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The *Arabidopsis* Aux/IAA Protein Family Has Diversified in Degradation and Auxin Responsiveness [W]

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Rapid, auxin-responsive degradation of multiple auxin/indole-3-acetic acid (Aux/IAA) proteins is essential for plant growth and development. Domain II residues were previously shown to be required for the degradation of several *Arabidopsis thaliana* Aux/IAA proteins. We examined the degradation of additional full-length family members and the proteolytic importance of N-terminal residues outside domain II using luciferase (LUC) fusions. Elimination of domain I did not affect degradation. However, substituting an Arg for a conserved Lys between domains I and II specifically impaired basal degradation without compromising the auxin-mediated acceleration of degradation. IAA8, IAA9, and IAA28 contain domain II and a conserved Lys, but they were degraded more slowly than previously characterized family members when expressed as LUC fusions, suggesting that sequences outside domain II influence proteolysis. We analyzed the degradation of IAA31, with a region somewhat similar to domain II but without the conserved Lys, and of IAA20, which lacks domain II and the conserved Lys. Both IAA20:LUC and epitope-tagged IAA20 were long-lived, and their longevity was not influenced by auxin. Epitope-tagged IAA31 was long-lived, like IAA20, but by contrast, it showed accelerated degradation in response to auxin. The existence of long-lived and auxin-insensitive Aux/IAA proteins suggeststhat they may play a novel role in auxin signaling.

INTRODUCTION

Auxin influences myriad developmental processes in plants, including but not limited to tropic responses, apical dominance, phyllotaxy, and embryogenesis (Muday, 2001; Jenik and Barton, 2005; Leyser, 2005; Reinhardt, 2005). To understand the primary transcriptional response to auxin, several laboratories identified a family of early response genes called the auxin/indole-3-acetic acid (*Aux/IAA*) genes (Guilfoyle, 1999), and subsequent biochemical and genetic analyses have indicated that regulated degradation of Aux/IAA proteins plays a crucial role in auxin-mediated signaling (reviewed in Dharmasiri and Estelle, 2004). Aux/IAA proteins are short-lived. Three family members, namely pea (*Pisum sativum*) IAA4 and IAA6 and *Arabidopsis thaliana* AXR2/IAA7 have half-lives of 5 to 12 min; a fourth member, AXR3/IAA17, exhibits a half-life of 80 min (Abel et al., 1994; Gray et al., 2001; Ouellet et al., 2001). In addition, Aux/IAA sequences from pea IAA6 and *Arabidopsis* IAA1 and AXR2/IAA7 target marker proteins for rapid degradation (Worley et al., 2000; Gray et al., 2001; Zenser et al., 2001). The current model hypothesizes that under basal (i.e., low-auxin) conditions, Aux/IAA proteins are able to repress the activity of Auxin Response Factor (ARF) transcription factors (Ulmasov et al., 1997, 1999; Tiwari et al., 2001, 2004). Increased auxin reduces the levels of Aux/IAA proteins by accelerating their degradation (Gray et al., 2001; Zenser et al., 2001), such that ARF activity is derepressed and numerous auxin-mediated transcriptional changes occur (Ulmasov et al., 1997, 1999; Tiwari et al., 2001, 2004).

Biochemical and genetic evidence suggests that conserved domain II, found in most Aux/IAA proteins, is required for rapid degradation and the auxin-mediated acceleration of degradation. To date, developmental defects caused by dominant and semidominant mutations in domain II have led to the identification of 10 different Aux/IAA family members (reviewed in Reed, 2001; Yang et al., 2004). Measurement of in vivo half-lives for pea IAA6 sequences fused to firefly luciferase (LUC) or AXR3/IAA17 fused to β-glucuronidase (GUS) revealed that domain II is required for the rapid degradation of these fusion proteins under basal conditions (Worley et al., 2000; Gray et al., 2001; Ramos et al., 2001), and *axr3-1/iaa17* with a domain II mutation has a slower degradation rate than endogenous wild-type IAA17 (Ouellet et al., 2001). In pulldown assays, domain II promotes interaction with the F-box protein Transport Inhibitor Response1 (TIR1) (Gray et al., 2001; Tian et al., 2003; Kepinski and Leyser, 2004, 2005; Yang et al., 2004; Dharmasiri et al., 2005a, 2005b). F-box proteins are a subunit of SCF (for Skp-Cullin/CDC53-F-box) ubiquitin ligases that typically catalyze the ubiquitination of substrates required for their subsequent degradation (reviewed in Reed, 2001; Deshaies, 1999). Domain II sequences are also sufficient to recapitulate the in vivo auxin-mediated acceleration of degradation first observed for a full-length IAA1:LUC fusion protein (Zenser et al., 2001, 2003). In vitro, auxin increases the interaction of TIR1 or related F-box proteins, Auxin Receptor F-Box Proteins (AFB1, AFB2, and AFB3), with domain II peptides or Aux/IAA proteins containing domain II (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005), suggesting that the auxin-mediated increased interaction speeds ubiquitination and subsequent degradation by the proteasome in vivo. The body of

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work on Aux/IAA proteolysis supports the conclusion that domain II acts as an essential region for promoting proteolysis, classifying it as a degron as defined by Varshavsky (1991).

The identification of domain II as an auxin-responsive degron has contributed substantially to our understanding of Aux/IAA regulation and function. However, it remains possible that domain II is not the sole determinant of the proper degradation of every Aux/IAA family member under all conditions. For example, p53 and p73, two closely related transcription factors, both bind Mdm2, a mammalian E3 ubiquitin ligase, but only p53 is targeted for degradation (Picksley et al., 1994; Haupt et al., 1997; Balint et al., 1999). p73 degradation is regulated independently by a short PY-containing degron that interacts with the Itch E3 ligase (Rossi et al., 2005). In addition, there are proteins with more than one degron, each promoting degradation under specific conditions or in certain cell types. For the Nrf2 transcription factor, one degron confers a half-life of <10 min on Nrf2 under homeostatic conditions, whereas under oxidative stress, a distinct degron causes Nrf2 to be degraded with a half-life of 40 min (McMahon et al., 2004). The Myc transcription factor contains one well-characterized degron in addition to a recently identified D-element capable of conferring different cell line–specific half-lives upon this protein (Herbst et al., 2004). Moreover, there is some evidence that degrons may compete with stabilons in regulating the proteolysis of certain substrates (reviewed in Dantuma and Masucci, 2002).

All of these examples suggest that regions outside of Aux/IAA domain II could independently regulate the proteolysis of specific Aux/IAA family members. And because the determinants of stability found in various proteins vary so drastically in size and amino acid composition, from the minimal R-X-X-L destruction box (Glotzer et al., 1991; King et al., 1996) to the 523–amino acid distributed degron of the yeast (*Saccharomyces cerevisiae*) Hmg2p protein (Gardner and Hampton, 1999), bioinformatics cannot be relied upon to identify novel degrons or stabilons present outside of domain II in Aux/IAA family members. Therefore, to gain a better understanding of regulated proteolysis among Aux/IAA family members, we initiated a search for additional residues required for normal Aux/IAA degradation through mutagenesis of a single family member and through comparison of diverse family members both under basal conditions and in response to increased levels of auxin.

RESULTS

The N-Terminal Portion of IAA17 Targets LUC for Rapid Degradation and Does Not Require Domain I

Twenty-three of the 29 *Arabidopsis* Aux/IAA proteins, referred to here as canonical members, contain four conserved domains (Abel et al., 1995; Reed, 2001; Liscum and Reed, 2002), including the core residues of domain II shown to be required for rapid proteolysis (Worley et al., 2000; Gray et al., 2001; Ramos et al., 2001). The N-terminal region of AXR3/IAA17 (Figure 1, underlined) was chosen to represent the canonical Aux/IAA family members in our degradation assays. The first 102 amino acids of the 230–amino acid protein, ending shortly after domain II, was previously shown to target GUS for rapid degradation (Gray et al., 2001). To facilitate the quantitative measurement of proteolytic rates, we fused the first 111 amino acids of IAA17 (Figure 1), which includes all residues N-terminal to conserved domain III, to nuclear-targeted LUC (LUC:NLS). Although amino acids shown to function as a bipartite nuclear localization signal (NLS) in *Arabidopsis* IAA3 and IAA9 and pea IAA4 (Abel and Theologis, 1995) are present in IAA17(1-111), an exogenous squash leaf curl virus NLS (Sanderfoot et al., 1996; Worley et al., 2000) was included to try to ensure the proper subcellular localization of the wild-type and mutant LUC fusion proteins. IAA17 (1-111):LUC:NLS showed a rapid rate of degradation under basal conditions of ~10 min (Figure 2A) in a cycloheximide-based degradation assay (Worley et al., 2000). This is very similar to the rate of degradation measured for full-length IAA17 fused to LUC (Figure 2A) and nearly identical to the degradation rate measured for endogenous IAA7 when this closely related family member (Remington et al., 2004) was analyzed using a pulse-chase assay (Gray et al., 2001). A reasonable estimate of the half-lives of these fusion proteins could also be obtained using a simplified 30-min cycloheximide treatment procedure (Figure 2B). In both assays, the half-lives of the fusion proteins were estimated to be between 8 and 11 min. This established that, as demonstrated previously for pea IAA6 (Ramos et al., 2001), the N-terminal region of IAA17 is sufficient to target LUC for the rapid proteolysis observed for the full-length protein.

Mutations were introduced into the IAA17(1-111):LUC:NLS expression construct to assess the importance of two conserved sequences outside of domain II that are shared by rapidly degraded family members: domain I and a Lys-Arg (KR) dipeptide (Figure 1). Domain I was recently shown to act as a transcriptional repressor domain (Tiwari et al., 2004). In several proteins (e.g., mammalian Myc), the degron overlaps with the transcriptional activation domain, suggesting than an important link can exist between transcriptional regulation and protein degradation (Salghetti et al., 1999, 2000). In addition, conversion of the conserved Thr and Glu residues in domain I to Val and Arg residues appeared to reduce the levels of an IAA17:LUC fusion protein in transfected carrot (*Daucus carota*) protoplasts (Tiwari et al., 2001). Therefore, to determine directly whether domain I sequences affect Aux/IAA:LUC degradation, the codons for nine amino acids of domain I (TELCLGLPG) were removed to generate an IAA17(1-111:ΔD1):LUC:NLS fusion protein (abbreviated ΔD1). When its degradation was measured in transgenic *Arabidopsis* seedlings, the ΔD1 protein showed a rapid loss of LUC activity after a 30-min treatment with cycloheximide, like the IAA17(1-111):LUC:NLS wild-type protein (Figure 2B), implying that both proteins are rapidly degraded. To determine whether the loss of domain I could compromise the auxin-mediated acceleration of degradation, steady state levels of wild-type and mutant fusion proteins were assayed after a 2-h incubation with 5 μM 2,4-D, a synthetic auxin that has been shown previously to accelerate the degradation of Aux/IAA:LUC proteins (Zenser et al., 2001). Both the wild-type and ΔD1 proteins showed similar losses of LUC activity (Figure 2C), suggestive of accelerated degradation in the presence of increased levels of auxin. Direct half-life measurements confirmed that both the wild-type and ΔD1 proteins showed similar auxin-mediated acceleration of degradation when compared in the same experiments (Figure 2D).

