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RAC GTPases in Tobacco and Arabidopsis Mediate Auxin-Induced Formation of Proteolytically Active Nuclear Protein Bodies That Contain AUX/IAA Proteins [W]

Li-zhen Tao,[a] Alice Y. Cheung,[a,b,c,1] Candida Nibau,[a,c] and Hen-ming Wu[a,b]

[a] Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003
[b] Molecular and Cellular Biology Program, University of Massachusetts, Amherst, Massachusetts 01003
[c] Plant Biology Graduate Program, University of Massachusetts, Amherst, Massachusetts 01003

Auxin signaling relies on ubiquitin ligase SCF^{TIR1}-mediated 26S proteasome-dependent proteolysis of a large family of short-lived transcription regulators, auxin/indole acetic acid (Aux/IAA), resulting in the derepression of auxin-responsive genes. We have shown previously that a subset of Rac GTPases is activated by auxin, and they in turn stimulate auxin-responsive gene expression. We show here that increasing Rac signaling activity promotes Aux/IAA degradation, whereas downregulating that activity results in the reduction of auxin-accelerated Aux/IAA proteolysis. Observations reported here reveal a novel function for these Rac GTPases as regulators for ubiquitin/26S proteasome-mediated proteolysis and further consolidate their role in auxin signaling. Moreover, our study reveals a cellular process whereby auxin induces and Rac GTPases mediate the recruitment of nucleoplasmic Aux/IAAs into proteolytically active nuclear protein bodies, into which components of the SCF^{TIR1}, COP9 signalosome, and 26S proteasome are also recruited.

INTRODUCTION

Auxin is critical for multiple cellular processes in plants, including cell division, growth, and differentiation, and its action underlies developmental processes ranging from embryogenesis to reproduction. Extensive genetic, molecular, and biochemical studies have established that auxin promotes E3 ligase SCF^{TIR1}– and 26S proteasome–mediated proteolysis of a large family of transcription regulators, auxin/indole acetic acid (Aux/IAA) proteins, thereby allowing the activation of auxin-responsive gene expression by auxin response transcription factors (Rogg and Bartel, 2001; Hagen and Guilfoyle, 2002; Hellmann and Estelle, 2002; Moon et al., 2004). Aux/IAAs act as repressors for auxin-inducible gene expression (Ulmasov et al., 1997; Tiwari et al., 2001), but they themselves are rapidly induced by auxin (Theologis et al., 1985). However, Aux/IAAs are among the most short-lived proteins in eukaryotes and have not been detected on protein blots by highly reactive antibodies, even under auxin-induced conditions (Abel et al., 1994; Oeller and Theologis, 1995). Auxin has been shown to enhance the interaction between SCF^{TIR1} and Aux/IAAs in seedlings (Gray et al., 2001). Recently, the auxin-enhanced TIR1–Aux/IAA interaction was observed in cell-free protein extracts (Dharmasiri et al., 2003; Kepinski and Leyser, 2004). This in vitro interaction involves auxin binding to SCF^{TIR1}, leading to the identification of TIR1 as an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Nevertheless, the mechanisms that relay auxin stimulation to the ubiquitination/26S proteasome machinery remain to be defined. Moreover, given the broad spectrum of auxin-induced responses, it is probable that multiple reception systems are used in auxin signaling in vivo (Rogg and Bartel, 2001).

Intracellularly, regulators for the ubiquitination/26S proteasome pathway significantly affect auxin signaling (Hellmann and Estelle, 2002; Moon et al., 2004). The COP9 signalosome (CSN), a highly conserved eight-subunit protein complex homologous with the lid subcomplex of the regulatory particle of the 26S proteasome, also critically affects auxin response (Schwechheimer et al., 2001; Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003). Phospho-regulatory mechanisms are also likely to play important auxin signaling roles (DeLong et al., 2002), and different mitogen-activated protein kinase cascades have been associated with regulating auxin response (Mizoguchi et al., 1994; Mockaitis and Howell, 2000; Kovtun et al., 1998, 2000). How various auxin signaling regulators interact with each other to mediate regulated Aux/IAA degradation remains to be determined.

Plant Rac-like GTPases are highly conserved across different species and together constitute a unique subfamily of the Rho family of GTPases (Gu et al., 2004). In *Arabidopsis thaliana*, they are encoded by at least 11 genes, *AtRac1* to *AtRac11* (Arabidopsis Genome Initiative, 2000; Winge et al., 2000) or *Rop1* to *Rop11* (Yang, 2002). Except for a high level of expression in pollen for a few of the AtRacs (Pina et al., 2005), mRNAs for these genes are constitutively present at low levels in all organs and throughout development (Schmid et al., 2005; see http://www.cbs.umn.edu/arabidopsis/). A clear revelation of the functional roles for individual Rac GTPases through analysis of single gene

[1] To whom correspondence should be addressed. E-mail acheung@biochem.umass.edu; fax 413-545-3291.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Alice Y. Cheung (acheung@biochem.umass.edu).
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knockout mutants has been rare, and mutants usually have subtle but pleiotropic phenotypes (Zheng et al., 2002; Arthur et al., 2003; C. Nibau, A.Y. Cheung, and H.M. Wu, unpublished data). This is probably attributable to functional redundancy among the coexpressed Rac GTPases and the multifunctional nature of these proteins. Nevertheless, functional analysis of plant Racs, like other Ras-related GTPases, has taken considerable advantage of the facts that the activity of these small GTP binding proteins is modulated by their shuttling between the GDP-bound inactive form and the GTP-bound active form and that this equilibrium can be easily manipulated. Negative regulatory molecules, guanine dissociation factors, and GTPase-activating proteins maintain these small GTPases in their GDP-bound inactive form, whereas positive regulators, such as guanine exchange factors in mammalian cells, shift the equilibrium to the activated GTP-bound form (LaMarche and Hall, 1994; Bar-Sagi and Hall, 2000; Etienne-Manneville and Hall, 2002). Constitutive active (CA) and dominant negative (DN) mutations at conserved positions in Ras-related GTPases render the mutant proteins predominantly in the GTP-bound state or locked in the GDP-bound state, respectively. Expression of these CA or DN mutant GTPases from transgenes perturbs endogenous Rac signaling, providing insights into the functional roles of the wild-type proteins. Accumulating evidence now suggests that plant Rac GTPases regulate a large number of intracellular signaling pathways, affecting growth, development, and interactions with the environment (Gu et al., 2004).

The involvement of Rac GTPases in several hormone signaling pathways has been suggested by studies in Arabidopsis that expressed CA or DN forms of these proteins (Gu et al., 2004). Selected Rac GTPases, such as AtRac1 (Lemichez et al., 2001) and Rop10 (Zheng et al., 2002), downregulate abscisic acid signaling. Moreover, abscisic acid apparently inactivates some Arabidopsis Rac GTPases (Lemichez et al., 2001) but represses the expression of Rop10 in root tips (Zheng et al., 2002). We have observed that increasing Rac signaling capacity by expressing a CA form of a tobacco (*Nicotiana tabacum*) Rac GTPase, NtRac1, and several AtRacs stimulated auxin-responsive gene expression (Tao et al., 2002). On the other hand, expressing DN forms of NtRac1 or negative regulators for Rac activity blocked the activation of auxin-induced expression of these genes, indicating that auxin signaling is dependent on normal Rac signaling activity. Moreover, Rac GTPases in seedlings are rapidly activated by auxin with a time frame compatible for mediating early auxin-inducible gene expression. Furthermore, transgenic seedlings with upregulated or downregulated Rac GTPase signaling activity, although pleiotropic, display some phenotypes closely mimicking those seen in mutants defective in auxin signaling. These observations suggest that a subset of Rac GTPases function as molecular links to mediate the auxin signal to downstream responsive gene expression.

The studies described here show that Rac GTPases that upregulate auxin-responsive gene expression are active in stimulating the 26S proteasome–dependent proteolysis of luciferase (LUC)- or green fluorescent protein (GFP)–tagged Aux/IAAs even in the absence of auxin, whereas downregulating Rac GTPase activity suppresses the auxin-accelerated proteolysis of these repressors. Microscopic observations reveal that auxin stimulates the recruitment of GFP-labeled Aux/IAAs from the nucleoplasm into protein particles that we refer to as nuclear protein bodies (NPBs) within which these substrates are degraded. Moreover, components of SCF^{TIR1}, CSN, and the 26S proteasome also are recruited into these NPBs. Rac GTPases likewise stimulate the formation of these NPBs in the absence of auxin, whereas their downregulation counteracts the auxin-induced response. Therefore, Rac GTPases that upregulate auxin-responsive gene expression apparently do so by mediating the auxin signal to Aux/IAA degradation in a process that involves regulated assembly of the ubiquitination/26S proteasome apparatus.

RESULTS

Aux/IAA Proteins Suppress Rac-Induced Auxin-Responsive Gene Expression

Increasing the level of Aux/IAA repressors by expression from transgenes suppresses auxin-induced gene expression (Tiwari et al., 2001). Using transfected protoplasts, a system used extensively in the study of auxin-regulated repressor proteolysis and gene expression as well as other signaling pathways (Ulmasov et al., 1999; Worley et al., 2000; Sheen, 2001; Tiwari et al., 2001), we observed that the expression of representative Aux/IAAs, IAA7 and IAA17, suppressed the NtRac1-stimulated expression of auxin-responsive DR5-GUS (Ulmasov et al., 1997) (Figure 1B), similar to how they suppressed auxin-induced gene expression (Figure 1A) (Tiwari et al., 2001). Moreover, the expression of proteolytically stable mutant forms of these repressors, IAA7(P88L) and IAA17(P88L) (Tiwari et al., 2001; Zenser et al., 2001), conferred further reduction in the ability of NtRac1 to stimulate DR5-GUS expression (Figure 1B). It seemed plausible that the Rac GTPase-stimulated auxin-responsive gene expression was mediated by the ability of these small GTPases to promote Aux/IAA protein degradation, thus serving as integral components for auxin-signaled gene derepression via regulated proteolysis.

