

REVIEW

Expansion and innovation in auxin signaling: where do we grow from here?

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ABSTRACT

The phytohormone auxin plays a role in almost all growth and developmental responses. The primary mechanism of auxin action involves the regulation of transcription via a core signaling pathway comprising proteins belonging to three classes: receptors, co-receptor/co-repressors and transcription factors. Recent studies have revealed that auxin signaling can be traced back at least as far as the transition to land. Moreover, studies in flowering plants have highlighted how expansion of the gene families encoding auxin components is tied to functional diversification. As we review here, these studies paint a picture of auxin signaling evolution as a driver of innovation.

KEY WORDS: *Marchantia polymorpha*, *Physcomitrium patens*, *Arabidopsis thaliana*, *Zea mays*, Evo-devo, Auxin response

Introduction

Auxins are a group of small molecules that regulate plant growth and development through a simple signaling pathway (Lavy and Estelle, 2016; Leyser, 2018; Woodward and Bartel, 2005). This pathway is composed of proteins that enable three functions: transcriptional activation of target genes, repression of this transcriptional activity in the absence of auxin, and perception of auxin that triggers de-repression (Fig. 1). Transcriptional activation is mediated by a group of transcription factors called A class AUXIN RESPONSE FACTORS (ARFs) that bind to AUXIN RESPONSIVE ELEMENTS (AuxREs) associated with auxin-regulated genes (Ulmasov et al., 1997). AUXIN/INDOLE-3-ACETIC ACID proteins (Aux/IAAs) then act as a bridge, connecting ARFs to members of the TOPLESS (TPL) family of co-repressors, thereby repressing auxin-responsive gene transcription when auxin levels are low (Szemenyei et al., 2008). Repression is relieved when auxin acts as a molecular glue, dramatically increasing the affinity of TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOXES (AFBs) for Aux/IAAs, both of which directly bind to auxin. TIR1/AFBs are the substrate-recognition component in an SCF-type E3 Ubiquitin Ligase complex. Association of an Aux/IAA with a TIR1/AFB leads to its polyubiquitylation and subsequent degradation by the 26S proteasome (Kepinski and Leyser, 2005). This auxin-dependent Aux/IAA degradation leads to increased transcription of ARF-associated target genes by removing TPL-mediated repression (Kim et al., 1997; Lavy and Estelle, 2016; Leyser, 2018).

The protein families involved in the auxin signaling pathway have expanded dramatically alongside major innovations in plant form and function (Mutte et al., 2018), and it has been theorized that, in fact,

expansion-enabled changes in paralog function drove innovation (Matthes et al., 2019). Here, we review the recent profusion of studies that are providing crucial insights into the evolutionary origins of auxin signaling, the likely original functions of auxin in shaping plant growth and morphology, and the impact of the expansion of gene families encoding auxin signaling components on novel functions. First, we delve into studies in the liverwort *Marchantia polymorpha* and the moss *Physcomitrium patens*. These models represent ancient land plant lineages with highly reduced auxin signaling repertoires, providing novel insights into protein origins and functions. As part of this molecular evolutionary approach to understanding auxin signaling, we also include insights obtained from studies of several algal species, which approximate the state of auxin signaling components in the last common ancestor before the transition to land. Second, we consider recent discoveries connecting the expansion of and diversification within each of the families encoding the major auxin signaling components – TIR1/AFBs, Aux/IAAs and ARFs – with innovations in development and physiology. Finally, we conclude with a brief perspective on unanswered questions in the field.

The auxin signaling pathway emerged with the transition to land

A fully functional auxin signaling pathway first appeared in land plants. Orthologs of TPL and TOPLESS-RELATED PROTEINS (TPRs) can be found in the earliest lineages of land plants, and they appear to have evolved from transcriptional co-repressor families found across eukaryotes (Causier et al., 2012). The evolutionary history of other core auxin components was, until now, somewhat contentious, with differing views on whether the auxin signaling pathway – as it exists in flowering plants – is truly restricted to land plants or whether a more limited version exists in algal lineages.

