Auxin Sensitivities of All Arabidopsis Aux/IAAs for Degradation in the Presence of Every TIR1/AFB

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Auxin plays a key role in regulation of almost all processes of plant growth and development. Different physiological processes are regulated by different ranges of auxin concentrations; however, the underlying mechanisms creating these differences are largely unknown. The first step of auxin signaling is auxin-dependent interaction of an auxin receptor with transcriptional co-repressors (Aux/IAA), which leads to Aux/IAA degradation. Arabidopsis has six homologous auxin receptors (TIR1 and five AFBs), 29 Aux/IAA proteins and two types of active auxins, IAA and phenylacetic acid (PAA). Therefore, a large number of possible combinations between these three factors may contribute to the creation of complex auxin responses. Using a yeast heterologous reconstitution system, we investigated auxin-dependent degradation of all Arabidopsis Aux/IAAs in combination with every TIR or AFB receptor component. We found that TIR1 and AFB2 were effective in mediating Aux/IAA degradation. We confirmed that the Aux/IAA domain II, which binds TIR1, is essential for degradation. IAA and other natural auxins, 4-chloroindole-3-acetic acid (4-Cl-IAA) and PAA, induced Aux/IAA degradation; and IAA and 4-Cl-IAA had higher activity than PAA. Effective auxin concentrations for Aux/IAA degradation depended on both Aux/IAAs and TIR1 or AFB2 receptors, which is consistent with the Aux/IAA–TIR1/AFB co-receptor concept.

Keywords: AFB • Arabidopsis thaliana • Aux/IAA • Auxin • Sensitivity • TIR1.

Abbreviations: ARF, auxin response factor; IBA, indole-3-butyr; 4-Cl-IAA, 4-chloroindole-3-acetic acid; EC50, 50% effective concentration; PAA, phenyl acetic acid.

Introduction

Auxin regulates many developmental processes, including embryogenesis (Frimer et al. 2003), apical dominance (Reinhart et al. 2000), cell elongation (Depuydt and Hardtke 2011), gravitropism (Wilson et al. 1990, Rashotte et al. 2000), phototropism (Liscum and Briggs 1996), cell division (Wightman and Lighty 1982), lateral root formation (Benkova et al. 2003), leaf and flower primordium formation (Benkova et al. 2003, Dubrovsky et al. 2008) and vascular tissue formation (Mattsson et al. 2003). These responses appear to be regulated by different ranges of auxin concentrations. For example, root elongation is induced by lower auxin concentrations than stem elongation (Thimann 1937), and the auxin concentration for root elongation is insufficient to initiate lateral root formation (Dubrovsky et al. 2008). The best studied auxin is IAA, but plants also have other molecules with auxin activity. Indole-3-butyr (IBA) is considered to be a storage form that is converted to the active form, IAA (Sauer et al. 2013). 4-Chloroindole-3-acetic acid (4-Cl-IAA) is found in several legumes and in Pinus sylvestris (Porter and Thimann 1965, Reinecke 1999), but to our knowledge there has been no report on the presence of 4-Cl-IAA in Arabidopsis. Exogenously applied 4-Cl-IAA can be more active than IAA (Katayama 2000, Karz and Burdach 2002). Auxin activity of externally applied phenylacetic acid (PAA) is weaker than that of IAA, IBA or 4-Cl-IAA; however, higher levels of PAA than IAA were detected in many plant species (Wightman and Lighty 1982), suggesting possible functions of endogenous PAA.

