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Protein ubiquitination in auxin signaling and transport

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Chapter 2

Auxin-induced, SCF^{TIR1}-mediated poly-ubiquitination marks AUX/IAA proteins for degradation

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Summary

The plant hormone auxin (indole-3-acetic acid or IAA) regulates plant development by inducing rapid cellular responses and changes in gene expression. Auxin promotes the degradation of Aux/IAA transcriptional repressors, thereby allowing auxin response factors (ARFs) to activate the transcription of auxin-responsive genes. Auxin enhances binding of Aux/IAA proteins to the receptor TIR1, which is an F-box protein that is part of the E3 ubiquitin ligase complex SCF^{TIR1}. Binding of Aux/IAA proteins leads to degradation via the 26S proteasome, but evidence for SCF^{TIR1}-mediated poly-ubiquitination of Aux/IAA proteins is lacking.

Here we used an *Arabidopsis* cell suspension-based protoplast system to find evidence for SCF^{TIR1}-mediated ubiquitination of the Aux/IAA proteins SHY2/IAA3 and BDL/IAA12. Each of these proteins showed a distinct abundance and repressor activity when expressed in this cell system. Moreover, the amount of endogenous TIR1 protein appeared to be rate-limiting for a proper auxin response measured by the co-transfected *DR5::GUS* reporter construct. Co-transfection with *35S::TIR1* led to auxin-dependent degradation, and excess of *35S::TIR1* even led to degradation of Aux/IAAs in the absence of auxin treatment. Expression of the mutant tir1-1 protein or the related F-box protein COI1, which is involved in jasmonate signaling, had no effect on Aux/IAA degradation. Our results show that SHY2/IAA3 and BDL/IAA12 are poly-ubiquitinated and degraded in response to increased auxin or TIR1 levels. In conclusion, our data provide experimental support for the model that SCF^{TIR1}-dependent poly-ubiquitination of Aux/IAA proteins marks these proteins for degradation by the 26S proteasome, leading to activation of auxin-responsive gene expression.

Introduction

The plant hormone auxin (indole-3-acetic acid or IAA) plays an essential role in a large variety of developmental processes throughout a plant's life cycle. Auxin is transported in a polar cell-to-cell manner, and this transport directs cell division and growth by generating dynamic auxin gradients in tissues and organs. At the cellular level, auxin concentrations are translated into a gene expression response by the complex and dynamic interaction between two major families of transcriptional regulators: the Auxin Response Factors (ARFs) and the labile Aux/IAA proteins (Guilfoyle *et al.*, 1998; Ulmasov *et al.*, 1999; Tiwari *et al.*, 2001). ARF proteins bind to specific sequences in the promoters of auxin-responsive genes through their N-terminal DNA-binding domain, and either activate or repress transcription. At the C-terminus they share the conserved domains III and IV with the Aux/IAA proteins, through which they homo- or heterodimerize with other ARFs or with the Aux/IAA proteins (Guilfoyle *et al.*, 1998) .

Aux/IAA proteins are short-lived transcriptional regulators that repress transcription controlled by auxin-responsive elements (AuxREs) by heterodimerizing with ARFs. The repressor activity of these proteins is located in the N-terminal domain I, whereas their stability is regulated by the central domain II. Auxin binds to the receptor TIR1 or the related Auxin signalling F-Box (AFB) proteins that are part of the E3 ubiquitin ligase complexes $SCF^{TIR1/AFB}$. Auxin enhances the affinity of TIR/AFB for domain II of Aux/IAAs (Gray *et al.*, 2001; Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Tan *et al.*, 2007). Mutations in either the Aux/IAA domain II or in one of the SCF components lead to auxin-resistant phenotypes that are mostly due to the stabilization of the Aux/IAA repressors (Worley *et al.*, 2000; Ouellet *et al.*, 2001). Moreover, treatment of plants with proteasome inhibitors leads to the accumulation of these proteins, indicating that Aux/IAA protein levels are controlled by the 26S proteasome (Gray *et al.*, 2001; Ramos *et al.*, 2001; Tian *et al.*, 2003). Taken together, this information leads to a model in which auxin-enhanced binding of TIR1/AFB to domain II of the Aux/IAAs results in

ubiquitination of these proteins, which marks them for degradation by the 26S proteasome. However, there is no direct experimental evidence for the $SCF^{TIR1/AFB}$-mediated ubiquitination of Aux/IAA proteins. Recent proteome-wide screens using a multi-dimensional protein identification technology were not able to detect Aux/IAAs among the ubiquitinated proteins in *Arabidopsis* (Maor *et al.*, 2007; Manzano *et al.*, 2008), suggesting that more direct methods may be necessary to detect the ubiquitinated Aux/IAAs.

Although implicated in a large variety of cellular responses, protein ubiquitination of plant transcriptional regulators has been demonstrated for only a few targets, including SLENDER RICE1 (SLR1) by SCF^{GID2} in gibberellin responses (Sasaki *et al.*, 2003), LONG HYPOCOTYL IN FAR RED (HRF1), LONG AFTER FAR-RED LIGHT1 (LAF1) and LONG HYPOCOTYL 5 (HY5) by CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) during photomorphogenesis (Xie *et al.*, 2002; Saijo *et al.*, 2003; Jang *et al.*, 2005), and LEAFY (LFY) by SCF^{UFO} (Chae *et al.*, 2008) in floral development. Targeted proteolysis by SCF-mediated ubiquitination has been implied for the transcription factor ETHYLENE INSENSITIVE3 (EIN3) by $SCF^{EBF1/EBF2}$ in ethylene signaling (Potuschak *et al.*, 2003; Guo and Ecker, 2003), for JASMONATE-ZIM-DOMAIN (JAZ) repressors by SCF^{COI1} in jasmonate signaling (Thines *et al.*, 2007; Chini *et al.*, 2007), and for many other proteins in diverse processes, but experimental evidence for ubiquitination of these proteins and for the specificity of the SCF-ligases for their targets is lacking.

SHORT HYPOCOTYL 2 (SHY2/IAA3) and BODENLOS (BDL/IAA12) are distantly related Aux/IAA proteins that regulate auxin responses in different stages of a plant's life cycle. Both have been shown to interact with TIR1 and to be stabilized by treatment with proteasome inhibitors. The mutant proteins shy2-2 and bdl carry the same Proline to Serine mutations in their domain II, leading to stabilized products show no or only residual interaction with TIR1/AFB (Tian *et al.*, 2003; Dharmasiri *et al.*, 2005b). BDL/IAA12 has been described as an interactor/regulator of the ARF MONOPTEROS (MP/ARF5) acting on embryo patterning (Hamann *et al.*, 2002). SHY2/IAA3 is also able to interact with MP/ARF5 and to inhibit its activity, but the expression patterns of

these two proteins do not overlap *in planta*. Recent studies have connected SHY2/IAA3 action to modulation of NON-PHOTOTROPHIC HYPOCOTYL4 (NPH4/ARF7)- and ARF19-regulated hypocotyl growth (Weijers *et al*., 2005).