Rac GTPases Stimulate 26S Proteasome–Dependent Degradation of Aux/IAA Proteins

To investigate whether Rac GTPases were indeed intermediaries for auxin-induced Aux/IAA degradation, we explored whether they were capable of activating the 26S proteasome–dependent proteolysis of these repressors. We adopted the use of LUC-Aux/IAA fusion proteins as reporters for Aux/IAA stability (Tiwari et al., 2001; Zenser et al., 2001, 2003) and examined whether Rac GTPases could effect the degradation of IAA7-LUC and IAA17-LUC, two prevalently used representatives for Aux/IAAs. Figures 1C and 1D show that coexpressing wild-type or CA NtRac1 significantly reduced the level of IAA-LUC in auxin-free medium and augmented its auxin-induced reduction in auxin-supplemented medium. Moreover, when included in the transfected protoplast cultures, the 26S proteasome inhibitor MG132 reduced the extent of NtRac1(CA)–induced decline in IAA17-LUC (Figure 1F), similar to its ability to suppress the auxin-induced degradation of IAA-LUCs (Ramos et al., 2001). Because results based on IAA7 and

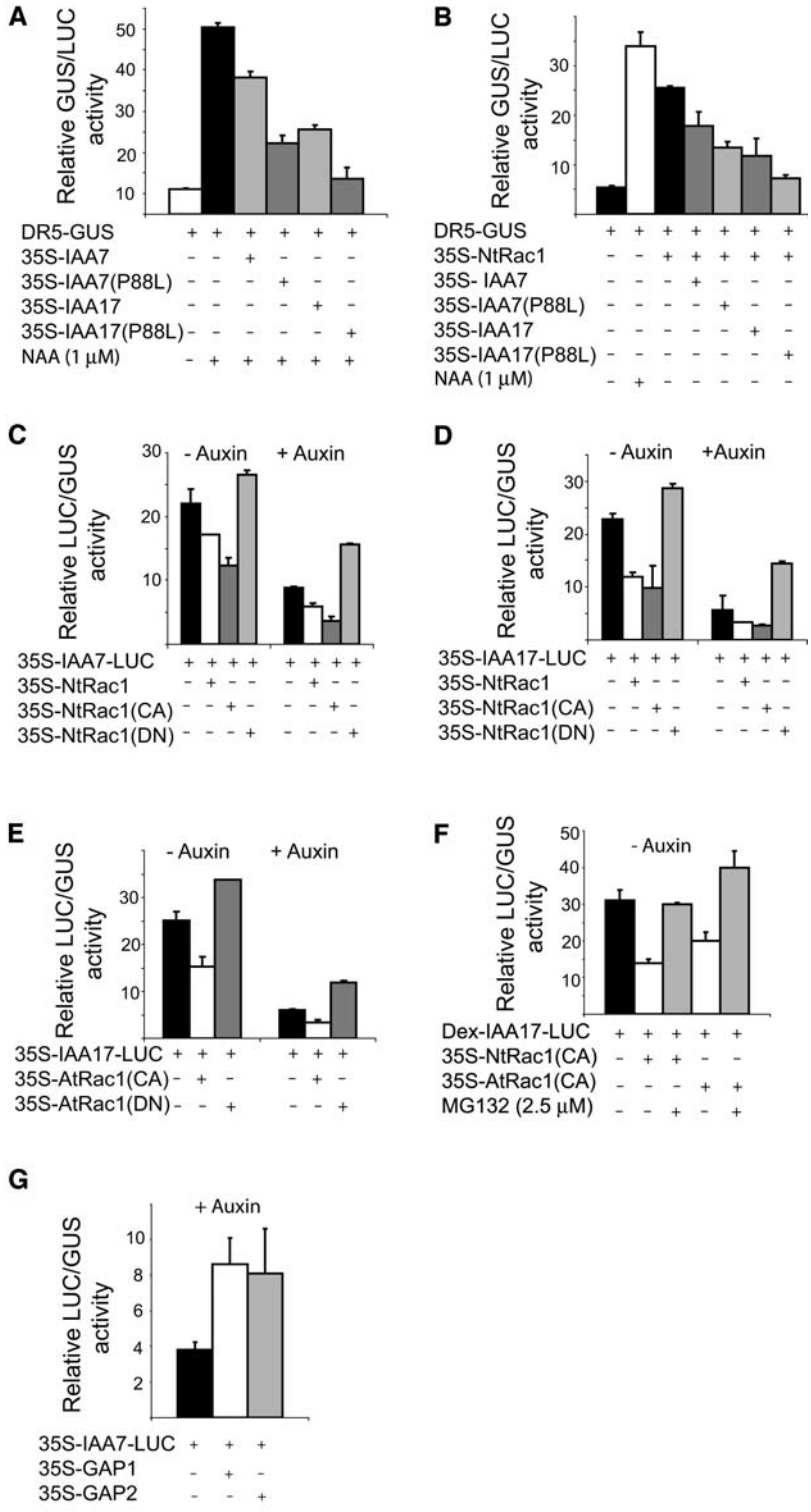

Figure 1. Rac GTPases Mediate Auxin-Induced Degradation of Aux/IAA Proteins.

Protoplasts were transformed by combinations of reporter and effector genes as indicated beneath each data bar. Effector genes NtRac1, AtRac1, and their CA and DN mutant variants were expressed from either the CaMV35S (35S) or a Dex-inducible (Dex) promoter (see Supplemental Figure 2 online). DR5-GUS (**[A]** and **[B]**) was a broadly used auxin-response reporter gene (Ulmasov et al., 1997; Tiwari et al., 2001). IAA7-LUC and IAA17-LUC (**[C]** to **[G]**) were commonly used reporter proteins for Aux/IAA stability (Ramos et al., 2001; Tiwari et al., 2001; Zenser et al., 2001, 2003). GUS and LUC

IAA17 were qualitatively similar in these assays and others described below, results based on IAA17 reporters will be shown predominantly in this report.

Similar to NtRac1, Arabidopsis Rac GTPases that are active in stimulating auxin-responsive gene expression (Tao et al., 2002) also stimulated the accelerated decline of IAA17-LUC (see Supplemental Figure 1A online). When one of these Rac GTPases, AtRac1, was examined in more detail, we observed that expression of AtRac1(CA) also stimulated IAA17-LUC degradation in the absence of exogenous auxin and augmented the auxin-accelerated response (Figure 1E). The AtRac1(CA)-stimulated repressor degradation was also sensitive to inhibition by MG132 (Figure 1F). Together, these observations suggest a signal, regulator, and response relationship for auxin, Rac GTPases, and 26S proteasome–dependent Aux/IAA degradation, which then leads to the derepression of auxin-responsive genes.

Downregulation of Rac GTPases Suppresses Auxin-Induced Aux/IAA Degradation

Constitutively GDP-bound forms of small GTPases interfere with endogenous signaling capacity and induce DN effects on downstream pathways (Bar-Sagi and Hall, 2000; Etienne-Manneville and Hall, 2002). Coexpression of either NtRac1(DN) or AtRac1(DN) efficiently suppressed the auxin-induced decline of IAA7-LUC and IAA17-LUC in transfected protoplasts (Figures 1C to 1E). Likewise, but in the absence of transgene-expressed Rac GTPases, the expression of GTPase-activating proteins to downregulate Rac signaling counteracted the ability of auxin to accelerate IAA17-LUC degradation (Figure 1G). These observations strongly support the notion that auxin-induced Aux/IAA degradation is dependent on properly regulated Rac signaling activity. Moreover, downregulation of Rac signaling capacity reproducibly enhanced IAA7-LUC and IAA17-LUC activity in transfected protoplasts maintained in the absence of exogenous auxin (Figures 1C to 1E, auxin panels). This suggests that the degradation of Aux/IAAs under endogenous auxin conditions is also dependent on Rac GTPase signaling. That downregulating Rac GTPases counteracts auxin action provides strong evidence for these small GTP binding proteins to be integral components of the auxin-activated 26S proteasome-dependent Aux/IAA proteolysis pathway.

