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Transcriptional Responses to the Auxin Hormone

Dolf Weijers[1] and Doris Wagner[2]

[1]Laboratory of Biochemistry, Wageningen University, 6703 HA Wageningen, The Netherlands; email: dolf.weijers@wur.nl

[2]Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104; email: wagnerdo@sas.upenn.edu

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Abstract

Auxin is arguably the most important signaling molecule in plants, and the last few decades have seen remarkable breakthroughs in understanding its production, transport, and perception. Recent investigations have focused on transcriptional responses to auxin, providing novel insight into the functions of the domains of key transcription regulators in responses to the hormonal cue and prominently implicating chromatin regulation in these responses. In addition, studies are beginning to identify direct targets of the auxin-responsive transcription factors that underlie auxin modulation of development. Mechanisms to tune the response to different auxin levels are emerging, as are first insights into how this single hormone can trigger diverse responses. Key unanswered questions center on the mechanism for auxin-directed transcriptional repression and the identity of additional determinants of auxin response specificity. Much of what has been learned in model plants holds true in other species, including the earliest land plants.

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This review examines recent advances in our understanding of how auxin sensing triggers changes in transcription or in cellular properties during plant development. Current challenges include identifying the mechanism by which auxin gradients are read and interpreted to enable to cell type–specific transcriptional or cellular responses. The individual contributions of the large number of paralogous factors that encode the key components of the auxin response pathway in flowering plants also remain to be determined. In addition, very few target genes are known to be directly controlled by auxin-responsive transcription factors. Finally, the cascade of events required for auxin-dependent changes in gene activation and repression needs to be elucidated.

TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX (TIR1/AFB) proteins: nuclear F-box proteins that—together with Aux/IAA proteins—form the auxin receptor

AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins: small nuclear proteins that physically interact with ARFs and prevent them from regulating transcription; they also serve as part of the auxin receptor

AUXIN RESPONSE FACTORs (ARFs): sequence-specific DNA-binding proteins that trigger transcriptional changes in responses to auxin

THE PLAYERS: CORE COMPONENTS THAT TRANSLATE AUXIN SENSING TO TRANSCRIPTIONAL RESPONSES

The path from auxin signal perception to altered gene expression is short in both the physical and genetic senses. The key components of this pathway are the TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX (TIR1/AFB) F-box proteins, the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional coregulators, and sequence-specific binding proteins called AUXIN RESPONSE FACTORs (ARFs). A coreceptor comprising a TIR1/AFB F-box protein and an Aux/IAA transcriptional coregulator senses auxin (21). Auxin promotes the interaction between TIR1/AFB and Aux/IAA, thereby triggering ubiquitin-mediated degradation of the Aux/IAA proteins via the proteasome (72, 234). Aux/IAA proteins generally act as corepressors to prevent auxin-responsive transcription (140, 196, 197, 203). *Arabidopsis* has 6 and 29 paralogous members in the TIR1/AFB and Aux/IAA families, respectively.

TIR1/AFB proteins are incorporated into a four-subunit $SCF^{TIR1/AFB}$ complex. Both this complex and the small Aux/IAA proteins are localized in the nucleus (2). All TIR1/AFB proteins bind auxin (46, 143). Four TIR1/AFB family members have been shown to promote auxin responses, and

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mutants in these factors are either subtly auxin resistant as single mutants (*tir1*, *afb2*, and *afb3*) or strongly resistant as higher-order mutants (*afb1*) (49, 143). The *tir1/afb* mutants also display morphological defects consistent with a role in auxin perception. Mutants in other components of the $SCF^{TIR1/AFB}$ ubiquitin ligase complex, such as ARABIDOPSIS SKP1 HOMOLOGUE (ASK1), CULLIN 1 (CUL1), or RING-BOX 1 (RBX1), also cause auxin resistance (67, 70–72, 86, 124). Members of the TIR1/AFB family have an N-terminal leucine-rich-repeat region and a C-terminal F-box domain (**Figure 1*a***). AFB4 and AFB5 have additional protein domains and bind auxin analogs differently than do TIR1 and AFB1–3 (21) (**Figure 1*a***). The crystal structure of TIR1-ASK1 in a complex with auxin and a small Aux/IAA peptide has been solved (193), revealing that the leucine-rich-repeat domain of TIR1/AFB contains the auxin-binding pocket, whereas the F-box domain contacts ASK1. The Aux/IAA peptide was also in contact with the leucine-rich-repeat domain at the auxin-binding site. These results suggest that auxin stabilizes the interaction between TIR1/ASK and the Aux/IAA proteins (reviewed in 144, 172). The structure of TIR1 with full-length Aux/IAA has not been solved. The TIR1/AFB proteins also contact the CUL1 subunit of the $SCF^{TIR1/AFB}$ complex via the F-box domain, which is linked to autocatalytic degradation (233).

Aux/IAA proteins do not have DNA-binding motifs and are instead recruited to genomic regions by ARF proteins, with whom they physically interact via shared C-terminal domains (196, 228) (**Figure 1*b***). Aux/IAA proteins in general have one or two N-terminal ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR–ASSOCIATED REPRESSOR (EAR) or EAR-like repressor motifs (domain 1); a central region that is required for the TIR1/AFB interaction and hence for degradation (the degron, or domain 2); and a C-terminal Phox and Bem 1 (PB1) protein-protein interaction domain that mediates both homo- and heterodimerization (reviewed in 75) (**Figure 1*b***). Two recent studies demonstrated that the multimerization of Aux/IAA proteins in a head-tail configuration is mediated by the PB1 domain (50, 100). Earlier studies had revealed strikingly different auxin sensitivities for different Aux/IAA proteins with half-lives from minutes to hours (52, 72, 139, 222, 234). An elegant follow-up study showed that this translates into different auxin-binding affinities of the coreceptor (K_d ranging from 10 to 300 nM) (21) and concluded that the auxin sensitivity of the coreceptor is determined largely by the Aux/IAA moiety. However, the TIR1/AFB moiety also contributes to the auxin sensitivity of the coreceptor (21, 143).

Individual Aux/IAA proteins differ significantly in the presence and conservation of domains important for auxin sensitivity/protein stability and association with TIR1 (52). Most important in this regard is the degron (domain 2) and a conserved pair of amino acids [lysine and arginine (KR)] between domains 1 and 2 (**Figure 1*b***). The presence of a canonical degron and the KR motif enhances the affinity of the coreceptor for auxin and causes lower stability (degradation at a lower auxin concentration) of the Aux/IAA protein (21, 52, 82, 125, 139, 154, 222). Some Aux/IAAs have variant degrons or entirely lack this domain, which leads to reduced sensitivity or insensitivity to auxin, respectively (21, 52, 125). Mutations in the degron render Aux/IAA proteins insensitive to auxin (reviewed in 156), presumably because the core residues in the degron directly interact with the TIR1-auxin complex (193). Aux/IAAs that carry a lysine-glutamine (KQ) motif or have no KR motif also exhibit decreased auxin sensitivity (21, 52, 125). The presence of polar amino acids downstream of the degron enhance TIR1/AFB interaction, and their mutation leads to gain-of-function phenotypes of Aux/IAA proteins that are similar to but less severe than mutations in the degron motif (125). The degron has been engineered into enhanced sensitivity auxin reporters (20, 107) and has been employed as a regulatory module in metazoans (88, 132).

Key for the transcriptional response to auxin are sequence-specific transcriptional regulators of the ARF family. ARF proteins bind to so-called auxin response elements (AuxREs), *cis*-regulatory sequences that at their core contain a TGTC motif that is sufficient to recruit ARF proteins (195, 199, 201). Recent elegant structural analyses as well as protein array data have indicated that

Auxin response elements (AuxREs): *cis*-elements bound by ARF proteins

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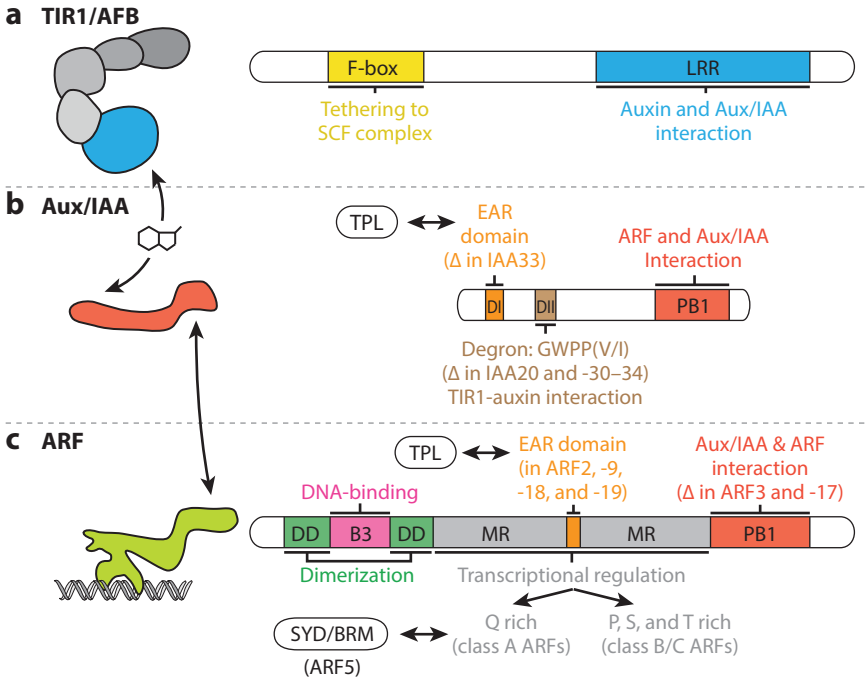

Figure 1

Domain architecture of central components of auxin-dependent gene regulation. Auxin responses are mediated by interactions (*arrows*) between three core components: (*a*) TIR1/AFB auxin receptors, (*b*) Aux/IAA transcriptional repressors, and (*c*) ARF transcription factors. TIR1/AFB proteins contain an F-box domain for tethering to the other subunits in the SCF E3 ubiquitin ligase complex and a leucine-rich-repeat (LRR) domain that carries the auxin-binding pocket and Aux/IAA contact site. Aux/IAA proteins consist of domain 1 (D1, missing in IAA33), which harbors an EAR motif that mediates interaction with TPL; domain 2 (D2, missing in IAA20 and -30–34), which carries the degron [the conserved amino acid sequence GWPP(V/I), which acts as the contact site with TIR1/AFB and auxin]; and a PB1 domain, which mediates oligomerization and Aux/IAA-ARF heterodimerization. ARFs have an N-terminal B3 DNA-binding domain flanked on either side by a dimerization domain (DD), followed by a middle region (MR) that mediates transcriptional regulation. This domain can contain an EAR motif (in ARF2, -9, -18, and -19) for interaction with TPL; it is glutamine (Q) rich in class A ARFs but proline (P), serine (S), and threonine (T) rich in class B and C ARFs. In ARF5, this domain mediates the interaction with SYD and BRM. At their C termini, ARFs (with the exception of ARF3 and -17) have a PB1 domain for oligomerization and Aux/IAA-ARF heterodimerization. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; BRM, BRAHMA; EAR, ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR–ASSOCIATED REPRESSOR; PB1, Phox and Bem 1; SYD, SPLAYED; TIR1/AFB, TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX; TPL, TOPLESS.

diverse *Arabidopsis* ARFs (ARF1, -3, and -5) preferentially bind the larger TGTCGG motif (17, 61). ARF proteins can be grouped into three classes from the early land plants onward (58, 59, 95) (**Figure 1*c***). Class A comprises ARFs with a glutamine (Q)–rich middle region that are classified as transcriptional activators based on transient gene expression assays in protoplasts (200). The Q-rich domain is present in all class A ARFs (58, 95). Recently, characterization of an allelic series of *monopteros* (*mp*) mutant alleles in the *Arabidopsis* Columbia ecotype has highlighted the importance of this domain (133). The remaining ARFs are classified as repressors based on the same protoplast

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assay or sequence homology (195, 200) and can be divided into the *microRNA 160* (*miR160*)–targeted ARFs (class C) and the remaining ARFs (class B) (**Figure 1*c***). Structural analyses of the ARF DNA-binding region revealed that the B3 DNA-binding domain is embedded in an ARF dimerization domain and that ARF proteins preferentially bind to inverted AuxRE repeats (17). Most but not all ARFs have a C-terminal PB1 domain (**Figure 1*c***), which is important for the physical interactions between ARFs and Aux/IAA proteins (100, 129). Aux/IAA proteins inhibit the transcriptional activity of ARFs specifically under low-auxin conditions (197). The PB1 domain may also contribute to ARF-ARF dimerization (75, 100, 129).