Aux/IAAs are short-lived proteins with hardly detectable endogenous levels (Abel *et al*., 1994; Ramos *et al*., 2001; Tian *et al*., 2003). This instability and rareness makes the study of their targeted degradation difficult to perform *in planta*. For a long time now, plant cell protoplasts have been used to assess hormone responses due to their amenability for transformation and their responsiveness to diverse stimuli (Abel and Theologis, 1998; Sheen, 2001). Much of the information gathered on the mechanisms of regulation of Aux/IAA stability has been performed using transiently transformed protoplasts (Tiwari *et al*., 2001; Ramos *et al*., 2001; Tiwari *et al*., 2004), and other important components of the auxin signaling pathway have been functionally characterized in this system including ARFs, SCF^{TIR1} and RAC GTPases (Guilfoyle *et al*., 1998; Tao *et al*., 2005; Wang *et al*., 2005).

In this study, we used *Arabidopsis* cell suspension protoplasts to demonstrate that auxin-enhanced TIR1-mediated ubiquitination of SHY2/IAA3 and BDL/IAA12 marks these proteins for degradation and leads to auxin-responsive gene expression. Our results show that auxin sensitivity of *Arabidopsis* protoplasts is strictly connected to the relative abundance of the TIR1 protein and Aux/IAA proteins, and that an excess of TIR1 leads to depletion of Aux/IAAs even in the absence of exogenous auxin. The mutant protein tir1-1 and the related F-box protein COI1, which is involved in jasmonate signaling, had no effect on Aux/IAA stability or ubiquitination, corroborating the specificity and importance of TIR1 in the process.

EXPERIMENTAL PROCEDURES

Protoplast isolation and transformation

Arabidopsis thaliana Col-0 cell suspension cultures were used for protoplast preparations. Culture maintenance, protoplast isolation and transfections were performed as previously described (Schirawski *et al.*, 2000) with minor modifications. Four-to-six days old cultures were diluted 5-fold in auxin-free Cell Medium (30 g /L saccharose, 3.2 g/L Gamborg's

B5 basal medium with mineral organics, adjusted to pH 5.8 with KOH and sterilized by autoclaving), incubated overnight and used for protoplast isolation in auxin-free solutions. Transfected cells were kept at 25°C in the dark for 16 hours before treatments. Where necessary, additional DNA of plasmid pART7 (Gleave, 1992) was added, to equalize the amount of DNA for each transformation.

DNA constructs

For the auxin-responsive GUS assays, a *DR5::GUS* reporter construct with 7 copies of the DR5 sequence cloned in the plasmid GusXX-47 (Pasquali *et al.*, 1994) was used. A plasmid carrying the *Renilla reniformis* luciferase (*LUC*) gene under the control of the *CaMV 35S* promoter was co-transfected as a control for transformation efficiency (De Sutter *et al.*, 2005). All effector plasmids are based on pART7 carrying the *CaMV 35S* promoter and *OCS* terminator. GATEWAY® (Invitrogen, www.invitrogen.com) destination cassettes derived from pEarleyGate 201 and 202 (Earley *et al.*, 2006) were transferred into pART7 to generate plasmids pART7-HA and pART7-FLAG for the expression of respectively N-terminally HA- or FLAG- tagged proteins in plant cells.

N-terminally HA-tagged cDNAs of *SHY2/IAA3* and *shy2*-2 (P69→S) were cloned from *pACT2-SHY2* and *pACT2-shy2-2* (kindly provided by Jason Reed, (University of North Carlolina, Chapel Hill, North Carolina) using *Xho*I/*Xba*I sites into *pART7*, generating *35S::HA-SHY2/IAA3* and *35S::HA-shy2-2*. The *BDL*/*IAA12* cDNA was excised with *BamH*I/*Xba*I from pET16H-BDL (Weijers *et*

al., 2006) and introduced into pENTR 3C (Invitrogen), and the resulting entry clone was used to create *35S::HA-BDL/IAA12* via LR recombination in pART7-HA (C.S Galvan-Ampudia and Offringa, unpublished) to generate *35S::HA-BDL/IAA12*. The *bodenlos* (P75→S) mutation (Hamann *et al.*, 2002) was introduced in this plasmid using the Quickchange Site-directed Mutagenesis kit (Stratagene) resulting in *35S::HA:bdl*.

The entry clone for *TIR1myc* (Gray *et al.*, 1999) was used for generating the *tir1-1*(G147→D) mutation by Quickchange site-directed mutagenesis. The deletions of the F-box motif in *TIR1* and *tir1-1*, which removes the first 50 amino acids from the original sequence substituting I50 for an alternative M as a start codon, were generated via PCR with primers: 5'-GAATTCATGGGGAACTGCTACGCCGTGAG-3' and 5'-GCGGATCCCTAAAACCTCATTGTTGAGTC-3'.The *COI1* cDNA was amplified from a leaf cDNA library with the primers 5'-CGAGCTCAAAATGGAGGATCCTGATATCAAG-3'and 5'-GGGGTACCGACTGACTCTATGTAATCTCC-3' and cloned into pENTR2B. Entry clones were used in an LR reaction with pART7-FLAG, generating *35S::FLAG-GFP*, *35S::FLAG-TIR1myc*, *35S::FLAG-tir1-1myc* and *35S::FLAG-COI1*.

GUS and LUC assays

In the *DR5::GUS* transactivation assays 10^6 protoplasts were transfected with 10 µg of the *DR5::GUS* reporter construct and 2 µg of *35S:Rluc* (p2rL7 (De Sutter *et al.*, 2005)) for experimental normalization. The DNA amounts of the effector constructs varied per experiment and are indicated in the figure legends. All transformations contained 10 µg of *35S::FLAG-GFP* as a control for transformation efficiency, and were split in 2 portions containing 5 x 10^5 protoplasts in a total volume of 2.5 mL of protoplast medium. After 16 h the samples were treated for 4 h either with 1 µM IAA or the same volume of the solvent DMSO. Treated cells were collected by centrifugation at 80 g for 1 minute and the pellets were frozen in liquid nitrogen for GUS (van der Fits and Memelink, 1997) and LUC measurements (Dyer *et al.*, 2000). Triplicate

transfections were assayed and mean GUS/LUC relative activities were analyzed by One-way ANOVA using SPSS 15.0 software.

