Small molecules unravel complex interplay between auxin biology and endomembrane trafficking

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Abstract

The establishment and maintenance of controlled auxin gradients within plant tissues are essential for a multitude of developmental processes. Auxin gradient formation is co-ordinated via local biosynthesis and transport. Cell to cell auxin transport is facilitated and precisely regulated by complex endomembrane trafficking mechanisms that target auxin carrier proteins to their final destinations. In turn, auxin and cross-talk with other phytohormones regulate the endomembrane trafficking of auxin carriers. Dissecting such rapid and complicated processes is challenging for classical genetic experiments due to trafficking pathway diversity, gene functional redundancy, and lethality in loss-of-function mutants. Many of these difficulties can be bypassed via the use of small molecules to modify or disrupt the function or localization of proteins. Here, we will review examples of the knowledge acquired by the use of such chemical tools in this field, outlining the advantages afforded by chemical biology approaches.

Key words: Auxin carriers, auxin gradients, auxin transport, chemical biology, endomembrane trafficking, phytohormones.

Introduction

Chemical biology is a powerful strategy, being employed ever more frequently, in which small molecules targeting specific functions are used as probes to dissect biological processes. This highly effective approach provides many advantages, especially for investigating complex and essential plant functions, in which lethality and redundancy problems may arise through the use of classical genetics. Chemical biology can often circumvent these problems, as well as allowing a high level of temporal, tunable, reversible control over the targets. Indeed, chemical biology has played a significant role in unravelling the complicated trafficking pathways operating in plant cells, and the list of useful chemical probes for this purpose is ever growing (Hicks and Raikhel, 2010; Mishev et al., 2013; Haeger and Robert, 2014).

The discovery and use of small molecules that selectively target and perturb specific proteins have enabled great advances in the field of hormone biology in general (Rigal et al., 2014), especially with regard to auxin (indole-3-acetic acid; IAA), including its biosynthesis, transport, and signalling (Hayashi and Overvoorde, 2014; Ma and Robert, 2014).
Within plant tissues, tightly regulated local biosynthesis, metabolism, and transport of auxin control the establishment and maintenance of differential auxin distributions or gradients, which are involved in many aspects of plant growth and development (Vanneste and Friml, 2009; Peer et al., 2011).

Directed auxin transport itself relies heavily on tightly controlled endomembrane trafficking pathways that regulate the localization and abundance of auxin carrier proteins (Kleine-Vehn and Friml, 2008; Grunewald and Friml, 2010). Once newly synthesized, these transmembrane proteins are sorted in the endoplasmic reticulum (ER), after which they must reach their cellular destinations via tightly regulated endomembrane trafficking of intracellular endosome compartments. Those proteins destined for the plasma membrane are transported there via early pre-Golgi and late post-Golgi secretory pathways (Luschnig and Vert, 2014). Plasma membrane-resident auxin carriers are frequently dynamically endocytosed and either recycled back to the plasma membrane, transcytosed to another plasma membrane, or sorted to a vacuolar-targeted pathway for degradation (Kleine-Vehn and Friml, 2008; Luschnig and Vert, 2014). The trans-Golgi network (TGN) functions as early endosomes (Dettmer et al., 2006), transporting auxin carriers between the Golgi and plasma membrane (Grunewald and Friml, 2010) and acting as important sorting stations and crossroads between these various trafficking routes (Gendre et al., 2014). Pre-vacuolar compartments (PVCs), otherwise known as multivesicular bodies (MVBs), function as late endosomes, transporting auxin carriers to the vacuole for degradation (Kleine-Vehn et al., 2008c).

In particular, polar positioning of plasma membrane-localized PIN-FORMED (PIN) auxin efflux carriers within individual cells directs polar cell to cell auxin transport (Wiśniewska et al., 2006). For example, PIN1 is localized basally (rootward) in vascular cells of the Arabidopsis root (Friml et al., 2002a), where auxin flow is directed downward. However, in epidermal root cells, PIN2 is localized apically (shootward) (Müller et al., 1998) and here auxin flow is directed upward. Auxin influx carriers may also be polarly localized, including to some extent ATP-BINDING CASSETTE SUBFAMILY B (ABCB) members (Geisler et al., 2005; Blakeslee et al., 2007) but especially AUXIN RESISTANT 1 (AUX1), which is enriched at both the apical and basal ends of epidermal root cells and is localized apically in protophloem vascular cells (Swarup et al., 2001). While distinct trafficking pathways regulate localization of different auxin carriers (Kleine-Vehn et al., 2006; Doyle et al., 2015), auxin can regulate its own transport by modulating these trafficking pathways (Paciorek et al., 2005), and the cross-talk of auxin with other phytohormones adds further complexity to this feedback regulation. Here we will review how chemical biology strategies have contributed considerably to our current understanding of these complicated interactions between auxin, endomembrane trafficking, and cross-talk with other hormones (Fig. 1).

Chemical biology dissects trafficking pathways controlling auxin carrier localization and dynamics

Investigations into the regulation of auxin transport via complicated endomembrane trafficking routes, which guide auxin carrier protein localization, have been greatly assisted by the use of chemical probes with specific effects within plant cells. Here we will provide a comprehensive review of the knowledge gained by such chemical biology approaches with regards to auxin carrier endocytosis, recycling, secretion, degradation, vesicle mobility, and cross-talk with other signalling pathways. The specific activities of the chemical tools discussed are summarized in Fig. 1.

