The Defective Proteasome but Not Substrate Recognition Function Is Responsible for the Null Phenotypes of the Arabidopsis Proteasome Subunit RPN10

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Ubiquitylated substrate recognition during ubiquitin/proteasome-mediated proteolysis (UPP) is mediated directly by the proteasome subunits RPN10 and RPN13 and indirectly by ubiquitin-like (UBL) and ubiquitin-associated (UBA) domain-containing factors. To dissect the complexity and functional roles of UPP substrate recognition in Arabidopsis thaliana, potential UPP substrate receptors were characterized. RPN10 and members of the UBL-UBA-containing RAD23 and DSK2 families displayed strong affinities for Lys-48-linked ubiquitin chains (the major UPP signals), indicating that they are involved in ubiquitylated substrate recognition. Additionally, RPN10 uses distinct interfaces as primary proteasomal docking sites for RAD23s and DSK2s. Analyses of T-DNA insertion knockout or RNA interference knockdown mutants of potential UPP ubiquitin receptors, including RPN10, RPN13, RAD23a-d, DSK2a-b, DDI1, and NUB1, demonstrated that only the RPN10 mutant gave clear phenotypes. The null rpn10-2 showed decreased double-capped proteasomes, increased 20S core complexes, and pleiotropic vegetative and reproductive growth phenotypes. Surprisingly, the observed rpn10-2 phenotypes were rescued by a RPN10 variant defective in substrate recognition, indicating that the defectiveness of RPN10 in proteasome but not substrate recognition function is responsible for the null phenotypes. Our results suggest that redundant recognition pathways likely are used in Arabidopsis to target ubiquitylated substrates for proteasomal degradation and that their specific roles in vivo require further examination.

INTRODUCTION

Ubiquitin/26S proteasome-mediated proteolysis (UPP) controls the half-lives of numerous critical regulatory proteins and thus plays a vital role in almost all aspects of plant growth and development, including hormone responses, flower development, disease resistance, self-incompatibility, circadian rhythm, and photomorphogenesis (Vierstra, 2009). A protein substrate targeted for UPP is first covalently modified with ubiquitin chains that are attached to substrates for proteasomal targeting via ubiquitin conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (Pickart and Eddins, 2004). The tagged protein is then recognized by various ubiquitin receptors and targeted to the 26S (E3) (Pickart and Eddins, 2004). The tagged protein is then recognized by various ubiquitin receptors and targeted to the 26S proteasome for proteolysis (Finley, 2009; Fu et al., 2010).

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Ubiquitin/26S proteasome-mediated proteolysis (UPP) controls the half-lives of numerous critical regulatory proteins and thus plays a vital role in almost all aspects of plant growth and development, including hormone responses, flower development, disease resistance, self-incompatibility, circadian rhythm, and photomorphogenesis (Vierstra, 2009). A protein substrate targeted for UPP is first covalently modified with ubiquitin chains that are attached to substrates for proteasomal targeting via ubiquitin conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (Pickart and Eddins, 2004). The tagged protein is then recognized by various ubiquitin receptors and targeted to the 26S proteasome for proteolysis (Finley, 2009; Fu et al., 2010).

It is generally accepted that the predominant signal that targets substrates for UPP is the Lys-48–linked ubiquitin chain with a minimum length of four ubiquitin units (Thrower et al., 2000). However, recent proteomic studies have indicated that the topology of the ubiquitin chains assembled on UPP substrates is more complicated than previously thought (Meierhofer et al., 2008; Xu et al., 2009), and the ubiquitin chains of other linkage types (e.g., Lys-11, Lys-29, and Lys-63) are competent targeting signals for proteasomal degradation in vitro and in vivo (Johnson et al., 1995; Hofmann and Pickart, 2001; Jin et al., 2008; Saeki et al., 2009). The primary structural determinants of ubiquitin chains that are attached to substrates for proteasomal targeting include a hydrophobic patch composed of Leu-8, Ile-44, and Val-70 on each of the individual ubiquitin units (Beal et al., 1996). The isopeptide linkers and the relative orientations of the ubiquitin units, or the chain conformation, of specific linkage types have also been shown to be critical in several studies (Trempe et al., 2005; Varadan et al., 2005; Zhang et al., 2009; Riedinger et al., 2010).

Recognition of ubiquitin chains that are assembled on proteasome substrates is mediated by multiple ubiquitin receptors; this recognition is an important mechanistic and regulatory component of UPP that controls substrate transfer from the ubiquitin conjugation machinery to the 26S proteasome (Finley, 2009; Fu et al., 2010). Although the substrate selectivity of UPP is primarily regulated by posttranslational modification and conformational changes of the substrates and by associations between the
substrates and their cognate conjugation enzymes (Petroski and Deshaies, 2005; Ravid and Hochstrasser, 2008), accumulating evidence has indicated that substrate selectivity can also be determined by various ubiquitin receptors during the proteasomal recognition of ubiquitylated substrates (Verma et al., 2004; Ivantsiv et al., 2006).

Three major classes of UPP ubiquitin receptors have been described and appear to be conserved among different species. The first class includes the intrinsic 26S proteasome base subunits RPN10 (van Noeker et al., 1996), RPN13 (Husnjak et al., 2008; Schreiner et al., 2008), and RPT5 (Lam et al., 2002), which directly recognize ubiquitylated substrates. The second class includes shuttle factors containing one N-terminal ubiquitin-like (UBL) domain and one or two C-terminal ubiquitin-associated (UBA) domains (e.g., RAD23, DSK2, DD1, and NUB1), which require an additional proteasomal docking step to target ubiquitylated substrates to the 26S proteasome. Evidence has also suggested that human p62 is involved in targeting ubiquitylated substrates for proteasomal proteolysis; this protein contains an N-terminal Phox and Bem1p (PB1) domain with a ubiquitin fold-like structure and a C-terminal UBA domain, which gives a domain organization very similar to UBL-UBA shuttle factors (Vadlamudi et al., 1996; Seibenhener et al., 2004). The UBL-UBA shuttle factors are capable of simultaneously binding to the 26S proteasome and to the ubiquitylated substrates through the UBL and UBA domains, respectively (Funakoshi et al., 2002; Saeki et al., 2002; Verma et al., 2004; Kaplun et al., 2005). Multiple docking sites for various UBL-UBA factors appear to be located on the base subcomplex of the regulatory particle, including sites on RPN1 and the ubiquitin receptors RPN10 and RPN13 (Elsasser et al., 2002; Wang et al., 2003b; Husnjak et al., 2008). The third class of UPP ubiquitin receptors includes CDC48-based complexes. In addition to a recently identified role in the degradation of the cytosolic protein hypoxia-inducible factor 1α (Alexandru et al., 2008), the CDC48 complexes are primarily involved in endoplasmic reticulum–associated degradation (Elsasser and Finley, 2005; Raasi and Wolf, 2007).

Distinct ubiquitin binding motifs or domains are used by the various ubiquitin receptors (Hicke et al., 2005; Hurley et al., 2006; Schreiner et al., 2008). The ubiquitin-interacting motif (UIM), the pleckstrin-like receptor of ubiquitin (PRU) domain, and the UBA domain are used by RPN10, RPN13, and UBL-UBA factors, respectively. Multiple ubiquitin binding sites are associated with different subunits of the CDC48 complexes, including the NPL4-zinc finger (Wang et al., 2003a) and UBA domains (Yuan et al., 2004) in NPL4 and p47, respectively, as well as the CDC48/p97 N-domain fold in CDC48 and UFD1 (Park et al., 2005).

The major recognition pathways for ubiquitylated UPP substrates appear to have diverged in different species in regards to the involved receptors and the associated interaction interfaces, suggesting a potential mechanistic and functional divergence (Fu et al., 2010). For the direct recognition of ubiquitylated UPP substrates, yeast (Saccharomyces cerevisiae) and humans use both Rpn10 (S5a) and Rpn13 as the major receptors (Deveraux et al., 1994; van Nocker et al., 1996; Husnjak et al., 2008; Schreiner et al., 2008; Fatimababy et al., 2010; Peth et al., 2010). For indirect recognition mediated by the UBL-UBA shuttle factors, yeast uses both Rad23 and Dsk2 as the major receptors (Elsasser et al., 2002; Fatimababy et al., 2010), but humans use hHR23 (the Rad23 homolog) as the major receptor (Fatimababy et al., 2010). For proteasomal docking of the UBL-UBA shuttle factors during indirect UPP substrate recognition, yeast primarily uses Rpn1 and Rpn10 to recognize Rad23 and Dsk2, respectively (Elsasser et al., 2002; Fatimababy et al., 2010), and Rpn13 also plays a minor role in Dsk2 docking (Husnjak et al., 2008; Fatimababy et al., 2010). By contrast, humans use both S5a and RPN13 to accept hHR23 (Schreiner et al., 2008; Fatimababy et al., 2010). A role for human RPN1 in recognizing hHR23 has not yet been demonstrated. Distinct substrate selectivity and structural determinants appear to be associated with the homologs of the major ubiquitin receptors (i.e., Rpn10, Rpn13, Rad23, and Dsk2) in different species (Fu et al., 2010). For example, using two UIMs in a cooperative manner, human S5a binds to Lys-48– and Lys-63–linked ubiquitin chains with equivalently high affinities (Young et al., 1998; Wang et al., 2005; Zhang et al., 2009; Fatimababy et al., 2010). By contrast, with a single UIM, yeast Rpn10 has a strong preference for Lys-48–linked ubiquitin chains (Fu et al., 1998; Fatimababy et al., 2010). Similarly, the interfaces for proteasomal docking of the UBL-UBA shuttle factors show clear divergence across species (Fatimababy et al., 2010). However, limited information is available on the detailed mechanisms, substrate specificities, regulation, and function of the distinct proteasomal recognition pathways for a given species or across species.