Immunoblotting and Immunoprecipitation

For the Aux/IAA degradation/ubiquitination assays, 10^6 protoplasts were transfected with 20 µg *35S::HA-Aux/IAA* construct and 10 µg of *35S::FLAG-GFP*. Depending on the experiment, plasmids encoding FLAG-tagged TIR1myc, tir1-1myc or COI1 were co-transfected in the amounts indicated in the figure legends. Treated protoplasts were resuspended by vortexing in cold Extraction Buffer (PBS, 1x Roche Complete Protease Inhibitor Cocktail) containing 1% Triton X-100, and the lysate was cleared by centrifugation at 20.000 g for 10 min. Total protein was quantified by Bradford assay (Bio-Rad) and 20 µg was mixed with sample buffer and separated on 15% SDS-PAGE minigels. PAGE-separated proteins were blotted onto nitrocellulose membranes, blocked with nonfat dry milk and incubated with the HRP-conjugated antibodies anti-HA High Affinity 3F10 (Roche) and anti-FLAG M2 (Sigma). For detection of ubiquitinated proteins, 10^6 transformed cells were resuspended in 100 µL Extraction Buffer containing 1% Triton X-100, 5 mM EDTA, 10 mM OPA (1,10-Phenanthroline monohydrate, Sigma), and 10 µM MG132 (Sigma). The lysate was cleared by centrifugation at 20.000 g for 10 min, and 5 µL was Western-analyzed as 5 % input control. The remaining total extract was diluted to a final volume of 900 µL with Extraction Buffer without Triton X-100 to bring the Triton concentration to 0.1%. This extract was then mixed with 40 µL of Anti-HA Affinity Matrix (Roche) and incubated for 2 h at 4^oC. The matrix was pelleted and washed 3x in Extraction Buffer, mixed with sample buffer, and the eluted proteins were separated on 12 % SDS-PAGE minigels. PVDF membranes containing transferred proteins were blocked with Qiagen Blocking Reagent and probed with 1000-fold diluted HRP-Anti-Ub P4D1 antibodies (Santa Cruz). After chemiluminescent detection (LumiGLO, Cell Signalling) the blots were stripped and reprobed with anti-HA High Affinity 3F10 antibodies (Roche). When necessary, quantification of the signal was

performed on scanned x-ray films using a BioRad™ GS-800 calibrated densitometer.

RESULTS AND DISCUSSION

Aux/IAAs repress auxin-responsive gene expression in Arabidopsis cell suspension protoplasts.

Leaf protoplasts have extensively been used to study auxin signaling and the regulation of auxin-responsive gene expression by ARF and Aux/IAA proteins (Ulmasov *et al.*, 1997; Kovtun *et al.*, 1998; Abel and Theologis, 1998; Ulmasov *et al.*, 1999). For our experiments we obtained protoplasts from *Arabidopsis* cell suspension cultures because of their continuous availability, the easy isolation procedure, and the high transformation efficiencies obtained. To establish the experimental conditions under which the repressive effect of Aux/IAA proteins on auxin-responsive gene expression could be detected in these cells, we transfected the auxin-responsive reporter construct *DR5::GUS* alone or in the presence of plasmids encoding HA-tagged Aux/IAA proteins. BDL/IAA12 or SHY2/IAA3 were selected for our studies, because they are well-characterized, but distantly related, and they are involved in different developmental processes and therefore representative for the other Aux/IAAs (Tian and Reed, 1999; Tian *et al.*, 2002; Tian *et al.*, 2003; Weijers *et al.*, 2005).

In order to identify working parameters for assaying transcriptional responses to auxin in protoplasts, we transfected one million cells with 10 µg of the auxin-responsive *DR5::GUS* reporter plasmid alone. GUS expression was induced by auxin in a concentration-dependent manner (Figure 1a), and a maximum response was obtained with 1 µM IAA. Co-transfection with 1 µg of the *35S::HA-BDL/IAA12* effector plasmid led to a significant reduction of this response, and when 5 or 10 µg effector plasmid was cotransfected the repression of the *DR5* promoter was saturated in that its activity remained at 30%, even when the cells were treated with 1 or 10 µM IAA (Figure 1a). Similar results were obtained with NAA (data not shown). Based on these results (Figure 1a), a reporter:effector plasmid ratio of 10 : 1 (in µg) and an auxin concentration of 1 µM IAA were used to study the repression activity on the

DR5 promoter. SHY2/IAA3 and BDL/IAA12 both showed repression activity on the DR5 promoter. The transfected amount of BDL/IAA12 plasmid resulted in a stronger repression than transfection with the same amount of SHY2/IAA3 plasmid. The auxin response was almost completely repressed by the stabilized mutant versions shy2-2 and bdl (Figure 1b).

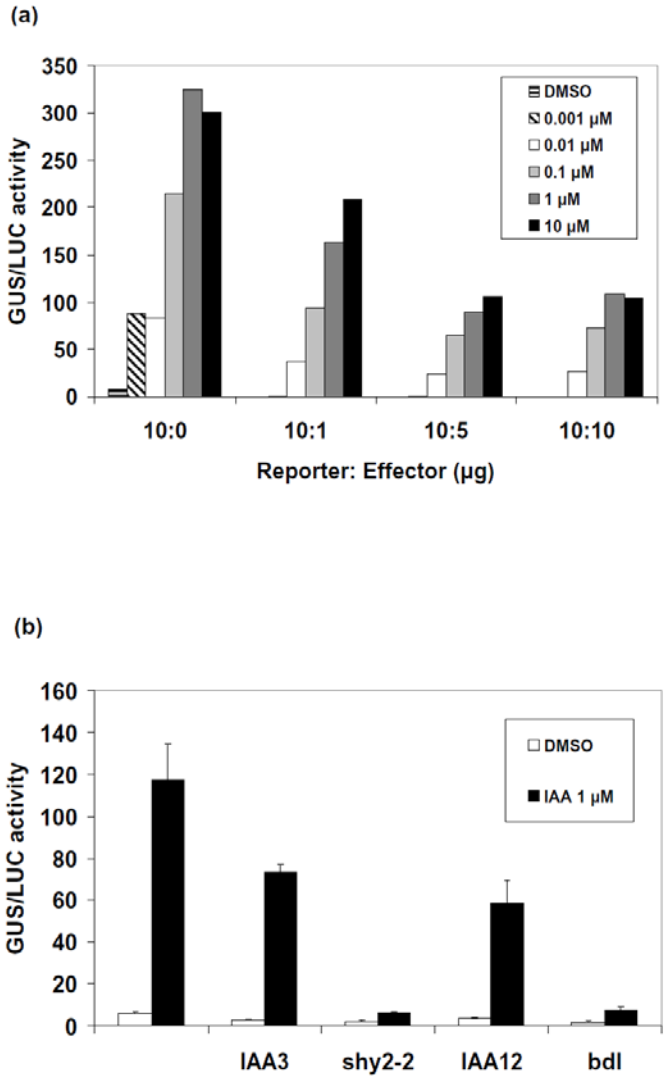

Figure 1. Auxin-responsive *DR5::GUS* reporter gene expression in Arabidopsis protoplasts is repressed by the Aux/IAA proteins BDL/IAA12 and SHY2/IAA3.

