Molecular mechanisms of auxin action

Loni Walker and Mark Estelle

The plant growth hormone auxin has an important role in a variety of plant growth and developmental processes. Identification of transcription factors, some with defined genetic function, has shed new light on the mechanisms of auxin regulated gene expression. In addition, the molecular characterization of genes required for auxin response indicates that regulated protein degradation by the ubiquitin–proteosome pathway has an important function in auxin action.

Address
Department of Biology, Indiana University, Bloomington IN 47405, USA

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Abbreviations

ABP	auxin BP
AuxRE	auxin response element
ARF	AUXIN RESPONSE FACTOR
BP	binding protein
MP	monopterous

Introduction

At the whole plant level, auxin is involved in the maintenance of apical dominance, regulation of tropistic responses, formation of lateral roots, development of vascular tissues, and senescence. At the cellular level, auxin has been implicated in cell division, cell elongation and cell differentiation. Although auxin effects have been extensively studied, the molecular mechanisms of auxin action remain poorly understood. Biochemical and pharmacological studies have implicated diverse signaling pathways in auxin response [1]. In the absence of genetic evidence, however, it has been difficult to establish a direct role for these pathways in auxin action. Similarly, the role of auxin binding protein ABP1, the best candidate for an auxin receptor, remains uncertain. In this review, we will focus on two complementary strategies for understanding auxin action: molecular studies of auxin-regulated gene expression, and the characterization of mutants deficient in auxin response. Both of these approaches have produced remarkable new results within the past year and, most gratifying, it is now possible to bring the two sets of results together in an integrated model for auxin response.

Auxin-regulated gene expression

Primary auxin-response genes are induced rapidly and specifically by auxin and do not require protein synthesis for their induction [2]. The auxin-induced genes consist of several multi-gene families that are expressed in a wide variety of tissues. The best characterized families are the *GH3* genes, the *SAUR* genes and the *AUX/IAA* genes [2]. Extensive analysis of promoter sequences has identified several auxin response elements (AuxREs) that function in auxin-regulated gene expression. Recently, the AuxREs have been used to identify transcription factors that mediate auxin response.

The *GH3* gene family was first identified in soybean. Related genes have now been identified in tobacco and *Arabidopsis* [2]. Although the function of the GH3 proteins remains unknown, the *GH3* promoter has been analyzed extensively. Within this promoter are three discrete and independently functioning AuxREs (D1, D4, E1). D1 and D4 are composite structures with a conserved element composed of TGTCTC that is required for auxin inducibility and an upstream element that confers constitutive activity to the promoter [3]. When auxin levels are low the TGTCTC element represses this constitutive promoter activity and when auxin levels are elevated the same element functions as a positive regulator of transcription [3]. The upstream constitutive element may delimit promoter activity to specific tissues and function as a coupling element that confers different patterns of auxin responsive gene-expression [3,4].