How ARFs execute their roles in gene repression is not well understood. Although many class B and C ARFs have PB1 domains, most appear to not interact very strongly with Aux/IAA proteins (146, 206). Richter et al. (162) have suggested that class B and C ARFs may interfere with the activity of class A ARFs in an auxin-independent fashion, for example, by competing for DNA-binding sites or blocking activating ARF activity via heterodimerization. However, genetic studies have linked class B and C ARFs to auxin-regulated processes (131, 180, 181), whereas transient transcription assays have suggested an Aux/IAA-independent mechanism for auxin-regulated gene expression also for class A ARFs (211). Several class B (repressive) ARFs (ARF2, -9, and -18) and a single class A ARF (ARF19) have an EAR domain, implicating them in recruitment of corepressor complexes (23). Because studies have also suggested that class A ARFs repress certain target genes (235, 237), there is at present no satisfying model for auxin-dependent gene repression.

Aux/IAA inhibits activating ARFs bound at their target loci by recruitment of corepressor complexes. The EAR repressor motif in domain 1 of the Aux/IAA proteins physically interacts with and recruits Tup1/Groucho/TLE family proteins called TOPLESS (TPL) or TOPLESS RELATED (TPR) (23, 96, 116, 192) (**Figure 2**). The cocrystal between TPL and the Aux/IAA EAR domain has recently been solved (96), showing that the interaction requires the so-called CTLH region at the TPL N terminus. The EAR motif–interacting region of TPL forms a tetramer that interacts more strongly with oligomerized EAR domain–containing partners, which may represent one biological role for the observed Aux/IAA protein oligomerization (100). Other components may also be needed for repression. In addition to its important function in preventing unlicensed gene expression in the auxin pathway, TPL also interacts with many other types of EAR domain–containing transcriptional repressors in diverse developmental and stress pathways (23, 135, 167).

Repression of auxin response gene expression in low auxin further requires histone deacetylases (HDACs) such as HDA19. Loss of *HDA19* activity partially rescues the phenotypes associated with gain-of-function mutations in Aux/IAA-encoding genes, and both TPL and HDA19 are recruited to activating ARF-binding sites specifically in low-auxin conditions (192, 224). TPL recruits HDAC complexes in plants (102) (**Figure 2**), as has been reported for its metazoan counterparts (24, 212). HDACs remove acetyl groups from lysines on histones (primarily histones H3 and H4), which leads to a more compact chromatin state and reduced accessibility of the genomic DNA for transcription factors or the general transcriptional machinery (53). The compacted chromatin prevents auxin-responsive gene expression when Aux/IAA levels are high and auxin levels are low. An increase in auxin levels leads to Aux/IAA protein degradation and eviction of TPL and HDAC from activating ARF target sites but leaves the compacted chromatin behind (224).

A recent study showed that another class of chromatin regulatory proteins, the SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin-remodeling ATPases, helps overcome this repressed chromatin state upon auxin sensing (**Figure 2**). SWI/SNF chromatin-remodeling complexes use the energy derived from ATP hydrolysis to alter the occupancy or positioning of nucleosomes on the DNA, thereby changing the accessibility of the genomic DNA in the context of chromatin (33). The Q-rich middle region of the class A ARF5 MP physically

TOPLESS (TPL): a corepressor protein that interacts with EAR domain–containing proteins (including Aux/IAA proteins), forms tetramers, and recruits chromatin regulators such as HDACs

Histone deacetylases (HDACs): enzymes that remove acetyl groups from lysines on histones and form a compact chromatin state refractory to transcription

SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin-remodeling complexes: large (2-MDa) complexes that utilize energy derived from ATP hydrolysis to alter the accessibility of genomic DNA in the context of chromatin

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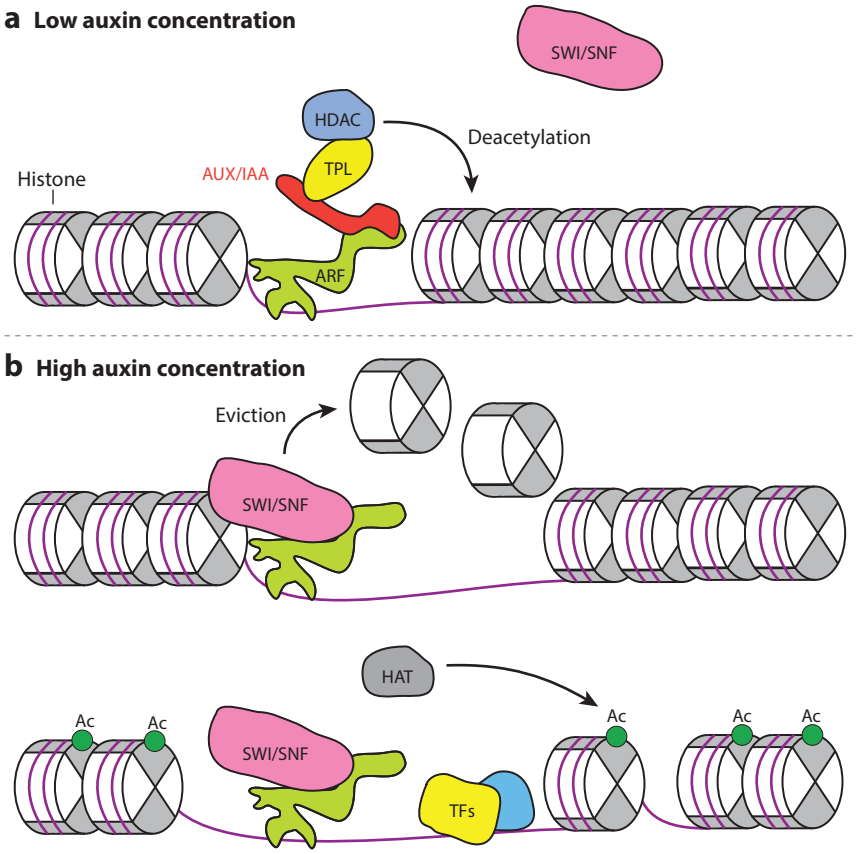

Figure 2

Control of gene expression by ARF proteins in a chromatin context. (*a*) Under low auxin concentrations, Aux/IAA proteins complex with ARFs bound at target loci and recruit the TPL corepressor and histone deacetylases (HDACs), resulting in a compact chromatin environment that prevents unlicensed auxin response gene expression. Aux/IAA proteins also block the physical interaction between MP and the BRM and SYD SWI/SNF chromatin-remodeling complexes. (*b*) Under high auxin concentrations, sensing of auxin causes a rapid degradation of Aux/IAA proteins, which leads to loss of TPL and HDAC complexes and allows SWI/SNF complexes to interact with ARFs (MP/ARF5). SWI/SNF-mediated chromatin remodeling destabilizes nucleosomes near the MP-bound sites, freeing binding sites for additional transcription factors (TFs). These factors bind to ARF target loci, which is followed by recruitment of histone acetyltransferases (HATs). Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; BRM, BRAHMA; MP, MONOPTEROS; SWI/SNF, SWITCH/SUCROSE NONFERMENTING; SYD, SPLAYED; TOP, TOPLESS. Additional abbreviation: Ac, acetylation.

interacts with and recruits plant SWI/SNF chromatin-remodeling complexes formed around BRAHMA (BRM) or SPLAYED (SYD) to MP target loci (224). BRM and SYD are necessary for auxin-responsive gene expression and execute this role by unlocking the repressed chromatin state at MP target loci, which opens up binding sites for additional transcription factors. Under low auxin, SWI/SNF remodeler recruitment is blocked by Aux/IAA proteins complexed with MP. Intriguingly, tethering SWI/SNF complexes to MP target loci rescued morphological defects of *mp* mutants, indicating that SWI/SNF complex recruitment is likely a major function of MP.

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Additional chromatin regulators, such as histone acetyltransferases (HATs), likely act after this step. Given that the chromatin-remodeling and histone (de)acetylation steps may take time to execute, an interesting question is whether fast auxin responses—those occurring within minutes (1)—are based on this mechanism.

Histone acetyltransferases (HATs): enzymes that add acetyl groups to lysines on histones, promoting transcriptional activation

QUANTITATIVE CONTROL OF TRANSCRIPTIONAL OUTPUT IN RESPONSE TO AUXIN

As discussed above, the core auxin response machinery leads to both qualitatively and quantitatively different responses in plant development. In this section, we summarize insights into how interactions between core components and other factors determine the quantitative output of auxin response.

Aux/IAA Degradation

Once auxin binds the pocket in the TIR1/AFB protein, the affinity for Aux/IAA proteins increases (193). Aux/IAA proteins are subsequently ubiquitinated (118) and degraded in the 26S proteasome (72, 234) (**Figure 3**). As the first step in Aux/IAA degradation, its binding by $SCF^{TIR1/AFB}$ has received much attention; later steps have not been studied in as much detail. For example, ubiquitination has been demonstrated in a protoplast assay (118), but the sites and extent of in vivo ubiquitination are not well understood. Gilkerson et al. (68) recently showed that this step may be more complex than initially thought. Even after mutating all 16 lysines in *Arabidopsis* IAA1 and thus eliminating all possible canonical ubiquitin acceptor sites, they found that the protein was still unstable and ubiquitinated. Drug treatments suggested that auxin-dependent IAA1 ubiquitination may involve noncanonical oxyester bonds to serines, threonines, or cysteines. As each of these residues can be changed by posttranslational modifications (such as phosphorylation or disulfide bridges), this work opens the exciting possibility that modifications to the Aux/IAA protein can influence the competence of Aux/IAA proteins for auxin-dependent degradation.

