Auxin Perception—Structural Insights

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The identity of the auxin receptor(s) and the mechanism of auxin perception has been a subject of intense interest since the discovery of auxin almost a century ago. The development of genetic approaches to the study of plant hormone signaling led to the discovery that auxin acts by promoting degradation of transcriptional repressors called Aux/IAA proteins. This process requires a ubiquitin protein ligase (E3) called SCF^{TIR1} and related SCF complexes. Surprisingly, auxin works by directly binding to TIR1, the F-box protein subunit of this SCF. Structural studies demonstrate that auxin acts like a "molecular glue," to stabilize the interaction between TIR1 and the Aux/IAA substrate. These exciting results solve an old problem in plant biology and reveal new mechanisms for E3 regulation and hormone perception.

The phytohormone auxin (indole-3-acetic acid) regulates many plant developmental processes including embryogenesis, root and stem elongation, phyllotaxy, apical dominance, photo- and gravitropism, and lateral root initiation (Muday and DeLong 2001; Reinhardt et al. 2003; Jenik and Barton 2005; Leyser 2005). Genetic and biochemical analyses in *Arabidopsis* have led to the identification of a number of genes involved in auxin perception, signaling and transport (reviewed in (Leyser 2006)). Mutations in one of these genes, *TIR1*, cause defects in several auxin mediated responses such as inhibition of root elongation and induction of lateral root formation (Ruegger et al. 1998). Further studies determined that the TIR1 protein is a component of the ubiquitin-proteasome system (UPS) that mediates protein degradation (Gray et al. 1999). TIR1 was one of the first characterized F-Box proteins (FBP) in plants. FBPs are the substrate receptors of SCF-type ubiquitin protein ligase (E3) complexes and therefore confer substrate specificity to the complex (Deshaies 1999; Skowyra et al. 1999; Cardozo and Pagano 2004). Through their F-box domain, FBPs bind to the SKP1 adaptor protein while their other diverse protein–protein interaction domains bind to the substrates (Zheng et al. 2002).

A number of genetic and biochemical analyses have revealed that SCF^{TIR1} is responsible for ubiquitination and subsequent degradation of the Aux/IAA transcriptional repressors

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in response to auxin (Gray et al. 2001) (Fig. 1) (Santner et al. 2009). Indeed, partial or complete loss of function mutants in SCF^{TIR1} subunits or its regulators exhibit auxin resistant phenotypes, showing that the targets of SCF^{TIR1} are negative regulators of auxin signaling (Gray et al. 1999; Hellmann et al. 2003). The recent demonstration that TIR1 also functions as an auxin receptor is one of the major successes in plant developmental biology during the last decade. Apart from the importance of this work to our understanding of auxin signaling, this was the first demonstration that a ubiquitin protein ligase is regulated by direct binding of a small ligand, in this case auxin. This article will provide an overview of the auxin signal cascade including the structural basis of auxin perception.

THE UBIQUITIN PROTEASOME SYSTEM

The ubiquitin proteasome system (UPS) plays a key role in regulation of cellular activities in all eukaryotes. In mammals, ubiquitin-mediated protein degradation is important in diverse processes including cell-cycle regulation, DNA repair, apoptosis, immune responses, and metabolism. The UPS is equally important in plants where regulated protein degradation impacts virtually every stage of development from embryogenesis through senescence (Vierstra 2009). Ubiquitin-mediated proteolysis is thought to occur in both the cytoplasm and nucleus and involves the attachment of multiple ubiquitin proteins to a protein substrate, which is then usually targeted for degradation by the 26S proteasome (Deshaies 1995; Bates and

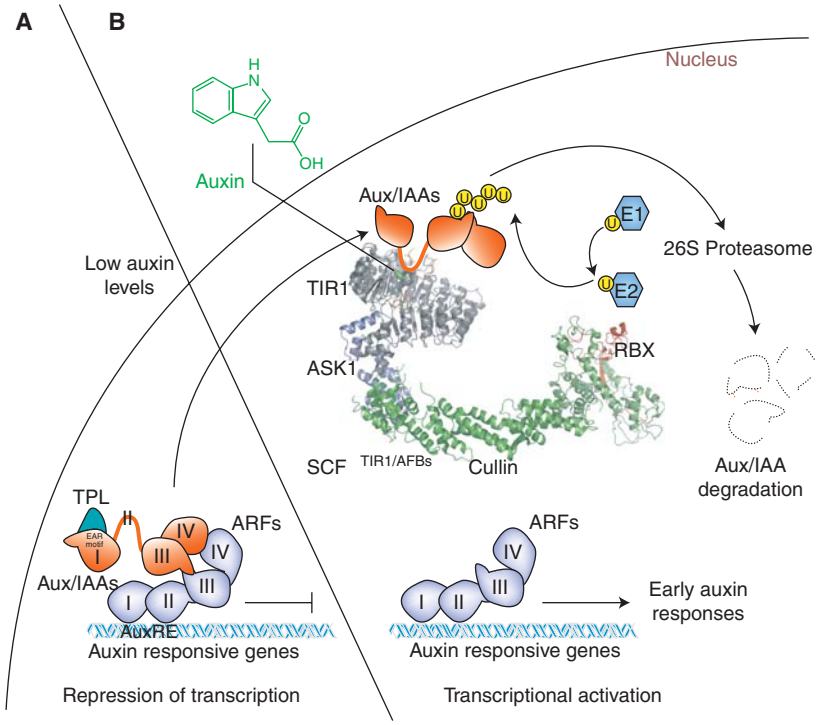

Figure 1. Model for auxin signaling. TIR1 is an F-box protein that binds auxin directly and targets auxin/indole acetic acid proteins (Aux/IAAs) for degradation. (*A*) At low auxin levels, ARF-dependent transcription of auxin response genes is repressed by the Aux/IAAs and the corepressor TPL. These proteins interact through the xxLXLXLxx (EAR motif) of Aux/IAAs and the carboxy-terminal to lissencephaly homology (CTLH) domain of TPL (*B*) Higher auxin levels result in the formation of the TIR1-Aux/IAA complex leading to Aux/IAA ubiquitination and subsequent degradation. Adapted from Santner et al. 2009.

