, NPA and IAA treatments result in opposite effects on actin organization (Rahman *et al.*, 2007; Zhu and Geisler, unpublished). Those results further support the hypothesis that NPA interferes with actin through an IAA-induced unbundling/severing complex.

Actin stability affects auxin transporter endocytosis and recycling by regulating vesicular trafficking

PINs

PIN trafficking is actin-dependent and mediated by BFA (brefeldin A)-sensitive GNOM action and clathrin-dependent endocytosis, which was found to be regulated by ROP GTPases (Robert *et al.*, 2010; Chen *et al.*, 2012; Kania *et al.*, 2014; Xu *et al.*, 2014). Studies of the PIN recycling mechanisms have redefined the models of plant membrane protein trafficking through endosomal compartments associated with the trans-Golgi network (Murphy and Peer, 2012; Grones and Friml, 2015). The actin cytoskeleton is involved not only in recycling/exocytosis processes, but also in endocytosis/internalization processes of PINs. As a consequence, interference with actin organization or dynamics either by chemical treatments with actin drugs, by plant hormones or ATIs, or by genetic mutations (including actin and actin regulator mutations) do have an impact on PIN trafficking.

Both cytochalasin D and latrunculin B (20 μM) inhibit BFA-induced intracellular PIN1 accumulation as well as its relocalization to the PM when BFA was washed out (Geldner *et al.*, 2001). Similarly, BFA-related effects were observed with TIBA (50 μM), which could stabilize and bundle actin filaments (Geldner *et al.*, 2001; Dhonukshe *et al.*, 2008; Higaki *et al.*, 2010b). As mentioned above, the concentration of NPA (200 μM), which was necessary to reduce PIN1 cycling to a similar magnitude as TIBA (25 μM), was higher and even much higher than the concentration of NPA required inhibiting auxin transport (Geldner *et al.*, 2001; Petrasek *et al.*, 2003). Moreover, it is important to recall that ATIs treatment in the absence of BFA had no significant effect on PIN1 locations (Geldner *et al.*, 2001; Muday and Murphy, 2002). However, the NBP was proposed to provide a bridge between efflux carriers, such as PIN1, and the actin network used to transport and/or localize these complexes (Muday and Murphy, 2002).

Although the maintenance of PINs on the PM is only partially dependent on actin, it is still someway affected by specific actin drug treatment in a cell-type dependent manner (Geldner *et al.*, 2001; Boutte *et al.*, 2006; Rahman *et al.*, 2007; Kleine-Vehn *et al.*, 2008). Lower concentrations of latrunculin B (17 nM, 2 d), which could reduce 50% of root elongation, did not alter polarized PIN localization but caused PIN2 to accumulate in bodies of unknown identity in the epidermis (Rahman *et al.*, 2007). Mislocalization of PIN2 in the root epidermis was also observed with 20 μM latrunculin B for 2 h (Kleine-Vehn *et al.*, 2008). In addition, apical localized PINs in the epidermis were more sensitive to disruption of actin filaments (Kleine-Vehn *et al.*, 2008). Furthermore, short-term treatments with cytochalasin D caused rapid internalization of PIN3, expressed in root caps and relocalized rapidly during gravitropism (Friml *et al.*, 2002). Some recently published genetic analyses further support the role of actin in PIN trafficking: a mutant allele of *ACT2* was shown to possess lower bundling of actin filaments and thus mislocalized PIN2 in *Arabidopsis* roots by an unclear mechanism (Lanza *et al.*, 2012). In addition, it was reported that in rice, OsARF-RMD-mediated actin dynamics play an essential role in regulating polarized OsPIN localization as well as regulating OsPIN vesicle trafficking (Li *et al.*, 2014). *rmd* was shown to promote endocytosis of FM4-64-labelled PM vesicles, but to inhibit FM4-64-labelled BFA compartment wash-out, which was proposed as a result of its disrupted actin organization (Li *et al.*, 2014). However, the mechanism,

by which actin itself modulates plant membrane protein trafficking, still needs further study.