Interestingly, the $SCF^{TIR1/AFB}$ E3 ligase complex is itself also subject to dynamic, auxin-dependent regulation. In a screen for mutations in TIR1 that change auxin-dependent interactions with Aux/IAA proteins in yeast, Yu et al. (233) identified a mutation that increases TIR1-Aux/IAA interactions. Closer inspection revealed that this mutation abrogated the incorporation of TIR1 in the SCF complex, and because TIR1 is itself ubiquitinated and degraded (188), the mutation stabilized the protein (**Figure 3**). Because mutant TIR1 interacted with Aux/IAA proteins without inducing their degradation, the mutation inhibited the auxin response in plants. This suggests that, in the absence of auxin, autocatalytic turnover of TIR1 protein maintains the homeostasis of $SCF^{TIR1/AFB}$ complexes.

Until recently, evidence that such quantitative output, defined by the Aux/IAA degradation rate (see The Players: Core Components That Translate Auxin Sensing to Transcriptional Responses, above), determines biological output had been lacking. By generating versions of IAA14 with different auxin-dependent degradation rates, Guseman et al. (76) tested the hypothesis that the degradation rate determines the pace of progression through subsequent stages of lateral root development. Previous studies had shown that this process depends on auxin response at several steps (43, 64) and that the progression of lateral root development is defined by a set beginning and several subsequent archetypical stages (117). After showing in a reconstituted yeast system that engineered Aux/IAA proteins indeed have a range of degradation rates, Guseman et al. (76) assessed the properties of the mutated proteins in plants. In transgenic plants, the Aux/IAA degradation rate strongly correlated with the developmental progression of lateral root development. Thus,

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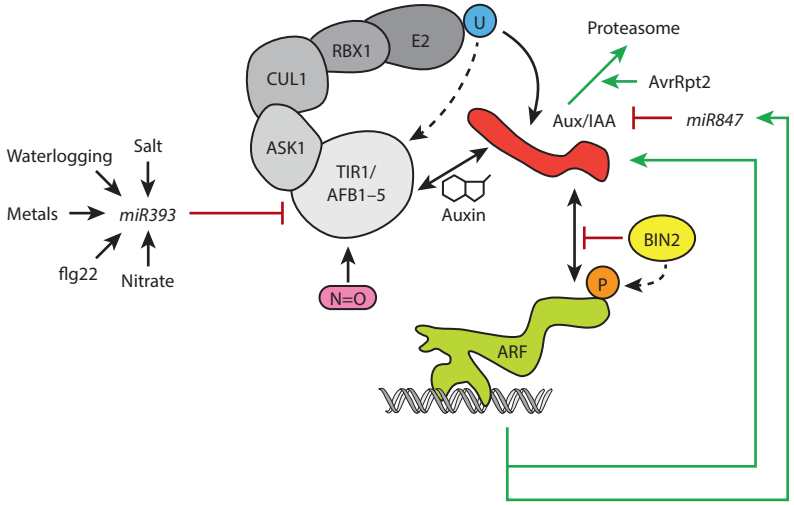

Figure 3

Regulation of auxin response output. Green arrows indicate positive regulation, and red lines indicate inhibition. Auxin promotes interaction between the $SCF^{TIR1/AFB}$ complex (*gray*) and Aux/IAA proteins (*red*), leading to the transfer of ubiquitin (U) (*blue*) to the latter and to degradation in the proteasome. Aux/IAA proteins bind to and inhibit ARF transcription factors (*green*), which can regulate transcription when released from inhibition. The auxin response pathway involves feedback loops: ARFs activate transcription of Aux/IAA proteins and trigger expression of *microRNA 847* (*miR847*), which inhibits Aux/IAA28 accumulation. In addition to ubiquitination, at least two other posttranslational modifications alter auxin response: TIR1 can be S-nitrosylated (*magenta*), and the kinase BIN2 can phosphorylate (*orange*) ARFs to inhibit their interaction with Aux/IAA proteins. Other signals also regulate auxin response: Levels of TIR1 are controlled by autoubiquitination in the absence of substrate (*dashed arrow*) as well as by *miR393*, which serves as an input hub for several environmental signals. Finally, the pathway can be hijacked by *Pseudomonas syringae*, as its secreted effector protein AvrRpt2 promotes Aux/IAA protein degradation. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; ASK1, ARABIDOPSIS SKP1 HOMOLOGUE; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; BIN2, BRASSINOSTEROID INSENSITIVE 2; CUL1, CULLIN 1; flg22, 22-amino-acid flagellin fragment; RBX1, RING-BOX 1; TIR1/AFB, TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX.

plants seem to leverage the quantitative nature of the TIR1/AFB-auxin-Aux/IAA interaction to generate quantitative auxin response output in development.

Transcriptional Regulation

Given the profound impact of auxin output on plant growth and development, it seems intuitive that this output must be buffered and balanced to prevent excessive response (**Figure 3**). Indeed, feedback control has been demonstrated at the level of auxin transport: PIN-FORMED (PIN) auxin efflux carrier genes are transcriptionally upregulated by auxin such that, when cellular auxin levels rise, excess auxin is transported out of the cell (208). A similar mechanism operates in auxin biosynthesis regulation. The *YUCCA* (*YUC*) auxin biosynthesis enzyme genes are transcriptionally repressed by auxin (191). Hence, high cellular auxin levels stall endogenous synthesis, and lower auxin levels (as occur, for example, in *yuc* mutants or upon pharmacological inhibition of YUC activity) lift transcriptional repression and elevate cellular auxin levels. Finally, Aux/IAA genes were initially identified because they are transcriptionally upregulated by auxin treatment, which suggested intrinsic feedback control (2). This feedback regulation has now been formally

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demonstrated using the MP/ARF5 protein: MP/ARF5 triggers activation of a subset of the 29 Aux/IAA genes through direct interaction with their gene promoters (101). The Aux/IAA proteins encoded by these same genes directly interact with MP/ARF5 and inhibit its activity.

Posttranscriptional Regulation

In systematic studies identifying microRNAs in *Arabidopsis* following their initial discovery, a microRNA specific to TIR1 and the AFB genes was also identified. This microRNA, *miR393*, negatively regulates TIR1, AFB2, and AFB3 transcript and protein accumulation (130, 143). Under normal growth conditions, *miR393* expression acts to restrict TIR1/AFB protein levels (143), and analysis of a *miR393a miR393b* double mutant, which has reduced levels of this microRNA, demonstrated that this restriction of TIR1/AFB protein levels is required for normal auxin response (219). Mutants showed a mildly increased response to auxin and subtle growth defects. Because some *miR393* remains even in the double mutant, it is difficult to evaluate the extent of microRNA regulation and its impact on auxin signaling, but it is clear that this regulatory node can act as a nexus for environmental control of auxin response (**Figure 3**). The *miR393* genes are controlled by a variety of environmental conditions, including salt stress (89), waterlogging in maize (113), metal toxicity (121), nitrate (207), and bacterial flagellin (130). Thus, *miR393*-TIR/AFB may represent a module for environmental control of auxin responses. In the case of flagellin regulation, Navarro et al. (130) showed that the increased *miR393* expression and concomitant decrease in auxin response restricted growth of the bacterial infection, and it is likely that other stimuli likewise tailor growth through a primary effect on auxin response.

The impact of small-RNA regulation on auxin response is not limited to the TIR1/AFB receptors. In fact, several small RNAs target *ARF* transcripts. *miR160* targets *ARF10*, *-16*, and *-17* (210), whereas *miR167* antagonizes *ARF6* and *-8* (109). Finally, *miR390* phases *trans*-acting small interfering RNAs from the *TAS3* locus targets *ARF2*, *-3*, and *-4* (3), which may contribute to auxin-controlled accumulation of *ARF2*, *-3*, and *-4* messages (30, 119, 230). For each of these small RNAs, it has been well established that regulation impacts the activity of the respective ARFs during development (reviewed in 147). Wang & Guo (209) recently found the first microRNA targeting an Aux/IAA protein: *miR847*, which is transcriptionally upregulated by auxin in a TIR1-dependent manner and targets *IAA28* mRNA for cleavage (**Figure 3**). Thus, auxin clears cells not only of IAA28 protein (166) but also of its mRNA, thereby prolonging the duration of the auxin response in leaf formation. This regulation is the opposite of that performed by all other small RNAs that target components of the auxin response, as those all antagonize positive regulators and thereby temporally restrict auxin response. It appears that a small RNA regulatory layer acts to sculpt the auxin response and integrate it with environmental cues.

Posttranslational Control

Since the discovery of the main components in nuclear auxin signaling, various studies have explored regulation by posttranslational control, including in vitro S-nitrosylation of TIR1 (194), in vitro phosphorylation of Aux/IAA proteins by phytochrome (36), and potential *cis-trans* isomerization of prolines in Aux/IAA proteins (47). However, none of these modification have been demonstrated to occur in vivo, and their impact on auxin responses remains to be determined. A phosphorylation event in ARF7 and ARF19 does appear to be important for their function in lateral root development (31): In a semi–in vivo assay, both ARF proteins are directly phosphorylated by the GLYCOGEN SYNTHASE KINASE (GSK) kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2), which acts in brassinosteroid signaling (**Figure 3**). Phosphorylation inhibits

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Aux/IAA-ARF interactions and potentiates ARF activity. Surprisingly, in this context BIN2 is activated not by brassinosteroids but by the peptide TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF). Intersignal crosstalk is widespread in plant signaling (45), yet this is this first example of a signal that acts through posttranslational modification of an ARF protein. An interesting question for future studies is whether this is a more general mode of regulation in the auxin signaling pathway.

Finally, pathogens have also found a way to tap into the auxin response machinery. *Pseudomonas syringae* injects pathogen effector proteins into infected host cells; among these injected proteins is AvrRpt2, which interferes with auxin responses (27). A detailed study showed that AvrRpt2 promotes Aux/IAA protein degradation and that this degradation-inducing activity acts independently of auxin-promoted degradation (40). Intriguingly, a mutation in IAA7 that prevents auxin-dependent degradation (*axr2-1*) reduces AvrRpt2 virulence, suggesting that Aux/IAA degradation is necessary for successful pathogen colonization and highlighting a successful strategy of hijacking the auxin response pathway. Similarly, the *Tobacco mosaic virus* replicase protein interacts with and inhibits *Arabidopsis* host IAA26 protein, and the resulting reprogramming of auxin response contributes to viral pathogenicity (142).

Protein Interactions and Oligomerization

Because the TIR1/AFB, Aux/IAA, and ARF families are all subject to transcriptional and posttranslational regulation, their relative levels in cells differ. If proteins have diversified in their affinity toward auxin and/or other proteins in the pathway, then interactions among components could potentially generate different responses. Interactions among components have been experimentally tested, and the results do indeed support diversification, at least in a quantitative sense.

