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REVIEW PAPER

Auxin: simply complicated

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Abstract

Auxin is a plant hormone involved in an extraordinarily broad variety of biological mechanisms. These range from basic cellular processes, such as endocytosis, cell polarity, and cell cycle control over localized responses such as cell elongation and differential growth, to macroscopic phenomena such as embryogenesis, tissue patterning, and *de novo* formation of organs. Even though the history of auxin research reaches back more than a hundred years, we are still far from a comprehensive understanding of how auxin governs such a wide range of responses. Some answers to this question may lie in the auxin molecule itself. Naturally occurring auxin-like substances have been found and they may play roles in specific developmental and cellular processes. The molecular mode of auxin action can be further explored by the utilization of synthetic auxin-like molecules. A second area is the perception of auxin, where we know of three seemingly independent receptors and signalling systems, some better understood than others, but each of them probably involved in distinct physiological processes. Lastly, auxin is actively modified, metabolized, and intracellularly compartmentalized, which can have a great impact on its availability and activity. In this review, we will give an overview of these rather recent and emerging areas of auxin research and try to formulate some of the open questions. Without doubt, the manifold facets of auxin biology will not cease to amaze us for a long time to come.

Key words: Auxin, metabolism, plant development, signalling, structure, transport.

The phytohormone auxin: a versatile regulator of plant development

Phytohormones are endogenous molecules occurring naturally in plants at very low concentrations. They do not have any nutritional function, but act as signalling compounds that promote and influence plant development and physiology. To date, structurally diverse phytohormones have been characterized, such as auxin, cytokinin, strigolactone, abscisic acid, ethylene, gibberellin, brassinosteroid, salicylic acid, and jasmonate. In 1880, Charles Darwin suggested the existence of moving growth regulators (Darwin and Darwin, 1880). Light-induced differential elongation in grass coleoptiles was proposed to be mediated by the root-ward transport of a signalling molecule (Darwin and Darwin, 1880), whose unequal distribution regulates plant curvature towards the light (Went, 1926; Cholodny, 1927). The underlying growth hormone was first isolated from fermentation media (Salkowski, 1885) and identified as indole-3-acetic acid (IAA) (Kögl *et al.*, 1934).

The term 'auxin' originates from the greek word '*auxein*', which means to enlarge/grow. Auxin activity was classically defined as the competence to stimulate elongation in coleoptile and stem sections, but also rooting (Went, 1934). Since then, auxin has been shown to be essential for plant development

mediating diverse responses, such as the control of senescence (Ellis *et al.*, 2005), response to pathogens (Kazan and Manners, 2009; Fu and Wang, 2011), and abiotic stress (Wang *et al.*, 2010). It also regulates fruit formation (De Jong *et al.*, 2009) and leaf abscission (Rubinstein, 1963). Auxin promotes the establishment and maintenance of polarity, apical dominance, and tropic response to light or gravity (Woodward and Bartel, 2005; Vanneste and Friml, 2009). At the cellular level, it controls cell division (e.g. regulation of meristem formation giving rise to new organs such as lateral and adventitious roots) and cell elongation by altering cell wall plasticity. In addition, auxin is not only acting through linear pathways, but is also involved in many cross-talk responses with other phytohormones (Swarup *et al.*, 2002; Vanstraelen and Benkova, 2012).

Endogenous auxins: it's all about the structure

Auxins are defined as low molecular weight organic acids containing an aromatic ring and a carboxyl group, which, to be active, need to be at a distance of 0.55 Å (George *et al.*, 1963). The most abundant endogenous auxin is IAA, which is able to fulfil most of the auxin actions involved in plant development and responses to the environment.

In addition to IAA, only three other naturally occurring compounds with auxin activity have been described in plants, namely indole-3-butyric acid (IBA) (Ludwig-Müller and Epstein, 1991), 4-chloroindole-3-acetic acid (4-Cl-IAA) (Engvild, 1985), and phenylacetic acid (PAA) (Okamoto *et al.*, 1967) (Fig. 1). They have been detected both as free acids and in conjugated forms (Ludwig-Müller, 2011).

IBA was originally found in potato tubers (Blommaert, 1954) but is present in diverse plant species (Ludwig-Müller, 2000). According to Ludwig-Müller and co-authors (1993), IBA may represent 25–30% of the total pool of auxins in *Arabidopsis thaliana* seedlings. However, recent studies suggest that endogenous IBA levels are below the detection limit in *Arabidopsis* (Novák *et al.*, 2012). Exogenously applied IBA induces rooting more efficiently than IAA itself (Zimmerman and Wilcoxon, 1935) and is widely used as a rooting agent in agricultural applications (Hartmann *et al.*, 1990). IBA is involved in other auxin-mediated developmental processes, such as leaf epinasty, cell division, stem bending (Zimmerman and Wilcoxon, 1935), root hair elongation (Strader and Bartel, 2009; Růžička *et al.*, 2010), and cell expansion in cotyledons (Strader *et al.*, 2010). IBA is both produced from and converted to IAA, and is, therefore, considered as a storage form of IAA, providing the active hormone when and where it is needed (Bartel *et al.*, 2001; Woodward and Bartel, 2005). Whether IBA itself is able to induce responses independently of IAA remains to be resolved.