To examine the main recognition pathways for ubiquitylated UPP substrates in Arabidopsis thaliana, we characterized the major Arabidopsis ubiquitin receptors. Based on their strong affinities for Lys-48–linked ubiquitin chains, Arabidopsis RPN10 and the UBL-UBA domain-containing factors RAD23s and DSK2s likely play important roles in the recognition of ubiquitylated UPP substrates. In addition, RPN10 contains the primary docking sites for RAD23s and DSK2s. Analyses of T-DNA insertion knockout or RNA interference (RNAi) knockdown mutants for the major ubiquitin receptors demonstrated that only the RPN10 null mutant shows pleiotropic vegetative and reproductive growth phenotypes. The RPN10 null mutant also has reduced double-capped proteasome complexes and increased 20S core complexes. Although in vitro interaction analyses suggest that Arabidopsis RPN10 plays a central role in both the direct and indirect recognition of ubiquitylated UPP substrates, surprisingly, the rpn10-2 phenotypes can be rescued by a RPN10 variant that is defective in ubiquitin and ubiquitin-like recognition, indicating that defects in substrate recognition are not responsible for the null phenotypes of RPN10. Together with the lack of obvious phenotypes for the other major ubiquitin receptor mutants examined, our results suggest that redundant recognition pathways are used in Arabidopsis to target ubiquitylated substrates for proteasomal degradation, and the specific roles of these receptors in vivo remain to be determined. The simultaneous restoration of growth phenotypes and of the two-capped proteasome expression level in rpn10-2 by complementing with a wild-type or a substrate recognition–defective RPN10 suggests that the null phenotypes might be caused by altered complex stability.
RESULTS

Isolation of *Arabidopsis* Genes Encoding Potential Receptors of Ubiquitylated UPP Substrates

To examine the functional roles and mechanistic details of individual recognition pathways for ubiquitylated UPP substrates, *Arabidopsis* genes encoding potential ubiquitin receptors were identified (see Supplemental Table 1 online). Full-length *Arabidopsis* cDNAs encoding these potential ubiquitin receptor homologs were isolated by PCR (see Supplemental Table 1 online), and the encoded proteins were characterized. The potential *Arabidopsis* ubiquitin receptors examined in this study include RPN10, RAD23a-d, DSK2a-b, DD1, NUB1, and a p62-like factor, LPS (LIKE HUMAN p62). The biochemical characterization of *Arabidopsis* RPN13 was described in our previous study (Fatimababy et al., 2010). Although the CDC48 complexes serve as the major receptors of endoplasmic reticulum–associated degradation (Elssasser and Finley, 2005; Raasi and Wolf, 2007), very weak ubiquitin chain binding activities were observed with the CDC48 complex subunits UFD1a-b and NPL4a-b, but not UFD1c-d, suggesting that full-strength chain binding activity requires the integration of these subunits into active complexes (see Supplemental Table 1 online).

**RPN10 and the UBL-UBA Factors RAD23b-d and DSK2a-b Strongly Associate with Lys-48-Linked Ubiquitin Chains**

To assess the involvement of the *Arabidopsis* ubiquitin receptors in the recognition of ubiquitylated UPP substrates, their ubiquitin chain binding properties, relevant structural domains or motifs, and critical residues were determined (see Supplemental Figures 1 to 4 online). The major *Arabidopsis* ubiquitin receptors RPN10, RAD23b-d, DSK2a-b, DD1, NUB1, and LPS were expressed and purified as glutathione S-transferase (GST)-tagged wild-type or mutated variants (see Supplemental Table 2 online). The ability of the tagged wild-type proteins to serve as receptors for ubiquitylated UPP substrates was first assessed by a pull-down assay using crude *Arabidopsis* extracts, which determined their capacity to associate with ubiquitin conjugates. As summarized in Table 1, endogenous ubiquitylated proteins with high molecular weights were readily pulled down by the GST-fused RPN10, RAD23b-d, DSK2b, DD1, and NUB1; however, GST-fused DD1 showed a clearly lower affinity for conjugates than the other GST-fused receptors (see Supplemental Figure 1 online). Under similar assay conditions, LPS was unable to pull down ubiquitylated proteins in contrast with the observation made for human p62, which strongly binds to Lys-63–linked chains (Seibenhenner et al., 2004).

Next, the ubiquitin chain binding properties, including linkage type and length preferences, of the *Arabidopsis* ubiquitin receptors were examined and compared using GST pull-down assays with Lys-48– or Lys-63–linked ubiquitin chains that each consisted of two to seven ubiquitin units. The profiles of the pulled-down and input chains were compared by immunoblotting (see Supplemental Figures 2A and 3 online). Whereas a Lys-48–linked ubiquitin chain of four or more ubiquitin units is the predominant UPP signal (Thrower et al., 2000), the Lys-63–linked ubiquitin chain plays major targeting roles in DNA repair, endocytosis, and signal transduction (Hicke and Dunn, 2003; Kanayama et al., 2004; Tsui et al., 2005).

As summarized in Table 1, RPN10 and the UBL-UBA factors RAD23b-d and DSK2a-b had clearly stronger affinities for Lys-48–linked ubiquitin chains than for Lys-63–linked chains (see Supplemental Figures 2A and 3A online). By contrast, distinct ubiquitin chain binding properties were associated with the single-member UBL-UBA factors DD1 and NUB1. DD1 displayed a relatively weaker affinity for both Lys-48– and Lys-63–linked ubiquitin chains and had only a slight preference for Lys-48–linked chains of three or more ubiquitin units over Lys-63–linked ubiquitin chains (see Supplemental Figure 3A online). An internal NUB1 fragment (NUB1-INT, 221 to 490) containing three UBA domains had affinities (rather weak) only for Lys-63– but not for Lys-48–linked ubiquitin chains (see Supplemental Figure 3B online).

Under the same pull-down assay conditions, *Arabidopsis* LPS was unable to bind to Lys-48– or Lys-63–linked ubiquitin chains, which agrees with the absence of binding activity with endogenous ubiquitylated conjugates. No association with Lys-48– or Lys-63–linked ubiquitin chains could be detected for RPN1a or RPT5a, which are the two other intrinsic 26S proteasome subunits that were potential ubiquitin receptors (Table 1). However, in our previous study, a potential base subunit RPN13 was shown to bind weakly to Lys-48– and Lys-63–linked ubiquitin chains (Fatimababy et al., 2010). Based on the strong in vitro affinities for Lys-48–linked long ubiquitin chains and ubiquitylated substrates, RPN10 and the UBL-UBA domain–containing shuttle factors RAD23s and DSK2s may play important roles in the direct and indirect recognition of ubiquitylated UPP substrates in *Arabidopsis*, respectively.

The Structural Motifs of Major *Arabidopsis* Ubiquitin Receptors Involved in Ubiquitin Chain Binding

We next determined the structural domains (or motifs) and residues that are critical for ubiquitin binding in the major *Arabidopsis* ubiquitin receptors RPN10, RAD23c, DSK2a-b, DD1, and NUB1. The structural domains (or motifs) in these major *Arabidopsis* ubiquitin receptors that are potentially involved in ubiquitin binding include three UIMs in RPN10 (see Supplemental Figure 2B online), three UBA s in NUB1, two UBAs in RAD23c, and a single UBA in DSK2a-b or DD1. Using site-specific mutations in the GST receptor fusions, we examined the involvement of these domains in ubiquitin chain binding by GST pull-down assays (see Supplemental Table 2 and Supplemental Figures 2C and 4 online). The ubiquitin receptor variants with mutated sites included RPN10-uim1-d5 (226 to 230; LALAL to NNNNN), RPN10-uim1-n5 (226 to 230; LALAL to NNNNN), RAD23c-uba1 (197 to 199; MGG to AAA), RAD23c-uba2 (385 to 387; MGF to AAA), DSK2a-uba (506 to 507; MG to AA), DSK2b-uba (519 to 520; MG to AA), DDI1-uba (386 to 387; MG to AA), NUB1-uba1 (271 to 272; MG to AA), NUB1-uba2 (343 to 344; MG to AA), and NUB1-uba3 (403 to 404; MG to AA) (see Supplemental Table 2 online). The mutated residues are highly conserved in the UIM and UBA domains of these major *Arabidopsis* ubiquitin receptors and have been shown to be...
critical for ubiquitin binding in some of the human and yeast homologs (Fatimababy et al., 2010).

As summarized in Table 1, UIM1, but not UIM2 or UIM3, of Arabidopsis RPN10 is critical for interactions with both Lys-48- and Lys-63-linked chains (see Supplemental Figure 2C online). The two UBA domains of Arabidopsis RAD23c contribute additively to Lys-48-linked ubiquitin chain binding (see Supplemental Figure 4A online). In addition, the single UBA domains in DSK2a-b and DD1i are required for ubiquitin chain binding (see Supplemental Figure 4B online). All of the three UBA domains of NUB1 also proved to be required simultaneously for binding to Lys-63-linked ubiquitin chains (Table 1).

### Table 1. Ubiquitin Chain Binding Properties and Associated Structural Domains of the Potential Arabidopsis Ubiquitin Receptors

<table>
<thead>
<tr>
<th>Name</th>
<th>Conjd</th>
<th>Lys-48e</th>
<th>Lys-63e</th>
<th>Domainf</th>
<th>Modeb</th>
<th>Referencesc</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPN10</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>UIM1</td>
<td>D</td>
<td>TS; Fatimababy et al. (2010)</td>
</tr>
<tr>
<td>RPN13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>PRU</td>
<td>i</td>
<td>Fatimababy et al. (2010)</td>
</tr>
<tr>
<td>RPN1a</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>TS</td>
</tr>
<tr>
<td>RPT5a</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>TS</td>
</tr>
<tr>
<td>RAD23bii</td>
<td>+</td>
<td>(+++)</td>
<td>(+)</td>
<td>ND</td>
<td>I (N10)</td>
<td>TS; Farmer et al. (2010)</td>
</tr>
<tr>
<td>RAD23c</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>UBA1 &amp; 2</td>
<td>ND</td>
<td>TS; Farmer et al. (2010)</td>
</tr>
<tr>
<td>RAD23d</td>
<td>+</td>
<td>(+++)</td>
<td>(+)</td>
<td>ND</td>
<td>I (N10)</td>
<td>TS; Farmer et al. (2010)</td>
</tr>
<tr>
<td>DSK2a</td>
<td>ND</td>
<td>+++</td>
<td>+</td>
<td>UBA</td>
<td>ND</td>
<td>TS</td>
</tr>
<tr>
<td>DSK2b</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>UBA</td>
<td>I (N10, N13)</td>
<td>TS</td>
</tr>
<tr>
<td>DD1i</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>UBA</td>
<td>?</td>
<td>TS</td>
</tr>
<tr>
<td>NUB1</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>UBA1, 2, and 3</td>
<td>?</td>
<td>TS</td>
</tr>
<tr>
<td>LPS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>TS</td>
</tr>
</tbody>
</table>

aPart of the ubiquitin binding properties of the major Arabidopsis ubiquitin receptors examined by a similar pull-down assay were shown in our previous studies (Farmer et al., 2010; Fatimababy et al., 2010); boldfaced items are properties shown in this study, regular font items were examined in the listed studies, and items in parentheses were examined by Farmer et al. (2010). NA, not applicable; ND, not determined.

b“D” and “I/i” indicate a direct or indirect role, respectively, in the recognition of ubiquylated substrates. The upper- and lowercase letters indicate a major and a minor role, respectively, based on the binding affinity for the Lys-48–linked ubiquitin chains. N10 and N13 (for RPN10 and 13, respectively) in parentheses are the docking subunits for either the RAD23 or DSK2 members. A “?” is added for DD1i and NUB1 as chain binding affinity, linkage specificity, and absence of a proteasomal docking site did not support roles in indirect UPP substrate recognition. NA, not applicable; ND, not determined.

cTS, this study.