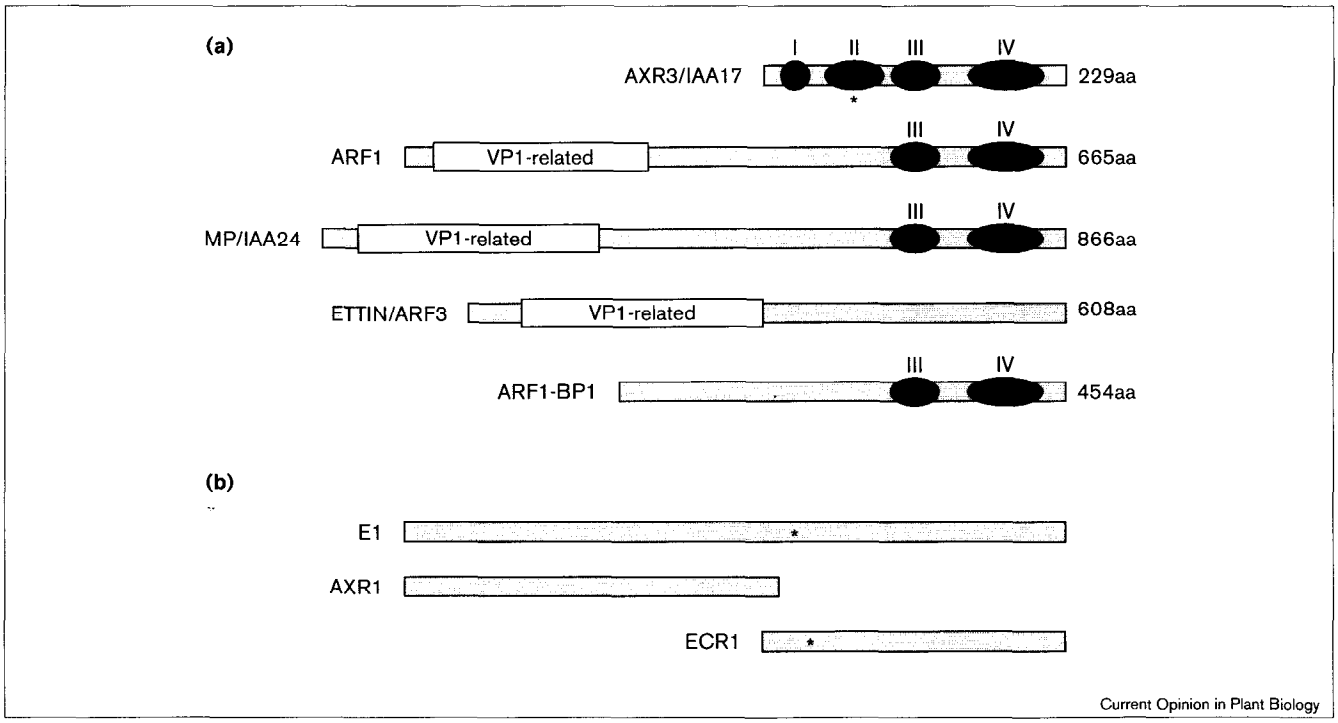
To identify *trans*-acting factors, Ulmasov *et al.* [5••] used an artificial AuxRE called P3(4X) as bait in a yeast one-hybrid screen of an *Arabidopsis* cDNA library. The P3(4X) element is composed of four tandem copies of inverted repeats of TGTCTC (present in D1 and D4) and confers auxin responsiveness when placed adjacent to a β-glucuronidase (GUS) reporter gene. The screen resulted in the recovery of a gene called *AUXIN RESPONSE FACTOR 1* (*ARF1*). Gel-shift experiments indicated that the ARF1 protein binds P3(4X) *in vitro*. Further, mutations in P3(4X) that reduce the activity of the element *in vivo*, also reduced ARF1 binding *in vitro*, implicating ARF1 as a transcriptional activator (transactivator) [5••]. The DNA binding domain of ARF1 includes a motif that is also found in the maize transactivator VP1 [5••,6]. In addition, the carboxy-terminus of ARF1 contains two of the four conserved domains found in the Aux/IAA proteins (see Figure 1) [5••,7]. Two additional ARF1-related genes were identified in *Arabidopsis*. One of these is called *IAA24* because it was also identified as a member of the Aux/IAA family (see below). IAA24 contains domains III and IV as well as the VP1-like motif [5••,8•]. The second related protein, called ARF3 and discovered by Ulmasov *et al.* [5••], has the VP1-like motif, but lacks domains III and IV. *ARF3* is identical to a gene identified by mutation called *ETTIN* (*ETT*) [9••]. IAA24 has the VP1-like motif as well as the two conserved domains from the Aux/IAA family, while ARF3 has the VP1-like motif, but lacks domains III and IV [5••,8•]. Although domain III of Aux/IAA proteins has been hypothesized to function as a DNA binding domain [10], it did not appear to play a role in ARF1

binding to the AuxRE. Instead, ARF1 binds specifically to the TGTCTC sequence through the VP-1 related domain [5••,10]. ARF1 also binds to a second synthetic auxin response element called DR5(8X). This AuxRE contains 8 tandem repeats of the TGTCTC sequence as well as flanking sequences. The interaction between ARF1 and DR5(8X) is weaker than that observed with P3(4X) suggesting that, *in vitro*, ARF1 prefers the inverted repeat found in P3 as opposed to the tandem repeat in DR5 [11•]. At this point it is clear that *ARF1* functions as a transcriptional activator during auxin response. Additional studies will be required to determine which promoters are activated by ARF1 *in vivo* as well as the physiological role of ARF1 during auxin response.