4-Cl-IAA was discovered in immature seeds of *Pisum sativum* (Gandar and Nitsch, 1967; Marumo *et al.*, 1968). Since then, the presence of 4-Cl-IAA has been unveiled in a large number of plants, mainly members of the Fabaceae (Engvild, 1975, 1980; Engvild *et al.*, 1978, 1980; Hofinger and Böttger, 1979; Katamayama *et al.*, 1987) and in developing seeds of several legumes and *Pinus sylvestris* (Reinecke, 1999). However, 4-Cl-IAA has not been detected in the main model plant *Arabidopsis*, which might explain the lack of detailed knowledge about its mode of action. 4-Cl-IAA stimulates pericarp growth in pea (Reinecke *et al.*, 1995), maize coleoptile

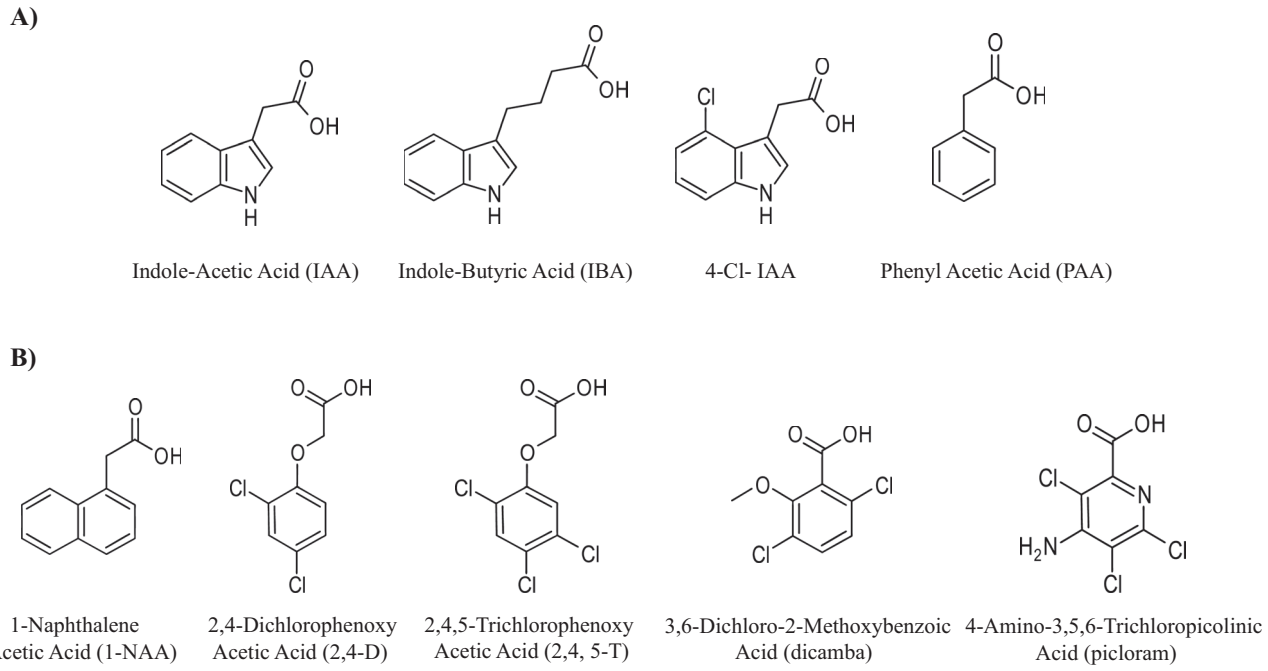

Fig. 1. Examples of naturally occurring (A) and some synthetic auxins (B) are presented. (A) indole-acetic acid (IAA), indole-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA), and phenyl-acetic acid (PAA). (B) 1-Naphthalene-acetic acid (NAA), 2,4-dichlorophenoxy-acetic acid (2,4-D), 2,4,5-trichlorophenoxy-acetic acid, 3,6-dichloro-2-methoxybenzoic acid (dicamba), and 4-amino-3,5,6-trichloropiconiölinic acid (picloram).

elongation (Rescher *et al.*, 1996), and protoplast swelling (Steffens and Lüthens, 2000). Interestingly, when applied exogenously, 4-Cl-IAA is active at lower concentrations compared with IAA (Böttger *et al.*, 1978), which might be explained by its greater chemical stability (Maruno *et al.*, 1973).

Finally, PAA is so far the only identified phenyl derivative endogenous auxin (Wightman and Lightly, 1982) and, compared with IAA, is active at much higher concentrations (Fitzsimons, 1989). PAA has been found in different plant species (Wightman and Lightly, 1996) and has been suggested to play a role in root interactions with soil microorganisms (Morris and Johnson, 1984; Slininger *et al.*, 2004; Somers *et al.*, 2005).

Synthetic auxins: the scientific and agronomic toolbox

Synthetic compounds with similar activities to plant hormones are termed 'plant growth regulators' (George, 1963). These synthetic analogues often diverge in structure, but share a range of comparable biological activities with the endogenous hormones. The analyses of the structure–activity relationship (SAR) of synthetic auxins allow a better understanding of natural auxin activity. A structural comparison of the compounds possessing auxin-like properties reveals that the indole group is not essential for auxin activity, and can be replaced by an aromatic ring or fused aromatic ring of a comparable size. These SAR studies also allowed the prediction of non-essential residues and just recently led to the development of fluorescently labelled auxin molecules (Strader and Nemhauser, 2013; Hayashi Laboratory).

Synthetic auxin analogues include 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 3,6-dichloro-2-methoxybenzoic acid (dicamba), 4-amino-3,5,6-trichloropicolinic acid (tordon or picloram), and many others. These synthetic auxins are more stable than IAA, presumably because these compounds show reduced metabolic turnover (Dunlap *et al.*, 1986). Nevertheless, synthetic auxins can be still inactivated via enzymatic conjugation with glucose (Barendse *et al.*, 1987; Klems *et al.*, 1998).