Auxin Regulates the Degradation of GFP-Tagged Aux/IAAs

Rather than just a homogenous collection of soluble components, specific structural domains are known to be assembled and disassembled in the eukaryotic nucleus in response to demands by different nuclear processes (Misteli, 2001, 2005). As auxin-stimulated proteolysis of Aux/IAAs depends on the interaction of several multimeric protein complexes, SCF^{TIR1}, CSN, and the 26S proteasome (Cope and Deshaies, 2003; Serino and Deng, 2003; Moon et al., 2004; Smalle and Vierstra, 2004), we were interested in examining whether the auxin-induced process involves nuclear events that could be observed in live cells. To accomplish this, we expressed GFP-labeled Aux/IAA proteins via a dexamethasone (Dex)-regulated promoter (Yanagisawa et al., 2003) (see Supplemental Figure 2B online) in transfected protoplasts so that their fate could be monitored microscopically. Among Dex-IAA17-GFP–transfected protoplast cultures, green fluorescence was readily detected in ~60 to 75% of all cells after ~4 to 5 h of Dex induction and in the absence of exogenous auxin (Figure 2B, data bar at left). The IAA17-GFP signal accumulated predominantly in the nuclei (Figure 2A), consistent with Aux/IAAs being nuclear proteins and transcription regulators (Abel and Theologis, 1995). After Dex withdrawal to minimize continued IAA17-GFP expression and 45 min of incubation in auxin-free medium, the green fluorescence levels in most cells diminished only slightly, reflecting the turnover of IAA17-GFP under endogenous auxin conditions. Typically, ~50% of all cells still maintained observable levels of IAA17-GFP (i.e., they were IAA17-GFP–positive) (Figures 2A and 2B, 0 μM naphthalene acetic [NAA] data). The addition of auxin to the medium accelerated IAA17-GFP decline in a concentration-dependent manner, reducing the level of IAA17-GFP–positive cells considerably within the same time frame (Figures 2A and 2B). Analogous to its effect on auxin-induced (Ramos et al., 2001) and Rac GTPase–mediated (Figure 1F) IAA17-LUC protein degradation, MG132 also suppressed the auxin-accelerated decline in IAA17-GFP–positive cells (data not shown; see Figure 4B below). Moreover, cells expressing the proteolytically stable IAA17(P88L)-GFP fusion proteins were not appreciably affected by auxin treatment (data not shown; see Figure 4D below). Similar to how they affected IAA7-LUC and IAA17-LUC stability (Figures 1C to 1E), coexpression of NtRac1(CA)

Figure 1. (continued).

activities were reported as a ratio of reporter gene expression to the expression level of an internal reference gene, 35S-LUC or Ubi10-GUS, respectively. Unless stated otherwise, + Auxin indicates that protoplasts were incubated in the presence of 1 μM NAA after transfection.

(A) and **(B)** Effect of IAA7, IAA17, and their proteolytically stable P88L variants on auxin-induced **(A)** (see also Tiwari et al., 2001) and NtRac1-induced **(B)** DR5-GUS expression.

(C) and **(D)** Effect of NtRac1, NtRac1(CA), and NtRac1(DN) on IAA7-LUC **(C)** and IAA17-LUC **(D)** activity.

(E) Effect of AtRac1(CA) and AtRac1(DN) on IAA17-LUC activity.

(F) Effect of the 26S proteasome inhibitor MG132 on NtRac1(CA)- and AtRac1(CA)-stimulated IAA17-LUC degradation. Auxin-stimulated Aux/IAA degradation was similarly suppressed by MG132 (Ramos et al., 2001).

(G) Effect of the Rac-negative regulators GAP1 and GAP2 (for GTPase-activating protein) on the auxin-induced degradation of IAA17-LUC.

Tobacco and Arabidopsis protoplasts were used in **(A)** to **(D)** and **(E)** to **(G)**, respectively. Throughout these and subsequent studies, IAA7 and IAA17 proteins, NtRac1, and AtRac1 produced similar results in both Arabidopsis and tobacco protoplasts. Data presented are averages from triplicate samples in one experiment; where no error bar is shown, SD was negligible. Each experiment was repeated at least three times with comparable results.

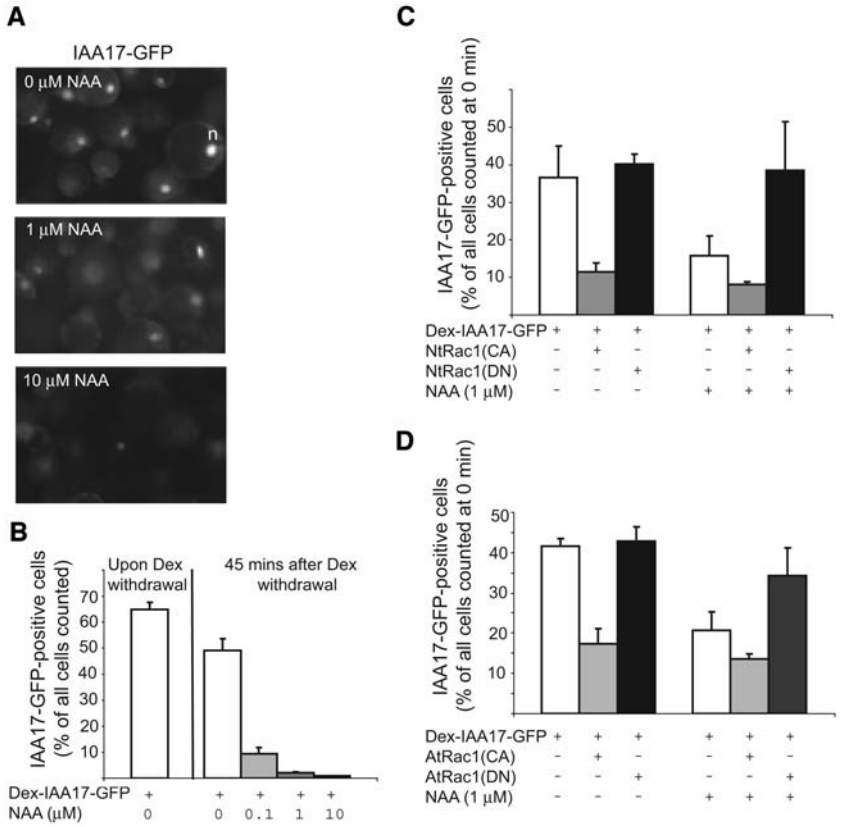

Figure 2. Auxin Signals and Rac GTPases Mediate Accelerated Degradation of Aux/IAA-GFP.

Protoplasts were transfected by Dex-IAA17-GFP (**[A]** and **[B]**), cotransfected by Dex-IAA17-GFP and 35S-NtRac1(CA) or 35S-NtRac1(DN) **(C)**, or cotransfected by Dex-IAA17-GFP and 35S-AtRac1(CA) or 35S-AtRac1(DN) **(D)**.

(A) Epifluorescence images of tobacco protoplasts after Dex-induced IAA17-GFP expression followed by 45 min of incubation in 0, 1, or 10 μM NAA-containing medium. n, nucleus.

(B) Decline profile of the level of IAA17-GFP–positive tobacco protoplasts upon auxin treatment. The data bar at left indicates the level of IAA17-GFP–positive cells upon Dex withdrawal. The other data bars show results from control (0 μM) and auxin-treated (0.1, 1, or 10 μM NAA) cultures at 45 min after Dex withdrawal. Triplicate aliquots of ~200 protoplasts from each sample were counted for the presence of nuclear GFP signal using a ×10 objective. The average percentages of IAA17-GFP–positive cells are presented, with 100% representing total protoplasts counted.

(C) Effect of NtRac1(CA) and NtRac1(DN) on IAA17-GFP in tobacco protoplasts cotransfected by Dex-IAA17-GFP and one of the 35S-NtRac1 mutants.

(D) Effect of AtRac1(CA) and AtRac1(DN) on IAA17-GFP in Arabidopsis protoplasts cotransfected by Dex-IAA17-GFP and one of the 35S-AtRac1 mutants.

Observations in **(C)** and **(D)** were made as described above for **(B)** except that they, and subsequent experiments based on the Dex system, were performed at 30 min after inducer withdrawal, when the residual level of IAA17-GFP–positive cells was higher in the auxin-treated cultures, permitting sampling of large numbers of cells.

(Figure 2C, gray bars) and AtRac1(CA) (Figure 2D, gray bars) also promoted the decline of IAA17-GFP–positive cells in auxin-free medium and augmented the auxin effect when it was provided exogenously. On the other hand, coexpressing their DN variants counteracted the auxin-induced decline in the level of IAA17-GFP–positive cells (Figures 2C and 2D, black bars). Similar observations were made using IAA7-GFP as substrate (data not shown; see Figure 4A below and Supplemental Figure 1B online). Therefore, both N- and C-terminally labeled Aux/IAA proteins are convenient reporters for the auxin-regulated Aux/IAA response in live cells.

Auxin Induces the Formation of Aux/IAA-Containing NPBs

When IAA17-GFP–expressing protoplasts were observed under high magnification, we noticed that auxin not only accelerated the decline of the overall IAA17-GFP signal, it also induced a signal relocalization within the nucleus. In contrast with the diffuse nucleoplasmic signal seen when cells were cultured in auxin-free medium (Figure 3A, top panel), a considerable percentage (see Figure 4 below) of auxin-treated cells showed discrete green fluorescent particles, referred to as NPBs (Figure 3A, bottom panel, left), even though the overall green fluorescence

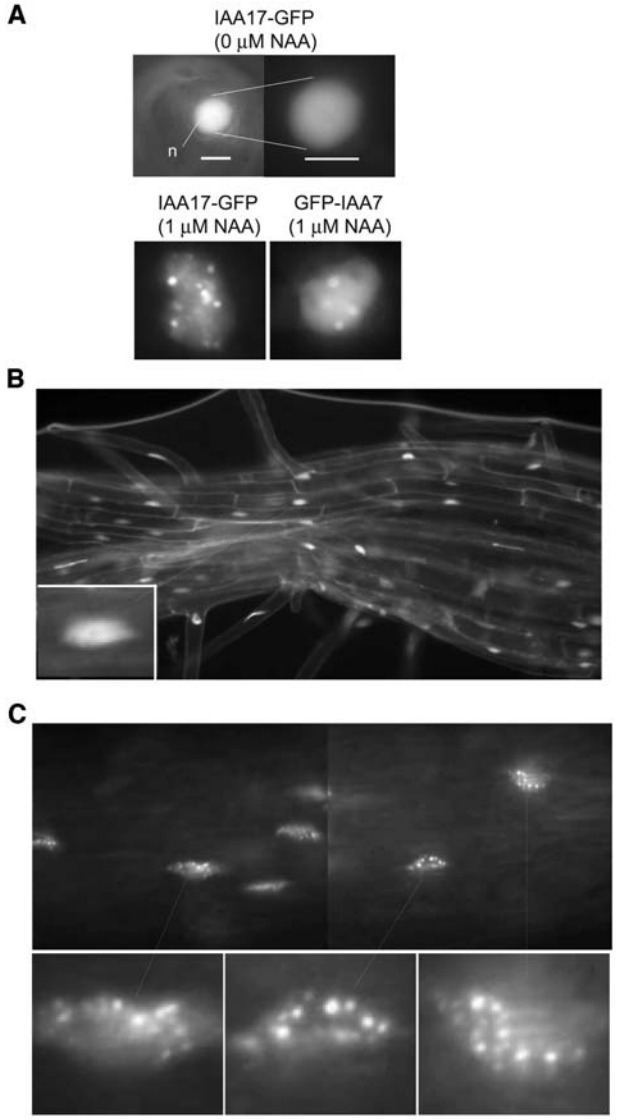

Figure 3. Auxin Induces the Formation of Substrate-Containing NPBs.

