Auxin modulates the degradation rate of Aux/IAA proteins

Nathan Zenser*†, Amanda Ellsmore*†, Colin Leasure*‡, and Judy Callis*†§

*Section of Molecular and Cellular Biology, †Graduate Group in Plant Biology, University of California, 1 Shields Avenue, Davis, CA 95616

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Aux/IAA **gene family members were first identified by their rapid transcriptional increase in response to auxin. Auxin/indole-3-acetic acid protein (Aux/IAA) luciferase (LUC) fusions expressed in *Arabidopsis* under control of a non-auxin-responsive promoter were used to monitor the effect of auxin on protein abundance independent of transcriptional regulation by auxin. After 2 hr in the presence of 1 μM exogenous dichlorophenoxyacetic acid (2,4D), a synthetic auxin, the levels of pea IAA6 (PSIAA6) and *Arabidopsis* IAA1 LUC activity were 35% and 67%, respectively, of mock-treated genetically identical seedlings, whereas the activity of LUC alone from equivalently treated seedlings remained unaltered. The steady-state level of an Aux/IAA fusion protein lacking domain II, one of the conserved domains found in all Aux/IAA proteins, was not reduced in the presence of auxin. Higher levels of exogenous auxin were required to affect the steady-state level of the PSIAA6::LUC fusion with a point mutation in domain II. A 13-aa consensus sequence from domain II fused to LUC created an auxin-responsive fusion protein. The change in steady-state levels in response to auxin is extremely rapid, with a decrease in LUC activity detectable by 2 min after auxin application. Direct half-life measurements show that the decrease caused by exogenous auxin is due to the decrease in fusion protein half-life. These results suggest that auxin rapidly modulates the degradation rate of Aux/IAA proteins, with higher levels of auxin increasing the proteolytic rate of Aux/IAA family members.**

Auxin is a classic phytohormone involved in a myriad of developmental and environmental processes: embryo patterning, cell division and elongation, vascular differentiation, lateral root initiation, gravitropism, and phototropism (1, 2). To help understand auxin-signaling pathways, a successful strategy was implemented to identify early-response gene families whose rapid transcriptional up-regulation was auxin-dependent, but independent of new protein synthesis. The auxin/indole-3-acetic acid (IAA) (*auxin/IAA*) gene family is one of the best characterized primary auxin-responsive families and has over 20 members in *Arabidopsis* (3). The physiological relevance of Aux/IAA proteins became apparent when several auxin-signaling mutants in *Arabidopsis* were found to have point mutations in an Aux/IAA member (4–6).

Aux/IAA proteins are characterized by four conserved domains (domains I–IV). Aux/IAA proteins are nuclear-localized and extremely short-lived (7). The role of domain I is unknown; however, domain II is required for the characteristic rapid degradation (8, 9). Similarity to Aux/IAA domains III and IV is found in another family of proteins involved in auxin signaling, the auxin-response factors (ARFs). Domains III and IV function as a protein–protein interaction domain, allowing Aux/IAA proteins to form homo- and hetero-dimers with other Aux/IAA proteins or with ARFs (10). ARF family members bind to auxin-responsive elements that are found in the promoters of many auxin-responsive genes (11). Some ARF members seem to activate transcription, whereas at least one, ARF1, has been shown to repress expression (12). In a transient assay, transfection of a plasmid expressing an Aux/IAA protein suppressed an ARF-dependent auxin response, suggesting that Aux/IAA proteins act as negative regulators of auxin signaling (13).

Proteolytic regulation is emerging as a paradigm in signaling pathways. Removal of regulators by proteolysis is a rapid, irreversible method of eliminating their activities. In addition to the well characterized loss of phytochrome A in red light, several other plant proteins recently have been shown to have their proteolytic rates tightly coupled to environmental cues. A blue-light receptor, CRY2, is degraded in high-fluence blue light but not in the dark or in low-fluence blue light, suggesting that CRY2 plays a role in low-fluence but not high-fluence blue-light signaling (14). HY5 is a transcription factor that binds to regulatory elements of light-induced genes to activate transcription. HY5 is present at low levels in the dark. HY5 protein accumulates in the light because of a decrease in its proteolytic rate (15). HY5 activity, and hence the genes it activates, is repressed in the dark by proteolysis (15).

The ubiquitin pathway is the major nuclear and cytosolic proteolytic pathway in eukaryotic cells. Proteolysis via the ubiquitin pathway requires three main sequential steps (16). A protein substrate must first interact with a ubiquitin E3/E2 (ubiquitin ligase/ubiquitin conjugating enzyme) complex. Ubiquitin attached to the E2 or E3 by a thiolester bond (initially catalyzed by ubiquitin-activating enzyme, E1) is then passed to a Lys in the substrate or to a Lys in a ubiquitin previously linked to the substrate. These subsequent additions of ubiquitin to attached ubiquityl moieties generate the multiubiquitin chain required for degradation (17). Finally, degradation of the ubiquitylated protein substrate is catalyzed by the 26S proteasome.

