The Pleiotropic Role of the 26S Proteasome Subunit RPN10 in Arabidopsis Growth and Development Supports a Substrate-Specific Function in Abscisic Acid Signaling

Jan Smalle,a Jasmina Kurepa,a Peizhen Yang,a Thomas J. Emborg,a Elena Babiychuk,b Sergei Kushnir,b and Richard D. Vierstra*a†

a Department of Horticulture and Cellular and Molecular Biology Program, University of Wisconsin–Madison, Madison, Wisconsin 53706
b Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

The 26S proteasome is an essential protease complex responsible for removing most short-lived intracellular proteins, especially those modified with polyubiquitin chains. We show here that an Arabidopsis mutant expressing an altered RPN10 subunit exhibited a pleiotropic phenotype consistent with specific changes in 26S proteasome function. rpn10-1 plants displayed reduced seed germination, growth rate, stamen number, genetic transmission through the male gamete, and hormone-induced cell division, which can be explained partially by a constitutive downregulation of the key cell cycle gene CDKA;1. rpn10-1 also was more sensitive to abscisic acid (ABA), salt, and sucrose stress and to DNA-damaging agents and had decreased sensitivity to cytokinin and auxin. Most of the phenotypes can be explained by a hypersensitivity to ABA, which is reflected at the molecular level by the selective stabilization of the short-lived ABA-signaling protein ABI5. Collectively, these results indicate that RPN10 affects a number of regulatory processes in Arabidopsis likely by directing specific proteins to the 26S proteasome for degradation. A particularly important role may be in regulating the responses to signals promulgated by ABA.

INTRODUCTION

The ubiquitin/26S proteasome pathway has been implicated in diverse aspects of eukaryotic cell regulation through its ability to rapidly remove intracellular proteins (Hershko and Ciechanover, 1998; Callis and Vierstra, 2000). In this pathway, proteins destined for degradation first become modified by the covalent attachment of polymeric ubiquitin chains. These chains are assembled on one or more Lys residues within the target via an ATP-dependent reaction cascade involving the sequential action of activating (E1), conjugating (E2), and ligating (E3) enzyme families (Hershko and Ciechanover, 1998; Gagne et al., 2002). The resulting polyubiquitinated proteins then are recognized by the 26S proteasome and degraded, with the concomitant release of the ubiquitin moieties for reuse. Through this conjugation/degradation cycle, the ubiquitin/26S proteasome pathway controls numerous physiological and developmental events by selectively removing key cell regulators (Hershko and Ciechanover, 1998). Although few of the targets are known in plants, genetic analyses have implicated the pathway in hormone regulation, embryogenesis, photomorphogenesis, circadian rhythms, floral homeosis, stress responses, senescence, and pathogen defense (Callis and Vierstra, 2000; Hellmann and Estelle, 2002).

The 26S proteasome is an ATP-dependent, self-compartmentalized protease (Voges et al., 1999; Glickman, 2000). Although most work on this 2-MD complex is derived from yeast and animals, evidence is accumulating that the higher plant version is similar in structure and function (Fu et al., 1998a, 1999a, 1999b; Shibahara et al., 2002). The 26S proteasome can be divided further into two particles, the 20S core protease (CP) and the 19S regulatory particle (RP). The CP is a broad-spectrum ATP- and ubiquitin-independent peptidase created by the assembly of four stacked heptameric rings of related α- and β-subunits in an α1,β1,β1,γ1,α2 configuration. The protease active sites within β1, β2, and β5 polypeptides are sequestered in a central chamber. Access to this chamber is restricted by a narrow gated channel created by the α-subunit rings that allows only unfolded proteins to enter (Glickman, 2000).

The RP binds to each end of the CP and confers both ATP dependence and substrate specificity to the holoenzyme, especially with respect to those substrates bearing the polyubiquitin tag (Voges et al., 1999; Glickman, 2000). The RP presumably helps identify appropriate substrates for breakdown, removes the attached ubiquitins, opens the α-subunit ring gate, and directs the entry of unfolded proteins into the CP lumen for degradation. Its 18 principal subunits can be divided further into two subparticles, the Lid and the Base. The Base contains three non-ATPase subunits (RPN1, RPN2, and RPN10) and a ring of six AAA-ATPase subunits (RPT1 to RPT6) that contacts the α-subunit ring and likely assists in target unfolding and transport (Voges et al., 1999; Glickman, 2000; Fu et al., 2001).

1To whom correspondence should be addressed. E-mail vierstra@facstaff.wisc.edu; fax 608-262-4743.

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RPT5 also may play a role in the recognition of polyubiquitinated proteins (Lam et al., 2002). The Lid binds to each end of the Base and contains nine additional RPN subunits (RPN3, RPN5 to RPN6, and RPN11 to RPN13). The functions of most of the RPN subunits are unknown. All but two (RPN9 and RPN10) are essential in yeast. RPN11 has proteolytic activities capable of removing bound ubiquitins, implicating it in the release of polyubiquitin chains from substrates (Verma et al., 2002). RPN1 has been proposed to play a role in the recognition of ubiquitin-like sequences (UBLs) that may help deliver substrates to the 26S proteasome (Elsasser et al., 2002).

Several roles have been ascribed to RPN10. It was identified originally by its ability to bind polyubiquitin chains in vitro (van Nocker et al., 1996a). Because its preference for ubiquitinated proteins bearing chains with four or more ubiquitins matches that of the 26S proteasome, RPN10 initially was considered to be the main ubiquitinated protein receptor in the 26S proteasome complex (van Nocker et al., 1996a; Piotrowski et al., 1997). van Nocker et al. (1996b) subsequently showed that a yeast Δrpn10 strain is viable but hypersensitive to amino acid analogs that create damaged proteins and is compromised in the degradation of only a subset of ubiquitin/26S proteasome analogs (Fu et al., 2001). It sits within an aspartate (Asp-11) near the N terminus of RPN10 (Glickman et al., 1998; Fu et al., 2001). A positionally conserved phobic patch near the C terminus of the protein (Fu et al., 1998b) suggests that it helps tether the two subcomplexes RPN10 allows the yeast Lid and Base to easily dissociate in vitro, suggesting that it helps tether the two subcomplexes.

Approximately 50% of the bound ubiquitin chain is released from the Lid and remains bound to the Base and contains nine additional RPN subunits (RPN3, RPN5 to RPN6, and RPN11 to RPN13). The functions of most of the RPN subunits are unknown. All but two (RPN9 and RPN10) are essential in yeast. RPN11 has proteolytic activities capable of removing bound ubiquitins, implicating it in the release of polyubiquitin chains from substrates (Verma et al., 2002). RPN1 has been proposed to play a role in the recognition of ubiquitin-like sequences (UBLs) that may help deliver substrates to the 26S proteasome (Elsasser et al., 2002).

The ubiquitin-interacting motif (UIM) was identified as a hydrophobic patch near the C terminus of the protein (Fu et al., 1998b). The UIM and the UBL domain appear important for the phenotypic function of one or more regulators of hormone action. Both the vWA domain and the UIM appear important for the phenotypic function of moss RPN10 (Girod et al., 1999). Double-stranded RNA interference of RPN10 in Drosophila cultured cells increased the levels of polyubiquitinated proteins even though the derived 26S proteasomes had increased peptidase activity (Wojcik and DeMartino, 2002). Similar interference showed that RPN10 is essential in Trypanosoma brucei (Li and Wang, 2002). Although the mutant still could assemble 26S proteasomes without the RPN10 protein, the proteozaan showed a strong growth arrest.