Auxins are perceived by two classes of receptors the ABP1 extracellular receptor (Xu et al. 2014) and intracellular TIR1/AFB class receptors (Mockaitis and Estelle 2008) in Arabidopsis. The molecular basis of auxin perception that regulates transcription is well known. In the absence of auxin, the transcriptional repressors Aux/IAA proteins bind to the transcription factors ARFs (auxin response factors) (Kim et al. 1997, Ulmasov 1997). Auxins act as an adaptor for binding Aux/IAA proteins to the F-box-containing TIR1, which is a component of the SCFTIR1 ubiquitination E3 complex (Ruegger et al. 1998, Gray et al. 2001). TIR1 has been proposed to be an auxin receptor (Dharmasiri et al. 2005a, Kepinski and Leyser 2005), but auxin does not bind to TIR1 without an Aux/IAA (Calderon Villalobos et al. 2012). Therefore, protein complexes containing SCFTIR1/AFB and an Aux/IAA are referred to as a co-receptor for auxin (Calderon Villalobos et al. 2012). Formation of the SCFTIR1/AFB–auxin–Aux/IAA complex leads to polyubiquitination and degradation of Aux/IAAs, which in turn allows a set of ARFs to activate their target genes. This process is essential for most physiological processes regulated by auxin.
Arabidopsis has 29 Aux/IAA proteins: IAA1–IAA20 and IAA26–IAA34 (IAA21–IAA25 belong to ARFs). Typical Aux/IAAs possess domains I–IV. Domain I binds to the TOPLESS corepressor (Long et al. 2002, Szemenyei et al. 2008, Guilfoyle and Hagen 2012). Domain II (Oeller et al. 1993, Abel et al. 1994) (Fig. 1) is the TIR1-binding domain, which is indispensable for auxin-induced destabilization of Aux/IAAs (Worley et al. 2000, Tan et al. 2007). Although the core 13 amino acid sequence of domain II (Fig. 1) is sufficient for auxin-dependent TIR1 binding (Tan et al. 2007), regions outside domain II also affect binding and degradation (Reed 2001, Calderon Villalobos et al. 2012). IAA20, IAA30 and IAA32–IAA34 lack domain II, IAA31 has a partial domain II sequence and others have well-conserved domain II sequences (Dreher et al. 2006). Domains III and IV are necessary for interactions with ARFs, or with the same or different Aux/IAAs (Reed 2001, Guilfoyle and Hagen 2012).

Arabidopsis has five TIR1 homologs, AFB1–AFB5 (Greenham et al. 2011, Calderon Villalobos et al. 2012), which interact with Aux/IAAs in an IAA-dependent manner (Parry et al. 2009, Greenham et al. 2011, Calderon Villalobos et al. 2012). There is direct evidence that AFB2 and TIR1 mediate auxin-dependent degradation of Aux/IAAs (Havens et al. 2012), and genetic evidence indicates that TIR1 and AFB2 are positive regulators of auxin signaling (Ruegger et al. 1998, Dharmasiri et al. 2005b, Parry et al. 2009). A T-DNA insertion mutant afb3-1 is resistant to auxin (Dharmasiri et al. 2005b), but another mutant, afb3-4, is not (Parry et al. 2009). The afb1-1 mutant adds auxin resistance to the tir1-1 afb2-1 afb3-1 triple mutant (Dharmasiri et al. 2005b), but the afb1-3 mutant does not add auxin resistance to the tir1-1 afb2-3 afb3-4 triple mutant (Parry et al. 2009). Thus, the in vivo roles of AFB1 and AFB3 are unclear (Parry et al. 2009). AFB4 (Parry et al. 2009, Calderon-Villalobos et al. 2010, Greenham et al. 2011) and AFB5 (Walsh et al. 2006) were suggested to be negative regulators of auxin-regulated processes.

The molecular basis for the complex nature of auxin responses is being uncovered by large-scale examination of interactions of Aux/IAAs, TIR1 or AFB receptor components, and auxins. IAA functions as an adaptor for association of Aux/IAAs with TIR1 or AFB2 (Parry et al. 2009, Calderon Villalobos et al. 2012).
Auxin sensitivity of Aux/IAA degradation depends on both Aux/IAAs and TIR1 or AFB2

To compare the IAA dose dependence of Aux/IAAs, we calculated the 50% effective concentration (EC50) of IAA for Aux/IAA degradation (Table 1). In yeast strains expressing TIR1 or AFB2, EC50 values differed for different Aux/IAAs, indicating that auxin sensitivity depends on particular Aux/IAAs. Auxin sensitivities of Aux/IAAs in the presence of TIR1 and AFB2 were correlated. However, there are some exceptions. For example, the EC50 value of IAA for IAA10 in the presence of AFB2 was lower than half that of EC50 in the presence of TIR1 (Fig. 3; Table 1), whereas EC50 values of IAA for IAA1, IAA12, IAA28, and IAA28 in the presence of TIR1 were lower than half those of the EC50 values in the presence of AFB2 (Fig. 3; Table 1). Similar tendencies were observed with 4-Cl-IAA or PAA (Supplementary Figs. S4, S5). Thus, auxin sensitivities are determined by combinations of particular TIR1/AFBs and Aux/IAAs. In several cases, the Aux/IAA–LUC levels were decreased by TIR1, OsTIR1 or AFB2 even in the absence of exogenous auxin (Supplementary Fig. S6).