In vivo inhibition of the catalytic core of the 26S proteasome affects Aux/IAA protein degradation (8), suggesting that the ubiquitin pathway is involved in Aux/IAA degradation. No ubiquitin pathway components have been shown to be required directly for Aux/IAA proteolysis, although one candidate protein is *Arabidopsis* TIR1. TIR1 is an F-box protein that has been shown to form a ubiquitin E3 ligase called the Skp1–Cullin–F-box (SCF) protein complex, SCF^{TIR1}. *tir1–1* phenotypes implicate the protein in auxin signaling (18). In addition, overexpression of TIR1 in wild-type *Arabidopsis* enhances their sensitivity to auxin (19). Finally, a mutant in *Arabidopsis* Skp1, called *ask1–1*, has alterations in auxin responses, again implicating an SCF in auxin signaling (19).

To assess the effect of auxins on Aux/IAA proteolysis directly, translational fusions of Aux/IAA amino acids with firefly luciferase (LUC) have been expressed under control of a non-auxin-responsive promoter in transgenic *Arabidopsis* plants. These fusions are enzymatically active and rapidly degraded (8). These features allowed us to observe changes in the degradation rate of Aux/IAA proteins by changes in LUC activity. In addition, we

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Abbreviations: IAA, indole-3-acetic acid; ARF, auxin-response factor; SCF, Skp1–Cullin–F-box; 2,4D, dichlorophenoxyacetic acid; LUC, luciferase; NLS, nuclear localization signals.

‡Present address: Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720.

§To whom reprint requests should be addressed. E-mail: jcallis@ucdavis.edu.

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directly measured the protein half-life in the presence and absence of exogenous auxin. Exogenous auxin alters the proteolytic rate of Aux/IAA LUC-fusion proteins. This effect is extremely rapid, possibly preceding auxin-induced transcriptional changes. Aux/IAA domain II is required for rapid proteolysis (8), and a mutation in this domain affects the response to exogenous auxin. Aux/IAA::LUC fusions respond to exogenous auxin equivalently in *TIR1* and *tir1–1* plants, indicating that *TIR1* is not required for this early response.

Methods

Plant Materials, Growth Conditions, and Protein Extracts. All transgenic *Arabidopsis* lines have been described (9). Multiple homozygous transgenic lines were analyzed. Each plant sample consisted of surface-sterilized seed in a dish immersed in 1 ml of liquid-culture medium (Murashige–Skoog salts, pH 6.5; GIBCO/BRL). After stratification at 4°C for 2 days, the samples were set at 22–24°C for 6–7 days under continuous light, except where noted. To obtain enough LUC activity for protein visualization, seedlings for Western analysis were grown initially on agar plates and then transferred to liquid medium. Dichlorophenoxyacetic acid (2,4D) stock was in 0.1 M KOH, and either this stock or an equivalent amount of 0.1 M KOH was added. The IAA stock solution was made fresh in 100% ethanol. The appropriate amount of IAA stock solution or 100% ethanol was added. Cycloheximide was added to a final concentration of 250 μg/ml. Before the initiation of the time course, the medium was removed and 700 μl of fresh medium was added. For the dark-grown seedlings, medium was changed under dim green light (F032T8 TL950 fluorescent tubes with T8/4G green tube covers; USA Plastics, Anaheim, CA). Protein extracts were prepared and assayed as described (9).

Seedling and yeast extracts were prepared for SDS/PAGE, and Western blotting was performed as described (20). *Saccharomyces cerevisiae* transformed with plasmids encoding the desired LUC-fusion proteins are described in ref. 10. Anti-LUC (Cortex Biochem, San Leandro, CA) polyclonal antibodies were used for chemiluminescence visualization (21). The signal was quantified on the Storm PhosphorImager system (Molecular Dynamics).

TIR1 Cleaved Amplified Polymorphism (CAPs) Test. *tir1–1* homozygous plants were identified by a CAPs test. The *tir1–1* mutation introduces a *Dpn*II site (18). Genomic PCR fragments spanning the *tir1–1* mutation were produced, incubated with *Dpn*II, and fractionated by agarose gel electrophoresis. The PCR fragment from wild-type DNA does not contain a *Dpn*II site, whereas the PCR fragment from *tir1–1* plants contains a single *Dpn*II site.

Results

Auxin Lowers Aux/IAA::LUC Levels. To investigate the role of auxin in posttranslational control of Aux/IAA abundance independent of changes in *Aux/IAA* transcription, *Aux/IAA* coding regions were fused to the 5′ end of the firefly LUC-coding region downstream of the *Arabidopsis UBQ10* polyubiquitin 5′ flanking region (9). *UBQ10* mRNA levels are neither developmentally regulated nor tissue specific (22), and do not change in response to exogenous auxin treatment (6). Luciferase activity provides an extremely sensitive and specific means for monitoring Aux/IAA::LUC protein levels. We previously demonstrated that LUC translational fusions with the *Arabidopsis* Aux/IAA protein IAA1 or the pea Aux/IAA protein PSIAA6 are enzymatically active and rapidly degraded in transgenic *Arabidopsis* plants (8).