Chemical tools to dissect endocytosis and recycling of auxin carriers

The fungal toxin brefeldin A (BFA) is to date the most widely used chemical probe for dissection of auxin carrier trafficking routes and is active in multiple plant species including Arabidopsis, maize, and tobacco (Geldner et al., 2001; Boutté et al., 2006; Tse et al., 2006). BFA was first used as an endomembrane trafficking inhibitor in mammalian cells where it is known to inhibit ADP ribosylation factor guanine nucleotide exchange factors (ARF-GEFs), essential regulators of vesicle formation in eukaryotes (Jackson and Casanova, 2000; Zehgouf et al., 2005). In the model plant Arabidopsis, there are eight ARF-GEFs, six of which are inhibited by BFA, while the remaining two are BFA resistant (Anders and Jürgens, 2008). These ARF-GEFs have different specificities, selectively regulating certain trafficking routes, many of which are important for auxin carrier trafficking. BFA thus provides a valuable tool for dissection of these different trafficking routes. Furthermore, BFA treatment of Arabidopsis cells results in easily visualized intracellular effects as it induces agglomerations of Golgi apparatus stacks and endosomal compartments, often referred to as BFA compartments or BFA bodies (Anders and Jürgens, 2008; Robinson et al., 2008).

The best studied of the Arabidopsis ARF-GEFs is GNOM, which is known to be important for PIN1 trafficking and sensitive to BFA (Steinmann et al., 1999). BFA therefore provides a powerful chemical tool to block GNOM activity at the desired stages of plant growth and development. BFA was used to show that PIN1 dynamically recycles at the plasma membrane in Arabidopsis root cells (Geldner et al., 2001). Roots were co-treated with BFA and cycloheximide, a protein translation inhibitor, to exclude any replenishment of the PIN1 pool with de novo produced protein via the secretory pathway. These co-treatments result in accumulation of PIN1 in BFA bodies, while subsequent washing out of the BFA treatment leads to disappearance of PIN1-labelled BFA bodies and reappearance of PIN1 at the plasma membrane (Geldner et al., 2001). Engineering of a BFA-resistant version of GNOM later confirmed that GNOM functions specifically in regulating recycling of basal PIN1 proteins at the plasma
membrane (Geldner et al., 2003). Meanwhile, recycling of apical PIN2 proteins is partially BFA resistant, suggesting the existence of differently regulated recycling routes for different proteins (Geldner et al., 2003). Constitutive recycling of proteins back and forth between the plasma membrane and TGN, through continuous endocytosis and exocytosis events, is thought to allow for a high level of monitoring and control of protein abundance and polar localization as well as the ability to redistribute proteins rapidly when needed (Kleine-Vehn and Friml, 2008; Luschnig and Vert, 2014).

Although recent evidence suggests the existence of clathrin-independent endocytosis in plants (Bandmann and Homann, 2012; Li et al., 2012), it is thought that the main endocytic route operating in plant cells is clathrin mediated (Chen et al., 2011). Tyrophostin A23 is a specific chemical tool that blocks uptake of endocytic cargo into clathrin-coated vesicles in animals and plants by inhibiting the necessary interaction of AP-2 adaptor complexes with the cytosolic domains of transmembrane proteins (Banbury et al., 2003; Ortiz-Zapater et al., 2006). This compound was valuable in demonstrating that internalization of PIN2 as vesicle cargo is clathrin dependent in Arabidopsis roots (Dhonukshe et al., 2007). Furthermore, visualization of these effects on PIN2 uptake was aided by co-treatment with tyrphostin A23 and BFA, due to induction of BFA body formation (Dhonukshe et al., 2007). BFA was later used to confirm the role of clathrin-mediated endocytosis in internalization of PIN1 and PIN2 in Arabidopsis, when overexpression of a dominant negative form of clathrin (Robert et al., 2010) prevented accumulation of these normally BFA-sensitive proteins in BFA bodies.
has also been useful in demonstrating the importance of sterols and very long chain fatty acids (VLCFAs) in auxin carrier endocytosis and recycling. PIN2 no longer accumulates in BFA bodies in a sterol biosynthesis mutant, implying a role for sterols in PIN2 endocytosis (Men et al., 2008). In contrast, PIN1 becomes hypersensitive to BFA-induced agglomeration in a VLCFA synthesis mutant, suggesting that VLCFAs regulate PIN1 recycling (Roudier et al., 2010).

BFA has also proven itself to be a valuable tool in enabling identification of proteins specifically regulating endocytosis or exocytosis events. In a forward genetic screen, Arabidopsis mutants were isolated in which accumulation of PIN1–green fluorescent protein (GFP) in BFA bodies is defective, suggesting PIN1 endocytosis defects (Tanaka et al., 2009). This led to the identification of the proteins BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE (BEN) 1 and 2 as regulators of PIN1 endocytosis. BEN1 was revealed to be an ARF-GEF otherwise known as BIG5 (Tanaka et al., 2009), whereas BEN2 was later shown to be VACUOLAR PROTEIN SORTING 45 (VPS45), a regulator of membrane fusion (Tanaka et al., 2013). Additionally, both ben1 and ben2 mutants were shown to be defective in many auxin-regulated developmental processes, emphasizing the importance of PIN endocytic events for auxin-regulated plant growth and development (Tanaka et al., 2013).