The intrinsic preference of TIR1/AFB members for auxin (21) and Aux/IAA substrates as well as differences in the degradation rates of the latter (82) suggest that different concentrations of auxin in the same cell, or the same concentrations of auxin in different cells, result in specific cellular concentrations of several Aux/IAA proteins (**Figure 4**). How are these differences translated to gene regulation? Aux/IAAs act by binding to and inhibiting ARF transcription factors (195). An important question is how specific levels of Aux/IAA proteins affect the ARF factors present in a cell. Systematic efforts have shown that almost all Aux/IAA proteins can interact with a subset of ARFs with a long, Q-rich middle region (i.e., activating ARFs), whereas interactions with other ARFs are limited and more specific (206). A recent study that incorporated coexpression data of ARF and Aux/IAA genes came to a similar conclusion (146). The emerging picture is a simple one: The totality of the accumulating Aux/IAA proteins converges on the Q-rich ARF proteins and collectively defines the degree of activity (**Figure 4**). However, the Aux/IAA-ARF assays that have been reported so far (146, 206) were not quantitative, and it is possible that the interaction affinities differ by orders of magnitude. Another important point is that most of the interactions (or lack thereof) have not been tested in plant cells, which misses the contribution of posttranslational modifications to the physical interactions. Significant differences in affinity could allow auxin to activate different ARF proteins at different concentrations. A quantitative relationship between auxin dose and gene activity has not yet been established, and whether there are separable gene sets that respond only to low or high auxin concentrations remains to be seen.

Both Aux/IAA and ARF proteins oligomerize in vitro, both in crystals and in solution. Mutations that prevent oligomerization affect the ability of Aux/IAA16 to inhibit auxin response (100), and analogous mutations in ARF5 make the protein less sensitive to auxin inhibition (129). Whether the biological activity of Aux/IAAs and ARFs requires oligomerization is an interesting open question, but proteins do have the potential to oligomerize. Intriguingly, structural studies

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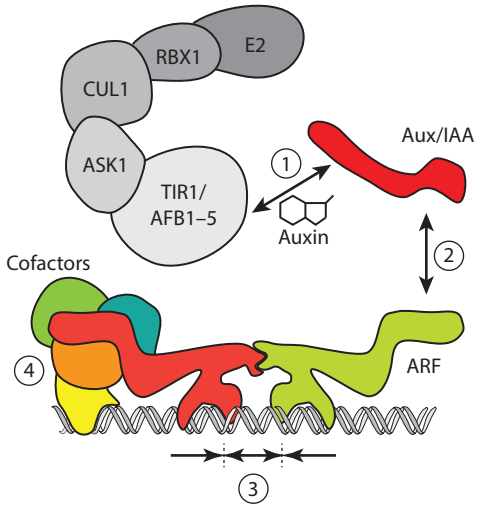

Figure 4

Specificity in auxin response. Transcriptional auxin output depends on interactions and regulation at various levels, ultimately leading to either quantitatively or qualitatively different gene expression profiles. (①) The affinity of the TIR1/AFB-auxin-Aux/IAA interaction depends on the identity of the receptor, the type of auxin molecule, and the identity of the Aux/IAA protein and can thus vary by orders of magnitude. (②) Aux/IAA-ARF interactions through their homologous C-terminal domains are likely selective. Aux/IAAs preferentially interact with class A ARFs, although interactions with class B and C ARFs have also been demonstrated. The affinities among the families likely depend on the exact pairs. (③) The selection of DNA target sites by ARF-DNA interactions can be selective not only by direct recognition of binding sites, but also by the spacing between two adjacent inverted binding sites to which ARF dimers can bind with high affinity. Although ARFs bind nearly identical motifs in vitro, there may be more selectivity in vivo. The optimal spacing between binding sites differs, at least in vitro, between ARFs, which adds selectivity. Furthermore, ARFs may theoretically heterodimerize, further expanding the range of binding specificities. (④) ARF-interacting cofactors can alter ARF activity or DNA-binding specificity. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; ASK1, ARABIDOPSIS SKP1 HOMOLOGUE; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; CUL1, CULLIN 1; RBX1, RING-BOX 1; TIR1/AFB, TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX.

of the TPL tetramer revealed that it preferentially complexes with oligomerized EAR motif–containing interaction partners (96); thus, Aux/IAA oligomerization may result in enhanced TPL and HDAC recruitment. Based on these observations, Farcot et al. (57) developed a model of the auxin response pathway composed of ordinary differential equations that describe interactions between TIR1/AFB, auxin, Aux/IAAs, and ARFs. Mathematical analysis of this network suggested that Aux/IAA-ARF interactions determine the response amplitude, whereas Aux/IAA-Aux/IAA interactions set the speed of the response and ARF-ARF interactions determine the sensitivity. Thus, oligomerization could significantly affect the output of auxin response, as all output parameters (amplitude, speed, and sensitivity) depend on interactions mediated by the domain that can oligomerize.

GENERATING SPECIFICITY IN AUXIN RESPONSES

Auxin triggers various distinct responses in plant development. Auxin accumulation in the shoot apex triggers floral organogenesis (85, 157, 206), in root pericycle cells induces lateral root formation (22), and in embryos induces either cotyledon or root initiation depending on whether the

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accumulation is at the top or bottom of the embryo (123). Thus, the response machinery must accommodate such local interpretation of auxin accumulation, and a key question is how hormone perception is locally translated to gene expression. Functional specificity can be generated at several levels (**Figure 4**), each of which we discuss below.

Protein-DNA Interactions Select Auxin-Responsive Genes

Auxin responses are functions of the genes that are activated or repressed in response to auxin. Thus, DNA recognition by ARF transcription factors is an important step in selecting the auxin-responsive gene repertoire. ARFs were originally identified based on their ability to bind a model AuxRE (TGTCTC) (199, 202) that was identified through promoter analysis of an auxin-responsive gene in soybean (114). Interestingly, following the identification of an AuxRE in the mid-1990s (202) and the subsequent demonstration that ARFs bind this sequence (199, 201), there had not been an exhaustive screen for DNA sequences bound by ARFs until recently. Clearly, the presence of the canonical TGTCTC AuxRE in promoters is insufficient to explain auxin-responsive transcription in vivo (97, 122), and unbiased bioinformatics analysis suggests that more complex motifs mediate auxin responsiveness (122). These analyses also suggest that auxin-regulated gene activation and gene repression are mediated by distinct motifs.

The first efforts to define the full range of sequences bound by ARFs were recently published. Both made use of protein-binding microarrays and a recombinant ARF protein (17, 61). Interestingly, in both studies, the optimal ARF binding site was distinct from the TGTCTC site identified 15 years earlier (201). In fact, Boer et al. (17) suggested that TGTCTC represents a medium-affinity target site and that the novel motif TGTCGG is a high-affinity binding site. Indeed, when Liao et al. (107) used the TGTCGG site to generate a gene expression reporter analogous to the *DR5* reporter based on TGTCTC, the high-affinity element showed broader activity in vivo, revealing suspected sites of auxin response, which could be explained by its approximately tenfold higher sensitivity to auxin.

Structural studies of the ARF DNA-binding domain suggested an additional level of control in selecting auxin-dependent genes (17). Although selection of the DNA element by the ARF DNA-binding domain is dictated by the DNA-contacting residues in the protein-DNA interface, DNA binding is defined by more than just these interactions. Because DNA-binding domains homodimerize, ARF dimers can bind complex motifs that have two binding sites in an inverted constellation with defined spacing (**Figure 4**). Ulmasov et al. (199) demonstrated almost two decades ago that such motifs are highly efficient in mediating auxin-dependent transcription. Structural biology has now suggested a basis for this efficiency, and Boer et al. (17) indeed showed that ARFs bind such complex sites cooperatively. High-affinity binding requires both two adjacent binding sites and an intact ARF dimerization interface (17), which suggests that ARFs can bind either as monomers to single motifs with low affinity or as dimers to complex motifs with high affinity.

A remaining question is how differences between ARF proteins help the selection of distinct sets of target genes, and in this sense, it was surprising that all ARFs tested with a protein-binding microarray showed nearly identical binding sites (17, 61). However, when tested for binding affinity in complex motifs, ARF1 and ARF5 dimers bound more or less strongly depending on the length of the spacer between the two inverted binding sites: ARF1 could bind only when the spacing was 7 or 8 based, whereas ARF5 bound a longer range of spacing (17). Thus, spacing may help discriminate ARF binding. This work has also raised many new questions: Do ARFs heterodimerize via the DNA-binding domain? Do ARFs differ in their in vivo binding sites? Does spacing between binding sites discriminate ARFs in vivo? Clearly, the answers to these questions will help researchers understand how ARFs select genes for local responses to auxin.

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Binding site selection can be influenced by the affinity of the transcription factor for a given site, the number of sites present, and their accessibility in the context of chromatin. Recent studies in *Drosophila* have uncovered an inverse relationship between the affinity of the binding site and its specificity for a given transcription factor (that is, low-affinity binding sites have the highest transcription factor specificity); moreover, clustered low-affinity sites contribute to the robustness of the response (39). Indeed, several reports have suggested that the AuxRE core (TGTC), which may represent a low-affinity binding site, is sufficient for ARF recruitment (127, 228). The evolutionarily conserved AuxRE core elements in the regulatory region of *LEAFY* (*LFY*) are bound only by MP during reproductive development, not in seedlings, even though MP binds to other target genes at this stage (228). One possible explanation for this finding is that sites in *LFY* are inaccessible during the vegetative phase; alternatively, a higher MP level may be necessary to bind these low-affinity sites (228). In agreement with the latter idea, MP levels increase during development (228).

Importantly, the ARF family has the potential to accommodate distinct outputs: The gene expression of the family is developmentally regulated, with cells expressing different combinations of ARFs (153). Furthermore, several ARFs mutate to create distinct loss-of-function phenotypes despite coexpression of (close) homologs (80, 153). Finally, promoter-swap experiments have shown that there is a limited potential for one ARF to replace a second, mutated ARF (152, 153, 214). Thus, auxin accumulation is locally translated to gene expression changes through an interaction network that culminates in functionally distinct ARF transcription factors.

Cofactors Can Modulate Auxin Output

Transcription factors often act in protein complexes, where cofactors can alter the activity or DNA-binding specificity of DNA-binding transcription factors (**Figure 4**). In some cases, cofactor binding can define novel specificities that enable the DNA-binding factors to bind DNA that it normally would not (184). Likewise, ARF-interacting proteins could conceivably modulate activity or specificity. Surprisingly few interactions with ARFs have been reported. These interactions likely mediate ARF repression without contributing to specificity. MYB77 interacts with ARF7 and is required for the auxin-dependent activation of several auxin-dependent genes (182). It is not clear how general this role of MYB77 in auxin signaling is, but mutant phenotypes suggest that it may be restricted to lateral root formation (182). Furthermore, MYB77 appears to connect abscisic acid signaling to auxin responses (236), which may be an example of modulation of a specific local auxin response. The basic helix-loop-helix (bHLH) factor BIG PETAL (BPE) interacts with ARF8, and the genes encoding these two proteins both regulate petal growth (205). How BPE modulates ARF function remains unclear.