Six-day-old light-grown transgenic seedlings expressing either unfused LUC as a control protein or IAA1::LUC were incubated in the presence of exogenous IAA, 2,4D, or buffer alone for 2 hr, and LUC activity was determined from extracts. Treatment

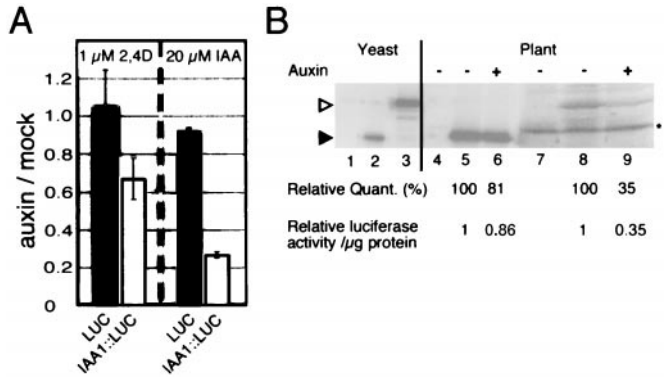

Fig. 1. Auxins lower LUC activity and protein accumulation for Aux/IAA::LUC fusions, but not for LUC alone. (*A*) Six-day-old light-grown seedlings expressing unfused LUC (negative control) or IAA1::LUC were incubated in fresh liquid medium with or without (mock treated) exogenous 1 μM 2,4D (*Left*) or 20 μM IAA (*Right*). After the 2-hr incubation period, LUC activity and total protein were determined from extracts. Results are expressed as a ratio of auxin-treated to mock-treated LUC activity per μg of protein. Data represent at least two experiments done in triplicate. Error bars are the standard deviation of the mean. (*B*) Anti-LUC Western analysis of yeast (lanes 1–3) and *Arabidopsis* (lanes 4–9) extracts. The samples were auxin (+) or mock treated (−) for 2 hr. Lanes 2, 5, and 6 and 3, 8, and 9 contain extracts from seedlings expressing unfused LUC or IAA1::LUC, respectively. Negative controls for yeast and plant samples are in lane 1 and lanes 4 and 7, respectively. Lanes 4–6 and 7–9 have 40 μg and 500 μg of protein, respectively. *, cross-reactive endogenous plant protein that reacts with anti-LUC Ab can be visualized in lanes 7–9; ▶, unfused LUC protein; ▷, IAA1::LUC protein. Band quantification and LUC activity expressed relative to the respective mock-treated samples are shown below the blot.

with 1 μM 2,4D or 20 μM IAA resulted in reductions in steady-state IAA1::LUC luciferase activity to 67 or 27%, respectively, of seedlings treated with buffer alone (Fig. 1*A*). In contrast, the level of LUC activity from transgenic seedlings expressing unfused LUC was not different (Fig. 1*A*). Western blots were performed on extracts from auxin-treated and control seedlings to demonstrate that changes in LUC activity in IAA1::LUC seedlings correlated exactly with changes in fusion-protein abundance (Fig. 1*B*, lanes 8 and 9) and not by lowering the fusion protein-specific activity. As expected, LUC protein levels remained the same in seedlings expressing unfused LUC (Fig. 1*B*, lanes 5 and 6).

Auxin regulation of LUC activity also was observed in *Arabidopsis* seedlings expressing PSIAA6::LUC. In these seedlings, LUC activity was ≈35% of the control after incubation in 1 μM 2,4D for 2 hr (Fig. 2*A*). The response of Aux/IAA::LUC fusions to exogenous auxin was independent of light. Six-day-old dark-grown seedlings expressing PSIAA6::LUC or LUC alone were incubated in the dark in 1 μM 2,4D for 2 hr. PSIAA6::LUC activity was reduced equivalently to light-grown seedlings (Fig. 2*A*).

Domain II Is Needed for Changes in Aux/IAA Luciferase Activity. To determine which regions of Aux/IAA proteins are sufficient for auxin regulation of Aux/IAA::LUC, LUC-fusion proteins containing portions of PSIAA6 were expressed in transgenic seedlings. PSIAA6(1–73)::LUC contains the first 73 amino acids of the protein, which includes domain I and II. This fusion protein has the same ability to respond to 1 μM 2,4D as does the full-length PSIAA6::LUC-fusion protein (Fig. 2*A*). To investigate the effect of smaller regions of Aux/IAA that would eliminate functional NLSs, a transferable NLS was added to the C terminus of LUC (9). As seen for LUC alone in Fig. 2*A*, unfused LUC::NLS abundance did not change in response to auxin. In contrast, the abundance of PSIAA6(18–73)::LUC::NLS, which contains amino acids 18–73 and spans domain