AUX1/LAXs

Although it was suggested that the subcellular trafficking of AUX1 is GNOM-independent, intracellular AUX1 dynamics and its polar localization have the strict requirement of an intact actin cytoskeleton (Geldner *et al.*, 2001; Kleine-Vehn *et al.*, 2006). It was shown that latrunculin B (30 µM, 2 h), but not oryzalin, led to intracellular agglomeration of AUX1 and affected the polarity of AUX1 localization in the protophloem (Kleine-Vehn *et al.*, 2006). Furthermore, the targeting of AUX1 was found to be more sensitive to latrunculin B than that of PIN1 (Kleine-Vehn *et al.*, 2006). Correspondingly, it was suggested that overexpression of the *Oryza* ARF-GTPase-activating protein, *OsAGAP*, could promote the delivery of AUX1 early endosomes from the PM to the cytoplasm by reducing the thickness and bundling of actin filaments (Du and Chong, 2011; Du *et al.*, 2011).

ATIs, such as TIBA and PBA, were shown to inhibit trafficking and mobility of AUX1 (Geldner *et al.*, 2001; Kleine-Vehn *et al.*, 2006; Dhonukshe *et al.*, 2008). TIBA (50 µM, 30 min), but not NPA, was shown to inhibit the BFA-induced (50 µM, 2 h) aggregation of AUX1 in the protophloem (Kleine-Vehn *et al.*, 2006). Further, the recovery of AUX1 from BFA compartments was completely inhibited when BFA was washed out in the presence of TIBA or PBA (Kleine-Vehn *et al.*, 2006). In addition, the direct effect of TIBA (50 µM) and PBA (10 µM) on AUX1 trafficking was confirmed by their ability to inhibit AUX1 dynamics within minutes. In contrast, although NPA (50 µM, 3 h) was shown to disrupt the polar localization of AUX1, it was considered to exert its role indirectly as an auxin-efflux inhibitor (Kleine-Vehn *et al.*, 2006). Similarly, NPA treatment was considered as an indirect auxin treatment in the experiment although auxin was not shown affect AUX1 dynamics (Kleine-Vehn *et al.*, 2006). Moreover, the possible role of NPA in inhibiting BFA-induced accumulation, as well as in the washout recovery of AUX1 has not yet been shown.

ABCBs

Compared to PIN proteins, ABCB proteins are localized to the PM in a widely non-polar manner (Geisler *et al.*, 2005; Titapiwatanakun and Murphy, 2009; Cho and Cho, 2012; Kania *et al.*, 2014). The trafficking of ABCBs is less investigated but was supposed to be regulated by differential mechanisms and to be less dynamic compared to the trafficking of PINs (Titapiwatanakun *et al.*, 2009; Cho *et al.*, 2012). In contrast to PINs, the trafficking of ABCB19 was shown to be insensitive to short-term treatments with BFA (50 µM, 30 min; Titapiwatanakun *et al.*, 2009), while other reports (Wu *et al.*, 2010) indicated that long-term BFA treatment (20 µM, 1.5 h) and even shorter-term BFA treatment (50 µM, 45 min) still induce ABCB19 aggregation in BFA compartments (Wang *et al.*, 2013). In contrast to ABCB19, ABCB1 was suggested to be less stable and more readily endocytosed since it aggregated in compartments with short-term (50 µM, 30 or 45 min) BFA treatment (Titapiwatanakun *et al.*, 2009; Wang *et al.*, 2013).

ABCB4 cycling between PM and endosomes seems to be less dynamic compared to other ABCBs because high concentration BFA treatments (50 µM, 2 h; 20 µM, 1.5 h) are needed to induce ABCB4 aggregation in BFA compartments (Cho *et al.*, 2007; Wu *et al.*, 2010). However, it was suggested that BFA-sensitivity of ABCB4 trafficking requires not only GNOM but also other BFA-sensitive ARF-GEFs (Cho *et al.*, 2007; Cho *et al.*, 2012). The trafficking of ABCB4 is actin-dependent because BFA-induced ABCB4 aggregation (50 µM, 45 min) is considerably inhibited by the treatment of cytochalasin D (30 µM; Cho *et al.*, 2012). Although prolonged cytochalasin D treatment could induce intracellular ABCB4 aggregation, the same treatment (30 µM, 2 h) inducing mislocalization of PIN1 and PIN2 could not affect the localization of ABCB4 (Cho *et al.*, 2012). This difference might be attributed to the less dynamic nature of ABCB4 trafficking. In addition, not only TIBA (50 µM) but also NAA (5 µM) could inhibit BFA-induced (50 µM) ABCB4 aggregation in compartments (Cho *et al.*, 2007, 2012).

In recent years, more and more studies reported that plant ROP GTPases play critical roles in regulating actin-dependent endocytosis of PM proteins. In mammalian cells, Rab proteins have been reported to be involved in the trafficking of human ABCB proteins (Fu *et al.*, 2007). Therefore it will be interesting to study the possible role of plant ROP GTPases in regulating actin-dependent trafficking of plant ABCB proteins.