Vierstra 1999). In *Arabidopsis*, nearly 6% of the encoded proteins are thought to participate in the UPS (Smalle and Vierstra 2004; Vierstra 2009). Ubiquitin and related small proteins are conjugated to other proteins through the sequential action of three enzymes called the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3) (Hershko and Ciechanover 1998). The 26S proteasome is a multisubunit complex responsible for the degradation of ubiquitin-tagged proteins and consists of the 19S regulatory particle and the 20S core particle (Baumeister et al. 1998). The 19S regulatory particle consists of two sub-complexes called a lid and a base. Polyubiquitynated substrates are first recognized by the 19S regulatory particle and then unfolded, deubiquitinated, and transferred to the 20S core particle, which is responsible for proteolysis of the target protein.

The E3-ubiquitin ligase subunit determines the specificity of ubiquitination through direct binding to the substrate protein. Typically, post-translational modification of the substrate protein occurs in response to intrinsic or external stimuli and is a prerequisite for recognition by the E3-Ubiquitin ligase. These events include phosphorylation, de-phosphorylation, hydroxylation, glycosylation, as shown in yeast and mammalian systems (reviewed in Pickart 2001).

Based on their structure and functional domains E3-ubiquitin ligases can be separated into several groups. The single subunit E3s include the RING-type E3, HECT (Homologous to E6-AP Carboxy-Terminus) domain E3s and Ubr1p (Ubiquitin amino-end Recognizing Protein 1). The multi subunit E3s present in plants include the APC/C (Anaphase Promoting Complex/Cyclosome and the Cullin-RING E3s, including the SCF (SKP1-Cullin-F-box protein) complexes (reviewed in Pickart 2001).

The largest E3 family in plants are the SCFs (Gagne et al. 2002). These complexes have been shown to ubiquitinate a broad range of proteins involved in cell cycle progression, signal transduction, and transcription (Deshaies 1999). SCF complexes consist of 4 subunits; SUPPRESSOR OF KINETOCHORE PROTEIN 1 (SKP1) (or ASK in plants), CULLIN (CUL), RING BOX1 (RBX1), and a substrate receptor called an F-box protein (FBP) (Deshaies 1999; Skowyra et al. 1999; Cardozo and Pagano 2004). The F-box domain is typically near the amino-terminus of the F-box protein and its function is to bind the SKP1 adapter protein (Cardozo and Pagano 2004). Different FBPs can associate in an interchangeable manner with SKP1 to form a large number of different SCF complexes with distinct substrate specificities (Schwechheimer and Calderón-Villalobos 2004). As in other species, most *Arabidopsis* FBPs contain a variable protein-interaction domain that serves to bind a variety of target proteins and thus confer specificity to the SCF complex (Zheng et al. 2002). Analysis of these proteins demonstrates an array of potential protein-interaction domains including leucine-rich (LRR), Kelch, WD-40, Armadillo (Arm), and tetratricopeptide (TPR) repeats, and Tub, actin, DEAD-like helicase, and jumonji (Jmj)-C domains. The largest class of plant FBPs are those containing LRRs (Gagne et al. 2002). These repeats consist of 20 to 29 amino acids and assemble in a multimeric fashion to form a helical structure.

The CUL and RBX1 subunits form the core of the SCF complexes, bind to diverse FBP-SKP1 subcomplexes and recruit the E2-ubiquitin conjugating enzyme.

The SCF complex is regulated by modification of the CUL subunit by the ubiquitin related protein RUB/NEDD8. RUB conjugation to CUL occurs in a manner similar to ubiquitin conjugation but requires specific E1 and E2 enzymes (Hori et al. 1999). In addition, RUB/NEDD8 is removed from the CUL subunit by the COP9 signalosome (CSN), an evolutionarily conserved multiprotein complex with similarity to the lid of the 26S proteasome (Hori et al. 1999; Osaka et al. 2000). Although the role of CUL modification is not fully understood, continuous rounds of neddylation and deneddylation seem to be essential for SCF assembly and activity (Lyapina et al. 2001; Schwechheimer and Deng 2001; Schwechheimer 2004; Duda et al. 2008; Saha and Deshaies 2008). In addition, another CUL regulator, CAND1 (cullin-associated and

neddylation-dissociated) was shown to preferentially interact with unneddylated cullins and negatively regulate the assembly of SCF-complexes by inhibiting SKP1-CUL interaction (Goldenberg et al. 2004; Duda et al. 2008; Saha and Deshaies 2008)).

The *Arabidopsis* genome encodes at least 6 CUL related proteins, 3 RBXs, 21 ASKs and nearly 700 FBPs (compared to 21 in *S.cerevisiae*, 31 in *D. melanogaster* and around 100 in *H. sapiens*). Together these families represent over 2% of the proteome, clearly demonstrating the importance of SCF-mediated protein degradation in cellular regulation (Vierstra 2009). So far the role of the vast majority of FBPs and therefore SCF type E3s in *Arabidopsis* is unknown.

THE UPS AND AUXIN RESPONSE

The connection between auxin response and the UPS was established through a series of genetic studies that began in the 1980s (Hobbie et al. 1994). The *auxin resistant* (*axr*) mutants were isolated by screening for auxin-resistant seedlings. Subsequent molecular characterization of these mutants revealed that several of the affected genes encode SCF subunits or proteins that regulate SCF function. For example, the *axr1* mutants exhibit a number of dramatic auxin-related phenotypes, including reduced apical dominance, reduced cell elongation, and defects in tropic responses. The cloning of *AXR1* resulted in the identification of the heterodimeric E1-like protein responsible for RUB/Nedd8 activation (Leyser et al. 1993; del Pozo et al. 2002). The second subunit in the dimer is ECR1 (E1 C-terminus Related). AXR1-ECR1 works with the E2 enzyme RCE1 (RUB1-Conjugating Enzyme) to mediate RUB/NEDD8 modification of CUL proteins. Loss-of-function mutants in *RCE1* and *ECR1* also exhibit reduced auxin responses (Leyser et al. 1993; del Pozo et al. 2002; Dharmasiri et al. 2003b; Woodward et al. 2007).