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eThe approximate binding affinities for either the Lys-48– or Lys-63–linked ubiquitin chains are designated qualitatively by +++, ++, +, and − for strong, moderate, and weak binding and the absence of the binding, respectively.

fThe ampersand indicates that the involved domains contribute additively to the binding. All three UBAs of NUB1 are required for ubiquitin binding.

### RPN10 Is Potentially the Major Docking Subunit for the RAD23 and DSK2 Shuttle Receptors in Arabidopsis

Indirect recognition of ubiquylated substrates by UBL-UBA factors requires an additional proteasomal docking step (Fu et al., 2010). The base subunits RPN1, RPN10, and RPN13 have been reported to be able to bind UBL-UBA shuttle factors and represent potential docking subunits (Eisasser et al., 2002; Ishii et al., 2006; Husnjak et al., 2008; Matushina et al., 2008). Due to its potential ubiquitin binding activity (Lam et al., 2002), the base subunit RPT5 might also interact with UBL-UBA factors. It is likely that distinct proteasomal docking sites are used for different UBL-UBA factors in a given species. Moreover, the docking sites and associated interfaces for various UBL-UBA factors may be divergent among species. To address these possibilities, we examined whether Arabidopsis RPN1a, RPN10, and RPT5a are involved in recognizing various UBL-UBA factors by analyzing potential direct interactions and associated domains and residues. The role of Arabidopsis RPN13 in the recognition of UBL-UBA factors has been described in a separate study (Fatimababy et al., 2010).

The GST-fused UBL-UBA factors RAD23b-d, DSK2b, DD1i, and NUB1 were tested for their abilities to interact with triple-HA-tagged RPN1a and T7-tagged RPT5a and RPN10 using pull-down assays (see Supplemental Table 2 and Supplemental Figure 5 online). As summarized in Table 2, RAD23b-d and DSK2b associate only with RPN10, but not RPN1a or RPT5a. As described previously, DSK2b associates with RPN13, but the interaction is significantly weaker than that with RPN10 (Fatimababy et al., 2010). By contrast, DD1i and NUB1 did not associate with any of the base subunits tested here (see Supplemental Figure 5 online) or with RPN13 (Fatimababy et al., 2010). In agreement with these results, the endogenous RPN10 proteins in crude Arabidopsis extracts can readily be pulled down by GST-fused RAD23b-d and DSK2b but not by GST-fused DD1i (see Supplemental Figure 5C online). These interaction assays suggest that RPN10, but not RPN1a or RPT5a, is potentially the major proteasomal recognition subunit for the RAD23 and DSK2 members in Arabidopsis. In addition, DD1i and NUB1 are likely not involved or play only minor roles in the indirect recognition of ubiquylated UPP substrates.
Arabidopsis RPN10 Employs Distinct Structural Motifs for the Recognition of RAD23 and DSK2 Members

We next determined the structural motifs in Arabidopsis RPN10 involved in the recognition of RAD23b-d and DSK2b. The possible roles of the three UIMs of RPN10 in recognizing RAD23b-d and DSK2b were tested with RPN10 variants containing single, double, or triple UIM mutations that were used in ubiquitin chain binding assays (see Supplemental Table 2 and Supplemental Figure 2B online). The GST-fused RAD23b-d and DSK2b proteins were tested for their ability to pull down the T7-tagged RPN10 variants (see Supplemental Figure 5B online). As summarized in Table 2, different structural motifs in RPN10 are responsible for the recognition of the DSK2 and RAD23 members. In addition to its role in recognizing ubiquitylated substrates, the UIM1 motif is responsible for DSK2 recognition; the UIM2 motif and, to a lesser extent, the UIM2 motif are responsible for recognizing RAD23 members.

Table 2. Distinct Proteasomal Docking Sites of the Arabidopsis RAD23 and DSK2 Members

<table>
<thead>
<tr>
<th>Name</th>
<th>Docking Subunita</th>
<th>Siteb</th>
<th>Interface on UBL-UBAc</th>
<th>Referencesd</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD23bii</td>
<td>RPN10</td>
<td>UIM2 and UIM3</td>
<td>UBL</td>
<td>TS; Fatimababy et al. (2010)</td>
</tr>
<tr>
<td>RAD23c</td>
<td>RPN10</td>
<td>UIM3</td>
<td>UBL</td>
<td>TS; Fatimababy et al. (2010)</td>
</tr>
<tr>
<td>RAD23d</td>
<td>RPN10</td>
<td>UIM2 and UIM3</td>
<td>UBL</td>
<td>TS; Fatimababy et al. (2010)</td>
</tr>
<tr>
<td>DSK2b</td>
<td>RPN10</td>
<td>UIM1</td>
<td>UBL</td>
<td>TS; Fatimababy et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>RPN13</td>
<td>PRU</td>
<td>UBL (Val-87)</td>
<td>Fatimababy et al. (2010)</td>
</tr>
</tbody>
</table>

Although the association of RAD23b-d and DSK2b with RPN10 or RPN13 was shown in our previous study for cross-species comparison (Fatimababy et al., 2010), the lack of involvement of RPN1 and RPT5 in recognizing these UBL-UBA factors was determined in this study. The boldfaced items are properties shown in this study, and regular font items were examined in the listed studies.

*Both RPN10 and RPN13 are involved in recognizing DSK2, but RPN10 may play a more important role as it has a stronger affinity for DSK2 than RPN13.

bUIM2 and UIM3 are involved in RAD23b-d recognition, in which UIM3 is more critical.

the interface regions on the UBL-UBA factors that are involved in binding with the docking subunit. The abbreviated amino acid indicated in parentheses is potentially divergent, as determined using mutagenesis.

Arabidopsis RP10, we tested the role of the potential hydrophobic patches on the UBL domains of the RAD23 and DSK2 members (equivalent to the hydrophobic patch in ubiquitin) in RPN10 interactions. Residues corresponding to Leu-8, Ile-44, and Val-70 in the hydrophobic patch of ubiquitin (Beal et al., 1996) are generally conserved in the UBL domains of the Arabidopsis RAD23 and DSK2 members (see Supplemental Figure 6 online). However, a slightly greater divergence in the corresponding residues in the UBL domain of DD1 was observed, which agrees with its inability to associate with RPN10. One or two conserved residues in the potential hydrophobic patches of the UBL domains of Arabidopsis RAD23b-d and DSK2b, including Leu-8 and Ile-47 for RAD23c, Leu-8 and Ile-46 for RAD23d, Ile-47 for RAD23b, and Ile-61 and Val-87 for DSK2b, were individually replaced with Ala residues (see Supplemental Table 2 online). The resulting variants lost their capability to associate with RPN10 (see Supplemental Figure 7 online). The results indicate that the conserved hydrophobic interfaces on the UBL domains of the RAD23 and DSK2 members are critical for their association with RPN10 (Table 2). Further structural analyses and computational modeling are required to determine the structural bases for recognition site specificity in the different UBL-UBA factors.

The Major Arabidopsis Receptors of Ubiquitylated UPP Substrates Are Generally Functional Redundant

Based on its strong affinities for Lys-48–linked ubiquitin chains and the major shuttle factors RAD23s and DSK2s, Arabidopsis RPN10 potentially plays a central role in both direct and indirect recognition of ubiquitylated UPP substrates. To examine the specific in vivo functions associated with different ubiquitin receptors and the potentially unique and central role associated with RPN10, we identified and established one or two Arabidopsis T-DNA insertion lines for each of the loci encoding the ubiquitin receptors of interest, including RPN10, RPN13, RAD23a-d, DSK2a-b, DD1, and NUB1 (see Supplemental Table 3 online). The T-DNA insertion lines were designated rpn10-2, rpn13-1, rad23a-2, rad23b-3, rad23c-2, rad23d-1, dsk2a-1, dsk2b-1, dd1-2, dd1-3, and nub1-1. The rpn10-2 line was established and initially characterized previously (Fatimababy et al., 2010). With the exception of dsk2a-1 and dsk2b-1, the established T-DNA insertion lines likely represent null mutations for the inserted loci because transcript and protein expression of the corresponding loci were generally not detected (Figure 1A; see Supplemental Table 3 online).

Wild-type phenotypes were associated with dsk2a-1 and dsk2b-1 under normal growth conditions, likely because protein expression was unaltered or only slightly reduced in these lines (Figure 1A; see Supplemental Table 3 online). However, wild-type phenotypes, as exemplified in 7- and 14-d-old seedlings, were also observed with multiple independent RNAi lines targeted to the two DSK2 loci (Figure 1B; e.g., dsk2i1-4 and dsk2i5-2), even though some of these lines had drastically reduced protein expression levels for both DSK2a and DSK2b.
dsk2-RNAi). Notably, except for rpn10-2, all of the other characterized T-DNA insertion null mutants of genes encoding ubiquitin receptors also displayed wild-type phenotypes under normal growth conditions, as exemplified in 7- and 14-d-old seedlings (Figure 1B). With the exception of a slightly reduced growth rate and partially defective ovules (36.4% ± 6.7%), near-wild-type phenotypes were associated with a quadruple null mutant of the RAD23 family (rad23a-2 rad23b-3 rad23c-2 rad23d-1) under normal growth conditions (Figures 1A and 1B, rad23abcd). However, as described previously (Fatimababy et al., 2010) and below, the rpn10-2 null mutant showed severe vegetative and reproductive growth phenotypes, suggesting that RPN10 has pivotal in vivo functions. The lack of clear phenotypes in the knockout or knockdown mutants of major ubiquitin receptors suggest their functional redundancy in Arabidopsis.

