

RESEARCH ARTICLE

Cytokinin Growth Responses in Arabidopsis Involve the 26S Proteasome Subunit RPN12

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The 26S proteasome is an ATP-dependent eukaryotic protease responsible for degrading many important cell regulators, especially those conjugated with multiple ubiquitins. Bound on both ends of the 20S core protease is a multisubunit regulatory particle that plays a crucial role in substrate selection by an as yet unknown mechanism(s). Here, we show that the RPN12 subunit of the Arabidopsis regulatory particle is involved in cytokinin responses. A T-DNA insertion mutant that affects *RPN12a* has a decreased rate of leaf formation, reduced root elongation, delayed skotomorphogenesis, and altered growth responses to exogenous cytokinins, suggesting that the mutant has decreased sensitivity to the hormone. The cytokinin-inducible genes *CYCD3* and *NIA1* are upregulated constitutively in *rpn12a-1*, indicating that feedback-inhibitory mechanisms also may be altered. *rpn12a-1* seedlings also showed changes in auxin-induced growth responses, further illustrating the close interaction between auxin and cytokinin regulation. In yeast, RPN12 is necessary for the G1/S and G2/M transitions of the cell cycle, phases that have been shown to be under cytokinin control in plants. We propose that RPN12a is part of the Arabidopsis 26S proteasome that controls the stability of one or more of the factors involved in cytokinin regulation.

INTRODUCTION

Regulated protein turnover provides a mechanism to adjust rapidly to changing ligand concentrations and/or environmental conditions and is essential for many signal response pathways. In eukaryotes, the ubiquitin/26S proteasome pathway is particularly important, being responsible for removing most short-lived intracellular proteins (Hershko and Ciechanover, 1998; Callis and Vierstra, 2000). In this proteolytic pathway, proteins committed for degradation first are modified by the covalent attachment of multiple ubiquitins. This conjugation is directed by an ATP-dependent reaction cascade involving the sequential action of E1s, E2s, and E3s, which ultimately attach one or more ubiquitins to appropriate targets. In most cases, the ubiquitinated proteins then are recognized and degraded by the 26S protea-

some, a multisubunit ATP-dependent protease with broad substrate specificity.

The 26S proteasome is a 2-MD complex assembled from two particles: the 20S core particle (CP) and the 19S regulatory particle (RP) (Voges et al., 1999). The proteolytic activities reside within the central chamber of the 28-subunit CP, whereas the functions that direct substrate recognition, unfolding, and subsequent entry into the 20S particle reside within the >18-subunit RP. The RP can be divided further in two subcomplexes, the lid and base (Glickman et al., 1998). The base contains six ATPase subunits, RPT1 to RPT6, which presumably use ATP hydrolysis to unfold target proteins, and three non-ATPase subunits, RPN1, RPN2, and RPN10. The lid contains nine additional RPN subunits (RPN3, RPN5 to RPN9, and RPN11 to RPN13). Many of the lid RPN subunits share sequence motifs with components of the COP9/signalosome and EIF3 complexes, implying a common ancestry (Glickman et al., 1998; Fu et al., 2001). To date, the roles of only two RPN subunits are known. RPN13 (also known as UCH37) can disassemble multiubiquitin chains, suggesting that it releases the ubiquitin moieties before target breakdown (Voges et al., 1999). RPN10 appears

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to help tether the lid to the base and may participate in recognizing multiubiquitinated proteins before digestion (Fu et al., 1998a; Glickman et al., 1998; Fu et al., 2001).

A growing body of evidence indicates that the ubiquitin/26S proteasome pathway controls the levels of many important fungal and animal regulatory processes. Targets include key checkpoint proteins within the cell cycle and components of numerous hormone signaling systems (Hershko and Ciechanover, 1998). Recent studies also have implicated the pathway in environmental and developmental responses in plants (Callis and Vierstra, 2000). Deletion of *RPN10* in *Physcomitrella patens* blocks the transition from the vegetative to the reproductive phase of the protonema (Girod et al., 1999). A number of Arabidopsis mutations in ubiquitin ligation have been described that affect floral development, male gametogenesis, photomorphogenesis, and circadian rhythms (Callis and Vierstra, 2000; Dieterle et al., 2001). Two mutants of particular interest for hormone regulation are *transport inhibitor response-1 (tir1)* and *coronatine insensitive-1 (coi1)*. They identify E3 genes that are required specifically for auxin and jasmonate sensitivity, respectively (Xie et al., 1998; Gray and Estelle, 2000). Furthermore, the detection of ubiquitin conjugates and the use of 26S proteasome inhibitors and dominant-negative ubiquitin mutants have implicated the pathway in leaf development, defense responses, abscisic acid signaling, and control of phytochrome A and cyclin A and B levels (Bachmair et al., 1990; Genschik et al., 1998; Clough et al., 1999; Becker et al., 2000; Lopez-Molina et al., 2001).

Another important signaling pathway in plants controls responses to the cytokinin family of hormones. Cytokinins regulate numerous developmental processes, including seed germination, photomorphogenesis, root/shoot differentiation, apical dominance, sink-to-source relations, senescence, and cell division (Mok and Mok, 1994). Although the phenotypic effects of exogenous cytokinins have been studied thoroughly, the underpinning sensory transduction chain(s) is not well understood. In Arabidopsis, cytokinins are detected by CRE1 and possibly other receptors related to bacterial two-component histidine kinases (Inoue et al., 2001). Several response regulators have been identified as cytokinin-inducible genes (Brandstatter and Kieber, 1998; Taniguchi et al., 1998) that could serve as the phosphoacceptors for the activated histidine kinases. Further downstream, cytokinin stimulates the G1/S transition of the cell cycle, which is possibly mediated in part by the transcriptional induction of the *CYCD3* gene encoding a D-type cyclin (Riou-Khamlichi et al., 1999). Because cytokinins also are required for the G2/M transition, it is expected that they activate or repress additional cell cycle regulators (Mironov et al., 1999). The hormone auxin also is known to affect plant cell division. In fact, auxins and cytokinins often act synergistically, and several of the auxin response mutants also have altered responses to cytokinins (Coenen and Lomax, 1997).

In an attempt to define how the ubiquitin/26S proteasome pathway participates in plant growth and development, we

have begun to systematically isolate and characterize Arabidopsis mutants affecting various components. In particular, we have focused on the 26S proteasome, given its central role in substrate breakdown. Initial studies identified all of the CP subunits and most of the RP subunits and showed that the Arabidopsis complex is remarkably similar in structure and function to those described in fungi and animals (Fu et al., 1999b). Here, we describe the RPN12a subunit within the lid of the Arabidopsis RP. RPN12 is potentially encoded by two genes, one that encompasses the entire polypeptide (*RPN12a*) and another that encodes an N-terminal truncated version (*RPN12b*). Studies on yeast *rpn12* mutants indicate that this essential subunit plays a special role in the cell cycle by helping to selectively degrade the Clb-specific Cdc28 kinase inhibitor Sic1, whose loss is required for the G1/S phase transition (Bailey and Reed, 1999). From analysis of the T-DNA mutant *rpn12a-1*, we show that Arabidopsis RPN12 is important for numerous cytokinin-regulated growth responses. The phenotypes suggest that RPN12a in particular, and the 26S proteasome in general, is required to degrade one or more repressors of cytokinin action in plants.

RESULTS

Arabidopsis 26S Proteasome Subunit RPN12

Using the subunit sequences of the *Saccharomyces cerevisiae* 26S proteasome as queries, we have identified the genes encoding all of the corresponding subunits of the Arabidopsis complex in the nearly complete genomic sequence (Fu et al., 1998b, 1999a, 1999b; H. Fu, J. Smalle, and R.D. Vierstra, unpublished data). For clarity, we have adopted the nomenclature recently proposed for subunits of the yeast 26S proteasome complex (Finley et al., 1998). Similar to what was found for subunits of the CP, most of the Arabidopsis RP subunits are encoded by two genes (the exceptions being RPT3, RPN6, RPN7, and RPN10).

An unusual pair of Arabidopsis genes is related to yeast *RPN12*. In addition to one locus encoding a full-length version (*RPN12a*), a second gene appears to synthesize a truncated form (*RPN12b*) missing a substantial portion from the N-terminal end (Figure 1A). The predicted *RPN12a* gene contains five introns and encodes a 267-amino acid protein. Comparison of the amino acid sequence of RPN12a with orthologs from various fungi and animals revealed similarity over their entire lengths (Figure 1B). For example, the overall amino acid similarity of Arabidopsis RPN12a to its yeast and human orthologs is 35 and 47%, respectively. Close relatives of RPN12a also were found in the expressed sequence tag (EST) databases of other plant species, including tomato, cotton, sugar beet, maize, wheat, barley, rice, potato, soybean, *Lotus japonicum*, and *Medicago truncatula* (data not shown). The presence of numerous cDNAs in various