The *AXR6* gene encodes the CUL1 subunit of the SCF. The original *axr6* alleles are homozygous seedling lethal but confer auxin resistance in the heterozygous condition (Hellmann et al. 2003). In addition, it was shown that expression of the auxin regulated genes *IAA5* and *IAA7* is reduced in *axr6* mutants compared to wild type, evidence that AXR6/CUL1 is required for auxin regulation of gene expression. Moreover, and consistent with the broad role of the SCF in plants, complete loss of CUL1 function results in lethality very early in embryogenesis (Shen et al. 2002).

A strong indication that ubiquitin-mediated protein degradation is crucial for auxin response came with the identification of the *transport inhibitor response1* (*tir1*) mutant. The *tir1* mutants were isolated based on their resistance to inhibitors of auxin transport, but were subsequently shown to be resistant to auxin itself (Ruegger et al. 1998). *TIR1* encodes an FBP of the LRR family. Because loss of TIR1 results in auxin resistance, SCF^{TIR1} was thought to mediate the degradation of negative regulators of auxin signaling. As we describe below, subsequent genetic studies showed that these negative regulators are the transcriptional repressors, the Aux/IAA proteins.

AUX/IAAs AND ARFs

The *Aux/IAA* genes were originally identified as auxin regulated genes in pea and soybean. The levels of *Aux/IAA* transcripts increase within minutes of auxin treatment, usually less than 60 min, and therefore were referred to as "early-induced" genes. Other early genes include the *SMALL AUXIN UP RNAs* (*SAUR*) and *GH3* families of genes (Abel and Theologis 1996; Hagen and Guilfoyle 2002). In general, rapid auxin-induction of these genes is not inhibited by protein synthesis inhibitors, as shown in various plant species including soybean, pea, tobacco, and *Arabidopsis* (Abel and Theologis 1996). The *Arabidopsis* genome contains 29 members of the *Aux/IAA* gene family. Expression and phenotypic analyses revealed that many members of the family have redundant function (Abel and Theologis 1996; Reed 2001; Remington et al. 2004; Overvoorde et al. 2005). Although most *Aux/IAAs* are auxin induced, some, such as *IAA28*, show little or no response to exogenous auxin (Rogg et al. 2001). These differences suggest that some Aux/IAAs

have distinct functions during auxin signaling and may interact differentially with other regulators of auxin dependent transcription.

Genetic studies provided important insight into the function of the *Aux/IAA genes.* Another group of *axr* mutants, including *axr2, axr3,* and *axr5*, are dominant and display a number of growth irregularities including defects in shoot and root gravitropism, reduced root length and lack of root hairs. In 1998, the *axr3* gene was cloned and shown to encode IAA17 (Rouse et al. 1998). This was closely followed by the discovery that other dominant auxin resistant mutants affect *Aux/IAA* genes including *shy2/iaa3, axr2/iaa7*, and *axr5/iaa1* and others (reviewed in Reed 2001; Mockaitis and Estelle 2008). In each case, the mutation results in increased stability of the Aux/IAA protein suggesting that they act as repressors and that their degradation is essential for normal auxin response.

During the last 10 yr various groups have clearly shown that auxin promotes degradation of the Aux/IAA proteins through the action of $SCF^{TIR1/AFB1-3}$. Aux/IAAs, including IAA7/AXR2, IAA12/BDL, and IAA17/IAA17 have been shown to interact directly with $SCF^{TIR1/AFB1-3}$ in an auxin dependent manner (Kepinski and Leyser 2004; Dharmasiri et al. 2005a; Dharmasiri et al. 2005b; Kepinski and Leyser 2005). In response to auxin, SCF^{TIR1} increases its affinity for Aux/IAAs, targeting them for degradation (Fig. 1) (Ulmasov et al. 1997; Worley et al. 2000; Gray et al. 2001; Tiwari et al. 2001; Zenser et al. 2001; Tiwari et al. 2004). Mutations in *TIR1* stabilize the Aux/IAA proteins IAA7/AXR2 and IAA17/AXR3, as do mutations in other subunits of the SCF, including CUL1 (Moon et al. 2007; Gilkerson et al. 2009).

The Aux/IAAs are nuclear proteins that contain four conserved domains (reviewed in Reed 2001; Mockaitis and Estelle 2008) (Fig. 2). Domain I is a repressor domain that contains the EAR (Ethylene Response Factor [ERF]-associated amphilic repression) motif (LxLxL), and is responsible for recruitment of the transcriptional co-repressor TOPLESS (TPL) (Long et al. 2006). The TPL protein is necessary for the repressor activity of IAA12/BDL during embryogenesis (Szemenyei et al. 2008), but it remains to be determined if TPL is involved in all aspects of Aux/IAA function. The 17 amino acids that constitute domain II function as a degron motif that confers auxin-dependent degradation by mediating interaction with the TIR1/AFBs. Many reports have clearly shown that domain II is required for the rapid degradation of Aux/IAAs (reviewed in Mockaitis and Estelle 2008) (Figure 2). Further analysis has also shown that a conserved lysine between domain I and domain II contributes to Aux/IAA degradation, since mutation of this residue decreases the turnover rate of AXR3/IAA17 nearly sevenfold (Ouellet et al. 2001). Also, the degradation rate of different Aux/IAAs varies significantly as shown using tagged fusions of the proteins. The half-life of IAA7 in the presence of auxin, for example, is 5 to 10 min whereas IAA28, which has a very similar domain II, has a half-life of 80 min (Dreher et al. 2006). IAA31, on the other hand, which has a domain II, but does not have the conserved lysine has a half-life of 4 hours after auxin treatment. This suggests that other regions in the Aux/IAA proteins might be required for recognition by the $SCF^{TIR1/AFBs}$ and therefore contribute to Aux/IAA degradation. Although, in general Aux/IAA proteins are short-lived proteins, it is worth noting that a small group of Aux/IAAs (IAA20, IAA30, IAA33, and IAA34) do not have the canonical domain II and therefore are auxin-insensitive and long-lived. The role of these proteins in auxin signaling is unclear. However a recent report indicates that overexpression of IAA20 or IAA30 results in strong auxin-related defects (Sato and Yamamoto 2008).