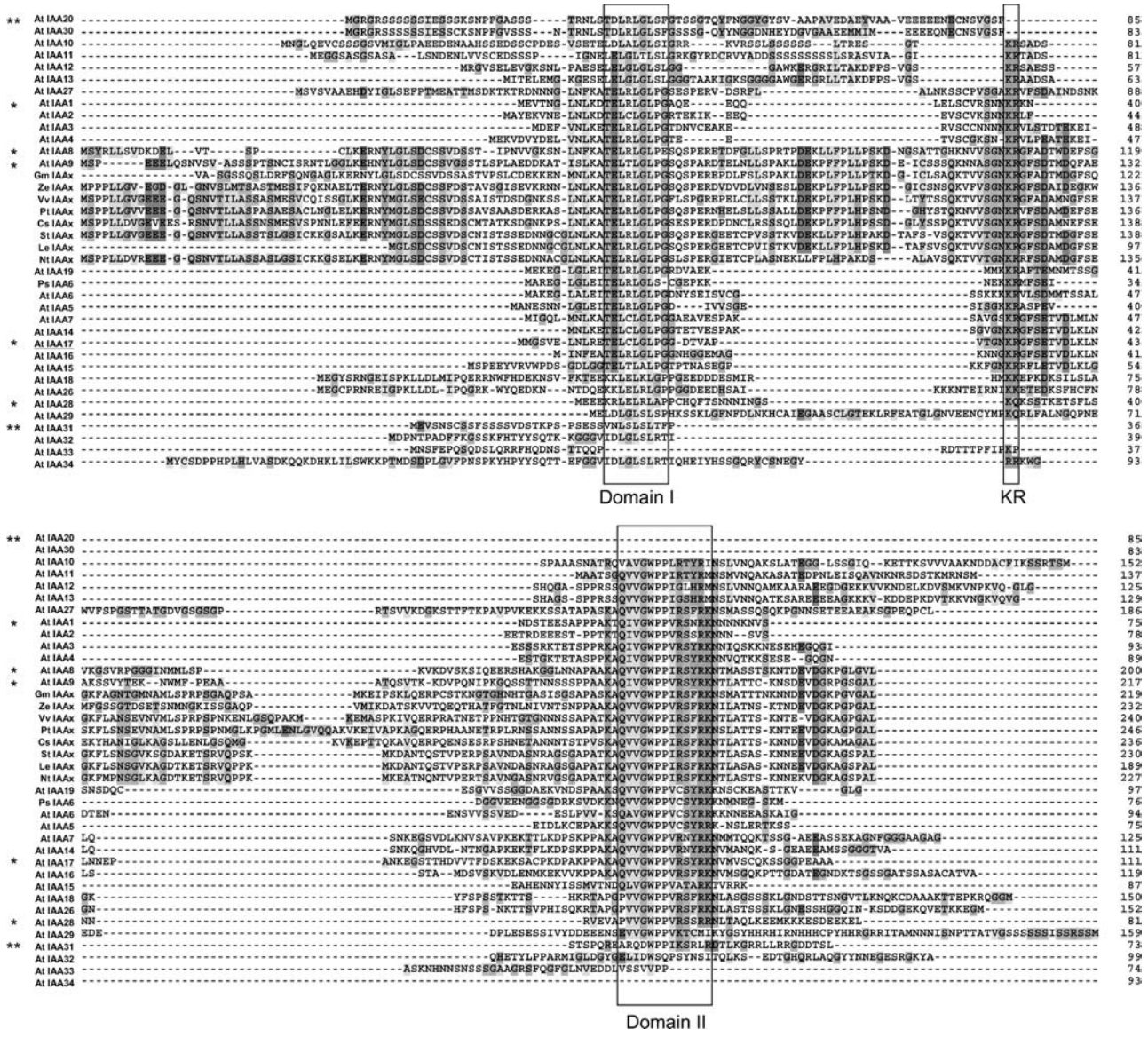

Figure 1. Alignment of Aux/IAA Family Members from the N Terminus to the Beginning of Domain III.

All Aux/IAA family members from *Arabidopsis* (Liscum and Reed, 2002), one rapidly degraded family member from pea, and several Aux/IAA proteins from other plant species were aligned using ClustalX (version 1.8) followed by manual editing using MacClade 4.05 OS X. All amino acids from the N terminus to the amino acid just before the beginning of domain III were included. Single asterisks mark canonical family members and double asterisks mark noncanonical family members analyzed in this study. Two groups of conserved amino acids within IAA17 (underlined name), a representative canonical family member, were changed for a series of experiments (see Figures 2 and 3). Conserved domains (Abel et al., 1995; Ramos et al., 2001; Tiwari et al., 2004) are boxed and vary slightly from domain I and domain II predictions from Abel et al. (1995) and Tiwari et al. (2004). Different shades of gray highlight specific subsets of amino acids according to default parameters in ClustalX. Sequences were obtained from The Arabidopsis Information Resource (TAIR) (*Arabidopsis* IAAs) or were identified using BLASTP at the National Center for Biotechnology Information (other species). At, *Arabidopsis thaliana*; Gm, *Glycine max*; Ze, *Zinnia elegans*; Vv, *Vitis vinifera*; Pt, *Populus tremula* × *Populus tremuloides*; St, *Solanum tuberosum*; Le, *Lycopersicon esculentum*; Cs, *Cucumis sativus*; Nt, *Nicotiana tabacum*; Ps, *Pisum sativum.*

A Lys in a Conserved Basic Patch between Domain I and Domain II Influences Degradation

Alignment of the Aux/IAA proteins shows that a conserved KR appears between domain I and domain II in 19 of the 29 family members in *Arabidopsis*, in 2 short-lived Aux/IAA proteins from pea, and in many other uncharacterized Aux/IAA proteins from other plant species (Abel et al., 1994) (Figure 1). In the four remaining canonical family members in *Arabidopsis*, a corresponding Lys residue is retained but the Arg is replaced by a Gln in IAA28

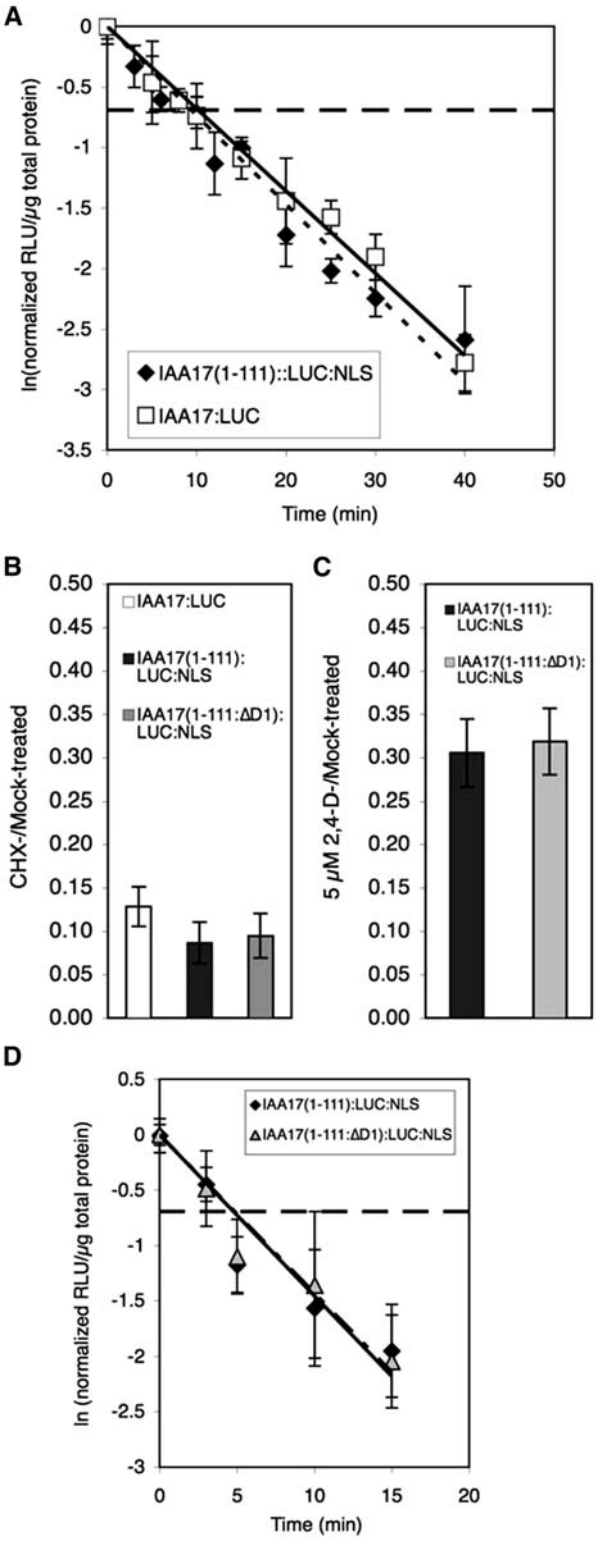

Figure 2. Degradation and Auxin Response of IAA17:LUC Fusion Proteins in *Arabidopsis* Seedlings.

(A) IAA17:LUC (solid line) and IAA17(1-111):LUC:NLS (dashed line) fusion proteins are degraded at similar rates in *Arabidopsis* seedlings in a cycloheximide-based assay. Values for the *y* axis are derived from measurements of relative light units (RLU) of LUC activity per microgram of total plant protein (see Methods for details of measurement and calculation). The horizontal dashed line represents the value expected when half of the fusion protein has been degraded; it intersects the degradation curve at the half-life of the fusion protein (e.g., 10 min). Error bars represent SD of all samples measured at a particular time point. Data for IAA17:LUC are based upon 2 independent transgenic lines (lines) in 6 experiments (exp) with an estimated half-life of 10.1 ± 0.5 min; data for IAA17(1-111):LUC:NLS are from 3 lines, 17 exp (half-life = 9.0 ± 0.3 min). Half-lives here and in all subsequent figures are presented as ±95% confidence intervals calculated using STATA.

(B) Full-length and truncated IAA17:LUC fusion proteins show similar loss of LUC activity after a 30-min incubation with cycloheximide (CHX). The values on the *y* axis represent the RLU/μg total protein in the cycloheximide-treated samples divided by the average RLU/μg total protein measured for all mock-treated samples in the same experiment. Error bars represent SD of all samples subjected to each treatment. Data for IAA17:LUC are from 2 lines, 6 exp; data for IAA17(1-111):LUC:NLS are from 3 lines, 8 exp; data for IAA17(1-111:ΔD1):LUC:NLS are from 3 lines, 6 exp.