Pleiotropic Vegetative and Reproductive Growth Phenotypes Are Associated with the Loss of RPN10 in Arabidopsis

A previous study reported a RPN10 T-DNA insertion line (rpn10-1) in the C24 ecotype background, in which RPN10 was C-terminally

Figure 1. Protein Expression and Phenotypes of Arabidopsis T-DNA or RNAi Lines for Loci Encoding Various UPP Ubiquitin Receptors.

(A) The expression levels in rosette leaves or seedlings for the proteins encoded by genes targeted in various Arabidopsis T-DNA or RNAi lines, including rpn10-2; rpn13-1; rad23a-2, rad23b-3, rad23c-2, rad23d-1, rad23abc, rad23abcd, rad23bcd, and rad23abcd (rad23s); dsk2a-1 and dsk2b-1 (dsk2s); dsk2-RNAi lines; ddi1-2 and ddi1-3 (ddi1s); and nub1-1, were determined by immunoblotting using antisera prepared separately with recombinant proteins, as indicated at the right. The expected mobilized positions of the various ubiquitin receptors are indicated with arrows, and the members’ alphabetical extensions are indicated when multiple members exist. The mobilized positions marked for RAD23a-d were determined using single, triple, and quadruple knockout mutants for four RAD23 gene members. The DDI1 and RPN10 expression levels or Brilliant Blue R (BBR)–stained gels are included to confirm that equal protein samples from Col-0 and insertion line(s) were loaded. For rpn13-1, the mobilized positions for protein size markers (in kilodaltons) are indicated at the left. The asterisks mark the positions of nonspecific proteins that were detected with the rpn13-1 protein sample.

(B) Representative 7- (D7) and 14-d-old (D14) seedlings from various T-DNA or RNAi lines.
truncated (all three UIMs deleted) and expressed at a very low level as a neomycin phosphotransferase (NPT-II) fusion protein (Smalle et al., 2003). This rpn10-1 line exhibited pleiotropic phenotypes, including reductions in germination, growth rate, stamen number, genetic transmission through male gametes, hormone-induced cell division, and seed set. It also displayed an increased sensitivity to abscisic acid. A slight accumulation of ubiquitylated substrates and a specific and drastic stabilization of ABI5 were also observed in the rpn10-1 mutant plants, indicating a defect in substrate degradation. However, whether the pleiotropic phenotypes were caused by loss of the UPP substrate recognition function (UIM deletion) or a nearly null mutation of RPN10 requires further investigation.

To address this question, we previously identified and established a new RPN10 T-DNA insertion line, rpn10-2, in the Columbia-0 (Col-0) ecotype background; the T-DNA in rpn10-2 was inserted in intron 4 of RPN10, and no RPN10 transcript or protein expression was detected, suggesting that rpn10-2 is a null mutant (Fatimababy et al., 2010) (Figures 1A and 2A; see Supplemental Table 3 online). Whereas rpn10-2 was initially characterized in the previous study, we performed more careful and extensive phenotypic analysis here. The homozygous rpn10-2 plants were sterile and were obtained by segregating heterozygous plants; only ~2% of progeny plants were homozygous. The vegetative and reproductive growth phenotypes of the homozygous mutant plants were then compared with those of wild-type Col-0 plants.

The rpn10-2 plants displayed a significantly reduced growth rate, as exemplified by plants at 22 and 45 d after stratification treatment (DAS) (Figure 2B). The rpn10-2 plants demonstrated significantly late flowering under long-day growth conditions. Whereas Col-0 plants flowered at ~20 DAS with an average of seven rosette leaves, the rpn10-2 plants flowered at ~40 DAS with an average of 18 rosette leaves (Figure 2C). The rpn10-2 plants had a significantly extended life cycle (from germination to senescence) and reached significantly higher final plant heights compared with Col-0 (Fatimababy et al., 2010) (Figure 2B, 76 DAS). The mature rpn10-2 plants also had larger, more lanceolar and epinastic rosette and cauline leaves than the Col-0 plants (Figures 2B [45 DAS] and 3A). In addition, the rpn10-2 rosette leaves were serrated (Figure 3A). The rosette leaves of the rpn10-2 plants were significantly thicker, and additional cell layers in both the palisade and parenchyma tissues were observed in the cross section (Figures 3B and 3C). A great portion of the trichomes on the upper epidermis of mature rpn10-2 rosette leaves at 55 DAS had increased branching (Figure 3D). Notably, delayed dark-induced senescence was associated with mature rpn10-2 rosette leaves (Figure 3E), which may partly account for the prolonged life cycle.

The rpn10-2 siliques were underdeveloped and completely sterile (Figure 4A). Although the average ovule number per developing carpel was slightly greater than that in Col-0, all ovules (100%) were aborted (Figures 4B and 4C). The male and female gamete function of rpn10-2 plants was tested by reciprocal crossing with Col-0. More than 50% of the ovules of crossed siliques were aborted (55.3% ± 15.2%) when a homozygous rpn10-2 plant served as the female parent; however, nearly all ovules (94.4% ± 6.4%) of crossed siliques were aborted when a homozygous rpn10-2 plant served as the male parent (Figure 4D). This result indicates that female and especially male gametes were defective.

The gamete defects of the rpn10-2 plants were further examined. As observed by cryo-scanning electron microscopy (cryo-SEM), the rpn10-2 anthers were clearly larger than those of the wild type (Figure 5A; see below). Compared with those of Col-0, the rpn10-2 anthers contained larger pollen grains (14.5 and 21.0% increases in the average height and width, respectively) and a greater amount of shrunk pollen (Figure 5B, red arrowheads). Accordingly, the average diameter of the rpn10-2 pollen grains that were visualized with Alexander’s stain was significantly increased compared with that of the wild type (Figure 5C). In anther cross sections, the rpn10-2 pollen grains were generally larger and more irregular in shape. In addition, the rpn10-2 pollen grains were full of small vacuoles and were frequently shrunk (Figure 5D). Furthermore, the rpn10-2 pollen grains that were stained with 4’,6-diamidino-2-phenylindole (DAPI) frequently displayed an absence of nuclei, diffuse nuclei, or (at best) two clear nuclei; these grains only rarely had the trinucleate pattern that is typically associated with Col-0 pollen (Figure 5E). The female gamete defect of the rpn10-2 plants was further examined by confocal laser scanning microscopy (CLSM). As summarized in Figure 5F, the rpn10-2 female gametophytes from ovules of different floral stages generally demonstrated a broader distribution of developmental stages and more frequently contained degenerated nuclei compared with the Col-0 female gametophytes of the corresponding floral stages.

Significant phenotypes during reproductive growth, including the aforementioned gamete defects, were clearly associated with the rpn10-2 mutation. The rpn10-2 plants had reduced auxillary inflorescences and increased accumulation of anthocyanin on the floral stem surface (Figures 6A and 6B). The rpn10-2 plants had significantly larger floral organs (Figure 6C) and extended pedicels (Figures 4A and 6D). The rpn10-2 petal surface area (2.88 ± 0.42 mm²) was approximately threefold larger than that of the wild-type plants (0.88 ± 0.13 mm²) (Figure 6E). Furthermore, cryo-SEM revealed that the increased petal surface area of rpn10-2 flowers could be largely attributed to increased cell size (e.g., the petal cell size of rpn10-2 flowers [177.56 ± 11.83 μm²] was approximately twofold greater than that of Col-0 [89.26 ± 14.17 μm²]) (Figures 6F and 6G). As examined by flow cytometry, only a small increase in polyploidy was observed in the petal and rosette leaf cells in rpn10-2 plants at various stages (see Supplemental Figure 8 online), indicating that polyploidy likely plays a minor role in the increased petal and leaf sizes. In the rpn10-2 plants, the stamen height and the anther and pistil size (both height and width) were significantly increased compared with those of Col-0 plants (Figures 6H to 6J). In addition to the increased floral organ size, the rpn10-2 plants exhibited abnormal floral organ numbers, particularly in the second and third whorls, the petals, and the stamens. Rather than the four petals and six stamens that are typical of Col-0 flowers, the rpn10-2 plants had numerous flowers containing three (2.3%), five (27.3%), or six (5.7%) petals and four (4.6%) or five (56.8%) stamens (see Supplemental Table 4 online).
Complementation Experiments Support That rpn10-2 Phenotypes Are Caused by a Loss of RPN10 Function

To confirm that the phenotypes associated with rpn10-2 were caused by the loss of RPN10 function, we performed complementation experiments using the RPN10 coding region driven by either the cauliflower mosaic virus 35S promoter or the endogenous RPN10 promoter. Multiple homozygous double transformants for rpn10-2 and the RPN10 coding region driven by 35S or the endogenous promoter were generated and assessed for complementation of the rpn10-2 phenotypes. The established complementation lines carrying the RPN10 coding region driven by the 35S promoter were designated c35S-1, c35S-2, and c35S-4, and the lines carrying the RPN10 coding region driven by the endogenous promoter were designated cN10-3, cN10-11, cN10-14, cN10-19, cN10-20, and cN10-25. These lines expressed RPN10 in rosette leaves at approximately equivalent (c35S lines) or lower (cN10 lines) levels than those observed in the Col-0 plants (Figure 2A) (Fatimababy et al., 2010). The c35S-1 and -2 lines were initially analyzed previously (Fatimababy et al., 2010). As 35S promoter–driven RPN10 failed to complement the gametophytic phenotypes (see below), complementation was further tested using endogenous RPN10 promoter.

With the exception of the male gamete defect, which was only fully complemented in the cN10 lines, all assessed rpn10-2 phenotypes were generally complemented in the cN10 and c35S lines, even in the lines with the lowest RPN10 expression (i.e., cN10-11 and cN10-20) (Figure 2A). The rescued phenotypes included the delayed growth rate exemplified by 22 and 45 DAS plants (Figure 2B), late flowering (Figures 2B [45 DAS] and 2C), altered leaf morphology (Figure 3A), increased leaf thickness (Figure 3C), increased leaf trichome branch number (Figure 3D), delayed dark-induced leaf senescence (Figure 3E), sterility (Figures 4A and 4C), reduced axillary inflorescences and accumulation of anthocyanin (data not shown), large flowers (Figure 6C), extended pedicels (Figures 4A and 6D), large petals (Figures 6C and 6E), large petal cells (Figure 6G), increased stamen length (Figure 6H), and increased anther and pistil size (Figures 6I and 6J). With the exception of the stamen of the c35S lines, the altered flower organ number was also generally complemented by both constructs (see Supplemental Table 4 online).