Domains III and IV of the Aux/IAAs share high homology with two domains on the ARF proteins, also called III and IV and mediate homo- and heterodimerization between Aux/IAAs and ARFs (Kim et al. 1997; Ulmasov et al. 1997). Aux/IAAs do not appear to bind DNA directly but exert their transcriptional repressor activity by binding to ARFs. The 23 ARF proteins in *Arabidopsis* act as DNA-binding transcription factors that can either activate or repress auxin responsive genes by binding

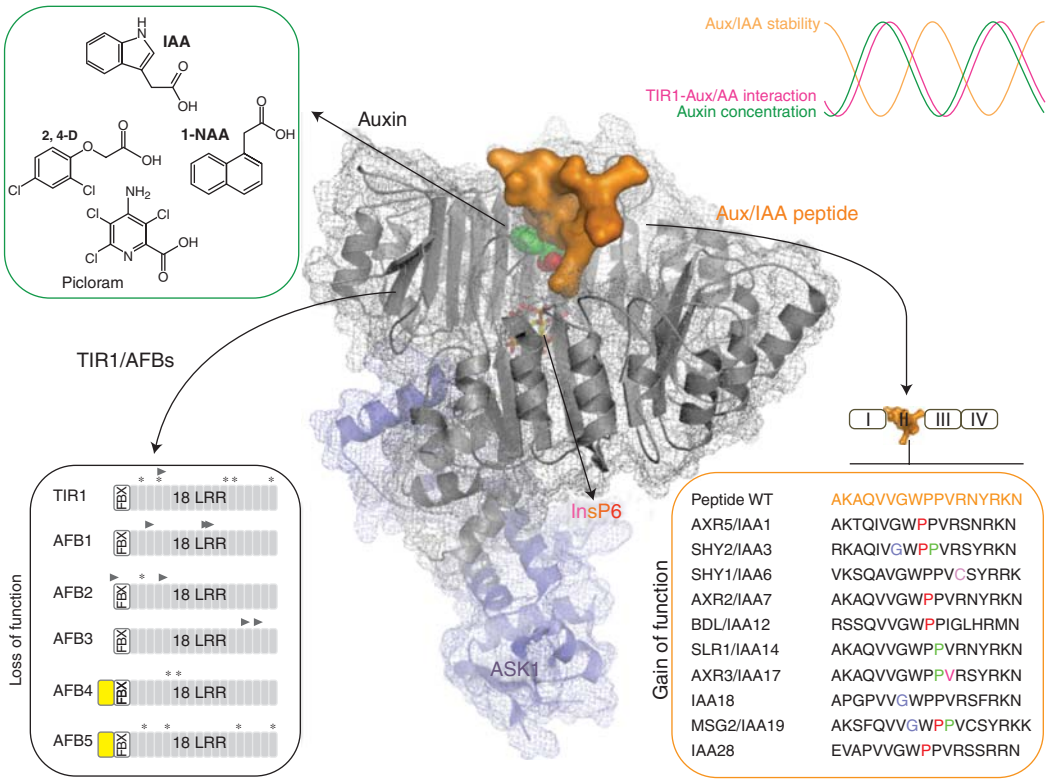

Figure 2. Regulation of ASK1-TIR1-Aux/IAA auxin receptor complexes. Auxin acts like "molecular glue" to stabilize the interaction between TIR1 (gray) and domain II of the Aux/IAA (orange). Gain-of-function mutations in domain II of several Aux/IAAs result in reduced binding to TIR1 and stabilization of the Aux/IAA. A variety of loss-of-function mutations in TIR1/AFB proteins have been characterized (T-DNA insertions (arrows) and point mutations (asterisks)) that result in an auxin-resistant phenotype. Indole-3-acetic acid (IAA) is the major natural auxin but other auxinic compounds, including α-Naphthalene acetic acid (1-NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 4-Amino-3,5,6-trichloropicolinic acid (picloram) promote auxin specific responses in the root or shoot of the plant. These compounds and other natural auxins may bind to the promiscuous auxin binding pocket of TIR1 with different affinities.

to *AuxREs* (auxin responsive elements) in the promoters of these genes. A unifying feature of ARF proteins is the presence of a B3-like DNA binding domain in the amino-terminal region, a variable domain II and domains III and IV dimerization and heterodimerization regions (Okushima et al. 2005). Because the phenotypes observed in Aux/IAA gain-of function mutants are the result of stabilization of Aux/IAAs and constitutive repression of ARF proteins, plants carrying loss of function mutations in *ARF* genes are predicted to have a similar phenotype to *Aux/IAA* mutants. Indeed, root meristem defects in *iaa12/bdl* mutants resemble the rootless phenotype of *arf5/mp* (Hamann et al. 2002; Weijers et al. 2005). In addition, the inhibition of lateral root formation in the *slr/iaa14* mutants is similar to the defects observed in *arf7 arf19* (Weijers et al. 2005; Muto et al. 2007). Further, the shoot phototropism defect observed in *arf7/tir5/nph4* mutants resembles the auxin-resistant phenotype in the hypocotyl in *msg2/iaa19* mutants (Tatematsu et al. 2004). These results illustrate the complexity of the Aux/IAA and ARF interactions and the transcriptional events downstream of auxin perception. ARFs appear to bind *AuxREs* independently of auxin levels,

implying that auxin regulation depends on interactions with Aux/IAAs or other ARFs (reviewed in (Chapman and Estelle 2009).