Auxin species effectiveness for degradation of Aux/IAAs

Although plants have multiple endogenous auxins, including IAA, 4-Cl-IAA, PAA and IBA, direct effects of these molecules on Aux/IAA degradation have been reported for only IAA (Havens et al. 2012). Therefore, we examined the effects of these auxins on all Arabidopsis Aux/IAAs in combination with every TIR1 or AFB protein. IBA at 1 μM was ineffective for any of the Aux/IAAs (data not shown). IAA and 4-Cl-IAA induced degradation of Aux/IAAs at similar concentrations (Fig. 4; Table 1; Supplementary Fig. S7). PAA was less effective compared with IAA or 4-Cl-IAA (Fig. 4; Table 1; Supplementary Fig. S7). Overall, sensitivities to IAA, 4-Cl-IAA and PAA were correlated (Fig. 4; Table 1). However, the effects of PAA on IAA12 and IAA13 in the presence of TIR1 were exceptional: IAA1–IAA4, IAA7–IAA9, IAA14, IAA16, IAA17 and IAA27 were similarly sensitive to IAA, but PAA sensitivities of IAA12 and IAA13 were higher than that of others of this group in the presence of TIR1 (Fig. 4; Supplementary Fig. S3).

Relationship between auxin sensitivity, auxin affinity and degradation rate

Auxin sensitivities of Aux/IAAs (EC50[IAA]) determined in this study and auxin affinities for the Aux/IAA–TIR1/AFB co-receptors (κd) (Calderon Villalobos et al. 2012) were correlated, except for IAA12 (Supplementary Fig. S8). Auxin sensitivities of Aux/IAAs and auxin-dependent maximum degradation rates (Havens et al. 2012) of Aux/IAAs were also correlated, including IAA12 (Supplementary Fig. S9). These results suggest that the EC50 depends not only on the affinity for auxin, but also on degradation rates.

Results

All Arabidopsis Aux/IAAs that have domain II can be degraded in an auxin-dependent manner

To evaluate the sensitivities of Aux/IAAs to auxins, we used an auxin perception system developed in yeast (Fig. 1). We established yeast strains co-producing each luciferase-fused Arabidopsis Aux/IAA (IAA1–IAA20 and IAA26–IAA34) and each TIR1/AFB (TIR1 and AFB1–AFB5) in all combinations. TIR1 or AFBs were translationally fused to yeast SKP1, a component of SCF complexes that binds F-box domains of yeast F-box-containing proteins. We used the fusion strategy because it was not known how effectively AFBs, which carry an F-box domain, could bind to yeast SKP1 and because a TIR1–SKP1 fusion was reported to function properly in Schizosaccharomyces pombe (Kanke et al. 2011). By using Western blotting, we confirmed that TIR1 or AFB proteins were produced in yeast (Supplementary Fig. S1). Luciferase activity was measured after the yeast strains were treated with different concentrations of auxins, in the presence of the protein synthesis inhibitor cycloheximide, for 3 h. We found that all Arabidopsis Aux/IAAs, except for those lacking domain II (IAA20, IAA30 and IAA32–IAA34), were degraded in an auxin-dependent manner when co-produced with Arabidopsis TIR1 (AtTIR1), AFB2 or rice OsTIR1 (Fig. 2; Supplementary Figs. S2, S3). AFB1 and AFB3–AFB5 could not mediate degradation of any Aux/IAAs (data not shown), which is consistent with the findings of a previous report (Havens et al. 2012). Degradation of IAA31, which carries an imperfect domain II, was induced only by 4-Cl-IAA (Supplementary Fig. S2).
Fig. 2 IAA dose-dependent degradation of Aux/IAAs in the presence of AtTIR1, OsTIR1 or AFB2. Luciferase activities are relative to the values obtained in the absence of IAA. Controls, yeast not carrying TIR1 or AFB. The data were fitted with a Richard’s five-parameter dose–response curve. Error bars represent standard errors.
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<tr>
<th>IAA</th>
<th>4-Cl-IAA</th>
<th>PAA</th>
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<tr>
<td></td>
<td>EC50 [IAA (μM)]</td>
<td>95% CI</td>
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<tr>
<td>TIR1</td>
<td>AFB2</td>
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<tr>
<td>IAA1</td>
<td>0.3626</td>
<td>0.2973–0.4422</td>
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<tr>
<td>IAA2</td>
<td>0.3711</td>
<td>0.2855–0.4823</td>
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<td>IAA3</td>
<td>0.3220</td>
<td>0.2915–0.3557</td>
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<td>IAA4</td>
<td>0.2967</td>
<td>0.2144–0.4107</td>
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<td>IAA5</td>
<td>0.3913</td>
<td>0.2257–0.6784</td>
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<td>IAA6</td>
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<td>IAA9</td>
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<td>IAA12</td>
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<td>IAA19</td>
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<td>IAA24</td>
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CI, confidence interval; ND, not determined because of an atypical dose–response curve, large data dispersions or low degree of auxin-induced degradation.
Fig. 3 (A) Sensitivities of Aux/IAAs to IAA in the presence of TIR1 or AFB2. The numbers on the graphs are Aux/IAA numbers (number x denotes the IAAx protein). (B) Close-up view of the low concentration range shown in (A). Error bars represent standard errors.