Oh et al. (134) recently showed that ARF6 interacts with the bHLH protein PHYTOCHROME INTERACTING FACTOR 4 (PIF4) and the transcription factor BRASSINAZOLE RESISTANT 1 (BZR1). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis of these three proteins showed a considerable overlap in genomic target sites, and auxin regulation of a large number of genes (presumably by ARF6) depends on PIF4 and BZR1 function. This work shows the interdependence of these proteins and suggests that ARF6 function may require the assembly of a transcription complex, at least in the context of hypocotyl growth. Again, it is unclear how PIF4 and BZR1 influence ARF6 activity or specificity, but the complex presents a good model to address mechanistic aspects of cofactor function. Strikingly, all three examples mentioned here involve closely related members of the ARF proteins with a Q-rich middle region, and an interesting question is whether interactions with other transcription factors are limited to this clade of ARFs.

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Chromatin-Level Control in Auxin Responses

Chromatin-level regulation may also provide a means to generate specificity in auxin responses. The interactions between auxin-dependent genes, response components, and chromatin have not been explored in detail, but there are several indications that such interactions occur. A new mechanism for modulation of auxin output was suggested by the recent discovery that the activating ARF MP unlocks chromatin at its targets together with SWI/SNF chromatin remodelers (224). In this study, Wu et al. (224) showed that *swi/snf* mutants phenocopy *mp* mutant phenotypes and that their activity is required for MP to activate its target genes. Chromatin unlocking allows transcriptional regulators to contact cognate binding sites that were previously occluded (**Figure 2**). These secondary transcriptional regulators can contribute to the specificity of the auxin response if their accumulation and activity are cell type or condition specific (224). One corollary of this finding is that evolutionarily conserved *cis*-regulatory elements should be present next to AuxREs or MP-bound sites. Although this has not been exhaustively tested, such co-occurrence has been reported (13, 224). The sequence-specific binding proteins, whose access to DNA is auxin gated, may also recruit additional chromatin regulators. Histone acetylation and transcriptional activation of MP targets is dependent on prior chromatin unlocking (224). Intriguingly, at a target locus that was tested in detail, an evolutionarily conserved basic leucine zipper (bZIP) motif was occluded by the chromatin prior to unlocking (224). In another study, the bZIP11 transcription factor, which binds near an AuxRE in the *GH3* promoter and helps activate the *GH3* gene, recruits a HAT to the promoter to bring about gene activation (216).

In addition, several PLANT HOMEODOMAIN (PHD) finger proteins—OBERON 1 (OBE1) and OBE2 and TITANIA 1 (TIT1) and TIT2—are required for normal development, and mutants show defects similar to *mp/arf5* and *bodenlos* (*bdl*)*/iaa12* mutants (170, 171). Although MP/ARF5 is still normally expressed in *obe* and *tit* mutants, several MP/ARF5 target genes are downregulated in a region-specific manner. OBE1 binds to the promoter of the MP/ARF5 target gene *TARGET OF MONOPTEROS 7* (*TMO7*) in a region that overlaps with the MP/ARF5 binding site (177). Hence, OBE and TIT proteins are required for the activation of ARF target genes. PHD fingers are known to bind methylated lysines on histone proteins (105), and it is thus likely that the histone modification status at target loci is integrated into auxin-dependent regulation. It will be interesting to assess what link (if any) exists between these histone-binding proteins and chromatin remodeling or histone modification.

TRANSCRIPTIONAL CONTROL OF AUXIN-DEPENDENT PROCESSES

Inspired by phenotypes in auxin response mutants (204), the identity of genetically defined developmental regulators as auxin response components (80), or the expression of auxin response reporter genes (62, 168), researchers have studied the involvement of auxin in several developmental processes. In this section, we focus on a few of these processes in which auxin action has been linked to individual and causal target genes. We discuss qualitative aspects of auxin-controlled development here. For dose-dependent outputs, see the sidebar Quantitative Auxin Output: A Plant Morphogen?

Embryogenesis

Life in flowering plants arguably begins at fertilization. Subsequent steps turn a zygote into a mature embryo that includes precursors for the major tissues as well as root and shoot meristems to sustain postembryonic growth (213). It has long been recognized that several steps in embryogenesis depend on normal auxin activity (**Figure 5**). Treatments of in vitro–grown embryos

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QUANTITATIVE AUXIN OUTPUT: A PLANT MORPHOGEN?

The auxin response machinery has the potential to generate different responses to different auxin concentrations: TIR1/AFB-Aux/IAA interactions have affinity constants that depend on both partners (21), and the binding equilibrium therefore varies with the auxin concentration in different ways for each Aux/IAA protein. In addition, the degradation rates of all Aux/IAA proteins are not the same (52, 82, 125), which means that after an auxin pulse, the differential clearance of Aux/IAA proteins in a cell can create a dynamically changing mix of Aux/IAA proteins over time. Finally, Aux/IAA-ARF interactions have some (perhaps limited) selectivity (146, 206, 214), and ARFs are biochemically distinct (152, 214) and expressed in different patterns (153). Thus, dynamic Aux/IAA landscapes can translate into different ARFs being activated to varying degrees.

Gradients of signaling molecules are known to control development in various animals, with famous examples being Decapentaplegic (Dpp) (161), Bicoid (148), and Sonic Hedgehog (Shh) (34). These molecules, collectively termed morphogens, locally induce responses along the gradient and thereby enable cells to "read" the local concentration. Auxin gradients have been widely discussed (204), but such gradients often do not extend over long ranges; instead, they are defined by a few cells with high *DR5* reporter activity and rapidly declining or absent reporter activity in neighboring cells (62, 85, 168). These are technically gradients, but the question that remains unanswered is whether distinct outputs are triggered by different auxin concentrations along such a gradient. No experiments have been reported that directly address the questions of whether different auxin concentrations trigger expression of unique gene sets and whether cells could therefore interpret their position in a gradient of auxin. Particularly with the advent of cell type–specific profiling of auxin responses (7), answers to these questions should soon be within reach.

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either with auxin or with auxin transport inhibitors cause distinctive defects in different species, including problems in the formation of the apical-basal axis, the formation of the root or shoot, and the formation or separation of cotyledons (77, 108). Indeed, both initial and improved gene expression reporters for auxin activity are active during several steps of embryogenesis and in different sites (62, 107) (**Figure 5**). Genetic analysis of auxin biology has revealed that all aspects of auxin action—synthesis, influx, efflux, and response—are critical for normal embryogenesis. Higher-order mutants in auxin biosynthesis genes cause a distinctive rootless defect (29, 187), and very similar defects occur in higher-order mutants in auxin influx carriers (164) and components of the $SCF^{TIR1/AFB}$ complex (48, 49, 87). Mutations in PIN auxin efflux regulators and in the PIN polarity regulator GNOM lead to overlapping but distinct phenotypes (220), consistent with the expected and observed dual effect of transport inhibition: Auxin does not sufficiently accumulate in target cells yet accumulates excessively in source cells.

Several defects induced by reduced auxin activity can be explained by altered activity of only a few of the 29 Aux/IAAs and 23 ARFs. A loss-of-function mutation in MP/ARF5 causes an absence of the embryonic root and hypocotyl as well as of much of the vascular tissue (14). An *mp/arf5 arf7* double mutant also eliminates cotyledon formation (81). Likewise, a stabilizing mutation in the BDL/IAA12 protein that prevents its auxin-dependent degradation (49, 78) causes a nearly identical spectrum of phenotypes (79), which suggests that the interacting MP/ARF5-BDL/IAA12 protein pair (215) is a major effector of auxin action in the embryo.

Beyond MP/ARF5, the *Arabidopsis* genome encodes 22 other ARF proteins, and a systematic analysis of transcription patterns revealed that several of these are expressed in the embryo (153). In fact, *ARF* gene expression patterns are diverse and suggest that most cell types express unique sets of ARF transcription factors. Given that ARF proteins are not simply interchangeable (see Generating Specificity in Auxin Responses, above), this finding suggests that auxin accumulation

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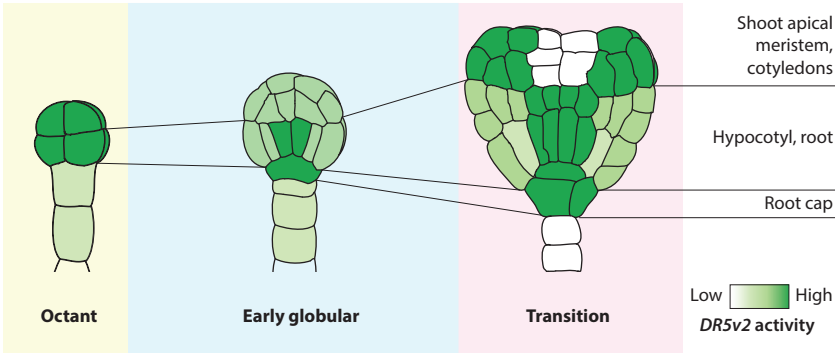

?	ARF5		ARF9/?	ARF5 (and -7?)		ARF(s)
?	*TMO5* → *LOG4* → *CK*	*TMO7* *NTT* *WIP4* *WIP5*	?	*DRN*	*WRKY23*	**Target(s) and pathway**
Cell division orientation	Vascular cell division	Hypophysis/ root formation	Suspensor identity	Cotyledon formation/ separation	Cotyledon/ root formation	**Process**

Figure 5

Auxin response during embryogenesis. The sites of auxin response vary at different stages of embryogenesis (shown chronologically from left to right), as shown by expression of the *DR5v2* reporter (107). These sites coincide with the establishment of regions destined to become organs in the seedling (*thin lines between stages* demarcate region boundaries). The table at the bottom summarizes the ARF or ARFs that act at each stage to control target genes and pathways, ultimately directing cellular or developmental processes. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; CK, cytokinin; DRN, DORNRÖSSCHEN; LOG4, LONELY GUY 4; NTT, NO TRANSMITTING TRACT; TMO, TARGET OF MONOPTEROS; WIP, WIP DOMAIN PROTEIN.

in different parts of the embryo triggers different transcriptional responses. To determine whether there are indeed multiple local auxin responses, Rademacher et al. (152) expressed the *bdl/iaa12* mutant protein from a range of tissue-specific promoters in the embryo. Because Aux/IAA-ARF interactions have limited specificity (146, 206, 214), this strategy allowed the authors to generically inhibit ARFs expressed in the target cell. The results confirmed all known auxin-dependent processes and revealed several novel ones (152). These include the prevention of embryo formation in suspensor cells and control of cell division orientation in the eight-cell embryo (231). An important question now is which target genes mediate local responses to auxin.