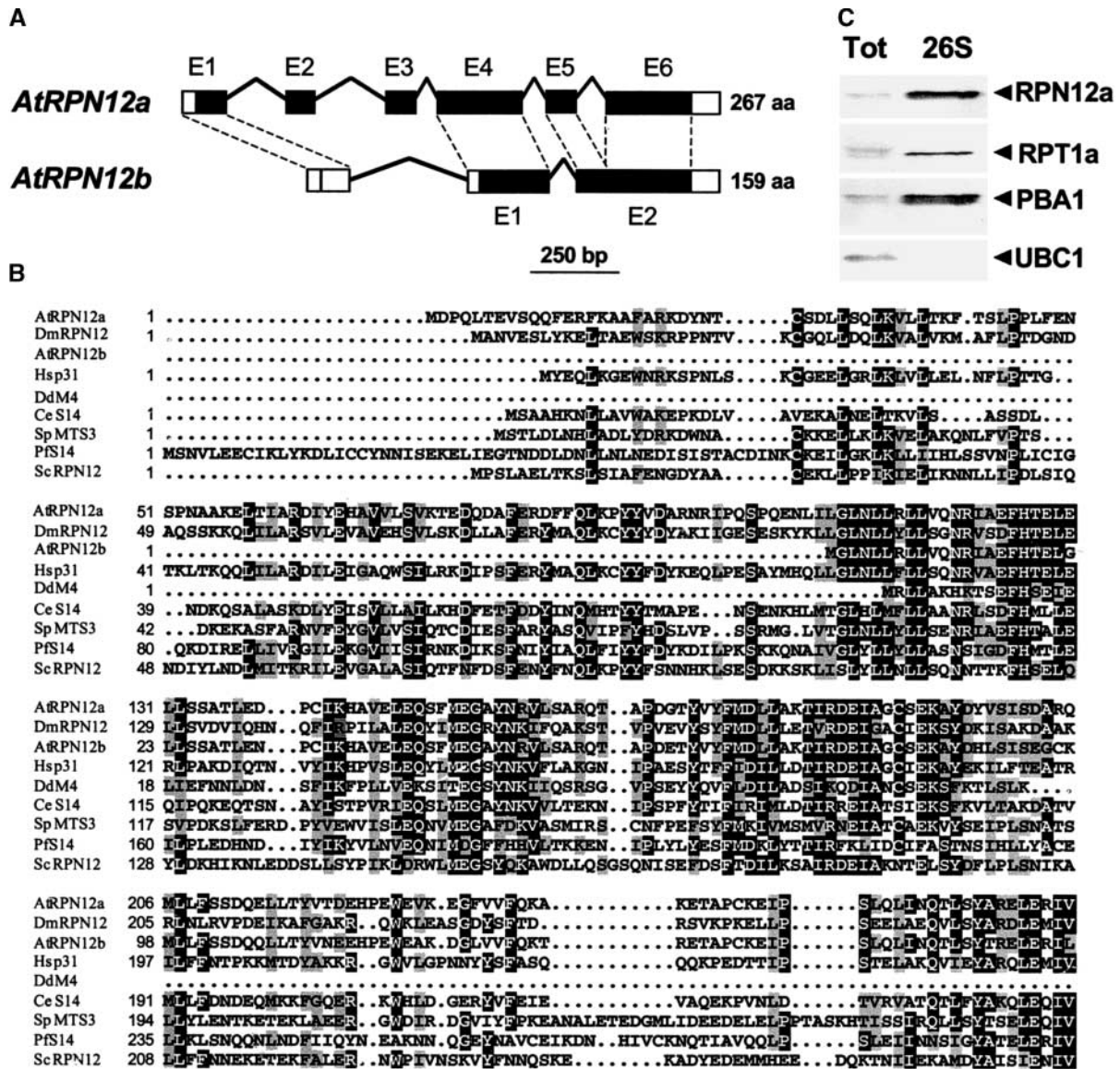


Figure 1. Organization and Derived Amino Acid Sequence Comparisons of the Arabidopsis *RPN12a* and *RPN12b* Genes and Association of the *RPN12a* Protein with the 26S Proteasome.

(A) Genomic organization of Arabidopsis *RPN12a* and predicted *RPN12b*. Boxes denote exons (designated E1 to E6), with open and black boxes indicating noncoding and coding regions, respectively. Lines show positions and lengths of introns. Dashed lines show exon relationships for *RPN12a* and *RPN12b*. aa, amino acids.

(B) Derived amino acid sequence comparison of *RPN12a* and *RPN12b* with possible orthologs from animals and fungi. Identical and similar residues are shown in reverse type and gray boxes, respectively. Sequences include those for *Drosophila melanogaster* DmRPN12, *Homo sapiens* Hsp31, *Dictyostelium discoideum* DdM4, *Caenorhabditis elegans* CeS14, *Schizosaccharomyces pombe* SpMTS3, *Plasmodium falciparum* PfS14, and *S. cerevisiae* ScRPN12.

(C) Copurification of *RPN12a* with other subunits of the Arabidopsis 26S proteasome. The Arabidopsis 26S proteasome was enriched by sequential polyethylene glycol precipitations followed by size exclusion fast protein liquid chromatography (FPLC). Both the total crude extract (Tot) and the FPLC fraction with highest CP peptidase activity (26S) were subjected to SDS-PAGE and immunoblot analysis with antibodies prepared against the Arabidopsis 26S proteasome subunits RPT1a, *RPN12a*, PBA1, and a control protein, the Arabidopsis E2 UBC1.

Arabidopsis EST collections indicated that *RPN12a* is transcribed actively in many tissue types. This was confirmed by RNA gel blot analysis, which detected the corresponding *RPN12a* mRNA of 1.1 kb in all Arabidopsis organs tested (Figure 2B and data not shown).

To verify that the RPN12a protein is part of the Arabidopsis 26S proteasome, the complex was partially purified from young green Arabidopsis seedlings by sequential polyethyl-

ene glycol precipitations followed by size exclusion fast protein liquid chromatography (FPLC) and assayed with anti-RPN12a antibodies. In accordance with previous mass determinations of the complex (Peng et al., 2001), the Arabidopsis 26S proteasome behaved as a 2-MD particle during size exclusion chromatography. The RPN12a protein of the expected size (31 kD) could be detected in fractions also enriched for CP peptidase activity (using the substrate Suc-

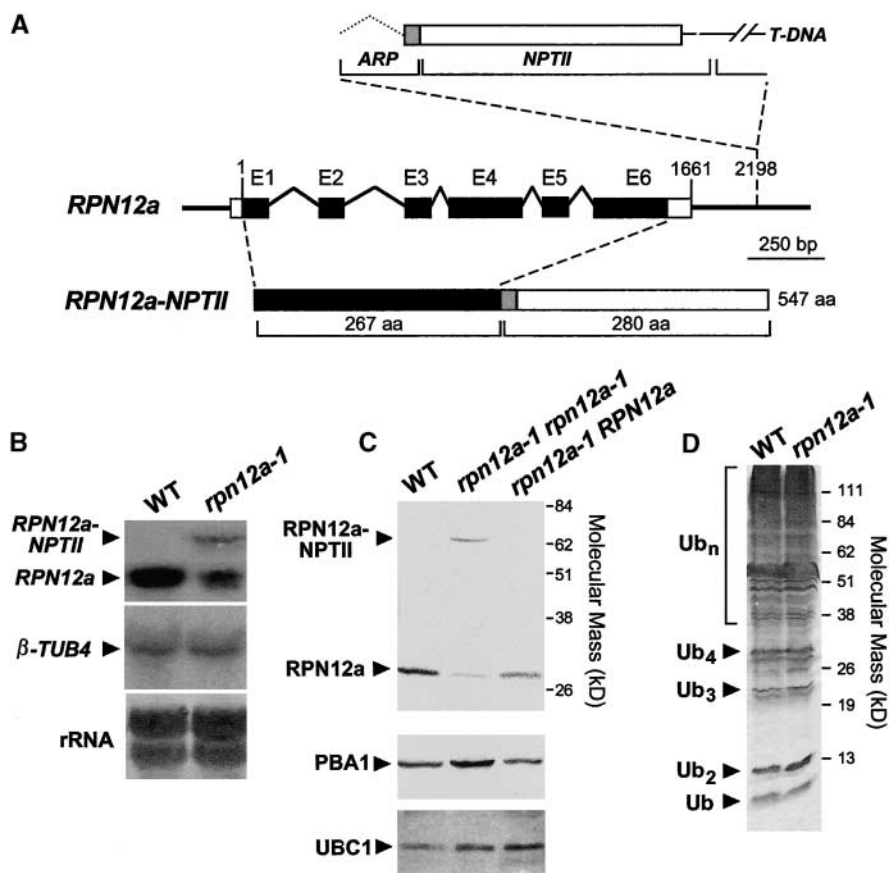


Figure 2. Molecular and Biochemical Descriptions of the Arabidopsis *rpn12a-1* Mutation.

(A) Scheme of the *RPN12a* gene and the predicted protein product of *rpn12a-1*, a RPN12a-NPTII translational fusion protein. Boxes and lines denote exons and predicted introns, respectively. Upon insertion of the T-DNA, the 3' untranslated sequence of the *RPN12a* gene (bp 1661 to 2198) and the 5' end of the *ARP-NPTII* gene from the T-DNA insertion form a chimeric intron that when spliced creates the translational fusion gene (*RPN12a-NPTII*) containing the full coding regions of *RPN12a* and the *ARP-NPTII* genes (Babychuk et al., 1997). aa, amino acid.

(B) Detection of *RPN12a* transcripts in wild-type (WT) and *rpn12a-1* seedlings by RNA gel blot analysis using a *RPN12a* gene probe. Arrowheads indicate the mRNA for wild-type *RPN12a* and the chimeric *RPN12a-NPTII* transcript. Equal amounts of total RNA were analyzed; loading was confirmed by staining for rRNA with methylene blue and by reprobing the blots with DNA encoding β -tubulin (β -TUB4).

(C) Immunoblot detection of the RPN12a protein. Total protein was extracted from wild-type seedlings and seedlings either hemizygous or homozygous for the *rpn12a-1* mutation and subjected to SDS-PAGE and immunoblot analysis with anti-RPN12a antibodies. Arrowheads indicate RPN12a and the RPN12a-NPTII fusion protein. Control immunoblot analysis was performed with antibodies against the CP β -subunit PBA1 and UBC1.

(D) Levels of ubiquitin (Ub) and ubiquitin-protein conjugates in wild-type and *rpn12a-1* seedlings. Equal amounts of total protein were subjected to SDS-PAGE and immunoblot analysis with anti-ubiquitin antibodies. The positions of the free ubiquitin, free multiubiquitin chains containing two to four monomers, and multiubiquitinated proteins are indicated.