(A) High-magnification (×100) observation of representative Dex-IAA17-GFP– or Dex-GFP-IAA7–transfected protoplasts (tobacco protoplasts shown) maintained in auxin-free medium (top panel) or in auxin-supplemented medium (bottom panel) after Dex induction in experiments similar to those shown in Figure 2. n, nucleus. Bars = 5 μm.

(B) A root segment from a 4-d-old Arabidopsis seedling transformed by 35S-IAA17-GFP. The small panel shows the nucleus from a typical IAA17-GFP–positive cell in the root cortex.

(C) A root segment of an auxin-treated (20 μM NAA for 30 min) 4-d-old transformed seedling. Images at two slightly different focal plane were merged. The bottom panels show three representative IAA17-GFP–positive cells.

level within the nuclei declined and eventually disappeared. IAA7-GFP behaved similarly (Figure 3A, bottom panel, right), suggesting that translocation of nucleoplasmic Aux/IAA proteins into these structures is likely to be a common response for these repressors upon auxin treatment. Nucleus-targeted GFP did not respond to auxin treatment and maintained a diffuse nucleoplasmic signal (data not shown; see Figure 7 below).

IAA17-GFP was an active repressor for DR5-GUS expression (see Supplemental Figure 1B online). Its accumulation in transformed Arabidopsis was extremely low and sporadic (see Supplemental Figure 3 online). When detectable by fluorescence microscopy in root epidermal and cortical cells, IAA17-GFP typically showed a diffuse nucleoplasmic signal (Figure 3B). When treated with auxin, IAA17-GFP–containing NPBs were induced in these cells (Figure 3C), indicating that the auxin-induced formation of repressor molecule–containing NPBs was not an anomaly specific to isolated protoplasts.

To better characterize the auxin-induced response of repressor-containing NPB formation, we quantified the localization property of IAA17-GFP in response to exogenous auxin in transfected protoplasts (Figure 4). The distribution of nuclear IAA17-GFP signal was examined at 30 min after Dex withdrawal with or without exogenous auxin. Within an auxin-treated culture, the number of IAA17-GFP–containing NPBs observed in each transformed cell was highly variable, from zero to too many to count, and the size and brightness of individual particles also varied with time. Therefore, we categorized the transfected protoplasts into two major classes, class I being those with diffuse nucleoplasmic signal and class II being those with fluorescent NPBs (Figure 4A; for a more detailed categorization and data distribution within class II cells, see Supplemental Figure 4 online). Class I cells were consistently bright. Class II cells varied in their overall green fluorescence level but invariably showed weaker nucleoplasmic signals than class I cells, suggesting that the IAA17-GFP in these cells had undergone a different extent of proteolysis.

In control cultures in which transfected cells expressed IAA17-GFP and were cultured in auxin-free medium, almost 100% of the IAA17-GFP–positive cells maintained a diffuse nuclear fluorescence signal (Figure 4B, class I cells, black data bar). When protoplasts were cultured with exogenous auxin, the level of IAA17-GFP–positive cells declined after 30 min (similar to Figure 3C). However, increasing numbers of the remaining IAA17-GFP–positive cells accumulated fluorescent NPBs in an auxin dosage–dependent manner (Figure 4B, class II cells, light and dark gray data bars). The increase in class II cells was correlated with a decline of cells maintaining nucleoplasmic green fluorescence (Figure 4B, class I cells, light and dark gray bars).

As in the case of Aux/IAA degradation (Zenser et al., 2003; Nemhauser et al., 2004) (see Supplemental Figure 1C online), the formation of IAA17-GFP–containing NPBs was stimulated specifically by exogenous auxin but not by the other hormones tested, including brassinosteroid, gibberellic acid, abscisic acid, cytokinin, and the ethylene precursor aminocyclopropane-1-carboxylic acid (Figure 4C). Moreover, this auxin-induced process was apparently dependent on substrates that were vulnerable to auxin-stimulated degradation, because only a low level of the proteolytically stable IAA17(P88L)-GFP was incorporated into NPBs in the presence of exogenous auxin (Figure 4D). Interestingly, MG132 suppressed the auxin-induced relocation of nucleoplasmic IAA17-GFP signal into NPBs (Figure 4B, white data bars), implying that proteolytic subunits of the 26S proteasomes may also be involved in the

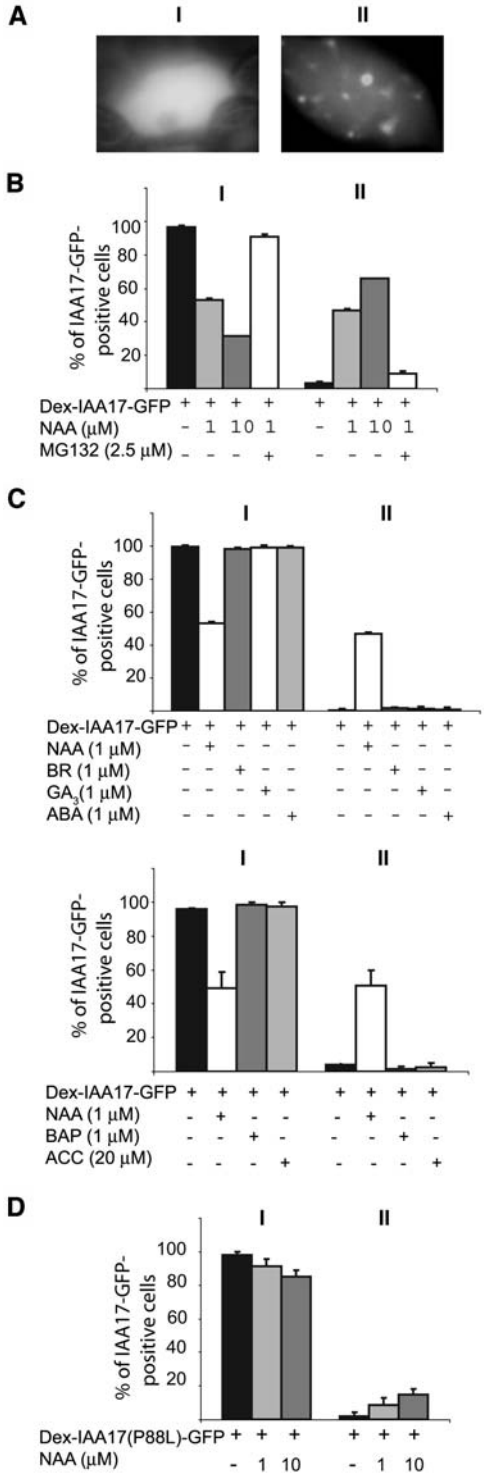

Figure 4. Characterization of the Auxin-Induced Formation of Substrate-Containing NPBs.

Arabidopsis protoplasts were transfected by Dex-IAA17-GFP (**[A]** to **[C]**) or Dex-IAA17(P88L)-GFP **(D)**. Transfected protoplasts were categorized based on the localization properties of IAA17-GFP or IAA17(P88L)-GFP after Dex induction followed by various treatments as indicated. (See Supplemental Figure 4 online for a more refined classification of cells within class II and data distribution into each of the subcategories.)

(A) Class I cells were those with diffuse nuclear IAA17-GFP signal. Class II cells were those with one to numerous IAA17-GFP nuclear bodies in a single focal plane. Images were captured by autoexposure and thus do not reflect relative fluorescence intensity between nuclei. Class I cells invariably had high levels of green fluorescence, whereas class II cells were of variable and declining overall fluorescence intensity relative to class I cells.

(B) to **(D)** Data bars show averages from three independent experiments. In each experiment, at least 200 IAA17-GFP–positive (**[B]** and **[C]**) or IAA17(P88L)-GFP–positive **(D)** cells maintained under each specified condition were observed. Where no error bar is shown, SD was negligible. Cells were grouped into class I or II.

(B) Effect of auxin (gray bars) and MG132 (white bars) on IAA17-GFP localization.

(C) Effect of different hormones on IAA17-GFP localization. ABA, abscisic acid; ACC, aminocyclopropane-1-carboxylic acid; BAP, benzyl amino purine; BR, brassinosteroid; GA_3, gibberellic acid. (See Zenser et al. [2001], Nemhauser et al. [2004], and Supplemental Figure 1C online for the effect of these hormones on IAA17-LUC stability.)

(D) Effect of auxin on the localization of proteolytically stable IAA17(P88L)-GFP.

formation of these substrate-containing protein conglomerates and that these structures may be proteolytically active.