As a valuable chemical probe for auxin carrier trafficking pathways, BFA demonstrates the wide level of control afforded by a chemical biology approach. The effects of short-term treatments with BFA are reversible upon washout (Geldner et al., 2001), and different application concentrations of this compound alter its activity. In Arabidopsis, 25 μM BFA preferentially targets recycling pathways, while 50 μM also affects vacuolar trafficking (Kleine-Vehn et al., 2008c; Robert et al., 2010). The advantage of tunable control offered by BFA can be demonstrated by altering the length of treatment time. While 1–2 h of BFA treatment induces PIN1 accumulation in BFA bodies, increasing the treatment length gradually leads to reduced PIN1 labelling of BFA bodies concomitant with transcytosis of PIN1 from basal to apical plasma membranes after 12 h of treatment (Kleine-Vehn et al., 2008a). This effect of long-term BFA treatment was exploited in another forward genetic screen in which an Arabidopsis mutant defective in PIN1 exocytosis was identified (Tanaka et al., 2014). After 12 h of BFA treatment in the mutant, PIN1 fails to relocate from BFA bodies to apical plasma membranes, which led to identification of BFA-VISUALIZED EXOCYTIC TRAFFICKING DEFECTIVE 1 (BEX1) as a regulator of PIN1 exocytosis. BEX1 was identified as the ADP ribosylation factor (ARF) ARF1A1C which, together with ARF-GEFs and other ARFs, regulates trafficking routes. ARF1A1C was confirmed as a regulator of PIN1 and PIN2 exocytosis by exploiting the reversibility of BFA upon wash-out. In the bex1 mutant, wash-out of BFA fails to remove PIN labelling of BFA bodies, indicating that exocytosis of PIN1 and 2 back to the plasma membrane is defective (Tanaka et al., 2014).

Chemical tools distinguish distinct auxin carrier transport routes including secretory trafficking pathways

BFA has also been useful for distinguishing the different populations of endosomes that exist, which transport distinct cargoes and are regulated by distinct proteins including different ARF-GEFs. AUX1 and PIN1 localize to the opposite polar ends of protophloem root cells in Arabidopsis and display different BFA sensitivities (Kleine-Vehn et al., 2006). PIN1 becomes strongly internalized into BFA bodies while AUX1 only partially labels BFA bodies and mostly remains at the plasma membrane, suggesting the involvement of a BFA-resistant ARF-GEF in AUX1 but not PIN1 trafficking (Kleine-Vehn et al., 2006). Like GNOM, the Arabidopsis ARF-GEFs GNOM-LIKE 2 (GNL2) and BIG1, 2, 4, and 5 are also BFA sensitive, whereas GNL1 and BIG3 are BFA resistant (Steinmann et al., 1999; Richter et al., 2007, 2012, 2014; Teh and Moore, 2007; Tanaka et al., 2009). The existence of distinct AUX1 and PIN1 trafficking pathways was confirmed by use of the chemical probe endosidin 1 (ES1), which disrupts trafficking of PIN2 and AUX1 but not PIN1 or PIN7 (Robert et al., 2008). Thus, some but not all auxin carriers are trafficked via ES1-sensitive routes. Similarly, PIN1 localization was recently shown to be disrupted by another endosidin, ES8, despite PIN2 being almost insensitive to this compound, suggesting that PIN1 but not PIN2 is trafficked via an ES8-sensitive route (Doyle et al., 2015). In addition, ABCB1 is BFA sensitive while ABCB19 is not (Titapiwatanakun et al., 2009), suggesting that trafficking of these auxin influx carriers also occurs via distinct trafficking routes. The VLCFA-sphingolipid biosynthesis inhibitor fumonisin B1 has also been used to distinguish distinct trafficking pathways for different auxin carriers, as this drug induces agglomerations of fluorescent-tagged PIN1, AUX1, ABCB4, and ABCB19, but not PIN2. LIKE-AUX1 3 (LAX3), ABCB1, or ABCB37, implying that VLCFA-containing sphingolipids regulate selective trafficking routes (Markham et al., 2011; Yang et al., 2013).

The partial labelling of BFA bodies with AUX1 further suggests that different pools of AUX1 exist that traffic through different routes (Kleine-Vehn et al., 2006). Intracellular pools of AUX1 are BFA sensitive in Arabidopsis roots, as shown previously (Grebe et al., 2002), but plasma membrane pools of AUX1 are BFA insensitive. It was additionally shown, through use of a BFA-resistant GNOM line, that the intracellular AUX1 pool is in fact not regulated by GNOM but by some other BFA-sensitive ARF-GEF (Kleine-Vehn et al., 2006). Moreover, auxin carriers that are basally polarized all tend to be BFA sensitive, while apically localized auxin carriers are mainly BFA resistant (Kleine-Vehn et al., 2008a) and BFA sensitivities of these proteins can vary according to the cell type (Kleine-Vehn et al., 2008b). The difference in BFA sensitivity of basal and apical auxin carriers has been useful for demonstrating the importance of phosphorylation for determining polar localization. PIN1 and PIN2 become BFA resistant when levels of the protein kinases PINOID (PID), WAG1, and WAG2 are elevated or when phosphatase 2A
(PP2A) function is lost (Kleine-Vehn et al., 2009; Dhonukshe et al., 2010). Similarly, cortical PIN2 becomes less or more sensitive to BFA-induced apical trancytosis in pid or pp2a mutants, respectively (Kleine-Vehn et al., 2009). These results demonstrate that phosphorylation of PINs determines their apical or basal targeting. Treatments with the protein kinase inhibitor staurosporine and the phosphatase inhibitor cantharidin helped to reveal the importance of auxin carrier phosphorylation for auxin-mediated gravitropism. Staurosporine treatment leads to intracellular PIN2 agglomerations, inhibited basipetal IAA transport, and agravitropic roots, as does the pid9 mutation, while cantharidin treatment restores gravitropism in pid9 mutants (Sukumar et al., 2009). The existence of such complicated and distinct trafficking routes guiding auxin carriers and regulated by different ARF-GEFs is likely to allow for precise control of localization and responsive, dynamic redistribution for these essential proteins, in turn enabling extreme fine-tuning of auxin gradients and auxin-directed developmental events.