Insights from algal ancestors

A protein with strong similarity to TIR1/AFBs has been identified in the charophyte algae *Coleochaete irregularis* genome, but closer examination found that the region homologous to the auxin-binding pocket of this protein is highly diverged (Mutte et al., 2018). In addition, although comparative genomics show that Aux/IAAs are found only in the genomes of land plants (Mutte et al., 2018), a group of non-canonical Aux/IAAs (ncIAAs) has been found in *C. irregularis*. However, these ncIAAs contain a PB1 domain, which enables interaction with ARFs, but lack the degron domain necessary for interacting with TIR1/AFB receptors (Mutte et al., 2018). ncIAA genes are also found in *M. polymorpha*, but loss of function and transcriptome studies have been unable to connect them to an auxin response (Flores-Sandoval et al., 2018a,b; Mutte et al., 2018). The preponderance of evidence to date suggests that, although algae do show some auxin responsiveness (Ohtaka et al., 2017), this responsiveness is unlikely to be mediated by the same pathway used in land plants.

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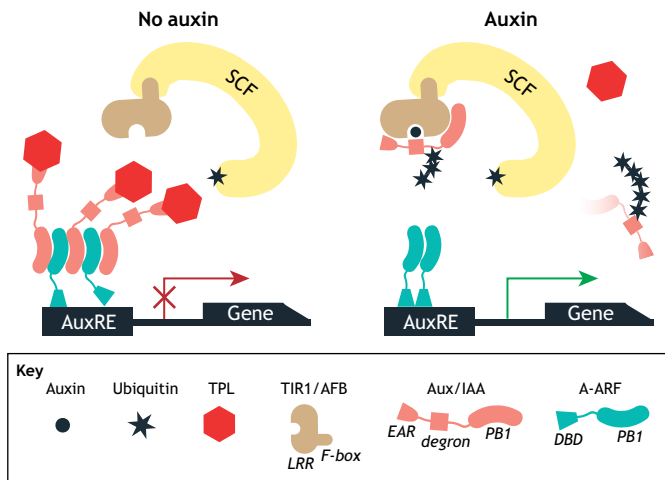


Fig. 1. A simplified representation of the canonical auxin signaling pathway. A highly simplified snapshot of the nuclear-localized, canonical auxin response pathway. In the absence of auxin, Aux/IAAs connect A-ARFs to TPL co-repressors, thereby repressing auxin-responsive gene transcription. Auxin then triggers the association of Aux/IAAs with the SCF^{TIR1/AFB} complex, leading to their polyubiquitylation and subsequent degradation. The removal of Aux/IAAs (and with them the TPL) activates A-ARFs and induces gene expression.

Charophytes have also been shown to express ARFs. Phylogenetic analysis of land plant genomes divides ARFs into three distinct clades (A, B and C); however, in multiple charophyte species, there is evidence of a combined A/B clade and a C clade (Martin-Arevalillo et al., 2019). This would date the separation of the A and B groups to after the establishment of land plants (Mutte et al., 2018). This phylogenetic analysis, combined with genetic studies, implies that C class ARFs play a fundamentally different role from A or B class ARFs, and may not be involved in auxin-dependent gene regulation (Flores-Sandoval et al., 2018a,b; Mutte et al., 2018). This is further supported by phylogenetic work, which shows that the sole C class ARF found in *M. polymorpha* has a unique, apparently auxin-independent, function in growth of the gemma – an asexually produced structure aiding in vegetative propagation (Flores-Sandoval et al., 2018a,b; Kato et al., 2020; Mutte et al., 2018). A recent study argued that the C class ARFs emerged in the earliest-diverging clades of streptophyte algae, *Mesostigma viridae* and *Chlorokybus atmophyticus*, while A/B class ARFs emerge later in *Coleochaete orbicularis* (Martin-Arevalillo et al., 2019). This study also showed that C and A/B class ARFs can directly bind TPL and AuxREs in promoters, allowing them to function in an entirely auxin-independent manner (Martin-Arevalillo et al., 2019) (Fig. 2A). This direct TPL interaction suggests that the loss of direct TPL interaction, and therefore the auxin-sensitive gene repression seen in A class ARFs, was among the last pieces of auxin signaling to arise. This appears to hold true in *M. polymorpha*, where the B and C class ARFs MpARF2 and MpARF3, respectively, but not the A class ARF MpARF1, have been shown to bind TPL (Fig. 2A) (Kato et al., 2020). Together, the evidence argues for land plant auxin signaling to have evolved from neofunctionalization of a simple ancestral auxin-independent pathway in algal ancestors (Martin-Arevalillo et al., 2019).