(a) Relative GUS/LUC activity (arbitrary units) in transfected Arabidopsis protoplasts treated for 8 hours with increasing concentrations of auxin (IAA). The numbers on the X-axis indicate the reporter (*DR5::GUS*) to effector (*35S::HA-BDL/IAA12*) ratio in µg plasmid DNA per transformation.
(b) Effect of co-transfecting Aux/IAA effector plasmids expressing SHY2/IAA3, BDL/IAA12 or the stabilized mutant proteins shy2-2 or bdl on the activity of the *DR5::GUS* reporter. Reporter and effector plasmids were co-transformed in a reporter to effector ratio of 10:1 µg plasmid DNA. Protoplasts were treated for 8 hours with DMSO or 1 µM IAA. Letters indicate significantly different groups based on One-way ANOVA ($p<0.05$).

Cellular auxin and TIR1 levels are interdependent parameters in Aux/IAA degradation

Although clearly active in the repression of transcription, the transiently expressed Aux/IAA proteins in the previous experiments could not be detected on Western blots (data not shown). To be able to correlate the level of transcriptional inhibition caused by the HA-tagged Aux/IAA proteins with the degree of protein turn over, we designed a different experimental set-up. Protoplasts were transfected with 20 µg of the effector plasmids *35S::HA-SHY2/IAA3* or *35S::HA-BDL/IAA12* and after auxin treatment total protein extracts were analyzed on Western blots using antibodies against the HA-epitope. HA-BDL/IAA12 and HA-SHY2/IAA3 were clearly detectable, but under these conditions, the latter failed to show a clear enhanced turn over after auxin treatment (Figure 2a, left panel). In fact, IAA3 protein levels increased during incubation, indicating that the *de novo* production was higher than the turn over rate. It is interesting to note that although the HA-SHY2/IAA3 protein was more abundant in figure 2a, BDL/IAA12 had higher repression activity in the *DR5* promoter assays in figure 1b. The facts that excessive amounts of repressor construct led to saturated repression of the *DR5* promoter, which could not be overcome by the addition of higher auxin concentrations (Figure 1a), and that the increased amount of SHY2/IAA3 protein failed to be degraded following auxin treatment (Figure 2a, left panel), led us to hypothesize that some component of the auxin-responsive protein degradation machinery in protoplasts was rate-limiting. To test if the amount of auxin receptor TIR1 was rate-limiting, we co-transfected the cells with 20 µg Aux/IAA effector construct and increasing amounts of *35S::FLAG-TIR1-c-Myc*. Co-transfection of 4 µg

TIR1 plasmid led to enhanced auxin-dependent turn over of SHY2/IAA3 (Figure 2a, IAA3/TIR panels). When 10 or 20 µg of the *TIR1* plasmid was added, the basal levels of SHY2/IAA3 became very low while at 20 µg the effect of auxin could not be visualized due to detection limitations (Figure 2a, IAA3 / TIR 20 µg panel). The stability of the mutant HA-shy2-2 protein was not affected by auxin without co-transfected *TIR1*, but with high *TIR1* amounts even the turn over of the stabilized HA-shy2-2 started to become evident (Figure 2a shy2-2 / TIR1 panels). For shy2-2 it has been reported that the mutant protein retains part of its TIR1 binding activity in the presence of auxin (Tian *et al.*, 2003). This may explain the enhanced turn over of the shy2-2 protein in the presence of additional TIR1 and exogenous auxin.

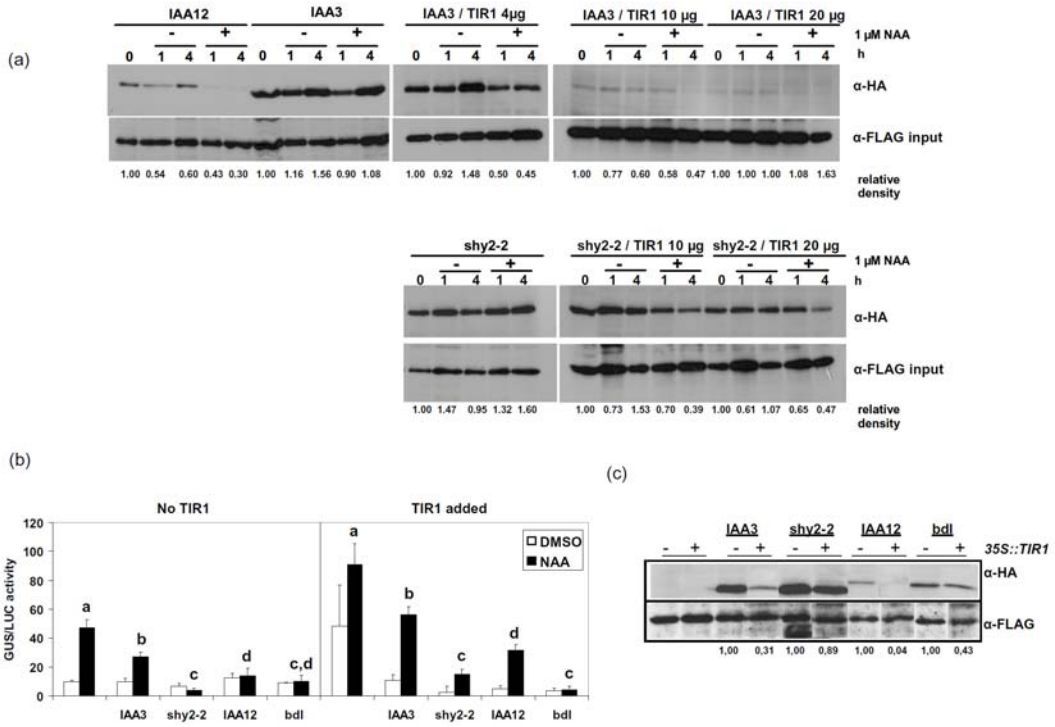

Figure 2. Auxin-induced TIR1-dependent degradation of Aux/IAA proteins leads to auxin-responsive gene expression in *Arabidopsis* protoplasts.