THE TIR1/AFB PROTEINS

The *Arabidopsis* genome encodes 5 TIR1-related proteins called AUXIN SIGNALING F-BOX1-5 (AFB1-5). AFB1 is most closely related to TIR1 and shares 70% identity, whereas AFB2 and AFB3 are over 80% identical to each other and 60% to TIR1 and AFB1 (Dharmasiri et al. 2005b; Parry et al. 2009). AFB4 and AFB5 are 76% identical to each other and approximately 50% identical to TIR1. The next most closely related protein is COI1 (Coronatine Insensitive) the jasmonic acid receptor (Chini et al. 2007; Thines et al. 2007; Katsir et al. 2008; Melotto et al. 2008). TIR1 and AFB1-5 share the same basic domain structure with the F-Box domain near the amino terminus. Much of the rest of the protein is composed of 18 LRRs. Further, structural studies showed that key residues for auxin and Aux/IAA binding are strongly conserved between TIR1 and AFB1-5 (see later discussion). However, the AFB4 and AFB5 proteins are distinct from the other members of the group because they contain an amino-terminal extension of unknown function (Fig. 2).

In vivo analysis has revealed that like TIR1, the AFB1-3 proteins interact with ASK1-CUL subunits to form SCF complexes (Dharmasiri et al. 2005b). In addition, based on GST pull-down assays, all three AFB proteins were shown to associate with Aux/IAA proteins in an auxin dependent manner, suggesting that they have a similar function to TIR1. Interestingly, single mutants in each of these genes display only mild auxin-related phenotypes. However, analysis of higher order mutant combinations shows that as the genes are progressively disrupted, the phenotypes become more severe with the most highly affected quadruple mutants arresting after germination with a single cotyledon and no root. Quadruple mutant seedlings that progress beyond the early seedling stage exhibit defects in hypocotyl elongation, apical hook and lateral root formation, tropic responses, root hair development, as well as reduced apical dominance (Dharmasiri et al. 2005b; Parry et al. 2009). Although these results indicate that TIR1 and the AFBs have overlapping function the analysis of various mutant combinations has shown that TIR1 and AFB2 have a greater role in root development than AFB1 and AFB3. Also, neither AFB1 nor AFB2 protein can completely replace TIR1 in the *tir1* mutant, even when expressed under the control of the TIR1 promoter (Parry et al. 2009).

The expression of the *TIR1/AFB* genes is also complex. The behavior of promoter-GUS fusion lines demonstrates that *TIR1* and *AFB1-3* promoters are active throughout plant development. However, the analysis of translational fusions lines reveals a more complex situation. While the AFB1-GUS protein is abundant throughout the *Arabidopsis* seedling, TIR1, AFB2, and AFB3 protein accumulation is highly restricted to growing organs, including root tips, leaf primordia and the shoot meristem (Parry et al. 2009). This pattern of regulation may relate to the fact that *TIR1*, *AFB2,* and *AFB3* are regulated by *miR393*, a pathogen induced *miRNA*, whereas *AFB1* is not.

Initial studies of the *AFB5* gene indicate that it may have a specialized function during plant development. Mutations in *AFB5* lack a morphological phenotype but display selective resistance to one class of synthetic auxins, called the picolinates (Walsh et al. 2006). This would suggest that AFB5 is part of an auxin receptor complex with higher binding affinity for picloram. The significance of this selectivity is not clear but it may reflect a difference in the biochemical activity of AFB5. It will also be interesting to see if the closely related AFB4 protein exhibits similar behavior.

Taken together, these results suggest that different members of the TIR1/AFB family may have specialized functions, which would be consistent with the fact that this subfamily diverged early during land plant evolution.

BIOCHEMICAL CHARACTERIZATION OF THE AUXIN CO-RECEPTOR COMPLEX

Although the TIR1 protein was identified in 1997, the connection between SCF^{TIR1},

Aux/IAA degradation, and auxin perception was not clearly understood for several years. In 2001, biochemical and genetic studies showed that SCF^{TIR1} is responsible for degradation of the Aux/IAA proteins (Gray et al. 2001). The interaction between the SCF and the Aux/IAA was demonstrated in a pull-down assay in which TIR1-myc was recovered from plant extracts using recombinant Aux/IAA proteins in the absence and presence of auxin. These experiments showed that the interaction between TIR1 and the Aux/IAA proteins is dramatically enhanced by auxin. Pulldown assays also showed that the TIR1-Aux/IAA interaction occurs through domain II of the Aux/IAA proteins. In addition, experiments with Aux/IAA-luciferase or GUS fusion proteins showed that domain II is required and sufficient for auxin-dependent degradation in plants (Zenser et al. 2001).

Similar studies also showed that TIR1-Aux/IAA binding does not require stable modification of either protein. As mentioned earlier, substrate recognition by an E3 ligase typically requires modification of the substrate. In contrast, pharmacological studies indicate that in the case of SCF^{TIR1}, substrate recognition does not require a stable modification of either protein (Dharmasiri et al. 2003a; Kepinski and Leyser, 2004). Instead these experiments suggested that auxin directly promotes the interaction between the Aux/IAAs and TIR1. Further support for this idea was obtained by showing that [^{3}H] IAA is recovered in the SCF^{TIR1}-IAA7 complex in a pulldown experiment. Auxin binding was strong and saturable, indicating that SCF^{TIR1} functions as a receptor. These experiments reported estimated dissociation constants (K_d) of 20 nM to 80 nM, which correlates closely with the biological activity of auxin in vivo. In addition, binding of TIR1 to the synthetic auxins 1-NAA and 2,4-D was shown to be one and two orders of magnitude weaker, respectively, than the natural auxin IAA. On the other hand, tryptophan, benzoic acid, and 2-NAA, inactive auxin-related molecules did not bind to TIR1-IAA7 (Dharmasiri et al. 2005a; Kepinski and Leyser 2005). These results suggested that IAA binds directly to the complex or to an unidentified protein in the plant extract that interacts with TIR1. To distinguish between these possibilities the authors performed pull-downs with TIR1 synthesized in two different heterologous systems, *Xenopus* embryos and Sf9 insect cells. In both cases, TIR1 bound to Aux/IAA proteins in an auxin dependent manner, confirming that TIR1 and not an unknown protein was responsible for auxin binding. These studies by two independent labs marked a major breakthrough in the search for the auxin receptor complex and significantly advanced our understanding of plant biology.