The secretory trafficking routes from the ER towards the plasma membrane clearly illustrate that different ARF-GEFs regulate distinct trafficking pathways in Arabidopsis. With the help of BFA, acting once again as a valuable chemical tool, it was shown that GN1 regulates early ER–Golgi secretion, probably together with GN0M, while late Golgi–plasma membrane secretion is regulated by BIG1–BIG4 (Richter et al., 2007, 2014; Teh and Moore, 2007). The inability of BFA treatments to modify Golgi apparatus structure in Arabidopsis roots implied that a BFA-resistant ARF-GEF regulates early secretory trafficking. This ARF-GEF was suggested to be GN1L, as introduction of an engineered BFA-sensitive version of GN1L results in Golgi fusion with the ER upon BFA treatment (Richter et al., 2007) and bodies of a secreted form of GFP (secGFP) that agglomerate in gnl1 mutants are not incorporated into BFA bodies (Teh and Moore, 2007). GN1L is likely to play important roles in auxin-regulated developmental processes via early secretion of auxin carriers, as accumulation of PIN1 and PIN2 in BFA bodies is reduced in gnl1 mutants, indicating defective PIN trafficking routes (Teh and Moore, 2007).

GN1L does not act alone in regulating early secretion in Arabidopsis, as gnl1 mutants are viable, although they contain somewhat abnormal Golgi (Richter et al., 2007). However, treatment of gnl1 mutants with BFA results in severe growth defects and causes the Golgi to fuse with the ER, revealing that another BFA-sensitive ARF-GEF also plays a role in early secretion (Richter et al., 2007; Teh and Moore, 2007). It was shown through the use of engineered BFA-resistant GN0M and BFA-sensitive GN1L that this ARF-GEF is probably GN0M (Richter et al., 2007). Recently, further evidence in favour of a role for GN0M in early secretion together with GN1L was provided using the chemical probe ES8, which specifically targets an early secretory pathway and to which gn0m and gnl1 mutants display resistance (Doyle et al., 2015). Moreover, ES8 revealed the important role of this route for auxin carrier localization and auxin-regulated plant development. Via interference with the GN1L/GN0M-mediated early secretory pathway, ES8 reduces PIN1 accumulation in BFA bodies and interferes with PIN1 polarity, leading to altered auxin response patterns and developmental defects in the root (Doyle et al., 2015).

BFA has proven to be an excellent tool for studying late secretion, as treatment of Arabidopsis big3 mutants with BFA inhibits the function of BFA-sensitive BIG1, 2, and 4, providing seedlings in which all of the BIG1–BIG4 ARF-GEFs are inactive. BFA treatment of big3 mutants results in secGFP becoming trapped in BFA bodies and unable to secrete to the plasma membrane, suggesting that all four of these ARF-GEFs act in late secretion (Richter et al., 2014). Interestingly, newly synthesized PIN1 also becomes trapped in BFA bodies, regardless of the presence of engineered BFA-resistant GN0M (Richter et al., 2014), revealing the importance of BIG1–BIG4 in late secretion of PIN auxin carriers. The sterol biosynthesis inhibitor fenpropimorph has also been used to investigate TGN to plasma membrane secretion. Fenpropimorph induces ABCB19–GFP agglomerations that partially co-localize with TGN markers, and FRAP (fluorescence recovery after photobleaching) analysis reveals that the drug increases intracellular recovery of photobleached ABCB19–GFP while decreasing its recovery at the plasma membrane, implying that sterols regulate late secretion of ABCB19 (Yang et al., 2013).

Cyloheximide was again used as a helpful tool to distinguish between secretory and recycling protein pools in a study on secretion of AUX1 during apical hook development in Arabidopsis seedlings. ECHIDNA (ECH) is a TGN-resident protein required for selective secretion in Arabidopsis (Gendre et al., 2013). FRAP analysis in the apical hook after whole-cell bleaching, in which fluorescence recovery at the plasma membrane reflects secretion of newly synthesized proteins, revealed greatly reduced recovery of AUX1–yellow fluorescent protein (YFP) but not PIN3–GFP in ech mutants compared with the wild type (Boutté et al., 2013). However, FRAP analysis after bleaching only part of the plasma membrane in the presence of cyloheximide, in which fluorescence recovery can only represent recycling, transcytosis, or lateral diffusion, revealed similar recovery of both AUX1–YFP and PIN3–GFP in ech and the wild type (Boutté et al., 2013). This suggests that ECH is not involved in recycling but is involved in selective secretion of AUX1 but not PIN3. Therefore, ECH-mediated secretion of AUX1 is important for establishing the auxin gradients needed for differential cell growth during apical hook formation.

Chemical tools reveal insights on trafficking pathways controlling degradation of auxin carriers

As well as BFA, other drugs such as the phosphatidylinositol-3-OH kinase inhibitor wortmannin have been useful in demonstrating the existence of distinct populations of endosomes (Jaillais et al., 2006). In Arabidopsis roots, fluorescent protein fusions of VPS29 and SORTING NEXIN 1 (SNX1) co-localize and label BFA-sensitive membranes that incorporate into BFA bodies (Jaillais et al., 2006, 2007). This suggests that VPS29 and SNX1, which together with other proteins constitute the endomembrane traffic-regulating retromer complex,
route through an endosome population regulated by at least one BFA-sensitive ARF-GEF. However, VPS29 and SNX1 do not co-localize with GNOM (Jaillais et al., 2007), which also labels BFA-sensitive membranes but resides in a different endosome population. The use of wortmannin confirmed these as separate endosome populations because SNX1 and VPS29 but not GNOM compartments are sensitive to this drug (Jaillais et al., 2006, 2007).