**Figure 2.** Reduced Plant Growth Rate and Delayed Flowering Time Are Associated with the rpn10-2 Line.

(A) The expression levels of RPN10 and 20S proteasome subunits in rpn10-2 and the complementation lines. The expression of RPN10 (α-N10) and 20S proteasome subunits (α-20S) in 7-d-old seedlings from Col-0, rpn10-2, and complementation lines cN10 and u123 were examined by immunoblotting. The Brilliant Blue R (BBR)–stained duplicate gels were examined to confirm equal loading. The obvious mobility shift of the triple-UIM RPN10 mutant likely is due to the UIM1 mutation, as noted previously (Fu et al., 1998).

(B) Representative 22, 45, and 76 DAS Col-0, rpn10-2, and complementation plants are shown. The reduced growth rate and increased final plant height associated with rpn10-2 plants were restored in the complementation lines.

(C) The flowering time, recorded by DAS (left) or rosette leaf numbers (right), for Col-0, rpn10-2, and complementation lines. The flowering time was recorded when the floral stalk reached ~1 cm. The numbers indicated above the histograms are the sample sizes. The error bars represent the SD. **P < 0.01, rpn10-2 was compared with Col-0; ++P < 0.01, the complementation lines were compared with rpn10-2 using Student’s t test.
Although fertility was completely restored in the cN10 lines, the number of seeds in the c35S siliques was only ~20% of the number observed for Col-0 plants. Reciprocal crossing with Col-0 was performed to determine if the male or female gametes of the c35S lines functioned normally. We found that the ovules were almost always aborted if one of the c35S lines was used as the male parent. By contrast, ~26% of the ovules were aborted if one of the c35S lines was used as the female parent, which was a rate that was only slightly higher than that of the Col-0 crossing control (15.7%) (Figure 4E). Among the c35S lines used as the female parent, the highest ovule abortion rate was found in c35S-1, which had a slightly lower rpn10-2 expression level in the rosette leaves than c35S-2 and c35S-4 (Figure 4E). As examined by cryo-SEM and Alexander’s stain, the pollen grain size appeared to be almost completely restored in the c35S and cN10 lines (Figure 5C). However, the DAPI-stained nuclear patterns of the c35S pollen were quite similar to those of the rpn10-2 pollen (Figure 5E). By contrast, the DAPI staining patterns of the cN10 pollen were similar to those of Col-0 (Figure 5E). Thus, the low fertility of the c35S lines was primarily due to the male gamete defect. The inability of the c35S construct to complement the male gamete defect associated with rpn10-2 is likely due to the absence of 35S promoter activity in pollen (Wilkinson et al., 1997). In agreement with this hypothesis, RPN10 expression was detected in pollen from the cN10 but not the c35S lines (Figure 4F, left).

The rpn10-2 Phenotypes Were Rescued by a RPN10 Variant Defective in Ubiquitin and Ubiquitin-Like Recognition Functions

Similar to other major UPP ubiquitin receptors (e.g., RPN13 and RAD23), RPN10 is a multifunctional protein with distinct structural domains (or motifs) that have different activities. Whereas the N-terminal vWA domain is critical for stable lid-base association (Fu et al., 2001), the three UIM motifs are involved in binding ubiquitylated proteins and UBL-UBA factors (see Supplemental Figures 2 and 5 online). The observed phenotypes...
associated with rpn10-2 (and rpn10-1) could be due to defects of any of these functions individually or in combination. One intriguing possibility is that the null phenotypes are caused by defective ubiquitin and ubiquitin-like recognition. To examine this possibility, the phenotypes were examined in multiple rpn10-2 lines expressing a triple UIM-site–mutated RPN10 variant that is expressed at levels equivalent to those of the wild-type protein in the cN10 lines (Figure 2A, u123-1 and -2). Surprisingly, all major phenotypes associated with rpn10-2 were rescued in these lines. The rescued rpn10-2 phenotypes included the reduced growth rate exemplified by 22 and 45 DAS plants (Figure 2B), late flowering (Figures 2B [45 DAS] and 2C), altered leaf morphology (Figure 3A), increased leaf trichome branch number (Figure 3D), delayed dark-induced leaf senescence (Figure 3E), sterility (Figures 4A and 4C), large flowers (Figure 6C), increased petal size (Figure 6E), extended pedicels (Figures 4A and 6D), increased stamen height (Figure 6H), increased anther and pistil size (Figures 6I and 6J), and large pollen (Figure 5C). The results indicate that the defects in ubiquitin and ubiquitin-like recognition are not responsible for the phenotypes associated with the loss of RPN10.

Increases of the Major Ubiquitin Receptors and the 20S Proteasome Subunits in rpn10-2 Plants

The lack of obvious growth phenotypes in the major ubiquitin receptor mutants and the irrelevance of ubiquitin and ubiquitin-like recognition defects of RPN10 to its null phenotypes suggest that the major ubiquitin recognition pathways in Arabidopsis are functionally redundant. Notably, slight increases in the protein expression levels of other potential UPP ubiquitin receptors were observed in rpn10-2 plants compared with Col-0 plants. Whereas an unaltered or slightly reduced expression level was detected for the Cop9/signalosome subunit CSN5 and the lid subunit RPN8, respectively, slight increases were detected with the intrinsic proteasome subunits RPN13 and RPT5 and the UBL-containing shuttle factors RAD23a (or bII), RAD23d, DSK2a (or b or both), and DDI1 (Figure 7A). Additionally, similar to the
Figure 5. Larger Anthers and Pollen and Distorted Developmental Stage Distribution of Female Gametes Are Observed with the *rpn10-2* Flowers.
(A) Representative anthers from stage 13 Col-0 and *rpn10-2* flowers are shown using cryo-SEM. Note that the images are shown with different scales; the *rpn10-2* plants have larger anthers than do the Col-0 plants. The red-boxed regions contain identical areas and are shown as close-ups in (B).
(B) The representative *rpn10-2* anther contains larger and shrunken pollen grains, marked by red arrowheads, compared with the typical wild-type Col-0 pollen grains.
(C) The relative diameters of pollen grains from the Col-0, *rpn10-2*, and complementation plants. The pollen grains were visualized by Alexander’s stain, and the numbers of pollen grains measured (n) are indicated. The error bars represent the SD. **P < 0.01, *rpn10-2* was compared with Col-0; ++P < 0.01, the complementation lines were compared with *rpn10-2* using Student’s t test.
(D) Representative anther cross-sections from stage 12 Col-0 and *rpn10-2* flowers. Larger, irregular, and shrunken pollen are associated with *rpn10-2* anthers, which often are full of small vacuoles.
(E) DAPI-stained pollen from Col-0, *rpn10-2*, *c35S-2*, and *cN10-25* plants. The typical trinucleate pattern is associated only with Col-0 and *cN10-25* plants; it is not associated with *rpn10-2* and *c35S-2* plants.
(F) The developmental stage distribution of the female gametophytes in flowers of different stages from Col-0 and *rpn10-2* plants. The ovules were collected from Col-0 and *rpn10-2* flowers at stage 12a, 12b, and 12c, from Col-0 flowers 2 d after emasculation (em) and from stage 13 *rpn10-2* flowers. The ovule stage distributions are indicated as color-coded horizontal bars with numbers of observed ovules. The female gametophyte developmental stages were determined using CLSM and were classified as FG0-7, as previously described (Christensen et al., 1998). Large portions of the *rpn10-2* female gametophytes from flowers of different stages contained only degenerated nuclei or lacked nuclei altogether (DG).
feedback regulation of proteasome subunits in proteasome mutants (Yang et al., 2004), the expression of 20S proteasome subunits was markedly increased in rpn10-2 plants (Figure 7A, α-20S).

Despite the different RPN10 expression levels in the various complementation lines, the altered expression levels of RPN13, RPT5, RAD23s, DSK2s, and DDI1 associated with rpn10-2 were restored to Col-0 levels (e.g., cN10-19 and c35S-2; Figure 7A). Notably, expression levels of 20S proteasome subunits in leaves (Figure 2A), pollen (Figure 4F, left), and flowers (Figure 4F, right) were generally inversely correlated with the RPN10 expression level in Col-0, rpn10-2, and the various cN10 lines.

Similar to the complementation lines expressing wild-type RPN10 driven by the 35S or endogenous promoter, the wild-type expression of the various UPP receptors examined was also observed in the rpn10-2 lines expressing the triple UIM-site-mutated RPN10 variant (Figure 7B, e.g., α-DDI1 and α-RAD23b).
However, the triple UIM-site RPN10 variant did not completely restore proper expression of the 20S proteasome. Whereas the RPN10 variant was expressed at levels approximately similar to those of the wild-type protein in Col-0 and cN10-19, the 20S proteasome subunits in complementation lines expressing the triple UIM RPN10 mutant were expressed at considerably higher levels (Figure 7B, \(\alpha\)-20S).

Reduced Abundance of Double-Capped Proteasomes in \textit{rpn10-2} Plants

Characterization of the 26S proteasomes purified from an \textit{rpn10Δ} yeast strain suggests that RPN10 plays a critical role in stable lid-base association (Glickman et al., 1998; Fu et al., 2001). As ubiquitin and ubiquitin-like recognition defects of RPN10 cannot account for its null phenotypes, the effect of RPN10 deletion in proteasome stability potentially becomes the primary cause of the various null phenotypes. To examine this possibility, the levels of core (CP), single-capped (RP1-CP), and double-capped (RP2-CP) proteasomes in \textit{rpn10-2} plants were examined and compared with those in Col-0 by native PAGE in conjunction with immunoblotting using the indicated antisera. CSN5 expression was included as a loading control. The slight mobility variation in different blots for major complexes is due to different PAGE run times.

This page contains a figure with images labeled A, B, C, D, and E, which are part of Figure 7. The figure describes the feedback regulation of ubiquitin receptors and altered proteasome complex abundance in \textit{rpn10-2} plants. It details the expression of various components and the effect of RPN10 deletion on proteasome stability.
an in-gel activity assay. The core proteasome was detected by immunoblotting with antisera against the moss (Physcomitrella patens) 20S proteasome and by an in-gel activity assay in the presence of SDS. Whereas the abundance of single-capped proteasomes in rpn10-2 plants was equivalent to that of Col-0 plants, the abundance of double-capped proteasomes was reduced (Figures 7C and 7D). In agreement with the results from the immunoblots of the SDS-PAGE gels (Figures 2A and 7A, RPN10 and RPN13, UBL-UBA shuttle factors (RAD23a-d, DSK2a-b, and DD1), and CDC48 complex subunits (CDC48a-c, UFD1a-d, and NPL4a-b). The potential importance of these Arabidopsis UPP ubiquitin receptors was assessed in vitro by determining the binding affinity and ubiquitin chain type preference of the purified recombinant proteins. The potentially primary docking sites on the proteasome for UBL-UBA factors were also determined by in vitro protein–protein interaction analyses.