From liverworts and mosses to angiosperms

Functional genetic studies on emerging bryophyte models have been a welcome complement to the long history of work in angiosperms. In *M. polymorpha*, there is a single TIR1/AFB

receptor, a single Aux/IAA, a single TPL co-repressor and three ARFs (Flores-Sandoval et al., 2015). In *P. patens* there are four TIR1/AFBs, three Aux/IAAs, two TPL proteins (Paponov et al., 2009) and 16 ARFs (Lavy et al., 2016). However, despite the highly reduced size of these gene families, auxin signaling appears to function similarly in these plants as it does in angiosperms (Flores-Sandoval et al., 2015, 2016; Kato et al., 2015, 2017, 2018). In *Arabidopsis*, for example, there are six TIR1/AFBs, 29 Aux/IAAs, 23 ARFs and four TPL relatives. The reduced auxin signaling network in bryophytes, along with their position as one of the earliest diverging groups of land plants (Harris et al., 2020; Morris et al., 2018), make them useful for inferring how the functions of this network have evolved.

In *M. polymorpha*, all the functional domains of MpTIR1, MpIAA, the MpARFs and MpTPL are intact (Flores-Sandoval et al., 2016; Kato et al., 2020). Translational fusions of MpTPL to either MpARF1 or the PB1 domain of MpIAA1 repress the auxin response and lead to severe stunting of growth (Flores-Sandoval et al., 2015, 2016). These experiments prove that MpTPL can repress auxin-responsive genes, and that MpARFs are able to dimerize with MpARF1 and MpIAAs (Flores-Sandoval et al., 2015, 2016). Although MpTIR1 function in auxin-dependent degradation of MpIAAs has not been measured directly, it has been shown that auxin-dependent growth and development require MpIAA, MpTPL and the A class ARF MpARF1 (Flores-Sandoval et al., 2015, 2016). A more recent study further detailed the specific role of MpARF1 in regulating the timing and position of cell divisions early in the development of gemma primordia, determining their axis of growth and ultimately their three-dimensional structure (Kato et al., 2017). This developmental function is highly reminiscent of the role of A class ARFs in controlling asymmetric cell divisions during early embryogenesis in the angiosperm *Arabidopsis thaliana* (Prigge et al., 2020). MpARF1 knockout plants are still able to develop structures necessary to survive (Kato et al., 2017). This initially seems surprising as MpARF1 is the only activator ARF in *M. polymorpha*, and MpARF2 and MpARF3 have been shown to be unable to complement ARF1 (Kato et al., 2020). However, it is likely that auxin-independent expression of these genes is mediated by other transcription factors (Kato et al., 2017). Together, these studies show that both the molecular functions and the developmental roles of auxin signaling circuit components are highly conserved across land plants.

Studies have employed the simplicity of the *P. patens* auxin signaling pathway to reveal functions of its components that were difficult to probe in angiosperms. For example, work in *P. patens* has shown that no auxin response is possible in full Aux/IAA knockouts, suggesting that Aux/IAAs are required for auxin response regulation (Lavy et al., 2016). These Aux/IAA null strains also show that repression is mediated by a combination of Aux/IAAs and B class repressor ARFs (Lavy et al., 2016). The B class ARF-mediated repression is weaker than that conferred by Aux/IAAs and does not require association with TPL, leading to the conclusion that it results from direct competition with the A class ARFs for DNA-binding sites (Lavy et al., 2016). Another study highlighted the complexities of Aux/IAA-induced repression using a *P. patens* strain in which PpIAA1a is the only functional Aux/IAA (Tao and Estelle, 2018). Importantly, PpIAA1a on its own is sufficient to enable wild-type auxin responses. When the interaction with TPL is removed, PpIAA1a is still able to repress the auxin response (Tao and Estelle, 2018). Moreover, further mutagenesis revealed that an Aux/IAA monomer retains function (Tao and Estelle, 2018). These suggest an additional dimension to Aux/IAA repression. In addition to multiple Aux/IAAs oligomerizing with