Wortmannin has proven to be a valuable tool in helping to identify the SNX1/VPS29-labelled endosome population as PVCs, which route PIN proteins to the vacuole for degradation in Arabidopsis (Kleine-Vehn et al., 2008c). PIN1 and PIN2 are wortmannin sensitive (Jaillais et al., 2006; Kleine-Vehn et al., 2008c), and interference with SNX1/VPS29 endosomes, via wortmannin treatment or mutation, leads to disturbed auxin distribution patterns and severe auxin-related developmental defects (Jaillais et al., 2006, 2007) while not affecting PIN1 or PIN2 recycling back to the plasma membrane after BFA wash-out, implying that these endosomes are not involved in recycling (Kleine-Vehn et al., 2008c). PVCs are also labelled by other members of the retromer complex in Arabidopsis, such as VPS35 (Nodzyński et al., 2013). PIN1–GFP is mislocalized in vps35 mutants, appearing in aggregations that are distinct from BFA bodies and which become larger after wortmannin treatment, suggesting their identity as PVCs (Nodzyński et al., 2013). Furthermore, VPS29 was shown to be involved in PIN1 trafficking in Arabidopsis through use of another drug, cycloheximide. In contrast to the wild type, exclusion of secretory contributions to protein pools by cycloheximide treatment leads to the disappearance of plasma membrane-labelled PIN1–GFP in vps29 mutants, implying that PIN1 is re-routed to the vacuole for degradation when not regulated by VPS29 (Jaillais et al., 2007).

Degradation of auxin carriers is important for controlling their abundance, localization, and redistribution in response to signals. For example, PIN2 is internalized and routed to the vacuoles for degradation in epidermis cells of the upper but not the lower side of gravistimulated Arabidopsis roots, allowing differential auxin gradients to form, leading to root gravitropic bending (Kleine-Vehn et al., 2008c). This PIN2 redistribution no longer occurs in gravistimulated Arabidopsis roots in the presence of BFA, which leads to an increase in PIN2 protein levels (Abas et al., 2006), suggesting interference of the drug with PIN2 degradation. Indeed, BFA was later revealed to be an inhibitor of vacuolar trafficking (Kleine-Vehn et al., 2008c). Interestingly, another chemical probe, the proteasome inhibitor MG132, also prevents PIN2 redistribution in gravistimulated roots, implying that routing of PIN2 to the proteasome for degradation is also important for gravitropic root bending (Abas et al., 2006).

The importance of auxin carrier degradation for gravitropic responses in Arabidopsis was also demonstrated through the use of the chemical probe ES5, which induces transport of PIN1 and PIN2 to the vacuole, leading to gravitropic defects (Drakakaki et al., 2011). Through BFA wash-outs it was shown that ES5 also blocks exocytosis of PIN proteins, suggesting that that re-routing of PIN proteins to the vacuo may occur by default when recycling is inhibited (Drakakaki et al., 2011). Another compound, trafficking and endocytosis inhibitor 1 (TE1), was recently identified as a reversible chemical tool interfering with PIN2 vacuolar trafficking and inhibiting gravitropism in both hypocotyls and roots in Arabidopsis (Paudyal et al., 2014). TE1 was shown to induce accumulation of PIN2 in PVCs and vacuoles, as well as inhibiting endocytosis, as shown by decreased internalization of the endocytic marker FM4-64, but not exocytosis, as shown by BFA wash-out experiments. Interestingly, two Arabidopsis ecotypes, Shahdara (Sha) and Heiligkreuztal 2 (HKT2-4), were identified with reduced sensitivity to TE1, including less PIN2 accumulation in PVCs and reduced gravitropic defects (Paudyal et al., 2014). Variability in sensitivity of natural Arabidopsis accessions to TE1 and other chemical probes could provide useful genetic means for determining their activities and targets.

Sorting of PIN proteins and other cargos to vacuolar trafficking routes for degradation has recently been shown to be mediated by TARGET OF MYB (TOM) 1-LIKE (TOL) proteins in Arabidopsis (Korbei et al., 2013). The TOL proteins partially co-localize with TGN and plasma membrane markers but not with PVC markers, and, as the TOL proteins are insensitive to both BFA and wortmannin, it could be concluded that they localize at or near the plasma membrane but do not recycle, indicating that they may be involved in early sorting of plasma membrane proteins (Korbei et al., 2013). BFA and wortmannin also proved useful in confirming that the TOL proteins selectively control vacuolar targeting of PIN2 but not PIN1. While PIN2 response to BFA is similar in control seedlings and tol mutants, the PIN2, but not PIN1, response to wortmannin is altered in tol mutants compared with control seedlings (Korbei et al., 2013). PIN2 no longer fully localizes to wortmannin-induced compartments in the mutants, indicating that the TOL proteins are involved in selectively targeting PIN2, but not PIN1, towards the PVC en route to the vacuole.

**Chemical tools to probe mobility of auxin carriers via the cytoskeleton**

The mobility of endomembrane compartments within the cell is facilitated by the cytoskeleton. The availability of several compounds acting on cytoskeletal integrity, such as cytochalasin D and latrunculin B, which depolymerize actin filaments, and oryzalin, which depolymerizes microtubules, has greatly assisted research on cytoskeletal roles in auxin carrier localization. Treatments with these drugs and sometimes in combination with BFA treatment revealed that actin filaments but not microtubules are important for recycling of PIN1 and AUX1 in Arabidopsis roots (Geldner et al., 2001; Kleine-Vehn et al., 2006) and that actin is also involved in PIN1 intracellular movement in isolated maize root cells (Boutté et al., 2006) and in PIN3 recycling but not ABCB19 trafficking in Arabidopsis roots (Friml et al., 2002b; Titapiwatanaikan et al., 2009). Interestingly, these drugs also showed that in contrast to recycling, trafficking of PIN1 towards the cell plate during cytokinesis in Arabidopsis root cells involves microtubules (Geldner et al., 2001). Importantly, sensitivity to these...
inhibitors varies depending on auxin carrier identity and polarity as well as the cell type (Kleine-Vehn et al., 2008b), once again confirming the existence of a multitude of distinctly regulated trafficking routes for auxin carriers, in which actin filaments and microtubules play different roles.