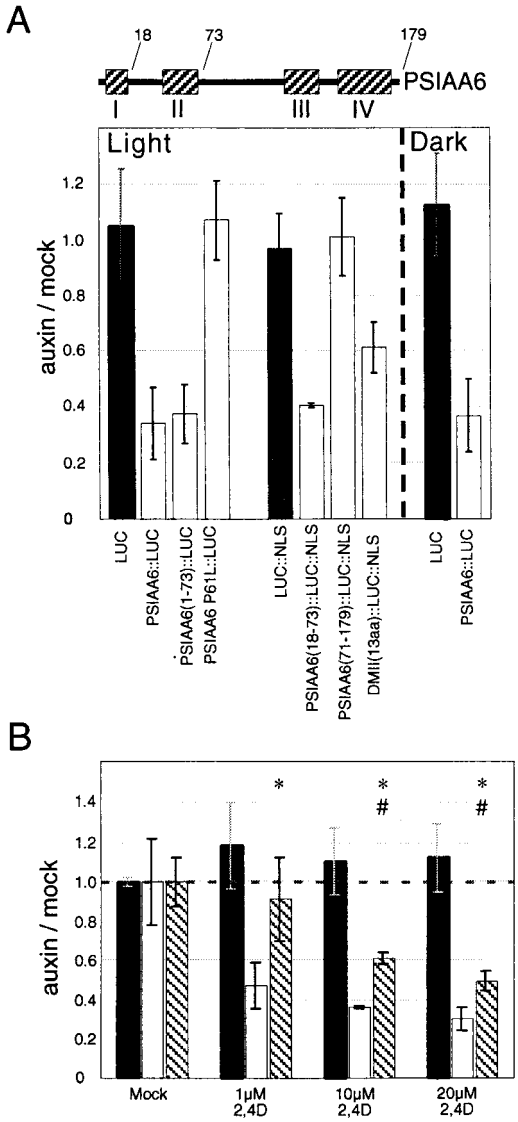

Fig. 2. Regulation by exogenous auxin requires the presence of Aux/IAA domain II. Six-day-old transgenic seedlings grown in liquid medium were incubated with 2,4D or without (mock treated) for 2 hr (*A*) or 12 hr (*B*). Data are represented as described in Fig. 1*A*. (*A*) A schematic of PSIAA6 and relevant amino acid positions (above the graph). Light-grown (Light) and dark-grown (Dark) seedlings expressing the negative controls [LUC or LUC::NLS (nuclear localization signal)] or the Aux/IAA::LUC/LUC::NLS fusions were incubated with and without 1 μM 2,4D. (NLS, squash leaf curl virus NLS.) (*B*) Light-grown seedlings expressing the designated LUC-fusion proteins (LUC alone, black bars; PSIAA6::LUC, white bars; PSIAA6^{P61L}::LUC, striped bars) were incubated with 2,4D (1 μM, 10 μM, and 20 μM) or without (mock treated). PSIAA6^{P61L}::LUC is significantly different from PSIAA6::LUC (*) and unfused LUC (#) as determined by a *t* test at a 95% confidence level.

II, showed a response to auxin equivalent to that of the full-length protein (Fig. 2*A*).

Completely removing domain II, as in the PSIAA6-(71–179)::LUC::NLS fusion protein, eliminated response to 1 μM 2,4D after either a 2-hr (Fig. 2*A*) or 12-hr incubation (data not shown). Finally, 13 amino acids from IAA17, corresponding to a domain II consensus sequence (13aa), have been shown to confer more rapid degradation to LUC::NLS (8). Transgenic seedlings expressing 13aa::LUC::NLS were treated with 1 μM 2,4D. The LUC activity measured after 2 hr was reduced to 61% of the genetically identical mock-treated seedlings (Fig. 2*A*). These 13 amino acids were sufficient to alter LUC steady-state activity in response to exogenous auxin.

A Point Mutation Analogous to the Point Mutation Seen in *axr3–1* Reduces, but Does Not Eliminate, PSIAA6::LUC Response to Auxin. Full-length PSIAA6 with a missense mutation in domain II, PSIAA6^{P61L}::LUC (Pro to Leu change analogous to the point mutation in the auxin response mutant *axr3–1*), eliminated completely the reduction in LUC activity seen for the wild-type protein after a 2-hr treatment in 1 μM 2,4D (Fig. 2*A*). This fusion protein has a half-life of $\approx$4 hr, which is much greater than the 8–10 min half-life observed for the equivalent wild-type fusion protein (8). After changes in the rate of protein synthesis or degradation, the time for a protein to reach a new steady state is a function of its half-life and not of its synthetic rate (23). Therefore, one reason for the lack of a detectable auxin response by PSIAA6^{P61L}::LUC is that the 2-hr treatment was not sufficiently long enough to reach a new steady-state level. To characterize the effect of long-term auxin incubations on the various LUC proteins, seedlings expressing either the auxin-responsive PSIAA6::LUC or the auxin-non-responsive LUC alone were incubated alongside PSIAA6^{P61L}::LUC for 12 hr in 1 μM 2,4D. After 12 hr, while PSIAA6::LUC activity was lower as previously observed at 2 hr, PSIAA6^{P61L}::LUC activity did not differ significantly between auxin-treated and control seedlings (Fig. 2*B*). In this length of time, which was three times longer than the half-life of the protein, PSIAA6^{P61L}::LUC levels should have accomplished 87% of the change to the new steady-state level. Therefore, a difference in LUC activity should have been observed in the longer time course if PSIAA6^{P61L}::LUC levels were responsive to this auxin treatment.