Fig. 4 Correlation between IAA and PAA sensitivities of Aux/IAAs. The numbers on the graphs are Aux/IAA numbers (number x denotes the IAAx protein). The EC50 of IAA and PAA in the presence of TIR1 (A and B) or AFB2 (C and D). Error bars represent standard errors.
**Discussion**

### Use of the reconstituted auxin perception system in yeast for examination of auxin sensitivities of the co-receptors

We utilized the heterologous system in yeast to examine auxin sensitivities of all Arabidopsis Aux/IAAs, TIR1, OsTIR1, AFB1, or AFB2 enhanced degradation of some Aux/IAAs in the absence or presence of auxins applied during the 3 h cycloheximide treatment (Supplementary Fig. S6). It is possible that some Aux/IAAs may respond to an endogenous auxin, as Saccharomyces cerevisiae produce IAA (Robinson and Stier 1941, Rao et al. 2010). Indeed, Aux/IAAs with high auxin sensitivities tended to be degraded in the absence of auxin. For analyses of auxin dose–responses, we used RLU (relative luminescence units) as relative values compared with the luciferase signals in the absence of auxin. Therefore, Aux/IAAs that are highly degraded in the absence of auxin (IAA5, IAA7, IAA9 and IAA17 in the presence of TIR1, AFB2 or OsTIR1) did not produce symmetric sigmoid curves (Fig. 2; Supplementary Fig. S2). So, the true EC$_{50}$ of these Aux/IAAs could be lower.

Another possibility is that Aux/IAAs and TIR1/AFBs with high auxin sensitivities tend to interact in the absence of auxin. It is unknown why IAA5 in the presence of AFB2 and IAA17 in the presence of OsTIR1 did not show typical sigmoid curves (Fig. 2; Supplementary Fig. S2).