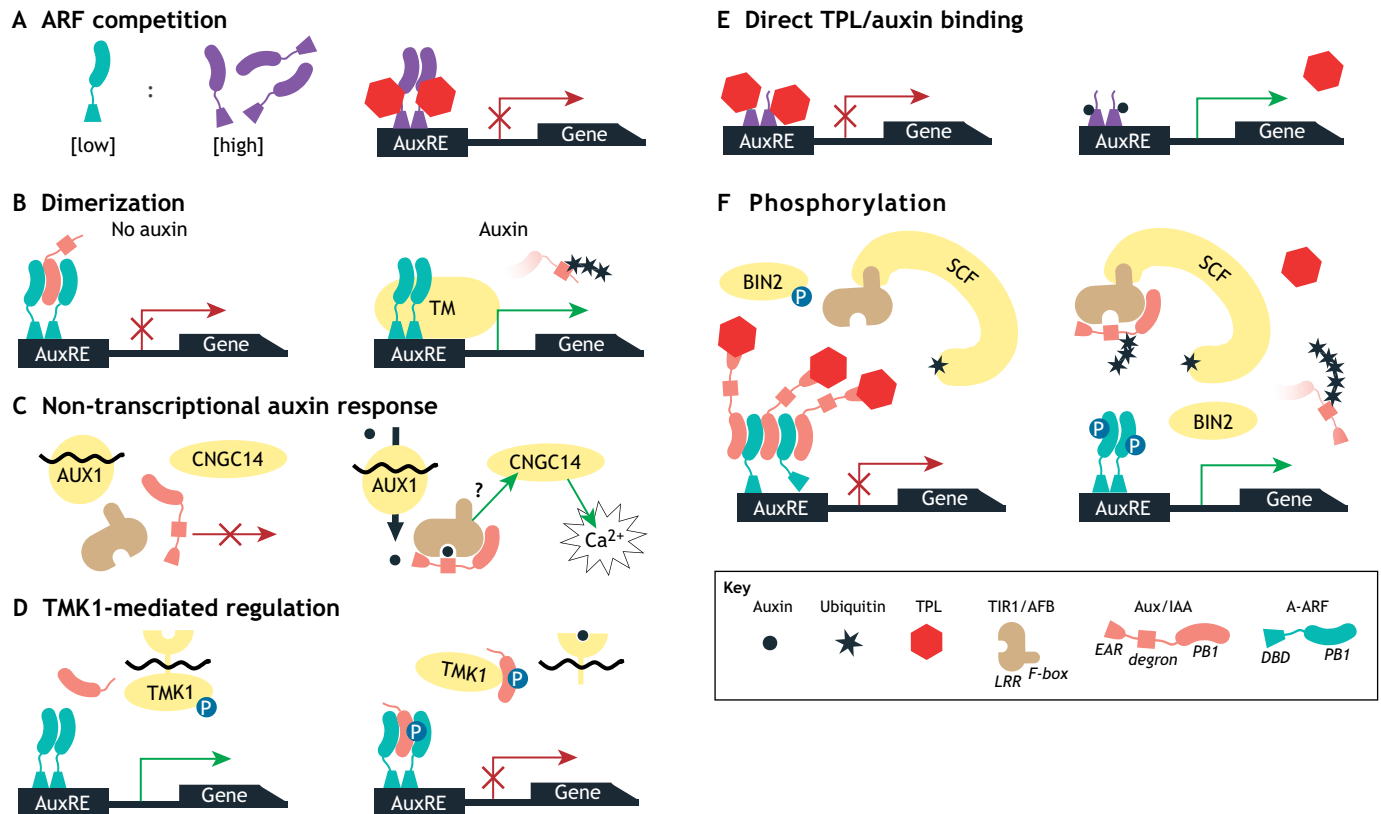


Fig. 2. A plethora of ways to regulate and signal through the simple auxin signaling pathway. (A) At high concentrations in the nucleus, A/B, B and C ARFs (purple) outcompete a low concentration of A-ARFs (blue) in binding AuxREs. This replaces the auxin-dependent repression of A-ARF with the auxin-independent repression of B and C ARFs, where these ARFs recruit TPL to AuxREs and repress auxin-inducible genes. (B) ARF dimerization is needed for full activation, acting at least in part by leading to increased affinity of class A ARFs for DNA. However, even without TPL, Aux/IAAs can contribute to repression of class A ARFs by disrupting their dimerization. (C) A rapid, non-transcriptional auxin response can also occur. This takes place outside the nucleus, where AUX1 transports auxin into the cytoplasm, triggering cytoplasmic TIR1 and likely Aux/IAAs to activate CNGC14 through an unknown pathway. This in turn allows calcium (Ca^{2+}) into the cell, rapidly depolarizing it. (D) TMK1 is a plasma membrane-localized, trans-membrane receptor-like kinase. When auxin is present, the kinase domain of TMK1 is cleaved and phosphorylates non-canonical IAAs (ncIAAs) that lack a degron. This phosphorylation then strengthens repression of some auxin-induced genes. (E) ETTIN/ARF3 (ETT, purple) is a B-class ARF that has no PB1 domain but is able to directly bind TPL. Auxin perception disrupts the interaction between ETT and TPL, thereby activating gene expression. (F) BIN2 phosphorylates (P) A class ARFs, preventing Aux/IAAs from binding to them, and strengthening their bond to AuxREs. BIN2 also facilitates Aux/IAA degradation in an auxin-independent manner, de-repressing auxin-inducible genes.

activator ARF pairs and recruiting TPL, single Aux/IAAs may disrupt A-ARF pairs by wedging themselves between ARF PB1 domains (Fig. 2B). In *A. thaliana*, A-ARF dimerization at the PB1 domain contributes to DNA binding (Pierre-Jerome et al., 2016). It is worth noting that quantifying this effect for *A. thaliana* proteins requires reconstructing the entire auxin pathway in a heterologous context, another argument in favor of making full use of bryophyte models.

Studies of bryophyte auxin signaling networks have therefore proven a useful tool, in both clarifying the mechanisms of nuclear auxin signaling, as well as for ascertaining the functions of individual components. Considering that the auxin nuclear response first appears early in land plant evolution, it is surprising that bryophyte auxin signaling is so similar to that in flowering plants at the molecular, cellular and organismal levels. This suggests that auxin signaling proteins have retained their fundamental functions throughout most of their evolutionary history.