At high concentrations, auxins are toxic and their activities target mainly dicots over monocot species, such as grasses and cereal crops. Because of these properties, many compounds with auxin-like activity have been developed and used as herbicides (Grossmann, 2010). Additionally, synthetic auxins are used as active molecules in commercial solutions for horticulture as they promote the initiation of adventitious roots and the synchronization of flowering and fruit set.

Auxin perception: affinities and whereabouts

To trigger a biological response, endogenous auxin and synthetic auxin-like compounds must be perceived by the plant and converted into a signal. Today, we know of three independent auxin receptors and their related signalling systems. This diversity at the level of auxin perception is very probably a key factor for the great variety of auxin responses.

TIR1, family and friends: a complex signalling network

Without doubt, the best-studied receptor is the F-box protein TRANSPORT INHIBITOR RESISTANT1 (TIR1). It was identified as a subunit of an E3 type ubiquitin-protein ligase and proposed as a player in auxin responses (Ruegger *et al.*, 1998; reviewed in del Pozo and Estelle, 1999). Some years later, TIR1 was unequivocally demonstrated to be an auxin receptor by two independent labs (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005). Crystallographic studies showed that IAA and other, synthetic auxin compounds fit into the core of a ring-like structure formed by TIR1 (Tan *et al.*, 2007). This binding does not change the conformation of TIR1, but it promotes its interaction with proteins of the AUXIN/INDOLE ACETIC ACID (Aux/IAA) family. This interaction triggers the ubiquitination of Aux/IAA proteins, which designates them for degradation by the 26S proteasome. In the absence of auxin, Aux/IAAs form inhibitory heterodimers with AUXIN RESPONSE FACTOR (ARF) family transcription factors. Thus, auxin-dependent Aux/IAA degradation leads to release of ARF transcription factors and subsequent transcriptional responses (for reviews, see Quint and Gray, 2006; Weijers and Friml, 2009).

Although conceptually straightforward (receptor binding leads to degradation of inhibitors and release of activators), this system of auxin perception is actually quite complex.

First, TIR1 is the F-box protein of a SKP-Cullin F-box (SCF) type ubiquitin E3 ligase and requires the other constituents of the complex, as well as the function of the 26S proteasome, to trigger downstream events. Crystallographic analysis of TIR1 revealed another, unexpected cofactor for TIR1 function, inositol (1,2,3,4,5,6) hexakisphosphate (InsP6) (Tan *et al.*, 2007). Mutation of amino acids required for InsP6 binding leads to disruption of the auxin-dependent TIR1–Aux/IAA interaction. Whether InsP6 has a role in modulating auxin signalling, or rather is a necessary structural component of the TIR1 protein is currently unknown (Calderon Villalobos *et al.*, 2012).

A second layer of complexity comes from the fact that the components of the TIR1–Aux/IAA pathway typically comprise large protein families. In *Arabidopsis*, there are five homologues of TIR1, termed AFB1–AFB5, which all bind auxin, but with different affinities (Calderon Villalobos *et al.*, 2012). Aux/IAA proteins form a large 29 member family that share a common structure of four domains (termed I–IV). Domain II (DII) directly participates in the interaction with TIR1: the auxin molecule fits into the auxin-binding site of TIR1 and the DII domain binds both TIR1 and the auxin molecule to form a lid-like structure, trapping the auxin molecule between TIR1 and Aux/IAA (Tan *et al.*, 2007). Since Aux/IAAs directly form part of the receptor–ligand interaction, they can be seen as auxin co-receptors. This co-receptor concept is biologically relevant, because the auxin interaction surfaces of the five TIR/AFB receptors and the 29 Aux/IAA proteins are not strictly conserved, which potentially gives rise to different TIR/AFB–Aux/IAA co-receptor pairs with distinct auxin affinities.

Although some of the potential co-receptor pairs might exist only theoretically, differences in auxin affinity of some

of them have been experimentally demonstrated. For example, in heterologous experiments performed in a yeast system, TIR1–Aux/IAA7 had a high affinity (K_d in the 10 nM range) while TIR1–Aux/IAA12 had a much lower affinity (K_d in the 300 nM range) (Calderon Villalobos *et al.*, 2012). While future *in planta* studies are needed to shed light on the naturally occurring co-receptor pairs, the concept could explain both the large dynamic range of auxin responses and the variety of processes in which auxin plays a role.

Although it seems that Aux/IAAs are the determining factor for auxin affinity rather than the type of TIR1/AFB (Calderon Villalobos *et al.*, 2012), there are, nevertheless, some differences in binding properties among TIR1/AFBs: it has been shown that AFB4 and AFB5 have a very high affinity towards the synthetic auxin picloram (Greenham *et al.*, 2011; Calderon Villalobos *et al.*, 2012), presumably due to changes in their auxin-binding pocket. Rather counter-intuitively, AFB4 has been recently reported as a negative regulator of auxin responses in seedlings (Greenham *et al.*, 2011). However, the molecular basis for this behaviour is currently not clear.

Synthetic yeast-based systems have proven useful to recreate and measure TIR1/AFB-dependent Aux/IAA degradation. For the same Aux/IAA, TIR1 and AFB2 confer a more rapid degradation than AFB1 and AFB3 (Havens *et al.*, 2012), in line with their stronger interaction with IAA *in vitro* (Parry *et al.*, 2009) and with genetic evidence that *tir1* and *afb2* mutants present more severe phenotypes than *afb1* and *afb3* mutants (Dharmasiri *et al.*, 2005b; Parry *et al.*, 2009). Studies of isolated signalling network components in heterologous systems seem a powerful way to overcome the inherent complexity of *in planta* approaches, which are hampered by multiple feedback mechanisms between perception, signalling, transport, and synthesis. They will be instrumental in clarifying the underlying molecular mechanisms in auxin–TIR1/AFB–Aux/IAA interactions. Eventually, they could lead to the development of synthetic auxin analogues or antagonists targeting distinct co-receptor complexes.