The carboxy-terminal fragment of ARF1, including domains III and IV of the Aux/IAA proteins, was used to recover an interacting protein called ARF1–BP in a yeast two-hybrid screen. ARF1–BP also contains Aux/IAA domains III and IV suggesting that these two proteins might interact through these domains (Figure 1) [5••]. Another study, by Kim *et al.* [8•], confirms that domains III and IV are important for protein–protein interactions. These researchers showed that the Aux/IAA proteins IAA1, IAA2, and PS-IAA4/5 proteins can form homo- and heterodimers *in vitro*. Dimer formation was dependent on a motif in domain III called the βαα motif [8•].

The ARF1 protein appears to function as a transactivator, at least for those promoters tested. What about the Aux/IAA proteins? The biochemical function of Aux/IAA was explored in carrot protoplasts. As stated above, P3 and DR5 reporter constructs are auxin responsive in these cells, presumably through the action of an endogenous ARF1-related protein. When the reporter construct is cotransfected with an *Aux/IAA* gene, however, the TGTCTC AuxRE is repressed [11•]. Other auxin-responsive proteins such as GH3 or SAUR6b do not have this activity. As previous studies demonstrated that Aux/IAA proteins do not bind directly to TGTCTC elements [5••], this repression probably results from interactions between Aux/IAA and ARF proteins that either prevent ARF binding to the AuxREs or prevent interaction between ARF and a cofactor that is required for promoter activity [11•]. Together these studies suggest that activity of the transactivator ARF1 may be regulated by interaction with other proteins that have domains III and IV, including members of the Aux/IAA class of proteins. In the case of the the P3 and DR5 promoters, the Aux/IAA proteins repress transcription. Genetic evidence described below, however, also suggests that these proteins can have a positive effect on their own expression. As the *AUX/IAA* family is large (> 25), it is likely that individual members will have specialized functions in auxin response.

Figure 1



Primary structure of proteins involved in auxin response. **(a)** IAA17/AXR3, ARF1, ARF3/ETT and IAA24/MP proteins of *Arabidopsis*. Domains I–IV are the four conserved domains of the Aux/IAA proteins. Domains III and IV are involved in protein–protein interactions. The asterisk indicates the location of the gain-of-function *axr3* mutations. **(b)** A comparison of conventional ubiquitin-activating enzyme (E1) and the two components of the bipartite RUB-activating enzyme (AXR1 and ECR1) from *Arabidopsis*. The asterisk indicates the position of the active-site cysteine in E1 and ECR1.

It is clear that genetic analysis of mutants with impaired *AUX/IAA* and *ARF* function will be integral to understanding how auxin effects plant growth and development. As mentioned previously, *ARF3* is identical to the *ETTIN* gene, shown to be involved in floral patterning. The *ett* mutants have increased numbers of sepals and petals, decreased numbers of stamens, as well as defects in carpel development. It is possible that *ETTIN* mediates auxin based signaling during patterning in the floral meristem. *IAA24* has recently been shown to encode the monopterous (MP) protein (Figure 1) [12•]. Mutations in the *monopterous* gene interfere with initiation of the embryo axis and formation of a continuous vascular system [12•,13]. These processes are believed to occur in response to directional cues, possibly involving auxin. Although auxin hasn't been summarily shown to be involved in vascular tissue formation and differentiation, it appears that at least one ARF functions as a transcription factor early in the process of vascular development and patterning.

Direct evidence implicating the Aux/IAA proteins as central players in auxin signaling was demonstrated when the auxin-response gene *AXR3* was shown to encode IAA17 [14••]. *Axr3* mutations effect auxin-regulated developmental processes, such as apical dominance, cell elongation, and gravitropic responses [15]. The phenotypes of mutant plants suggest that the semi-dominant mutations confer auxin hypersensitivity, as mutant plants exhibit increased apical dominance, reduced root elongation, agravitropic hypocotyl and roots, and ectopic expression from the *SAUR-AC1* promoter [15]. *SAUR-AC1* is an *Arabidopsis* member of the *SAUR* family of auxin regulated genes. Like GH3, the biochemical function of the SAUR proteins is unknown. As transcription of *SAUR-AC1* is very rapidly induced by auxin, activity of the promoter has proven to be a useful marker for auxin response. The three original *axr3* mutations affect adjacent residues in domain II of Aux/IAA proteins (Figure 1). Four revertant alleles, none of which restores a total wild-type phenotype, all have secondary mutations in the other three conserved domains present in Aux/IAA proteins [14••]. The original semi-dominant *axr3* alleles are probably gain-of-function mutations since the revertants almost certainly result in the loss of AXR3 function [14••]. Because Aux/IAA proteins can form homo- and heterodimers with each other and with ARFs, the axr3 mutant protein may act in a dominant negative fashion by forming nonfunctional complexes with other members of the family. Alternatively, the mutations may increase stability of the protein resulting in ectopic auxin response. This second hypothesis is particularly attractive because the Aux/IAA proteins are known to be short-lived [10] and other studies (see below) implicate regulated protein degradation in auxin response.

