Function of the ubiquitin–proteasome pathway in auxin response

William M. Gray and Mark Estelle

The plant hormone auxin regulates many aspects of growth and development. Despite the importance of this hormone, the molecular basis for auxin action has remained elusive. Recent advances using molecular genetics in Arabidopsis have begun to elucidate the mechanisms involved in auxin signaling. These results suggest that protein degradation by the ubiquitin pathway has a central role in auxin response.

SELECTIVE PROTEIN DEGRADATION has emerged as a regulatory mechanism for a wide variety of cellular processes. In eukaryotic cells, the ubiquitin system is a major pathway for regulated protein degradation. Ubiquitin is a highly conserved 76-amino-acid protein that covalently modifies target proteins, marking them for degradation by the 26S proteasome. This pathway regulates key biological processes such as cell division, metabolism, immune response and apoptosis.

Recent studies with Arabidopsis thaliana have revealed that the ubiquitin-proteasome system plays a central role in the auxin-response pathway. The plant hormone indole-3-acetic acid (IAA or auxin) controls many aspects of plant growth and development. Some of the best-characterized examples are tropic growth responses, stem elongation, lateral branching of roots and shoots, and vascular development. These processes are controlled by auxin-mediated changes in cell division, cell expansion and cell differentiation. Given the prominent role of auxin in these basic cellular events, it is hardly surprising that plant biologists have long been intrigued by this hormone and have compiled an enormous amount of physiological data concerning the responses of plants to IAA. Nonetheless, fundamental aspects of auxin biology such as auxin biosynthesis, perception and response are still poorly understood. This review will focus on recent advances that implicate the ubiquitin pathway in the auxin response.

The ubiquitin-conjugation pathway

Ubiquitin conjugation involves an enzymatic cascade in which ubiquitin is first activated by the formation of a thiol-ester bond between its C terminus and a cysteine residue within the ubiquitin-activating enzyme (E1). Ubiquitin is then transferred to a member of a family of ubiquitin-conjugating enzymes (E2). Finally, with the assistance of a ubiquitin ligase (E3), ubiquitin is covalently attached to an eNPH2 group of a lysine residue within the substrate protein. Ubiquitin ligases have a crucial role in determining substrate specificity. E3 enzymes are very diverse and the least understood components of the ubiquitin-conjugation pathway. In some cases, the E3 forms a catalytic intermediate with ubiquitin, whereas in others, the major function of the E3 might be to bring the E2 and the substrate into close proximity. Reiteration of these reactions using specific lysine residues within the conjugated ubiquitin results in the generation of a polyubiquitin chain. The 26S proteasome recognizes this chain and degrades the tagged protein, releasing free ubiquitin in the process.

In recent years, a number of ubiquitin-like proteins (Ubls) have been identified. These proteins are conjugated to a lysine residue of target proteins by a mechanism very similar to ubiquitin conjugation. However, in marked contrast to ubiquitination, Ubl conjugation does not generate a polyUbl chain and does not appear to affect target protein stability. The SUMO-1 protein (for small ubiquitin-related modifier; also known as PIC1/Ubl/Sentrin, and Smg3p in yeast) is approximately 20% identical to ubiquitin and is conjugated to several proteins, including RanGAP1, PML, and hS in mammals and the septin proteins of budding yeast. In several cases, modification by SUMO-1 appears to regulate subcellular localization of the target protein. The RUB1 (related to ubiquitin 1) family (NEEDH in mammals) of Ubls is 50-60% identical to ubiquitin. Less is known about the function of these proteins. Genetic analysis of the yeast cell cycle and the Arabidopsis auxin-response pathway suggests that RUB1/NEEDH modifications might regulate the activity of a subset of E3 ubiquitin ligases (see below).

Approaches to studying auxin action

Two strategies have been employed to identify genes involved in auxin-mediated growth and development. The first approach is the recovery of mutants that exhibit resistance or reduced response to applied auxin. The second strategy has been to identify genes that are rapidly induced by auxin and then link these to factors acting upstream in the response pathway. Both of these approaches have been successful, and it appears that the two might be about to converge on the ubiquitin pathway.

Studies of auxin-response mutants

In Arabidopsis, several mutants have been isolated that display diminished response to auxin. Genetic analysis suggests that four of these genes (AXR1, TIR1, AXR4 and SAR1) act in the same or overlapping pathways. Loss-of-function mutations in AXR1, AXR4 and TIR1 confer diminished auxin response, and double mutant combinations between these genes display synergistic interactions.