Here, we describe the isolation and characterization of a T-DNA mutant from the higher plant Arabidopsis expressing an aberrant form of RPN10. The rpn10-1 line exhibited a pleiotropic phenotype, indicating that a number of processes were compromised selectively. Most strikingly, the postgerminative development of rpn10-1 seedlings was highly sensitive to the hormone abscisic acid (ABA), salt, and sucrose stress. Many of the phenotypes appear to be caused by a hypersensitivity to ABA, reflected at the molecular level by a failure to rapidly degrade the ABA response protein ABA INSENSITIVE-5 (ABI5). Collectively, the mutant phenotypes indicate that RPN10 plays a role in delivering a specific subset of short-lived targets to the Arabidopsis 26S proteasome for degradation.

RESULTS

The T-DNA Insertion Mutant rpn10-1

The Arabidopsis RPN10 protein is encoded by a single gene located on chromosome 4 (TAIR locus At4g38630). To help define its functions, we searched the various mutation databases for insertion mutants in the RPN10 locus. A T-DNA insertion of Arabidopsis ecotype C24 within the transcribed region of RPN10 was generated fortuitously as part of an exon-trapping project that exploited a promoterless chimeric apurinic endonuclease (ARP)–neomycin phosphotransferase (NPTII) gene to identify expressed loci (Babiychuk et al., 1997). Sequence analysis of the 5’ rapid amplification of cDNA ends product from the kanamycin-resistant line SL41-45 (now designated rpn10-1) detected a chimeric mRNA that encoded the N-terminal part of RPN10 (residues 1 to 186) followed by the full-length ARP-NPTII protein sequence of 280 amino acids (Figure 1A). Analysis of the genomic sequence revealed that the T-DNA inserted within the fifth intron of the rpn10-1 locus, thus creating a chimeric intron that, when spliced out, would generate a fused coding region (Figure 1A). Notably, the rpn10-1 protein product retained the N-terminal vWA fold of RPN10 that is essential for RP stability in yeast but was missing the C-terminal half that contains...
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The UIM responsible for binding polyubiquitin chains (Fu et al., 1998b, 2001). Kanamycin resistance was tightly linked to the rpn10-1 mutation, indicating that this line contained a single T-DNA insert (J. Smalle, unpublished data).

RNA gel blot analysis showed that the T-DNA insertion severely attenuated the accumulation of the RPN10 mRNA. Whereas the 1.4-kp RPN10 transcript was detected easily in wild-type plants, just a trace of the predicted 1.6-kp RPN10-NPTII transcript was seen in homozygous rpn10-1 plants using a RPN10 probe (Figure 1B). This species also was detected using a NPTII probe, demonstrating that the NPTII sequence was part of the mRNA. Accordingly, a much reduced amount of the RPN10-NPTII fusion protein accumulated in the rpn10-1 plants. Using anti-NPTII antibodies, a protein with the expected apparent molecular mass of 50 kD was detected in total protein extracts from rpn10-1, indicating that the predicted full-length fusion protein was expressed (Figure 1C). The NPTII domain also was active and could confer kanamycin resistance to the seedlings. However, when anti-RPN10 antibodies were used, the level of fusion protein was nearly undetectable compared with RPN10 in the wild type (Figure 1C). (We note that wild-type RPN10 migrates anomalously during SDS-PAGE, with an apparent mass similar to that of the RPN10-NPTII fusion protein [∼50 kD], even though its actual mass is 41 kD [van Nocker et al., 1996a]). Because the anti-RPN10 antibodies easily recognized the N-terminal half of the protein (Figure 2A) (Fu et al., 1998b), we concluded that this low apparent level reflects diminished synthesis and not decreased antigenicity of the RPN10-NPTII fusion protein.

Reduced levels of RPN10 have been shown in other organisms to substantially increase the levels of ubiquitinated proteins, likely by attenuating 26S proteasome activity (van Nocker et al., 1996a). Kanamycin resistance was tightly linked to the rpn10-1 mutation, indicating that this line contained a single T-DNA insert (J. Smalle, unpublished data).

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**Figure 1.** Molecular and Biochemical Descriptions of the Arabidopsis rpn10-1 Mutation.

(A) Structure of the RPN10 gene and the effect of the T-DNA insertion containing ARP-NPTII on the predicted protein product. Boxes and lines denote exons and introns, respectively. Upon insertion of the T-DNA into the fifth intron, the remaining intron sequence of the RPN10 gene (bp 940 to 950) and the 5′ end of the ARP-NPTII gene form a chimeric intron that when spliced expresses the translational fusion product (RPN10-NPTII) containing the N-terminal 267 amino acids of RPN10 appended to the 280 amino acids of ARP-NPTII. aa, amino acids.

(B) Detection of RPN10 and RPN10-NPTII transcripts in 2-week-old wild-type (WT) and rpn10-1 seedlings by RNA gel blot analysis using probes for the RPN10 and NPTII sequences. Equal loading of total RNA was confirmed by staining for rRNA with methylene blue (data not shown) and by reprobing the blots with β-tubulin (βTUB4).

(C) Detection of RPN10 and the RPN10-NPTII fusion protein. Total protein from 2-week-old wild-type and homozygous rpn10-1 seedlings was subjected to SDS-PAGE and immunoblot analysis with anti-RPN10 and anti-NPTII antibodies. Equal protein loads were confirmed by immunoblot analysis with anti-UBC1 antibodies.

(D) Ubiquitin (Ub) conjugate levels are increased in rpn10-1 seedlings. Equal amounts of total protein from (C) were subjected to SDS-PAGE and immunoblot analysis with anti-ubiquitin antibodies. The positions of free polyubiquitin chains and ubiquitinated proteins are indicated.
et al., 1996b; Girod et al., 1999; Wojcik and DeMartino, 2002). Arabidopsis rpn10-1 seedlings displayed a similar effect. In addition to increased levels of high molecular mass polyubiquitinated proteins, increased levels of free polyubiquitin chains were evident (Figure 1D). This increase was not caused by reduced amounts of 26S proteasomes. In fact, immunoblot analysis indicated that the amounts of 26S proteasome subunits other than RPN10 were higher in rpn10-1 seedlings than in wild-type seedlings (J. Smalle, unpublished data).