Given their strong affinities for Lys-48–linked ubiquitin chains, which is the major targeting signal of UPP substrates, Arabidopsis RPN10 and the RAD23 and DSK2 UBL-UBA factors may play major roles in the direct and indirect recognition of ubiquitylated UPP substrates, respectively (Table 1; see Supplemental Figures 2 and 3 online). Other base subunits, including RPN1 and RPT5, that are potentially involved in direct UPP substrate recognition were similarly tested but did not interact with ubiquitin chains. Arabidopsis RPN13, however, has been shown to possess weak affinity for Lys-48–linked ubiquitin chains (Fatimababy et al., 2010), suggesting that it plays a minor role in direct UPP substrate recognition. Moreover, although RPN13 is an integral subunit of the 26S proteasome in yeast and humans (Husnjak et al., 2000; Schreiner et al., 2008), Arabidopsis RPN13 was not detected in single- or double-capped proteasomes by native PAGE and immunoblotting. Arabidopsis RPN13 was also not detected with affinity purified 26S proteasomes (Book et al., 2010). These results suggest that RPN13 may not be an integral 26S proteasome subunit or that it associates only weakly and transiently with the Arabidopsis complex. Pairwise yeast two-hybrid interaction analyses using all yeast regulatory particle subunits and Gal4-BD–fused monomeric or pentameric ubiquitin as baits detected no additional RP subunits that interact with ubiquitin.

Among the examined Arabidopsis UBL-UBA factors, only RAD23b-d and DSK2a-b had strong affinities for Lys-48–linked ubiquitin chains, suggesting that they may play major roles in the indirect recognition of ubiquitylated UPP substrates (Table 1; see Supplemental Figure 3 online). By contrast, whereas DD1 displayed relatively weaker affinities for both Lys-48– and Lys-63–linked ubiquitin chains, NUB1 had a weak affinity for Lys-63– but not Lys-48–linked ubiquitin chains, indicating that these factors make only minor contributions to UPP substrate recognition. Likewise, the lack of ubiquitin chain binding activity exhibited by the p62-like Arabidopsis LPS protein suggests that it is also not involved in the recognition of ubiquitylated substrates.

The UBL-UBA factor-mediated indirect recognition of ubiquitylated UPP substrates requires an additional proteasomal docking step (Fu et al., 2010). The four base subunits most likely to be involved in this process (RPN1, RPT5, RPN10, and RPN13) were examined here (Table 2; see Supplemental Figure 5 online) or previously (Fatimababy et al., 2010) for their ability to interact with the UBL-UBA factors RAD23b-d, DSK2b, DD1, and NUB1. RPN10 interacts with DSK2b and RAD23b-d (but not DD1 or NUB1) through distinct structural elements; DSK2s associate with RPN10 via UIM1 and RAD23s primarily through UIM3 (UIM2 has a minor role). This structural separation in RPN10 may be a critical mechanistic component, or it may allow for cooperative action during UPP substrate recognition. In comparison with RPN10, RPN13 likely plays a minor role in recognizing DSK2 factors but does not interact with RAD23s or DD1 (Fatimababy et al., 2010).

**DISCUSSION**

To delineate the functional and mechanistic complexity of the ubiquitylated UPP substrate recognition pathways in Arabidopsis, we characterized the major Arabidopsis ubiquitin receptors. Although in vitro interaction analyses and mutant phenotypes suggest that RPN10 potentially plays a unique and central role in UPP substrate recognition, surprisingly, the various rpn10-2 phenotypes were rescued by a RPN10 variant that is defective in ubiquitin and ubiquitin-like recognition. Notably, double-capped and core proteasome complexes were reduced and increased, respectively, in the RPN10 null mutant, and the former could be restored to approximately wild-type levels by the RPN10 variant defective in ubiquitin and ubiquitin-like recognition. Our results suggest that defective 26S proteasome stability might be the primary cause of the rpn10-2 phenotypes. Moreover, because near-wild-type growth phenotypes were associated with most of the examined ubiquitin receptor mutants, functionally redundant UPP substrate recognition pathways are likely utilized in Arabidopsis to target ubiquitylated substrates for proteasomal degradation.

**Major UPP Ubiquitin Receptors Found in Other Species Are Present in Arabidopsis**

The Arabidopsis genome contains conserved sequences encoding all of the major UPP ubiquitin receptors that are found in other species, including the intrinsic proteasome base subunits RPN10 and RPN13, UBL-UBA shuttle factors (RAD23a-d, DSK2a-b, and DD1), and CDC48 complex subunits (CDC48a-c, UFD1a-d, and NPL4a-b). The potential importance of these Arabidopsis UPP ubiquitin receptors in the recognition of ubiquitylated UPP substrates was assessed in vitro by determining the binding affinity and ubiquitin chain type preference of the purified recombinant proteins. The potentially primary docking sites on the proteasome for UBL-UBA factors were also determined by in vitro protein–protein interaction analyses.

Given their strong affinities for Lys-48–linked ubiquitin chains, which is the major targeting signal of UPP substrates, Arabidopsis RPN10 and the RAD23 and DSK2 UBL-UBA factors may play major roles in the direct and indirect recognition of ubiquitylated UPP substrates, respectively (Table 1; see Supplemental Figures 2 and 3 online). Other base subunits, including RPN1 and RPT5, that are potentially involved in direct UPP substrate recognition were similarly tested but did not interact with ubiquitin chains. Arabidopsis RPN13, however, has been shown to possess weak affinity for Lys-48–linked ubiquitin chains (Fatimababy et al., 2010), suggesting that it plays a minor role in direct UPP substrate recognition. Moreover, although RPN13 is an integral subunit of the 26S proteasome in yeast and humans (Husnjak et al., 2000; Schreiner et al., 2008), Arabidopsis RPN13 was not detected in single- or double-capped proteasomes by native PAGE and immunoblotting. Arabidopsis RPN13 was also not detected with affinity purified 26S proteasomes (Book et al., 2010). These results suggest that RPN13 may not be an integral 26S proteasome subunit or that it associates only weakly and transiently with the Arabidopsis complex. Pairwise yeast two-hybrid interaction analyses using all yeast regulatory particle subunits and Gal4-BD–fused monomeric or pentameric ubiquitin as baits detected no additional RP subunits that interact with ubiquitin.

Among the examined Arabidopsis UBL-UBA factors, only RAD23b-d and DSK2a-b had strong affinities for Lys-48–linked ubiquitin chains, suggesting that they may play major roles in the indirect recognition of ubiquitylated UPP substrates (Table 1; see Supplemental Figure 3 online). By contrast, whereas DD1 displayed relatively weaker affinities for both Lys-48– and Lys-63–linked ubiquitin chains, NUB1 had a weak affinity for Lys-63– but not Lys-48–linked ubiquitin chains, indicating that these factors make only minor contributions to UPP substrate recognition. Likewise, the lack of ubiquitin chain binding activity exhibited by the p62-like Arabidopsis LPS protein suggests that it is also not involved in the recognition of ubiquitylated substrates.

The UBL-UBA factor-mediated indirect recognition of ubiquitylated UPP substrates requires an additional proteasomal docking step (Fu et al., 2010). The four base subunits most likely to be involved in this process (RPN1, RPT5, RPN10, and RPN13) were examined here (Table 2; see Supplemental Figure 5 online) or previously (Fatimababy et al., 2010) for their ability to interact with the UBL-UBA factors RAD23b-d, DSK2b, DD1, and NUB1. RPN10 interacts with DSK2b and RAD23b-d (but not DD1 or NUB1) through distinct structural elements; DSK2s associate with RPN10 via UIM1 and RAD23s primarily through UIM3 (UIM2 has a minor role). This structural separation in RPN10 may be a critical mechanistic component, or it may allow for cooperative action during UPP substrate recognition. In comparison with RPN10, RPN13 likely plays a minor role in recognizing DSK2 factors but does not interact with RAD23s or DD1 (Fatimababy et al., 2010).
Near-Wild-Type Growth Phenotypes of Major Ubiquitin Receptor Mutants Suggest Functional Redundancy

Among the ubiquitin receptor mutants examined, only rpn10-2 displayed severe vegetative and reproductive growth defects, and near-wild-type growth phenotypes were observed for the rest of the ubiquitin receptor mutants. As the defects in ubiquitin and ubiquitin-like recognition of RPN10 could not account for the rpn10-2 phenotypes, these results clearly suggest that the ubiquitylated substrate recognition pathways mediated by these major ubiquitin receptors are functionally redundant in Arabidopsis.

A clear absence of protein expression was associated with the T-DNA insertion mutants rpn13-1, ddi1-2, ddi1-3, and nub1-1, indicating that these lines are null mutants for the single gene-encoded UPP ubiquitin receptors RPN13, DDI1, and NUB1, respectively (Figure 1A; see Supplemental Table 3 online). However, near-wild-type growth phenotypes under normal growth conditions were observed for these null mutants, suggesting that the functional roles (if any) of RPN13, DDI1, and NUB1 in UPP substrate recognition overlap with those of other ubiquitin receptors. Although in vitro ubiquitin chain binding analysis indicated that Arabidopsis RAD23s and DSK2s likely play major roles in the indirect recognition of ubiquitylated UPP substrates (Table 1), the single null rad23a-2, rad23b-3, rad23c-2, and rad23d-1 mutants demonstrated near-wild-type growth phenotypes. Strikingly, with the exception of a partial ovule defect, even an established quadruple mutant (rad23a-2 rad23b-3 rad23c-2 rad23d-1) also showed near-wild-type growth phenotypes. These results indicate that the contribution to indirect UPP substrate recognition provided by RAD23s is likely not essential or overlap with that of other major recognition factors, such as DSK2s or RPN10. However, two single RAD23b mutants, rad23b-1 and -2, were previously demonstrated to have mild phyllotaxy and sterility defects. Furthermore, a quadruple mutant containing rad23b-1 was unable to thrive, indicating that RAD23 members have redundant functions such that only all four RAD23 members together provide an essential function (Farmer et al., 2009); larger and serrated rosette leaves were associated with this hypothesis, a slight accumulation of ubiquitylated proteins was observed in rpn10-1 and -2 plants, and significant stabilization of a specific regulatory protein, ABI5, was observed in rpn10-1 (Smalle et al., 2003; Fatimababy et al., 2010).