To determine whether incubation at higher levels of exogenous auxin could elicit alterations in the steady-state level of PSIAA6^{P61L}::LUC, transgenic seedlings expressing PSIAA6::LUC or PSIAA6^{P61L}::LUC were incubated in 10 or 20 μM 2,4D for 12 hr. Both PSIAA6::LUC and PSIAA6^{P61L}::LUC luciferase activity decreased as the auxin concentration increased (Fig. 2*B*). Thus, the auxin response of PSIAA6^{P61L}::LUC is not eliminated but requires more exogenous auxin than does PSIAA6::LUC to have a detectable effect. The P61L mutation in PSIAA6 makes the fusion protein less responsive to auxin.

Auxin Increases the Rate of Degradation of Aux/IAA Proteins. To determine the mechanism responsible for the change in Aux/IAA::LUC abundance, the rate of proteolysis of IAA1::LUC was compared between auxin-treated and mock-treated seedlings grown under identical conditions. Transgenic seedlings expressing IAA1::LUC were incubated for 30 min in the presence of 5 μM 2,4D or buffer alone, and the LUC activity was determined (Fig. 3*A*). As expected, LUC activity of the chimera was reduced by 50% after the addition of auxin. Cycloheximide then was added to both auxin- and mock-treated seedlings, and the fusion protein half-life was determined by the rate of loss of LUC activity (8). IAA1::LUC half-life is $\approx$2-fold shorter in the presence of 5 μM 2,4D compared with buffer alone (Fig. 3*B*). This difference in half-life correlates exactly to the difference in the steady-state LUC activities (Fig. 3*A*). Thus, the differences in LUC activity can be accounted for entirely by a change in the degradation rate of Aux/IAA::LUC fusion proteins.

Auxin Rapidly Alters Aux/IAA::LUC Activity Levels. The kinetics of auxin regulation of Aux/IAA transcription, a primary response to auxin, is well documented. The transcripts of many *Arabidopsis* AUX/IAA proteins including IAA1 increase several-fold by 10 min of a 20 μM IAA application after a 4–6 min lag (24). The kinetics of changes in IAA1::LUC abundance in response to

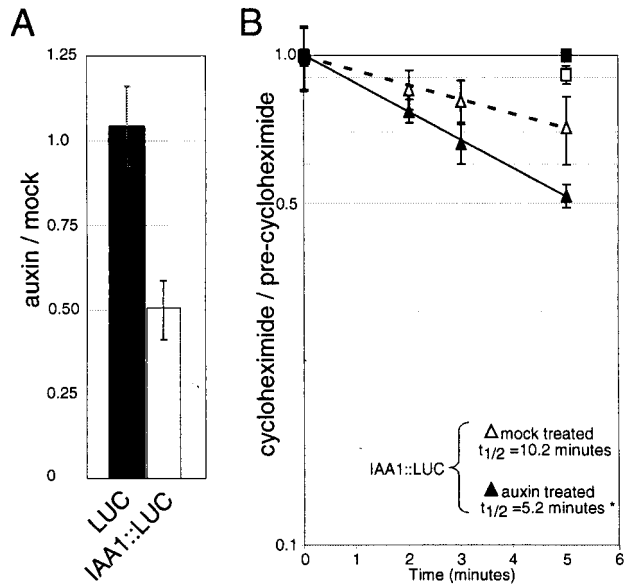

Fig. 3. Auxin increases the proteolytic rate of IAA1::LUC. (*A*) Six-day-old light-grown seedlings expressing unfused LUC or IAA1::LUC were incubated with 5 μM 2,4D or mock treated for 30 min. Data are represented as described for Fig. 1*A*. (*B*) Seedlings from the same experiment as *A* were treated additionally after the 30-min preincubation with 5 μM 2,4D with cycloheximide or buffer alone for the indicated amount of time. Results are expressed as a ratio of LUC activity per μg of protein from cycloheximide-treated seedlings relative to seedlings before cycloheximide treatment. Squares represent LUC; triangles represent IAA1::LUC. Black symbols represent auxin-treated samples; white symbols represent mock-treated samples. Data represent two experiments each with duplicate time points. The half-life values were determined by the best-fit line equations. *, Auxin-treated line is significantly different from the mock-treated line as determined by a linear regression test at a 95% confidence level.

exogenous auxin were determined. IAA1::LUC-expressing seedlings were incubated in 20 μM auxin for various time intervals, and LUC activity was measured and compared with untreated seedlings (Fig. 4). A change in LUC activity could be detected by the earliest time point (at 2 min), and by 4 min, LUC activity from IAA1::LUC-expressing seedlings was 45% of that present in untreated seedlings. By 20 min of 20 μM IAA incubation, IAA1::LUC activity was at its new, lower steady