Loss of RPN10 significantly alters the stability of the yeast RP in vitro, allowing the Lid to dissociate from the Base under mild salt conditions (Glickman et al., 1998; Fu et al., 2001). To test for a similar effect with the Arabidopsis rpn10-1 mutant, we partially purified 26S proteasomes from wild-type and rpn10-1 seedlings and compared their size and stability by chromatographic methods. The CP was assayed by peptidase activity using the synthetic substrate N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin and by immunoblot analysis with antibodies against the α3-subunit PAC1 and the β6-subunit PBF1. The RP was assayed by immunoblot analysis with antibodies against RPT1 and RPN5 that detect the Base and Lid subcomplexes, respectively. Superose HR6 size-exclusion chromatography did not reveal any significant size differences between the wild-type and mutant 26S proteasome preparations (P. Yang, unpublished data). Likewise, Resource Q anion-exchange chromatography, which can detect subtle effects on RP stability (Glickman et al., 1998; Fu et al., 2001), also failed to detect differences between the two preparations. Both wild-type and mutant complexes eluted at the same salt concentration with similar profiles (Figures 2A and 2B). In both wild-type and rpn10-1 preparations, the RP (as identified by the RPT1 and RPN5 proteins) eluted with a broader profile than the CP, with some eluting at slightly lower salt concentrations, consistent with its dissociation from the CP under these chromatographic conditions (Fu et al., 2001).

Despite the much reduced levels of the RPN10-NPTII protein in total extracts from the homozygous rpn10-1 plants compared with wild-type plants (Figure 1C), the fusion protein was detected easily in the enriched 26S proteasome fractions using either anti-RPN10 or anti-NPTII antibody (Figure 2B). Thus, even with the NPTII appendage, the fusion protein readily incorporated into the 26S complex. This incorporation indicates that the C-terminal half of RPN10 is not necessary for RP binding and is consistent with the importance of the N terminus for

Figure 2. Association of the RPN10-NPTII Fusion Protein with the Arabidopsis 26S Proteasome.

The 26S proteasome was enriched from 10-day-old wild-type (WT) and homozygous rpn10-1 seedlings by sequential polyethylene glycol precipitations and subjected to anion exchange fast protein liquid chromatography using a NaCl gradient for elution. 
(A) Column fractions were assayed for peptidase activity of the CP using N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin as the substrate and are expressed in relative fluorescence units (F). Only the portions of the fast protein liquid chromatography elution profiles centered on the peaks of peptidase activity are shown.
(B) Column fractions were subjected to SDS-PAGE and immunoblot analysis with antibodies against NPTII, the RPN10, RPN5, and RPT1 subunits of the RP, and the PBF1 and PAC1 subunits of the CP. C, control.
RP stability (Fu et al., 1998b, 2001). The structural integrity of the 26S proteasome from the rpn10-1 mutant strongly suggests that the resulting fusion protein does not alter the incorporation/association of the other principal subunits into the Arabidopsis 26S complex.

**rpn10-1 Shows Developmental Changes throughout Its Life Cycle**

Consistent with the importance of the ubiquitin/26S proteasome pathway in plants (Callis and Vierstra, 2000; Hellmann and Estelle, 2002), Arabidopsis seeds homozygous for the rpn10-1 mutation displayed numerous developmental defects. rpn10-1 seeds have markedly decreased germination, especially for seeds sown soon after reaching maturity. If germinated within 1 week after maturation, only 7 ± 6% of rpn10-1 seeds germinated compared with 98 ± 2% for wild-type seeds (Figure 3A). This poor germination improved as the seeds aged, with 83 ± 5% of the rpn10-1 seeds germinating compared with nearly 100% for wild-type seeds after 1 month of storage at room temperature.

After germination in the dark, etiolated rpn10-1 seedlings grew similar to wild-type seedlings, except for a slight reduction in hypocotyl and root extension (Figure 3D). When grown in the light, a substantial decrease in growth rate was observed, including a slower rate of leaf initiation and expansion and a decrease in root elongation (Figure 3B and data not shown). Although the cotyledons appeared normal, the rosette leaves senesced prematurely, especially at the leaf edges (Figure 3C). This chlorosis was more pronounced in leaves that emerged within the first few days after sowing (Hensel et al., 1993). Surprisingly, most rpn10-1 seeds germinated with nearly 100% for wild-type seeds after 1 month of storage at room temperature.

To further understand the roles of RPN10 in cytokinin signaling, we examined by RNA gel blot analysis the expression of a constitutive downregulation of CDKA;1 Expression

We previously showed that the Arabidopsis RPN12a subunit plays a role in cytokinin responses (Smalle et al., 2002). Given that RPN10 interacts with RPN12 in yeast (Kominni et al., 1997; Wilkinson et al., 2000) and that some of the rpn10-1 phenotypes could be explained superficially by defects in cytokinin signaling (reduced growth rate and accelerated senescence), we tested for a similar decrease in cytokinin sensitivity in rpn10-1. As can be seen in Figure 5A, root elongation of rpn10-1, like that of rpn12a-1 (Smalle et al., 2002), was substantially less inhibited by the cytokinin kinetin than that of the wild type. Whereas root growth of wild-type seedlings was suppressed strongly by as little as 0.05 μM kinetin, 1 μM kinetin was needed to elicit the same effect in the mutant.

The rpn10-1 mutation also decreased the ability of cytokinins to stimulate cell division and shoot induction. As in rpn12a-1 (Smalle et al., 2002), greening and shoot induction from hypocotyl segments by appropriate ratios of the auxin indoleacetic acid (IAA) and the cytokinin 2-isopentyladenine (2iP) were reduced markedly in the rpn10-1 mutant (Figure 5B). However, after prolonged exposures of 3 weeks or more, which produced green calli and stimulated the emergence of shoots from the wild type and a modest induction of calli from rpn12a-1, cell division and callus formation were not evident from rpn10-1 (Figure 5C). Although this response suggested that rpn10-1 is more insensitive to cytokinins than rpn12a-1, the response of intact seedlings exposed continuously to kinetin implied a more complex effect. The growth of rpn12a-1 seedlings was affected only mildly by 0.1 μM kinetin, whereas the growth of wild-type and rpn10-1 seedlings was repressed dramatically (Figure 5D).
battery of cytokinin-regulated genes in the rpn10-1 background. The cytokinin early-response gene ARR5, which is upregulated dramatically but transiently within 15 min of cytokinin treatment, is thought to act as a feedback inhibitor of cytokinin action (D’Agostino et al., 2000; Hwang and Sheen, 2001). The basal and cytokinin-induced expression of ARR5 is unaffected by the rpn12a-1 mutation (Smalle et al., 2002). For the rpn10-1 mutation, the basal ARR5 mRNA levels were suppressed by approximately twofold compared with wild-type levels and increased by approximately twofold after a 30-min treatment with a 5-μM concentration of the cytokinin benzyladenine (BA) (Figure 5E). Expression of cytokinin late-response genes, like those that encode nitrate reductase (NIA1) and cyclin D3 (CYCD3), is upregulated after prolonged exposure of wild-type seedlings to cytokinins (Yu et al., 1998; Riou-Khamlichi et al., 1999). Smalle et al. (2002) found that the mRNA levels for both NIA1 and CYCD3 are increased constitutively in rpn12a-1. By contrast, rpn10-1 seedlings expressed normal levels of NIA1 and CYCD3 in the absence of BA but failed to upregulate their expression after a 12-h BA treatment (Figure 5F). This long exposure of rpn10-1 to BA elicited a mild increase in the steady state level of ARR5 mRNA (Figure 5F). Expression of the RPN10 gene also was induced by BA (approximately threefold), similar to the cytokinin induction of RPN12a and other genes that encode 26S proteasome subunits (Figure 5F) (Smalle et al., 2002).