Phenotypic analyses indicate that ARF (auxin response factor) genes are important regulators of auxin signaling (Fig. 1a). axr1 mutants exhibit defects in essentially all processes thought to be mediated by auxin, including meristem function, tropic growth responses and cell elongation. Additionally, axr1 plants exhibit reduced expression of the AxrinIAA and SAUR (for small auxin up RNA) auxin-inducible genes. The AXR1 protein is related to the Wnt/ß-catenin or Wnt signaling pathway. AXR1 interacts with the ECR1 (E3 C terminus-related 1) protein to form a bipartite enzyme that activates the E3 Ubl pathway in auxin signaling. Arabidopsis contains at least three members of the RUB family. RUB1 and RUB2

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is a two-step process. The first step involves the activation of ubiquitin by the RUB (for Rub1) protein. The second step involves the conjugation of ubiquitin to the protein substrate. The RUB protein acts as an adaptor subunit that recruits specific substrates to the SCF complex, targeting them for ubiquitination by Cdc34p. In S. cerevisiae, SCF^Cdc4 is required for the formation of the SCF^Cdc4 ubiquitin-ligase complex. Mutations in the yeast Rub1p conjugation pathway confer no obvious phenotype, a synthetic lethal phenotype is observed with double mutants between genes in the pathway and conditional alleles of components of SCF^Cdc4. This suggests that Rub1p conjugation to Cdc34p has a role in SCF^Cdc4 function. Possible functions include facilitating SCF complex assembly, improving SCF catalytic efficiency or altering substrate specificity. Unlike mutations in the yeast Rub1 conjugation pathway, mutations in the Arabidopsis RUB conjugation pathway have quite dramatic consequences, suggesting that the RUB pathway has a much more prominent role in higher eukaryotes.

Molecular characterization of the TIR1 (for auxin transport inhibitor gene 1) auxin-response gene has greatly strengthened the hypothesis that Rub1p conjugation regulates some aspect of SCF function. Mutations in TIR1 confer an auxin-resistance phenotype that is similar to that caused by mutations in AXR1, although less severe. TIR1 encodes an F-box protein with a series of leucine-rich repeats. Recent findings have confirmed that TIR1 interacts with plant orthologs of Skp1p (called ASK1 and ASK2) and a ubiquitin ligase (UBR1), although less severe. TIR1 encodes an F-box protein with a series of leucine-rich repeats. Recent findings have confirmed that TIR1 interacts with plant orthologs of Skp1p (called ASK1 and ASK2) and a ubiquitin ligase (UBR1), although less severe. TIR1 encodes an F-box protein with a series of leucine-rich repeats. Recent findings have confirmed that TIR1 interacts with plant orthologs of Skp1p (called ASK1 and ASK2) and a ubiquitin ligase (UBR1), although less severe. TIR1 encodes an F-box protein with a series of leucine-rich repeats. Recent findings have confirmed that TIR1 interacts with plant orthologs of Skp1p (called ASK1 and ASK2) and a ubiquitin ligase (UBR1), although less severe. TIR1 encodes an F-box protein with a series of leucine-rich repeats.
SCFTIR1 in auxin response has been obtained by analysing ask1-1 mutants and transgenic lines that overexpress TIR1. The ask1-1 mutation was identified in a screen for male sterile plants. Mutant plants also exhibit several phenotypes consistent with reduced auxin response, including decreased lateral root development and resistance to applied auxin. ASK1 is a member of a family of Skp1-like proteins in Arabidopsis that includes at least ten members. The isolation of ask1 mutants, along with the discovery that TIR1 does not interact with all of the Skp1-like proteins in a two-hybrid assay, implies that individual family members have distinct functions. This could provide an additional layer of SCF combinatorial control in higher eukaryotes.

The auxin-regulated genes

The Aux/IAA genes are rapidly (10–60 minutes) and specifically induced by auxin. There are at least 20 members of the Aux/IAA gene family in Arabidopsis, and many members display distinct basal and induced expression levels, induction kinetics and patterns of expression. Because cycloheximide treatment also induces many of these genes, it has been proposed that a short-lived repressor could negatively regulate their transcription. The Aux/IAA proteins are 20–35 kDa and share four highly conserved domains (Fig. 3a). Domain III is related to the b-ribbon DNA-binding domains of the Arc and MetJ prokaryotic transcriptional repressor proteins, suggesting that these factors might function as transcriptional regulators. Consistent with this possibility, several of these proteins have been localized to the nucleus.