### TIR1 and AFB2 can mediate auxin-dependent degradation of Aux/IAAs

In our reconstitution system in yeast, TIR1 and AFB2 mediated auxin-dependent degradation of all Arabidopsis Aux/IAAs that have domain II, but AFB1 and AFB3–AFB5 did not. Havens et al. (2012) also detected Aux/IAA degradation when co-expressed with TIR1 or AFB2, but not with AFB1 or AFB3. TIR1 and all AFBs have been shown to bind several Aux/IAAs in an IAA-dependent manner in pull-down assays or yeast two-hybrid assays (Dharmasiri et al. 2005b, Parry et al. 2009, Greenham et al. 2011, Calderon Villalobos et al. 2012). Loss-of-function $\text{tir1}$, $\text{afb1}$, $\text{afb2}$ and $\text{afb3}$ mutations can cause auxin resistance, suggesting that the corresponding proteins are positive regulators of auxin signaling (Dharmasiri et al. 2005b). However, in another study (Parry et al., 2009), mutation in AFB1 or AFB3 had an unclear effect on auxin responses. The reason for the inability of AFB1 and AFB3 to mediate Aux/IAA degradation in yeast is unclear. AFB1 and AFB3 may not participate in the canonical auxin signaling pathway in plants, or they may not fold properly or they may require additional components in yeast to function. Loss-of-function $\text{afb4}$ or $\text{afb5}$ mutants are hypersensitive to IAA, but resistant to the picolinate class of artificial auxins (Walsh et al. 2006, Greenham et al. 2011). Although AFB4 and AFB5 bind Aux/IAAs in the presence of auxins (Greenham et al. 2011, Vernoux et al. 2011, Calderon Villalobos et al. 2012), this binding may not destabilize Aux/IAAs.

### Combinations of Aux/IAAs and TIR1 or AFBs determine auxin sensitivities

Our results indicate that auxin sensitivity of Aux/IAA for degradation is determined by the combinations of Aux/IAAs and TIR1 or AFBs. This is consistent with the ‘co-receptor concept’, which implies that an Aux/IAA, auxin and an SCF$_{TIR1/AFB}$ form a ternary complex (Calderon Villalobos et al. 2012). The core 13 amino acid sequence (consensus: QVGWPPVRSYRK) of domain II in Aux/IAA binds to TIR1 (Tan et al. 2007) in an auxin-dependent manner. In our study, only Aux/IAAs carrying domain II were degraded in an auxin-dependent manner, confirming that domain II is essential for auxin-dependent degradation. IAA31 has a highly diverged domain II, in which only three central residues (WPP) are conserved. IAA31 was shown to be slowly degraded in the presence of 5 µM 2,4-D (a synthetic auxin) in Arabidopsis (Dreher et al. 2006). In our study, IAA31 was only partially degraded in the presence of 4-Cl-IAA (Supplementary Fig. S2).

In many dominant auxin-resistant mutants, mutations affected the amino acid residues between the fourth amino acid, glycine, and the eighth amino acid, valine (Reed 2001). Thus, the fourth to eighth amino acid sequences (GWPP[V/I]) are essential for auxin-induced degradation, but its C-terminally flanking sequence in the core domain II and perhaps regions outside domain II also contribute to auxin-induced degradation. EC$_{50}$[IAA] values of all Aux/IAAs that have RxxRKN (x denotes any amino acid residue) following GWPP[V/I], in the presence of either TIR1 or AFB2, are <1 µM. Also, all Aux/IAAs with an EC$_{50}$[IAA] of >1 µM, in the presence of TIR1 or AFB2 (except for the EC$_{50}$ for IAA5, which is unreliable), do not have the RxxRKN sequence. However, this sequence is not essential because IAA6, IAA12 and IAA13 have diverged sequences but exhibit an EC$_{50}$ < 1 µM.

### Aux/IAA degradation in response to three different auxin species

As a whole, IAA and 4-Cl-IAA had higher activity than PAA, and the sensitivities of pairs of Aux/IAA and TIR1/AFB to these three auxins correlated. Interestingly, IAA12/BDL, and its close homolog, IAA13, have relatively high sensitivities to PAA (Fig. 4B; Supplementary Figs. S3, S5) in the presence of TIR1. IAA12/BDL is implicated in embryogenesis (Weijers et al. 2005). It would be interesting to examine the tissue-specific distribution of PAA.

### Possible functions of relatively stable Aux/IAAs

IAA20, IAA30 and IAA32–IAA34 lack domain II, and are not degraded by auxins. IAA20, IAA30 and IAA34 have the LxLxL motif in domain I, which binds the TOPLESS co-repressor (Loker and Weijers 2009). Repressors not degradable by auxins can limit the magnitude of auxin responses. IAA10, IAA11, IAA15, IAA28, IAA29 and IAA31 also have the LxLxL motif and are relatively less sensitive to auxins, suggesting that they are potentially limiting factors of auxin sensitivity.
Multiple mutants for genes encoding these proteins will be needed to test these hypotheses.