The reduced growth rate of the rpn10-1 plants suggested that the expression and/or action of one or more cyclin-dependent kinases (CDKs) were affected. Of particular interest was the CDKA;1 gene (previously named CDC2a), whose expression has been linked to plant growth rate and cell division activity (Hemerly et al., 1993; Beemster et al., 2002). Whereas the expression of CDKA;1 is normal in rpn12a-1 plants (Smalle et al., 2002), its expression was downregulated selectively in the rpn10-1 mutant (Figure 5G). In contrast to the levels of two other Arabidopsis CDKs, CDKC;1 and CDKD1;3 (Vandepoele et al., 2002), we found that the mRNA level for CDKA;1 was reduced at least twofold to threefold in rpn10-1 seedlings with or without BA treatment (Figures 5E and 5G).

Rpn10-1 Roots Have Decreased Auxin Sensitivity

The rpn10-1 mutant, like rpn12a-1 (Smalle et al., 2002), exhibits a slight decrease in auxin sensitivity, detected by a diminished responsiveness of roots to low concentrations of 2,4-D. Auxins are known to inhibit root elongation while at the same time stimulating lateral root formation. As can be seen in Figures 6A and 6C, inhibition of root elongation and promotion of lateral roots were less pronounced in rpn10-1 compared with the wild type on medium containing 5- to 10-nM concentrations of the

Figure 3. Developmental Changes Induced by the rpn10-1 Mutation. (A) Decreased germination of rpn10-1 seeds compared with wild-type (WT) seeds. (B) Decreased growth rate of rpn10-1 plants during the first 4 weeks of development. (C) Premature chlorosis of mature rpn10-1 leaves. Shown is a rosette leaf from a 6-week-old rpn10-1 plant exhibiting chlorosis at the edges next to a wild-type rosette leaf of comparable age. (D) Decreased hypocotyl and root growth of etiolated rpn10-1 seedlings. (E) Reduced stamen number of rpn10-1 flowers. Whereas wild-type flowers contained six stamens, the rpn10-1 flowers typically contained four stamens (arrowheads). (F) Prolonged longevity of rpn10-1 inflorescences. Arrowheads indicate active floral meristems after 8 weeks of growth under a long-day photoperiod.
Figure 4. The rpn10-1 Mutant Is Hypersensitive to UV-B Light and the DNA-Damaging Agent Mitomycin C.
(A) rpn10-1 seedlings are hypersensitive to long-day versus short-day photoperiods. Wild-type (WT) and rpn10-1 plants were grown for 3 weeks under long days (16 h of light and 8 h of dark) or for 4 weeks under short days (8 h of light and 16 h of dark).
(B) Hypersensitivity of rpn10-1 plants to UV-B light. Seedlings were irradiated daily with 0.5 min of UV-B light. Images are of 14-day-old seedlings.
(C) Effect of daily UV-B irradiations on the accumulation of fresh weight. Seedlings were irradiated daily for 14 days with a dose of 0.5 or 3 min of UV-B light. Values shown are means from 10 seedlings (± SD).
(D) rpn10-1 seedlings are hypersensitive to mitomycin C. Seedlings were grown for 2 weeks under short days on medium containing the indicated concentrations of mitomycin C.

Rpn10-1 Is Hypersensitive to ABA

In addition to cytokinins and auxins, the ubiquitin/26S proteasome pathway regulates responses to a variety of other plant hormones (Callis and Vierstra, 2000; Hellmann and Estelle, 2002). Thus, it was conceivable that other hormone signaling pathways were affected in rpn10-1, given its overall increase in ubiquitinated proteins (Figure 1D). Especially relevant was ABA, based on its role in suppressing seed germination (Finkelstein et al., 2002) and the poor germination of rpn10-1 seeds (Figure 3A).

Using effects on overall seedling growth and root elongation as the assays, we found that rpn10-1 plants responded normally to 22(S),23(S)-homobrassinolide, gibberellic acid 3, methyl jasmonate, salicylic acid, and the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (J. Smalle, unpublished data). By contrast, rpn10-1 seedlings exhibited a strong hypersensitivity to ABA. When germinated for 1 day in the absence of ABA and then transferred to medium containing increasing amounts of ABA, wild-type seedlings showed an increasing inhibition of leaf emergence and expansion, with higher concentrations (4 μM) also blocking expansion and greening of the cotyledons (Figure 7A) (Lopez-Molina et al., 2001). Relative to other Arabidopsis ecotypes, such as Columbia, Landsberg erecta, and Wassilewskija, we noted that C24 wild type appeared to be more sensitive to ABA by this assay. When rpn10-1 seedlings were tested similarly, an ~10-fold increase in ABA sensitivity was observed, with as little as 0.4 μM ABA needed to arrest cotyledon development completely (Figure 7A). Even after weeks of incubation on ≥1 μM ABA, the rpn10-1 seedlings remained arrested at this early developmental stage. A similar increase in ABA sensitivity was not seen for rpn12a-1 (Figure 7B), suggesting that this effect on postgerminative development specifically requires RPN10. ABA also inhibited root elongation, with our results indicating that rpn10-1 roots are hypersensitive. For seedlings grown for 4 days without ABA and then transferred to medium containing 1 μM ABA, the growth rate of rpn10-1 roots was reduced by 50% compared with that of wild-type roots (Figure 7C). By comparison, the rpn12a-1 mutant showed normal ABA sensitivity by this root assay (Smalle et al., 2002).

ABA also is important for regulating plant growth responses to osmotic stress, especially early in development, suggesting that it plays a role in preventing premature germination and growth under unfavorable conditions (Finkelstein et al., 2002; Rolland et al., 2002). In agreement, we found that rpn10-1 seedlings were hypersensitive to NaCl and sucrose stress. Concentrations of NaCl (100 mM) or sucrose (5%) that did not visibly deter the early development of the wild type effectively inhibited the greening and expansion of rpn10-1 (Figure 7D). rpn10 mutants from other eukaryotes are hypersensitive to amino acid analogs (Girod et al., 1999; Li and Wang, 2002; Wojcik and DeMartino, 2002). Similarly, we found that rpn10-1 has increased sensitivity to the Arg analog canavanine by the root elongation assay (J. Smalle, unpublished data).

Several reports have suggested that ABA arrests plant growth in part by directly influencing the cell division machinery (Finkelstein et al., 2002). Given the constitutive downregulation of CDKA;1 in rpn10-1 seedlings (Figures 5E to 5G), we tested hormone. However, the ability of 2,4-D to promote root hair elongation (Pitts et al., 1988) was unaffected by the rpn10-1 mutation, indicating that not all auxin responses were altered. Although untreated root hairs were shorter in rpn10-1, 2,4-D still was able to stimulate their elongation to an extent comparable to that in the wild type (Figure 6B).
by RNA gel blot analysis whether the expression of this CDK gene is regulated by ABA. Wild-type seedlings showed a selective reduction of CDKA;1 mRNA (compared with CDKC;1 and RPN10 mRNA) when treated with 10 μM ABA for 6 h (Figure 7E). In rpn10-1 seedlings, this level was reduced substantially even in the absence of ABA, possibly reflecting a constitutive repression of the CDKA;1 gene. It has been reported that high concentrations of ABA (50 μM) transcriptionally upregulate the CDK inhibitor ICK1/KRP1, suggesting that increased levels of this protein could mediate some of the growth inhibition by ABA (Wang et al., 1998). However, ICK1/KRP1 mRNA levels were not altered in rpn10-1 seedlings, nor did treatment with an ABA concentration (10 μM) sufficient to downregulate CDKA;1 change these levels (Figure 7E). As a result, we concluded that this CDK inhibitor is not involved in the growth rate reduction and ABA hypersensitivity of rpn10-1. Interestingly, the level of ARR5 mRNA was reduced by ABA in both wild-type and mutant plants (Figure 7E). As a consequence, it is more likely that the reduction in ARR5 mRNA in rpn10-1 seedlings seen in Figure 5F was caused by increased sensitivity to ABA and not by decreased sensitivity to cytokinins.