(a) Western blot detection of transiently expressed HA-BDL/IAA12, HA-SHY2/IAA3, HA-bdl and HA-shy2-2 in *Arabidopsis* protoplasts co-transfected with control plasmid *35S::FLAG-GFP* and increasing amounts of plasmid *35S::FLAG-TIR1c-myc*. One million transfected protoplasts were were split in two halves and each was treated either with 0.1% DMSO (-) or 1 µM NAA (+) and samples were harvested on the indicated time points (in hours). The labels on top indicate the Aux/IAA construct and the TIR1 plasmid amount. The panels indicated with "α-HA" represent the

detection of HA-tagged Aux/IAA with anti-HA antibodies and "α-FLAG input" indicates detection of FLAG-GFP with anti-FLAG antibodies. Numbers on the bottom of each lane indicate the HA versus FLAG signal density ratio relative to time point zero in each transformation.

(b, c) Correlation between auxin-responsive *DR5::GUS* expression and TIR1-dependent Aux/IAA degradation. Protoplasts were transfected with 10 µg *DR5::GUS*, 10 µg *35S::RLuc*, 10 µg *35S*::*FLAG-GFP* and where indicated with 20 µg *35S::HA-Aux/IAA* effector plasmid. The graph in (b) shows relative GUS/LUC activity (in arbitrary units) in protoplasts after 4 hours treatment with DMSO or 1 µM NAA, in the absence (No TIR1) or presence of 10 µg *35S::FLAG-TIR1-c-Myc* (TIR1 added). Letters indicate significantly different groups based on One-way ANOVA ($p<0.05$). (c) Western blot analysis showing the level of HA-tagged Aux/IAA protein in the NAA-treated samples in (b). Addition of *35S::TIR1* is indicated (-/+). Detection of the FLAG-GFP control (α-FLAG) is used to demonstrate comparable transformation efficiencies and loading.

TIR1-dependent Aux/IAA degradation coincides with auxin-responsive gene expression

To correlate the TIR1-dependent turn over of Aux/IAAs with auxin-responsive gene expression, we repeated the *DR5::GUS* trans-activation experiments, but now using 20 µg of effector plasmid, and measuring both GUS activity and protein levels. Again, in these experiments the BDL/IAA12 protein showed equal or even stronger repression activity on *DR5::GUS* expression as SHY2/IAA3 (Figure 2b), whereas it accumulated to a much lower level than SHY2/IAA3 (Figure 2c). In the absence of additional TIR1, auxin treatment led to a weak activation of the *DR5* promoter (Figure 2b, "No TIR1" panel and Figure 2c). Co-transfection of 10 µg *TIR1* plasmid enhanced the responsiveness of the *DR5* element (Figure 2b, "TIR1 added" panel) and this effect correlated with an increased turn over of the Aux/IAA repressors (Figure 2c). TIR1 alone resulted in an increase of GUS activity in DMSO-treated cells without co-transfected Aux/IAAs (Figure 2b, first bar in "TIR1 added" panel). This effect is probably due to the degradation of the endogenous pool of Aux/IAAs. As expected, the levels of the mutant shy2-2 and bdl proteins were less sensitive to overexpression of TIR1. The slight increase in *DR5::GUS* activity observed after auxin treatment in the samples co-transfected with *shy2-2* and *TIR1* might reflect the residual binding of the mutant shy2-2 protein to TIR1 (Tian *et al.*, 2003) and its enhanced turn over rate at higher TIR1 levels

(compare to Figure 2a, shy2-2 / TIR1 20 µg panel). Additionally, comparative analysis of BDL/IAA12 and SHY2/IAA3 indicated that optimized ARF and Aux/IAA interaction pairs are active in specific auxin-regulated developmental processes (Weijers *et al.*, 2005). It is therefore likely that the specific ARFs that are responsible for the activation of the *DR5* element in cell suspension protoplasts interact more efficiently with BDL/IAA12 or endogenous Aux/IAAs than with SHY2/IAA3. This may also explain the stronger repressor activity of BDL/IAA12.

The observation that excessive amounts of TIR1 led to reduction of detectable Aux/IAAs even in the absence of auxin treatment is in agreement with the effect of overexpressing TIR1 in plants, which mimics auxin treatment and causes auxin hypersensitivity (Gray *et al.*, 1999). It also corroborates the finding that in *in vitro* pull-down experiments Aux/IAA proteins do interact with TIR1 in the absence of auxin, albeit at low efficiency (Dharmasiri *et al.*, 2003; Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Tan *et al.*, 2007), and that Aux/IAA degradation and auxin-responsive gene expression are severely affected in the *tir afb2 afb3* triple mutant (Dharmasiri *et al.*, 2005b). All these data indicate that TIR1 and AFB protein levels are important determinants in the cellular auxin responsiveness.

TIR1 differentially regulates SHY2/IAA3 and BDL/IAA12 abundance

Interestingly, there is a clear and significant difference in abundance and repressor activity of the two wild type Aux/IAA proteins, with BDL/IAA12 being the stronger but less abundant repressor (Figure 2b). To assess the roles of auxin and TIR1 in this different behavior of the two Aux/IAA proteins, we tested the auxin-induced degradation of SHY2/IAA3 and BDL/IAA12 with increasing TIR1 levels (Figure 3a). HA-BDL/IAA12 levels were auxin-sensitive when cells were transfected with 20 µg of *35S::HA-BDL/IAA12* effector plasmid alone. The observed variation between experiments in the effect of auxin treatment on IAA12 turn over (compare Figures 2a and 3b) possibly relates to differences in endogenous TIR1 levels. In the same experiment, auxin treatment did not lead to a clear reduction in HA-SHY2/IAA3 levels, corroborating our observation that

SHY2/IAA3 has a longer half-life than BDL/IAA12 (Figure 2b). Co-transfection of the effector plasmids with increasing amounts of *35S::FLAG-TIR1-c-Myc* made the levels of both proteins more sensitive to auxin treatment (Figure 3a). As a control, effector plasmids were also transfected with plasmids expressing mutant versions of TIR1 (tir1-1 [G147→D]), ΔF-TIR1 lacking the F-box, or ΔF-tir1-1 carrying both mutations (Figure 3b), or the related F-box protein COI1, which is involved in jasmonate signaling (Figure 3c). Neither the mutant versions of TIR1 (Figure 3b) nor COI1 (Figure 3c) affected the abundance of the Aux/IAA proteins (compare to 20/10 HA-IAA/TIR1 treatments in Figure 3a), corroborating the specificity of TIR1 in Aux/IAA degradation.