Embryogenesis is a fundamentally important phase of plant life, yet very few auxin-dependent genes have been described in this process, likely because of the embryo's small size and lack of accessibility. However, studies of postembryonic functions of MP/ARF5 have isolated several direct target genes, some of which are functionally important for auxin-dependent embryogenesis. In an effort to isolate genes that are controlled by both MP/ARF5 and BDL/IAA12, Schlereth et al. (177) identified a set of likely direct transcriptional targets (**Figure 5**); these targets included a large number of uncharacterized factors, and their detailed study of four of these factors (TMO3, -5, -6, and -7) showed that some indeed contribute to MP/ARF5-dependent embryonic root

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formation. Expression of all four genes is strongly downregulated in *mp/arf5* mutant embryos; at least the *TMO3*, *-5*, and *-7* promoters are bound by MP/ARF5 in vivo; and restoring expression of TMO5 or TMO7 partially rescued the phenotype of a mutant with a weak allele of *mp/arf5*. Downregulation of TMO7 caused *mp/arf5*-like embryonic root defects. Finally, TMO7 appears to be transported from its domain of expression to neighboring cells to induce the formation of the future quiescent center (177).

Other transcription factors have been linked to MP/ARF5 function in a more directed approach. Both *DORNRÖSSCHEN* (*DRN*) and *WRKY23* are expressed in the embryo in a subdomain of the MP/ARF5 domain (35, 74), and because *DRN* gene expression is auxin dependent (35) and *WRKY23* loss-of-function phenotypes resemble those of the *mp/arf5* mutant (74), Cole et al. (35) and Grunewald et al. (74) analyzed their regulation by MP/ARF5. In both cases, expression was downregulated in *mp/arf5* mutant embryos, and in one case (*DRN*), MP/ARF5 bound the target promoter. Thus, a handful of direct MP/ARF5 targets have been identified, but there remains a large gap in our understanding of how auxin triggers embryo development through MP/ARF5.

An intriguing facet of MP/ARF5 action in embryogenesis is that it appears to act non-cell-autonomously. MP/ARF5 is strongly expressed in cells in the lower half of the embryo, adjacent to the hypophysis cell, which later forms the quiescent center (80, 215). Yet cell division defects in *mp/arf5* mutants occur in both the proembryo and the hypophysis (14). Two parsimonious explanations could account for this discrepancy: (*a*) MP/ARF5 is expressed at very low (nearly undetectable) levels in the hypophysis and triggers hypophysis specification locally, or (*b*) MP/ARF5 triggers a nonautonomous signal that moves to the adjacent hypophysis. A recent paper favored the former hypothesis (37). The *NO TRANSMITTING TRACT* (*NTT*), *WIP DOMAIN PROTEIN 4* (*WIP4*), and *WIP5* genes (collectively termed *NWW*) act redundantly in embryonic root formation; an *nww* triple mutant is rootless (37). All three genes are expressed in the hypophysis and its descendants. Expression is lost in the *mp/arf5* mutant, and ChIP suggests that the genes may be direct MP/ARF5 targets. Both in situ hybridization and a functional fluorescent protein fusion to MP/ARF5 reveal low-level expression in the hypophysis, and a model has been proposed in which MP locally activates *NWW* genes to control root initiation (37). Reality is probably more complex, though; Weijers et al. (215) had previously shown that inhibition of MP/ARF5 in the proembryo alone is sufficient to cause the mutant hypophysis defect, and complementation of the mutant by expression of MP/ARF5 only in the proembryo domain completely rescued the *mp/arf5* mutant defect. Thus, although MP/ARF5 may be expressed at low levels in the hypophysis (37), it is clearly not required in this cell (215). The identification of more direct target genes should help resolve whether MP/ARF5 acts directly on hypophysis formation or through cell-cell signaling.

Vasculature

Auxin has long been known to promote vascular tissue formation and differentiation. Classical experiments by Sachs (169) showed that new vascular strands are formed from a local external source of auxin applied to a stem segment and that these strands eventually connect to the main stem vascular system. Thus, an auxin source is sufficient to direct vascular tissue formation. Likewise, veins in leaves form along paths of increased auxin concentration defined by PIN-dependent transport (175). In another classical model for vein formation, during graft union establishment, cells express the *DR5-GFP* auxin response reporter well before new vascular tissue is formed (120). Genetic evidence has shown that an auxin response is indeed also required for vascular tissue formation: The *mp/arf5* mutant makes very little vascular tissue (151).

One key question is how auxin, through MP/ARF5, triggers vascular tissue formation. Some of its functions may be mediated by activation of PIN1 transcription, thus promoting vein continuity

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(178, 217). However, MP/ARF5 activity must also somehow trigger a gene expression program that defines vascular tissue. Several direct MP/ARF5 target genes are specifically expressed in vascular tissue and may mediate auxin-dependent vascular tissue formation. *ARABIDOPSIS THALIANA HOMEOBOX 8* (*ATHB8*) is directly activated by MP/ARF5 and is expressed early in procambial cells throughout the plant. A mutation in *ATHB8* affects normal leaf vein formation but is certainly not critical for the process. Rather, vascular domains are wider in the mutant, which suggests that it may restrict vascular tissue formation to define narrow veins (51). Similarly, *Dof5.8* is activated by MP/ARF5 and expressed in procambial cells (98). A mutation in Dof5.8 enhances a weak *mp/arf5* allele, which suggests a functional contribution to leaf vascular tissue formation. Interestingly, overexpression of the transcriptional repressor Dof5.8 reduces vascular tissue complexity (99), which means that this factor is not an important MP/ARF5 output in vascular tissue formation.

Finally, among the genes isolated as MP/ARF5 targets (177) were two closely related bHLH genes—*TMO5* and *TMO5-LIKE 1* (*T5L1*)—that showed vascular-specific gene expression (42). Mutant analysis showed that TMO5, T5L1, and their close homologs T5L2 and T5L3 are collectively required for the promotion of oriented, periclinal divisions in the vascular tissue of the embryo and root (42). Such periclinal divisions are necessary to generate a vascular bundle from the four precursor cells in the early embryo, and importantly, these divisions are strongly reduced in the *mp/arf5* mutant (42). TMO5 and its paralogs act as heterodimers with the bHLH factor LONESOME HIGHWAY (LHW) (42, 136). The overlap of the auxin-dependent TMO5 and auxin-independent LHW transcription patterns defines a small zone in which periclinal divisions occur (42), and joint overexpression of TMO5 and LHW can induce periclinal divisions ubiquitously (42, 137). Recently, two studies showed that TMO5 and LHW promote periclinal divisions by activating local cytokinin biosynthesis through the *LONELY GUY 4* (*LOG4*) gene (41, 137). Thus, an important output of MP/ARF5 activity in vascular cells is the activation of periclinal division through its target TMO5 and subsequent cytokinin biosynthesis. Nonetheless, the auxin-dependent vascular genes so far have not shed light on how auxin can trigger vascular tissue identity, which remains a future challenge.

Organogenesis from the Reproductive Shoot Apex

A classical auxin response is organogenesis from the reproductive shoot apex. At the shoot apex, the central stem cell pool gives rise to stem cell descendants at its flanks (8, 213). These cells become competent to give rise to new lateral organs (leaves or flowers) when they perceive an auxin maximum (157) (**Figure 6**). The most dramatic mutant phenotypes have been observed in flower primordium initiation. Mutants in auxin biosynthesis, transport, and response all form pin-like inflorescences that lack flowers (28, 138, 151, 206). These auxin maxima, which presage the sites of future primordium initiation, are generated by the combined activities of the auxin efflux carrier PIN1, the auxin influx carrier AUX1, and the PINOID kinase, which regulates PIN1 localization (11, 32, 63, 138, 158). The relative positions of the newly initiated primordia (the phyllotaxis) are species specific, and modeling, cell biological, and genetic studies have shown that they rely on both a local auxin maximum and a region of low auxin concentration around it (6, 93, 185). The only ARF linked to flower primordium initiation thus far is the activating MP/ARF5 (151).

Auxin likely is not solely responsible for selecting the site of organogenesis. Two cytokinin-upregulated genes, encoding the response regulators ARABIDOPSIS RESPONSE REGULATOR 7 (ARR7) and ARR15, were identified as MP target genes in the shoot apex (237) (**Figure 6**). MP bound to the promoter of *ARR15* in vivo, and auxin and *N*-1-naphthylphthalamic acid (NPA) treatment caused decreased and increased expression of *ARR7* and *ARR15*, respectively, suggesting that both genes are repressed upon auxin sensing. Accordingly, mutations in the AuxREs of the

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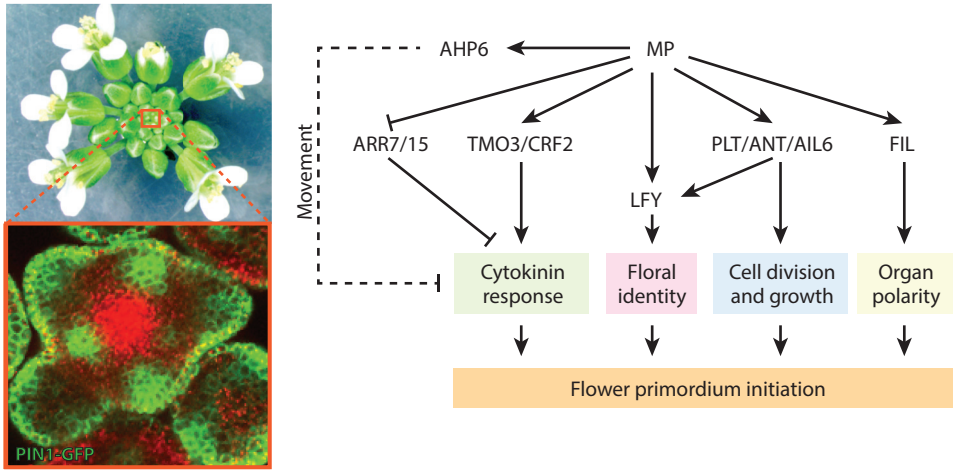

Figure 6

MP-dependent flower primordium initiation. In the *Arabidopsis* inflorescence (*top left*), flowers arise in a regular phyllotactic pattern from stem cell descendants at the shoot apex. The position of each primordium is determined by a local maximum of the hormone auxin, marked by PIN1-GFP (*green fluorescence in bottom left panel*; image reproduced from Reference 228). In primordia, auxin activates MP, which induces the expression of several direct targets whose activities converge on flower primordium initiation. *AHP6*, an inhibitor of cytokinin signaling, is activated by MP and moves outside of the primordium zone to restrict the cytokinin response to the primordium. MP also represses the expression of two negative regulators of cytokinin response, *ARR7* and *ARR15*, in the central region of the shoot apex and induces expression of an AP2 family transcription factor gene, *TMO3/CRF2*, that has been linked to the promotion of cytokinin responses in incipient flower primordia. MP promotes cell division and growth through direct activation of two members of the *PLT* family of transcription factor genes, *ANT* and *AIL6*. MP induces expression of the helix-turn-helix transcription factor gene *LFY*, which directs establishment of floral identity. Finally, MP activates expression of the *YABBY* transcription factor gene *FIL*, which plays a role in specifying abaxial or peripheral fate. Protein abbreviations: AHP6, ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER 6; AIL6, AINTEGUMENTA-LIKE 6; ANT, AINTEGUMENTA; AP2, APETALA 2; ARR, ARABIDOPSIS RESPONSE REGULATOR; CRF2, CYTOKININ RESPONSE FACTOR 2; FIL, FILAMENTOUS FLOWER; GFP, green fluorescent protein; LFY, LEAFY; MP, MONOPTEROS; PIN1, PIN-FORMED 1; PLT, PLETHORA; TMO3, TARGET OF MONOPTEROS 3.