Relatedly, the two-component nature of the TIR1/AFB–Aux/IAA co-receptor system has been exploited to generate synthetic compounds that block this signalling pathway. The anti-auxins tert-butoxycarbonylaminohexyl-IAA (BH-IAA), α-[phenylethyl-2-oxo]-IAA (PEO-IAA), and α-[2,4-dimethylphenylethyl-2-oxo]-IAA (auxinol) bind TIR1/AFBs the same way as endogenous IAA, but they are unable to promote the interaction with the DII domain of Aux/IAA proteins, because of their bulky side groups that hinder DII access. Thus, they effectively compete with endogenous IAA and render the TIR1/AFB signalling pathway inactive (Hayashi *et al.*, 2008, 2012). It will be interesting to see whether endogenous auxin derivatives can also act as natural attenuators of this signalling pathway.

At the end of the signalling chain, repression of ARF transcription factors by Aux/IAAs adds another level of complexity: in *Arabidopsis*, there are 23 ARFs with conserved domains that allow interaction with Aux/IAAs. Yeast two-hybrid-based interaction studies showed that the network of possible Aux/IAA–ARF interactions is potentially vast and complex (Vernoux *et al.*, 2011). It remains to be seen how many of these interactions can be validated *in planta* and in which developmental context they play a role. In this setting, it is interesting to note that the majority of ARFs actually act as transcriptional repressors and do not interact with Aux/IAAs. They instead seem to compete with activating ARFs for binding the *cis*-elements of auxin-regulated genes, which adds yet another layer of regulation in this pathway.

The sheer amount of data on TIR1/AFB in the current literature might lead to the belief that this pathway can explain all auxin responses. Nevertheless, there exists strong evidence for other, independent auxin signalling pathways that co-exist with TIR/AFB-mediated auxin perception (reviewed in Badescu and Napier, 2006)

SKP2A, an emerging auxin receptor?

In mammals, the SCF^{SKP2} E3 ubiquitin ligase is an important player for the degradation of cell cycle factors (Carrano *et al.*, 1999; Tsvetkov *et al.*, 1999). An SKP2 orthologue in *Arabidopsis*, the S-Phase Kinase-Associated Protein 2A (SKP2A), is also a regulator of the cell cycle and is involved in the degradation of at least two cell cycle factors, DPB and E2FC (Del Pozo *et al.*, 2006; Jurado *et al.*, 2008). Recently, it was shown that auxin can bind directly to SKP2A; and, by structural modelling using TIR1 as template, a novel auxin-binding pocket in SKP2 was suggested. Mutations of the core amino acids of this predicted pocket abolished auxin binding and also the biological activity of SKP2A (Jurado *et al.*, 2010). Similar to TIR1, high auxin levels promote the interaction between SKP2A and DPB or E2FC, which is required for their degradation, but, in contrast, SKP2A is degraded itself under high auxin conditions (Del Pozo, 2006; Jurado *et al.*, 2010).

Although our knowledge about SKP2A signalling is far less detailed than what we know about TIR1/AFB, SKP2A seems to fulfil the basic requirements for an auxin receptor, although more detailed studies will be needed to satisfy the classical criteria for a receptor [specific and saturable binding, specific physiological responses, and rate-limiting function in these responses (see also the review by Jones and Sussman, 2009)].

Cell cycle control is to a large extent governed by precisely timed degradation of key regulators. In contrast to the output signal of the TIR1/AFB pathway, which is the transcriptional activation of target genes, the SKP2A pathway leads to rapid degradation of key regulators and might thus represent a plausible candidate for the molecularly still poorly understood link between auxin and cell cycle control. Future studies will reveal more details of the SKP2A pathway and its biological relevance in bridging auxin and the cell cycle.

AUXIN-BINDING PROTEIN1 (ABP1): 40 years old and still enigmatic

ABP1 is the longest known auxin receptor. It was first purified in maize from a cell fraction that showed auxin binding activity (Löbler and Klämbt, 1985), cloned (Hesse *et al.*, 1989; Inohara *et al.*, 1989; Tillman *et al.*, 1989) and its auxin