Several members of the Aux/IAA family are capable of forming homo- and heterodimers through domains III and IV. Additionally, some can interact with members of a second protein family, including the DNA-binding protein auxin-responsive factor 1 (ARF). Members of the ARF protein family share domains III and IV with the Aux/IAA proteins (Fig. 3a). The ARFs bind to a 6-bp auxin-response element (AuxRE) found upstream of several auxin-inducible genes and apparently function to regulate transcription of these genes. TIR1, as well as other Aux/IAA proteins (e.g. auxin-inducible gene expression13), have shown that TIR1 is localized to the nucleus, implying that individual family members have distinct functions.

Recent genetic evidence has solidified the importance of the Aux/IAA gene family in auxin response. Mutations in the Aux/IAA gene were isolated in a screen for seedlings resistant to low concentrations of applied auxin. The semi-dominant aux3 mutants exhibit elevated auxin responses, including increased apical dominance, adventitious rooting and ectopic auxin-related growth defects. SHY2 and AXR2 were recently cloned and found to encode Aux/IAA proteins (P. Nagpal, L. Walker, M. Estelle, J. Reed, unpublished). Curiously, mutations in ARD1, SHY2 and AXR2 all occur within a small, highly conserved region of domain II. Analysis of in transgenic revertants of AXR3 and SHY2 indicates that the original mutations in these genes confer a gain of function. This could be a dominant-negative effect resulting from the formation of non-functional dimers with ARF or Aux/IAA proteins. An alternative explanation favored by these
Regulation of RUB1 conjugation?Aux-dependent kinase?

A model for auxin response. In response to auxin, SCF\(^{\text{TIR1}}\) (for S\text{F\text{C}}, \text{Cullin and E1-terminus-repressor}) gene expression, thus altering auxin response. A model for auxin response.

Figure 4 presents one possible model for the AXR1–TIR1 pathway in auxin signaling. In response to hormone, TIR1 recruits one or more repressors of auxin response to an SCF ubiquitin-ligase complex. Ubiquitination of this repressor is dependent upon the AXR1–E1R1-mediated RUB1 modification of the AUC11 gene. This modification derepresses the auxin-responsive pathway resulting in the expression of auxin-regulated genes and auxin-mediated growth and development. The putative repressor targeted for degradation by SCF\(^{\text{TIR1}}\) is unknown. One possible candidate is the SAR1 (for suppressor of auxin resistance 1) gene product. Recessive mutations in SAR1 were isolated in a screen for suppressors of axr1 (Ref. 34). In addition to suppressing nearly every aspect of the axr1 phenotype, sar1 mutations also confer a distinct phenotype that is epistatic to auxr1, suggesting that SAR1 acts downstream of AXR1 in the auxin-responsive pathway. Given that loss of SAR1 largely alleviates the need for AXR1 in auxin response, one attractive possibility is that SAR1 is the target of the AXR1–TIR1 pathway. This seems somewhat unlikely, however, given that sar1 mutants do not display any constitutive auxin-response phenotypes, and mutations in sar1 do not suppress mutations in tir1 (A. Cernac and M. Estelle, unpublished). Thus, SAR1 could act at a point between AXR1 and TIR1. One possibility is that SAR1 encodes a protein that removes RUB modifiers from their substrates. Li and Hochstrasser\(^{37}\) recently identified a yeast protease that is required for cell-cycle progression. This protease specifically cleaves the Sm3p/SMO1 Ubl from substrates, thus demonstrating that Ubl removal is an important cellular function.

Where is the regulation?