(C) IAA17(1-111):LUC:NLS and IAA17(1-111:ΔD1):LUC:NLS fusion proteins show similar reduction in LUC activity after a 2-h incubation with the synthetic auxin 2,4-D (5 μM). The value measured on the *y* axis represents the RLU/μg total protein in the 2,4-D–treated samples divided by the average RLU/μg total protein measured for all mock-treated samples in the same experiment. The samples have statistically indistinguishable ratios at P = 0.05 by Student's *t* test. Error bars are as described for **(B)**. Data for IAA17(1-111):LUC:NLS are from 2 lines, 2 exp; data for IAA17 (1-111:ΔD1):LUC:NLS are from 3 lines, 6 exp.

(D) IAA17(1-111):LUC:NLS (solid line) and IAA17(1-111:ΔD1):LUC:NLS (dashed line) fusion proteins degrade at similar rates after a 2-h pretreatment with 5 μM 2,4-D, graphed as in **(A)**. The 95% confidence intervals for these two lines, as determined using STATA, overlap. Data for IAA17(1-111):LUC:NLS are from 1 line, 2 exp (half-life = 4.8 ± 0.7 min); data for IAA17(1-111:ΔD1):LUC:NLS are from 1 line, 2 exp (half-life = 4.8 ± 0.7 min).

and IAA29 or a second Lys in IAA18 and IAA26. This high level of conservation could be attributed to the fact that it is part of a bipartite NLS. However, many of the family members have a C-terminal NLS in domain IV, and some possess a third putative NLS at the N terminus (Abel and Theologis, 1995), suggesting that proper localization may not be the sole function of the KR in the bipartite NLS. To determine whether the KR residues affect proteolytic regulation independent of their role in localization, the codon for Lys-31, or the codons for Lys-31 and Arg-32, were mutated to encode Arg (RR) or Gln-Gln (QQ), respectively, within the context of the rapidly degraded and auxin-responsive IAA17 (1-111):LUC:NLS wild-type fusion protein. All three of these constructs should have been targeted to the nucleus by the exogenous C-terminal NLS, even if the endogenous NLS were disrupted by the KR-to-QQ or the K-to-R substitutions.

Interestingly, IAA17:LUC fusion proteins with either QQ or RR had roughly equivalent degradation rates that were slower than that of the wild-type control. These amino acid substitutions increased the half-lives of these fusion proteins by approximately threefold, from ~9 min to 30 min (Figure 3A). To determine the importance of these residues in the auxin-mediated modulation

of degradation, the steady state levels of these wild-type and QQ fusion proteins after a 2-h treatment with 5 μM 2,4-D were compared with the levels present after a mock treatment. The steady state levels of the QQ fusion proteins decreased sharply after auxin treatment (see Supplemental Figure 1 online), and direct half-life measurements demonstrated that both the QQ and RR fusion proteins had degradation rates very similar to that of the wild-type fusion protein in the presence of increased levels of auxin (Figure 3B). This finding suggests that the conserved Lys is dispensable for rapid degradation in the presence of high levels of auxin but is necessary for rapid basal degradation.

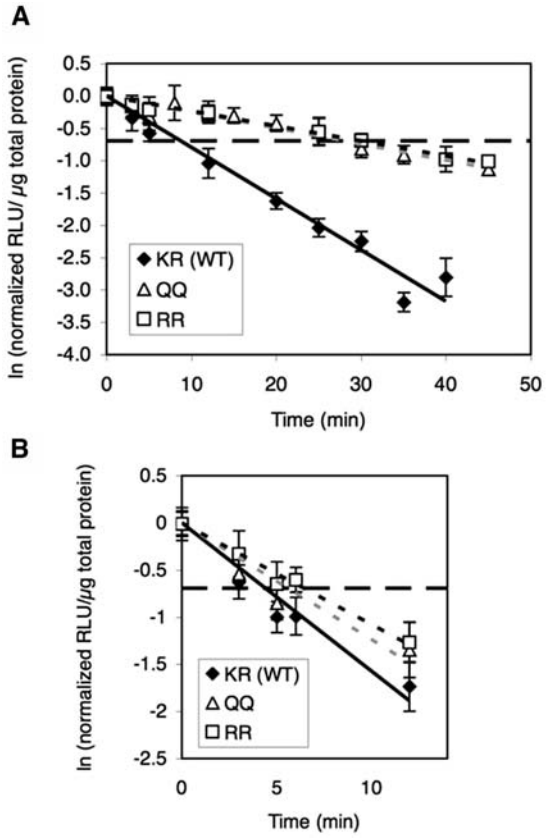

Figure 3. Degradation of Aux/IAA Fusion Proteins with Mutations in a Conserved KR Motif.

(A) IAA17(1-111):LUC:NLS [KR (WT)] (solid black line) is degraded more quickly under basal conditions than IAA17(1-111:K31Q,R32Q):LUC:NLS (QQ) (gray dashed line) and IAA17(1-111:K31R):LUC:NLS (RR) (black dashed line) fusion proteins (graphed as in Figure 2A). Data for KR (WT) are from 3 lines, 8 exp (half-life = 8.8 ± 0.3 min); data for QQ are from 3 lines, 5 exp (half-life = 28.2 ± 2.6 min); data for RR are from 3 lines, 6 exp (half-life = 29.5 ± 2.3 min).

(B) KR (WT), QQ, and RR fusion proteins all show similar rates of degradation after a 2-h incubation with 5 μM 2,4-D (graphed as in Figure 2A). Data for KR (WT) are from 3 lines, 8 exp (half-life = 4.6 ± 0.2 min); data for QQ are from 1 line, 2 exp (half-life = 5.6 ± 0.7 min); data for RR are from 3 lines, 5 exp (half-life = 6.4 ± 0.5 min).

Aux/IAA Family Members Containing Domain II Target LUC for Different Rates of Degradation

Both domain II and the conserved Lys found in most Aux/IAA family members appear to be required for the proper degradation of IAA17 and, potentially, other Aux/IAA proteins. It is also possible that sequences outside of these two conserved regions could additionally contribute to the proteolytic regulation of Aux/IAA proteins and yield yet another level of control in auxin signaling. To date, only the half-lives of pea IAA4 (Abel et al., 1994), pea IAA6 (Abel et al., 1994; Worley et al., 2000; Ramos et al., 2001), *Arabidopsis* IAA1 (Zenser et al., 2001, 2003), *Arabidopsis* AXR2/IAA7 (Gray et al., 2001), and *Arabidopsis* IAA17 (Ouellet et al., 2001; Tao et al., 2005) have been determined experimentally using endogenous proteins or fusion proteins. The degradation of more divergent family members has not been measured. To explore the possibility of variable degradation among canonical but diverged *Arabidopsis* Aux/IAA proteins, IAA8, IAA9, and IAA28 were selected for proteolytic analyses. These proteins could contain novel degrons or stabilons outside of the conserved domains, because their sequences vary markedly in these regions (Figure 1). For example, when considering the full-length proteins, IAA9 is 32% identical to IAA17 at the amino acid level. When the C-terminal portions of the proteins, composed primarily of conserved domain III and domain IV, are excluded, the level of identity between IAA9 and IAA17 decreases to 16%. Based on a whole protein comparison, IAA28 shares only 26% amino acid identity with IAA17, and the identity decreases to 17% when the comparison is limited to the N-terminal portion (Figure 1). On the other hand, as a result of genomic duplications (Blanc et al., 2000; Vision et al., 2000), there are 10 pairs of highly similar Aux/IAA family members in *Arabidopsis*, including IAA8 and IAA9 (Remington et al., 2004) (Figure 1). Full-length IAA8 and IAA9 proteins share 57% amino acid identity, and they are still 48% identical when the analysis is confined to the more divergent N-terminal region, suggesting that their degradation rates might be similar if shared sequences outside of the conserved domains affect degradation.

To test this hypothesis, the full-length open reading frames of IAA8, IAA9, and IAA28 were placed upstream of the LUC open reading frame to generate LUC fusion proteins. Treatment with cycloheximide or a mock solvent for 30 min revealed that although IAA1:LUC and IAA17:LUC showed a very significant protein loss, indicative of rapid degradation, the IAA8:LUC, IAA9:LUC, and IAA28:LUC protein levels remained higher (Figure 4A). Notably, lines expressing IAA8:LUC and IAA9:LUC behaved similarly to each other, but they showed statistically significant differences from lines expressing IAA1:LUC, IAA17:LUC, and IAA28:LUC. Direct half-life measurements were performed with multiple IAA9:LUC-expressing lines, and they substantiated the findings of the 30-min experiments. Although IAA9:LUC consistently appeared to be degraded at a slower rate than IAA17:LUC (Figure 4B), the results were variable. These fluctuations could not be directly attributed to any specific differences in experimental conditions (e.g., light, temperature, etc.), but they raise the possibility that an unknown factor may influence IAA9 degradation. IAA28:LUC levels barely decreased after a 30-min cycloheximide treatment, and direct half-life experiments