Evolving complexity in signaling components enables diversity in plant form

In parallel to the evolution of morphological and physiological complexity in land plants, the gene families encoding auxin signaling

components have undergone sizable expansions (Mutte et al., 2018). Several recent studies bolster the argument that this expansion contributed to evolutionary innovations (Lavy and Estelle, 2016; Matthes et al., 2019; Mutte et al., 2018; Wang and Estelle, 2014; Israeli et al., 2020). The 1001 Genome Project (Weigel and Mott, 2009), with its ever-growing number of sequenced *A. thaliana* accessions, has been a tremendous boom to plant evolutionary studies. This community resource greatly facilitates the linking of morphological or physiological variation to differences in molecular expression and/or function caused by changes in genome sequence. In one study, a natural hypermorphic variant of AtTIR1 was identified (Wright et al., 2017). This variant alters root architecture when expressed in a commonly used lab accession, and the roots of most accessions carrying this variant were found to be more sensitive to exogenous auxin. A follow-up to this work was the development of a webtool called Visualizing Variation (ViVa), which aims to make use of the 1001 Genomes data a routine and readily accessible part of every molecular biologist's toolkit (Hamm et al., 2019). When ViVa was applied to auxin pathway components, several interesting patterns emerged, including the observation that recently duplicated sister proteins in the large *Arabidopsis* Aux/IAA family frequently have very different rates of sequence diversification, suggesting that

one sister was becoming neo- or sub-functionalized, while the other was retaining a more ancestral function (Hamm et al., 2019). This is consistent with the detailed analysis of the diversification in sequence, expression pattern and function of one such sister pair: *IAA6* and *IAA19* (Winkler et al., 2017). Together, these and other approaches are beginning to shed light on how expanding auxin signaling networks are evolving, and how this expansion might have driven diversification in the dynamics of auxin signaling pathways and plant form.