binding activity confirmed (Jones and Venis, 1989; for a comprehensive overview, see Napier *et al.*, 2002). Classical experiments revealed a requirement for ABP1 in very rapid responses close to the plasma membrane, such as auxin-triggered ion fluxes or rapid (within a few minutes) cell elongation responses (reviewed in Sauer and Kleine-Vehn, 2011). For a long time, however, the biological importance of ABP1 was unclear, until an *Arabidopsis* T-DNA insertion mutant of ABP1 was shown to be embryo lethal (Chen *et al.*, 2001) and ABP1 therefore an essential gene. Novel tools to alter endogenous ABP1 levels *in planta* permitted the study of the roles of ABP1 and its relationship to auxin in more detail. Nevertheless, there is still no full consensus about the exact physiological processes controlled by ABP1: some studies provided evidence for a role in auxin-dependent cell cycle and cell expansion control (David *et al.*, 2007; Braun *et al.*, 2008), thus confirming earlier reports. An entirely new perspective came with the discovery that ABP1 is required for the auxin-induced inhibition of clathrin-mediated endocytosis (Robert *et al.*, 2010) and the simultaneous finding that ABP1 is linked to a cell polarity-generating mechanism in which it activates the Rho-GTPases ROP2 and ROP6, which are implied in control of endocytosis and cytoskeleton reorganization via their effectors RIC4 and RIC1, respectively (Xu *et al.*, 2010). Recent work on ABP1-related ROPs revealed that ROP6 interacts with the Rho-GEF SPIKE1 (SPK1), and SPK1 is needed for the auxin-dependent activation of ROP6 and the inhibitory effect of auxin on endocytosis. (Lin *et al.*, 2012). Whether SPK1 and ABP1 are connected by a direct signalling mechanism is currently not clear. ROP6 and RIC1 regulate clathrin-mediated endocytosis of PIN-FORMED (PIN) auxin efflux transporters, and genetic analyses suggest that their action is downstream of ABP1 (Chen *et al.*, 2012; for an overview of the PIN family, see Paponov *et al.*, 2005; Krecek *et al.*, 2009). Taken together, these studies demonstrate a link between ABP1 activity and abundance of membrane proteins, such as PIN auxin efflux carriers, at the plasma membrane. Thus, ABP1 could form the auxin receptor for a signalling network that is independent of *de novo* gene transcription, but operates directly at or in close vicinity to the plasma membrane and controls protein abundance and/or activity.

In this respect, it is important to note that ABP1 is, although mainly localized in the endoplasmic reticulum (ER), secreted to some extent into the extracellular space and seems to be active as an auxin receptor there. The binding affinities of ABP1 for auxin have been studied under different pH and found to be highest at a slightly acidic pH of ~5.5, which is the pH of the extracellular space. In contrast, at pH 7.0 of the ER lumen, ABP1 has almost zero affinity for auxin (Tian *et al.*, 1995), adding further weight to the notion that ABP1 is an extracellular auxin receptor. If this is correct, then there is the question of how an extracellular ABP1–auxin signal is relayed across the plasma membrane to downstream factors, such as ROPs. ABP1 is not a transmembrane protein; thus, it requires (an) accessory protein(s), which transmits a signal to the cell interior, and at the same time holds ABP1 in place close to the membrane. Currently, the best candidate for a docking protein is an extracellular, glycosylphosphatidylinositol (GPI)-anchored protein with similarity to *Arabidopsis* SKEWED5 (SKU5), which was identified in maize as a putative ABP1 interactor (Shimomura, 2006). SKU5, however, is unlikely to act as a facilitator for ABP1-related signalling events across the plasma membrane, as it does not contain a transmembrane domain. Its role could be rather that of a scaffold or anchor required to hold ABP1 in place. Recently, Zhenbiao Yang's group presented preliminary evidence for a leucine-rich repeat receptor-like protein kinase (LRR-RLK) involved in relaying an ABP1 signal across the membrane and potentially also in augmenting ABP1 sensitivity (meeting report by Strader and Nemhauser, 2013). Lastly, it was speculated that phospholipases might play a role in signalling events downstream of ABP1 (Scherer *et al.*, 2012). Further studies are required to verify these hypotheses, and the identification of the interaction partners of ABP1 for coupling extracellular perception to intracellular signalling is currently the greatest challenge in the ABP1 field.

Although the extracellular nature of ABP1 is not unambiguously clarified and many questions are still unanswered, ABP1 remains the best candidate for sensing differential local auxin concentrations in the immediate cellular surrounding. Especially in self-organizing, auxin-dependent tissue patterning processes predicted by the canalization hypothesis (Sachs, 1981, 1991), such as vein formation during leaf development, wound responses, or during organ primordia establishment (Sauer *et al.*, 2006; Scarpella *et al.*, 2006), where cells react to local tissue gradients, ABP1 could therefore play an important role as a kind of 'directional sensor'. Unlike the nuclear receptors TIR1/AFB or SKP2A, the peripheral localization of ABP1 could provide the spatial information required for sensing not only the concentration, but also the direction of an auxin signal. This might feed into a mechanism that locally controls events close to the plasma membrane, such as rates of clathrin-mediated endocytosis, cytoskeleton arrangement, and cell expansion.

Whether ABP1 also fulfils a role as an auxin receptor in the ER is speculative at this point, but the definite answer to this question is not yet known.

Auxin metabolism: how much and is it active?

Several endogenous compounds with auxin activity (Fig. 1), plus distinct auxin receptors have been suggested (Fig. 2). However, the complexity of auxin does not stop here: upon closer inspection, the metabolism of the most abundant auxin IAA also emerges as a surprisingly complicated affair.

Auxin biosynthesis: how much complexity is needed?

IAA can be produced via tryptophan (Trp)-independent and Trp-dependent pathways (Chandler, 2009; Normanly, 2010; Zhao, 2010). The Trp-independent auxin biosynthesis pathway is not well characterized, but seems to be operational in plants, although its biological relevance is not clear (Wright