LLVY-MCA) and other subunits of the 26S proteasome, including the RP subunit RPT1a and the CP subunit PBA1 (Figure 1C and data not shown).

Compared with *RPN12a*, the *RPN12b* gene is missing the second and third exons along with two introns (Figure 1A). Conceptual translation of this gene suggested that the resulting RPN12b protein would start at a methionine 102 residues downstream from the start of RPN12a, thus generating a truncated 18-kD protein containing just 159 amino acids (Figure 1B). When this shorter sequence was aligned with RPN12a, the two proteins displayed 87% amino acid identity and 98% amino acid similarity. There is no direct evidence that *RPN12b* is expressed, because we have been unable to find *RPN12b* cDNAs in the Arabidopsis EST collections or to detect the *RPN12b* transcript and protein in green Arabidopsis seedlings (data not shown).

T-DNA Insertion Mutation Affecting *RPN12a* Expression

To help identify the function(s) of RPN12, we characterized a T-DNA insertion mutant affecting *RPN12a* that was created by exon-trap mutagenesis (Babiychuk et al., 1997). The exon-trap vector pSL4 used here contained a fusion of the first intron of the apurinic endonuclease gene (*ARP*) lacking its 5' donor splice site followed by a coding region containing exon 2 of *ARP* appended in frame to that for neomycin phosphotransferase II (*NPTII*) lacking its translation initiation codon (Figure 2A). The *ARP-NPTII* fusion gene was placed immediately after the right border of the T-DNA. Previous studies showed that upon transformation of Arabidopsis with the pSL4 T-DNA, kanamycin resistance created by the expression of *NPTII* was possible only when the T-DNA integrated into a host gene that provided the promoter activity, the translation start site, and a 5' donor intron splice site. This splice site enabled removal of the *ARP* intron to allow translation of the ARP-NPTII protein using the upstream, host-derived ATG codon. From 5' rapid amplification of cDNA ends polymerase chain reaction (PCR) analysis of ~400 independent kanamycin-resistant transgenic lines of Arabidopsis ecotype C24, ~90% had the T-DNA integrated within known or predicted gene-coding loci (Babiychuk et al., 1997; data not shown).

Sequence analysis of the 5' rapid amplification of cDNA ends product from the kanamycin-resistant line SL42-38 (now designated *rpn12a-1*) revealed the presence of a chimeric mRNA containing the *ARP-NPTII* coding region fused to the full coding region for *RPN12a*. RNA gel blots showed a single *NPTII* mRNA, indicating that *rpn12a-1* contains only one transcriptionally active insertion. PCR analysis of genomic DNA using *RPN12a*- and *NPTII*-specific primers showed that the T-DNA was inserted 531-bp downstream of the *RPN12a* stop codon. We assume that this insertion created a chimeric intron in which the end of the *RPN12a* coding region provided the 5' donor splice site (AG/GT) for the *ARP*

intron (Figure 2A). RNA gel blot analysis of *rpn12a-1* plants using *RPN12a* or *NPTII* probes detected the predicted fusion mRNA of 1.8 kb (Figure 2B and data not shown). The *RPN12a* probe also hybridized to a smaller, more abundant transcript indistinguishable in size from that of the wild-type *RPN12a* mRNA (Figure 2B). We assume that the two mRNAs were created by differential transcript processing and/or termination using either the polyadenylation signal within the 3' untranslated region of the *RPN12a* gene or the signal downstream of the *NPTII* coding region.

Immunoblot analysis with anti-RPN12a antibodies detected both the RPN12a-NPTII fusion and wild-type RPN12a proteins in homozygous *rpn12a-1* plants (Figure 2C), consistent with the presence of two mRNAs. Despite an apparently lower level of the *RPN12a-NPTII* mRNA, the levels of the corresponding proteins were nearly equal. This similar abundance was observed in all tissues and developmental stages examined (data not shown), suggesting that the mutation affected most, if not all, cell types. Interestingly, the RPN12a-NPTII fusion protein assembled into the 26S proteasome. As can be seen in Figure 3, the fusion protein coeluted during size exclusion FPLC in a 2-MD complex with CP peptidase activity and with wild-type RPN12a and other subunits of the 26S complex, including RPN10, RPN5a, and RPT1a of the RP and PBA1 and PAC1 of the CP. (The slight tailing of RPN10 and RPN5a suggested that they partially dissociate from the lid during FPLC, an effect observed previously for yeast RPN10 [van Nocker et al., 1996b].) The fact that RPN12a-NPTII coeluted with both the lid (RPN12a and RPN5a) and base (RPT1 and RPN10) subunits and with the CP suggested that the *rpn12a-1* mutation did not appreciably alter the structure and subunit composition of the allied 26S complex.

Whereas both forms of RPN12a could be detected in homozygous *rpn12a-1* plants, only the wild-type version could be seen in hemizygous plants (Figure 2C). Overexpression of wild-type *RPN12a* in plants homozygous for *rpn12a-1* also resulted in the disappearance of the RPN12a-NPTII fusion protein (see below). These observations suggested that wild-type RPN12a can competitively block the association of the RPN12a-NPTII fusion protein with the 26S complex; the free fusion protein then is subjected to rapid degradation. However, even hemizygous *rpn12a-1* plants are kanamycin resistant, indicating that these plants retained sufficient amounts of NPTII activity even though the RPN12a-NPTII fusion protein was undetectable.

The incorporation of both forms of RPN12a into the 26S proteasome of homozygous *rpn12a-1* plants implied that two populations of 26S complexes existed. Thus, instead of causing a strong 26S proteasome defect, it is likely that the *rpn12a-1* mutation causes a more subtle change in proteasome activity. This possibility was supported by immunological measurements with anti-ubiquitin antibodies. Whereas other ubiquitin/26S proteasome pathway mutants often increase the steady state levels of ubiquitinated proteins (Kominami et al., 1995; Bailly and Reed, 1999; Girod et al.,

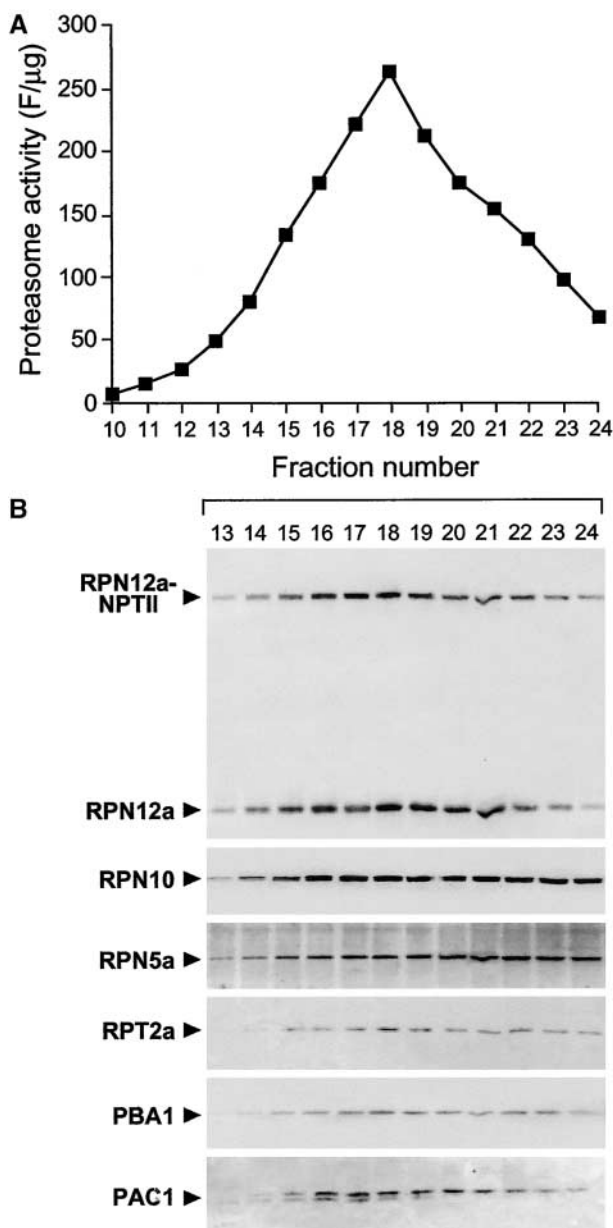


Figure 3. Association of the RPN12a-1-NPTII Fusion Protein with the 26S Proteasome.

The 26S proteasome was partially purified from Arabidopsis seedlings homozygous for the *rpn12a-1* mutation by sequential polyethylene glycol precipitations and subjected to size exclusion FPLC.

(A) Column fractions were assayed for CP peptidase activity. Only the portion of the FPLC elution profile around the peak of peptidase activity is shown.

(B) Column fractions subjected to SDS-PAGE and immunoblot analysis with antibodies against RPN12a, RPN10, RPN5a, RPT2a subunits of the RP, and PBA1 and PAC1 subunits of the CP. The positions of the wild-type RPN12a and the RPN12a-NPTII fusion protein are indicated by arrowheads.

1999; Doelling et al., 2001), these levels remained indistinguishable from wild-type levels in homozygous *rpn12a-1* plants (Figure 2D). This lack of effect suggested that the mutation does not perturb the function of the Arabidopsis 26S proteasome globally and thus may affect RPN12a-related functions more specifically.