Evolution of the rapid auxin response

One evolutionary innovation observed in *Arabidopsis* roots enables auxin to induce very rapid growth responses that are too fast to require *de novo* protein production but still require *AtTIR1* (Shih et al., 2015). The key to pinpointing this response, and connecting it to *AtTIR1*, was the development of two new technologies. The first allowed researchers to measure rapid changes in auxin response in combination with a means to rapidly apply and remove exogenous auxin. Work with this system provided conclusive evidence that the first auxin-regulated growth responses precede transcriptional effects (Fendrych et al., 2018). By combining this set-up with a second technology – an engineered *Arabidopsis* TIR1 (*ccvTIR1*) that responds exclusively to an engineered auxin molecule (*cvxIAA*) (Uchida et al., 2018) – researchers could illustrate unambiguously that auxin sensing by *AtTIR1* is sufficient for triggering the rapid response (Fendrych et al., 2018). Additional work in root hairs connects this rapid response to AUXIN RESISTANT 1 (*AUX1*)-mediated auxin uptake followed by CYCLIC NUCLEOTIDE-GATED CHANNEL 14 (*CNGC14*)-mediated Ca^{2+} depolarization of cells (Dindas et al., 2018). MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascade-mediated regulation of auxin-induced cell expansion via RHO-LIKE GTPASES FROM PLANTS (*ROPs*) (Enders et al., 2017) may also be connected to this mode of auxin signaling. Given work in *P. patens* showing that Aux/IAAs are required for an auxin response (Lavy et al., 2016), the non-transcriptional auxin response likely requires both TIR1/AFBs and Aux/IAAs, but may not need ARFs or TPL (Fig. 2C). However, this hypothesis only holds true if the rapid auxin response exists in *P. patens*, which remains to be determined.

A recent genetic tour-de-force combining all *TIR1/AFB* loss of function mutants in *A. thaliana* revealed that *AtTIR1* and its closest paralog *AtAFB1* have been sub-functionalized to elicit these rapid, non-transcriptional auxin responses (Prigge et al., 2020). *AtAFB1* and *AtTIR1* arose from a duplication at the base of Brassicales – a group of flowering plants including *A. thaliana* (Prigge et al., 2020). Although *AtAFB1* is highly conserved in *A. thaliana* (Hamm et al., 2019), it is divergent across plant species, evolving three times more rapidly than *AtTIR1* (Prigge et al., 2020). It also has a substitution in a residue crucial for full function in auxin-dependent Aux/IAA degradation (Yu et al., 2015), leading to the hypothesis that it has undergone non-functionalization. However, when the function of all TIR1/AFBs was measured during rapid bending in response to a change in gravity vector, *AtAFB1* function was seen to be essential for normal growth kinetics involving the rapid response (Prigge et al., 2020). Whether this special role for *AtTIR1* and *AtAFB1* among the receptors represents an early bifurcation in function among the receptor family, or instead reflects a division of labor seen only in some species, will be exciting to investigate in future work.

Evolution of Aux/IAA degradation rates

Expansion of the gene families encoding auxin components raises the question of whether there are unique roles for other members, as

seen for *AtAFB1* and *AtTIR1*. The heterologous expression of auxin pathway components in yeast has enabled direct testing of many components in isolation from one another (Pierre-Jerome et al., 2014). One insight gleaned from this approach is that the identity of both TIR1/AFB and Aux/IAA components can have profound impacts on auxin-induced degradation rates (Havens et al., 2012). In addition, experiments using the yeast system to study auxin signaling components from *Zea mays* (maize) found fairly limited differences in degradation rates between *ZmAux/IAAs* when expressed with a maize receptor, whereas a broad range of rates could still be detected when the assays included a receptor from *A. thaliana* (Ramos Báez et al., 2020).

Aux/IAAs act as co-receptors with TIR1/AFBs, directly binding auxin along their degron domain. Although mutations within the degron can dramatically stabilize Aux/IAAs, their degradation rate is also impacted by sequences outside this region. Degron domains that enable fast or slow degradation in *A. thaliana* confer different behaviors in *P. patens* (Tao and Estelle, 2018). Swapping the domains of two *A. thaliana* Aux/IAAs showed that most domains play a role in the auxin-induced affinity of the Aux/IAAs to the *AtTIR1* receptor (Niemeyer et al., 2020). The same study demonstrated that *AtTIR1* has specific residues outside the auxin-binding region that greatly facilitate Aux/IAA interaction (Niemeyer et al., 2020). Importantly, changing the rate of auxin-induced degradation was able to alter the pace of lateral root development in transgenic *A. thaliana* plants (Guseman et al., 2015), suggesting that this property may be optimized during evolution. Future work that includes more species, and especially more receptors, could shed light on how important auxin sensitivity/degradation rate is in different contexts.