Interestingly, it was shown that ABCB19 could stabilize PIN1 on the PM and that ABCB19 is stably associated with BIG/TIR3-containing membrane fractions (Titapiwatanakun *et al.*, 2009). BIG/TIR3 is an orthologue of the *Drosophila* calossin and *tir3* (*transport inhibitor response 3*) mutants were isolated in a screen for resistance to the inhibitory effects of NPA on root elongation (Ruegger *et al.*, 1997). *big/tir3* mutants have, beside being resistant to NPA, reduced PAT as well as reduced NPA binding sites and are hypersensitive to NPA with respect to PIN1 localization (Gil *et al.*, 2001). These results suggest that TIR3 either encodes an NBP or is required for their expression, localization, or stabilization (Ruegger *et al.*, 1997; Gil *et al.*, 2001).

Conclusions

NPA targets and mechanisms are still unclear

For a long time it has been accepted that ATIs, such as NPA, inhibit PAT at the export level (Muday *et al.*, 1993; Cox and Muday, 1994; Bernasconi *et al.*, 1996; Jensen *et al.*, 1998; Murphy *et al.*, 2002). Likewise, they are used in probably all labs that work on auxin transport or auxin-controlled development. Therefore, it is all the more remarkable that the individual number and the identity of NBPs, as well as their membrane association, is still under discussion (Luschnig, 2002; Muday and Murphy, 2002). As a consequence, molecular mechanisms and downstream targets of NPA action

are obviously also unclear. Biochemical work has suggested the existence of at least two NPA-binding sites with different binding affinities, low- and high-affinity binding sites (Michalke *et al.*, 1992). Despite the fact that these findings might be species and tissue-dependent, the affinities might be provided by ABCBs and TWD1 (Fig. 2), both shown to bind NPA (Bouchard *et al.*, 2006; Bailly *et al.*, 2008; Kim *et al.*, 2010). However, until now, individual binding affinities have not been quantified. But both actions make sense from a transport point of view because ABCB inhibition by NPA and disruption of TWD1-ABCB1 interaction, leading to block of ABCB1 transport, have been demonstrated (Geisler *et al.*, 2005; Bouchard *et al.*, 2006; Bailly *et al.*, 2008; Kim *et al.*, 2010). Physical interaction of ABCBs and TWD1, and demonstrated interdependence of NPA binding capacities (Bailly *et al.*, 2008) on the PM also provide a good explanation of why some groups found only one binding affinity (Muday *et al.*, 1993). The physiological relevance and the advantage of having two NPA-binding affinities remain unclear. Finally, one should not forget that all the ATIs used are synthetic and that plant-endogenous ATIs are still largely unknown (Peer and Murphy, 2007; Geisler *et al.*, 2014).

More importantly, NPA (and auxins) affect actin bundling (Fig. 1), which has a striking impact on PAT. While it seems clear that the effect of ATIs on F-actin is indirect (Dhonukshe *et al.*, 2008), it requires the action of an integrating protein, the identity of which is unknown. Further, it is unclear if both actions of ATIs on actin and auxin transporters, respectively, are independent or connected.

In our eyes there are two likely scenarios on the action of NPA on actin and PAT. In scenario 1, low concentrations of NPA would bind to two NBPs with different affinities for NPA that both function in PAT directly; these could be auxin transporters and regulatory proteins, such as ABCBs and TWD1. The effect of high concentrations (>50 µM) of ATIs on actin would thus be unspecific. In scenario 2, NPA would bind to one NBP formed by components of the auxin-exporter complex (built for example by ABCB/TWD1) that might share NPA binding. The latter idea is supported by the finding that interacting surfaces on ABCBs and TWD1 (NBDs of ABCBs and the FKBD of TWD1) are thought to build domains of NPA binding (Bouchard *et al.*, 2006; Bailly *et al.*, 2008; Kim *et al.*, 2010). A second PM NBP would thus integrate ATI action on the actin cytoskeleton. This NBP could be an actin-binding protein itself or, more likely, interact with an ABP conferring the final effect of NPA on actin organization.