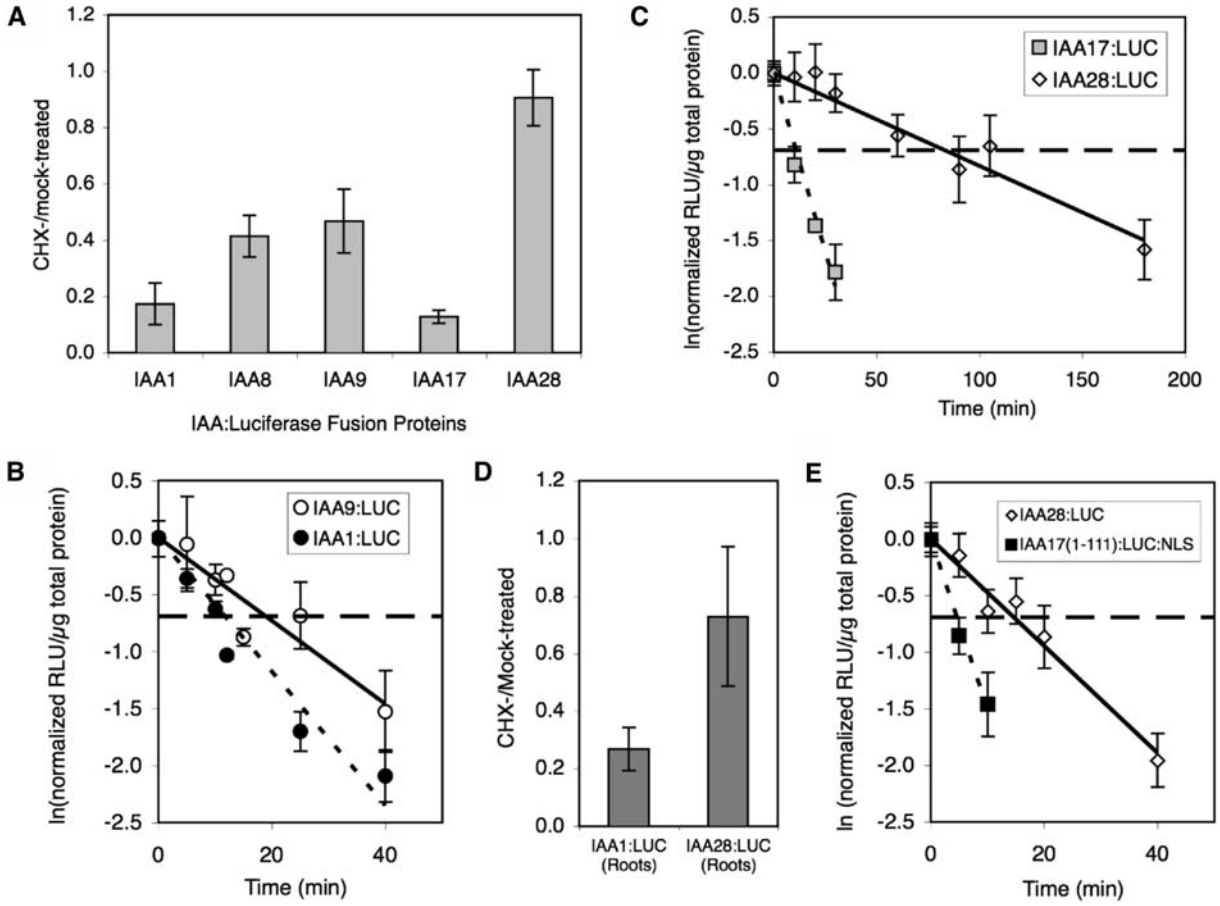

Figure 4. Degradation of Canonical Aux/IAA:LUC Fusion Protein Family Members.

(A) Aux/IAA:LUC fusion proteins exhibit differential loss of LUC activity after a 30-min treatment with cycloheximide (graphed as in Figure 2B). IAA8:LUC and IAA9:LUC have statistically indistinguishable ratios from each other at $P = 0.05$ by Student's *t* test. Data for IAA1:LUC are from 3 lines, 14 exp; data for IAA8:LUC are from 3 lines, 5 exp; data for IAA9:LUC are from 3 lines, 9 exp; data for IAA17:LUC are from 2 lines, 6 exp; data for IAA28:LUC are from 3 lines, 6 exp.

(B) IAA9:LUC is degraded more slowly than IAA1:LUC (graphed as in Figure 2A). Data for IAA9:LUC are from 3 lines, 4 exp (half-life $= 19.0 \pm 2.3$ min); data for IAA1:LUC are from 1 line, 2 exp (half-life $= 11.8 \pm 0.9$ min).

(C) IAA28:LUC has a half-life of >1 h in whole seedlings (graphed as in Figure 2A). Data for IAA28:LUC are from 3 lines, 13 exp (half-life $= 79.3 \pm 4.4$ min); data for IAA17:LUC are from 1 line, 3 exp (half-life $= 11.1 \pm 0.9$ min).

(D) IAA28:LUC is degraded more slowly in roots than IAA1:LUC (graphed as in Figure 2B). The ratios are statistically different at $P = 0.05$ by Student's *t* test. Data for IAA28:LUC are from 3 lines, 7 exp; data for IAA1:LUC are from 2 lines, 5 exp. CHX, cycloheximide.

(E) IAA28:LUC degradation is greatly accelerated by a 3-h treatment with 5 μM 2,4-D (graphed as in Figure 2A). Data for IAA28:LUC are from 3 lines, 8 exp (half-life $= 14.8 \pm 1.4$ min); data for IAA17(1-111):LUC:NLS are from 2 lines, 4 exp (half-life $= 4.6 \pm 0.4$ min).

resulted in the measurement of a half-life of ~80 min for this fusion protein, compared with ~10 min for the IAA17:LUC protein (Figure 4C).

In these experiments, all of the transgenes were under the control of the *UBQ10* promoter to ensure sufficient expression of the fusion proteins. It is possible that cell type– or organ-specific factors regulate the degradation of certain family members. If an ectopically expressed Aux/IAA:LUC fusion protein were rapidly degraded only in specific parts of the plant and thus remained long-lived outside of these parts, then the half-life of the fusion protein would be overestimated in whole seedling degradation assays. Both visualization of GUS activity in transgenic seedlings expressing GUS under the control of the *IAA28* promoter and RNA gel blot analysis suggested that IAA28 is expressed primarily in the roots of young plants (Rogg et al., 2001). Because IAA28 had the most dramatically different half-life under our experimental conditions, IAA28:LUC protein degradation was measured using only the roots from treated intact seedlings. IAA1:LUC was used as a control in this experiment because RNA gel blot analyses (Abel et al., 1995) and RT-PCR analysis (Yang et al., 2004) detect IAA1 expression in a number of different organs, including seedling roots, mature stems, leaves, and flowers.

The loss of LUC activity for IAA1:LUC and IAA28:LUC was measured in roots removed from *Arabidopsis* seedlings after a 30-min treatment with cycloheximide or a mock solvent (Figure 4D). The results revealed that although IAA1:LUC still disappeared quite rapidly in roots, IAA28:LUC appeared to be degraded more slowly. Although IAA28:LUC seemed to degrade somewhat more rapidly in roots than in whole seedlings, its half-life was still ~60 min (Figure 4D). This finding suggested that the long half-life observed for IAA28:LUC in whole seedlings (Figure 4C) could not be attributed solely to ectopic expression.

Auxin-mediated acceleration of degradation is another hallmark of Aux/IAA proteolysis (Gray et al., 2001; Zenser et al., 2001), resulting from increased interaction between domain II of the Aux/IAAs and TIR1 (Gray et al., 2001; Kepinski and Leyser, 2004, 2005; Dharmasiri et al., 2005a). The degradation of full-length IAA1 and the N-terminal portion of pea IAA6 and IAA17 fused to LUC all show an approximately twofold increase in degradation after treatment with 5 μM 2,4-D (Zenser et al., 2001, 2003). The rate of IAA28:LUC degradation also increased after a 3-h incubation with 5 μM 2,4-D. Its half-life of ~15 min is still longer than that of the control IAA17(1-111):LUC:NLS subjected to the same auxin treatment (Figure 4E). However, compared with its basal rate of degradation, IAA28:LUC proteolysis accelerated nearly fivefold in response to the same concentration of auxin that doubled the rate of degradation for IAA17 (1-111):LUC:NLS, and this effect was observed in whole seedlings.

IAA20, an Aux/IAA Protein Lacking Domain II, Does Not Contain Any Transferable Degrons

To date, all Aux/IAA proteins examined, either biochemically or physiologically, meet the criteria we used to identify canonical family members. However, several Aux/IAA proteins lack some of the conserved regions found in canonical family members (Figure 1). For instance, IAA20 and IAA30 completely lack domain II and the conserved KR, although they retain the other three domains. Not surprisingly, based on the functional characterization of these domains, IAA20 has been shown to repress the transcription of an auxin-responsive reporter gene in a carrot protoplast assay, similar to IAA17, IAA1, and other canonical family members (Tiwari et al., 2001). Although it does not possess domain II, other regions of IAA20 could act as novel degrons. To investigate the degradation of IAA20, an IAA20:LUC fusion protein was introduced into *Arabidopsis*. LUC activity was not diminished after a 12 h treatment with cycloheximide (Figure 5A) in seedlings expressing IAA20:LUC. In these assays, IAA17(1-111):LUC:NLS levels decreased significantly, confirming that the cycloheximide treatment did block de novo protein synthesis. Protein gel blot analysis demonstrated that the remaining LUC activity observed after cycloheximide treatments ranging from 3 to 12 h could be attributed to IAA20:LUC and not to the release of free, long-lived LUC (Figure 5C; see Supplemental Figure 2A online). Additional experiments were performed to assess the effect of auxin on IAA20:LUC degradation. Twelve-hour treatments with exogenous auxin (5 μM 2,4-D [Figure 5B] or 25 μM 2,4-D [see Supplemental Figure 2B online]) also failed to reduce the levels of IAA20:LUC activity in the seedlings, providing evidence that not all Aux/IAA family members show accelerated degradation in response to auxin.

LUC is a large tag, with a predicted molecular mass of 61 kD. Attachment of LUC to the C terminus of IAA20 could interfere with normal IAA20 degradation and auxin response, presumably by blocking interactions with the proteolytic machinery. To address these concerns, an additional set of lines was generated using a significantly smaller (~17 kD) 10xMyc tag, fused to the N terminus of IAA20. For all lines, two forms of the 10xMyc:IAA20 protein reacted with the anti-Myc antibody: one at the predicted size of ~38 kD and a second, slower-migrating form with an apparent molecular mass of ~50 kD (Figures 5D and 5E). Further work will be required to determine the reason for the presence of two forms of 10xMyc:IAA20 with different electrophoretic mobilities. However, both forms behaved similarly in our assays and revealed that the 10xMyc:IAA20 protein expressed under the control of the *35S* promoter was long-lived (Figure 5D, quantitation in Figure 5F) and insensitive to auxin (Figure 5E, quantitation in Figure 5G). Similar results were obtained for an IAA20:4xMyc fusion protein (see Supplemental Figure 3 online), but unfortunately, the fusion protein could only be detected by protein gel blotting in one transgenic line. Nevertheless, the results obtained demonstrate that IAA20 does not possess a transferable degron capable of targeting LUC for rapid degradation in basal or increased levels of auxin, and IAA20, with a smaller epitope tag at either the N or C terminus, is similarly long-lived and insensitive to auxin.

Several components of the SCF^{TIR1} ubiquitin ligase, implicated in the degradation of the canonical Aux/IAA proteins, reside primarily in the nucleus (Dharmasiri et al., 2005b; Tao et al., 2005). IAA20 does not possess any experimentally verified NLSs, and it contains a Ser-rich N-terminal extension (Figure 1) predicted to send it to the chloroplast, according to the TargetP 1.1 server (Emanuelsson et al., 2000). However, IAA20 can repress transcription from an auxin-responsive promoter in a carrot protoplast assay (Tiwari et al., 2001), and an sGFP:IAA20 fusion protein enters the nucleus more readily then GFP alone (see Supplemental Figure 4 online). Therefore, the longevity of IAA20 most likely results from its failure to interact with SCF^{TIR1} and related ubiquitin ligases rather than from a spatial separation of these proteins.