The ubiquitin pathway and auxin signaling

The auxin response genes *TIR1* and *AXR1* are required for normal response to auxin in *Arabidopsis* [16,17,18••]. Recent studies have suggested that both of these genes are involved in protein modification by ubiquitin or a related protein. Conjugation of ubiquitin to a target protein involves three enzymes or enzyme complexes called the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2), and the ubiquitin protein ligase (E3). In most, but not all cases, ubiquitin modification results in degradation by the 26s proteosome [19]. In all species examined there are a small number of E1 isoforms, a larger number of E2 isoforms, and diverse E3s and E3 complexes. The specificity of the pathway resides in the E2 and E3 enzymes. One class of E3 in particular, called the SCF complex, has been the focus of considerable attention [20]. The SCF consists of SKP1, CDC53, and an F-box protein. Most species have small families of related SKP1 and CDC53 genes. In contrast, the F-box proteins are structurally diverse. By definition, F-box proteins contain an F-box domain that is required for binding to SKP1 [21,22,23]. In addition, F-box proteins often have a repeated motif such as the leucine rich repeat or the WD40 repeat [20]. SCFs are named for their F-box proteins and are required for a variety of cellular processes. For example, yeast SCF^{Cdc4} includes the F-box protein Cdc4p, and is required for ubiquitination of the cyclin dependent kinase inhibitor Sic1p during the G1 to S phase transition. Also in yeast, SCF^{Grr1} is required for the degradation of the G1 cyclin CLN2 and for the transcriptional activation of glucose transporter genes, while SCF^{Met30} is required for repression of methionine biosynthesis genes. Different F-box proteins appear to recruit specific phosphorylated substrates (like Sic1p) to the E3 complex for subsequent ubiquitination [20,24].

AXR1 encodes a protein with homology to the amino terminal half of the E1 enzyme, implicating *AXR1* in some aspect of ubiquitin-mediated processes [25] (Figure 1). Analysis of the *axr1* mutant phenotype indicates that the *AXR1* gene has a fundamental role in auxin response. Some of the evidence in support of this conclusion is summarized below. First, the *axr1* mutants are deficient in most, and perhaps all growth processes thought to be mediated by auxin, including hypocotyl elongation, meristem function, tropic responses, and root hair elongation [16,26,27]. Second, mutant seedlings are less sensitive to IAA at all concentrations tested from 10^{-12} to 10^{-5} M [28]. It is important to note that very low concentrations of IAA stimulate root elongation and that *axr1* seedlings are less sensitive than wild-type seedlings [28]. Furthermore, the *axr1* mutations act to suppress the effects of increased IAA levels in transgenic plants expressing the *iaaM* gene from *Agrobacterium tumefaciens* [29]. Finally, mutant tissues are deficient in auxin-regulated gene expression. This is true for both the *SAUR* [17] and *AUX/IAA* [7] families of auxin-regulated genes.

Although *AXR1* encodes an E1-like protein, it is half the size and lacks the active site cysteine required for E1 activity [25]. In an attempt to understand the biochemical function of AXR1, Lammer *et al.* [30•] identified a yeast

homolog of *AXR1*, called *ENR2*. Deletion of the *ENR2* locus had no affect on yeast cell growth; however, the *enr2*Δ mutation was shown to enhance the phenotype of mutations in *CDC4*, *CDC53*, and *SKP1*, the three components of SCF^{Cdc4}. Further, *enr2*Δ is synthetically lethal with a temperature sensitive allele of *CDC34*, which encodes the E2 known to function together with SCF^{Cdc4}. These data indicate that Enr2p can affect function of SCF^{Cdc4} [30•].