Development of *rpn12a-1* Seedlings

Phenotypic analysis of homozygous *rpn12a-1* seedlings indicated that the T-DNA insertion altered Arabidopsis seedling development substantially. In all cases, the mutation behaved as a recessive trait, consistent with the presence of the RPN12a-NPTII fusion protein only in homozygous *rpn12a-1* plants. Young *rpn12a-1* seedlings grown under continuous white light accumulated 2.5 times more anthocyanins in both hypocotyls and petioles (Figure 4A) ($A_{530} - A_{650}$ for 10 seedlings was 0.032 ± 0.001 for the wild type versus 0.083 ± 0.003 for *rpn12a-1*). An increased percentage of *rpn12a-1* seedlings developed with a single cotyledon compared with wild type (2.4% versus <0.05%) (Figure 4B). This higher percentage was observed in all *rpn12a-1* lines created after backcrossing to wild type, indicating a linkage to the mutation. The *rpn12a-1* plants showed a slower rate of rosette leaf emergence (Figure 4C) and a delay in flowering time (33.3 ± 3.1 days for *rpn12a-1* versus 25.2 ± 2.1 days for wild type). During the first week of growth, *rpn12a-1* roots grew more rapidly than wild-type roots. Thereafter, they elongated more slowly, such that after 14 days *rpn12a-1* roots were significantly shorter than wild-type roots (Figure 4D). Dark-grown *rpn12a-1* seedlings showed a delay in skotomorphogenesis, with a reduced rate of cotyledon opening and petiole elongation (Figure 4E).

rpn12a-1 Plants Have Altered Growth Responses to Exogenous Cytokinins

Some of the phenotypes associated with the *rpn12a-1* mutation were suggestive of an attenuated cytokinin response (e.g., delayed shoot development and skotomorphogenesis, increased percentage of monocotyledonous seedlings [Chaudhury et al., 1993; Chory et al., 1994; Mok and Mok, 1994]). Therefore, we tested the effect of exogenous cytokinins on *rpn12a-1* seedlings. As can be seen in Figure 5A, wild-type seedlings were strongly inhibited in their growth and eventually became chlorotic when germinated on medium containing 0.1 μ M kinetin. In contrast, *rpn12a-1* seedlings were much less affected. This difference in growth inhibition was not restricted to germinating seedlings. Shoot growth of *rpn12a-1* seedlings germinated first in the absence of cytokinins was less sensitive to kinetin when the seedlings were transferred subsequently to medium containing the hormone (Figure 5B).

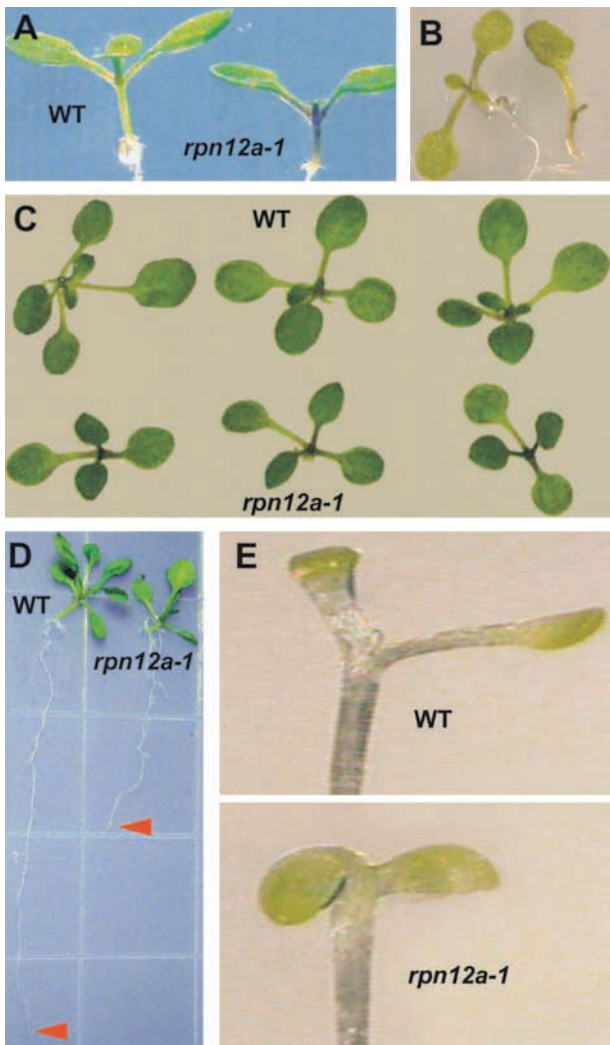


Figure 4. Developmental Changes Induced by the *rpn12a-1* Mutation.

- (A) Increased accumulation of anthocyanins in hypocotyls of 1-week-old *rpn12a-1* seedlings. WT, wild type.
- (B) Example of a monocotyledonous seedling in an *rpn12a-1* population. Left, normal seedling; right, seedling with one cotyledon.
- (C) Delayed leaf emergence of *rpn12a-1* plants after 14 days of growth. Both wild-type and *rpn12a-1* seedlings germinated at the same time.
- (D) Reduced root elongation of an *rpn12a-1* seedling after 14 days of growth. Red arrowheads indicate the root tips.
- (E) Reduced cotyledon opening and petiole growth of an etiolated *rpn12a-1* seedling after 10 days of growth in darkness.

Cytokinins are inhibitors of root elongation growth (Cary et al., 1995). When wild-type plants germinated in the absence of cytokinin were transferred to plates containing 200 nM or more kinetin, a dramatic reduction in root elongation was evident (Figures 5C and 5D). Surprisingly, the *rpn12a-1*

mutant displayed the opposite effect. Whereas *rpn12a-1* roots were significantly shorter than wild-type roots without kinetin treatment, the addition of low concentrations increased root growth substantially, with 0.2 μ M kinetin restoring elongation to nearly the level seen for wild-type plants without hormone (Figures 5C and 5D). Higher concentrations became inhibitory, such that *rpn12a-1* roots treated with 0.8 μ M kinetin were as short as wild-type roots treated with the same concentration. Another root response to cytokinin treatment is the production and elongation of root hairs, probably via the induction of ethylene biosynthesis (Cary et al., 1995). Whereas the roots of the wild type became densely covered with longer hairs after treatment with 0.2 μ M kinetin, *rpn12a-1* roots did not show such a dramatic effect (Figure 5E).

Cytokinin application is known to induce deetiolation in dark-grown seedlings, characterized by accelerated opening of the cotyledons, elongation of the petioles, and development of true leaves (Chory et al., 1994). Whereas wild-type *Arabidopsis* seedlings showed these effects when treated with 20 μ M concentrations of the cytokinin benzyladenine (BA), this response was delayed severely in *rpn12a-1* seedlings (Figure 5F). However, there was no difference between mutant and wild type for the ability of cytokinins to inhibit hypocotyl elongation of etiolated seedlings (data not shown).

Although most of the growth response assays suggested a decrease in cytokinin sensitivity for *rpn12a-1* seedlings, root elongation was more complicated to interpret. To further analyze the cytokinin insensitivity, we tested the ability of 2-isopentenyladenine (2iP) to induce greening and shoot formation in hypocotyls and root segments (Mok and Mok, 1994). Wild-type hypocotyls and root segments responded to the 2iP by becoming green, which was reduced noticeably in the *rpn12a-1* mutant (Figures 5G and 5H). Whereas 1 to 4 μ M 2iP also strongly stimulated shoot formation in wild-type hypocotyls, the mutant hypocotyls failed to develop shoots at any 2iP concentration tested (Figure 5G). Thus, it appears that both roots and shoots from *rpn12a-1* have decreased sensitivity to cytokinins.

Cytokinin-Inducible Genes *CYCD3* and *NIA1* Are Upregulated in the *rpn12a-1* Mutant

To analyze the cytokinin response at the molecular level, we measured the abundance of the cytokinin-inducible transcripts *CYCD3*, *NIA1*, and *ARR5*. Based on the reduced cytokinin sensitivity of the *rpn12a-1* seedlings, we predicted that the cytokinin-induced accumulation of all three mRNAs would be attenuated as well. *CYCD3* and *NIA1* encode a D-type cyclin and nitrate reductase, respectively, and both show prolonged transcriptional upregulation by cytokinins (Yu et al., 1998; Riou-Khamlichi et al., 1999). Figure 6A confirmed this effect in wild-type plants. Compared with control mRNAs for *CDC2a* and β -*TUB4*, 20 μ M kinetin increased

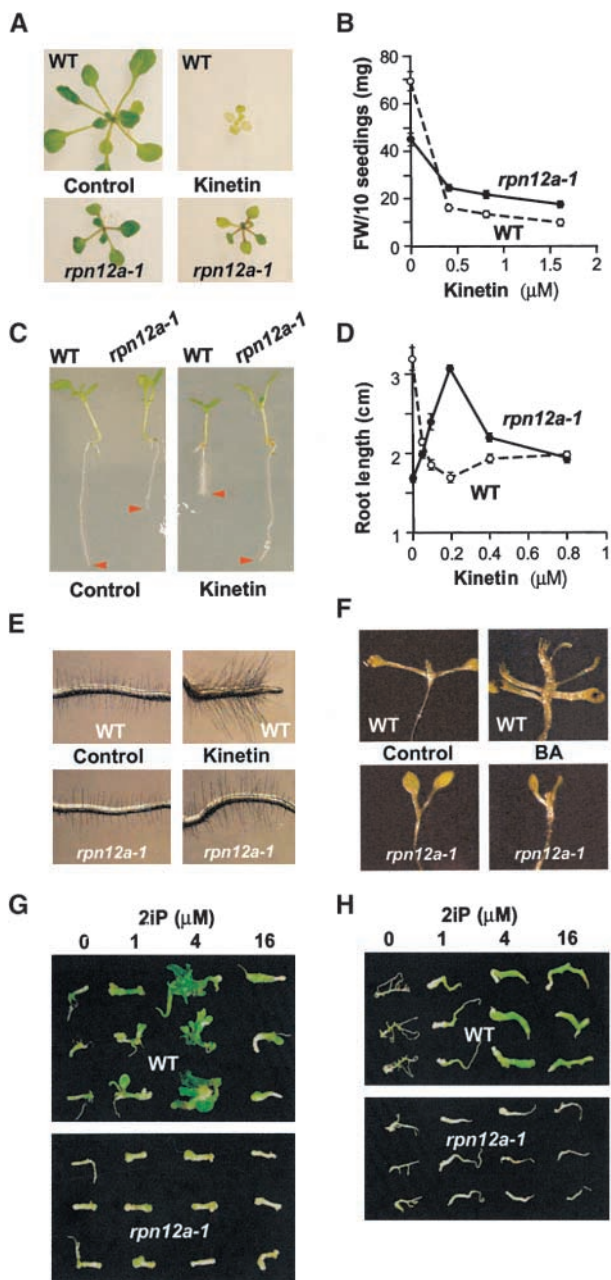


Figure 5. The *rpn12a-1* Mutant Has Decreased Sensitivity to the Cytokinins Kinetin, BA, and 2iP.