IAA31, with a Partial Domain II, Is Long-Lived but Auxin-Responsive

IAA31, another noncanonical Aux/IAA protein, shares a similar domain structure with IAA20. Like IAA20, IAA31 retains conserved domain III and IV in the C terminus, maintains the minimal LxLxL motif of domain I shown to be crucial for Aux/IAA-mediated transcriptional repression (Tiwari et al., 2004), and completely lacks the interdomain conserved Lys. But, unlike IAA20, IAA31 possesses a domain II–like region (ARQDWPPIKSRLR) that differs from the consensus domain II sequence (QVVGWPPVRSYRK) (Ramos et al., 2001) at several positions, including the first amino acid of the important GWPPV core residues (Figure 1). Comparable G-to-E substitutions in the context of the canonical domain II regions in SHY2/IAA3 and IAA18 are found in the *shy2-3* and *iaa18-1* mutants (Tian and

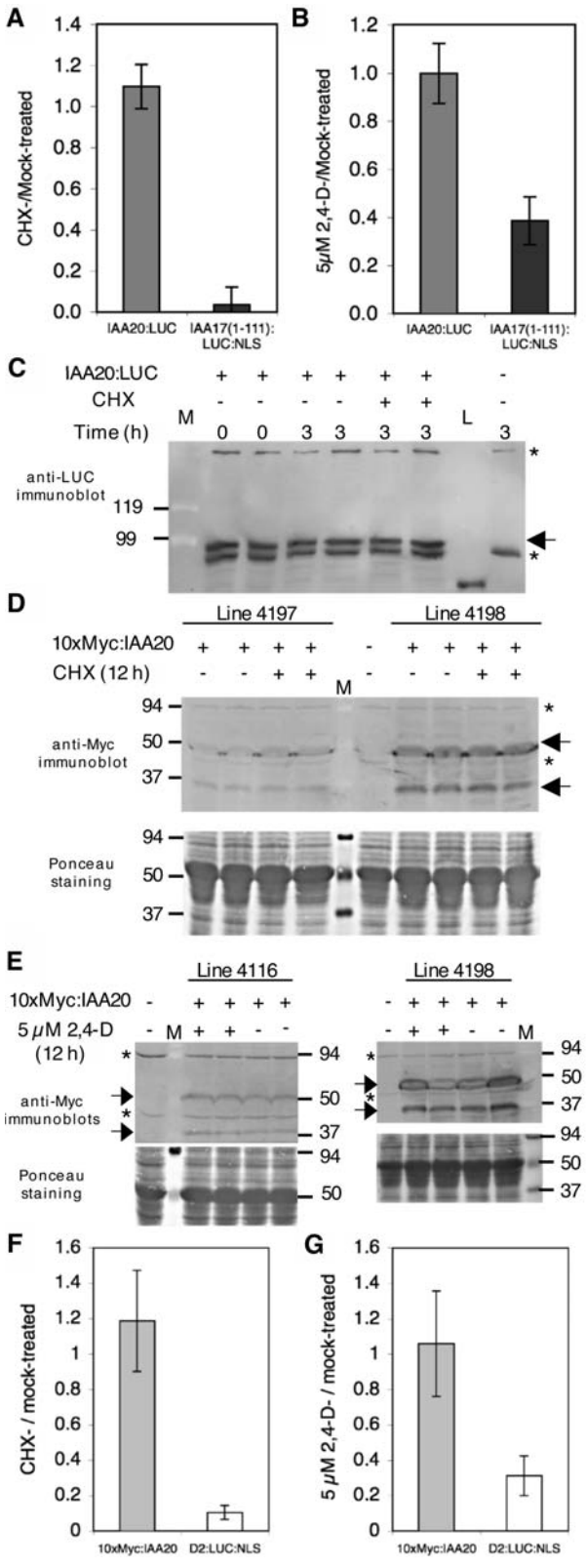

Figure 5. Degradation and Auxin Response of the Noncanonical, Domain II–Less IAA20 Protein Fused to LUC and a Myc Epitope Tag.

Reed, 1999; Reed, 2001). Their mutant phenotypes presumably are attributable to the longevity of these proteins. In addition, recapitulation of this mutation increased the accumulation of a D2:LUC:NLS fusion protein by >20-fold in a transient transfection assay (Ramos et al., 2001).

To examine the proteolytic profile of IAA31, the 10xMyc epitope tag that allowed the visualization of IAA20 was added to the C terminus of IAA31. Several lines bearing a *Pro$_{35S}$:IAA31:10xMyc* insertion were generated. As in the case of 10xMyc:IAA20, two forms of the IAA31:10xMyc protein were detected in all lines using an anti-Myc antibody (Figures 6A, 6C, and 6E). The faster-migrating form ran slightly ahead of a 37-kD marker,

(A) IAA20:LUC levels do not decrease after a 12-h treatment with cycloheximide (CHX) (graphed as in Figure 2B). Data for IAA20:LUC are from 4 lines, 7 exp; data for IAA17(1-111):LUC:NLS are from 1 line, 2 exp.

(B) IAA20:LUC levels do not decrease after a 12-h treatment with 5 μM 2,4-D (graphed as in Figure 2C). Data for IAA20:LUC are from 3 lines, 4 exp; data for IAA17(1-111):LUC:NLS are from 1 line, 2 exp.

(C) A 3-h treatment with cycloheximide does not diminish the levels of the IAA20:LUC fusion protein or promote LUC cleavage. Immunoblot analysis was performed with anti-LUC antibodies. The arrow points to the IAA20:LUC protein. Asterisks mark nonspecific cross-reacting bands used as loading controls. Lane 1 contains markers (M), lane 8 contains recombinant LUC (L), and lane 9 contains extract from untreated wild-type Columbia seedlings. Approximate molecular masses of markers are given in kilodaltons at left. The IAA20:LUC fusion protein runs larger than its predicted size of ~80 kD. The predicted size of LUC is ~61 kD. There is no evidence of free LUC in the IAA20:LUC samples.

(D) and **(F)** 10xMyc:IAA20 levels do not decrease after a 12-h treatment with cycloheximide.

(D) Immunoblot analysis was performed with anti-Myc antibodies. Arrows point to two forms of the 10xMyc:IAA20 protein with different mobilities. Asterisks mark nonspecific cross-reacting bands used as loading controls. Results from two independent lines are shown (4197 and 4198), and data from a third line are included in **(F)**. The lane without 10xMyc:IAA20 contains extract from mock-treated domain II (D2):LUC:NLS seedlings treated in parallel to verify cycloheximide effectiveness. Ponceau S staining shows total protein levels present on the membranes subjected to immunoblotting.

(F) Quantification of the data shown in **(D)**. The values on the *y* axis represent the average intensity of the bands in cycloheximide-treated samples divided by the average intensity of the bands for all mock-treated samples in the same experiment. Error bars represent SD of all samples subjected to each treatment. Data for 10xMyc:IAA20 are from 3 lines, 6 exp; data for D2:LUC:NLS are from 1 line, 4 exp.

(E) and **(G)** 10xMyc:IAA20 levels do not decrease after a 12-h treatment with 5 μM 2,4-D.

(E) Immunoblot analysis was performed with anti-Myc antibodies; labeling is as in **(D)**. The lane without 10xMyc:IAA20 contains extract from mock-treated D2:LUC:NLS seedlings treated in parallel to verify 2,4-D effectiveness. Results from two independent lines are shown (4116 and 4198), and data from a third line are included in **(G)**.

(G) Quantification of the data shown in **(E)**. The values on the *y* axis represent the average intensity of the bands in 2,4-D–treated samples divided by the average intensity of the bands for all mock-treated samples in the same experiment. Error bars represent SD of all samples subjected to each treatment. Data for 10xMyc:IAA20 are from 3 lines, 4 exp; data for D2:LUC:NLS are from 1 line, 2 exp.

closely matching the 36-kD predicted size for IAA31:10xMyc. The slower form, with an apparent molecular mass of slightly less than 50 kD, comigrated with the cross-reactive band identified previously (Figures 5D, 5E, 6A, 6C, and 6E). Again, the reason for the differential mobility is still under investigation, but there was no substantial difference in the behavior of the two species of IAA31:10xMyc protein. Interestingly, levels of the IAA31:10xMyc fusion protein barely decreased after a 12-h incubation with cycloheximide (Figure 6A). Quantification of the band intensities and normalization to the most abundant band present for each line revealed some variability across experiments. When averaged, cycloheximide-treated samples had only ~15% less IAA31:10xMyc protein than comparable mock-treated samples after the 12-h cycloheximide incubation (Figure 6B). Therefore, the partial domain II found in IAA31 was not sufficient to allow the rapid degradation of the epitope-tagged protein.

In the case of IAA20, protein longevity was coupled to auxin insensitivity. By contrast, a 12-h treatment with auxin did decrease the steady state levels of IAA31:10xMyc (Figures 6C and 6D). Because IAA31:10xMyc was expressed under the control of the non-auxin-responsive cauliflower mosaic virus *35S* promoter, these results suggested that IAA31:10xMyc degradation could be accelerated by auxin. Direct half-life experiments confirmed this hypothesis. Ten-hour cycloheximide assays were performed on seedlings expressing IAA31:10xMyc after a 2-h incubation with 10 μM 2,4-D or a mock solvent control. In the presence of increased levels of auxin, IAA31:10xMyc had an apparent half-life of ~4 h compared with an average half-life of >20 h for seedlings incubated with the solvent control (Figures 6E and 6F). IAA31:10xMyc thus appears to be both long-lived and auxin-responsive, demonstrating the existence of a novel proteolytic profile for another noncanonical family member.

DISCUSSION

Domain II of the Aux/IAA proteins has been implicated as an important determinant of Aux/IAA degradation through biochemical, genetic, and physiological experiments. Notably, a 17–amino acid synthetic peptide spanning domain II alone can bind to the F-box protein TIR1 in vitro (Kepinski and Leyser, 2004, 2005). However, a 13–amino acid segment containing domain II alone cannot confer a very rapid half-life on LUC in planta unless it is placed within a full-length or N-terminal region of an Aux/IAA protein (Worley et al., 2000; Ramos et al., 2001; Zenser et al., 2001, 2003). Many factors could account for the discrepancy between the two experimental systems, such as differences in the number of amino acids included, protein folding of these different peptide and fusion proteins, and/or accessibility of the degron in the different configurations. Additionally, the assays have different readouts, the former in vitro binding and the latter in vivo proteolysis, and the latter could be affected by in vivo factors. Therefore, it is possible that other residues outside of domain II in the canonical Aux/IAA proteins could be required for efficient degradation in vivo. Truncation mapping of pea IAA6 (Worley et al., 2000) and of AXR3/IAA17 (Gray et al., 2001) suggested that these amino acids would reside at the N-terminal portion of canonical family members.