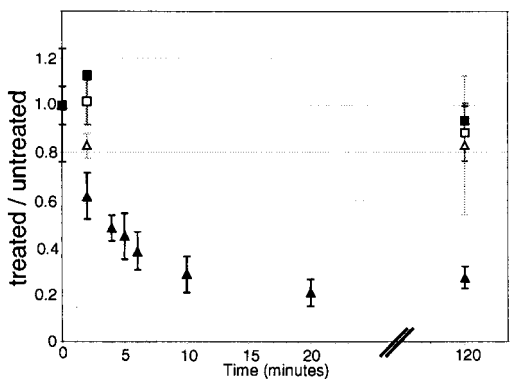

Fig. 4. Auxin rapidly lowers IAA1::LUC luciferase activity. A time course of LUC activity from LUC (squares) and IAA1::LUC (triangles) expressing seedlings after incubation in 20 μM IAA (black) or mock treated (white). Results are expressed as a ratio of treated to untreated LUC activity per μg of protein. Data represent two experiments done in duplicate.

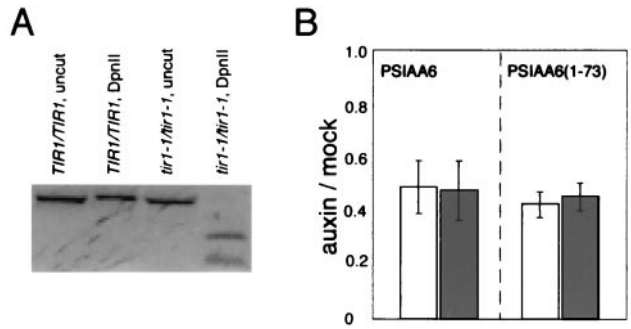

Fig. 5. *tir1–1* does not alter the auxin response of Aux/IAA::LUC activity. Transgenic plants expressing either PSIAA6::LUC or PSIAA6(1–73)::LUC were crossed to *tir1–1* plants. Plants homozygous for the transgene were identified by the absence of segregation for kanamycin resistance and LUC activity in progeny tests. *TIR1* genotype was determined [see *A* and *Materials and Methods*] to obtain homozygous lines and in a portion of the experimental material to ensure that the proper genotypes were analyzed. (*A*) The *tir1–1* mutation creates a *Dpn*II restriction enzyme site. The DNA was visualized by ethidium bromide staining. (*B*) Seedlings homozygous for PSIAA6::LUC (*Left*) or PSIAA6(1–73)::LUC (*Right*) in either *TIR1*/*TIR1* (black) or *tir1–1*/*tir1–1* (white) background were tested for changes in LUC activity in response to 1 μM 2,4D, as described in Fig. 1*A*. Data are represented as described for Fig. 1*A*.

state. As observed (Figs. 1 and 2), LUC activity did not change in seedlings expressing LUC alone (Fig. 4).

Auxin-Mediated Regulation of an Aux/IAA::LUC Half-Life Is Not TIR1-Dependent. Aux/IAA proteolysis has been shown to be affected by inhibitors of the proteasome, implicating the involvement of the ubiquitin pathway in Aux/IAA degradation (8). One enzyme proposed to be participating in Aux/IAA protein degradation is the F-box protein TIR1 (25). To test whether the response of Aux/IAA LUC fusions to auxin depends on TIR1, the PSIAA6::LUC and PSIAA6(1–73)::LUC transgenes were introduced into the *tir1–1* background by crossing. Doubly homozygous lines for *tir1–1* and the transgene were generated. The *TIR1* genotype of the experimental plants was verified by a CAPs test (see *Material and Methods*, Fig. 5*A*). PSIAA6::LUC in *tir1* plants had a similar response to 1 μM 2,4D after 2 hr as in TIR1 sibling seedlings (Fig. 5*B*). However, slight alterations in Aux/IAA regulation could be missed in this time interval. A time course of PSIAA6::LUC-fusion activity vs. incubation in 20 μM IAA was performed simultaneously with TIR1 sibling and *tir1–1* seedlings. No differences in the timing of the auxin response between TIR1 and *tir1–1* were seen (data not shown).

Discussion

The increase of *Aux/IAA* mRNA after auxin addition is a well characterized auxin response. This study has revealed an additional mechanism by which the *Aux/IAA* family is regulated by auxin. We demonstrate here that exogenous auxin increased the degradation rate of Aux/IAA::LUC proteins, resulting in a decrease in Aux/IAA::LUC activity and protein. We hypothesize that the response of endogenous Aux/IAA proteins to auxin is identical to that observed here for introduced Aux/IAA::LUC proteins, and we hypothesize further that endogenous auxin are also capable of modulating Aux/IAA degradation rates. The rapid change in LUC activity after auxin application suggests that the change in degradation rate is extremely rapid. This mechanism is the quickest one revealed to date whereby Aux/IAA protein levels can be modulated *in vivo*. Given the importance of Aux/IAA proteins in auxin signaling as revealed by genetic screens (4–6), modulation of Aux/IAA abundance by auxin could be an important point of control for downstream auxin-regulated events.