Despite these advances in our understanding of auxin action, the mechanisms of auxin perception and regulation of the AXR1–TIR1 pathway are still largely a mystery. Although several auxin-binding proteins have been identified, convincing evidence for receptor function has remained elusive. Nonetheless, recent findings with the Arabidopsis auxin-binding protein 1 (ABP1) are encouraging. Using an inducible expression system, Alan Jones and colleagues have demonstrated that ABP1 promotes auxin-dependent cell expansion when overexpressed in tobacco and maize leaf cells\(^{38}\). It will be interesting to determine whether this effect is dependent on the AXR1–TIR1 pathway. If ABP1 does indeed function as an auxin receptor in the AXR1–TIR1 pathway, it is still unclear how the signal is transduced. The sequence of ABP1 resembles no other proteins in the database, thus providing no information on what types of signaling mechanisms might be employed. The identification of an SCF complex in the auxin response suggests
that phosphorylation-based signaling pathways might be involved. All known substrates of SCF ubiquitin ligases must be phosphorylated to trigger their association with the SCF (Refs 5, 37). Perhaps, an auxin-activated kinase phosphorylates substrates of SCF ubiquitin ligases must be phosphorylated to trigger their association with the SCF (Refs 5, 37). Perhaps, an auxin-activated kinase phosphorylates substrates of SCF ubiquitin ligases must be phosphorylated to trigger their association with the SCF (Refs 5, 37). Perhaps, an auxin-activated kinase phosphorylates substrates of SCF ubiquitin ligases must be phosphorylated to trigger their association with the SCF (Refs 5, 37). Perhaps, an auxin-activated kinase phosphorylates substrates of SCF ubiquitin ligases must be phosphorylated to trigger their association with the SCF (Refs 5, 37). 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A novel FeS cluster in Fe-only hydrogenases

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Many microorganisms can use molecular hydrogen as a source of electrons or generate it by reducing protons. These reactions are catalysed by metalloenzymes of two types: NiFe and Fe-only hydrogenases. Here, we review recent structural results concerning the latter, putting special emphasis on the characteristics of the active site.

IN 1931, Stephenson and Stickland showed that the facultative anaerobic colon bacterium Escherichia coli could ac- tivate hydrogen thanks to enzymes they termed hydrogenases 1. More recently, these enzymes have been shown to play a central role in the hydrogen metabolism of many microorganisms of great biotechnological interest, such as methanogenic, acetogenic, nitrogen-fixing, photosynthetic and fermentative. Most of the hydrogenases described to date are metalloproteins containing Ni or Fe, or both. Of these, the NiFe hydrogenases are the most extensively studied and the three-dimensional structures of several enzymes belonging to this group have been reported 2. A surprising feature of these enzymes is that their active sites contain, in addition to a Ni or Fe atom, an FeS cluster. The hydrogenases catalyse the reversible reduction of molecular hydrogen to generate ATP and reducing power; electrons, which are subsequently used in a fermentation. The unrelated Fe-only hydrogenases are at the Dept of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300, USA.

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Structural comparisons

Both amino acid sequence analyses and electronic paramagnetic resonance (EPR) studies have indicated that Fe-only hydrogenases generally contain two [4Fe–4S] clusters (the F-clusters) in a ferredoxin-like domain. In addition, an unusual EPR signal has been attributed to a novel [8Fe] cluster that was proposed to be the active site and was called the H-cluster 3. The positions of the F- and H-clusters in the respective F- and H-domains of C. pasteurianum hydrogenase I (CpI) and D. desulfuricans hydrogenase (DdH) are shown in Fig. 1. DdH is a dimeric periplasmic protein of 53 kDa (43 kDa for the large and small subunits, respectively) and CpI is a monomeric cytoplasmic enzyme of 61 kDa. In addition to the clusters described above, CpI contains two FeS centers coordinated by amino acid residues that are found at the N-terminus of the molecule. One of these domains bears a striking structural similarity to [2Fe–2S] plant-type ferredoxins 4. A short domain (pink in Fig. 1b,c) connects the N-terminal [2Fe–2S] ferredoxin-like module with the F-domain and consists of just two a helices separated by a loop region that coordinates an additional [4Fe–4S] cluster through three cysteinyl ligands and the Ne ring atom of a histidine residue.

As was expected from the high degree of amino acid sequence identity (Fig. 1a) 5,6, CpI and DdH display extensive structural similarities at the H- and F-domains (Fig. 1b,c) 6,7. The root-mean-square deviation (rmsd) for the superposition between the H-domains is 1.0 Å (338 Cα atoms) and that between the F-domains is 1.7 Å (57 Cα atoms). However, the relative orientations of the H- and F-domains are not the same in the two enzymes: the N-terminus of H- and F-domains (381 Cu’s) is 2.2 Å. This is probably a consequence of differences elsewhere in the molecules, such as the two extra Nterminal residues in CpI. The numerous insertions in the F-domains in CpI and the longer C-terminal region of