We first examined the role of domain I because it is well conserved among rapidly degraded family members and resides in the N-terminal portion of the proteins. Domain I's ability to affect degradation might differ among family members. However, using the N-terminal region of IAA17 as representative of canonical Aux/IAA proteins containing all four domains, we found that domain I does not appear to be required for the rapid degradation of IAA17(1-111):LUC:NLS under normal conditions or in response to increased levels of auxin. Therefore, there does not seem to be a functional overlap between the transcriptional repressor domain and the degron in the case of IAA17, even though this association has been observed for the transcriptional activation domain and the degron of transcriptional activators such as Myc (Salghetti et al., 2000). However, it should be noted that the IAA17(1-111):LUC:NLS fusion protein would not be predicted to bind to ARF transcription factors, because it lacks domains III and IV. Further experimentation is required to ascertain whether domain I affects Aux/IAA degradation rate in full-length proteins that can bind to ARFs.

Further mutational analysis in the IAA N terminus revealed that a conserved Lys found between domain I and domain II may form part of the functional IAA17 degron, because a conservative substitution of Lys-31 to an Arg slows the degradation of IAA17(1-111):LUC:NLS nearly threefold. Interestingly, this effect is seen only under basal conditions, because the mutant fusion protein degrades at almost the same rate as the wild-type fusion under conditions of increased auxin. To date, there are no other known mutations found within Aux/IAA proteins that selectively affect their degradation in this manner, prompting the question of how this conserved Lys might act specifically as part of a basal degron.

All canonical Aux/IAA family members in *Arabidopsis* possess a small basic patch in the variable region between domain I and domain II that contains at least one Lys residue, often directly in front of an Arg. A similar motif is present in the rapidly degraded pea IAA4 and IAA6 proteins and can also be found in Aux/IAA homologs from many other species (Figure 1). These residues can function as part of a bipartite NLS in pea IAA4 and IAA6 and *Arabidopsis* IAA1, IAA3, IAA5, and IAA9 (Abel and Theologis, 1995). Therefore, the slowed degradation of IAA17(1-111:K31R):LUC:NLS could possibly result from a disruption of normal subcellular targeting. This seems unlikely, first because conservative substitution of an Arg for a Lys would not necessarily interfere with the activity of the bipartite NLS. For instance, the $RR_{(11)}$RLRKK sequence from the TGA-1A protein appears to function as part of a NLS (van der Krol and Chua, 1991). More importantly, however, both the wild-type and K31R fusion proteins possess the same exogenous NLS, suggesting that they should localize to the same subcellular compartment. Attempts to directly demonstrate the subcellular localization of proteins bearing this mutation have not been successful. If the K31R mutant is impaired in some aspect of nuclear localization, further studies would be needed to determine a previously unidentified relationship between auxin levels, Aux/IAA subcellular localization, and Aux/IAA degradation.

The conserved Lys might also act as part of a functional degron if it lies on the surface that interacts with the F-box proteins that target Aux/IAA proteins for degradation. To date, no structures have been determined for any Aux/IAA proteins, but it

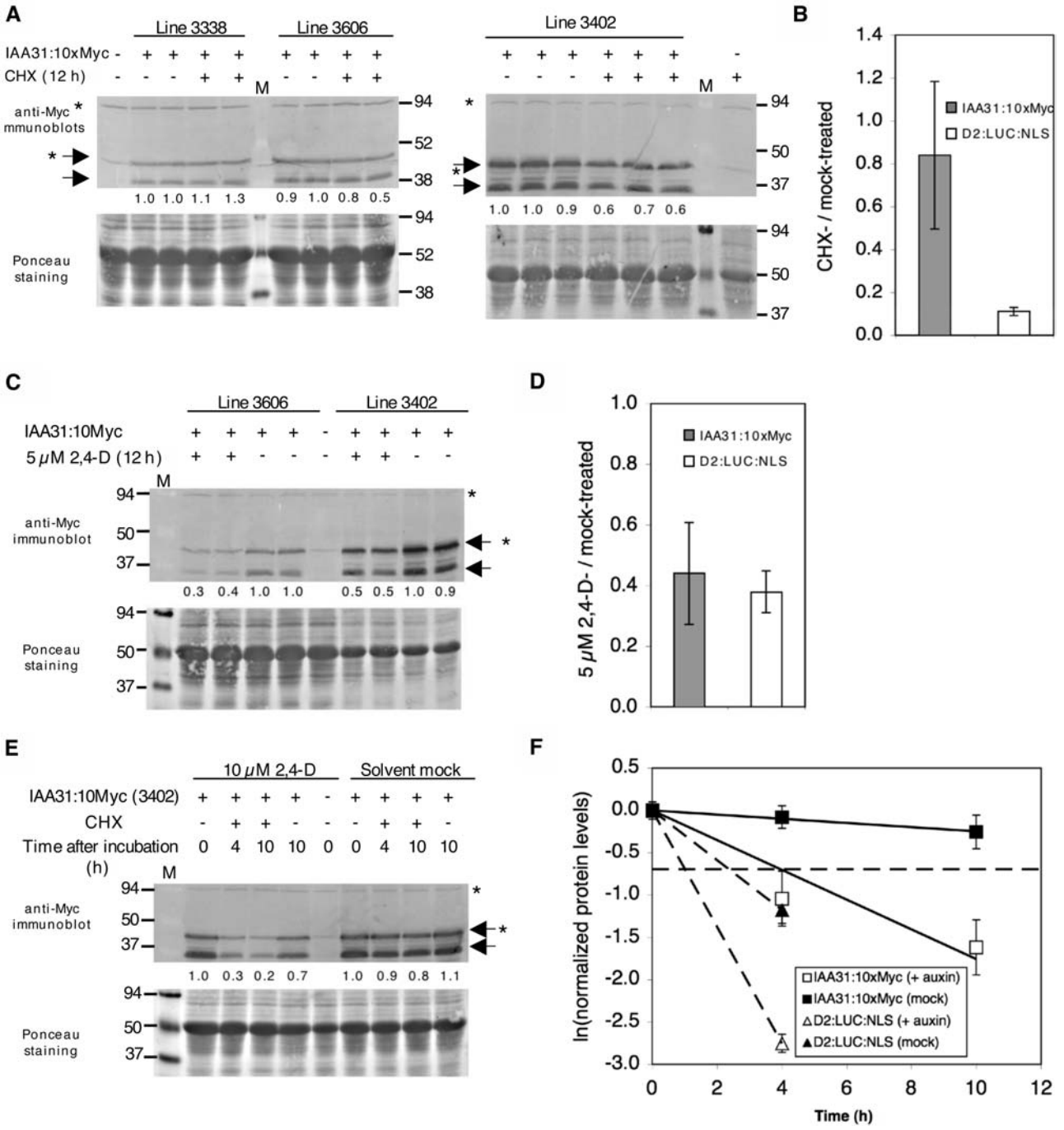

Figure 6. Degradation and Auxin Response of the Noncanonical IAA31 Protein Fused to a Myc Epitope Tag.

(A) and **(B)** IAA31:10xMyc levels remain relatively constant after a 12-h treatment with cycloheximide (CHX).

(A) Immunoblot analysis was performed with anti-Myc antibodies. Arrows point to two forms of the IAA31:10xMyc protein with different mobilities. Asterisks mark nonspecific cross-reacting bands. The upper band was used as a loading control, and the lower band visible in Figure 5 comigrates with IAA31:10xMyc. Results from three independent lines are shown (3338, 3606, and 3402). On the blots for lines 3338 and 3402, the lanes without IAA31:10xMyc contain extract from D2:LUC:NLS seedlings used in parallel to verify cycloheximide effectiveness. Levels of immunoreactive protein were quantified and then normalized to the most intense mock-treated sample for each line; these values are listed below each lane. Both forms of IAA31:10xMyc were included in the analyses. Ponceau S staining shows total protein levels present on the membranes subjected to immunoblotting.

(B) Quantification of the data shown in **(A)** (graphed as in Figure 5F). Data for IAA31:10xMyc are from 3 lines, 7 exp; data for D2:LUC:NLS are from 1 line, 3 exp.

is possible that the basic patch containing the Lys lies in close spatial proximity to domain II in properly folded Aux/IAA proteins. The crystal structure of the nuclear import factor karyopherin-α indicates that it can simultaneously bind the two basic regions separated by 10 amino acids in the prototypical nucleoplasmin bipartite NLS (Conti et al., 1998), and doubling this spacer region through the addition of a polyalanine tract did not disrupt the nuclear localization of a nucleoplasmin fusion protein (Makkerh et al., 1996). These findings suggested that the two basic clusters could still come together in space to interact with a small surface on the nuclear import machinery, despite their separation in the primary amino acid sequence. The same could occur for Aux/IAA proteins, and in fact, basic patches in a bipartite NLS separated by 24 residues (IAA1) to 70 residues (IAA9) all targeted GUS to the nucleus (Abel and Theologis, 1995). So, if the conserved Lys and domain II are in close spatial proximity, both could be required for optimal binding of an F-box protein under basal conditions. However, this Lys appears dispensable for the accelerated rapid degradation observed under high-auxin conditions. Because auxin appears to directly promote interaction between the Aux/IAA proteins and TIR1 family members, it has been hypothesized that auxin could promote a change in the three-dimensional structure of one or both of these binding partners (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Perhaps these changes alter the Aux/IAA-TIR1 binding surface and render Lys-31 unnecessary for rapid proteolysis under high-auxin conditions.

Although change of a Lys to an Arg could disrupt surface interactions, it also eliminates a potential ubiquitination site that could be an essential component of the IAA17(1-111) degron. Although Aux/IAA proteins interact with an SCF-type E3 ubiquitin ligase, no ubiquitination sites have been identified in the Aux/IAA proteins. Clearly, Lys-31 cannot serve as the sole ubiquitination site in IAA17(1-111):LUC:NLS, because the K31R mutant fusion protein can still be degraded. However, it remains possible that Lys-31 serves as a preferred ubiquitination site under low-auxin conditions, such that when Arg is present instead proteolysis slows but is not prevented. The observation that nearly wild-type levels of degradation occur in the Arg-31 mutant under high-auxin conditions suggests that different Lys residues become favored.

Finally, it also remains formally possible that the Lys contributes to the Aux/IAA degron by interacting with some unidentified component of the proteolytic machinery that affects Aux/IAA degradation. Future experiments must be performed to assess its impact on degradation in the context of several full-length family members.

Differential Degradation among Aux/IAA Family Members

Of the 29 Aux/IAA proteins present in *Arabidopsis*, degradation rates for only three family members were previously determined directly in seedlings, either endogenous AXR2/IAA7 or AXR3/IAA17 (Gray et al., 2001; Ouellet et al., 2001) or IAA1 and AXR3/IAA17 sequences in fusion with a marker protein (Worley et al., 2000; Gray et al., 2001; Zenser et al., 2001). With the exception of endogenous IAA17, degradation rates were similarly rapid at ~8 to 12 min. The reasons for the differences in half-lives between endogenous IAA17 reported by Ouellet et al. (2001) and the IAA17:LUC fusion protein reported here are not known.