Complementation of rpn10-1

To confirm that the rpn10-1 phenotype was caused by alteration of the RPN10 gene, we attempted to complement the mutation by ectopic expression of the wild-type cDNA under the control of the 3SS promoter of Cauliflower mosaic virus (CaMV). When a number of independently transformed rpn10-1 3SS-RPN10 seedlings were examined, all of the aberrant traits associated with the rpn10-1 mutation were restored to those of the wild type. These include the reduced growth rate and sta-

Figure 5. Cytokinin Responses and the Expression of Cytokinin-Regulated Genes Are Altered in the rpn10-1 Mutant.

(A) Effect of a range of kinetin concentrations on root elongation in wild-type (WT) and homozygous rpn10-1 seedlings. Seedlings were germinated and grown for 4 days in the absence of kinetin and transferred to medium containing the indicated kinetin concentrations. After an additional 9 days of growth, the mean root lengths of 10 plants were determined and are expressed as percentages of the values from the respective untreated controls (±SE).

(B) and (C) The induction of greening and shoot formation by 2iP is blocked in the rpn10-1 mutant. Hypocotyl segments of 5-day-old wild-type, rpn10-1, and rpn12a-1 seeds were germinated directly on 0.1 μM kinetin and grown for 3 weeks.

(D) mRNA levels for various cell cycle proteins in wild-type, rpn10-1, and rpn12a-1 seedlings. Total RNA from seedlings grown in liquid medium for 10 days was subjected to RNA gel blot analysis with probes for the CDK genes CDKA;1, CDKC;1, and CDKD1;3 and the cyclin gene CYCD3. Equal amounts of total RNA were confirmed by staining for rRNA with methylene blue (data not shown) and by reprobing the blots with βTUB4 or CDKC;1.
bidopsis by acting as both an activator and a repressor of gene expression (Finkelstein et al., 2002). Of interest are the observations that transgenic plants that constitutively overexpress ABI5 have an ABA response phenotype similar to rpn10-1 (arrested postgerminative development [Lopez-Molina et al., 2001]), whereas abi5 null mutants respond in an opposite manner from rpn10-1 (they are insensitive to ABA [Finkelstein and Lynch, 2000]). Lopez-Molina et al. (2001) previously showed that ABA can increase the levels of ABI5 through both transcriptional induction of the ABI5 gene and increased accumulation of the protein, possibly by a mechanism that blocks its degradation by the 26S proteasome. Consequently, it is plausible that the ABA hypersensitivity of rpn10-1 is mediated wholly or in part by the upregulation of ABI5 expression and/or the selective stabilization of the ABI5 protein.

To test these possibilities, we compared the levels of the ABI5 mRNA and protein in wild-type and rpn10-1 seedlings before and after treatment with ABA. The ABI5 mRNA was expressed at nearly undetectable levels in young Arabidopsis seedlings. As shown in Figure 7E, these levels were not increased significantly by the rpn10-1 mutation or by treatment of wild-type and mutant seedlings with ABA. By contrast, the steady state level of the ABI5 protein was increased dramatically by the mutation, even in the absence of exogenous ABA (Figure 9A). Accumulation was highest in younger seedlings, in accord with previous reports showing that ABI5 synthesis is restricted to a short interval during early seedling development (Lopez-Molina et al., 2001). Five-day-old rpn10-1 seedlings had the greatest enrichment of ABI5; its accumulation then decreased such that the level of ABI5 in 9-day-old rpn10-1 seedlings was just slightly more than that in the wild type (Figures 7A and 9A). A doublet of ABI5 protein was detected (Figure 9A); the lower apparent molecular mass species was consistent with the size of the unmodified protein, with the higher, more diffuse species potentially representing a phosphorylated form (Lopez-Molina et al., 2001). In the presence of ABA, levels of ABI5 increased even further in rpn10-1 plants and could be elicited by a concentration of ABA (0.2 μM) too low to affect ABI5 levels in wild-type plants (Figure 9B). Notably, the same low level of ABA also was sufficient to inhibit the postgerminative development of rpn10-1 but not wild-type seedlings (Figure 7A), implicating the stabilization of ABI5 in the ABA-hypersensitive phenotype.

Analysis of two other ubiquitin/26S proteasome pathway substrates demonstrated that the increased amount of ABI5 in rpn10-1 was caused by a selective stabilization of ABI5 and not by a general stabilization of all pathway substrates. Both phytochrome A (phyA) and long hypocotyl-5 (HY5) are regulators of Arabidopsis photomorphogenesis but are degraded by two different mechanisms within the pathway (Clough et al., 1999; Osterlund et al., 2000). Whereas phyA is stable in dark-grown plants but is degraded rapidly once plants are exposed to light, HY5 is degraded rapidly in dark-grown plants but stabilized once plants are exposed to light. As shown in Figures 9C and 9D, both phyA and HY5 were degraded normally in the rpn10-1 background. phyA was removed rapidly during red light irradiation of dark-grown rpn10-1 seedlings, with a half-life (~1 h) indistinguishable from that of the wild type. Likewise, ABI5 is a member of a family of basic domain/Leu zipper transcription factors that helps confer ABA responsiveness in Ara-

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**Figure 6.** Roots of the rpn10-1 Mutant Are Less Sensitive to the Auxin 2,4-D.

(A) Effect of the rpn10-1 mutation on the inhibition of root elongation by 2,4-D. After 7 days on 2,4-D, the mean root lengths of 10 wild-type (WT) and rpn10-1 plants were determined and are expressed as percentages of the lengths of the respective untreated controls (±SD).

(B) Effect of the rpn10-1 mutation on lateral root formation after 10 days of treatment with various concentrations of 2,4-D.

(C) Effect of the rpn10-1 mutation on 2,4-D-stimulated root hair elongation. Wild type (open circles) and rpn10-1 (closed squares) seeds were germinated on medium containing 2,4-D, and the mean lengths of 40 root hairs were determined (±SD) after 7 days of growth.

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HY5 did not accumulate in dark-grown rpn10-1 and wild-type seedlings but did accumulate in both when the seedlings were exposed to light. The normal turnover rates of phyA and HY5 are consistent with the absence of photomorphogenic defects for the rpn10-1 mutant (Figures 3A and 3D) expected if either or both proteins were stabilized (Clough et al., 1999; Osterlund et al., 2000).