Organ-dependent expression or activation of a putative auxin-actin integrator

Although it is still possible that NPA affects actin organization indirectly by disturbing IAA accumulation (Rashotte *et al.*, 2000), there are several reports supporting the idea that NPA affects actin in a way distinct from IAA (Rahman *et al.*, 2007; Dhonukshe *et al.*, 2008). This would suggest the existence of integrating auxin/ ATI-binding proteins, which we will call *auxin-actin integrators* (see Fig. 3). In the simplest model, there would be two integrators, one for ATIs and one for auxins that have opposite effects on actin organization (Fig. 3A). An alternative model could involve both ATIs and auxin bound to the same integrator but to different binding sides, each with opposite read-outs. Finally, both ATIs and auxins bind competitively to the same integrator pocket with different affinities and thus opposite read-outs (Fig. 3A).

In principle, ABP1 is a very good candidate for such a proposed auxin-actin integrator because ABP1 was shown to alter actin organization via the ROP-RIC pathway (Chen *et al.*, 2012). Auxin sensing seems to occur by TMK receptor-like kinases interacting with ABP1, forming a cell-surface auxin-perception complex that activates downstream ROP-RIC signalling pathways (Xu *et al.*, 2014). Interestingly, besides auxins, ABP1 also seems to bind NPA: NPA was reported to inhibit IAA-binding and has a k_D for NPA ~1000-fold higher than for that for NAA (Shimomura *et al.*, 1986). Based on competition with a monoclonal antibody, NPA (and TIBA) is thought to bind to ABP1 thereby inducing a conformational change (Venis and Napier, 1990).

In rice it was shown that IAA induces actin bundling in roots but actin unbundling in shoots at high (µM) concentrations (Nick *et al.*, 2009; Li *et al.*, 2014). A similar effect was also observed in *Arabidopsis* roots but at lower IAA concentrations (Rahman *et al.*, 2007). At very high concentration (50 µM) however, IAA can induce actin unbundling in 1 h in *Arabidopsis* roots (Lanza *et al.*, 2012). Work from multiple labs suggests that auxin-induced cell elongation is accompanied by debundling of actin filaments in the shoot, while auxin-inhibited cell elongation in root cells is associated with enhanced F-actin bundling, emphasizing the importance of actin filament bundling in auxin-controlled cell growth (Holweg *et al.*, 2004; Nick, 2010; Rahman *et al.*, 2007; Li *et al.*, 2014).

This obvious root-shoot enigma might be explained by root- or shoot-specific expression of proposed auxin-actin integrators causing opposite downstream effects on actin stability and dynamics (Fig. 3B). Alternatively, one auxin-actin integrator could regulate root-shoot specific downstream pathways leading to opposite read-outs on actin, which would be oppositely regulated in an organ-specific manner by a second signalling molecule, which we call here a modulator (Fig. 3B).

A good candidate for such a modulatory input might be intracellular calcium. It has been shown that application of auxin can induce a detectable increase in calcium concentrations (Ayling *et al.*, 1994; Monshausen *et al.*, 2011). Further, it was reported that *Arabidopsis* VILLIN3 not only bundles actin filaments in a calcium-independent manner, but also severs actin filaments and bundles when intracellular calcium is elevated to micromolar levels (Tominaga *et al.*, 1998). Thus, we hypothesize that auxin/ATIs modulate actin organization in a concentration-dependent manner and that species-/organ-/tissue-dependent variable conditions are responsible for a functional change of a putative auxin-actin integrator.