One year later, in 2007, structural studies revealed a stunning image of TIR1 (Tan et al. 2007). ASK1-TIR1 purified from insect cells was crystallized in a complex with the 17 amino acids that constitute domain II of Aux/IAA proteins in the presence of various auxins including IAA, 1-NAA, and 2,4-D. The structure showed that auxin enhances the affinity of TIR1 for the Aux/IAA proteins, and that auxin and Aux/IAA binding sites are spatially connected. These results, together with the fact that TIR1 and domain II of Aux/IAA contribute to high affinity auxin binding, suggested that TIR1 and the Aux/IAA protein together form a coreceptor complex (Fig. 2) (Calderon-Villalobos, unpublished).

STRUCTURE AND FUNCTION OF TIR1 IN AUXIN PERCEPTION

The unprecedented dual functions of TIR1 as a subunit of the SCF ubiquitin ligase complex and the receptor of a small molecule hormone hint at a novel molecular mechanism of hormone perception. The high-resolution atomic structures of the TIR1-ASK1 complex in different functional states paint detailed pictures of how the TIR1 E3 ligase recognizes auxin and its many analogs, and how the hormone, in turn, mediates the interaction between TIR1 and Aux/IAAs, transmitting a chemical signal through protein ubiquitination and degradation.

OVERALL STRUCTURE OF THE TIR1-ASK1 COMPLEX

The crystal structure of the TIR1-ASK1 complex reveals a mushroom-like overall structure with

the 18 Leucine-Rich-Repeat (LRR) domain of TIR1 forming the "cap" and the F-box domain of TIR1 bound with ASK1 being the "stem" (Fig. 2b) (Tan et al. 2007). The F-box domain is a ~40 residue domain in the amino terminus of TIR1 that forms a three-helix-bundle, extensively interacting with the carboxy-terminal four helices of ASK1. Immediately following the F-box domain, the 18 LRRs of TIR1 fold into a twisted horseshoe-shaped solenoid. The top surface of the TIR1 LRR domain has a single surface pocket that is responsible for binding both auxin and the domain II peptide. In the crystal structure, auxin docks to the bottom of the TIR1 pocket, whereas the Aux/IAA peptide sits on the top of auxin and essentially covers up the pocket. Overall, TIR1 and the conserved Aux/IAA degron peptide sandwich auxin in the middle, which nucleates a hydrophobic core among the three molecules. Unexpectedly, an inositol hexakisphosphate molecule (InsP6) was found in the crystal structure tightly binding to TIR1 right below the auxin-binding pocket.

A structural model of the complete SCF^{TIR1} complex indicates that the relative orientation of the two structurally coupled domains of TIR1, LRR, and F-box, is important. In this model, the top surface of the LRR domain is positioned facing the ubiquitin-activating enzyme (E2), which is docked on the Rbx1 subunit of the SCF complex. Such a spatial arrangement will facilitate the transfer of ubiquitin from the E2 to the Aux/IAA substrate recruited by the LRRs (Fig. 2b).

THE AUXIN-BINDING POCKET OF TIR1 IS PROMISCUOUS

The structure-activity relationship (SAR) of auxin has been extensively investigated in the mid-20th century (Jonsson 1961). Among more than 200 auxinic compounds identified in these studies, only two common features can be recognized as critical for auxin activity—a planar aromatic ring structure and a carboxyl group-containing side chain. A more precise description of the auxin SAR remains elusive (Kaethner 1977; Farrimond et al. 1978). On the one hand, the ring structure and its attached atoms on known auxinic compounds can vary significantly, suggesting a large degree of promiscuity. On the other hand, the two common features alone do not necessarily give rise to an auxin-like molecule. In fact, in many cases, the position of a single atom attached to the planar ring can dictate the activity, indicating a fine level of specificity.

The crystallographic analysis of TIR1 elucidates the structural determinants of the hormone-binding site on the receptor and help rationalize the puzzling SAR of auxin. The auxin and substrate-binding pocket of TIR1 is formed between a long loop projecting out from the second TIR LRR and the inner concave surface of the carboxy-terminal half of the TIR1 LRR domain (Fig. 3A). In the absence of the Aux/IAA degron, the auxin-binding pocket of TIR1 can be likened to a three-walled room with an open ceiling (Fig. 3A,B and Fig. 4). The planar ring of auxin stacks on top of the floor, occupying the lower half of the room. Meanwhile, a conserved basic residue on the floor helps anchor the hormone by forming a salt bridge and hydrogen bonds with the carboxyl group of auxin. Importantly, the lower half of the three walls, which holds the aromatic ring of auxin, is characterized by two hydrophobic phenylalanine residues and the parallel polypeptide backbones of the β-strands found in TIR1 LRRs. Together, they define an overall hydrophobic binding site with a specific shape. Comparison of three auxin molecules (IAA, 2,4-D, and 1-NAA) bound in the pocket reveals the partial promiscuity of the auxin-binding site (Tan et al. 2007). Although the three compounds are diverse in their chemical structures, they all bind to the TIR1 pocket in a similar manner. Their common carboxyl group interacts with the same positive charged residue at the bottom of the TIR1 pocket. Their different ring structures are accommodated by the same part of the TIR1 pocket, although with different degrees of surface complementation. Furthermore, the differences in the ring structures of auxin analogs might account for their