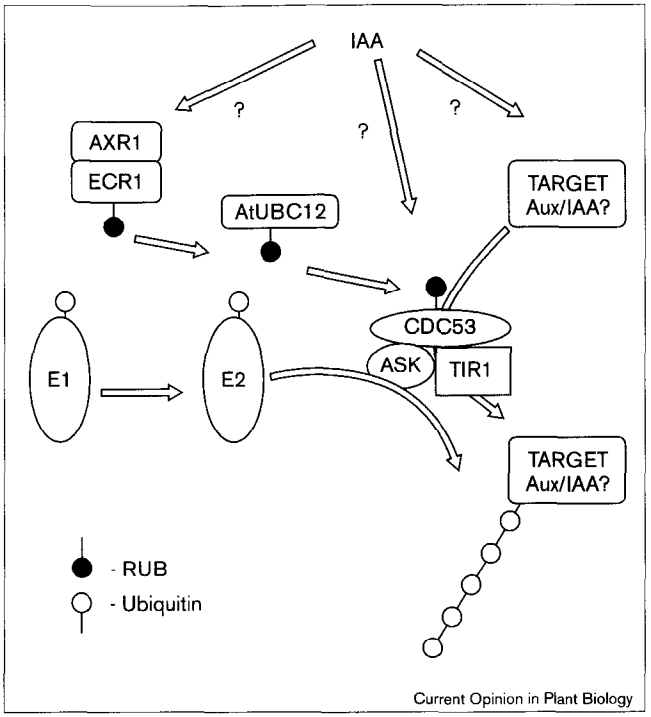
In the course of these studies it was observed that *ENR2* deletion strains lack the larger of two Cdc53p isoforms [30•]. It was subsequently shown that the larger isoform is produced by conjugation of a ubiquitin-like protein called Rub1p to Cdc53p. Conjugation of Rub1p to Cdc53p required *ENR2* and *SKP1* gene function. Thus the basis for the interaction between *enr2*Δ and mutations in components of the SCF^{Cdc4}, is the loss of the modified form of Cdc53p. The modification of Cdc53p doesn't target it for degradation since Rub1p-Cdc53p is stable for long periods of time. Rub1p appears to be important for regulation of some aspect of Cdc53p function [30•].

How does Enr2p facilitate Rub1p conjugation of Cdc53p? Several recent studies provide an answer [31,32]. Two families of ubiquitin-related proteins have been identified, Smt3p/SUMO and RUB/NEDD8 [33]. Each of these molecules are activated by a bipartite enzyme consisting of one protein with sequence similarity to the amino-terminal half of the E1 enzyme and a second protein with sequence similarity to the carboxy-terminal half of E1. In yeast, Smt3p is activated by an E1-like heterodimer of Aos1p and Uba2p [31]. Aos1p is related to the amino-terminal half of E1 (and AXR1) and Uba2p is related to the carboxy-terminal half of E1 and contains the active site cysteine [31]. Liakopoulos *et al.* [32] have shown that yeast Rub1p is also activated by a bipartite E1-like enzyme composed of Enr2p and Uba3p.

On the basis of these results del Pozo *et al.* [34••] hypothesized that AXR1 may be part of a similar E1-like enzyme. A search of dBEST revealed an expressed sequence tag (EST) encoding a protein with similarity to the carboxy-terminal half of E1 (Figure 1B). When this protein (called ECR1) was synthesized in bacteria and mixed with recombinant AXR1, *Arabidopsis* RUB1, and ATP, the RUB1 protein became bound to ECR1 by a thiolester linkage [34••]. Substitution of the putative active-site cysteine in ECR1 with alanine abolished this activity. Our results indicate that AXR1/ECR1 functions as a RUB activating enzyme in a manner similar to Enr2p/Uba3p in yeast.

Confirmation that AXR1/ECR1 functions as a RUB-activating enzyme was obtained using extracts from wild-type and mutant seedlings. When ^{32}P-labeled RUB1 was added to extracts from wild-type seedlings, a labeled protein the size of ECR1–RUB1 was observed. When extracts prepared from *axr1-12* seedlings were used, no ECR1–RUB1 was formed suggesting that AXR1 is responsible for the majority of RUB1 activation in seedlings [34••]. These

Figure 2



Speculative model for AXR1 and TIR1 function in auxin response. According to this model TIR1 is part of an SCF complex required for degradation of a negative regulator of auxin response. As indicated, one candidate target of the pathway is one or more members of the AUX/IAA family of proteins. The activity of SCF^{TIR1} is regulated by RUB modification via the AXR1/ECR1 pathway. Auxin may regulate the activity of the RUB-conjugation pathway and/or target recognition by SCF^{TIR1}, perhaps via phosphorylation.

data indicate that decreased auxin response in *axr1* plants is due to the loss of RUB1 activation and that auxin response is mediated at least in part, through the modification of a protein or proteins by RUB. Efforts to identify RUB-modified proteins in plants are underway. One possibility is that CDC53 is also modified in plants.