**DISCUSSION**

Previous studies have implicated the ubiquitin/26S proteasome pathway in the perception of various environmental signals and hormones (Callis and Vierstra, 2000; Hellmann and Estelle, 2002). Here, we provide additional support for its role in ABA responses through the analysis of an exon trap mutant of
RPN10, a 26S proteasome subunit within the RP Base subcomplex. The rpn10-1 allele synthesized a chimeric protein containing the N-terminal half of RPN10 appended to the sequence of NPTII. This truncation preserved the vWA domain identified as being important for tethering the Lid to the Base (at least in vitro) but was missing the C-terminal domain containing the UIM that binds polyubiquitin chains (Fu et al., 1998b, 2001). Consistent with this protein organization, the RPN10-NPTII fusion protein retained its ability to associate with the Arabidopsis 26S proteasome. However, this association was diminished greatly in the presence of wild-type RPN10, indicating that the fusion protein does not effectively compete with the native protein for binding to the complex. We obtained a similar result for an analogous NPTII fusion with RPN12a (Smalle et al., 2002), suggesting that appendages to various RP polypeptides can be useful in creating weak alleles for essential subunits.

Analysis of the resulting 26S proteasomes demonstrated that rpn10-1 does not appreciably alter the structure, core subunit composition, and peptidase activity of the Arabidopsis holoenzyme. This finding implies that the observed phenotypes associated with rpn10-1 most likely are caused by a specific loss of RPN10 activity(ies) rather than by more general defects in RP structure, activity, or levels. As such, the analysis of rpn10-1 should provide insights into the specific function(s) of the RPN10 protein.

Two mutually compatible mechanisms may explain biochemically the phenotypic defects observed here for rpn10-1 plants. The most obvious is that substitution of the C-terminal domain of RPN10 for NPTII removes or interferes with a critical activity in RPN10. In particular, the absence of the UIM may reduce the ability of the RP to deliver polyubiquitinated cargo to the 26S proteasome. Although the UIM does not appear to be critical in yeast RPN10 (Fu et al., 1998b), complementation of a Physcomitrella rpn10Δ strain with UIM mutants suggests that this domain plays a more important role in higher order eukaryotes (Girod et al., 1999). The second possibility relates to the decreased abundance of the fusion protein compared with the wild-type protein. Among the RP subunits, RPN10 is unusual because a large fraction in crude extracts exists in an apparently free form not associated with the 26S complex (Haracska and Udvardy, 1995; van Nocker et al., 1996a). If this free RPN10 pool exist in vivo, one conceivable function is that it helps shuttle targets to the 26S proteasome by reversible binding. Lower amounts of the RPN10-NPTII fusion protein may affect this free pool and thus its shuttle function preferentially. It also is possible that the lower level of RPN10 concomitantly reduces the

Figure 8. Complementation of the rpn10-1 Mutation with RPN10.

Lines tested include the wild type (WT), rpn10-1, and rpn10-1 transformed with a cDNA containing the RPN10 coding region expressed under the control of the CaMV 35S promoter (rpn10-1 35S-RPN10).

(A) Growth for 2 weeks under a short-day photoperiod.
(B) Hormone-induced cell division in hypocotyl segments. Hypocotyl segments of 5-day-old seedlings were incubated for 3 weeks in the presence of 1 μM IAA and 4 μM 2iP.
(C) Effect of ABA on the expansion and greening of cotyledons. Seeds were stratified for 4 days at 4°C. After a 1-day incubation at 22°C, the seeds were transferred to medium containing 0.8 μM ABA and grown for 14 days.
(D) Levels of RPN10, RPN10-NPTII fusion protein, and polyubiquitinated proteins. Crude protein extracts were prepared from 7-day-old seedlings and subjected to SDS-PAGE and immunoblot analysis with antibodies against RPN10, NPTII, and ubiquitin (Ub). Analysis with anti-UBC1 antibodies was used to confirm equal protein loads.
level of the 26S proteasome by limiting the supply of this subunit. However, we found that the levels of 26S proteasome actually increased slightly in the rpn10-1 background, likely attributable to a coordinated increase in the synthesis of the other subunits (Wojcik and DeMartino, 2002; P. Yang and J. Smalle, unpublished data). Moreover, it is unlikely that a dramatic reduction in 26S proteasome levels would generate the specific defects observed here.

The pleiotropic phenotype of rpn10-1 plants indicates that the mutation affects a number of responses in Arabidopsis, a substantial portion of which can be explained by a flaw in ABA signaling. ABA plays numerous roles, including preventing precocious germination of seeds, arresting postgerminative development, delaying chloroplast development, inhibiting cell division, controlling pollen development, and protecting plants from salt and sucrose stress (Lopez-Molina et al., 2001; Finkelstein et al., 2002; Rolland et al., 2002). Importantly, defects in all of these processes were observed with the rpn10-1 mutant and are consistent, in most cases, with an increase in ABA sensitivity. The reduced germination of rpn10-1 seeds, especially soon after maturation, agrees with a role of ABA in preventing germination too early after seed drop or under unfavorable conditions (Finkelstein et al., 2002). For example, the maize viviparous1 mutant, with accentuated precocious germination, affects a key component in ABA signaling (Suzuki et al., 2001). The enhanced chlorosis of rpn10-1 plants is consistent with a stimulatory role of ABA in the senescence processes (Finkelstein et al., 2002).

Although the decreased cytokinin sensitivity of rpn10-1 superficially links RPN10 to cytokinin signaling, this insensitivity is atypical and may be explained more easily by a change in ABA responsiveness. For example, rpn10-1 seedlings are phenotypically less sensitive than rpn12a-1 seedlings to cytokinin based on the ability of the hormone to stimulate cell division. However, rosette growth of rpn10-1 is more sensitive to cytokinin than that of rpn12a-1. These differences also were seen at the molecular level, where rpn12a-1 constitutively upregulated the late-cytokinin-induced genes NIA1 and CYCD3 but rpn10-1 failed to induce both genes in the presence or absence of cytokinin (Figures 5E and 5F) (Smalle et al., 2002). In addition, mRNA levels for the cytokinin early-response gene ARR5 were unaffected in rpn12a-1 (Smalle et al., 2002) but were downregulated in rpn10-1 in the absence of cytokinin (Figure 7E). The ARR5 protein was described initially as a repressor of both cytokinin responses and ARR5 expression and appears to function as a feedback inhibitor to suppress cytokinin responses after the initial burst in cytokinin signaling (Hwang and Sheen, 2001). However, we show here that ABA, consistent with its ability to antagonize many cytokinin responses, also can decrease ARR5 expression and that this repression is accentuated by the rpn10-1 mutation. Thus, it is conceivable that altered ARR5 expression in rpn10-1 actually is caused by the ABA hypersensitivity. Furthermore, the observations that ABA inhibits cell division and CYCD3 expression (Murray et al., 2001) also are consistent with the attenuated cytokinin-induced

Figure 9. Selective Stabilization of the ABA Signaling Protein ABI5 in rpn10-1 Seedlings.