To determine whether diversity in degradation exists among Aux/IAA proteins, we decided to use uniform systems to test the effect of naturally occurring sequence differences present outside of conserved domains on Aux/IAA degradation. In these experiments, we constitutively expressed Aux/IAA proteins fused to LUC or Myc epitope tags, under the assumption that this would allow us to directly and quantitatively compare their degradation rates in whole *Arabidopsis* seedlings. With this approach, we made several findings that reiterate the importance of domain II and highlight the contribution of residues outside of it.

Twenty-three Aux/IAA proteins possess a complete domain II and a conserved Lys between domain I and domain II. Although these conserved regions could target all of the family members for equally rapid degradation, our results indicate that this is not the case. Within the context of our experimental conditions, different rates of degradation were observed among canonical Aux/IAA proteins fused to LUC, varying from 10 min for IAA1 to ~80 min for IAA28. In addition, varying degrees of auxin-responsiveness were observed. For instance, IAA1:LUC (Zenser et al., 2001) and IAA17(1-111):LUC:NLS degrade approximately twofold faster in the presence of 5 μM 2,4-D. However, the

Figure 6. (continued).

(C) and **(D)** IAA31:10xMyc levels decrease after a 12-h treatment with 5 μM 2,4-D.

(C) Immunoblot analysis was performed with anti-Myc antibodies; labeling is as described for **(A)**. The lane without IAA31:10xMyc contains extract from mock-treated D2:LUC:NLS seedlings used in a parallel control experiment. Results from two independent lines are shown (3606 and 3402), and data from a third line are included in **(D)**. Higher levels of total protein were loaded for line 3606 because it had lower levels of IAA31:10xMyc expression. Bands from two representative experiments were quantified and normalized as described for **(A)**; their values are shown below the immunoblot.

(D) Quantification of the data shown in **(C)** (graphed as in Figure 5G). Data for IAA31:10xMyc are from 3 lines, 4 exp; data for D2:LUC:NLS are from 1 line, 3 exp.

(E) and **(F)** IAA31:10xMyc degrades much more rapidly after a 2-h incubation with 10 μM 2,4-D.

(E) Immunoblot analysis was performed with anti-Myc antibodies; labeling is as described for **(A)**. The lane without IAA31:10xMyc contains extract from mock-treated D2:LUC:NLS seedlings used in a parallel control experiment. Bands were quantified as described for **(A)** and normalized to the 0-h time point for each treatment; their values are shown below each lane.

(F) Quantification of IAA31:10xMyc protein loss over time (graphed as in Figure 2A). For IAA31:10xMyc, normalized protein levels are based on band intensities of immunoreactive proteins, whereas for D2:LUC:NLS, normalized protein levels are based on RLU/μg total protein, as described for Figure 2A. Data for IAA31:10xMyc are from 1 line, 2 exp; data for D2:LUC:NLS are from 1 line, 2 exp.

half-life of IAA28:LUC is nearly fivefold faster than its degradation rate under basal conditions or in the presence of a solvent control, decreasing from ~80 min to ~15 min in the presence of 5 μM 2,4-D. It seems unlikely that differences within domain II can explain these differential proteolytic patterns. For instance, the only difference between domain II in IAA17 and IAA9 is a conservative Val to Ile substitution at position 3, and substitution of this residue to Ala within domain II of a D2:LUC:NLS construct did not increase its accumulation in a transient expression assay (Ramos et al., 2001). Similarly, IAA28 differs from IAA17 at three residues (1, 10, and 13) within domain II, but none of these changes would be predicted to interfere with degradation based on mutagenesis within the context of the D2:LUC:NLS reporter. More specifically, substitution of Ala residues at position 1 and 10 did not affect the accumulation of D2:LUC:NLS, nor did changing the final Lys to the Arg found in IAA28 (Ramos et al., 2001).

If differences within domain II do not cause the differential degradation of Aux/IAA proteins, alternative explanations must be sought. Perhaps misexpression of the fusion proteins outside of their normal biological milieu affects their longevity. If their degradation depends upon tissue- or organ-specific degradation machinery, then the proteins would be long-lived wherever they are ectopically expressed. To date, the only proteolytic factors demonstrated to interact with Aux/IAA proteins are TIR1 and the other AFBs. Based upon MPSS and GENEVESTIGATOR data (Meyers et al., 2004; Zimmermann et al., 2004) and published data for *TIR1* (Gray et al., 1999) and *AFB1*, *AFB2*, and *AFB3* (Dharmasiri et al., 2005b), there are no strong organ- or cell-specific expression patterns for *TIR1* or any of the other *AFB*s, but this does not preclude the existence of organ-specific regulatory proteins. A preliminary attempt was made to determine whether root-specific factors regulated IAA28:LUC degradation, because endogenous IAA28 is predicted to be produced primarily in the root. IAA28:LUC was degraded more slowly than IAA1:LUC even when only root-expressed proteins were measured. If cell-specific factors are required for rapid IAA28 degradation, assays at the whole root level would still not be able to accurately measure biologically relevant IAA28 degradation. However, it seems clear that even if localized factors control IAA28 degradation in the roots under basal conditions, IAA28 may be rapidly degraded throughout the seedling under high-auxin conditions, suggesting that root-specific factors are definitely not required under these circumstances. Nevertheless, further work could reveal the existence of tissue- or organ-specific regulatory components of Aux/IAA degradation.

The different rates of degradation observed among Aux/IAA proteins could also result from the presence of additional residues that form parts of degrons or stabilons outside of domain II. Interestingly, large differences in stability were observed in proteins that differed substantially outside of the conserved domains (e.g., IAA17 and IAA28 are only 26% identical), whereas more closely related family members, such as IAA8 and IAA9 (57% identical), targeted LUC for very similar rates of degradation. If conservation of novel degrons or stabilons is required for the proper function of each family member in vivo, then it would be reasonable to expect to find these residues preserved in paralogs present within *Arabidopsis* and putative orthologs found in other plant species. For instance, the closely related IAA8 and IAA9 proteins also share a high degree of similarity to Aux/IAA proteins found in many other dicot species (Figure 1). Mutations within or deletions of these highly similar regions that are found exclusively in IAA8, IAA9, and their putative orthologs could be used to learn whether they are important determinants of IAA8 and IAA9 degradation.

New Kids on the Auxin Block

The current model for Aux/IAA function relies upon auxin-mediated accelerated degradation of short-lived Aux/IAA proteins. To date, domain II and, as reported here, potentially a conserved Lys have been linked to rapid degradation in canonical Aux/IAA family members. However, these features are wholly or partially missing from the IAA20, IAA30, IAA31, IAA32, IAA33, and IAA34 proteins in *Arabidopsis* (Figure 1). Transcripts have been detected for all of the noncanonical Aux/IAAs except IAA33 (Remington et al., 2004), indicating that they are expressed. And there is also evidence for the presence of noncanonical family members in other plant species (Remington et al., 2004). A putative rice (*Oryza sativa*) ortholog of IAA20/30/31, Os IAA8, that contains domains I, III, and IV but lacks domain II and the conserved KR, appears to be expressed (Jain et al., 2006), and two predicted proteins expressed in aspen (*Populus tremula* × *Populus tremuloides*; GenBank accession numbers AJ306827 and AJ306826) contain the DWPPI found in the domain II–like region of *Arabidopsis* IAA31. It remains an open question whether these proteins transduce auxin signals similar to canonical family members or whether their divergent sequences lead them to perform novel functions in plant growth and development.

Within our experimental system, IAA20 and IAA31 do not possess any novel transferable degrons that can substitute for domain II to render them short-lived in whole seedling assays. This immediately suggests that these proteins occupy a novel niche in the auxin signal transduction cascade. However, even these two long-lived proteins seem unlikely to occupy the same niche, because IAA20 longevity was completely unchanged in response to increased levels of auxin, whereas these same conditions accelerated IAA31 proteolysis.

Before drawing final conclusions about the stability of IAA20 and IAA31 and postulating new regulatory functions for these and related proteins, it is important to note that noncanonical family members, such as IAA20 and IAA31, seem to be transcribed at very low levels in *Arabidopsis* plants, based on microarray data available at GENEVESTIGATOR, and might be preferentially expressed in small subsets of cells. For instance, IAA31 expression may be enhanced specifically in seeds and siliques (Zimmermann et al., 2004). If specific factors required to promote the degradation of noncanonical family members reside in a similarly limited set of cells, additional work will be required to identify these factors and to characterize the novel degrons that they recognize in the noncanonical family members. However, it also remains possible that the loss of domain II and the interdomain basic region from these family members renders them refractory to TIR1-dependent degradation in all organs and cell types and allows them to function as long-lived Aux/IAA family members. Initial characterization of IAA20 and IAA31 indicates that more exploration of noncanonical Aux/IAA degradation is warranted.

For more than a decade, we have known that Aux/IAA proteins vary substantially in their amino acid sequences and transcription profiles. Here, we provide evidence that their degradation profiles vary as well. More experiments have been initiated to understand the biological function of the noncanonical IAA family members, now shown to be long-lived and, in the case of IAA20, unresponsive to auxin. Further analysis of Aux/IAA proteolysis will enhance our understanding of how these proteins manage to regulate numerous aspects of plant growth and development.

METHODS

Plant Material and Growth Conditions

All transgenes were expressed in *Arabidopsis thaliana* ecotype Columbia, and experiments were performed using homozygous T3 or T4 seeds, with one exception. For IAA17(1-111:K31R):LUC:NLS, T3 seeds from one line bearing two insertions were used, even though the seedlings were not necessarily homozygous at both loci. Seeds were surfaced-sterilized and grown for 6 to 8 d under continuous light in 1 mL of liquid growth medium (GM) (4.3 g/L Murashige and Skoog basal salts [Sigma-Aldrich], 1% sucrose, 2.5 mM MES, and 1× B vitamin, pH 5.7). For some experiments, seedlings were stratified at 4°C for 1 to 3 d. Liquid medium was replaced the day before each experiment, or on the day of the experiment when overnight incubations were performed.

