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Letter to the Editor

Auxin does not inhibit endocytosis of PIN1 and PIN2 auxin efflux carriers

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Dear Editor,

The phytohormone and morphogen auxin regulates essentially all aspects of plant development from embryogenesis to fruit formation (e.g. Moubayidin and Ostergaard, 2014; Robert et al., 2018). Uneven or unidirectional cell-to-cell transport is an important property of auxin's mode of action and, together with local auxin biosynthesis or degradation, result in the formation and maintenance of auxin maxima and minima pivotal for proper patterning and development (e.g. Chen et al., 2014; Brumos et al., 2018). Canonical "long" PIN-FORMED auxin exporters (PINs) are essential for this function because their polar plasma membrane distribution and their ability to repolarize within a cell make them ideal to directionally transport auxin through simple and complex tissues (Bennett, 2015). The abundance of polarly distributed PINs at the plasma membrane, together with mechanisms regulating PIN activity, determines the absolute amount of auxin being exported from a cell in a given direction (Barbosa et al., 2018).

Classical studies on polar auxin transport and its role in vascular differentiation have suggested a positive feedback loop between auxin and its polar transport (Berleth and Sachs, 2001). The polar distribution of some PINs in the same orientation along cell files suggested a polar signal, which could be auxin itself, facilitates PIN localization and, in turn, polar auxin transport. In 2005, Paciorek et al. (2005) reported that auxin could inhibit PIN endocytosis, and in fact endocytosis in general, from the plasma membrane and that auxin in this way promoted its own efflux from cells (Figure 1, A). The authors thereby described a mechanism that could be part of a process regulating PIN abundance at the plasma membrane and directional auxin export from a cell.

These observations were made possible based on the application of the transport inhibitor Brefeldin A (BFA) leading to the accumulation of PINs and many other integral plasma membrane proteins in intracellular, albeit non-physiological, BFA compartments (Figure 1, A; Geldner et al., 2001). Paciorek et al. (2005) examined BFA compartment formation after a 90-min BFA treatment as proxy for the activity of PIN endocytosis and its impact on auxin efflux. Since treatments with the protein synthesis inhibitor cycloheximide (CHX) did not influence PIN accumulation in BFA compartments, the authors concluded PIN protein accumulation in BFA compartments was the result of PIN endocytosis from the plasma membrane (Paciorek et al., 2005). A further aspect of their study found the natural auxin indole-3-acetic acid (IAA) had only weak effects when compared with the synthetic auxins 1-naphthalene-1-acetic acid (1-NAA) or 2,4-dichlorophenoxyacetic acid (2,4D). IAA effects were, however, enhanced when IAA was applied together with the antioxidant butylated hydroxytoluene (BHT), leading to the conclusion that IAA was unstable *in planta* unless prevented from oxidizing (Figure 1, A).

Several of these findings have been questioned in recent years. In three publications, Jásik and colleagues examined a version of Arabidopsis (*Arabidopsis thaliana*) PIN2 tagged with the green-to-red photoconvertible fluorescent protein

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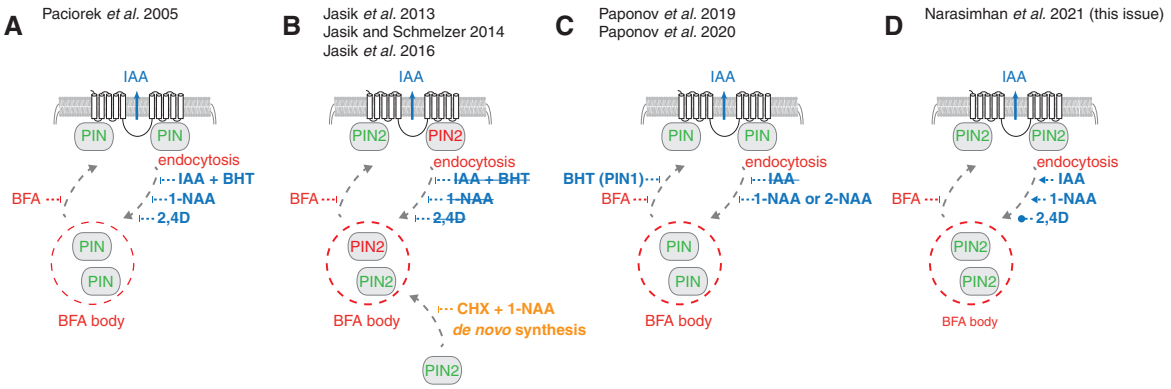

Figure 1 Contradictory observations reveal the complexity of chemical signaling on PIN intracellular transport and *de novo* synthesis. (A–D) Summary of the key results from the publications discussed in this commentary as specified. Specifically, new observations are highlighted in bold; PIN identity is specified where possible or considered necessary. BFA, Brefeldin A; CHX, cycloheximide; IAA, indole-3-acetic acid; 1-NAA, 1-naphthalene-1-acetic acid; 2,4D, 2,4-dichlorophenoxyacetic acid; BHT, butylated hydroxytoluene.