(A) Shoot growth inhibition and chlorosis induced by kinetin is stronger in wild-type (WT) than in *rpn12a-1* seedlings. Plants were germinated and maintained for 14 days on 0.1 μM kinetin.

(B) Inhibition of shoot growth of wild-type and *rpn12a-1* seedlings by a range of kinetin concentrations. Seedlings were germinated in the absence of kinetin and then transferred to medium containing the indicated kinetin concentrations. After 21 days, the mean fresh weights of rosettes from 10 seedlings were determined (\pm SD). FW, fresh weight.

(C) The *rpn12a-1* mutation alters the effect of kinetin on root elonga-

tion. The mRNA levels substantially for *CYCD3* and *NIA1* in seedlings after a 4-hr treatment. Surprisingly, when the *rpn12a-1* seedlings were tested, the levels of *CYCD3* and *NIA1* mRNAs were upregulated even in the absence of hormone (Figure 6A). In fact, the addition of 20 μM kinetin did not further increase the level of the *CYCD3* mRNA and only increased the level of *NIA1* mRNA modestly compared with that in untreated *rpn12a-1* plants.

Analysis of the *ARR5* mRNA showed that this constitutive upregulation does not occur for all cytokinin-regulated transcripts. *ARR5* encodes a response regulator presumably associated with the cytokinin sensory transduction chain and displays a strong and rapid, but transient, increase in mRNA accumulation after exposure to the cytokinin BA (Brandstatter and Kieber, 1998). For this mRNA, *rpn12a-1* plants responded like the wild type. Both contained low levels of the *ARR5* mRNA without hormone and showed a strong increase after a 15-min exposure to 5 μM BA (Figure 6B).

RNA gel blot analysis revealed that the level of the *RPN12a* transcript also was upregulated modestly (approximately twofold) by kinetin (Figure 6A). To determine whether this effect was specific for *RPN12a* or included other subunits of the 26S proteasome, we measured the expression level of the *PBA1* and *PBF1* genes, which encode β -subunits of the CP (Figure 6C). Levels of both mRNAs, like that of *RPN12a*, were increased by the treatment of seedlings with 20 μM kinetin.

Seeds were germinated on growth medium, and after 4 days seedlings were transferred to plates containing 0.2 μM kinetin. The red arrowheads indicate root tips after an additional 4 days of growth. **(D)** Effect of a range of kinetin concentrations on root elongation. Wild-type and *rpn12a-1* seeds were germinated on growth medium, and after 4 days seedlings were transferred to plates containing the indicated kinetin concentrations. After an additional 14 days of growth, the mean root lengths of 10 plants were determined (\pm SD). **(E)** The promotion of root hair formation and elongation by kinetin is reduced by the *rpn12a-1* mutation. Seeds were germinated on growth medium. After 4 days, seedlings were transferred to medium containing 0.2 μM kinetin and grown for an additional 4 days. **(F)** Promotion of deetiolation by BA is delayed by the *rpn12a-1* mutation. Wild-type and *rpn12a-1* seedlings were grown for 28 days in the dark in the presence or absence of 20 μM BA.

(G) The induction of greening and shoot formation by 2iP is decreased in *rpn12a-1* hypocotyls. Hypocotyl segments of 5-day-old seedlings were incubated for 2 weeks in the presence of 1 μM indole-3-acetic acid supplemented with a range of 2iP concentrations. **(H)** The induction of greening by 2iP is decreased in *rpn12a-1* roots. Root segments of 5-day-old wild-type and *rpn12a-1* seedlings were incubated for 2 weeks in the presence of 1 μM indole-3-acetic acid supplemented with a range of 2iP concentrations.

In both **(G)** and **(H)**, representative segments from separate 2iP treatments were aligned before photography.

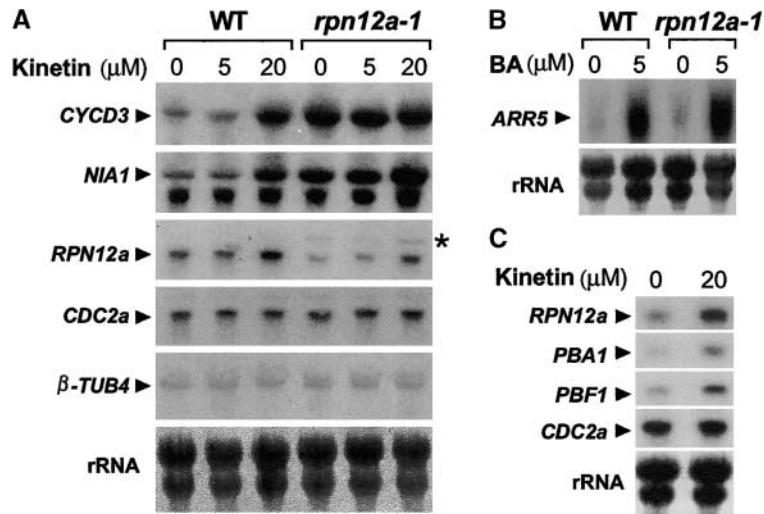


Figure 6. Expression of Specific Cytokinin-Inducible Genes Is Altered by the *rpn12a-1* Mutation.

Wild-type (WT) and *rpn12a-1* seedlings were grown in liquid medium for 14 days and then treated with the cytokinin kinetin or BA. After the treatments, total RNA was extracted and subjected to RNA gel blot analysis. Probes included sequences for the 26S proteasome genes *RPN12a*, *PBA1*, and *PBF1*, the cytokinin-inducible genes *NIA1*, *ARR5*, and *CYCD3*, and the cytokinin-noninducible genes *CDC2a* and β -*TUB4*. Equal loading of RNA was verified by methylene blue detection of rRNA. Autoradiographic exposure times were varied to best reflect the changes in expression patterns. For each panel, representative blots from at least three independent experiments are shown.

(A) and **(C)** Seedlings treated for 6 hr with various concentrations of kinetin. The asterisk indicates the position of the *RPN12a-NPTII* fusion transcript.

(B) Seedlings treated for 15 min with 5 μ M BA.

rpn12a-1 Roots Have Decreased Auxin Sensitivity

Several plant hormone response mutants have altered sensitivity to more than one hormone, indicating a close interplay among the associated signaling pathways (Coenen and Lomax, 1997). Consequently, we tested the response of *rpn12a-1* seedlings to other hormones, including 1 to 20 nM of the synthetic auxin 2,4-D, 0.01 to 10 μ M abscisic acid, 0.01 to 1 μ M of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, 0.5 to 10 μ M methyl jasmonate, and 0.2 to 5 μ M 22(S),23(S)-homobrassinolide. By using the inhibition of root growth as a parameter, no differences were observed between wild-type and *rpn12a-1* seedlings for all concentrations of all hormones except 2,4-D (data not shown). For 2,4-D, the response was remarkably similar to that observed with cytokinins; *rpn12a-1* roots actually grew better on low concentrations of 2,4-D rather than being inhibited (Figure 7A). Auxins also are known to stimulate lateral root growth. As can be seen in Figure 7B, 20 nM 2,4-D enhanced lateral root formation in the wild type, but this effect was delayed in *rpn12a-1* seedlings. In contrast to the effects with cytokinins, the decreased auxin sensitivity of *rpn12a-1* seedlings appeared restricted to roots, because the responses

of other tissues to exogenous 2,4-D (e.g., shoot growth, hypocotyl elongation) were like those of the wild type (data not shown).

Complementation of *rpn12a-1* by Overexpressing the *RPN12a* cDNA

To confirm that the *rpn12a-1* phenotype was caused by the disruption of the *RPN12a* gene, we attempted to complement the mutation by ectopic expression of the wild-type *RPN12a* cDNA under the direction of the 35S promoter of *Cauliflower mosaic virus*. When the transformed *rpn12a-1* plants were examined phenotypically, they regained their wild-type morphology and sensitivity to cytokinins and 2,4-D (Figures 8A and 8B; data not shown). All 21 35S-*RPN12a* transgenic lines containing high levels of the RPN12a protein looked indistinguishable from the wild type, indicating that overexpression of the RPN12a protein was not deleterious to Arabidopsis development. Like the results with hemizygous *rpn12a-1* plants (Figure 2C), overexpression of the wild-type protein resulted in the loss of the RPN12a-NPTII fusion protein (Figure 8C), presumably by competitively blocking the association of the fusion protein with the 26S

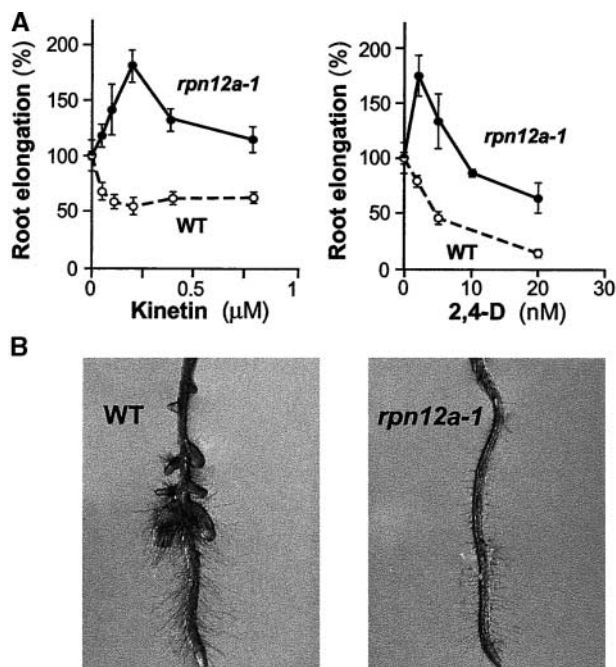


Figure 7. Roots of *rpn12a-1* seedlings are less sensitive to the Auxin 2,4-D.