ARR15 promoter caused increased expression. Finally, knockdown of these two negative regulators of cytokinin response partially rescued organ initiation in *mp* null mutants (198). These and additional data led to the hypothesis that cytokinin and auxin act in concert during organogenesis in the shoot (9, 218, 232). Further support for this idea comes from more recent studies demonstrating that MP directly activates the ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER 6 (AHP6) cytokinin signaling inhibitor, which non-cell-autonomously lowers the cytokinin response in the region surrounding the auxin maximum (**Figure 6**). Loss of *AHP6* caused the initiation of multiple flowers from a single auxin maximum, suggesting that a reduction of cytokinin response contributes to the generation of an inhibitory field that restricts the potential for organogenesis (15). These data are consistent with the idea that reduced cytokinin signaling and reduced auxin signaling in the zone surrounding an initiating primordium are required for the stereotypic spatial initiation of flower primordia.

How is the auxin cue transmitted from auxin-activated MP to direct the cell identity changes that culminate in the formation of a flower primordium? This reprogramming requires establishing the primordium developmental program and erasing the meristem program. Expression of

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key regulators of plant development changes dramatically during the early stages of flower initiation; for example, expression of the polarity gene *FILAMENTOUS FLOWER* (*FIL*) and the floral identity gene *LFY* increases, whereas that of the organ boundary gene *CUP-SHAPED COTYLEDON 2* (*CUC2*) and the meristem regulator *SHOOT MERISTEMLESS* (*STM*) decreases (85). Genetic analyses had not identified factors acting directly downstream of MP in this process. The first insight into why this was the case came from identification of the genes encoding the LFY transcription factor and the PLETHORA (PLT) family transcription factors *AINTEGUMENTA* (*ANT*) and *AINTEGUMENTA-LIKE 6/PLETHORA* 3 (*AIL6/PLT3*) as direct MP-regulated target genes with a role in flower primordium initiation (228). The authors found that MP acts in a single input module to directly control the expression of multiple factors that each contribute to flower formation; this gene regulatory network architecture precludes the identification of individual factors with roles in flower primordium initiation by genetic means.

LFY was identified as a direct MP target through the discovery of evolutionarily conserved AuxREs in a key region (16) of the *LFY* promoter. These AuxREs are bound by MP in vivo (228) (**Figure 6**). In *mp* mutants, inhibition of polar auxin transport and nuclear accumulation of auxin-insensitive Aux/IAA proteins lead to reduced *LFY* expression, whereas auxin treatment and nuclear accumulation of MP (even in the presence of a protein synthesis inhibitor) lead to increased *LFY* expression (224, 228). Because *lfy* null mutants do not have defects in flower primordium initiation, additional direct MP targets may act in parallel with LFY. A candidate approach identified *ANT* and *AIL6* as being directly MP upregulated at the time of flower primordium initiation (228). Genetic rescue and genetic enhancer tests demonstrated that LFY, ANT, and AIL6 indeed promote flower primordium initiation (228). In agreement with these findings, PLT family members were shown to be important for phyllotaxis (149). Interestingly, both LFY and PLT transcription factors feedback-regulate auxin accumulation (106, 145, 228). An additional feed-forward loop, in which ANT/AIL6 directly induce LFY expression, may serve as a signal persistence detector during flower primordium initiation (227).

lfy ant ail6 triple mutants still initiate rudimental organs but form pin-like inflorescences when treated with low doses of an auxin transport inhibitor (228), suggesting that addition MP targets exist that contribute to flower primordium initiation. Recent studies have identified the genes encoding the polarity regulator and YABBY family transcription factor FIL (56, 174, 183) and the APETALA 2 (AP2) transcription factor TMO3 [also called CYTOKININ RESPONSE FACTOR 2 (CRF2)] (155, 177) as direct MP-regulated target genes (**Figure 7**). Wu et al. (224) found that MP binds to the promoters of both genes in vivo and that both genes are induced upon increased nuclear MP accumulation and repressed upon increased nuclear Aux/IAA accumulation. Several genetic enhancer tests confirmed that FIL plays a role in flower primordium initiation (224). Whether TMO3/CRF2 also contributes to primordium initiation at the reproductive shoot apex remains to be determined. The combined findings indicate that during flower primordium initiation, MP directly activates diverse developmental processes, including floral fate specification (LFY), cell proliferation and growth (ANT/AIL6), organ polarity (FIL), and cytokinin responses (ARR15 and -17, AHP6, and TMO3/CRF2) (15, 224, 228, 237). Their activation by MP logically couples these disparate processes. It is possible that MP induces additional, as yet undiscovered pathways.

During the last stage of flower primordium differentiation, expression of the pluripotency gene WUSCHEL (WUS) must be repressed in the center of the flower meristem in order for the carpel (a part of the female reproductive structure) to form. The floral homeotic gene *AGAMOUS* (*AG*) and Polycomb repression play critical roles in this process (111, 112, 190). Recently, a genetic enhancer screen linked the repressive ARF ETTIN (ETT)/ARF3 to this pathway. The AG antagonist AP2, which has nonoverlapping domains of activity with AG in the flower primordium,

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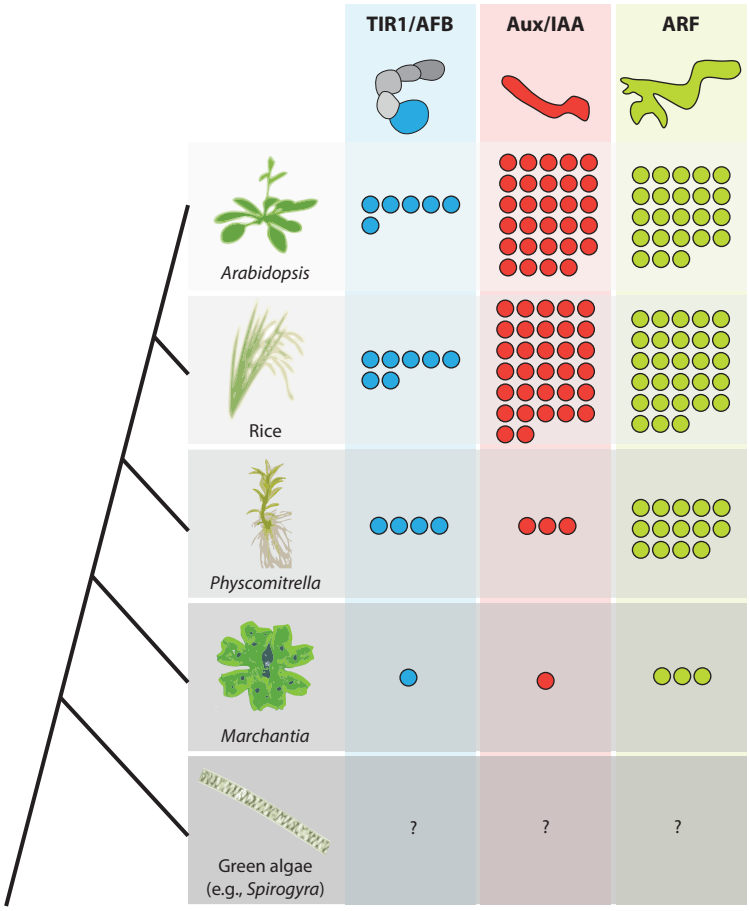

Figure 7

The evolution of the auxin response pathway, showing the distribution of genes encoding TIR1/AFB, Aux/IAA, and ARF proteins in published plant genomes for several plant species. These species represent eudicots (*Arabidopsis*), monocots (rice), mosses (*Physcomitrella*), liverworts (*Marchantia*), and green algae (*Spirogyra*, as an example of charophytes). The tree on the left-hand side indicates the divergence order but is not drawn to scale. Protein abbreviations: ARF, AUXIN RESPONSE FACTOR; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; TIR1/AFB, TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX.

directly represses *ETT*. ETT in turn directly represses *WUS*. The ETT-bound site at the *WUS* promoter is close to a region occupied by *AG*, and ARF3 binding to WUS (but not to a previously defined ETT target) was shown to be dependent on AG (110, 112). Hence, AG modulates the activity of this repressive ARF.

Auxin Response During Gynoecium, Ovule, and Pollen Development

Local auxin maxima and auxin responses play important roles during the formation of the male and female reproductive structures (reviewed in 83, 103, 163). The female reproductive structure, the gynoecium, consists of multiple tissue layers with distinct functions, such as the gynophore at the base, the two valves with the transmitting tract and ovules arising from the valve margins, the

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style, and the stigmatic papillae. Genetic or pharmacological disruption of polar auxin transport or loss of function of two repressive ARFs (ETT and the closely related ARF4) causes severe gynoecium patterning defects and female sterility (12, 131, 180, 181). ETT/ARF3 and ARF4 have been proposed to repress expression of the bHLH transcription factors SPATULA and HECATE (73, 84), although whether this effect is direct is unknown. ETT is required for carpel valve/ovary formation, and the carpels of the *ett arf4* double mutant have polarity defects (131, 180, 181). Likewise, MP plays a role in carpel development, and weak *mp* mutants form flowers with carpels that lack carpel margin tissue and ovules (35, 65, 115, 151). These phenotypes are partially recapitulated by loss of function of the direct MP target LFY (228). A pair of class A ARFs, ARF6 and ARF8, together regulate both gynoecium and stamen maturation, but their direct targets remain unknown (128, 223). ARF6 and ARF8 promote expression of HALF FILLED, a bHLH transcription factor important for transmitting tract development (38). Several of the transcription factors involved in gynoecium development directly regulate auxin transport and biosynthesis; these include the bHLH transcription factors SPATULA, INDEHISCENT, and HECATE as well as a family of B3 transcription factors called NGATHA (69, 126, 179).

MP also plays a role in ovule formation. Defects in ovule formation have been described for mutants in *ANT* and double mutants in *CUC1* and *CUC2*, which are known regulators of organ boundaries (54, 90). A recent study employed conditional *cuc1 cuc2* double mutants to further dissect this pathway and showed that ANT and CUC1/CUC2 act in parallel (65). *MP*, *ANT*, *CUC1*, and *CUC2* were expressed in the placenta before ovules form, and expression of *ANT*, *CUC1*, and *CUC2* was reduced in *mp* mutants; in addition, MP bound to the *ANT*, *CUC1*, and *CUC2* promoters (65). These data indicate that MP directs ovule formation by inducing at least two distinct processes: increased cell proliferation and growth (ANT) in the central region and establishment of an organ boundary (CUC2) at the periphery of the ovule primordium (65). Finally, ARF17, a class C repressive ARF, was recently implicated in callose synthesis during pollen development (229). In *arf17* mutants, microspores did not form an exine layer and subsequently died. The authors traced this defect to reduced callose deposition and reduced expression of *CALLOSE SYNTHASE 5* (*CalS5*) and provided evidence that ARF17 directly binds to the *CalS5* promoter. The *arf17* mutant microspore defect is more severe than that in *cals5*, suggesting that additional ARF17 targets in this pathway remain unidentified. This evidence suggests that ARF17 plays a role in activating gene expression.