Evolution of distinct auxin signaling circuits

Auxin can produce a multitude of distinct transcriptional responses depending on which cells or tissues are involved. One way to explain such context-specific auxin functions would be if each ARF had a distinct DNA-binding preference, as ARFs vary in their expression patterns (Truskina et al., 2021). Several recent studies have provided evidence that this is at least partially true when comparing DNA-binding preferences of A and B class ARFs (Boer et al., 2014; Galli et al., 2018; Freire-Rios et al., 2020). However, a recent study in *P. patens* revealed that both classes of ARFs have the same binding preferences (Lavy et al., 2016; Kato et al., 2020). Moreover, in all species examined to date, ARFs within a single class share binding preferences. A large-scale analysis of *ZmARF* binding sites (Galli et al., 2018) and a synthetic study in yeast that used clade A ARFs from both *A. thaliana* and maize (Lancot et al., 2020) point to nearly identical binding site preferences within the class A ARFs. When comparing binding specificities of A, B and C class ARFs, as well as those of the charophyte algae *C. atmophyticus* ARF, C class ARFs are seen to bind to a broader array of promoters (Martin-Arevalillo et al., 2019). These differences in DNA specificity could be due to observed differences in residues within the DNA-binding domain (Kato et al., 2020), as well as to differences in ARF interactors. Recent *in vivo* analysis and transcriptome analysis has also shown that differences in DNA architecture, such as AuxRE repeat organization, affect the affinity of *AtARF5* (class A) and *AtARF1* (class B) for different promoters (Freire-Rios et al., 2020). Because significant differences in DNA specificity have not been shown in A class ARFs, it remains challenging to resolve how distinct transcriptional modules are encoded.

One emerging model poses that the ratios of ARFs and other signaling components provide specificity. In *Marchantia*, MpARF1

(class A) and MpARF2 (class B) have similar DNA affinity and binding preferences (Kato et al., 2020). In addition, it has been observed that inducing the overexpression of MpARF1 alone maintains auxin responsiveness but inhibits growth, while overexpressing MpARF2 leads to auxin insensitivity (Kato et al., 2020). Overexpression of both ARFs together restores auxin sensitivity. Thus, the relative stoichiometry and not absolute levels of the different ARF classes is proposed to be the key parameter for determining the amplitude, and perhaps duration, of auxin-induced transcriptional effects (Fig. 2A) (Kato et al., 2020). The same study was also able to quantify the levels of the two ARFs across gemma cells, revealing differences in the ratio of protein accumulation in different cells (Kato et al., 2020). Moreover, the expression of A class ARFs in *Arabidopsis* was recently shown to be regulated by a network of repressors, with the interaction networks varying for different ARFs (Truskina et al., 2021). This provides a mechanism by which different A class ARFs might vary in their accumulation across different plant tissues, leading to context-specific auxin responses.

The ratio of ARFs and Aux/IAAs also contributes to signaling dynamics. A comprehensive molecular genetic study of A class ARFs in *Solanum lycopersicum* (tomato) has linked subfunctionalization within the family to different aspects of leaf shape determination (Israeli et al., 2019), revealing that a specific expression ratio of SLAux/IAAs and SLARFs in the developing leaf primordia is required to ensure the appropriate formation of discrete leaflets. Specifically, SLAux/IAAs act to suppress growth between leaflets, whereas SLARFs stimulate expansion. Changing the ratios of these proteins results in leaves that range from simple and fully expanded to extremely reduced and needle-like (Israeli et al., 2019). Interestingly, gene redundancy and subsequent robustness are also showcased in this work. In double mutants for an SLAux/IAA and an SLARF, the average number of leaflets per leaf is similar to that observed in wild-type plants, although the variation in leaflet number between leaves increases (Israeli et al., 2019, 2020).