It has been shown that some compounds traditionally used as auxin transport inhibitors, such as 2,3,5-triiodobenzoic acid (TIBA) and 1-pyrenylbenzoic acid (PBA), in fact act as general inhibitors of cytoskeletal mobility. TIBA and PBA interfere with PIN1 and AUX1 recycling in Arabidopsis roots, as visualized via co-treatment with BFA (Geldner et al., 2001; Kleine-Vehn et al., 2006). These compounds inhibit the uptake of PIN1 and AUX1 into BFA bodies as well as their transfer back to the plasma membrane upon BFA wash-out, suggesting that both endocytosis and exocytosis events are inhibited (Geldner et al., 2001; Kleine-Vehn et al., 2006).

It was later shown that TIBA and PBA interfere similarly with general plasma membrane-resident proteins, in different eukaryotic organisms, by interfering with actin filament dynamics (Dhonukshe et al., 2008). Combined treatments with other chemical tools proved useful in understanding the activity of TIBA and PBA. Pre-treatment of Arabidopsis roots with either of these compounds inhibits the actin-stabilizing effect of latrunculin B treatment, indicating that both TIBA and PBA interfere with actin by stabilizing actin filaments (Dhonukshe et al., 2008). Furthermore, these two actin stabilizers were shown to interfere with auxin gradients and the gravitropic response, highlighting the importance of the cytoskeleton for auxin carrier trafficking and, in turn, auxin-regulated plant development.

**Chemical tools reveal complicated regulation of auxin carrier transport**

It is clear that a complicated array of cellular processes and signals orchestrate trafficking and polarity of auxin carrier proteins. For example, research has suggested that components of the cell wall are involved in regulating PIN polarity, and chemical inhibitors were useful in demonstrating this. Isoxaben and dichlobenil, inhibitors of cellulose biosynthesis, induce PIN1 polarity defects in Arabidopsis roots similar to those found in cellulose mutants, suggesting that cellulose in the cell wall plays a role in PIN polarity regulation (Feraru et al., 2011). Interestingly, cellulase treatment, which partially degrades the cell wall via digestion of cellulose, results in total loss of PIN1 and PIN2 polarity and reveals partial attachment of these but not apolar proteins to the cell wall (Feraru et al., 2011). This suggests that cellulose in the cell wall may play a role in restricting the localization of PIN proteins to their polar domains.

Among other cellular processes that have been shown to play roles in PIN localization and polarity are the Rho GTP hydrolases of plants (ROPs)/ROP interactive CRIB motif-containing proteins (RICs) signalling pathways. For example, the ROP2/RIC4 and ROP6/RIC1 pathways counteract each other during co-ordination of auxin-regulated lobing in Arabidopsis leaf epidermal pavement cells, in which PIN1 is polarly localized at lobe tips (Xu et al., 2010). The endosidin ES3, which induces PIN1 and PIN2 localization defects in Arabidopsis roots, additionally alters pavement cell lobe shape (Drakakaki et al., 2011). ES3 was shown to target the ROP6/RIC1 pathway, revealing a strong link between ROP/RIC signalling and PIN localization necessary for auxin-regulated lobing (Drakakaki et al., 2011).

**Auxin and other phytohormones as regulators of auxin carrier trafficking**

Apart from their well-studied effects on plant development and gene regulation, auxin and other phytohormones have also been shown to regulate the distribution of auxin carrier proteins directly via endomembrane trafficking pathway modulation. In recent studies, the use of chemical tools has been indispensable for identifying the rapid effects of hormones on protein localization (Fig. 1).

**Auxin activity on localization of auxin carriers**

Auxin has been shown to stabilize PIN proteins locally at the plasma membrane by inhibiting clathrin-mediated endocytosis (Paciorek et al., 2005). Interestingly, the endogenous auxin IAA, and synthetic auxins, such as 1-naphthalencacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), inhibit endocytosis (Paciorek et al., 2005) but with different potency (Simon et al., 2013). NAA is the most potent in this regard and has been used as a convenient chemical tool to dissect the signalling pathway by which auxin mediates endocytosis (Robert et al., 2010; Chen et al., 2012). The characterization and use of the auxin analogues α-(phenylethyl-2-oxo)-IAA (PEO-IAA) and 5-fluoro-IAA (5-F-IAA) demonstrated that auxin inhibition of endocytosis specifically requires the auxin signalling pathway mediated by AUXIN BINDING PROTEIN 1 (ABP1). PEO-IAA inhibits the expression of the synthetic auxin-responsive promoter DR5 and has even been characterized as an auxin antagonist, which blocks the function of the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALING F-BOX (AFB) auxin receptors (Hayashi et al., 2008; Nishimura et al., 2009). However, PEO-IAA is still able to inhibit clathrin-mediated endocytosis, as this compound prevents PIN accumulation in BFA bodies (Robert et al., 2010). In contrast, 5-F-IAA mimics an auxin effect via the TIR1/AFB signalling pathway by inducing the expression of DR5, but is inactive on clathrin-mediated PIN endocytosis (Robert et al., 2010). The activity of auxin on endocytosis via the ABP1- but not the TIR1/AFB-mediated signalling pathway was confirmed by a genetic approach. In the quadruple tir1/afb mutant, in which four of the six nuclear auxin receptors are deficient, NAA fails to induce DR5 expression but does inhibit PIN endocytosis; however, abp1 knockout lines display PIN endocytosis defects, thus enabling this function of auxin to be assigned to a specific receptor, ABP1 (Robert et al., 2010). Despite ABP1 having been shown to bind auxin and to be involved in many auxin-related developmental processes (Grones and Friml, 2015), recent work with abp1 knock-out mutants suggests...
that ABP1 is not essential for *Arabidopsis* development (Gao et al., 2015).