Substrate-Containing NPBs Are Proteolytically Active

Consistent with the possibility that the auxin-induced NPBs may be proteolytically active is the observation that the translocation of nucleoplasmic IAA17-GFP into NPBs was always accompanied by the decline and ultimate disappearance of the IAA17-GFP signal from within these nuclear structures (Figures 5B to 5D). When the IAA17-GFP signal was monitored in individual cells treated with auxin, the decline in the diffuse nucleoplasmic signal followed a considerably faster kinetics than signals in control cells in auxin-free medium (Figures 5A to 5C). The auxin-induced rapid decline in nucleoplasmic IAA17-GFP was accompanied by the appearance of green fluorescent NPBs within the nucleus (Figures 5B to 5D). In many of these cells, the number of NPBs increased with time after auxin addition. The IAA17-GFP intensity within some of these particles increased initially (Figures 5B and 5D, dots 1 and 2), although with time the overall fluorescence signal within these NPBs also declined, eventually to undetectable levels. Nevertheless, tiny NPBs also became visible with time in control, non-auxin-treated cells (Figure 5A), implying that the turnover of Aux/IAA proteins under endogenous auxin conditions also engaged in a similar process, which was apparently amplified and accelerated by auxin. Therefore, these observations reveal that auxin-signaled proteolysis of Aux/IAAs involves a cellular process whereby substrates are recruited from the nucleoplasm into proteolytically active megaprotein complexes, within which they are degraded.

Rac GTPases Mediate the Auxin-Stimulated Formation of Substrate-Containing NPBs

To explore whether a signaling relationship exists between auxin and Rac GTPases in the formation of substrate-containing NPBs,

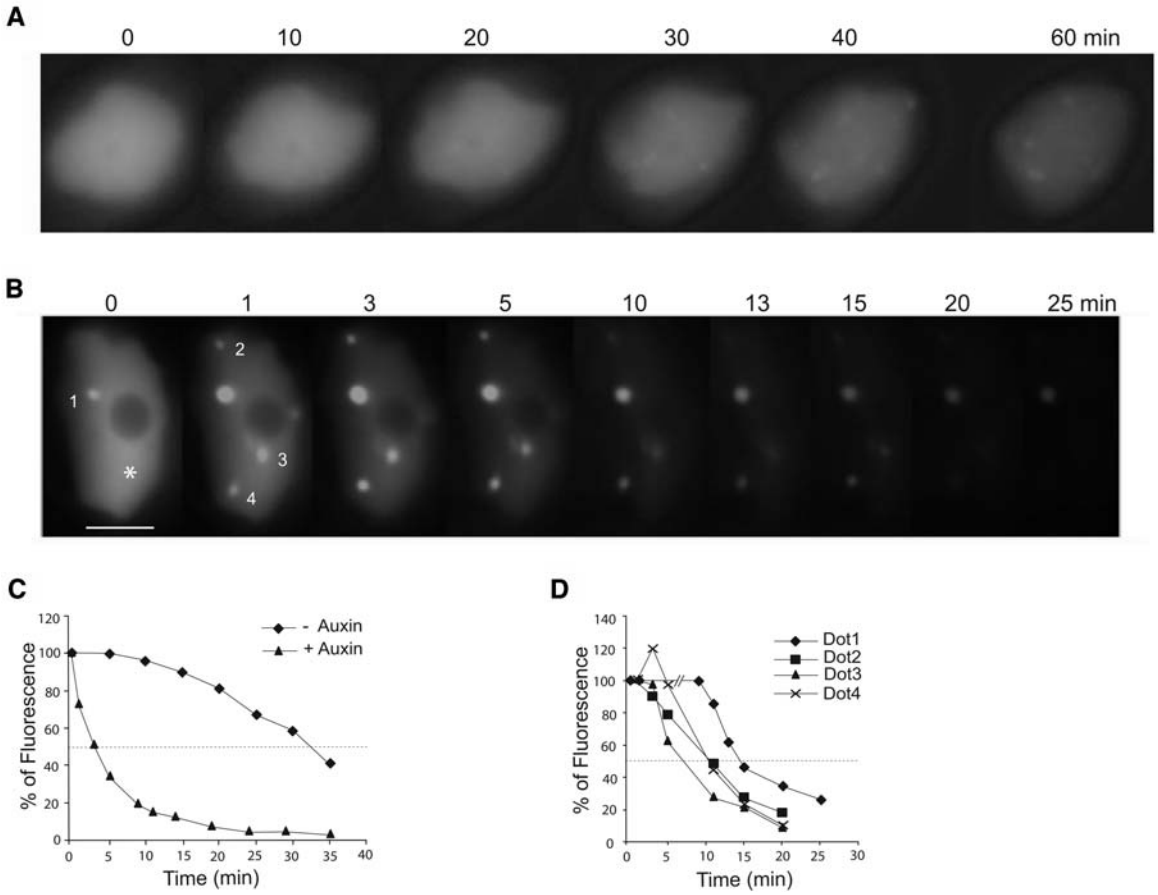

Figure 5. Kinetics of the Auxin-Induced Relocation of Nucleoplasmic IAA17-GFP into NPBs and Their Decline from within These Structures.

Single cells in Dex-IAA17-GFP–transfected Arabidopsis protoplast cultures were observed under high magnification (×100) to monitor the localization and level of IAA17-GFP in the nucleus.

(A) Nucleus from a representative control cell maintained in auxin-free medium and observed immediately after Dex withdrawal for 60 min.

(B) Nucleus from a representative cell maintained in auxin-supplemented (20 μM NAA) medium immediately after Dex withdrawal. Observations were made over the 30 min that followed.

Images in **(A)** and **(B)** were made with the same exposure time used for the 0-min image for each series and so reflect fluorescence intensity relative to that seen at the beginning of the observation.

(C) and **(D)** Fluorescence intensity in the nucleoplasm (asterisk) and in individual NPBs (dots 1 to 4) in the auxin-treated cell shown in **(B)** was estimated by pixel levels and plotted to show the kinetics of nucleoplasmic signal decline (**[C]**, closed triangles) and the appearance of NPBs and the decline of fluorescent signal from within the NPBs **(D)**. In **(C)**, the pixel level in the nucleoplasm of cells shown in **(A)** and **(B)** at 0 min was set as 100%. Similarly, the pixel level in each of the dots shown in **(D)** as they were first detected was set at 100%. The level of fluorescence remained flat for ~10 min for dot 1 (closed diamond plot in **[D]**), because exposure from this dot was saturating under the imaging condition in these initial minutes. Initial increases in IAA17-GFP were obvious in some of the NPBs (e.g., dots 1 and 4), reflecting the initial recruitment of IAA17-GFP into these structures before their accumulation levels began to decline (at ~5 min) and eventually became too weak to be detected (starting at ~20 min) (see images in **[B]** also). The signal decline half-life of nucleoplasmic IAA17-GFP was ~3.5 min for this auxin-treated cell **(C)**. Approximately 15 similarly auxin-treated protoplasts from three independent experiments were observed, and they showed nucleoplasmic IAA17-GFP decline half-lives ranging from 3 to 4 min to 12 to 13 min. The decline half-life of IAA17-GFP within the NPBs ranged between 5 and 10 min for this **(D)** and other similarly observed cells in several independent experiments. The decline profile of nucleoplasmic IAA17-GFP in the representative control protoplast shown in **(A)** is also shown here (**[C]**, closed diamonds). The nucleoplasmic IAA17-GFP decline half-life observed in 10 control cells ranged between 25 and ~40 min. Tiny NPBs consistently became visible during the course of observation among the still relatively high nucleoplasmic signal, but they were too weak for additional analysis.

the effect of CA and DN Rac GTPases on the auxin-regulated process was assessed and quantified. Coexpressing NtRac1 (CA) or AtRac1(CA) stimulated the formation of IAA17-GFP–containing NPBs in a large number of cells, even without exogenous auxin (Figures 6A and 6B). On the other hand, expression of NtRac1(DN) or AtRac1(DN) diminished the ability of auxin to stimulate these NPBs (Figure 6C). Moreover, transiently transformed leaf epidermal cells coexpressing IAA17-GFP and NtRac1(CA) accumulated substrate-containing NPBs in the absence of auxin (Figure 6G), mimicking the auxin-induced