(A) Effect of the *rpn12a-1* mutation on the inhibition of root elongation by kinetin (left) and 2,4-D (right). Wild-type (WT) and *rpn12a-1* seedlings were grown at a range of concentrations of kinetin or 2,4-D. After 14 days, the mean root lengths of 10 plants were determined and are expressed as percentages of the lengths of the respective untreated controls (\pm SD).

(B) Effect of the *rpn12a-1* mutation on 2,4-D-stimulated lateral root formation. Seedlings were grown for 10 days on 20 nM 2,4-D.

proteasome. From this finding, we conclude that the observed phenotypes for the *rpn12a-1* seedlings were the direct result of the T-DNA insertion affecting the *RPN12a* gene and not another gene located nearby.

DISCUSSION

Recent studies have implicated the ubiquitin/26S proteasome proteolytic pathway in many environmental and developmental responses in higher plants (Callis and Vierstra, 2000). Here, we extend its importance to cytokinin regulation with the demonstration that an Arabidopsis mutation affecting the synthesis of the RPN12a subunit of the 26S proteasome alters development consistent with a reduction in cytokinin responses. Previous data implicated the ubiquitin/26S proteasome system in the response to hormones, auxins, jasmonate, and abscisic acid (Xie et al., 1998; Gray and Estelle, 2000; Lopez-Molina et al., 2001). Given the

complexity of this pathway in plants (in Arabidopsis, it includes more than 1100 genes), it is likely that most if not all hormone responses in plants are regulated at some level by such proteolysis.

In agreement with data from other eukaryotes, RPN12a is a component of the Arabidopsis 26S proteasome. At present, its biochemical function is unknown. RPN12a does not have any recognizable sequence motifs present in other RPN subunits that could provide hints of a function (Glickman et al., 1998; Fu et al., 1999b). It also is distinct from other RP subunits in that it displays the greatest dissimilarity compared with orthologs from other species and by being one of the few Arabidopsis RPN subunits that fails to complement a corresponding yeast deletion (Fu et al., 1999b; H. Fu and R.D. Vierstra, unpublished data). Yeast RPN12 maps to the lid subcomplex of the RP, where it possibly interacts with RPN10 (Kominami et al., 1995; Glickman et al., 1998). Given the predicted role of the RP in substrate selection, RPN12 orthologs could have a unique role in recruiting specific targets.

In addition to *RPN12a*, we also detected a second Arabidopsis gene (*RPN12b*) encoding a truncated version of the protein. Although the expression of *RPN12b* has not yet been demonstrated, three lines of evidence suggest that it is a functional gene. First, the high nucleotide sequence identity between *RPN12a* and *RPN12b* exists only within the exons of the coding region, indicating selective evolutionary pressure for the maintenance of the correct coding sequence. Moreover, the predicted beginning of the RPN12b protein coincides with the beginning of a highly conserved stretch (GLNLLXLL) within all RPN12 orthologs from different animal and fungal species (Figure 1B). Second, when the *RPN12b* genomic sequence was expressed ectopically in Arabidopsis using the 35S promoter of *Cauliflower mosaic virus*, reverse transcriptase-mediated PCR analysis of the resulting mRNA indicated that the transcript was spliced correctly at the predicted exon/intron junctions to produce a mRNA encoding this 159-amino acid protein (data not shown). However, we were unable to detect immunologically a RPN12b protein of the expected size (18 kD) in these transgenic seedlings using anti-RPN12a antibodies. Third, searches of various databases revealed a *Dictyostelium discoideum* gene (designated *DdM4*) encoding a truncated form of RPN12 beginning at a similar position as RPN12b (Kimmel and Firtel, 1985). It was identified as a mRNA of unknown function bearing a short interspersed repeat appended to its 5' end. The predicted DdM4 protein starts six amino acids downstream of AtRPN12b and terminates 66 residues before AtRPN12b, generating a protein of only 88 residues (Figure 1B). The possibility that the *RPN12b* locus also encodes a functional subunit raises the intriguing possibility that the RPN12a and RPN12b subunits are interchangeable, thus creating 26S proteasome populations with distinct properties.

The exon-trap T-DNA mutant *rpn12a-1* was created by a fortuitous insertion of the *ARP-NPTII* gene downstream of *RPN12a*. Transcription, processing, and translation of the

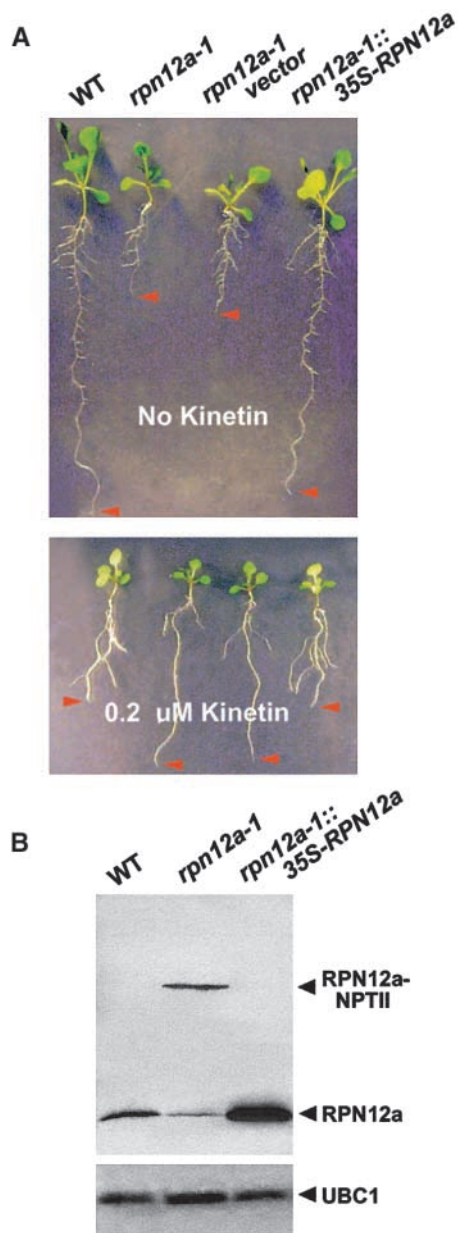


Figure 8. Complementation of the *rpn12a-1* Mutation with Wild-Type RPN12a.

The *rpn12a-1* mutant was transformed with a cDNA containing the entire RPN12a coding region expressed under the control of the 35S promoter of *Cauliflower mosaic virus*.

(A) Growth of wild type (WT), the homozygous mutant (*rpn12a-1*), or the homozygous *rpn12a-1* mutant harboring either the empty pGSVE9 vector T-DNA or the 35S-RPN12a transgene. Seedlings were grown for 14 days without (top) or with 0.2 μM kinetin (bottom). Arrowheads identify the tips of the longest roots.

(B) Levels of the RPN12a and RPN12a-NPTII proteins in total crude extracts from 7-day-old seedlings from wild type, *rpn12a-1*, or the complementation line *rpn12a-1::35S-RPN12a*. Immunoblots were probed with antibodies against RPN12a or UBC1.

rpn12a-1 locus directed the simultaneous synthesis of two proteins, one indistinguishable from wild-type RPN12a and another organized as a RPN12a-NPTII fusion protein. Like its counterparts in budding and fission yeast (Nisogi et al., 1992; Gordon et al., 1996), we expect that null alleles of RPN12a will be lethal in Arabidopsis. Given the relatively subtle phenotypes elicited by the *rpn12a-1* mutation and the presence of both the RPN12a and RPN12a-NPTII fusion proteins in homozygous plants, it is reasonable to conclude that *rpn12a-1* represents a weak allele. In fact, biochemical fractionation of the 26S proteasome detected complexes containing either the wild-type or fusion protein, indicating that only part of the RP pool is compromised. Additional results support the possibility that *rpn12a-1* confers a relatively subtle effect. First, unlike strong mutants affecting other 26S proteasome subunits, including yeast RPN12 (Kominami et al., 1995; Bailly and Reed, 1999; Girod et al., 1999), increased levels of ubiquitinated proteins, characteristic of a backup in protein breakdown, were not observed in *rpn12a-1* plants (Figure 2). Second, preliminary biochemical analysis of 26S proteasomes containing RPN12a-NPTII indicate that they are similar in size to those bearing wild-type RPN12a, suggesting that integration of the fusion protein does not radically destabilize the complex (Figure 3). Third, Kominami and Toh-e (1994) demonstrated that RPN12-LacZ fusion proteins can complement a yeast *rpn12* mutant, showing that this protein can tolerate large C-terminal additions.