Half-Life and Auxin Response Experiments

A mock solution of GM or 10× cycloheximide (Sigma-Aldrich) dissolved in GM was added to each plate of seedlings to a final concentration of 200 μg/mL for standard cycloheximide experiments. For auxin treatments, 2,4-D (Sigma-Aldrich) dissolved in filter-sterilized 0.1 M KOH and diluted using GM was added to a final concentration of 5, 10, or 25 μM 2,4-D, or an equivalent amount of 0.1 M KOH diluted in GM was added as a mock treatment. Cycloheximide was dissolved in an appropriate concentration of 2,4-D in GM for half-life experiments after auxin treatments. For 30-min, 3-h, or 12-h cycloheximide assays, comparable plates of seedlings were incubated with 200 μg/mL cycloheximide or GM for the indicated periods. For assays using roots, the treatment was applied to whole seedlings in liquid culture, and the roots were sliced off using a razor before collection. A comparable procedure was performed for auxin response assays in whole seedlings, except that the two sets of plates were incubated with a set amount of 2,4-D or a mock 0.1 M KOH solvent control. For half-life experiments, seedlings were incubated with cycloheximide for the times indicated in the figures. A 0-h time point was generated by incubating seedlings with a mock GM treatment for an amount of time equivalent to the shortest cycloheximide treatment. For half-life experiments using LUC fusion proteins with an auxin pretreatment, the 0-h time point was generated by incubating seedlings with a mock 5 μM 2,4-D treatment for an amount of time equivalent to the shortest cycloheximide + 5 μM 2,4-D treatment. Control half-life experiments performed using a mock 0.1 M KOH solution in place of auxin indicated that the solvent alone did not affect Aux/IAA:LUC degradation rates (see Supplemental Figure 5 online). To measure the half-life of IAA31:10xMyc, all samples were treated with 10 μM 2,4-D or an equivalent amount of the 0.1 M KOH solvent for 2 h. Some samples were harvested at this time. Additional samples were treated with 200 μg/mL cycloheximide dissolved in 10 μM 2,4-D or 0.1 M KOH solution for an additional 4 or 10 h. A final set of samples were treated solely with the auxin or KOH solution for 10 h after the 2-h incubation. For all experiments performed to analyze the degradation or auxin response of Myc-tagged proteins, comparable control experiments were performed using seedlings expressing D2:LUC:NLS (Zenser et al., 2001), and losses of LUC per microgram of total protein were measured to ensure the efficacy of the cycloheximide and auxin applications. After treatment in all of these experiments, samples were frozen in liquid nitrogen and ground in LUC extraction buffer (Ramos et al., 2001). The supernatant was harvested and used for subsequent LUC assays or protein gel blot analysis. Total protein was measured using a Bradford assay (Bio-Rad). In the figure legends, the term ''experiment'' is used to describe an assay performed on one independent transgenic line on a specific date; therefore, multiple lines analyzed on the same day and compared with the same control are counted as separate experiments.

LUC Assays

The Microlumat LB 96 P luminometer (EG&G Berthold Instruments) was used to inject 100 μL of LUC assay buffer (25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 7 mM β-mercaptoethanol, 1 mg/mL BSA, and 5 mM ATP) and 40 μL of 0.5 mM D-luciferin (BD PharMingen) into a sample of plant extract and then immediately measure total RLU emitted over an 8-s interval. At least three measurements were performed for each sample.

Half-Life Calculations

RLU/μg total protein was calculated for each sample. An average RLU/μg total protein value was calculated for all of the mock-treated samples. The RLU/μg total protein for each sample was then divided by the average for the mock-treated samples to generate a normalized RLU/μg total protein value. To display the data using a linear scale, the natural log of the normalized RLU/μg total protein for each sample was determined and plotted on the *y* axis versus time on the *x* axis. Values obtained for mock-treated samples were plotted at time 0. The equation from the linear best-fit line that passes through the origin was used to calculate the half-life based on the equation ln(normalized RLU/μg total protein) = *m*(time), where *m* is the slope. To find the half-life when half of the original protein is remaining, ln(0.5) was divided by the slope of the degradation curve. The heavy dashed line on the half-life graphs is plotted at the value of ln(0.5), −0.693. This line intersects the degradation curve at half-life. This same protocol was used to estimate an approximate half-life in the 30-min cycloheximide assays. Ninety-five percent confidence intervals for half-lives were determined by linear regression analysis using STATA (release 7.0, 2001; Stata Corporation). A similar method was used to calculate an approximate half-life for IAA31:10xMyc, but normalized band intensity was substituted for normalized RLU/μg total protein.

Protein Separation and Immunoblotting

Plant extracts were mixed with 5× loading sample buffer (Laemmli), boiled, and separated using SDS-PAGE. Equal amounts of total protein were loaded in each lane. Proteins were transferred to an Immobilon-P polyvinylidene difluoride transfer membrane (Millipore) and detected using polyclonal anti-LUC antibodies (Cortex Biochem) or monoclonal anti-c-Myc antibodies (Roche) followed by horseradish peroxidase–coupled secondary antibodies. Proteins were visualized using the ECL plus Western Blotting Detection system (Amersham Biosciences) and the Storm PhosphorImager system (Molecular Dynamics). AlphaEaseFC software, version 3.1.2, was used to quantify the intensity of protein bands visible on immunoblots (Alpha Innotech). Immunoblots were stained with Ponceau S reagent (Sigma-Aldrich) to examine protein loading and transfer efficiency.

Molecular Techniques

Standard digestion and ligation techniques were used to generate all clones for the expression of LUC fusion proteins (Sambrook et al., 1989). Quikchange site-directed mutagenesis was used to introduce a silent codon change to eliminate an internal *Nco*I site in an *IAA17* cDNA and to

eliminate domain I and change the KR residues in the IAA17(1-111) coding region according to the manufacturer's instructions (Stratagene). The mutagenized *IAA17* coding region, flanked by *Kpn*I and *Nco*I sites, was inserted downstream of a *UBQ10* 5′ flanking region and upstream of a LUC coding sequence followed by a polyadenylation sequence from the *Agrobacterium tumefaciens* nopaline synthase gene in a pBIN19-based plant transformation vector, as described previously (Worley et al., 2000). IAA17(1-111) wild-type and mutant fragments were placed into a similar binary transformation vector that possessed an NLS downstream of the LUC coding sequence (Worley et al., 2000). This whole cassette, flanked by *Hin*dIII and *Not*I sites, was then inserted into the pGreenI 0029 binary plant transformation vector (Hellens et al., 2000). The coding sequences for IAA8, IAA9, IAA20, IAA31 (ABRC), and IAA28 (a gift from B. Bartel) were amplified by PCR to add restriction sites for cloning for LUC fusion proteins, or to add Gateway attB sequences for Myc-tagged proteins. An IAA20 fragment was ligated into the pBIN19-based Pro_{UBQ10}:*LUC*:*nos* cassette. Site-directed mutagenesis was used to remove an internal *Nco*I site in IAA8 and to correct a PCR-induced error in IAA9. IAA8, IAA9, and IAA28 fragments bearing only silent mutations were subsequently ligated into the Pro_{UBQ10}:*LUC*:*nos* cassette in pGreenI 0029 or a modified version of pGreenI 0029 with an altered multiple cloning site. IAA20 and IAA31 flanked by attB sites were recombined into the pDONR201 Gateway entry vector (Invitrogen). The stop codon was eliminated in constructs used for C-terminal fusions. The IAA20 and IAA31 coding regions were then recombined through Gateway cloning (Invitrogen) into the pGWB 17, 20, and 21 binary transformation vectors (a gift from T. Nakagawa) to allow the expression of 4xMyc- and 10xMyc-tagged transcripts under the control of the cauliflower mosaic virus *35S* promoter. The 10xMyc:IAA20 construct possesses a TEV protease cleavage site (ENLYFQS) between the tag and the IAA20 protein that was incorporated during PCR amplification. Construction of the vectors for fluorescent fusion proteins is described in Supplemental Figure 4 online. All constructs were introduced into *Arabidopsis* plants, ecotype Columbia, using the floral dip method (Clough and Bent, 1998). Kanamycin-resistant seedlings that segregated 3:1 in the T2 or T3 generation were propagated to homozygosity with the one exception noted above. The identities of transgenes in planta were confirmed by PCR amplification of genomic DNA followed by diagnostic digests or automated sequencing.

In Silico Analysis

Expression patterns for Aux/IAA family members, TIR1, and other AFB family members were examined at the MPSS (http://mpss.udel.edu/) (Meyers et al., 2004) and GENEVESTIGATOR (https://www.genevestigator.ethz.ch/) (Zimmermann et al., 2004) websites. The subcellular localization for IAA20 was predicted by the TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/).

Phylogenetic Analysis

All sequences for *Arabidopsis* Aux/IAA genes listed by Remington et al. (2004) were obtained from TAIR, and AtNgxxxxx.1 gene models were used. Sequences for Aux/IAA proteins from other organisms were obtained from GenBank. Sequences were aligned using ClustalX (version 1.8) with default parameters followed by manual editing in MacClade 4.05 OS X (Sinauer Associates). Percentage amino acid identity values were calculated using AlignX in the Vector NTI 7 version 10.3.7 suite (Invitrogen) on the same alignments, or comparable alignments of the full-length proteins.

Microscopic Analyses

Light-grown *Arabidopsis* seedlings expressing fluorescent fusion proteins were treated with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) in PBS to stain nuclei and examined using a Zeiss Axioskop2 plus fluorescence microscope (Carl Zeiss MicroImaging). Additional details are provided with Supplemental Figure 1 online.

Accession Numbers

Sequence data from this article can be found at TAIR under the following accession numbers: IAA1 (AT4G14560.1), IAA2 (AT3G23030.1), IAA3 (AT1G04240.1), IAA4 (AT5G43700.1), IAA5 (AT1G15580.19), IAA6 (AT1G52830.1), IAA7 (AT3G23050.1), IAA8 (AT2G22670.1), IAA9 (AT5G65670.1), IAA10 (AT1G04100.1), IAA11 (AT4G28640.1), IAA12 (AT1G04550.1), IAA13 (AT2G33310.1), IAA14 (AT4G14550.1), IAA15 (AT1G80390.1), IAA16 (AT3G04730.1), IAA17 (AT1G04250.1), IAA18 (AT1G51950.1), IAA19 (AT3G15540.1), IAA20 (AT2G46990.1), IAA26 (AT3G16500.1), IAA27 (AT4G29080.1), IAA28 (AT5G25890.1), IAA29 (AT4G32280.1), IAA30 (AT3G62100.1), IAA31 (AT3G17600.1), IAA32 (AT2G01200.1), IAA33 (AT5G57420.1), and IAA34 (At1g15050.1), or at GenBank/EMBL under the following accession numbers: Gm IAAx (AAB70005.1), Ze IAAx (AAM12952.1), Vv IAAx (AAL92850.1), Pt IAAx (CAC84706.1), St IAAx (AAM29182.1), Le IAAx (CAI77628.1), Cs IAAx (BAA85821.1), Nt IAAx (CAD10639.1), Ps IAA6 (CAA48300), and Ps IAA4/5 (CAA48297).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Mutations in Conserved Basic Residues Outside of Domain II Affect the Auxin-Responsiveness of an IAA17(1-111):LUC:NLS Protein.

Supplemental Figure 2. Full-Length IAA20:LUC Is Long-Lived and Not Responsive to Auxin.

Supplemental Figure 3. IAA20:4xMyc Is Long-Lived, and Its Levels Do Not Decrease in Response to Auxin.

Supplemental Figure 4. Subcellular Localization of GFP:IAA20.

Supplemental Figure 5. Mock Solvent Control for Auxin Treatment Does Not Affect Aux/IAA:LUC Degradation.

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