(A) Immunoblot detection of ABI5 at various times after seed germination. Total protein was extracted from wild-type (WT) and rpn10-1 seedlings of the indicated ages and subjected to SDS-PAGE and immunoblot analysis with anti-ABI5 antibodies. (B) Immunoblot detection of ABI5 in response to ABA treatment. Seeds were transferred 1 day after stratification to medium containing 0.2 μM ABA and incubated for another 8 days. Immunoblot analysis was performed as described for (A), except that a shorter exposure time was required to visualize the ABA-induced accumulation of ABI5 in rpn10-1. Analysis with anti-UBC1 antibodies was used to confirm equal protein loads. (C) Turnover of phyA in wild-type and rpn10-1 seedlings grown for 6 days in the dark and then exposed to continuous red light (RL). Immunoblots were probed with an anti-phyA monoclonal antibody. (D) Levels of HY5 in wild-type and rpn10-1 seedlings grown for 6 days in the dark followed by a 15-h exposure to white light. Immunoblots were probed with anti-HY5 antibodies.
cell division and the absence of CYCD3 induction shown here for rpn10-1 plants.

Changes in ABA and not cytokinin signaling also may be responsible for the repression of rpn10-1 growth. In Arabidopsis, as in yeast, the levels and/or activities of CDKs appear to be important determinants of cell division and thus growth. For example, plant growth rates have been correlated with the levels of CDKs, in particular the CDKA subfamily (Granier et al., 2000; Beemster et al., 2002). Inhibiting CDKA activity subsequently inhibits growth by attenuating meristem activity (Wang et al., 1998; De Veylder et al., 2001). Interestingly, the CDKA1 gene has been reported to contain a putative ABA response element in its promoter region, suggesting that its expression is under the control of ABA (Chung and Parish, 1995). Hemerly et al. (1993) found that ABA can suppress CDKA1 expression using promoter-β-glucuronidase fusions in Arabidopsis protoplasts and roots. In agreement, we found here that ABA can suppress the accumulation of the natural CDKA1 mRNA and that this suppression is accumulated constitutively in the rpn10-1 mutant. Together, these findings suggest that ABA exerts its action on cell division by suppressing CDKA1 expression. Heightened sensitivity to ABA could exacerbate this suppression and thus negatively regulate growth even in the presence of cytokinins.

Heightened ABA responsiveness also may play a role in the hypersensitivity of rpn10-1 plants to DNA-damaging agents. For example, the Arabidopsis uvs66 mutant, identified first as a UV light-hypersensitive mutant, was found subsequently to have increased sensitivity to ABA (Albinsky et al., 1999). The DNA repair process may be connected to RPN10 via its potential association with RAD23 (Chen and Madura, 2002). Similar to the rpn10-1 defect in Arabidopsis, the loss of RAD23 function in yeast leads to UV light and mitomycin C hypersensitivity and the accumulation of ubiquitinated proteins (Lamberton et al., 1999; Chen and Madura, 2002). The Arabidopsis genome encodes four putative RAD23 homologs, at least two of which can complement yeast rad23Δ mutants (Sturm and Lienhard, 1998). Their connection to ABA and DNA damage in plants is not yet known. However, a rice homolog of RAD23 has been reported to contain a putative ABA response element (1993) found that ABA can suppress CDKA1 expression using promoter-β-glucuronidase fusions in Arabidopsis protoplasts and roots. In agreement, we found here that ABA can suppress the accumulation of the natural CDKA1 mRNA and that this accumulation is accumulated constitutively in the rpn10-1 mutant. Together, these findings suggest that ABA exerts its action on cell division by suppressing CDKA1 expression. Heightened sensitivity to ABA could exacerbate this suppression and thus negatively regulate growth even in the presence of cytokinins.

Assuming that many of the phenotypes of rpn10-1 are induced by a heightened sensitivity to ABA, we considered it likely that RPN10 plays a role in the degradation of one or more activators/repressors of ABA action in Arabidopsis. Previous studies have suggested that the ABA response regulator ABI5 is a target of the 26S proteasome by showing that the CP inhibitor MG132 increased the level of the protein dramatically (Lopez-Molina et al., 2001). Here, we confirmed this notion by demonstrating that the level of ABI5 was increased specifically by the rpn10-1 mutation. By contrast, two other targets of the Arabidopsis ubiquitin/26S proteasome pathway, phyA and HY5, were degraded normally. Intriguingly, ABA treatment of the rpn10-1 seedlings further enhanced ABI5 levels, suggesting that ABA can increase ABI5 half-life directly. Given the fact that many phenotypes of rpn10-1 plants can be explained by ABI5 protein regulation (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001), it is possible that ABI5 is one of the prime targets of RPN10. However, we consider it unlikely that ABI5 is the sole target, because of the general increase in ubiquitinated proteins in the rpn10-1 background. Additional targets could include other components of the ABA-signaling cascade, such as ABI3 and other members of the ABI5 family (Gampala et al., 2002; Kim et al., 2002).

One revealing property of ABI5 is that its action requires ABA. For example, plants that overexpress ABI5 behave like wild-type plants in the absence of exogenous ABA and only elicit their characteristic phenotypes upon treatment with the hormone (Lopez-Molina et al., 2001). This finding suggests that the enhanced synthesis of ABI5 protein alone is insufficient for its function but must be combined with repressed degradation, presumably to allow the accumulation of an ABA-activated form. Such a two-part mechanism could explain the differences in developmental phenotypes of ABI5-overexpressing plants with a mutant such as rpn10-1 that stabilizes the protein. The rpn10-1 phenotype also suggests a more general role for ABI5 in Arabidopsis development, as opposed to the very limited function in postgerminative development ascribed to it previously (Lopez-Molina et al., 2001). In fact, several studies have documented ABI5 expression throughout plant development, in agreement with the highly pleiotropic phenotype of rpn10-1 plants (Signora et al., 2001; Brocard et al., 2002).

How ABI5 is degraded selectively is not yet known. The kinetics of ABI5 accumulation with and without ABA suggest that ABI5 normally is short-lived. Although other mechanisms are possible, the simplest is that ABI5 turnover is achieved through the interaction of ABI5 with RPN10. This interaction could be direct or indirect, could be dependent or independent of prior ubiquitination, and/or could exploit adaptor proteins such as RAD23 or a RAD23/ABI3 complex. ABI5 could stabilize ABI5 by preventing this association. One possible mechanism for the ABA-induced stabilization of ABI5 is via phosphorylation of the protein (Lopez-Molina et al., 2001). As a consequence, ABI5 may be related to a number of other regulatory proteins that exploit phosphorylation to control their turnover by the ubiquitin/26S proteasome pathway (Hershko and Ciechanover, 1998). Although phosphorylation most often stimulates ubiquitination and 26S proteasome–dependent degradation, ABI5 may behave...
like the mammalian c-MOS mitogen-activated protein kinase kinase kinase, in which phosphorylation promotes its stabilization (Sheng et al., 2002).