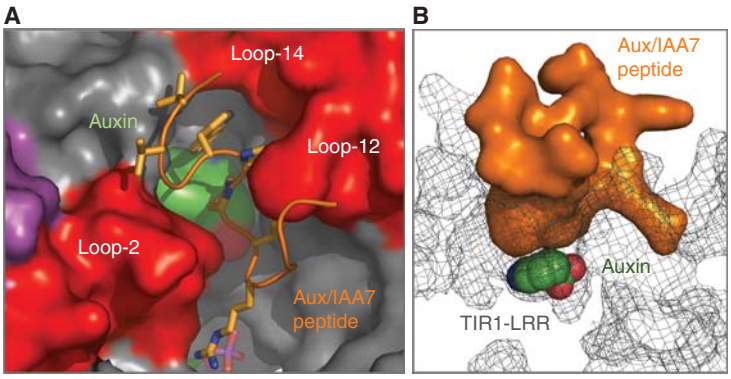

Figure 3. (*A*) Overall view of the TIR1 surface pocket. (*B*) A slab view of the TIR1–auxin–IAA7 peptide complex, showing that IAA7 peptide covers the auxin binding site from the top. The molecular surface of TIR1 is shown in grey mesh. Adapted from (Tan et al. 2007).

different binding affinities to TIR1. For example, IAA, the most potent auxin, has a unique NH group in the indole ring, which forms a hydrogen bond with a nearby carbonyl group of TIR1 backbone. Such an interaction is missing in 2,4-D and 1-NAA and might explain the higher affinity of IAA to TIR1 in comparison to the other two. Overall, the binding mode of auxin on TIR1, in conjugation with its unique functional role at the protein interface as discussed next, provides a plausible explanation for both the selectivity and

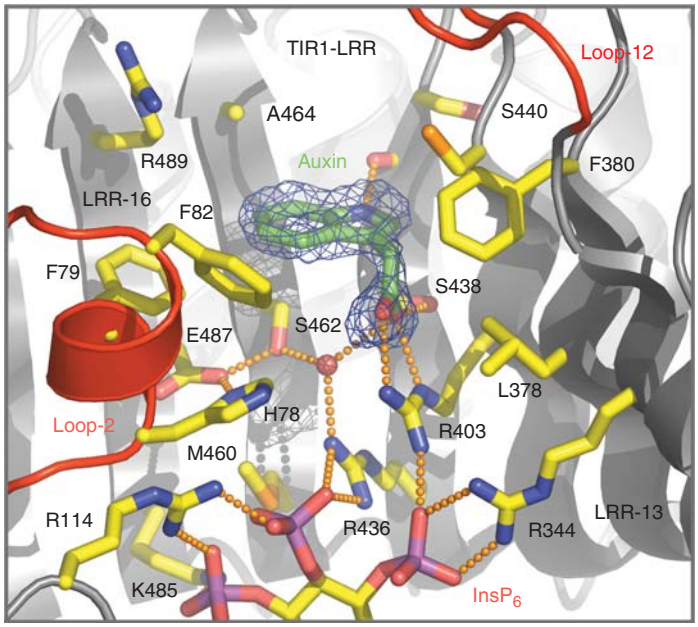

Figure 4. InsP6 binds with a few key residues that form the binding site of auxin. The auxin molecule (IAA) is shown as a green stick model, together with its electron density map. The TIR1 residues surrounding auxin and right underneath the auxin-binding site are shown as a yellow stick model. A central water molecule as part of the pocket floor is shown as a red sphere. The hydrogen-bond and salt-bridge network connecting auxin and InsP6 are indicated by orange dashed lines. Adapted from (Tan et al. 2007).

plasticity in the structural determinants of auxinic compounds.

AUX/IAA DEGRON BINDING AND THE ROLE OF AUXIN AS A MOLECULAR GLUE

Comparison among the structures of TIR1-ASK1 in the free, auxin-bound, and auxin-Aux/IAA degron peptide-bound forms reveals a new hormone action mechanism distinct from allosteric regulation. Superposition analysis of the TIR1 molecule in the presence and absence of auxin shows that auxin binding does not result in a conformational change in the protein, suggesting that, unlike most animal hormones, auxin does not alter the shape of its receptor to modulate its substrate-binding activity. In fact, auxin is also different from most known small molecule hormones by directly interacting with both its receptor and its receptor's substrate. Upon binding to TIR1, the Aux/IAA degron peptide is docked right on top of auxin, completely enclosing the three-walled TIR1 pocket (Fig. 3B). The Aux/IAA degron has predominantly hydrophobic residues that form extensive hydrophobic interactions with the auxin-bound TIR1 surface pocket. It adopts a highly coiled conformation so that the central hydrophobic consensus motif GWPPV is positioned to cover the entire auxin molecule. As described above this signature motif of the Aux/IAA degron is strictly conserved among all Aux/IAA proteins and mutations in this motif result in Aux/IAA stabilization, reduced auxin response and a variety of growth defects. At the center of this motif, the side chains of the tryptophan and the second proline residues pack directly against auxin as well as the surrounding hydrophobic wall of the TIR1 pocket. Their conformation is partially maintained by the first proline, which also forms hydrophobic interaction with TIR1. Overall, auxin nucleates a hydrophobic core together with the degron and the TIR1 pocket, which provides the energy basis for enabling the high affinity interaction between TIR1 and Aux/IAAs. By filling the gap between two proteins, auxin acts as a "molecular glue" that tightly sticks the two proteins together. Such a mechanism is entirely different from the canonical "conformational switch" mechanism adopted by most known hormones.

INOSITOL HEXAKISPHOSPHATE AND ITS POTENTIAL ROLE IN AUXIN PERCEPTION

Inositol hexakisphosphate, InsP6, was first identified in plants because of its abundant presence in the seeds (Irvine and Schell 2001; Stevenson-Paulik et al. 2005). It has later been found to exist across eukaryotic kingdoms. The crystal structure of TIR1 unexpectedly reveals an InsP6 molecule tightly bound to the protein at a functionally important position of the hormone receptor. Surrounded by more than 10 conserved positively charged residues at the concave surface of the TIR1 LRR domain, InsP6 interacts with the auxin-binding pocket from underneath and is in direct contact with the basic residue binding to the carboxyl group of the hormone (Fig. 4). The high affinity and the binding mode of InsP6 at the core of the auxin receptor strongly suggest that it is a functional cofactor of TIR1. Sitting adjacent to the auxin-binding pocket, it appears to perform an organizing and supporting function of the auxin-binding site. Whether InsP6 has a signaling role beyond a structural cofactor will be a very interesting subject for future study.