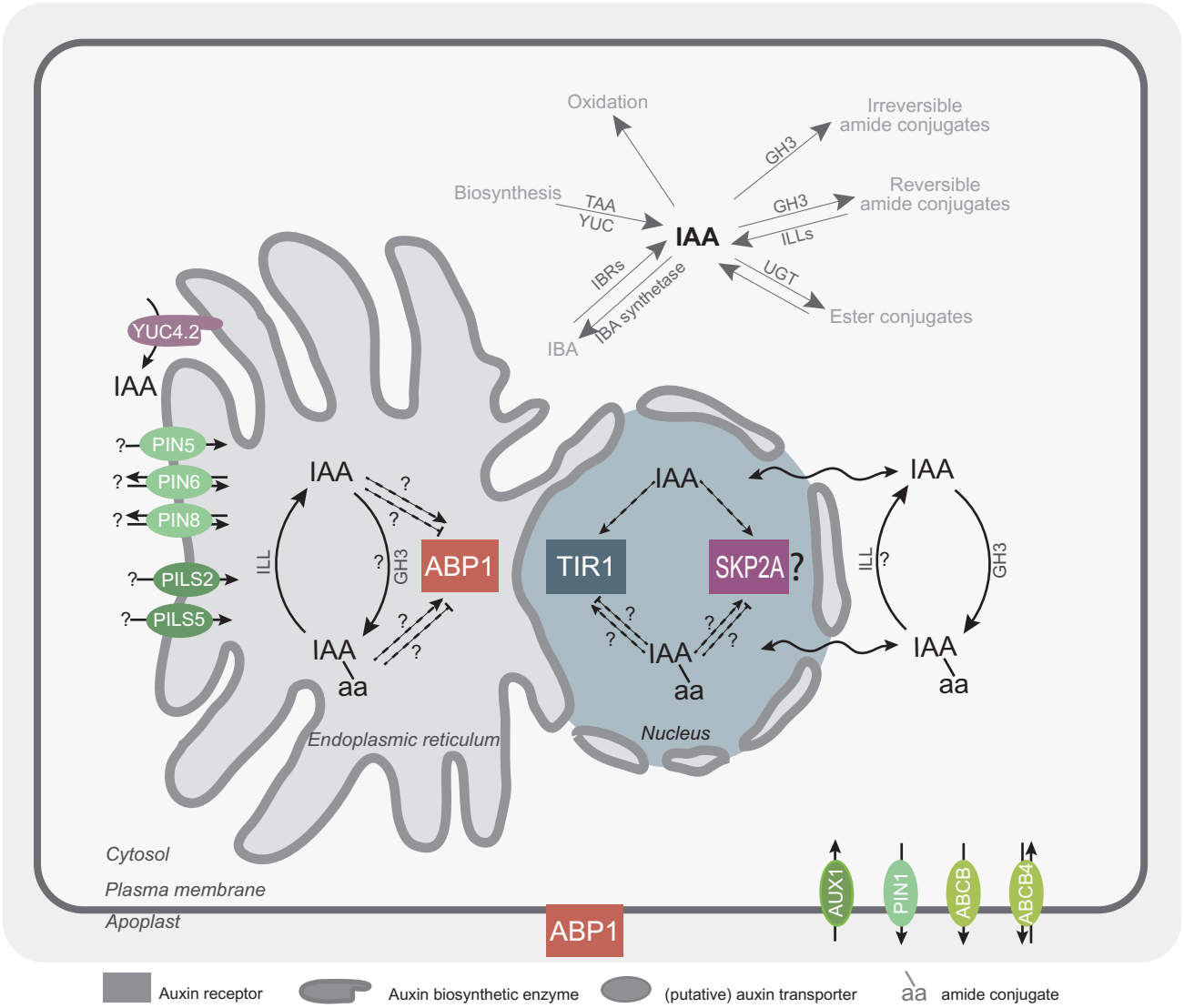

Fig. 2. Schematic working model on IAA conversion and compartmentalized auxin transport/signalling. ATP-binding cassette B (ABCB), auxin-binding protein1 (ABP1), auxin resistant1 (AUX1), Gretchen Hagen3 (GH3), indole-3-acetic acid (IAA), indole butyric acid (IBA), IAA-leucine resistant1-like (ILL), IBA-resistant (IBR) UDP-glucosyl transferase (UGT), PIN-formed (PIN), PIN-likes (PILS), S-Phase Kinase-Associated Protein 2A (SKP2A), Tryptophan aminotransferase of *Arabidopsis* (TAA), transport inhibitor response1 (TIR1), Yucca (YUC).

et al., 1991; Normanly *et al.*, 1993; Ouyang *et al.*, 2000; Ehlert *et al.*, 2008). The Trp-dependent pathways are better defined and appear to be the developmentally important source of auxin. Four Trp-dependent pathways can be categorized into indole-3-acetaldoxime (IAOx), indole-3-acetamide (IAM), tryptamine (TAM), and indol-3-ylpyruvic acid (IPA) pathways, based on their major intermediates. In the IPA pathway, the *YUCCA* (*YUC*) gene family encodes flavin monooxygenase-like proteins, and genetic studies suggested its function downstream of *WEAK ETHYLENE INSENSITIVE8* (*WEI8*)/*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*). These protein families jointly form a two-step biosynthetic route and constitute the main auxin biosynthesis pathway in *Arabidopsis* and maize (Mashiguchi *et al.*, 2011; Phillips *et al.*, 2011; Won *et al.*, 2011). A recent biochemical study on auxin biosynthesis shows that YUC6 catalyses the oxidative decarboxylation of α-keto acids including IPA and phenyl pyruvate (PPA), providing the biochemical proof for the TAA/YUC-mediated two-step auxin biosynthesis pathway in *Arabidopsis* (Dai *et al.*, 2013).