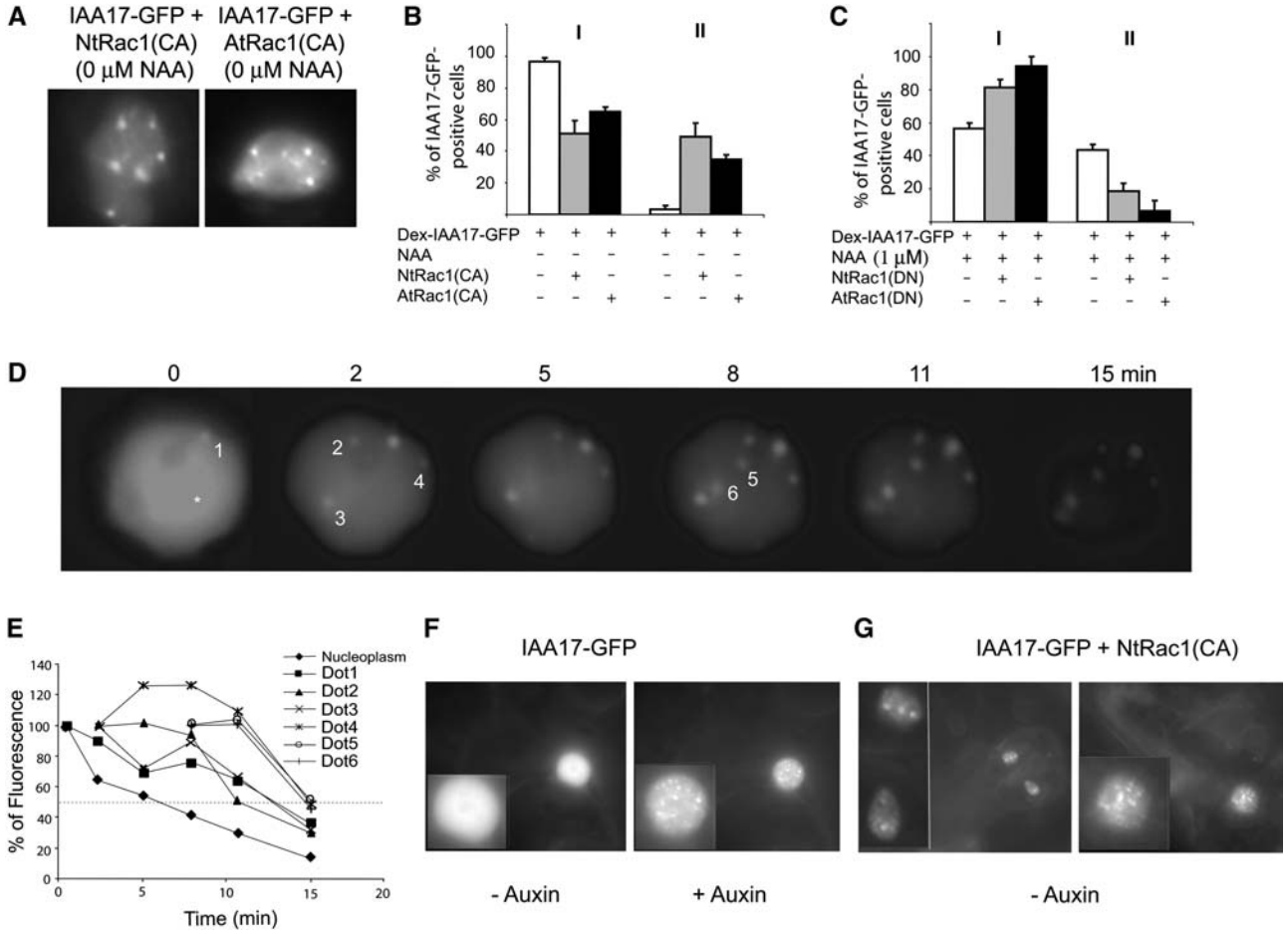

Figure 6. Rac GTPases Mediate the Auxin-Stimulated Formation of Substrate-Containing NPBs.

Protoplasts were cotransfected by Dex-IAA17-GFP and 35S-NtRac1(CA), NtRac1(DN), AtRac1(CA), or AtRac1(DN) as indicated.

(A) Tobacco protoplasts coexpressing IAA17-GFP and NtRac1(CA) or AtRac1(CA) maintained in the absence of exogenous auxin. Images were captured by autoexposure and so do not reflect relative fluorescence intensity between samples.

(B) and **(C)** Effect of NtRac1 and AtRac1 on the auxin-regulated process in Arabidopsis protoplasts. Data bars show averages from three independent experiments. In each experiment, at least 200 IAA17-GFP–positive cells maintained under each specified condition were observed. Where no error bar is shown, SD was negligible. Cells were grouped into class I or II.

(B) Effect of NtRac1(CA) and AtRac1(CA) on IAA17-GFP localization in the absence of exogenous auxin.

(C) Effect of NtRac1(DN) and AtRac1(DN) on the auxin-induced formation of IAA17-GFP NPBs.

(D) and **(E)** Effect of NtRac1(CA) on IAA17-GFP relocalization to NPBs and its decline from within these structures. **(D)** shows the nucleus of a single Arabidopsis protoplast cotransfected by Dex-IAA17-GFP and 35S-NtRac1(CA) observed under high magnification (×100) immediately after Dex withdrawal but without exogenous auxin supplementation. The cell shown was among the class I cells still remaining at the time of sampling (see **[B]**). The nuclear images were captured at the indicated minutes after Dex withdrawal with the same exposure conditions used for the 0-min image. Fluorescence intensity in the nucleoplasm (asterisk in **[D]**) and in individual NPBs (dots 1 to 6) was measured by pixel level and plotted in **(E)** to show the kinetics of nucleoplasmic signal decline (closed diamonds) and the appearance of NPBs and the decline of fluorescent signal from within these structures. The decline half-life of nucleoplasmic IAA17-GFP was ~6 min for this cell. Ten other similarly transfected protoplasts showed comparable responses, with IAA17-GFP decline half-life ranging between 4 and 15 min. [See Supplemental Figure 5 online for the AtRac(CA)-induced effect on IAA17-GFP localization and signal decline.]

(F) Effect of auxin on IAA17-GFP localization in tobacco leaves transformed by Dex-IAA17-GFP by agroinfiltration. The same cell is shown before (−) and 30 min after (+) auxin treatment (20 μM NAA). Images were made by autoexposure.

(G) Effect NtRac1(CA) on the localization of IAA17-GFP in tobacco leaves cotransformed by 35S-NtRac1(CA) and Dex-IAA17-GFP by agroinfiltration. Insets in **(F)** and **(G)** show nuclei from the cells in the main panels to reveal details.

response seen in IAA17-GFP–expressing cells in similarly treated leaves (Figure 6F). Therefore, the auxin-induced cellular pathway that assembles substrate molecules into NPBs is also mediated by Rac GTPases. Accordingly, when individual cells coexpressing Dex-induced IAA17-GFP and NtRac1(CA) (Figures 6D and 6E) or AtRac1(CA) (see Supplemental Figure 5 online) maintained in auxin-free medium were followed for the time course of IAA17-GFP signal distribution, the decline of nucleoplasmic IAA17-GFP signal, the appearance of fluorescent NPBs, and the subsequent disappearance of fluorescent signals from within these structures occurred more prominently and rapidly than in control cells (Figure 5A).

SCF^{TIR1}, CSN, and 26S Proteasome Components Colocalize with Substrates in the Auxin-Induced NPBs

The current model of auxin-induced Aux/IAA protein proteolysis suggests that these transcriptional regulators interact with the F-box protein TIR1 and are then tagged for degradation by the SCF^{TIR1} complex (Hellmann and Estelle, 2002; Moon et al., 2004). CSN interacts with cullin and Rbx subunits of SCF E3 ligases and mediates multiple SCF-dependent processes, including auxin signaling (Schwechheimer et al., 2001; Cope and Deshaies, 2003; Serino and Deng, 2003; Smalle and Vierstra, 2004). Together with our observations that the level of GFP-labeled Aux/IAAs declined from within NPBs (Figures 5 and 6), these data suggest that not only substrate molecules but also components of SCF^{TIR1}, CSN, and 26S proteasomes coexist in these auxin-induced nuclear structures. When TIR1-YFP, YFP-Rbx1, ASK2-YFP, and CUL1-YFP were expressed alone, predominant nucleoplasmic signals were observed among a low YFP signal in the cytosol, and auxin did not noticeably alter these localization properties (Figure 7A, left column). When IAA17-CFP was coexpressed with each of these YFP-labeled SCF^{TIR1} components, they all became colocalized with IAA17-CFP in NPBs in auxin-supplemented medium (Figure 7A). The localization of selected subunits of CSN and 26S proteasome core particle (CP) was also examined. Like the SCF^{TIR1} components, YFP-labeled CSN3, CSN5, CSN7, and CSN8, YFP-CP-α1, and YFP-CP-β2 localized predominantly in the nucleoplasm (Figures 7B and 7C, left columns), with a low level of the protein remaining in the cytosol when each was expressed alone. When coexpressed with IAA17-CFP, all of these YPF-labeled proteins were recruited to colocalize with these substrates in NPBs (Figures 7B and 7C). On other hand, nucleus-targeted YFP, CFP, and GFP were not recruited into NPBs under similar conditions when IAA17-CFP, IAA17-YFP, and IAA17-dsRFP were coexpressed, respectively (Figure 7D). Because CSN and the 26S proteasome are known to exist as protein complexes in vivo (Serino and Deng, 2003; Smalle and Vierstra, 2004), the presence of selected components of these complexes in the substrate-containing NPBs and the fact that the NPBs are proteolytically active (Figures 5 and 6) suggest that in all likelihood a majority, if not all, of the constituents necessary to support the ubiquitination/26S proteasome activity are present in these megaprotein structures.

DISCUSSION

Rac GTPases Regulate Auxin-Induced 26S Proteasome–Dependent Proteolysis of Aux/IAA

We have shown previously that a subset of Rac GTPases mediate auxin-induced gene expression (Tao et al., 2002). The results reported here show that Rac GTPases activate the ubiquitin/26S proteasome–mediated proteolysis of Aux/IAAs, a process uniquely induced by auxin but not other major plant growth regulators (Zenser et al., 2003; Nemhauser et al., 2004), resulting in derepression of auxin-inducible genes. Observations showing that Rac GTPases are rapidly activated by auxin (Tao et al., 2002), mediate the auxin-specific effect of accelerated Aux/IAA degradation, and turn on downstream responsive genes (Tao et al., 2002) together provide strong evidence that these small GTPases are integral components of the auxin-signaled pathway of Aux/IAA degradation and auxin-responsive gene expression.