IMPLICATIONS OF THE AUXIN ACTION MODEL

Auxin regulates plant physiology through multiple pathways and different levels of the hormone might specifically induce the degradation of different Aux/IAAs via different TIR1/AFB family members. Although the crystallographic studies of the minimal TIR1-auxin-Aux/IAA degron peptide complex help reveal the fundamental mechanism underlying auxin perception by TIR1, our understanding of how the system works is far from complete. A number of new concepts and new hypotheses derived from the structural analyses need to be tested. Nonetheless, the significance of these studies is far reaching and will surely

catalyze more investigation in auxin research and beyond.

TIR1 AND AUX/IAAS FUNCTION AS CORECEPTOR FOR AUXIN

The "molecular glue" mechanism of auxin perception represents a novel hormone sensing mechanism. In most known cases of animal hormones, the hormone-binding site is usually located at a position distant from the active site of the receptor. In contrast, the two sites in TIR1 are within the same surface pocket. Aux/IAA binds on the top of auxin and seals the auxin-binding site so that auxin will remain trapped until the Aux/IAA substrate polypeptide is ubiquitinated and released from the TIR1 E3 ligase. The highest affinity of hormone binding, therefore is expected to be achieved when an Aux/IAA substrate is bound to auxin-loaded TIR1. In this sense, TIR1 and Aux/IAAs might be regarded as coreceptors for auxin (Calderon-Villalobos, unpublished). Given the high conservation of the auxin binding site amino acids in TIR1/AFB family, this coreceptor mechanism should be conserved throughout the family. However, differences in TIR1/AFB family members and the Aux/IAA proteins might allow coreceptors of different hormone affinities to form when the F-box proteins are paired with different Aux/IAA proteins (Fig. 2).

SPECIFICITIES AMONG TIR1/AFBS AND AUX/IAAS

As mentioned previously, TIR1 and AFB1-3 have been shown to perceive auxin in a redundant fashion, which is consistent with the high sequence conservation of the four proteins. AFB4 and AFB5, on the other hand, have a higher degree of sequence divergence from the rest and may have different specificity in binding Aux/IAAs and auxin analogs. Indeed, the floor of the predicted hormone-binding pocket of AFB4 and AFB5 has two residues that are different from the other members of the family. How these and other structural elements determine the differential functions of members of the auxin receptor family remains to be investigated.

In addition, the 29 Aux/IAA family members are involved in different functions throughout plant growth and development and have different half-lives in the presence of auxin. It is not difficult to imagine that the more diverse region outside of the their common degron sequence might contribute to their differential binding affinity to TIR1 and therefore their differential responses to the ever-changing hormone level. A full structural model of Aux/IAAs in complex with TIR1 and auxin, together with quantitative understanding of the interaction system will be needed to address this important specificity issue.

AUXIN AGONISTS/ANTAGONISTS AND IMPLICATIONS IN DRUG DISCOVERY

The structural model of auxin perception by TIR1 provides a valuable platform for designing and developing auxin agonists and antagonists. The partial promiscuity of the auxin-binding pocket on TIR1 presents opportunities for altering the pharmacokinetic properties of IAA by changing its chemical structure without sacrificing its auxinic activity. Meanwhile, an auxin agonist with potency higher than IAA might be developed if it can form additional or better interaction to either TIR1 or Aux/IAAs. Because auxin functions as "molecular glue," it has to fill the gap at the imperfect protein interface without causing steric hindrance. To take advantage of such a requirement, an auxin antagonist can be in theory developed if it can bind to TIR1 with high enough affinity and at the meantime introduce structural hindrance to block Aux/IAA binding. In fact, a recent study has elegantly demonstrated the feasibility of these approaches. By adding an aliphatic chain with an increasing length to the α carbon of IAA, it has been shown that agonists of the hormone can be made and eventually converted into antagonists when the additional chain reaches a certain length (Hayashi et al. 2008). Structural analysis of these IAA-derived compounds show that they bind TIR1 in the same pocket as auxin and probably elicit their

differential effects through their variable side chains.

In addition to the impact in plant biology, the discovery of the remarkable molecular glue mechanism of auxin perception points to an exciting new direction for drug discovery and development targeting ubiquitin ligases in humans. Human ubiquitin ligases are hailed as the next generation of drug targets because of their important roles in diverse cellular functions. The conventional drug development strategies of searching for small molecule inhibitors of ubiquitin ligase have had very limited success due to the major obstacle of finding small molecule compounds that can potently disrupt protein–protein interactions, which underlie the functions of most ubiquitin ligases (reviewed in Nalepa et al. 2006). In contrast, auxin acts to regulate ubiquitin ligases by promoting protein–protein interaction. This principle might be directly applicable to ubiquitin ligases in other organisms. In fact, many human disorders such as cancer and Parkinson disease are associated with defective ubiquitin ligases that can no longer bind and ubiquitinate their natural substrates. Their activities might be restorable by small molecules following the same principle that auxin employs. Such compounds can be both small and effective, therefore, more feasible to develop.

CONCLUDING REMARKS

During the last 10 years our understanding of the molecular mechanisms of auxin perception and response has improved dramatically. In the future a major challenge will be to understand how the TIR1/AFB-AUX/IAA-ARF core auxin-signaling module regulates so many disparate processes. Part of this complexity is probably related to differences in the function of individual members of the TIR1/AFB, Aux/IAA and ARF families. For example IAA might be perceived differentially by different receptor complexes, which in turn may trigger different ARF-dependent transcriptional responses. As we learn more about the activities of individual signaling proteins, our ability to generate predictive models of auxin regulated growth will improve. Simple models for auxin-dependent regulation of shoot and root growth have already been developed (Jonsson et al. 2006; Grieneisen et al. 2007; Hamant et al. 2008; Kramer et al. 2008). Ultimately these models will incorporate the diversity of hormone and environmental signals and thus enable an integrated view of plant growth and development.

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The first two authors of this work contributed equally.

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