In these experiments TIR1, tir1-1 and COI1 were not detectable by Western blot analysis of total protein extracts using the anti-FLAG antibody, even though their expression was driven by the strong *35S* promoter. This observation suggests that TIR1 and COI1 themselves are short-lived proteins, which is in line with the observation that several F-box proteins, including TIR1 and COI1, are targets for ubiquitination (Maor *et al.*, 2007; Jurado *et al.*, 2008; Stuttmann *et al.*, 2009). To demonstrate that the F-box proteins were expressed to similar levels in our model system, we transfected 5 times more cells than usual (5 x 10^6) and immunoprecipitated the FLAG-tagged F-box proteins from total cell extracts. Western blot analysis of the concentrated eluates showed that the FLAG-tagged versions of TIR1, tir1-1 and COI1 were expressed at similar levels (Figure 3d). As expected, transfection with 2 times more plasmid led to the production of more TIR1 protein (Figure 3e). Treatment with MG132 for four hours did not lead to elevated TIR1 levels nor to the appearance of additional modified bands (Figure 3e), which is in contrast to the conclusion by Stuttmann and coworkers (2009) that the protein is a target of the 26S proteasome.

TIR1 was shown to act as an auxin receptor together with other F-box family members ABF1, ABF2 and ABF3 (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b). Interestingly, a *tir1 afb1 afb2 afb3* quadruple loss-of-function mutant shows a variable phenotype, but several of these mutant plants are able to flower and set seed, suggesting further functional redundancy. We have not tested the effect of AFB1, AFB2 or AFB3 in our

system, but it is likely that they will show similar effects as TIR1 overexpression, since they were found to physically interact with GST-tagged BDL/IAA12 in an auxin-dependent manner in pull-down assays, and the BDL/IAA12 protein was stabilized in the *tir1-1 afb2 afb3* triple mutant (Dharmasiri *et al.*, 2005b).
The related F-box proteins AFB5 and AFB4 have not been studied in detail yet, but the specific resistance of *afb5* mutants to picolinate auxin analogs indicates that AFB5 is involved in the response pathway to these herbicides (Walsh *et al.*, 2006). COI1 is the closest relative of the TIR/AFB auxin receptors in the F-box family tree. The similarity in sequence and the ability of different proteins from this clade to associate with the same SCF components (Gray *et al.*, 1999; Xu *et al.*, 2002) raised the possibility that there is cross-recognition of targets among related F-box proteins and that COI1 may also be actively involved in Aux/IAA protein degradation. The results in Figure 3c clearly show that the presence of overexpressed FLAG-COI1 did not affect the stability of either SHY2/IAA3 or BDL/IAA12. Based on our results, we conclude that COI1 is not involved in the process of Aux/IAA proteolysis.

SCF^{TIR1}-mediated ubiquitination marks Aux/IAA proteins for degradation

The previous experiments demonstrated that the cell suspension protoplast system reproduces the *in planta* action of Aux/IAA proteins and the receptor F box protein TIR1 in auxin responses. The same system was used to establish whether auxin- and TIR1-enhanced degradation of Aux/IAA proteins is connected to SCF^{TIR1}-mediated ubiquitination. A plasmid encoding HA-tagged versions of BDL/IAA12 or SHY2/IAA3 together with a plasmid carrying TIR1 or its mutant version *tir1-1* were transfected into cells. Transformations of one million protoplasts were performed in triplicate and one of the samples was pre-treated for 1 h with 50 µM of the proteasome inhibitor MG132. Samples were subsequently treated for one additional hour with either DMSO or 1 µM NAA. Five percent of the protein input was analyzed by Western blotting using anti-HA antibodies, and the remaining sample was immunoprecipitated with anti-HA antibodies conjugated to agarose beads. The recovered proteins were analyzed by Western blotting using anti-Ubiquitin and anti-HA antibodies.

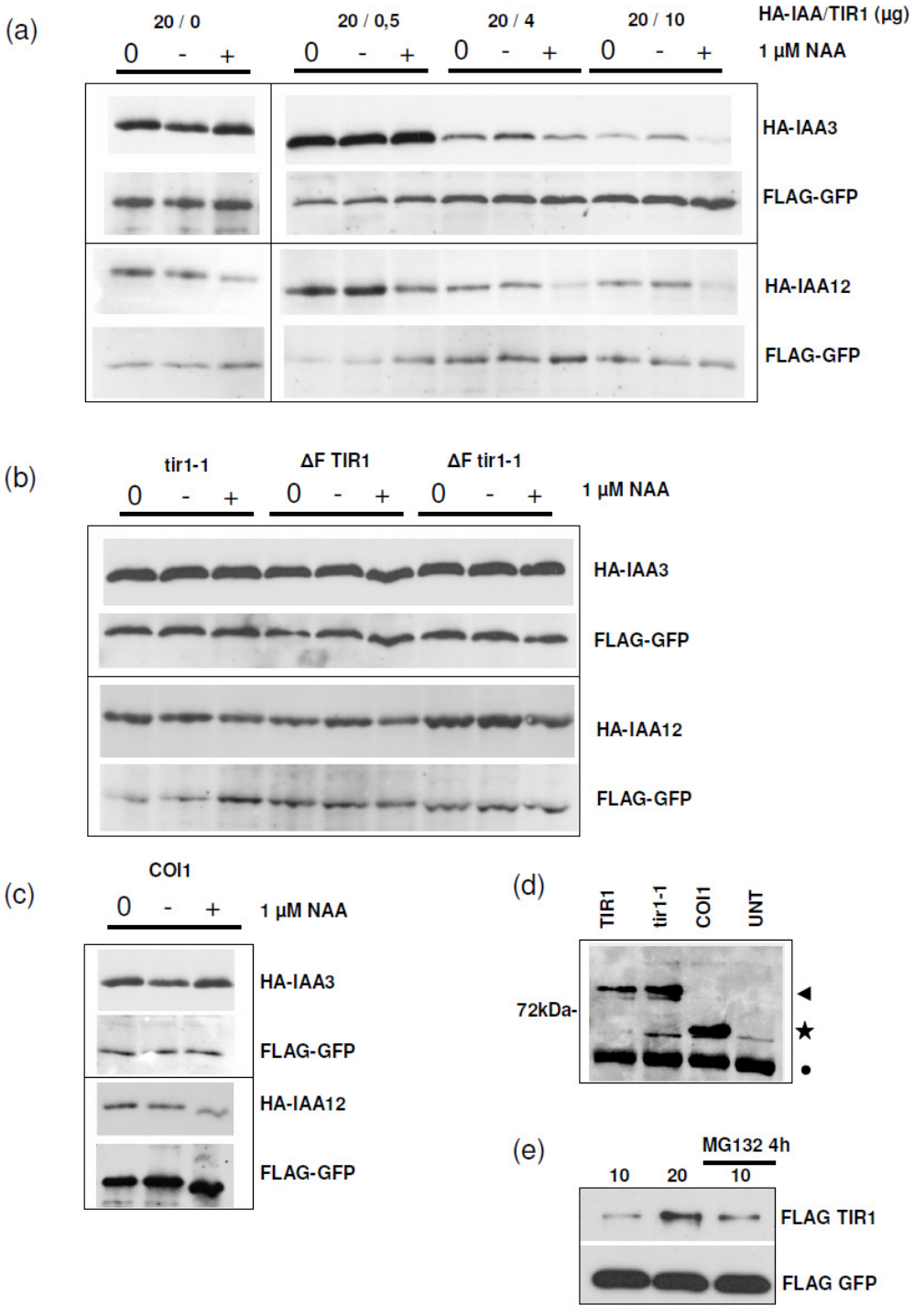
(a)
20 / 0
20 / 0,5
20 / 4
20 / 10
HA-IAA/TIR1 (µg)
0 - + 0 - + 0 - + 0 - +
1 µM NAA
HA-IAA3
FLAG-GFP
HA-IAA12
FLAG-GFP
(b)
tir1-1
ΔF TIR1
ΔF tir1-1
0 - + 0 - + 0 - +
1 µM NAA
HA-IAA3
FLAG-GFP
HA-IAA12
FLAG-GFP
(c)
COI1
0 - +
1 µM NAA
HA-IAA3
FLAG-GFP
HA-IAA12
FLAG-GFP
(d)
TIR1
tir1-1
COI1
UNT
72kDa-
◄
★
●
(e)
MG132 4h
10 20 10
FLAG TIR1
FLAG GFP