Rac GTPases in plants are known to regulate a large number of cellular target systems, including gene expression, multiple pathways that converge on the cytoskeleton, and oxidative stress-induced reactions, and they also intersect membrane trafficking, thus influencing exocytosis and endocytosis (Camacho and Malho, 2003; Gu et al., 2004; Bloch et al., 2005). These studies also revealed direct effectors (Wu et al., 2001; Fu et al., 2005), secondary messengers such as Ca^{2+} (Li et al., 1999) and H_2O_2 (Kawasaki et al., 1999; Ono et al., 2001), and downstream effectors such as actin-depolymerizing factor (Chen et al., 2003a) that regulate the ultimate target systems. However, the role in activating 26S proteasome–dependent proteolysis revealed here is previously unknown for members of the Rho GTPase family. Given the prevalent dependence on regulated proteolysis and the broad involvement of Rac GTPases in plant growth and developmental processes (Gu et al., 2004; Smalle and Vierstra, 2004), these small GTPases may turn out to be key regulators for a large number of signaling pathways using regulated proteolysis as checkpoints.

The apparatus that relates auxin to Rac GTPases and signaling molecules between these regulators and the SCF^{TIR1}/26S proteasome machinery remains to be identified. When activated, Rac GTPases are known to associate with the cell membrane. The stimulation of auxin-responsive gene expression by NtRac1(CA) correlates with a strong cell membrane localization for GFP-NtRac1(CA). On the other hand, the cell membrane association of GFP-NtRac1 was substantially diminished when signaling activity was downregulated, which correlates with reduced auxin-signaled gene expression (Tao et al., 2002). Together, these results suggest that Rac GTPases most likely interact with a cell membrane–associated auxin reception mechanism that has yet to be identified. However, it was shown recently that TIR1 acts as an auxin receptor in cell-free soluble protein extracts to mediate the auxin-enhanced Aux/IAA–TIR1 interaction (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Nevertheless, ubiquitin/26S proteasome–dependent proteolysis is known to be regulated on multiple levels (Pickart, 2001; Cope and Deshaies, 2003; Serino and Deng, 2003; Smalle and Vierstra, 2004), and auxin signaling per se involves numerous regulators and modulators (Schwechheimer et al., 2001; Ullah et al., 2001;

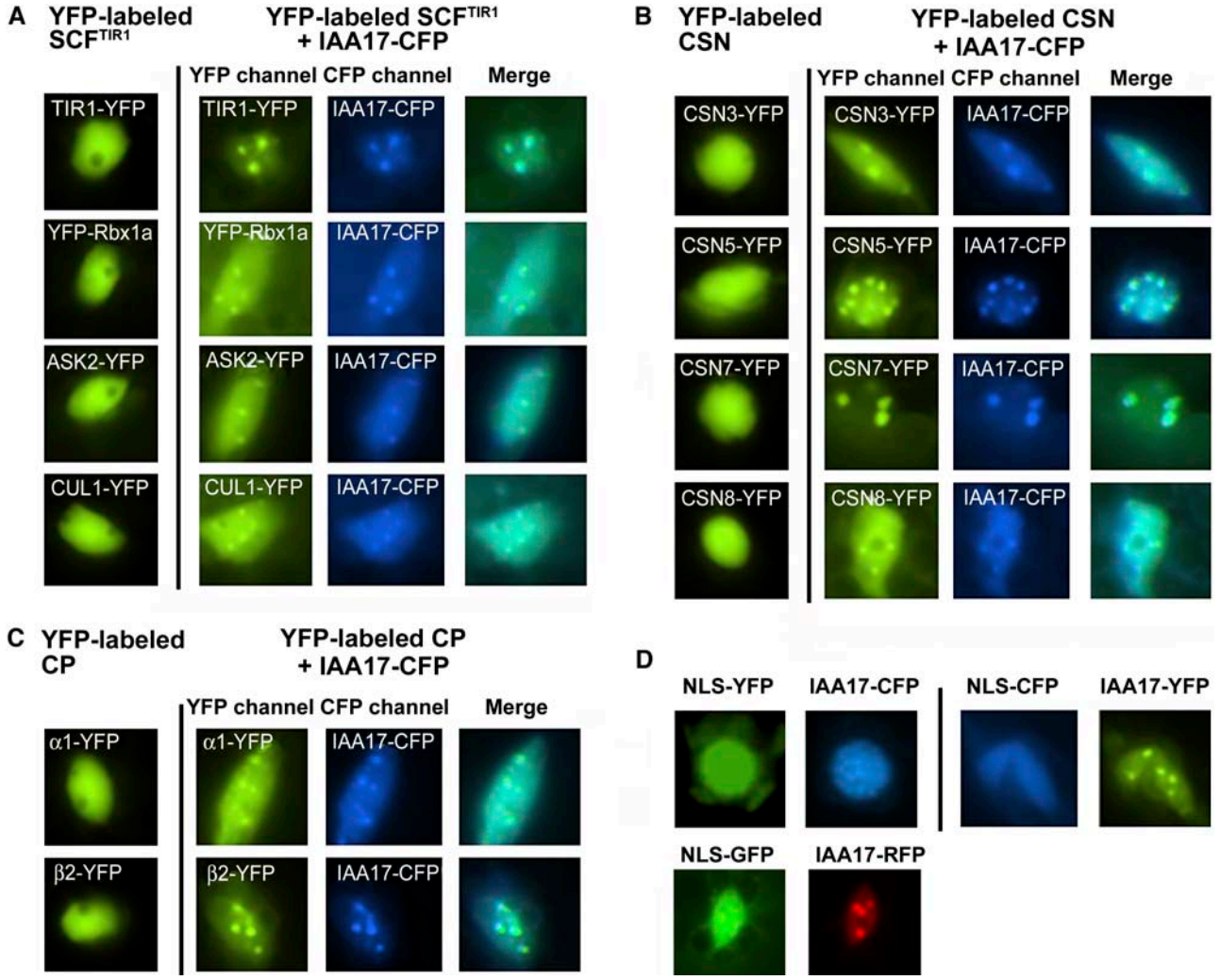

Figure 7. Colocalization of SCF^{TIR1}, Components of CSN, and the 26S Proteasome with IAA17 in NPBs.

(A) to **(C)** Arabidopsis protoplasts were transfected by 35S-YFP–labeled SCF^{TIR1}, CSN, or 26S proteasome component proteins or cotransfected by 35S-IAA17-CFP and one of these YFP-labeled protein genes as indicated. Images were obtained after overnight culturing in auxin-free medium and treated with 10 μM NAA for 30 min before observation. The image at left in each row shows the nucleus from a transfected protoplast expressing just the indicated YFP-labeled subunit from SCF^{TIR1}, CSN, or the 26S proteasome. The second to fourth images in each row show the nucleus of a single protoplast coexpressing the indicated YFP-labeled subunit and IAA17-CFP observed individually in the YFP channel or the CFP channel (second and third images) and their merged images (fourth image). Images were captured by autoexposure. The hue in each image was adjusted to accentuate the signals.

(A) Localization of YFP-labeled TIR1, Rbx1a, ASK2, CUL1, and components of SCF^{TIR1} with coexpressed IAA17-CFP.

(B) Localization of YFP-labeled selected subunits of CSN (3, 5, 7, and 8) with coexpressed IAA17-CFP.

(C) Localization of YFP-labeled 26S proteasome CP-α1 and -β2 subunits with coexpressed IAA17-CFP.

(D) Localization of 35S-expressed nucleus-targeted YFP (NLS-YFP), NLS-CFP, and NLS-GFP with coexpressed IAA17-CFP, IAA17-YFP, and IAA17-dsRFP, respectively. Cells were treated with 10 μM NAA for 30 min before observation.

The constitutive 35S promoter was used in these experiments to express the various tagged IAA17s to ensure a sustained supply of these substrates during the 30 min of auxin treatment. (See Supplemental Figure 6 online for similar observations made with IAA17-dsRFP and GFP-labeled components of the three functional complexes.)

del Pozo et al., 2002a, 2002b; Gray et al., 2003; Moon et al., 2004). Therefore, in addition to the SCF^{TIR1}–Aux/IAA interaction, auxin may also regulate, for example, SCF, CSN, and proteasome assembly, interactions between these functional complexes, and necessary enzymatic activities that lead to substrate degradation. It is possible that multiple pathways relate auxin (Rogg and Bartel, 2001) and converge on regulating the SCF^{TIR1}–Aux/IAA interaction to effect gene expression via repressor proteolysis. How Rac GTPases interact with some of these and other possible regulatory pathways, such as various

mitogen-activated protein kinase and other phospho-regulatory cascades, to convey the auxin signal to the ubiquitination/26S proteasome machinery remains to be explored.