Although the expression of fusion proteins often confers a dominant phenotype, the *rpn12a-1* mutation resulting in the RPN12a-NPTII fusion protein behaved as a recessive trait. This can be explained by the ability of the wild-type protein to outcompete the RPN12a-NPTII fusion protein for binding to the 26S proteasome. Accordingly, when sufficient levels of wild-type protein are available (as is the presumed case of hemizygous *rpn12a-1* plants or homozygous *rpn12a-1* plants expressing a 35S-RPN12a transgene), the fusion protein is blocked from binding and few mutant 26S proteasomes are assembled. Our failure to detect RPN12a-NPTII in both situations implies that the free protein is degraded rapidly. However, for the homozygous *rpn12a-1* plants, the wild-type protein created by alternative splicing of the chimeric mRNA is limiting, allowing the RPN12a-NPTII protein to assemble and thus generate a pool of altered 26S proteasomes. The phenotype of the *rpn12a-1* plants would be indicative of a specific reduction in RPN12a function and not in the activity of the whole 26S proteasome complex.

The phenotype of *rpn12a-1* plants is consistent with a decreased sensitivity of both roots and shoots to cytokinins. Although it is possible that the mutant also is impaired in cytokinin biosynthesis, the fact that *rpn12a-1* plants cannot be rescued by exogenous cytokinins suggests otherwise. *rpn12a-1* plants also are affected in their response to auxin, indicating that auxin signaling is affected as well. Interestingly, several of the auxin-resistant mutants also are less sensitive to cytokinins, suggesting that these hormone signaling pathways share one or more components (Coenen

and Lomax, 1997). The *rpn12a-1* mutation appears less severe than the strong cytokinin-resistant mutant *cyr1* and may reflect the mild biochemical effect on the RPN12a protein. Thus, it is possible that a stronger defect in *RPN12a* will further reduce cytokinin sensitivity, leading to a stronger morphological phenotype, as was observed for the *cyr1* mutant. The *rpn12a-1* phenotype also is different from that of *amp1*, which is characterized by an increase in endogenous cytokinin levels and by a developmental phenotype consistent with a constitutive cytokinin response (Chaudhury et al., 1993). Instead of a delay in leaf formation in the light and a delayed opening of cotyledons in the dark, *amp1* seedlings have accelerated leaf formation and constitutive photomorphogenesis, mimicking wild-type seedlings treated with cytokinin (Chin-Atkins et al., 1996). Although *amp1* increases the likelihood of seedlings with more than two cotyledons (Chin-Atkins et al., 1996), we found that *rpn12a-1* increased the percentage of monocotyledonous seedlings.

The ability of low cytokinin concentrations to stimulate the elongation of *rpn12a-1* roots was unexpected, because exogenous cytokinins are known to inhibit wild-type root growth (Cary et al., 1995). Because untreated *rpn12a-1* roots were much shorter than wild-type roots, one possible explanation is that endogenous cytokinins normally promote root growth at low concentrations but become inhibitory at high concentrations. The altered sensitivity of the *rpn12a-1* plants could reflect a shift in the dose-response curve toward hormone concentrations higher than those found naturally in the plant. Another apparent contradiction is the enhanced accumulation of anthocyanins in young *rpn12a-1* seedlings. Because anthocyanin accumulation is augmented by exogenous cytokinins, a decrease in hormone sensitivity was expected to decrease anthocyanin accumulation, as was observed for the *cyr1* mutant (Deikman and Ulrich, 1995). However, decreased cytokinin sensitivity combined with increased anthocyanin accumulation also has been observed in the *fus6* mutant (Castle and Meinke, 1994). Thus, it is possible that *cyr1* on one side and *rpn12a-1* and *fus6* on the other side affect different branches of a cytokinin signaling pathway. Interestingly, the *FUS6* gene encodes a subunit of the COP9/signalosome complex; this complex is related to the RP and appears to modulate RP subunit composition (Peng et al., 2001). Recent data indicate that the COP9/signalosome and the 26S proteasome work in concert to affect auxin signaling in addition to cytokinins (Schwechheimer et al., 2001).

Although the developmental phenotype of *rpn12a-1* plants indicated a decreased cytokinin response, transcript levels of the cytokinin-inducible genes *CYCD3* and *NIA1* implied an accentuated cytokinin response. Both of these transcripts were upregulated even in the absence of hormone treatment. *CYCD3* overexpression was shown previously to lead to an increase in the rate of leaf formation (Riou-Khamlichi et al., 1999). Given the fact that a decreased rate of leaf formation was evident in the *rpn12a-1* mutant, this increased expression of *CYCD3* appeared inef-

fective in the *rpn12a-1* background. How can this be explained? One possible model suggests the participation of an inhibitory feedback loop requiring RPN12a (Figure 9). Such feedback loops are used by many signaling systems to desensitize organisms to the input stimulus and thus adapt. In fact, negative feedback control of gene expression has been shown to be part of both the cytokinin and auxin response pathways (Dominov et al., 1992) and to be involved in the desensitization of plant cell cultures to cytokinins (Mok and Mok, 1994). For cytokinins, it is possible that the pathway that leads to the final responses also activates this negative feedback loop to repress continued activation. Mutations that attenuate both the output response and the feedback pathway could enhance the response of intermediate steps. The increased levels of *CYCD3* and *NIA1* transcripts in *rpn12a-1* seedlings could indicate that both steps are upstream of the event(s) controlled by RPN12a, a possibility also suggested by the absence of any *CYCD3* overexpression phenotype in *rpn12a-1* plants (Figure 9).

In contrast to *NIA1* and *CYCD3*, the expression level of the early response gene *ARR5* remained unaffected by the *rpn12a-1* mutation in the absence of as well as after a short (15-min) cytokinin treatment (Figure 6B). This lack of effect could imply that RPN12a does not control the cytokinin response leading to early *ARR5* induction (Figure 9). Alternatively, we may have used too much BA for the analysis of *ARR5*. Although Brandstatter and Kieber (1998) reported previously that 5 μ M BA was sufficient to induce expression, it is possible that much lower concentrations are effective as well.

How could RPN12a participate in regulating cytokinin growth responses? RPN12a appears to be involved specifically, because T-DNA mutations in other Arabidopsis subunit genes encoding 26S proteasome lid subunits did not lead to a similar phenotype (J. Smalle and R.D. Vierstra, unpublished data). Based on the function of the 26S proteasome, RPN12a likely helps degrade one or more factors that repress cytokinin signaling; stabilization of these regulatory proteins in *rpn12a-1* plants would decrease hormone sensitivity. Given the role of cytokinins in promoting cell division (den Boer and Murray, 2000), possible targets include inhibitors of the cell cycle. In yeast, RPN12 has a specific role in the G1/S and G2/M transitions of the cell cycle (Kominami et al., 1995). Although its participation in the G2/M transition is unknown, one function in the G1/S phase is to degrade the cyclin-dependent kinase inhibitor SIC1 (Bailey and Reed, 1999). Disruption of yeast *RPN12* leads to the stabilization of SIC1; SIC1 then prevents entry into the cell cycle by associating with and inhibiting the Cln/Cdc28 kinase complex. Interestingly, the Arabidopsis functional homolog of Cdc28 (Cdc2a) interacts with cyclin D3, a mediator of cytokinin-activated cell division and possibly of other cytokinin responses, such as chloroplast development and senescence (Riou-Khamlichi et al., 1999; Healy et al., 2000). Assuming a similar role for Arabidopsis RPN12a, we can expect that it also participates in the breakdown of SIC1-like proteins.

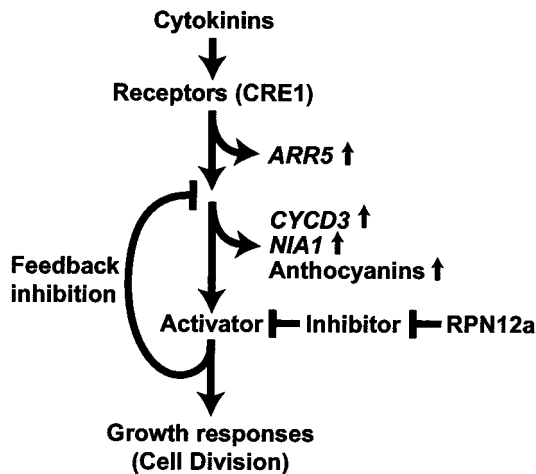


Figure 9. Possible Role for RPN12a in Cytokinin Signaling.

Cytokinins interact with receptors such as CRE1, resulting in the early induction of *ARR5* transcription and subsequent increases in anthocyanin accumulation and *CYCD3* and *NIA1* expression. The proteasome subunit RPN12a degrades an inhibitor of the pathway immediately upstream of steps that activate cell division, thus allowing cytokinin-induced growth responses to proceed. Destabilization of this inhibitor also activates a negative feedback loop repressing upstream components such as *CYCD3* and *NIA1* expression. The *rpn12a-1* mutation decreases the activity of RPN12a, stabilizing the inhibitor and attenuating both growth responses and feedback inhibition. As a result, *CYCD3* and *NIA1* expression and anthocyanin synthesis are upregulated but growth responses are downregulated.

Degradation of SIC1 analogs triggered by cytokinins then would activate the *CYCD3/CDC2a* complex and increase cell division and growth rate. Conversely, their stabilization, resulting from the loss of RPN12a function, would attenuate *CYCD3/CDC2a* activity and subsequently decrease cytokinin-related growth responses. As yet, no sequence relatives of yeast SIC1 are apparent in the nearly complete Arabidopsis genome. However, several unrelated Arabidopsis cyclin-dependent kinase inhibitors have been identified that could function as inhibitors of the *CYCD3/CDC2a* complex (Wang et al., 1998; Lui et al., 2000). Whether these are stabilized in the *rpn12a-1* mutant awaits testing.