THE EVOLUTION OF THE AUXIN RESPONSE PATHWAY

Auxin is a major regulator of growth and development in land plants, and a key question is when its response machinery evolved. Phylogenetic analysis of the Aux/IAA and ARF families has shown that both are represented by multiple members in most plants studied [including, for example, *Arabidopsis* (156, 159), poplar (94), and rice (91, 173)], suggesting that these species are capable of complex auxin responses (**Figure 7**). The genome sequences of the moss *Physcomitrella patens* (160) and the lycophyte *Selaginella moellendorffii* (5) also encode auxin response systems of considerable complexity. Functional information on Aux/IAAs and ARFs is lacking for most species. However, given sequence conservation, auxin responses were likely present in early land plants.

Only recently have studies begun to genetically dissect auxin responses in early-diverging land plants. Ashton et al. (4) isolated several auxin-resistant *P. patens* mutants in the 1970s. Following the sequencing of the *P. patens* genome (160), Prigge et al. (150) mapped the causal genes to *P. patens* auxin response mutants and found that they were caused by mutations in *PpAux/IAA* genes. These mutations were analogous to those found in gain-of-function *aux/iaa* mutants in *Arabidopsis* (156) and indeed prevented interaction with the *P. patens* TIR1/AFB co-orthologs

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(150). Thus, Aux/IAA proteins act in a similar auxin- and TIR1/AFB-dependent manner in a moss. The *P. patens* genome encodes multiple TIR1/AFBs, Aux/IAAs, and ARFs (160) and thus has an elaborate network of auxin response components (**Figure 7**). However, this species may have undergone gene losses or duplications and may not be representative of other early-diverging land plants.

Indeed, recent analysis of the auxin response machinery of the liverwort *Marchantia polymorpha* revealed the slimmest system thus far. This species encodes a single TIR1/AFB ortholog, a single Aux/IAA, and three ARFs (59, 95) (**Figure 7**). Interestingly, the three ARFs each represent a different class (A, B, and C), which suggests that the functional diversification of ARFs occurred early during plant evolution, before the diversification of the Aux/IAA and TIR1/AFB families. Analyses of *M. polymorpha* lines with a Cas9-induced mutation in MpARF1 (189), a gain-of-function mutation in the MpAux/IAA protein (95), or reduced MpAux/IAA expression (artificial microRNA) (59) all showed a profound involvement of auxin in the growth and development of this species. Lines with reduced auxin responses showed a variety of defects (60, 95), suggesting that these responses are essential in multiple aspects of development.

The *M. polymorpha* auxin response systems appear to operate in much the same way as was described in *Arabidopsis*: Aux/IAA and the ARFs can all interact, and ARFs can both homo- and heterodimerize (95). Whereas an *Aux/IAA* gain-of-function mutation (95) or *ARF* knockout (189)

NONTRANSCRIPTIONAL AUXIN RESPONSES

Auxin not only profoundly influences gene transcription, but also affects cell function via nongenomic pathways. Classical auxin responses include rapid hyperpolarization of the plasma membrane following auxin addition to protoplasts (10), which is believed to be mediated by activation of the plasma membrane proton pump. Auxin-induced cell growth can also occur very quickly, and this response is intact in TIR1/AFB quadruple receptor mutants (176). Thus, auxin affects cell growth through a pathway that may not depend on gene regulation. Paciorek et al. (141) showed that auxin inhibits endocytosis of plasma membrane proteins, again without involving components of the nuclear response pathway.

For decades, researchers have investigated proteins with auxin-binding properties in plant extracts, with most of the attention captured by AUXIN-BINDING PROTEIN 1 (ABP1). ABP1 resides primarily in the endoplasmic reticulum but is also secreted and present in the apoplast (92). Crystal structures confirmed specific auxin binding but revealed no conformational change upon binding (221). Antibodies raised against ABP1 inhibited auxin-induced plasma membrane hyperpolarization (186), suggesting that this protein may serve as a receptor for nongenomic auxin responses. Embryo-lethal phenotypes in T-DNA mutants with an insertion in the *ABP1* gene (25) precluded straightforward genetic analysis of its role, and alternative strategies were used instead. Expression of a fragment of the anti-ABP1 antibody (104) or an *ABP1*-antisense RNA fragment (19) induced strong growth defects, suggesting that ABP1 plays a role in mediating nontranscriptional responses. Likewise, a point mutation in the auxin-binding pocket derived from targeting induced local lesions in genomes (TILLING) (226) led to defects consistent with a reduced auxin response. These genetic resources have been used to elucidate a pathway involving ABP1 as an extracellular receptor, interacting TRANSMEMBRANE KINASE (TMK) receptor kinases, and intracellular ROP protein activation to alter cytoskeletal properties, cell shape, and endocytosis (26, 165, 225).

Strikingly, though, new knockout alleles generated through genome editing were viable and did not show obvious phenotypes (66). In addition, Enders et al. (55) showed that the *abp1-5* TILLING allele harbors many other mutations that might condition the auxin response phenotypes in this line. These findings cast doubt on the importance of ABP1 in mediating important auxin responses. Thus, although it is clear that auxin can trigger nongenomic responses, the question of whether ABP1 mediates this process is still largely open.

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creates auxin-insensitive plants, *ARF* overexpression or *Aux/IAA* downregulation causes auxin hypersensitivity (59). Finally, MpAux/IAA also appears to act by recruiting the MpTPL protein. Targeting MpTPL to auxin-responsive genes by fusing it to the DNA-binding domain of MpARF1 induced the same range of phenotypes as the gain-of-function MpAux/IAA mutation (59).

Thus, land plants that diverged as early as *M. polymorpha* possessed an auxin response system that displays features of the logic and regulation described in the more complex higher plants. This finding now allows researchers to address many questions related not only to the wiring and dynamics of the auxin response system, but also to the evolution of novel specificities, regulation, and functionality during land plant radiations. Given that *M. polymorpha* is considered a representative of the earliest-diverging land plants (18) and encodes three different ARFs, a fundamentally important question is whether functional auxin responses predated the water-to-land transition. Largely owing to the small number of available genome sequences for algae, knowledge of algal auxin function and response is fragmented (44). The availability of additional genome sequences in the future should help clarify when the auxin response system first evolved.

SUMMARY POINTS

1. A core mechanism for nuclear auxin responses has been identified. This mechanism involves binding of auxin to both the $SCF^{TIR1/AFB}$ ubiquitin ligase and its AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) substrate protein. The subsequent ubiquitination and degradation of Aux/IAA proteins releases interacting, DNA-binding AUXIN RESPONSE FACTOR (ARF) transcription factors from inhibition and allows these to regulate gene transcription.
2. Transcriptional regulation by auxin involves chromatin-level control to sustain the repressed state and promote the activated state. Repression involves histone deacetylation upon recruitment of the respective enzyme by the TOPLESS (TPL) corepressor and the Aux/IAA repressor. Activation requires recruitment of SPLAYED/BRAHMA (SYD/BRM) chromatin remodelers to the ARF transcription factor.
3. The auxin response machinery has the potential to confer quantitatively different responses to varying auxin concentrations. All core components are represented by multiple copies in plant genomes. Interactions among these core factors, each of which has unique biochemical properties, creates a range of possible response outcomes. Whether cells respond to different auxin concentrations by regulating distinct sets of genes remains an open question.
4. Despite the brevity of the auxin response pathway, transcriptional, posttranscriptional, and posttranslational control over core components allows tuning of the pathway by feedback regulation, during development, or by other hormonal or environmental signals.
5. Specificity in response is critical to the ability to trigger multiple, distinct responses in different contexts during plant development. Selection of context-specific target genes is governed by ARF-DNA interactions, which depend on cooperative binding of complex DNA motifs by ARF dimers. ARF-binding cofactors have been identified that may mediate specificity in gene regulation. A role for ARFs in controlling access of other transcription factors to *cis*-elements in the context of chromatin may also contribute to specificity.

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6. Several target genes have been isolated that mediate auxin responses in specific developmental processes. Several of these target genes, often directly regulated by ARF transcription factors, are expressed only in a specific context, suggesting that auxin indeed directs local activation of defined subsets of ARF target genes.
7. Analysis of the auxin response system in early-diverging land plants has shown that the mechanism of signaling has deep roots, going back at least to the liverworts. Simpler auxin response networks appear to share the same regulatory principles, and the presence of multiple ARFs in a liverwort suggests that auxin responses may have evolved even earlier.

FUTURE ISSUES

1. The potential for specific auxin responses at different concentrations remains unexplored. Understanding whether auxin gradients can carry meaningful information across their length (see sidebar Quantitative Auxin Output: A Plant Morphogen?) will require defining the sensitivity that cells have to distinguish different auxin concentrations and trigger distinct sets of genes. Given that cell types differ in their response to auxin, it will be important to address this question in several well-defined populations of specific cell types.
2. A structural model exists that explains the binding of ARF transcription factors to a canonical DNA element in vitro, but in vivo binding preferences have not been systematically investigated for the ARF family. In vivo binding information will be essential to determine whether structural properties of ARF proteins themselves are sufficient to explain binding preferences in vivo. This will also be critical to determine the degree of specialization of ARFs at the level of target recognition in vivo.
3. Although we now have a better understanding of the activation of gene expression by auxin sensing, the precise temporal series of events that lead from signal perception to increased transcription has not yet been elucidated, nor is it known whether all of the players have been identified. It will be highly informative to dissect this event further and determine whether it follows the same general logic at different target loci or in different contexts.
4. A well-grounded model exists to explain auxin-dependent gene activation, but how auxin represses genes is essentially unknown. Does this process use the same components, or does it involve other, as yet unknown proteins? Is repression based on inhibitory interactions between activating and repressing ARFs and/or competition for DNA sites? Are Aux/IAA proteins involved? A full biochemical and genetic description of gene repression will shed light on these questions.
5. When did auxin responses evolve, and what were the earliest targets and biological processes regulated by the hormone? The origin may lie before the evolution of the first land plants, and exploration of genome information from organisms at the transition from water to land (e.g., charophytes) will be essential to reconstruct the early evolution of the pathway.

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6. What does AUXIN-BINDING PROTEIN 1 (ABP1; see sidebar Nontranscriptional Auxin Responses) do? Is this indeed an extracellular auxin receptor, or do nongenomic responses require different binding sites? Do nongenomic responses intersect with the nuclear auxin signaling pathway? And if so, at what station do they do so? Scrutiny of the role of ABP1 and identification of components mediating fast, nonnuclear auxin effects will help solve this puzzle.

DISCLOSURE STATEMENT

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