There are also reports of unexpected avenues of diversification of auxin pathway components. One recent study showed that AtIAA32 and AtIAA34 – both highly diverged Aux/IAAs with minimal, if any, response to auxin – interact with TRANSMEMBRANE KINASE 1 (TMK1) to activate auxin-responsive growth in the apical hook (Cao et al., 2019) (Fig. 2D). A second study focused on AtARF3/ETTIN (ETT), which directly binds to auxin (Kuhn et al., 2020) (Fig. 2E), thereby bypassing the ubiquitylation of Aux/IAAs and directly disrupting TPL binding to relieve repression on target genes. This appears to be a textbook example of neofunctionalization. A third study connects the kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2) to auxin-independent regulation of auxin-inducible genes. BIN2 was found to phosphorylate A class ARFs in *Arabidopsis* AtARF7 and AtARF19 with multiple auxin response-promoting results, including a facilitation of their interaction with AuxREs, a weakening of their interaction with Aux/IAAs and a strengthening of the Aux/IAA interaction with the SCF^{TIR1} complex (Fig. 2F) (Cho et al., 2014). Phosphorylation by BIN2 also results in loss of DNA binding and repression of activities of AtARF2, a class B ARF (Cho et al., 2014). It is exciting to ponder whether more of this type of radically unexpected variation will be uncovered as auxin responses are studied in more detail in more species.

Conclusions and future perspectives

We still have much to learn about the evolutionary origins of auxin signaling, especially with regard to how individual genes are co-opted to serve new functions and how crosstalk is minimized

between auxin modules. Outside of the model that A and B class ARFs bind to and compete for the same AuxREs, there remain many mysteries about B and C class ARF function. Learning more about the roles of A/B class ARF in algae might help us to better understand what makes A and B class ARFs fundamentally different from C class ARFs and provide clues into the origins of ARF classes. Intriguingly, some flowering plant C class ARFs interact with specific Aux/IAAs (Piya et al., 2014; Vernoux et al., 2011). Have some of these ARFs become neofunctionalized to perform an auxin signaling function in other plants? In addition, there are tantalizing unanswered questions about the rapid auxin response. Is it conserved in all land plants? If, as the work in bryophytes suggests, all auxin signaling requires Aux/IAAs, are Aux/IAAs necessary for the non-transcriptional auxin response? What is the relationship between events at the plasma membrane and those in the nucleus, and is that relationship different in different cell types or plant lineages? More studies in *Marchantia* and *Physcomitrium*, as well as studies in a broader array of angiosperms, would help to answer these questions.

Despite its deceptive simplicity, the nuclear auxin signaling pathway continues to reveal complexity in functions. Studies in tomato, *Arabidopsis* and maize have shown that many of the fundamental functions of auxin signaling components are highly conserved across land plants. They have also revealed a number of new signaling mechanisms. Included in these mechanisms are: differences in protein ratios (Israeli et al., 2019); non/sub-functionalization of components (Israeli et al., 2019); binding competition between ARFs as well as with other signaling proteins (Kato et al., 2018); and the conservation and extent of non-canonical functions, such as direct auxin interactions and direct TPL interactions (Causier et al., 2012; Simonini et al., 2016). These novel functions translate into diversity in plant architecture, and highlight subtle control of the auxin response beyond a simple on/off growth signal.

Although genetics has enabled great advances in identifying the function of auxin pathway components, many single mutants are lethal, highly pleiotropic or have no phenotype at all. Moving forward, it would be particularly useful to observe phenotypes in specific cell types or at crucial time points in development (Decaestecker et al., 2019). Although studies have shown similarities in circuit function between angiosperms and flowering plants, there are many other plant families that remain understudied. CRISPR/Cas9-based genome editing makes it feasible to build multi-knockout lines in Aux/IAAs and ARFs in diverse species. In combination with new technologies that allow for easier comparative analysis of natural or engineered variation (Ramos Báez et al., 2020), generating genetic resources in a phylogenetically informed collection of plants should ultimately make it possible to connect changes in specific amino acid sequences in auxin signaling components to key innovations in development. It will also be fascinating to learn whether evolution has re-parameterized auxin signaling with compensatory changes across the network when confronted with novel hyper- or hypomorphic variants.

Finally, work on the auxin pathway could be used as a scaffold to better understand other plant signaling pathways, especially those with similar structures. Jasmonic acid (JA) is of particular interest, as the evolutionary history of auxin and JA perception are closely linked (Blázquez et al., 2020; Wang et al., 2015). What was the function of the shared ancestral signaling pathway in early land plants? Genomics studies of charophyte algae and bryophytes, alongside ancestral sequence reconstruction of critical nodes combined with functional analysis in bryophytes and angiosperms, could help us reconstruct

this pivotal moment in plant evolution. By understanding how plants came to survive a completely new existence on land, we may find clues for engineering plants that are able to meet the challenges of tomorrow.

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Competing interests

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