Concluding remarks

Auxin sensitivities measured in this study and half-lives (Havens et al. 2012) of all Arabidopsis Aux/IAAs, interaction between Aux/IAAs and ARFs (Vernoux et al. 2011), and relatively fine expression data for Aux/IAAs and TIR1 genes (Brady et al. 2007, Yadav et al. 2009) are now available. Knowledge of ARF target genes is also increasing (Okushima et al. 2005, Okushima et al. 2007). Integration of this body of knowledge will contribute to understanding of the systems biology of auxin-regulated processes.

Materials and Methods

Vector construction

A DNA fragment containing the TEF promoter, a multicloning site and the CYC1 terminator was excised from p415TEF (Mumberg et al. 1995) by using ScaI and KpnI, and inserted into the ScaI and KpnI sites of pRS304 and pRS306 (Sikorski and Hieter 1989) to produce pRS304TEF and pRS306TEF, respectively. The luciferase-coding sequence was amplified by PCR from the pG3-3Basic vector (Promega) and inserted into the SpeI and BamHI sites of the pRS304TEF vector to produce pRS304TEF::Luc. The SKP1-coding sequence was amplified by PCR from genomic DNA of the yeast strain W303A and inserted into the SpeI and BamHI sites of the pRS306TEF vector to produce pRS306TEF::SKP1. cDNAs encoding Aux/IAAs were amplified by PCR from the pGL3-Basic vector (Promega) and inserted into the 3′ terminus of luciferase-Aux/IAA constructs, whereas mating type α was used for pRS306TEF::SKP1-TIR1 or AFB constructs. Integration was confirmed by PCR. Strains to be mated were co-inoculated into YPAD (1% yeast extract, 2% bacto-peptone, 2% glucose, and 0.01% adenine) for 8 h, and plated out to single colonies on SC-Trp-Ura to select for diploids.

Luciferase assays

The levels of luciferase–Aux/IAA fusion proteins were determined by using the Steady-Glo Luciferase Assay System (Promega). Yeast strains were cultured in YPAD in 96-deep-well plates (DW-20SQ, Ina-optika) (500 µl per well) at 20°C until the stationary phase. The cultures were diluted 1:50 with YPAD, cultured at 20°C for 5 h to release them from the stationary phase, dispensed into 384-well plates (5 µl per well) that contained 5 µl of YPAD per well with 400 µg ml⁻¹ cycloheximide and varying concentrations of one of the tested auxins or dimethylsulfoxide (DMSO; the concentrations of auxins are listed in Supplementary Table S2), and incubated at 20°C for 3 h before luciferase assay. Steady-Glo reagent (10 µl) was added to the culture (10 µl) and, after 10 min, the luciferase signal was measured with a TriStar LB 941 Multimode Microplate Reader (Berthold) at 20°C. Light signals were captured for 0.25 s. Values relative to the mock treatment with 0.1% DMSO were used for the data analyses. To calculate the EC₅₀, the data were imported into the GraphPad Prism 6 package (GraphPad Software, Inc.) and fitted with a Richards five-parameter logistic equation (Giraldo et al. 2002), in which logarithmically converted data are used for calculation. Using the fitted line, the EC₅₀ of IAA was determined as the IAA concentration that induces 50% degradation of Aux/IAAs compared with degradation at 1 mM IAA.

Western blotting

Protein extracts were prepared by the trichloroacetic acid extraction method. Yeast cells (10 ml) in the log phase (OD₅₆₀ = 0.6) were centrifuged for 10 min at 1,000×g, 4°C, and pelleted cells were disrupted in 20% trichloroacetic acid (600 µl) using glass beads. Then the homogenates were centrifuged for 10 min at 100,000×g, 4°C. The pellets were dissolved in 200 µl of High-pH Laemmli buffer (150 mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue), and denatured at 100°C for 5 min. Lysates were centrifuged at 1,000×g for 10 min, and the supernatants were diluted 10-fold with a liquid containing 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue, and aliquots...
(5 μl) were separated by SDS–PAGE. The presence of proteins of SKP1-fused TIR1 or AFBs were checked by Western blot analysis using primary S. cerevisiae SKP1 antibody (polyclonal YC-20, Santa Cruz Biotechnology) and secondary anti-goat/sheep IgG–peroxidase (monoclonal A9452, Sigma-Aldrich).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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