Whether all Aux/IAA family members respond to exogenous auxin is currently unknown; however, we demonstrated that the protein degradation rates of two different full-length Aux/IAA::LUC fusions are auxin responsive. The degradation rate for an Aux/IAA::LUC protein with only 13 amino acids of domain II from IAA17 (13aa::LUC::NLS) responded to auxin. The amino acids in IAA17 domain II represent the consensus sequence, suggesting that the degradation rates of most family members are auxin responsive. It is possible, however, that changes from the consensus found in some family members and/or flanking amino acids of any member antagonize and/or modulate this response.

The degradation rate of an Aux/IAA::LUC protein with a single amino acid substitution in domain II corresponding to *axr3–1* was less responsive to auxin. Application of higher levels of exogenous auxin was required to see an effect on PSIAA6^{P61L}::LUC activity. Although PSIAA6^{P61L}::LUC is degraded more slowly than wild-type PSIAA6::LUC (8), the slower degradation rate cannot explain the reduced response. The degradation rates of PSIAA6^{P61L}::LUC and 13aa::LUC::NLS, at 4 and 2 hr, respectively (8), are comparable, yet the latter responds to 1 μM 2,4D whereas the former does not respond. Because half-life determines the rate of change to a new steady state after a change in synthesis or degradation (23), an incubation time 3-fold longer than the PSIAA6^{P61L}::LUC half-life was performed. No detectable change was seen for PSIAA6^{P61L}::LUC after a 12-hr incubation in 1 μM 2,4D, while the change in PSIAA6::LUC degradation rate was maintained during this time, and the response of 13aa::LUC::NLS was detectable after one half-life. These results indicated that changes in the PSIAA6^{P61L}::LUC degradation rate did not occur at this concentration of exogenous auxin. However, changes in LUC activity were detected after increasing the exogenous auxin levels. Thus, at least this domain II mutation results in a protein that requires a higher level of auxin than the wild-type protein. This fact raises the interesting question whether the auxin-response phenotypes seen in *axr3–1* (4) are caused by higher iaa17 levels from the slower degradation rate *per se*, from the requirement for higher levels of auxin, or from alterations in the time to reach a new steady state.

The changes in LUC activity in response to exogenous auxin were extremely rapid, with significant differences visible by 2 min, the earliest time point tested. This time is faster than the reported induction of primary auxin-responsive mRNAs in multiple systems (24). A well characterized family of early auxin-response genes is the *Aux/IAA* genes themselves. After a lag of 5–10 min, IAA1 mRNA is elevated in the presence of exogenous auxin (24).

It is paradoxical that auxin both down-regulates Aux/IAA abundance and up-regulates *Aux/IAA* transcription. By itself, the increase in transcription of *Aux/IAA* genes should lead to a corresponding increase in protein abundance. Interestingly, in previous studies, increases at the protein level were not as dramatic as increases seen in mRNA levels (26). Our data provide an explanation for this discrepancy and for the lag time seen before an increase in transcription (Fig. 6). Aux/IAA proteins are thought to function as negative regulators of the auxin response (13). Aux/IAA proteins could negatively regulate positive factors, for example, ARFs and possibly others, by protein–protein interactions as demonstrated *in vitro* (10), suppressing auxin-regulated transcription in low auxin (Fig. 6, *Top*). In higher auxin, the increased proteolytic rate lowers Aux/IAA abundance, derepressing transcription (Fig. 6, *Middle*).

A prediction from this model is that Aux/IAA proteins should begin to inhibit their own synthesis as they accumulate because of increases in mRNA abundance. This phenomenon has not been seen in the majority of *Aux/IAA* family members in wild-type plants. Of the 14 *Aux/IAA* genes analyzed, only *IAA3*