Dendra2 expressed from a *PIN2* promoter fragment (Figure 1, B; Jasik et al., 2013, 2016; Jasik and Schmelzer, 2014). PIN2-Dendra2 allowed tracing the fate of distinct cellular PIN2 pools through Dendra2 photoconversion, e.g. to distinguish between *de novo* synthesized unconverted red PIN2-Dendra2 from preexisting photoconverted green PIN2-Dendra2 (Jasik et al., 2013). In a Letter to the Editor, Jasik and Schmelzer (2014) then reported in Molecular Plant that PIN2-Dendra2 accumulating in BFA compartments originated not only from endocytosed plasma membrane PIN2-Dendra2 but also from *de novo* synthesized PIN2-Dendra2. In an extension of this study, Jasik et al. (2016) then showed that neither natural nor synthetic auxins affected PIN2-Dendra2 endocytosis in root epidermal cells. They further provided evidence that the effects of auxin on PIN2-Dendra2 accumulation in BFA compartments was a result of auxin acting on the *de novo* synthesized pool of PIN2-Dendra2, rather than on PIN2-Dendra2 originating from the plasma membrane (Jasik et al., 2016). Thus, the studies by Jasik and colleagues directly questioned the inhibitory effects of auxin on PIN endocytosis as originally reported in Paciorek et al. (2005).

Whether the inactivity of the natural auxin IAA as PIN endocytosis inhibitor could be explained by its *in planta* instability was reexamined by Paponov et al. (2019) (Figure 1, C). In contrast to the original finding, they found IAA remained chemically stable in the incubation medium and biologically active *in planta*. Thus, chemical instability could not sufficiently explain the reduced inhibitory effect of IAA on PIN endocytosis. They also found BHT alone seemingly promoted PIN1 and PIN2 internalization after BFA treatment, with a more substantial effect on PIN1 than on PIN2. The high sensitivity of PIN1 to BHT was subsequently explained by an inhibitory effect of BHT on PIN1 exocytosis (Paponov et al., 2019, 2020). Thus, the presumably IAA-stabilizing compound BHT interfered by itself with the process of cellular PIN trafficking. Finally, the active auxin 1-NAA, as well as the inactive auxin 2-NAA had comparable inhibitory effects on PIN endocytosis, leading to the conclusion that the observed effects did not stem from the actual auxin activity of the compounds (Figure 1, C; Paponov et al., 2019). Unfortunately and despite of their importance, the studies by Jásik et al. and Paponov et al. received hardly any attention from the plant development and cell biology community.

Perhaps as a result of work published by Jasik et al., Narasimhan et al. (2021) have now re-evaluated the effects of natural and synthetic auxins on PIN endocytosis. Contrary to the original conclusions (Paciorek et al., 2005), Narasimhan *et al.* find both types of auxins have general effects on the aggregation of the endomembrane system and that treatments with the synthetic auxin 1-NAA affected early endosome/*trans*-Golgi network composition (Figure 1, D; Narasimhan et al., 2021). They also found no general effect of the auxins on endocytosis when using total internal reflection fluorescence (TIRF) microscopy to specifically trace the formation of clathrin-containing endosomes at the plasma membrane. Rather, IAA and 1-NAA would promote specifically the endocytosis of PIN2, but not that of PIN1, even at low concentrations (Narasimhan et al., 2021). Thus, the effects of auxin that may be considered physiologically relevant are, according to this study, opposite to those originally proposed and, at the same time, possibly much more specific (Figure 1, D; Paciorek et al., 2005).

All research is done in the light of the available knowledge and with the range of methods available at the time and place of experimentation. Due to space constraints, it is impossible to list and discuss the multitude of studies, including some very recently published, that do not only refer to but actually built on the original Paciorek *et al.* report. In view of the recent and now corrected findings, the results and conclusions of many of these studies will require re-evaluation. Such re-evaluations are even more urgent since also other, seemingly firmly established mechanisms of auxin sensing, auxin transport regulation, and PIN polarity control have been questioned in recent years (Dai et al., 2015; Enders et al., 2015; Gao et al., 2015; Michalko et al., 2015; Weller et al., 2017; Gelova et al., 2021).

An encouraging lesson from the above-summarized studies is reflected by the fact that the field of plant cell biology has become significantly more quantitative and qualitative over the past 15 years. At the same time, they demonstrate the immense importance of such carefully performed quantitative studies to fully understand the complexity of cell biological processes.

Another lesson from this odyssey of findings is that caution is required when small chemicals, be they native or synthetic, are used in biological experiments. Although we all are aware of the risks associated with their use in our experiments, we tend to forget about these risks when we have catchy results in hands that originate from experiments with such compounds. In their sum, the above-summarized publications provide several warning examples of misinterpretations that can result from the use of chemical compounds in biological studies.

Finally, one should not overlook that the studies by Jásik et al. and Paponov et al. were, by far, not as visibly published as the Paciorek et al. paper. This is possibly due to the fact that they were in conflict with the latter or because they were, unfortunately until now, considered of secondary importance. What is more, these studies received hardly any attention in the relevant community even after publication, as judged by their low citation numbers. As we understand now, these critical findings turned out to be very insightful and, without doubt, were much more quantitative and possibly also more focused and dedicated to the study of individual aspects when compared with the earlier report. For us as authors, the lesson is that we should pay proper attention to such critical findings, include them in our experimental planning, and discuss them appropriately in our own publications. As scientists, we should encourage ourselves and our coworkers to trust our own results, even or especially when they are not in line with published knowledge. Better we learn from these conflicting findings rather than to move on or leave them unexplained. As reviewers and editors, we may want to give more freedom and space to authors to discuss and cite papers that are in conflict with the predominant views in the field to strengthen the field at its entire breadth.

Whether auxin indeed regulates its own transport via PIN proteins or other mechanisms, and whether directional auxin transport impacts on PIN polarization remain open questions.

Feedback mechanisms, as described in the Paciorek et al. paper, have become an integral part of studies trying to explain auxin-dependent processes through mathematical modeling. In a separate commentary in this issue of *Plant Physiology*, Kirsten Ten Tusscher explains, in light of findings and revised concepts presented here, the importance of such mechanisms for the modeling of auxin distribution (Ten Tusscher, 2021).

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