It has been accepted generally that the specificity of ubiquitin/26S proteasome-dependent degradation is controlled to a large extent by the enzymes that conjugate ubiquitin. However, evidence is accumulating that 26S proteasome subunits also have roles in target recognition. Specific RP subunits may function to recognize substrates without previous ubiquitination, may serve as scaffolds to recruit specific E3s, or may identify directly specific ubiquitinated proteins (Murakami et al., 1992; van Nocker et al., 1996b; Sheaff et al., 2000; Xie and Varshavsky, 2000). In budding yeast, for example, the loss of *RPN12* stabilizes SIC1, whereas the loss of *RPN3* stabilizes the G1 cyclin Cln2 and

the anaphase inhibitor PDS1 (Bailey and Reed, 1999). Therefore, instead of a single entry point for all substrates, multiple routes to the CP may exist, each involving different RP subunits. The effects of the *rpn12a-1* mutation on Arabidopsis support such a possibility. Instead of conferring a general defect in proteolysis, alteration of the protein led to a specific defect in cytokinin regulation. Certainly, continued investigation of other RPN subunits will reveal how selective each is to the recognition and degradation of the various ubiquitin/26S proteasome targets.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* C24 ecotype, obtained from Dr. M. Jacobs (Vrije Universiteit, Brussels, Belgium), was used as wild type for all phenotypic assays of *rpn12a-1*. For sterile conditions, plants were grown on Gamborg's B-5 medium (growth medium) (Gibco BRL, Gaithersburg, MD). Unless noted otherwise, plants were irradiated with continuous white fluorescent light. The hormones kinetin, benzyladenine (BA), 2-isopentenyladenine (2iP), indole-3-acetic acid, 2,4-D, abscisic acid, 1-aminocyclopropane-1-carboxylic acid, methyl jasmonate, and 22(S),23(S)-homobrassinolide were obtained from Sigma (St. Louis, MO). For root elongation assays, seedlings were transferred 4 days after germination onto solid medium containing hormones and placed vertically. Root elongation was measured after 9 days on a minimum of 10 seedlings per treatment. For fresh weight analyses, seedlings were germinated on growth medium, transferred after 4 days to medium containing different kinetin concentrations, and grown for an additional 21 days. For the shoot induction experiments, root and hypocotyl segments were dissected from 5-day-old light-grown seedlings, transferred to medium containing 1 μ M auxin with or without a range of (2iP) concentrations, and incubated for another 2 weeks in continuous light. Anthocyanin content was measured in hypocotyls of 7-day-old seedlings according to Kubasek et al. (1992) after extraction in methanol containing 1% (v/v) HCl.

Analysis of the Arabidopsis *RPN12* Genes and Proteins

RPN12a and *RPN12b* were located on the lower arms of chromosomes I and V and were sequenced as part of bacterial artificial chromosomes F1N19 and MJC20, respectively. Exon/intron junctions for *RPN12a* were identified by alignment of the genomic sequence with that for a full-length cDNA (obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus). DNA sequencing was performed using dye terminator chemistry (Perkin-Elmer, Foster City, CA). Amino acid sequence comparisons were performed using ClustalW (<http://www2.ebi.ac.uk/clustalw>) and displayed using Mac BoxShade (Institute of Animal Health, Pirbright, UK).

Proteasome Purification and Activity Assays

The 26S proteasome was partially purified from wild-type or *rpn12a-1* seedlings grown in a 16-hr-light/8-hr-dark photoperiod. Plants were frozen to liquid nitrogen temperatures, pulverized, and ground in 4

volumes of buffer A (50 mM Na phosphate, pH 7.5, 2 mM MgCl₂, 5% glycerol, 1 mM 2-mercaptoethanol, 1 mM ATP, and 5% polyvinylpyrrolidone-360). After filtering through cheesecloth, the extract was clarified for 15 min at 3000g and polyethylene glycol 8000 was added to a final concentration of 2% (w/v). The extract was reclarified for 15 min at 3000g, and the supernatant was adjusted to 10% polyethylene glycol 8000. The precipitate was collected by centrifugation for 15 min at 3000g, and the pellet was resuspended in buffer B (buffer A with 20% glycerol but without polyvinylpyrrolidone). Proteins were separated by fast protein liquid chromatography (FPLC) using a 1- × 20-cm Sephacryl S-400 column equilibrated with buffer B. 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.

Antibody Production and Immunoblot Analysis

The *RPN12a* coding region was amplified by polymerase chain reaction (PCR) from the full-length cDNA using the primers 5'-AAAACC-ATAGAACATATGGATCCGCAGCTA-3' and 5'-CAGTGAGAAAAGTCGACTCGAGCAGCAGATACGCTCCA-3' designed to contain 5' and 3' flanking *Nde*I and *Xho*I sites, respectively. Upon *Nde*I-*Xho*I digestion, this product was cloned into pET28b (Novagen, Madison, WI) for expression in *Escherichia coli* strain BL21 (DE3). After 3 hr of induction with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, the RPN12a protein was collected in the inclusion body fraction by centrifugation. The pellet was dissolved in SDS-PAGE sample buffer and subjected to preparative SDS-PAGE. The portion of the gel containing the 31-kD RPN12a protein (visualized by Coomassie Brilliant Blue R-250) was excised, emulsified, and injected directly into rabbits (Polyclonal Antibody Service, Madison, WI). Anti-RPN12a antibodies were affinity purified from serum by adsorption to nitrocellulose coated with recombinant protein (Sambrook et al., 1989). Antibodies against RPN5a, RPT1a, RPT2a, PAC1, and PBA1 were prepared similarly except that recombinant PBA1 protein remained soluble and was purified using nickel-nitrilotriacetic acid agarose chromatography under nondenaturing conditions, as recommended (Qiagen, Valencia, CA). Anti-RPN10, anti-ubiquitin, and anti-UBC1 antibodies were as described previously (Sullivan et al., 1994; van Nocker et al., 1996a).

For immunoblot analyses, proteins subjected to SDS-PAGE were transferred to nitrocellulose membranes (Immobilon NC; Millipore, Bedford, MA) and the membranes were blocked using 10% milk powder (Sambrook et al., 1989). Membranes probed with anti-ubiquitin antibodies were autoclaved before blocking. Both primary and secondary antibodies were diluted 1:1000 in PBS containing 0.2% Tween 20 and 1% BSA. Secondary antibodies used were alkaline phosphatase- or peroxidase-labeled goat anti-rabbit immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

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

Total RNA was extracted using Trizol reagent (Gibco BRL) from 14-day-old seedlings grown in liquid growth medium. 5' rapid amplification of cDNA ends PCR was conducted as described by Babychuk et al. (1997). For RNA gel blot analysis, 20 μg of total RNA was electrophoresed on 1% agarose-formaldehyde gels and transferred to Zeta-Probe GT genomic membranes (Bio-Rad, Hercules, CA). Bound

RNA was stained using methylene blue to verify loading and transfer. Probe hybridization, washing, and autoradiography were performed as described previously (Kurepa 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). The *CYCD3* template used the pJSCYCD3 plasmid linearized with *Sal*I-*Spe*I. This plasmid was created by PCR amplification of the *CYCD3* cDNA from a cDNA library using primers 5'-GATCTTATCTTTCTTCTCATTCTTGAGTTT-3' and 5'-AACAGTAAATATCATATAAACGAAATCGAG-3' and insertion of the product into pGEM-T (Promega). The *NIA1* template used the pJSNIA1 plasmid linearized with *Sph*I-*Aat*II. This plasmid was created by PCR amplification of the *NIA1* cDNA using primers 5'-GACCTCCGTCGATAACCGCCATTATCCAC-3' and 5'-ACGATGATTCTCTTAACCATTTAACCATC-3' and insertion of the product into pGEM-T. The *ARR5*, *RPN12a*, and *CDC2a* templates used expressed sequence tag clones 103N10T7, 198J17T7, and 205H2T7, respectively, linearized with *Kpn*I-*Pst*I. The β-*TUB4*, *PBA1*, and *PBF1* probes were described previously (Fu et al., 1998b). RNA gel blot signal intensities were estimated using the NIH Image program (National Institutes of Health, Bethesda, MD).

rpn12a-1 Complementation

The *RPN12a* cDNA was amplified by PCR using primers 5'-AGAAGAGAAAAAACTCTAGAAGCGATGGAT-3' and 5'-TGAACACTTAAACTCTAGAATATAACGTAA-3' designed to contain an *Xba*I site at both ends. The resulting product was digested with *Xba*I and inserted into the binary T-DNA vector pGSVE9 that was digested similarly (E. Babychuk and S. Kushnir, unpublished data). The resulting construction allowed for expression of the *RPN12a* coding sequence driven by the 35S promoter. The vector was introduced into *Agrobacterium tumefaciens* strain AtC58Rif^r (pMP90), and the strain was used to transform homozygous *rpn12a-1* seedlings by the floral dip method (Clough and Bent, 1998). Transformants were identified by hygromycin resistance. Initial transformants (T1) were selfed, and the progeny (T2) were tested for hygromycin resistance. These hygromycin-resistant plants were again selfed, and the progeny (T3) were tested for both kanamycin and hygromycin resistance. Lines that were homozygous for both kanamycin resistance (i.e., containing the *rpn12a-1* T-DNA) and hygromycin resistance (i.e., containing the 35S-*RPN12a* transgene) were analyzed for phenotypic complementation.

Accession Numbers

The accession numbers for the sequences described in this article are AC009579 (*RPN12a*) and AB017067 (*RPN12b*). The accession numbers for the bacterial artificial chromosomes described are AC009519 (*F1N19*) and AB017067 (*MJC20*), and the accession numbers for the sequences shown in Figure 1 are AE003527 (*DmRPN12*), AC005783 (*Hsp31*), P02889 (*DdM4*), Q23449 (*CeS14*), X92682 (*SpMTS3*), CAA15600 (*PfS14*), and D10515 (*ScRPN12*).

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