As suggested previously for rpn10-1 (Smalle et al., 2003), the various defects observed in the rpn10-2 plants could be attributed to a major deficiency in the recognition of ubiquitylated substrates, given that RPN10 plays a central role in UPP substrate recognition as revealed by in vitro interaction analyses and the integration into the 26S proteasome of the C-terminal truncated RPN10 marker fusion lacking the three UIMs. However, the extremely low expression level of the RPN10 fusion in rpn10-1 did not preclude the severely compromised proteasome stability function of RPN10 in this mutant. As all examined rpn10-2 phenotypes were rescued by the triple UIM site–mutated RPN10 variant, we have now clearly demonstrated that the recognition defect of ubiquitin and UBL-UBA factors is not responsible for RPN10 null phenotypes. From these results, it is clear that the UPP substrate recognition functions mediated by RPN10 are likely dispensable. Although the major direct and indirect recognition routes are lost in rpn10-2 plants, RPN13 could potentially substitute for the loss of RPN10 function by mediating indirect recognition through interactions with DSK2 (Fatimababy et al., 2010). However, similar to the RAD23 and DSK2 UBL-UBA factors, only a slight increase in RPN13 expression was detected in rpn10-2 plants (Figure 7A). Additionally, the CDC48 complexes are also capable of targeting ubiquitylated substrates to the proteasome (Alexandru et al., 2008).

Most intriguingly, defective proteolysis could be attributed to the altered structural integrity of the 26S proteasome because RPN10 has also been found to be critical for stable lid-base associations (Glickman et al., 1998; Fu et al., 2001). Notably, double-capped proteasomes were markedly reduced (Figures 7C to 7E) in the rpn10-2 plants, possibly due to the altered structural stability of the 26S proteasome, and they were clearly restored by the triple UIM site–mutated RPN10 variant (Figure 7E). Vegetative and reproductive growth defects similar to those associated with rpn10-2 were observed in proteasome RP subunit mutants, suggesting that defective proteolysis of common regulatory factors could be involved. Null, truncated, or chimeric fusion mutations are often associated with these proteasome subunit mutants and possibly altered the 26S proteasome structure, although the precise defects likely differ among the various mutants. For example, rpn1a and rpn12a also presented a reduced growth rate (Kurepa et al., 2009b; Wang et al., 2009); larger and serrated rosette leaves were associated

Underlying Mechanisms That Cause the Pleiotropic Phenotypes of rpn10-2

The pleiotropic phenotypes and complementation experiments performed with the rpn10-2 null mutant clearly indicate that RPN10 is essential for vegetative and reproductive growth in Arabidopsis (Figures 2 to 6). Similar to other UPP ubiquitin receptors, such as RPN13 (Chen et al., 2010), RPN10 is a multifunctional factor that performs several distinct biochemical activities through different structural domains or motifs. Whereas the N-terminal vWA domain is critical for stable lid-base association (Glickman et al., 1998; Fu et al., 2001), the three C-terminal UIM motifs are involved in recognition of ubiquitylated substrates and the major UBL-UBA factors (Tables 1 and 2). A defect in any of these functions could potentially affect proteasomal proteolysis, and it is possible that proteolysis of multiple regulatory factors involved in various growth and developmental processes may be affected by the loss of RPN10. In agreement with this hypothesis, a slight accumulation of ubiquitylated proteins was observed in rpn10-1 and -2 plants, and significant stabilization of a specific regulatory protein, ABI5, was observed in rpn10-1 (Smalle et al., 2003; Fatimababy et al., 2010).

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with rpt2a (Sonoda et al., 2009); lanceolar rosette leaves were associated with rpn5a, rpn8a, and rpt2a (Huang et al., 2006; Book et al., 2009; Kurepa et al., 2009b); an increased trichome branch number on the adaxial leaf epidermis was associated with rpt2a and rpn1a (Kurepa et al., 2009b; Sonoda et al., 2009); and short root phenotypes were associated with rpn12a, rpt2a, and rpn5a (Smaille et al., 2002; Ueda et al., 2004; Gallois et al., 2009; Kurepa et al., 2009b). In terms of reproductive growth defects, delayed flowering was observed for rpn5a, rpn8a, and rpn12a (Smaille et al., 2002; Huang et al., 2006; Book et al., 2009); longer pedicels and abnormal floral organ numbers were associated with rpn8a (Huang et al., 2006); defects in the male and/or female gametophytes caused by postmeiotic cell divisions were associated with rpt5a and rpn5a/5b (Book et al., 2009; Gallois et al., 2009); and larger floral organs were associated with rpt2a and rpn12a (Kurepa et al., 2009b). Similar growth phenotypes are potentially caused by defective proteolysis of different regulatory factors, but some of the shared phenotypes of the proteasome subunit mutants likely are due to defective proteolysis of common regulatory factors.

The 26S proteasome subunit mutants that shared similar phenotypes with rpn10-2 also often displayed distinct phenotypes that were not observed in rpn10-2. For example, small rosettes were associated with rpt5a, rpn1a, and rpn5a (Book et al., 2009; Gallois et al., 2009; Kurepa et al., 2009b); leaf abaxialization and aberrant phyllotaxy were associated with rpn8a (Huang et al., 2006); defective embryogenesis was associated with rpn1a and rpn5a (Brukhin et al., 2005; Book et al., 2009); and abnormal cellular organization in the postembryonic shoot and root apical meristems was associated with rpt2a (Ueda et al., 2004). The distinct phenotypes that were associated with various RP subunit mutants indicate that the structural details of the proteasome defects vary among the different mutants, and the sensitivity of various substrates to different mutations probably also differs. Moreover, some of the subunit-specific phenotypes might be due to the involvement of individual proteasome subunits in direct substrate recognition (Hoyt and Coffino, 2004).

As recently proposed (Kurepa et al., 2009a), the imbalanced steady state levels of the 26S and 20S proteasomes could potentially contribute to some of the rpn10-2 phenotypes observed. As in other RP subunit mutants (Yang et al., 2004; Book et al., 2009), we observed a drastic increase in the 20S proteasome activity that was likely due to increased steady state levels of the 20S proteasome subunits and complex (Figures 2A and 7). The delayed dark-induced leaf senescence observed in rpn10-2 (Figure 3E) and rpn10-1 (Kurepa et al., 2009a) plants could potentially be due to the enhanced capacity to degrade oxidized proteins catalyzed by the increased 20S proteasome activity (Kurepa et al., 2008). However, the possibility that imbalanced 26/20S complexes cause the null phenotypes is relatively remote as the phenotype-rescued lines carrying the triple UIM site RPN10 variant expressed high levels of 20S proteasomes (Figures 7B and 7E). In yeast, feedback regulation of proteasome function is largely regulated at the transcriptional level by the transcription factor Rpn4 (Dohmen et al., 2007). A similar transcriptional feedback regulation system employing the nuclear factor erythroid-derived 2-related factor Nrf1 was recently observed in mammals (Radakrishnan et al., 2010). However, no Nrf1 or Rpn4 homologs have been detected in Arabidopsis, and little information is available on the mechanisms or the components involved in feedback regulation in this organism.

METHODS

Recombinant Protein Expression, Purification, and GST Pull-down Analyses

The coding sequences for wild-type, site-mutagenized, and deletion variants of the Arabidopsis thaliana ubiquitin receptors were generated by PCR and inserted into either pET42 or pET28 (Novagen) to yield constructs encoding GST/HIS- and T7/HIS-tagged proteins, respectively (see Supplemental Table 2 online). The HA tag of the RPN1a prey was derived from an intermediate vector, p1239 (Li et al., 1998). Site-directed mutagenesis was performed using PuTurbo and paired primers (see Supplemental Table 2 online) that were centered at the mutation sites, according to the manufacturer’s protocols (Stratagene). Multiple-site mutants were generated by sequential mutagenesis. The GST fusion construct of human S5a (U51007) has been described previously (Fatimababy et al., 2010). All of the expression constructs were confirmed by DNA sequencing using an ABI Prism 3700 DNA analyzer (Applied Biosystems). The recombinant proteins expressed in Escherichia coli BL21 (DE3; Novagen) were purified as previously described (Fatimababy et al., 2010). The GST pull-down assay, including the detection of pulled-down products by immunoblotting, has been described previously (Fatimababy et al., 2010).

To visualize the pulled-down ubiquitin chains, conjugates, and T7- or HA-tagged recombinant proteins, we used rabbit polyclonal antiserum against rabbit polyclonal antisum against human ubiquitin (Santa Cruz Biotechnology) or mouse monoclonal antibodies against T7 (Novagen) or the HA tag (Santa Cruz Biotechnology). The horse radish peroxidase–conjugated goat anti-rabbit (or mouse) IgG secondary antisum was used (Santa Cruz Biotechnology). The Lys-48– and Lys-63–linked ubiquitin chains (Ub2-7) were purchased from Boston Biochem.

Plant Materials and Growth Conditions

The sources and stock numbers of the T-DNA lines for loci encoding the various Arabidopsis ubiquitin receptors are listed in Supplemental Table 3 online. Col-0 seeds were purchased from Lelehe Seeds. To grow the Arabidopsis plants, the seeds were surface sterilized using 20% bleach and 0.1% Tween 20 and stratified on half-strength Murashige and Skoog plates (0.8% agar, pH 5.8) supplemented with 1% sucrose at 4°C for 3 d in the dark. The seedlings were then germinated in a growth chamber at 22°C (16 h light/8 h dark). Twelve-day-old seedlings were transferred to soil (a 6:1:1 mixture of humus:vermiculite:perlite) and grown using a 16-h-light/8-h-dark photoperiod with a light intensity of ~120 μmol·m⁻²·s⁻¹ at 22°C. For dark-induced leaf senescence, rosette leaves were detached, placed on Petri dishes containing two layers of 3MM chromatography paper (Whatman) soaked in sterile water, and incubated in the dark at 22°C. To perform crosses, the sepals, petals, and stamens were removed from stage 12 flowers (Smyth et al., 1990), and the pistils were protected by covering with plastic wrap 2 d before hand-pollination of the pistils with anthers from the male parent. Homozygous rpn10-2 plants were identified at the early seedling stage from the progeny of selfed heterozygous plants by their reduced growth rate and leaf morphology and subsequently by genotyping.

Transgenic Constructs and Arabidopsis Transformation

For DSK2 RNAi, two DSK2b (At2g17200) coding fragments (nucleotides 384 to 1024 and 1102 to 1655 as measured from the first base of the start
samples were postfixed in 1% OsO4 in the same buffer for 4 h and rinsed.