It has been assumed previously that the selectivity of the ubiquitin/26S proteasome pathway is achieved largely by the ubiquitination steps (Hershko and Ciechanover, 1998; Gagne et al., 2002). The 26S proteasome was expected to provide little specificity, using the same route to degrade its varied substrates. However, recent genetic analyses of the yeast complex have shown that the loss of individual RP subunits can lead to the selective stabilization of specific targets, demonstrating that the 26S proteasome also can distinguish among its substrates and presumably use different routes for entry (van Nocker et al., 1996b). The 26S proteasome was expected to provide little specificity, using the same route to degrade its varied substrates and presumably use different routes for entry (van Nocker et al., 1996b; Bailly and Reed, 1999). The combined analyses of Arabidopsis mutants of the RPN12a and RPN10 subunits are consistent with this notion. Each appears to affect plant growth bidispersity and presumably use different routes for entry (van Nocker et al., 1996b). The 26S proteasome was expected to provide little specificity, using the same route to degrade its varied substrates and presumably use different routes for entry (van Nocker et al., 1996b). The ability of RPN10 to promote the selective recruitment of substrates may be related to its ability to bind polyubiquitin chains and/or potentially help adaptor proteins such as RAD23 and DSK2 associate with the protease. Clearly, identifying the proteins that interact with RPN10 will be critical to defining how it helps deliver appropriate cargo to the 26S proteasome.

**METHODS**

**Plant Materials and Growth Conditions**

The Arabidopsis thaliana C24 ecotype was used as wild type for all phenotypic assays of rpn10-1. Unless noted otherwise, plants were grown under sterile conditions on solid Gamborg’s B5 growth medium (GM) (Gibco BRL, Gaithersburg, MD) containing 1% sucrose. Hormones were obtained from Sigma (St. Louis, MO). To reduce the poor germination of rpn10-1 seeds, phenotypic assays were performed with seeds stored at 24°C for at least 1 month before sowing. For monitoring of root elongation, lateral root formation, and root hair elongation, a minimum of 10 seedlings were transferred at 4 days after germination on GM, and the plates were held in the vertical position for 7 days. For the shoot induction experiments, root and hypocotyl segments were dissected from 5-day-old light-grown seedlings, transferred to GM containing 1 μM indoletal acetic acid with or without a range of 2-isopentyladenine concentrations, and incubated for another 2 weeks in continuous light. To test the effects of abscisic acid on postgermination development, seeds first were stratified in the dark at 4°C for 4 days on GM. The seeds then were placed under continuous irradiation at 22°C. After 1 day, the seeds were transferred to GM containing a range of abscisic acid concentrations and incubated for another 2 weeks. To test the response to NaCl and sucrose, seeds were sown directly on GM containing these compounds; after stratification, the plates were placed under continuous irradiation at 22°C for 1 week. For responses to UV-B light and mitomycin C, seedlings exposed to an 8-h-light/16-h-dark photoperiod (short days) were either placed on GM containing mitomycin C (Sigma) or irradiated every day for 14 days with 30-s or 3-min exposures to UV-B light provided by a UV Transilluminator 2000 (Bio-Rad Laboratories).

**26S Proteasome Purification and Assay**

The 26S proteasome was partially purified from 3-week-old seedlings grown in a 16-h-light/8-h-dark photoperiod. Plants were frozen to liquid nitrogen temperatures, pulverized, and ground in 1.25 volumes of buffer A (50 mM sodium phosphate, pH 8.0, 2 mM MgCl2, 10% glycerol, 1 mM 2-mercaptoethanol, 1 mM ATP, and 5% polyvinylpyrrolidone 360). After filtering through cheesecloth, the extract was clarified for 15 min at 3,000g and twice more for 20 min at 35,000g. The supernatant was subjected to fast protein liquid chromatography using a 1 × 1-cm Resource Q column (Bio-Rad) equilibrated with buffer B (buffer A without 5% polyvinylpyrrolidone 360) (Fu et al., 2001). The proteins were eluted using a gradient of 0.1 to 0.5 M NaCl in buffer B at a flow rate of 0.3 mL/min. Fractions containing the 26S proteasome were identified by peptidase activity using the substrate N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Sigma) and by immunoblot analysis with appropriate antibodies (Smalle et al., 2002).

**Immunoblot Analysis**

Immunoblot analysis was performed according to Smalle et al. (2002). Antibodies against ABI5, HY5, RPN5, RPT1, PBF1, PAC1, RPN10 (previously designated MBP1), ubiquitin, and UBC1 were as described (van Nocker et al., 1996a; Osterlund et al., 2000; Lopez-Molina et al., 2001; Smalle et al., 2002). Anti-NPTII antibodies were obtained from U.S. Biological (Swampscott, MA). Arabidopsis phytochrome A was detected with the monoclonal antibody 073D generated against oat phytochrome A (Clough et al., 1999).

**RNA Isolation, 5′ Rapid Amplification of cDNA Ends PCR, and RNA Gel Blot Analysis**

Total RNA was isolated and subjected to gel blot analysis according to Smalle et al. (2002). 5′ rapid amplification of cDNA ends was conducted as described by Babychuk et al. (1997). 32P-labeled riboprobes were synthesized with T7 or SP6 RNA polymerase using the appropriate linearized plasmids and the Riboprobe Gemini II core system (Promega, Madison, WI). The CYCD3, βTUB4, ABR5, NIA1, and CDKA;1 templates were as described (Smalle et al., 2002). The RPN10 template used the pJSRN10 plasmid linearized with SpeI-PstI. This plasmid was created by PCR amplification of the RPN10 cDNA from a cDNA library using primers 5′-TATGCAGACGATGTTCTGGAGGCGATCTAT-3′ and 5′-AATTCAGTTCAACTCTATCACAACAAAG-3′ and insertion of the product into pGEM-T (Promega). The NPTII template used the pJSNPTII plasmid linearized with Apal-Ncol. This plasmid was created by PCR amplification of the NPTII coding frame from the genomic DNA of the rpn12a-1 mutant (Smalle et al., 2002) using primers 5′-TGCCCTGAATGAACTGCTTCGTGAGAAGG-3′ and 5′-AATTCAGTTCAACTCTATCACAACAAAG-3′ and insertion of the product into pGEM-T. The ABI5 plasmid used the pJSABI5 plasmid linearized with Nott-Ncol. This plasmid was created by PCR amplification of the ABI5 coding region from Columbia genomic DNA using primers 5′-TCTTACTGAGAAGAAGA-3′ and 5′-TGTCAGTGTGGTCTCAGAAGGAC-3′ and insertion of the product into pGEM-T. The CDK1;1, CDKD1;3, and ICK1/KRP1 templates used the EST clones 105H14, 122J8, and 156J24, respectively, linearized with KpnI-PstI. RNA gel blot signal intensities were estimated using the NIH Image program (National Institutes of Health, Bethesda, MD).

**rpn10-1 Complementation**

The RPN10 cDNA was amplified by PCR using primers 5′-TCTCTC-TGCTATGACGAGATGTTCTTG-3′ and 5′-AATTCAGTTCAACTC-

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TAGATCAAACAAAGAAACCA-3′ designed to contain an XbaI site at both ends. The resulting product was digested with XbaI and inserted downstream of the Cauliflower mosaic virus 35S promoter into the binary T-DNA vector pGSVE9, which was digested similarly (E. Babychuk and S. Kushnir, unpublished data). The vector was introduced into Agrobacterium tumefaciens strain AGL1(pMP90), which was used to transform homozygous rpnl1-1 seedlings by the floral-dip method. The presence of the 3SS-RPN10 transgene and the rpnl1-1 mutation were followed by hygromycin and kanamycin resistance, respectively. T3 progeny resulting from self-crosses that were homozygous for both kanamycin and hygromycin resistance were analyzed for phenotypic complementation.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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