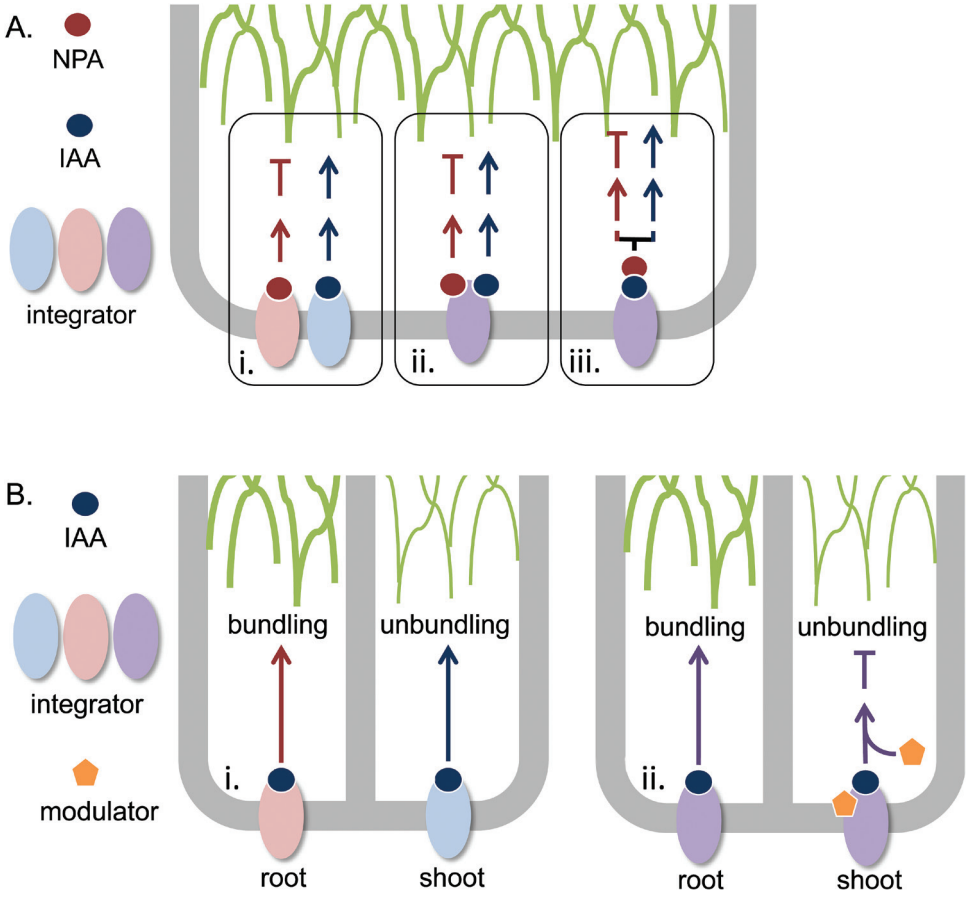

Fig. 3. Action of a putative auxin-actin integrator. (A) Hypothetic working model on a putative auxin-actin integrator. An auxin-actin integrator is thought to provide integration of downstream signalling actions of auxins (here: IAA) and auxin transport inhibitors (here: NPA) on actin cytoskeleton bundling and dynamics. In the simplest case, IAA and NPA employ independent integrators that are specific for them, respectively, leading to inverse outputs (i). IAA and NPA might also only utilize one integrator with distinct binding sites that each have opposite outputs (ii). Alternatively, IAA (and NPA) might influence NPA- (and IAA-) binding and/ or action allosterically. Finally, IAA and NPA might also compete for a single binding site on the integrator, where binding of NPA might block IAA action (iii). Note that the auxin-actin integrator is not essentially a transmembrane protein. (B) Tissue-specific action of a putative auxin-actin integrator. In the simplest case, two oppositely acting auxin-actin integrators providing opposite regulatory outputs on the actin cytoskeleton (here: bundling) are expressed in a tissue (here: root-shoot) specific manner (i). Alternatively, one integrator is expressed throughout the plant body but oppositely regulated by an unknown modulator that either interferes with the integrator directly (allosterically) or with down-stream components (ii). Note that for simplicity, only the action of auxins (IAA) but not of auxin transport inhibitors is depicted. (This figure is available in colour at *JXB* online.)

Guideline suggestions

The current findings suggest that auxin-actin regulation is species and organ-dependent, which has made a comparison of individual studies very difficult. Moreover, this process is dose- and time-dependent: the concentrations of exogenous auxin/ATI vary from nM to mM and treatment times differ from <1 h to days (Maisch and Nick, 2007; Rahman *et al.*, 2007; Nick *et al.*, 2009; Lanza *et al.*, 2012; Li *et al.*, 2014). Furthermore, long-time auxin treatment might influence actin organization in a different manner compared to short-time treatments because of transcriptional regulation of auxin on growth-related genes, including actin (such as *ACT7*) and actin-binding proteins.

Although the lack of experimental standards is not a new problem, we suggest the community adhere to the following simple experimental guidelines in the future: (i) Plant growth condition as well as style (type of application, solvents etc.), concentration and duration of auxin/ATI applications need to be clearly indicated throughout the publication (not only in the Methods). (ii) Data for short (1–3 h) *and* long-term application (12–24 h) should be provided and clearly indicated. (iii) Auxin and ATI concentrations should be chosen to match a physiological context. For auxin we suggest low nM concentrations (1–10 nM), while for ATIs (such as NPA) we recommend using multiple, defined concentrations, such as 5 µM and 50 µM, covering described relevant NPA sensitivities. Following these guidelines will allow for a better reproducibility and cross-comparison of individual studies, which will push forward this exciting field.

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