Figure 3. TIR1-dependent degradation of transiently expressed SHY2/IAA3 and BDL/IAA12 in *Arabidopsis* protoplasts.

(a) Western blot analysis of total extracts from protoplasts co-transfected with increasing amounts of *35S::FLAG-TIR1-c-Myc*, *35S::FLAG-GFP* control plasmid and *35S::HA-SHY2/IAA3* (upper panel) or *35S::HA-BDL/IAA12* (lower panel). Transfected protoplasts were either untreated (0), or treated for 1 hour with DMSO (-) or 1 µM NAA (+). The numbers on top indicate the *35S::HA-AuxIAA* to *35S::FLAG-TIR1-c-Myc* plasmid ratio in µg DNA. Blots were probed with anti-HA or anti-FLAG antibodies.

(b) As in (a), following co-transfection with 10 µg *35S::FLAG-tir1-1-c-Myc*, *35S::FLAG-ΔF-TIR1-c-Myc* or *35S::FLAG-ΔF-tir1-1-c-Myc*.

(c) As in (a) , following co-transfection with 10 µg *35S::FLAG-COI1*.

(d) Western blot detection of FLAG-tagged TIR1, tir1-1 and COI1 immunoprecipitated using anti-FLAG antibodies from pooled extracts from five transformations of 10^6 protoplasts with 20 µg DNA of *35S::FLAG-TIR1-c-Myc*, *35S::FLAG-tir1-1-c-Myc* or *35S::FLAG-COI1*, respectively. The position of the FLAG-tagged TIR1-c-Myc and *tir1-1*-c-Myc proteins (79 kDa) is indicated by an arrowhead, the position of FLAG-COI1 (70 kDa) with a star. The lane marked UNT contained protein from untransformed protoplasts. An unspecific band recognized by the anti-FLAG antibody in all samples (circle) shows equal loading.

(e) Increasing amounts of 35S:FLAG-TIR1myc plasmid leads to higher expression of the recombinant protein and TIR1 is not stabilized by inhibition of the 26S proteasome. Anti-FLAG western blot of FLAG-immunoprecipitated samples from one million protoplasts transfected with 1 µg of 35S:FLAG GFP and 10 or 20 µg of 35S:FLAG-TIR1myc as indicated over each lane. One sample transfected with 10 µg was treated for 4 h with 10 µM MG132. Total protein was immunoprecipitated with anti-FLAG affinity matrix and analyzed by western blot. FLAG TIR1 and FLAG GFP bands are indicated.

The relative amounts of Aux/IAA proteins recovered by immunoprecipitation correlated well with those present in the input extracts (Figure 4, α-HA: 5% input versus IP-HA). Samples from protoplasts that were treated with auxin and MG132 showed additional anti-HA reactive bands migrating slower than the unmodified SHY2/IAA3 and BDL/IAA12 monomers (Figure 4a and b, IP-HA α-HA, +/+ lanes). Anti-Ubiquitin antibodies detected bands of sizes larger than 40 kDa (IP-HA α-Ub panels) that overlapped with the additional bands detected with anti-HA antibodies (marked with black arrow heads), and thus represent poly-ubiquitinated versions of Aux/IAA proteins. The combined auxin and MG132 treatment enhanced the amount of detectable ubiquitinated Aux/IAA proteins. Interestingly, when TIR1 was co-expressed,

auxin treatment resulted in an increase of the ubiquitinated signal for both HA-SHY/IAA3 and HA-BDL/IAA12 (Fig 4a and b, +/+ lanes), corroborating our previous observation that SCF^{TIR1}-directed degradation of Aux/IAA proteins is dependent on a fine balance between auxin and TIR1 levels (Figure 3a and b). As expected, the co-expression of the mutant tir1-1 protein had no effect on the turn over rate or the ubiquitination level of SHY2/IAA3 (Figure 4a, tir1-1 lanes).