The presumed storage form of auxin, IBA, is largely synthesized from IAA by the action of IBA synthetase via a chain elongation reaction similar to those found in fatty acid biosynthesis (Ludwig Müller and Hilgenberg, 1995; Woodward and Bartel, 2005). A possible IAA-independent pathway for IBA biosynthesis has been suggested (Ludwig Müller, 2000), but it is unlikely to be the major route. IBA conversion back to IAA is catalysed by the action of peroxisomal β-oxidation enzymes IBRs (INDOLE-3-BUTYRIC ACID RESPONSE) (Epstein and Ludwig Müller, 1993; Zolman *et al.*, 2008). In contrast to IBA, biosynthesis of the auxins PAA and 4-Cl-IAA has been suggested to be independent of IAA. 4-Cl-IAA seems to originate from 4-Cl-Trp (Reinecke, 1999), and PAA production might require a nitrilase pathway

with benzylglucosinolate as a precursor (Ludwig Müller and Cohen, 2002). However, very recent evidence (discussed at the last auxin meeting in Hawaii) suggests that PAA may be produced via a YUC-dependent pathway (Dai *et al.*, 2013; Strader and Nemhauser, 2013)

The auxin biosynthesis pathways are differentially controlled in response to environmental stimuli, such as light, drought, cold, and wounding (Rapparini *et al.*, 2002; Tao *et al.*, 2008; LeClere *et al.*, 2010; Lehmann *et al.*, 2010), and intrinsic cues, such as hormones and nutrients (Stepanova *et al.*, 2008; Mishra *et al.*, 2009; Zhou *et al.*, 2011; Hentrich *et al.*, 2013). Notably, the joint utilization of a precursor seems to coordinate the amounts of the two vital hormones auxin and ethylene (Zheng *et al.*, 2013).

In conclusion, the multiple biosynthesis pathways seem to allow for flexible responses to diverse and often simultaneous endogenous and exogenous triggers.

Auxin conjugation and oxidation: just temporal inactivation and decay of auxin?

Plants have multiple auxin molecules on hand with potentially distinct signalling capacity. Compared with IAA, IBA has (if at all) only a very weak activity and is considered to be rather a temporal storage form of IAA (Fig. 2) (Strader and Bartel, 2011). Notably, IBA has been suggested to be below the detection limit in *Arabidopsis* (Novák *et al.*, 2012), questioning the endogenous function of IBA in at least some species. Intriguingly, both IAA and IBA are transported from cell to cell by distinct transport systems (Strader and Bartel, 2009, 2011; Ruzicka *et al.*, 2010). However, while IAA is certainly active during its cell to cell transit, IBA seems largely inactive while in transit and its defined conversion to IAA in particular (competent) cells seems to be the developmentally important step (De Rybel *et al.*, 2012). As a consequence, the polar transport of active IAA has direct impact on the transporting tissue by triggering cellular identity, such as vascularization. In contrast, IBA seems to be a mobile, but largely inactive messenger, and only competent cells can initiate the readout of this spatially defined auxin signal.

Ester and amide conjugation are other possibilities to inactivate auxins temporally (Fig. 2) (Sztein *et al.*, 1995; Tam *et al.*, 2000; Kowalczyk and Sandberg, 2001; Ljung *et al.*, 2002; Seidel *et al.*, 2006). Only a small fraction of auxin appears in its free form and is mostly conjugated to sugar moieties, amino acids, peptides, or proteins (Sztein *et al.*, 1995; Tam *et al.*, 2000; Kowalczyk and Sandberg, 2001; Ljung *et al.*, 2002; Seidel *et al.*, 2006). A recent study reveals the tissue-specific distribution of the auxin metabolome in *Arabidopsis* and highlights a complex regulation of auxin metabolism (Novák *et al.*, 2012).

IAA conjugation to amino acids seems to be the strategy of choice in *Arabidopsis*, and so far has drawn most attention. IAA–alanine (IAA–Ala), IAA–leucine (IAA–Leu), IAA–aspartate (IAA–Asp), and IAA–glutamate (IAA–Glu) are the most abundant amino acid auxin conjugates in *Arabidopsis* (Tam *et al.*, 2000; Kowalczyk and Sandberg, 2001); however, other amide conjugates, including IAA–valine (Val), IAA–phenylalanine (Phe), and IAA–tryptophan (Trp), are also present in lower amounts (Kai *et al.*, 2007; Staswick, 2009). The auxin-inducible GRETCHEN HAGEN3 (GH3) family facilitates IAA conjugation to amino acids (Hagen and Guilfoyle, 1985; Staswick *et al.*, 2005), while on the other hand the IAA-LEUCINE RESISTANT 1 (ILR1)-like family of amidohydrolases release IAA (Bartel and Fink, 1995; Davies *et al.*, 1999; LeClere *et al.*, 2002; Rampey *et al.*, 2004). The IAA–Ala hydrolase IAR3 is under the evolutionarily conserved regulation of microRNA miR167 (Kinoshita *et al.*, 2012), highlighting the auxin conjugation-dependent mechanism to cope with environmental stress conditions (Park *et al.*, 2007; Du *et al.*, 2012).

Beside conjugation-based temporal inactivation, the excess cellular auxin can also be degraded via decarboxylative (Barcelo *et al.*, 1990) or non-decarboxylative oxidation pathways (Östin *et al.*, 1998). Notably, conjugation to IAA–Asp and IAA–Glu is considered to be irreversible and they have been therefore suggested to be precursors for degradation (Östin *et al.*, 1998; Ljung *et al.*, 2001; Kowalczyk and Sandberg, 2001).

However, the option to conjugate auxin might not only function as pure auxin storage (Fig. 2). IAA or IBA conjugation might also be a strategy to limit its spatial distribution within a tissue by interfering with its cell to cell transport. Moreover, some of the auxin conjugates might still have a certain signalling function (Staswick, 2009) and could thus provide a spatially restricted signal.