Proteolytically Active NPBs and Regulated Proteolysis

NPBs enriched in substrates and 26S proteasomal proteins, referred to as clastosomes, have been observed in mammalian cells by immunofluorescence (Lafarga et al., 2002). In plants, SCF, CSN, and 26S proteasome components have been detected biochemically in protein conglomerates (Peng et al., 2003). Nucleus-located protein bodies or speckles have been shown to associate with several hormone- and light-regulated signaling pathways (Wang et al., 2001; Chen et al., 2003b; Lopez-Molina et al., 2003; Seo et al., 2003; Ng et al., 2004). In particular, the ring motif E3 ligase COP1 has been observed to colocalize in nuclear protein particles with its substrate LAF1 and another regulatory protein for photomorphogenesis, SPA1, when they were expressed as fluorescent protein–tagged fusion proteins in transiently transformed onion epidermal peel (Seo et al., 2003). Similarly, COP1 also localizes to protein bodies in the nucleus along with ABI5 and its interacting protein AFP (Lopez-Molina et al., 2003). Although microscopically observed protein bodies in the nucleus and biochemically detected protein conglomerates have been postulated to be sites for regulated proteolysis (Wang et al., 2001; Lafarga et al., 2002; Lopez-Molina et al., 2003; Peng et al., 2003), proteolytic activity has not been demonstrated in these protein ensembles. The observations reported here reveal a novel pathway of signal-regulated and substrate-dependent formation of proteolytically active NPBs composed of substrates, SCF, CSN, and 26S proteasome components. Our results show that auxin induces the recruitment of substrates from the nucleoplasm into NPBs (Figures 3 and 4) and, together with substrates, also induces the recruitment of nucleoplasmic SCF, CSN, and 26S proteasome components into these NPBs (Figure 7). This and the observation that a P88L substitution in the substrates rendered the proteolytically stable protein substantially less responsive to auxin (Figure 4D), correlating with the reduced ability of IAA17(P88L) (Figure 1A) and repressors with an analogous mutation to suppress the auxin response (Zenser et al., 2001), suggests a process whereby auxin induces changes in the substrates, which in turn triggers the assembly of the necessary components for the machinery needed for substrate removal.

The involvement of nuclear protein particles in various signaling pathways reported to date was revealed under conditions in which cellular levels of the proteins examined were increased by expression from transgenes (Wang et al., 2001; Chen et al., 2003b; Lopez-Molina et al., 2003; Seo et al., 2003; Ng et al., 2004), suggesting that the endogenous processes must have been accentuated, facilitating their observation. The auxin-induced formation of substrate-containing NPBs is likewise observed under conditions of increased Aux/IAA levels (Figure 4). The endogenous levels of Aux/IAAs are extremely low, rendering their detection on immunoblots or by immunofluorescence difficult to accomplish (Abel et al., 1994; Oeller and Theologis, 1995). Moreover, under normal cellular conditions, a very low percentage of the cellular pool of SCF, CSN, and 26S proteasome components was biochemically detected in megaprotein complexes, suggesting that the molecular interactions involved may be transient or highly dynamic (Peng et al., 2003) and thus precluding the formation of protein particles of sizes detectable by fluorescence microscopy. The increasing substrate levels reported here might actually have driven the protein conglomerate assembly process to a very high level and/or stabilized these structures, allowing them to be observed as NPBs. Nevertheless, several lines of evidence support the notion that the NPBs observed here did not simply arise from the overaccumulation of substrates or other constituents in the protein conglomerates. Under similar expression conditions, the formation of IAA17-GFP–containing NPBs did not occur invariably but was dependent on auxin, substrate degradability, and proteolytic activity and was regulated by Rac GTPases (Figures 3, 4, 6B, and 6C). Similarly, nucleoplasmic YFP-labeled subunits of SCF^{TIR1}, CSN, and the 26S proteasome did not form NPBs spontaneously, nor were they induced by auxin alone; rather, they were dependent on coexpressed IAA17-CFP for their recruitment into NPBs (Figure 7). These findings, together with the fact that these NPBs are proteolytically active (Figures 5, 6D, and 6E), provide strong support for their biological relevance in the auxin-regulated Aux/IAA proteolysis pathway. Moreover, their formation is apparently also dependent on proper SCF^{TIR1} and CSN functions, because downregulating components in these complexes and mutations that regulate SCF assembly have been observed to compromise this auxin-induced process (L.Z. Tao, A.Y. Cheung, and H.M. Wu, unpublished data). How the multitude of factors that constitute the ubiquitin/26S proteasome–mediated pathway respond to auxin and Rac GTPases to achieve the assembly of proteolytically active NPBs will need to be examined to gain further insight into the contribution of this signal-regulated process to auxin signaling.

The dynamic assembly and disassembly of biologically active protein conglomerates within the interphase nucleus is being recognized as a strategy to compartmentalize functional components within the nucleoplasm to support diverse nuclear processes (Misteli, 2001, 2005). The observations reported here suggest that auxin signaling has adopted a strategy whereby the assembly of proteolytically active megaprotein complexes are stimulated in the nucleus upon demand by elevation of auxin level. The rapidity of auxin signaling, with the early responsive genes induced within the first minutes of stimulation, suggests that this mechanism may provide the benefit of rapid removal of transcription repressors in situ within these structures. Perhaps the high local concentrations of substrates, regulators, and proteases achieved in these protein conglomerates allow for the rapid turnover of the major components of this regulated process in a highly coordinated manner not achievable if they are all randomly distributed in the nucleoplasm. In addition to their assembly in response to the need for proteolytic removal of Aux/IAAs, it will be interesting to examine whether the disassembly of these NPBs and the recycling of their component proteins upon substrate depletion represent regulatory checkpoints for this process. Although the functionality remains to be demonstrated for NPBs and biochemically detected protein conglomerates observed in other regulated proteolysis-dependent signaling pathways, their existence suggests that strategies similar to those described here for auxin signaling may

be used by a broader range of ubiquitination/26S proteasome–dependent cellular and developmental processes.

METHODS

cDNA Isolation, Mutagenesis, and Chimeric Gene Construction

cDNAs for *Arabidopsis thaliana* proteins were obtained by RT-PCR from seedling mRNAs. Tobacco (*Nicotiana tabacum*) NtRac1 cDNAs were described previously (Tao et al., 2002). Mutagenesis and chimeric gene construction were performed using PCR-based methods (Tao et al., 2002). The backbone vector used to clone various chimeric genes is shown in Supplemental Figure 2A online.

Protoplast Transfection, Agroinfiltration, and Arabidopsis Transformation

Arabidopsis and tobacco (SR1) protoplast preparation and transfection followed previously described procedures (Tao et al., 2002) and yielded similar results; the source of protoplasts for each experiment is indicated in the figure legends. NAA, a commonly used auxin, was used as exogenous auxin. Briefly, 0.2 mL of protoplast suspension ($\sim 2 \times 10^5$ cells) was transfected with DNA for various constructs (10 μg each). For internal reference genes, 5 μg of 35S-LUC or 1 μg of Ubi10-GUS was used in combination with the reporter genes DR5-GUS or IAA7/17-LUC, respectively. Reporter activity is shown relative to activity from these internal standards to account for differences in transfection efficiency and cell recovery.

After transfection, cells were cultured in protoplast medium (K3 + 0.4 M sucrose) under different conditions, as indicated in the figures. In transfections that involved chimeric genes expressed from a Dex-inducible promoter (Yanagisawa et al., 2003) (for data shown in Figures 1F, 2, 3A, and 4 to 6; see also Supplemental Figure 2B online), transfected protoplasts were cultured overnight ($\sim$14 h) in auxin-free K3 medium. Dex (10 μM) was added to induce IAA17-GFP the next morning. After Dex treatment (4 to 5 h) and removal, protoplasts were cultured with or without auxin for defined periods of time as indicated in the figures and text. When used, the 26S proteasome inhibitor MG132 (2.5 μM) was added to protoplasts during the last hour of Dex treatment and was included for the duration of culturing time that followed Dex withdrawal. When other hormones were examined, they were also added at the time of Dex withdrawal.

Tobacco leaf epidermal cells were transiently transformed by Dex-IAA17-GFP alone or together with 35S-NtRac1(CA) using agroinfiltration according to Batoko et al. (2000). Arabidopsis was transformed by 35S-IAA17-GFP using the floral dip method (Clough and Bent, 1998).

GUS and LUC Assays

GUS and LUC assays followed previously described procedures (Tao et al., 2002). All biochemical analyses were repeated at least three times with comparable results, and the data presented are averages of triplicate assays for each condition in one representative experiment.

Microscopic Analyses

Observations were made on a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan), and images were captured with a SPOT camera. Excitation and emission filters Ex460-500/DM505/BA510-560, Ex426-446/DM455/BA460-500, Ex490-510/DM515/BA520-550, and Ex516/10/DM575/BA590 (from Nikon) were used for GFP, CFP, YFP, and dsRFP (Campbell et al., 2002), respectively. To tally the number of GFP-positive cells among transfected cultures (Figure 2), protoplasts were observed under the $\times$10 objective. The $\times$100 objective was used to observe localization patterns within the nuclei.

Conditions used to monitor the localization and decline kinetics of IAA17-GFP in transfected protoplasts were as follows. IAA17-GFP was expressed by Dex induction (10 μM for 4 to 5 h). For auxin-treated cells, 20 μM NAA was added to an aliquot of the transfected culture after Dex withdrawal. Dex withdrawal minimized the contribution from continued gene expression to the substrate pool. Cells were observed immediately with the $\times$100 objective. The nucleus of the cell of interest was photographed as soon as it was located, usually within 1 min after auxin addition, and these are designated the 0-min images in Figures 5A, 5B, and 6D. Observations were made at intervals over the next 30 to 40 min as indicated in the figures. To examine the effect of Rac GTPases on IAA17-GFP localization and decline, protoplasts were cotransfected with Dex-IAA17-GFP and 35S-NtRac1(CA) or AtRac1(CA). Cells that had not yet or had just begun to show NPB formation were followed after Dex withdrawal and resuspension in auxin-free medium as described above. Images were made over the course of 15 to 60 min, depending on the rapidity of signal decline. For both sets of experiments, images at later time points were captured with the same exposure conditions as the 0-min images and were not photographically enhanced, so they reflect relative fluorescence intensity in each nucleus over time. IAA17-GFP levels were monitored by fluorescence signal intensity (as pixels) in the nucleoplasm (designated with asterisks in Figures 5B and 6D) and at the NPBs (designated by numbers in the figures). Cells expressing IAA17-GFP and not treated with auxin served as controls for both sets of experiments.

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