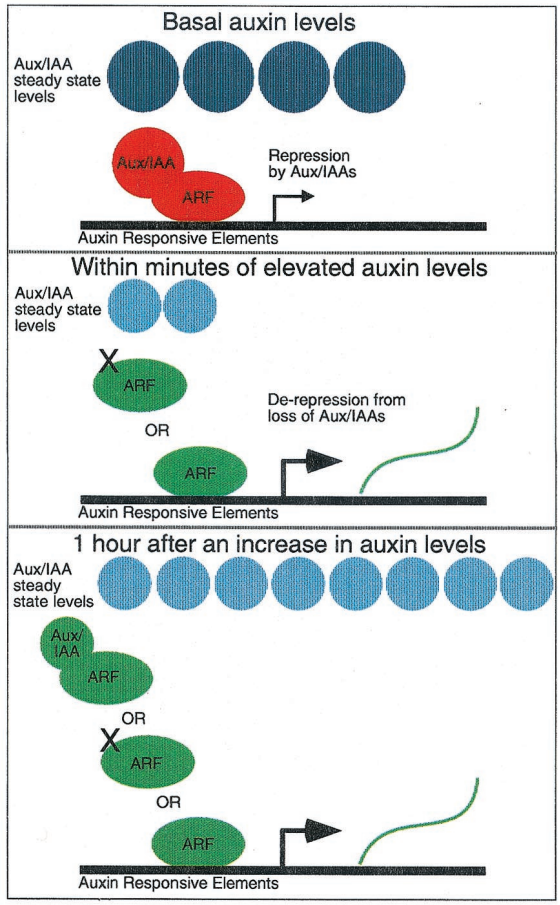

Fig. 6. Model for the alteration in primary auxin-responsive transcription. Aux/IAA proteins, encoded by auxin-responsive genes, are shown in dark and light blue circles. Generic ARF members interacting with Aux/IAA and possible unknown (X) proteins regulating an auxin-responsive gene are shown schematically. Green-filled protein complexes symbolize positive complexes promoting transcription, whereas the red-filled complex represents complexes with repressor activity. The number of circles represents the relative steady-state levels. (*Top*) At low auxin concentrations, auxin-responsive transcription is low because of the Aux/IAA repressor activity. (*Middle*) The presence of auxin increases Aux/IAA proteolysis, lowering the steady-state levels of the Aux/IAA proteins (shorter-lived proteins are represented by lighter blue circles). The complexes are freed from repressor activity and primary auxin-responsive transcription occurs. (*Bottom*) The steady-state levels of Aux/IAA proteins increase because of auxin up-regulating their transcription. However, auxin-responsive transcription is still present because of Aux/IAA latent repressing activity.

and *IAA6* mRNA levels diminished after 4 hr of auxin incubation (24). This fact suggests that at least some Aux/IAA proteins synthesized in the presence of auxin are not able to repress the activity of a positive transcription factor (Fig. 6, *Lower*). In addition to increased Aux/IAA proteolysis, auxin-treated cells could posttranslationally modify newly synthesized Aux/IAA proteins, thereby affecting their function. Also, additional proteins synthesized in the presence of auxin could affect Aux/IAA activity. Finally, the balance of Aux/IAA proteins synthesized in the presence of auxin may be different, causing an altered composition of Aux/IAA proteins, which effects a different biological result. The advantage of regulating Aux/IAA activity in the presence of auxin is striking. The presence of inactive repressors provides a pool that can be activated rapidly when auxin levels decline.

How does auxin modulate Aux/IAA proteolysis? Regulating proteolysis through the ubiquitin pathway can occur through mod-

ulating interactions between the ubiquitin E3 and its protein substrate(s) and/or with ancillary proteins by posttranslational modification(s) of any of these components (16). Because TIR1 is implicated in auxin responses (18) and is a component of a ubiquitin E3 ligase (19), the substrate-recognition component of the ubiquitin pathway, and because Aux/IAA proteolysis is sensitive to proteasome inhibition (8), SCF^{TIR} is a logical target for modulation by auxin. Our data indicating that this rapid response does not require TIR1 do not eliminate involvement of TIR1 in Aux/IAA degradation or modulation of its activity by auxin. However, our data indicate that additional factors (such as other members of the large F-box family) are able to compensate for loss of TIR1 function in *tir1–1* plants. This conclusion is consistent with the mild phenotype of *tir1* plants (25). Alternatively, TIR1 may interact with a subset of Aux/IAA proteins that does not include PSIAA6. The importance of TIR1 might also be masked in these transgenic plants because Aux/IAA::LUC proteins are expressed in a greater number of cells than is TIR1 (19, 22).

Several modifications of degradation machineries have been linked to auxin signaling. One component of the SCF, *At*CUL1, has been shown to be modified by the addition of the ubiquitin-like protein, RUB1 (27). Significantly, *axr1* plants with an inactive RUB-activating enzyme are insensitive to auxin (25, 28). Transgenic plants containing little COP9 signalosome (CSN) have decreased Aux/IAA degradation (29). The CSN has been shown to interact with an SCF complex, and this interaction is thought to facilitate proteolysis (29, 30). Aux/IAA proteins have been shown to be phosphorylated (31). Therefore, auxin could theoretically increase Aux/IAA proteolysis by altering: (*i*) the SCF-activation state through RUB addition or other modification; (*ii*) CSN activity/interaction with SCF; and/or (*iii*) Aux/IAA modification.

The nature of polar auxin transport allows for large differences in auxin concentrations between and within organs. Developmental patterning, vascular canalization, and the Cholodny–Went phototropism model all depend on an auxin gradient (1, 2). Our results predict that a cell with a higher auxin concentration than its neighboring cells would have a higher Aux/IAA proteolytic rate. In addition to auxin, it is important to determine whether other signals regulate Aux/IAA proteolysis. We have demonstrated that auxin can increase Aux/IAA proteolysis independently of light. It will be important to understand what other phytohormones and whether environmental cues can modulate Aux/IAA proteolysis; this finding could be the critical mechanism for integrating the complex signaling networks involved in plant developmental and tropic responses.

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