At this point the function of RUB modification is not known. There is no evidence that modification by either RUB/NEDD8 or Smt3p/SUMO results in protein degradation. Only a small number of targets have been analyzed so far, however. In addition to Cdc53p–Rub1p in yeast (described above), SUMO has been shown to be conjugated to RanGAP1, a component of the nuclear pore and a nuclear protein called PML [35,36]. In the case of RanGAP1, the modification appears to be essential for correct localization to the nuclear pore complex, whereas for PML, only nuclear forms are modified suggesting a function in localization. In *Arabidopsis*, AXR1 is strongly localized to the nucleus, suggesting that the targets for RUB modification are nuclear proteins [34••]. It is also striking that expression of the *AXR1* gene is largely restricted to dividing and elongating cells (JC del Pozo, M Estelle, unpublished data). For example, there is no

detectable *AXR1* mRNA or AXR1 protein in mature regions of the root except for sites of lateral root formation. At these sites, expression of *AXR1* precedes the earliest cell divisions in formation of the lateral root primordia (JC del Pozo, M Estelle, unpublished data). Unlike ubiquitin modification, RUB modification appears to have a restricted function in cellular metabolism.

The *tir1* mutants of *Arabidopsis* are also defective in several auxin-regulated growth processes including auxin induced cell division, the formation of lateral roots and auxin dependent hypocotyl elongation [18••]. *TIR1* encodes an F-box protein that contains 16 degenerate leucine rich repeats and is most closely related to the yeast protein Grr1p and human protein SKP2 [18••]. Using TIR1 as bait in a yeast two-hybrid screen, two *Arabidopsis SKP1* homologs called *ASK1* and *ASK2* have been isolated (WM Gray, M Estelle, unpublished data). Thus it is likely that TIR1 functions within an SCF complex to promote ubiquitination of one or more regulatory proteins required for auxin response (Figure 2). Theologis and co-workers [2] have observed that transcription of the *AuxIAA* auxin regulated genes is induced by the protein synthesis inhibitor cycloheximide. Based on this result, these workers propose that transcription of these genes is normally repressed by short-lived repressor proteins and that auxin stimulates the degradation of the repressor(s) [2]. *TIR1* may function in auxin signaling by targeting this repressor protein for degradation. There are at least three *TIR1*-like genes in *Arabidopsis*. One of these is identical to the *COI1* gene [37•]. The recessive *coi1* mutants are completely insensitive to jasmonic acid suggesting that the SCF and ubiquitin-mediated protein degradation are essential for jasmonate signaling [38]. These exciting results suggest that SCF complexes and regulated protein degradation have a general role in plant hormone action.

It is striking that both *AXR1* and *TIR1* encode proteins that function in a ubiquitin pathway. Analysis of double mutants suggest that *TIR1* and *AXR1* function in overlapping pathways [18••], reminiscent of the relationship between *ENR2* and *CDC4* in yeast. These results suggest a model in which *AXR1* mediates RUB1-modification of CDC53 in a SCF that includes TIR1 (SCF^{TIR1}). RUB1-modifiction may regulate SCF^{TIR1} possibly specifying the ubiquitin-mediated proteolysis of an unknown protein such as the repressor mentioned above (Figure 2). One interesting possibility is that abundance of the Aux/IAA proteins, known to be extremely unstable, is mediated by the *AXR1/TIR1* pathway(s). Auxin may regulate this process through phosphorylation of a protein in the AXR1/ECR1-SCF^{TIR1} pathway, or by phosphorylation of the putative target protein. It is noteworthy that auxin has been reported to activate a MAP kinase pathway [39].

Conclusions

The identification of transcription factors and the molecular characterization of genes required for auxin response have made significant contributions to our understanding of how auxin regulates plant growth and development. The elucidation of the interactions between ARF and Aux/IAA proteins, their role in regulating auxin response, and the identification of target genes will provide valuable information in understanding the molecular action of auxin. This, in combination with the identification and cloning of auxin-response genes with defined functions, will produce important new insights in auxin regulated growth and development.

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- •• of outstanding interest

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