Intracellular auxin transport: tuning compartmentalized auxin metabolism or more?

Temporal and spatial regulation of auxin metabolism gives important impulses for flexible plant development. However, the intercellular cell to cell transport machinery further extends the complexity and can build up auxin gradients within plant tissues. A detailed treatment of this intriguing carrier network is beyond the scope of this review and we would like to refer the reader to several excellent reviews on this matter (Kramer and Bennett, 2006; Grunewald and Friml, 2010; Zazimalova *et al.*, 2010; Peer *et al.*, 2011). Instead, we want to highlight a rather surprising connection between putative auxin carrier activity and auxin metabolism. Just recently, presumed auxin carriers, such as PIN5/PIN6/PIN8 and the PIN-LIKES (PILS)2/PILS5 have been shown to reside at the ER and seem to limit nuclear auxin signalling by an auxin sequestration mechanism (Mravec *et al.*, 2009; Barbez *et al.*, 2012; Dal Bosco *et al.*, 2012; Ding *et al.*, 2012; Sawchuck *et al.*, 2013). Moreover, the activity of the evolutionarily distinct PIN5 and PILS2/PILS5 at the ER reduces the levels of free IAA at the expense of increased IAA conjugation to amino acids and glucose (Mravec *et al.*, 2009; Barbez *et al.*, 2012; Feraru *et al.*, 2012; Viaene *et al.*, 2013), suggesting a link between auxin compartmentalization and auxin conjugation-based metabolism (Fig. 2). The current data suggest that putative auxin carriers at the ER regulate auxin accumulation in the ER lumen, where compartmentalized auxin metabolism might take place (Barbez

and Kleine-Vehn, 2012). Some ILR1-like amidohydrolases, but not GH3 conjugases, display an *in silico* defined ER targeting signal (Campanella *et al.*, 2003; Ludwig Müller *et al.*, 2009). How the presumed carrier-dependent compartmentalization of auxin leads to higher auxin conjugation rates remains therefore molecularly ill defined.

Notably, a particular splice variant of YUCCA4 localizes to the outer surface of the ER (Kriechbaumer *et al.*, 2012) and might produce auxin in the vicinity of the ER. As mentioned earlier, the potential auxin receptor ABP1 localizes mainly to the ER. Although experimental evidence predicts its activity rather for the extracellular space (reviewed in Sauer and Kleine-Vehn, 2011), it nevertheless remains a theoretical possibility that ABP1 perceives the ER-compartmentalized auxin or even auxin conjugates (Barbez and Kleine-Vehn, 2012).

In summary, increasing evidence proposes that the ER might have a role in cellular IAA homeostasis (Fig. 2). This is in accordance with the finding that ER-derived peroxisomes have a role in compartmentalized IBA metabolism (Strader and Bartel, 2011). Recent studies reveal that intracellular auxin transport is indeed required and biologically relevant in regulation of cellular growth, pollen development, flowering time, *de novo* organogenesis, and vascularization (Mravec *et al.*, 2009; Barbez *et al.*, 2012; Dal Bosco *et al.*, 2012; Ding *et al.*, 2012; Sawchuck *et al.*, 2013). Auxin canalization and, hence, intercellular auxin transport has been traditionally linked to vein patterning (Sachs, 1981; Sauer *et al.*, 2006), but in fact requires convergent intercellular and intracellular transport mechanisms (Sawchuck *et al.*, 2013). Theoretical studies previously suggested that intracellular auxin transport could also lead to auxin canalization in evolutionarily older species (Wabnik *et al.*, 2011). In accordance with these computational assumptions, Sawchuck and colleagues revealed an ER-localized PIN-dependent mechanism to select cell files specialized for vascular function that seems to pre-date evolution of plasma membrane-localized PIN proteins.

We assume that research on putative auxin carriers at the ER will further reveal unexpected developmental aspects. Nevertheless, further insight into biosynthesis, transport, metabolism, and perception in and around the ER is needed to understand fully the actual mechanistic role of intracellular auxin transport for cellular auxin homeostasis.

Concluding remarks and open eminent questions

The recent insights into auxin-dependent plant development suggest that not only do a multitude of auxin molecules, distinct biosynthesis routes, and several signalling pathways add to the complexity of auxin biology, but also the spatiotemporal regulation of auxin conjugation and carrier-dependent subcellular distribution of auxin matter for the actual cellular responsiveness to auxin.

In the last decades, the field has made substantial progress in the mechanisms of auxin biology at many different levels. It seems that we now face a period that requires the systematic integration of the multiple classical auxin research approaches as proposed by Teale *et al.* (2008). Besides our emerging understanding of the dazzling complexity of auxin signalling and response, several very eminent, basic questions still remain to be solved. For instance, it is tempting to speculate that the multitudes of endogenous auxin compounds have distinct affinities for the diversified auxin receptors or co-receptor pairs. Who binds whom and what is the developmental consequence? Do endogenous auxin-like molecules, or even other hormones compete for receptor attention? Besides the interesting question of whether the endogenous auxin IBA has auxin signalling competence itself, it remains to be shown if other auxin metabolites, such as conjugates, are able to bind and attenuate the different auxin receptors. To decipher auxin perception mechanisms further, it appears that more quantitative data and computational modelling are needed to describe the signal integration of TIR/AFB-, SKP2a-, and ABP1-based auxin perception. How the different receptors and co-receptor pairs jointly coordinate decisions on cellular division, cell elongation, and cell fate, and how all the pathways are interconnected, are the questions which guarantee that auxin biology will stay a fresh, rich, and fascinating topic for many years to come.

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