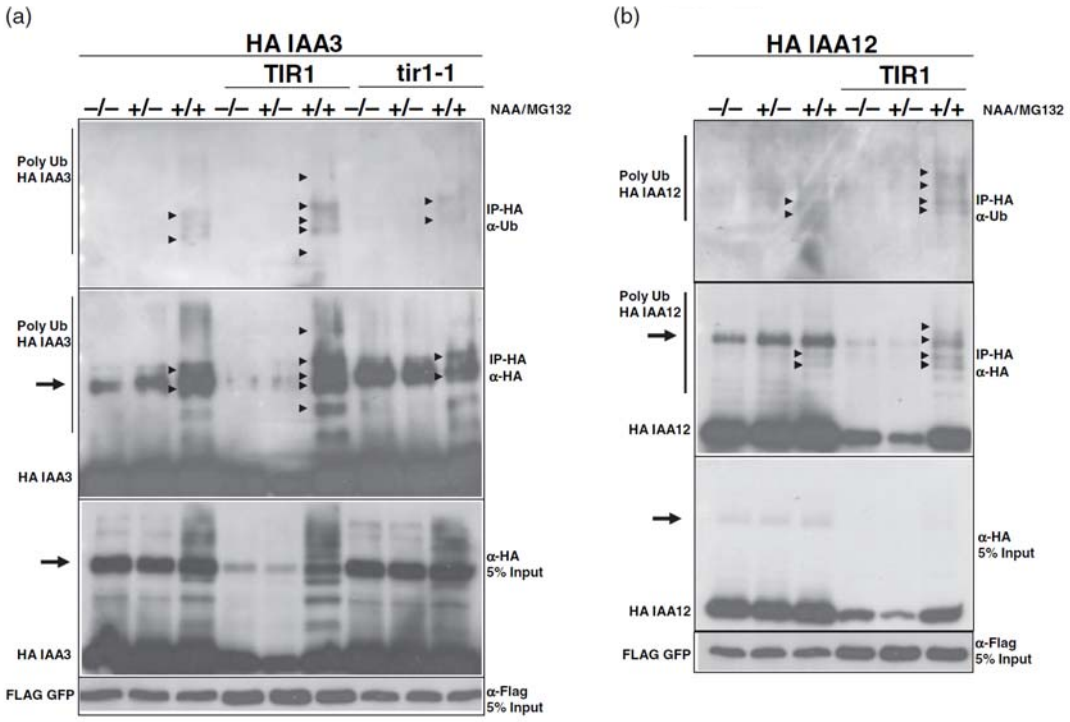

Figure 4. Aux/IAA proteins are poly-ubiquitinated and degraded in an auxin- and TIR1-dependent manner.

(a-b) Western blot analysis of anti-HA immunoprecipitations (IP-HA) or total extracts (5% input) from *Arabidopsis* protoplasts transfected with:

(a) *35S::FLAG-GFP* and *35S::HA-SHY2/IAA3* with either 10 µg of *35S::FLAG-TIR1-c-Myc* (TIR1) or *35S::FLAG-tir1-1-c-Myc* (tir1-1).

(b) *35S::HA-BDL/IAA12 and 35S::FLAG-GFP* co-transfected with 10 µg *35S::FLAG-TIR1-c-Myc*.

Each transformation was performed in triplicate. One sample was treated for 1 h with 0.1% DMSO (-/-), the second sample for 1 h with 1 µM NAA (+/-), and the third replicate was pre-treated for 1 h with 50 µM MG132 followed by 1 h treatment with 1 µM NAA (+/+). Five percent of the total protein extract of each transfection was analysed as input (5% input). The remaining sample was used in an immunoprecipitation with anti-HA antibodies conjugated to agarose beads and these samples

(IP-HA) were first probed with anti-Ubiquitin antibodies (α-Ub) to detect ubiquinated proteins, and subsequently with anti-HA antibodies (α-HA) to detect HA-tagged proteins. The bands corresponding to unmodified HA-IAA3 and HA-IAA12 monomers are indicated. The poly-ubiquitinated forms of IAA12 and IAA3, visible as higher molecular size bands/smears in the α-Ub and α-HA blots, are indicated by Poly-Ub. Arrow heads mark the bands overlapping in the α-Ub and α-HA blots. The arrow indicates the position of a putative homo-dimer of the HA-tagged Aux/IAA proteins.

As the goal of this experiment was to detect the rare ubiquitinated Aux/IAAs forms, significantly higher protein amounts were loaded on gel, and this prevented us to observe the auxin-induced turn over of Aux/IAA proteins in the absence of cotransfected *35S::FLAG-TIR1-c-Myc*.

One constant observation in the Western blots with anti-HA antibodies was the presence of two distinct bands: one at ~30 kDa corresponding to the size of unmodified Aux/IAA monomers (indicated in Figure 4 on the left of each panel by HA IAA3 or HA IAA12) and a fainter band around 60 kDa size (Figure 4, indicated by arrows, α-HA panels). This band was not detected with the anti-Ub antibodies, and although we can not exclude other pos-translational modifications such as phosphorylation or sumoylation, based on the size shift we believe this band to represent Aux/IAA homo-dimers. The 60kDa band was also detected even when protein samples were prepared and gel separated under strong denaturing conditions (boiled in Laemmli loading buffer prior to Urea-SDS-PAGE, data not shown), suggesting a covalent coupling, or a denaturation-resistant association of the Aux/IAA proteins. One possibility is that the dimers are stabilized by intermolecular disulfide bonds, which is a common mechanism in redox control of transcription factor activity (Benezra, 1994; Zheng *et al.*, 1998; Mou *et al.*, 2003). However, the fact that the band is not dissolved by the thiol reducing agent 2-mercaptoethanol in the Leammli buffer suggests that the Aux/IAA dimers are stabilized by another mechanism. The 60 kDa band putatively representing Aux/IAA homodimers almost disappeared in TIR1 co-transfected samples, suggesting that an additional consequence of Aux/IAA degradation is the dissolution of Aux/IAA dimers. Hypothetically, the dimerized forms might be more accessible for interaction

with SCF-^{TIR1} than the DNA-ARFs associated ones and hence be more easily degraded.

Repressor poly-ubiquitination: a paradigm for plant hormone signaling pathways

Our results indicate that auxin-responsive gene expression in *Arabidopsis* protoplasts depends on a fine tuning of the intracellular concentrations of different elements that participate in the auxin perception pathway. Auxin sensitivity and Aux/IAA stability are directly correlated with intracellular levels of TIR1. Previous reports showed that auxin responses in seedlings are enhanced by *TIR1* overexpression (Gray *et al.*, 1999) and repressed by TIR1/AFB loss-of-function (Dharmasiri *et al.*, 2005b). In addition, our experiments directly correlate the TIR1-enhanced auxin response with the increased turn over of Aux/IAA proteins, and suggest that TIR1, when present at sufficiently high levels, sensitizes the protoplast cells to endogenous auxin levels and can mediate Aux/IAA degradation without the need for exogenous auxin application. We demonstrate that the SHY2/IAA3 and BDL/IAA12 proteins behave differently in protoplasts, the latter being less stable but more active in the repression of the auxin response in this system. The simplest explanation is that IAA12 is a more efficient repressor than IAA3, but we can not rule out that the effect is indirect through the efficient interaction of overexpressed IAA12 with the SCF$^{TIR1/AFB}$ complexes, which sequesters these complexes and thereby stabilizes endogenous Aux/IAAs.

It is well established that several plant hormonal signaling pathways act through proteasomal degradation of transcriptional repressors, and that the hormones show similar roles in enhancing the association of SCF E3 ligase complexes with their targets (e.g. auxin/TIR1/Aux/IAA, JA-Ile/COI1/JAZ and GA/GID/DELLA.) (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Griffiths *et al.*, 2006; Thines *et al.*, 2007). Our data provide experimental support for the model that hormone-responsive gene expression is mediated by hormone-enhanced poly-ubiquitination and subsequent proteolytic degradation of repressor proteins by the 26S proteasome.

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