The use of PEO-IAA and 5-F-IAA has also been determinant in identifying the involvement of the TIR1/AFB signalling pathway in auxin-mediated PIN targeting to the vacuole, independently of ABP1-mediated signalling pathway activation (Baster et al., 2013). Interestingly, auxin mediation of endocytosis and vacuolar trafficking act with different kinetics, as auxin-mediated endocytosis is observed within a few minutes through ABP1 (Robert et al., 2010) while longer is required for auxin-mediated PIN degradation (Baster et al., 2013). Treatments with IAA and 5-F-IAA, which induce *DR5* expression, promote targeting of plasma membrane-localized PIN proteins to the vacuole, while PEO-IAA is inactive on both TIR1/AFB-dependent signalling and vacuolar PIN targeting. This is in accordance with the resistance of the quadruple *tir1afb* mutants to NAA-induced vacuolar PIN targeting, to which *abp1*-5 mutants are still sensitive (Baster et al., 2013). The reduction of the intracellular auxin level by inhibition of tryptophan-dependent auxin biosynthesis with 1-kyurenine treatment (He et al., 2011) reduces the abundance of plasma membrane-localized PIN proteins and promotes their targeting to the vacuole (Baster et al., 2013). When co-administered with NAA, the effect of 1-kyurenine on PIN2 abundance at the plasma membrane is reversed, as auxin stabilizes PIN proteins at the plasma membrane (Paciorek et al., 2005; Baster et al., 2013). The effect of 1-kyurenine in targeting PINs to the vacuole reveals the importance of finely balanced auxin levels and timing in controlling PIN quantity at the plasma membrane (Baster et al., 2013). In auxin research, such chemical tools have been invaluable in selectively targeting signalling pathways within a controlled time scale during the investigation of PIN trafficking regulation. PIN turnover at the plasma membrane by two opposite auxin-regulated trafficking effects—inhibition of endocytosis and promotion of vacuolar targeting—ensures fine-tuned transient auxin redistribution during the root gravitropic response (Baster et al., 2013) and probably many other aspects of plant developmental as well.

**Cross-talk with other phytohormones regulating auxin carrier localization**

Transcriptomic response analysis has revealed how phytohormones act independently to control plant development but also regulate the transcription of genes related to other hormones (Nemhauser et al., 2006; Dello Ioio et al., 2008; Růžička et al., 2009; Sun et al., 2009). At the cellular level, the distribution of auxin carrier proteins may also be regulated by hormones other than auxin at specific trafficking steps to fine-tune auxin carrier distribution and auxin gradient formation during organogenesis and developmental responses.

The phytohormone cytokinin acts antagonistically to auxin in several developmental processes (Skoog and Miller, 1957; Laplaze et al., 2007; Vanstraelen and Benková, 2012). The activity of the aromatic cytokinin **N**<sub>6</sub>-benzyladenine (BA) on PIN trafficking has been investigated during lateral root primordium organogenesis. A specific depletion of PIN1 and PIN3 proteins from the plasma membrane and targeting to the vacuole occurs upon BA treatment, whereas PIN7 and AUX1 are either stabilized or not affected by BA (Marhavý et al., 2011). Although *PIN1* and *PIN3* expression is reduced upon BA treatment (Dello Ioio et al., 2008; Růžička et al., 2009), plasma membrane-localized PIN1 is still depleted upon BA treatment when *PIN1* is expressed under the constitutive 35S promoter, indicating the contribution of post-transcriptional regulation during BA-induced PIN1 depletion (Marhavý et al., 2011). BA-dependent PIN1 targeting to the vacuole is inhibited when BA is applied in co-treatment with either wortmannin or latrunculin B, indicating, respectively, the requirement for functional transport to the vacuole and a dynamic actin network (Marhavý et al., 2011). Through a reverse genetic strategy, it was further shown that the cytokinin receptor ARABIDOPSIS HISTIDINE KINASE 4 (AHK4) participates in the BA-mediated degradation of PIN1, thus uncovering a new layer in auxin-cytokinin cross-talk (Marhavý et al., 2011). This study demonstrated that although hormonal cross-talk with auxin often occurs via transcriptional regulation of auxin-related genes, hormones can also directly influence auxin carrier trafficking pathways.

Salicylic acid (SA), a phytohormone involved in stress responses (Vlot et al., 2009) and regulation of growth and development (Rivas-San Vicente and Plasencia, 2011), has been shown to act as a potent inhibitor of clathrin-mediated endocytosis (Du et al., 2013). Consequently, SA reduces the accumulation of PIN2 proteins in BFA-induced agglomerations and thus limits the establishment of asymmetric auxin distribution during the root gravitropic response (Du et al., 2013). Interestingly, the inhibitory effect of SA on PIN2 endocytosis still occurs in *abp1* mutants, suggesting that SA acts through a different signalling pathway from auxin itself (Du et al., 2013). Moreover, the active synthetic SA analogue benzothiadiazole **S**-methylester (BTH) does not affect PIN internalization, demonstrating that SA targets a BTH-insensitive signalling pathway to inhibit endocytosis (Du et al., 2013). Additionally, SA-mediated PIN endocytosis still occurs in the presence of cycloheximide and cordycepin, translation and transcription inhibitors, respectively, revealing that SA acts through an undescribed post-translational signalling pathway (Du et al., 2013). Therefore, a cross-talk between SA and auxin controls stabilization of PIN proteins at the plasma membrane, thereby participating in the regulation of plant development and responses to environmental stimuli.

The stress-related hormone methyl jasmonate (MeJA) interacts with auxin during lateral root formation (Sun et al., 2009). MeJA has been shown to display a dose-dependent effect on the distribution of PIN2 proteins. A low concentration of MeJA reduces PIN2 protein internalization into BFA bodies and this effect requires the proteins ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1 (ASA1), TIR1/AFB, and CORONATINE INSENSITIVE 1 (COI1), which are related, respectively, to auxin biosynthesis and auxin- and jasmonate-dependent signalling pathways (Sun et al., 2011). However, a high concentration of MeJA induces a decrease in PIN2 abundance at the plasma membrane, which requires a functional AUXIN RESISTANT 1
(AXR1) and COI1 signalling pathway and is enhanced in *asa1*-1 and quadruple *tir1*/*laf5* mutants (Sun et al., 2011). Moreover, MeJA has been shown to activate auxin biosynthesis rapidly through the COI1-dependent signalling pathway, by promoting the expression of the *ASA1*, *YUCCA8*, and *YUCCA9* genes (Sun et al., 2009; Henrich et al., 2013), and PIN2 abundance at the plasma membrane is tightly regulated by an optimal auxin level (Baster et al., 2013). Thus, a complicated cross-talk between MeJA and auxin exists whereby MeJA-induced auxin biosynthesis alters intracellular auxin levels (Sun et al., 2009; Henrich et al., 2013), controlling PIN2 abundance at the plasma membrane (Sun et al., 2011) by either auxin-mediated endocytosis (Paciorek et al., 2005; Robert et al., 2010) or auxin-mediated PIN2 degradation (Baster et al., 2013). A high MeJA concentration inhibits root growth (Sun et al., 2009) and MeJA is likely to contribute to auxin redistribution during the root gravitropic response by altering PIN2 distribution (Sun et al., 2011).

Gibberellic acid (GA), a plant growth-promoting hormone, has been shown to regulate root growth curvature in response to gravistimulation, through the modulation of PIN-dependent auxin transport (Willige et al., 2011). In the presence of the GA biosynthesis inhibitor paclobutrazol or in the GA biosynthesis-deficient mutant *gal1*, PIN abundance at the plasma membrane is reduced, revealing a GA-dependent stabilization of PIN proteins (Willige et al., 2011). Interestingly, this effect is specific to PIN proteins, as paclobutrazol does not affect AUX1 protein quantity at the plasma membrane. Treatment of *gal1* with the vacuolar ATPase inhibitor concanamycin A increases vacuolar accumulation of PIN2, suggesting that GAs inhibit PIN vacuolar targeting (Willige et al., 2011). Furthermore, PIN2-labelled BFA body size is increased in the wild type upon GA treatment, but decreased in the *gal1* mutant or after treatment with uniconazole, another GA biosynthesis inhibitor, placing GA action downstream of BFA action in the trafficking route to the lytic vacuole (Löfke et al., 2013). In the gravistimulated root epidermis, an asymmetric distribution of GA has been shown specifically to stabilize PINs at the plasma membrane, revealing an important role for GA during PIN-mediated auxin asymmetric redistribution (Löfke et al., 2013). Together with cytokinin, GA is one of only two non-auxin hormones identified as acting directly on the targeting of PIN proteins to the vacuole.

Ethylene is a phytohormone involved in a plethora of plant developmental processes, including regulation of apical hook formation, as ethylene treatment leads to exaggerated apical hook bending (Guzman and Ecker, 1990). Cross-talk between auxin and ethylene, as well as occurring via effects on gene expression, has also been shown to act through the activity of ethylene on auxin carrier trafficking pathways. Due to the volatile nature of ethylene, this hormone can be conveniently replaced with its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) for experimental treatments. Interestingly, *AUX1* expression and AUX1 protein turnover are increased by ACC treatment at the concave (inner) side of the apical hook, where an auxin maximum is established (Vandenbussche et al., 2010). Meanwhile, during ACC-induced exaggerated apical hook formation, PIN3 is specifically stabilized polarly at the plasma membranes of hook epidermal cells (Žádníková et al., 2010). Moreover, ACC treatment is inactive in the AUX1 secretion-defective mutant *ech*, confirming the importance of ECH-dependent AUX1 secretion during ethylene-regulated apical hook formation (Boutté et al., 2013). Thus, auxin-ethylene cross-talk regulates the formation of asymmetrical auxin gradients necessary for hook bending via precise modulations in the distributions of specific auxin influx and efflux carriers.

**Conclusions and future perspectives**

Small molecules interfering with endomembrane trafficking have greatly enhanced our understanding of auxin carrier trafficking regulation in plants over the past decade, overcoming many of the inherent difficulties associated with investigations of such complex processes. The recent discoveries identifying the roles of endogenous plant compounds as players in this regulation illustrate another level of complexity, providing clues as to how plants achieve hormonal homeostasis at the cellular level. Further identification and characterization of chemical inhibitors of these essential processes will continue to assist this research in the future. Systems approaches in cell biology are required for the further characterization of such chemical tools. Developments in microscopy techniques and the design of automated analysis will lead to improved screening strategies towards the further identification of more potent and specific chemicals actively interfering with regulation of auxin carrier dynamics.

The emergence of phytohormone-related chemicals targeting auxin-specific trafficking, signalling, and biosynthetic pathways have also been, and will continue to be, essential tools in investigations of such rapid biological processes (Fonseca et al., 2014; Ma and Robert, 2014; Rigal et al., 2014). Indeed, considering the redundancy and robustness of both hormone and trafficking regulation, the emergence of chemicals displaying functional selectivity is needed to guide the association of signalling pathways with cellular processes. The recent development of fluorescent auxin analogues that do not induce auxin signalling have allowed visualization of auxin transport and confirmed the presence of intracellular auxin gradients (Hayashi et al., 2014). Moreover, the exciting development of genetically encoded fluorescent RNA sensors of specific small molecules may also provide useful tools to reveal the subcellular dynamics of endogenous compounds (Paige et al., 2012). In conclusion, chemical biology approaches have contributed greatly to our current understanding of auxin carrier traffic regulation and will most certainly continue to provide means of further enhancing this understanding as advances in the identification and design of active chemical tools continue.

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