Glutaraldehyde and 4% paraformaldehyde in 100 mM sodium phosphate respectively, using an upright Axioplan microscope or an upright Axio Imager Z1. Stained pollen samples were observed by white and UV light, respectively, using first-strand cDNAs and cloned into pBl121 (Jefferson et al., 1987) downstream of the cauliflower mosaic virus 35S promoter (c35S). Alternatively, the RPN10 coding region was placed in pBluescript KS+ (Stratagene) downstream of a PCR-amplified 2267-bp fragment of the endogenous RPN10 promoter (with restriction sites for SacI and Smal added to the 5′ and 3′ ends, respectively). The fused RPN10 promoter–RPN10 coding region was then cloned into pBl101.2 (cN10) (Jefferson et al., 1987). These binary plasmids were separately transformed into Agrobacterium tumefaciens GV3101 using a freeze-thaw method. Arabidopsis transformation was performed as described previously (Clough and Bent, 1998). Homozygous DSK2 RNAi lines were selected from segregating T2 seedlings, which gave 100% BASTA resistance of the corresponding T3 progenies. T1 seedlings containing the complementation construct and rpn10-2 were first selected by kanamycin resistance and genotyping. The homozygous rpn10-2 lines containing the c35S or cN10 complementation constructs were obtained from T2 (or T1 for cN10-25) seedlings by kanamycin resistance and confirmed by genotyping. Double homozygous lines for rpn10-2 and the c35S or cN10 complementation construct were obtained from T3 or T4 seedlings with 100% kanamycin resistance and confirmed by genotyping.

Genotyping and RT-PCR

The preparation of genomic DNA and total RNA for genotyping and RT-PCR has been described previously (Fatimababy et al., 2010). The PCR primer pairs that were used to detect the wild-type loci, the T-DNA junctions, and the encoded transcripts for various T-DNA insertion lines, to amplify the RPN10 coding region and promoter and DSK2b fragments for constructing complementation and RNAi plasmids, and to detect the RPN10 complementation constructs are listed in Supplemental Table 5 online.

Leaf and Anther Cross Sections and Pollen Staining

Fresh, stage 13-14 pollen was incubated in a 10-fold dilution of Alexander’s stain (Alexander, 1969) and directly observed. The Alexander’s stain was made with 10 mL 95% ethanol, 1 mL 1% malachite green (in 95% ethanol), 5 mL 1% fuchsin acid (in water), 0.5 mL 1% orange G (in water), 5 g phenol, 5 g chloral hydrate, 2 mL glacial acetic acid, 25 mL glycerol, and 50 mL distilled water. For DAPI staining, four to 10 opened flowers were placed in an Eppendorf tube containing a 3:1 mixture of ethanol:acetic acid. After brief vortexing and centrifugation, the supernatant was removed, and 10 μL DAPI staining solution (100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, and 1 μg/mL DAPI) was added to the pollen pellet for ~30 min at room temperature. The Alexander’s- and DAPI-stained pollen samples were observed by white and UV light, respectively, using an upright Axioplan microscope or an upright Axioscop imaging system (Carl Zeiss).

To obtain the semithin cross sections of anthers from stage 12 flowers (Smyth et al., 1990) and rosette leaves, the samples were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.0, at room temperature for 4 h. The leaf sections were collected from the midpoint between the midvein and the right or left leaf edge of the central part of the leaf blade. After three rinses with buffer, the samples were postfixed in 1% OsO4 in the same buffer for 4 h and rinsed with buffer three times. The fixed samples were dehydrated as described for CLSM and embedded in Spurr’s resin. Semi-thin cross sections (1 μm) were made using a Reichert Ultrcut S or EM UC6 ultramicrotome (Leica). The sections were stained with 1% toluidine blue O in 1% Borax and observed using an upright Axioplan microscope (Carl Zeiss). The images were captured with a charge-coupled device, such as Cool Snapix (Photometrics) or Axiscan IC1 (Carl Zeiss). The organ size measurements were made using ImageJ 1.38x (National Institutes of Health). The size of the pollen that was visualized by Alexander’s stain and cryo-SEM was manually calculated from the output images.

CLSM and Cryo-SEM

The developmental stages of female gametophytes from different floral stages (Smyth et al., 1990) in ovules were determined using CLSM as previously described (Christensen et al., 1998). The pistils were fixed for 4 h at room temperature using 4% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.0. The fixed samples were dehydrated in a 30 to 100% graded ethanol series (five 5 to 20% reduced increments, each for 30 min). The pistils were then cleared in an 8:1:2 mixture of chloral hydrate:glycerol:water. The ovules were dissected and released from the pistils, mounted, and sealed under a cover slip with fingernail polish. A 488-nm laser line and a long-pass 505-nm filter were used to observe the autofluorescence exhibited from the cytoplasm, nucleoplasm, and nucleoli of the ovule cells on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss).

Cryo-SEM was conducted using a Quanta 200 equipped with a cryo system Quorum PP2000TR (FEI). Freshly dissected samples were loaded on the stub, frozen in liquid nitrogen slush, and transferred to the preparation chamber at ~180°C. The samples were sublimed for 10 to 15 min at ~85°C, coated with Au at ~130°C, transferred to the cryo stage in the SEM chamber, and imaged at ~160°C and 20 kV.

Crude Arabidopsis Protein Extracts and Immunoblotting

To detect specific proteins, crude Arabidopsis protein extracts were prepared as described previously (Fatimababy et al., 2010). Rabbit polyclonal antibodies were raised against purified recombinant full-length Arabidopsis RPN8a, RPN10, RPN13, RAD23b-c, DSK2b, DD1, and NUB1 (custom-made by Genesis Biotech or Cashmere Biotech). Primers, restriction sites, and vectors used for recombinant protein expression are listed in Supplemental Table 2 online. Purification of the His-tagged recombinant proteins was described previously (Fatimababy et al., 2010). Except α-RPN10, α-RAD23c, α-DSK2b, and α-NUB1, which used 2000-, 25,000-, 500-, and 500-fold dilutions, respectively, 1000-fold dilution was used for the rest of antisera. Detection of the immunoblots following SDS-PAGE was performed using either chemiluminescence (Perkin-Elmer) or color development for horseradish peroxidase– or alkaline phosphatase–labeled secondary antisera, respectively. To quantify the protein expression levels, the signals from the chemiluminescent blots were recorded and analyzed using a BioSpectrum 600 configured with VisionWorksLS software, version 6.8.

Abundance Analyses of Various Proteasome Complexes

The proteasome complexes (core, single-capped, and double-capped) from mature rosette leaves were partially purified with 10% (w/v) polyethylene glycol 8000 (Sigma-Aldrich) at 4°C as described previously (Yang et al., 2004). The polyethylene glycol precipitates were dialyzed with extraction buffer supplemented with 1 mM ATP. The various proteasome complexes were separated by 4% native PAGE at 4°C and detected by immunoblotting using antisera against the msp2 20S proteasome, the lid subunits RPN6 and RPN8a, and the base subunits RPT5a and RPN10. The antisera against RPT5a, RPN6, and CSN5 were purchased from Enzo Life Sciences. The antisera against the msp2 20S

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codon) were amplified by PCR using primer pairs dDSK2b-1F/dDSK2b-1R and dDSK2b-2F/dDSK2b-2R (see Supplemental Table 5 online). Each of these fragments was subsequently cloned in the vector orientation, separated by the intron sequence of the *Petunia hybrida* Chalcone Synthase gene in pFGC5941 (Kerschen et al., 2004), to give constructs dDSK2-1 and dDSK2-2. To complement the rpn10-2 phenotypes, the *Arabidopsis* RPN10 coding region was amplified by PCR (with the Smal and SstI restriction sites added to the 5′ and 3′ ends, respectively) from first-strand cDNAs and cloned into pBl121 (Jefferson et al., 1987) downstream of the cauliflower mosaic virus 35S promoter (c35S). Alternatively, the RPN10 coding region was placed in pBluescript KS+ (Stratagene) downstream of a PCR-amplified 2267-bp fragment of the endogenous RPN10 promoter (with restriction sites for SacI and Smal added to the 5′ and 3′ ends, respectively). The fused RPN10 promoter–RPN10 coding region was then cloned into pBl101.2 (cN10) (Jefferson et al., 1987). These binary plasmids were separately transformed into *Agrobacterium* tumefaciens GV3101 using a freeze-thaw method. Arabidopsis transformation was performed as described previously (Clough and Bent, 1998). Homozygous DSK2 RNAi lines were selected from segregating T2 seedlings, which gave 100% BASTA resistance of the corresponding T3 progenies. T1 seedlings containing the complementation construct and rpn10-2 were first selected by kanamycin resistance and genotyping. The homozygous rpn10-2 lines containing the c35S or cN10 complementation constructs were obtained from T2 (or T1 for cN10-25) seedlings by kanamycin resistance and confirmed by genotyping. Double homozygous lines for rpn10-2 and the c35S or cN10 complementation construct were obtained from T3 or T4 seedlings with 100% kanamycin resistance and confirmed by genotyping.
proteasome was a kind gift from P.-A. Girod (University of Lausanne, Switzerland). The antisera against RPN8a and RPN10 were custom-made as described above. All antisera were diluted 1000-fold. The complexes were also detected by an in-gel proteasome activity assay using 50 μM N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Enzo Life Sciences) as the substrate. Two duplicate gels were incubated with buffer (50 mM Tris, pH 7.5, 5 mM MgCl₂, and 1 mM ATP) containing the substrate at 30°C for 20 min, and one of the gels was then incubated for 20 min after addition of SDS to 0.02%. The in-gel proteasome activity was visualized using a built-in UV illuminator on a BioSpectrum 600 (UVP).

Accession Numbers

The sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the accession numbers listed in Supplemental Table 1 online.

Supplemental Data

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

Supplemental Figure 1. Arabidopsis RPN10 and UBL-UBA Factors Associate with Endogenous Ubiquitylated Proteins.

Supplemental Figure 2. Arabidopsis RPN10 Binds Lys-48-Linked Long Ubiquitin Chains via UIM1.

Supplemental Figure 3. Various Arabidopsis UBL-UBA Factors Have Distinct Ubiquitin Chain Binding Properties.

Supplemental Figure 4. The UBA Domains of Various Arabidopsis UBL-UBA Factors Are Critical for Ubiquitin Chain Binding.

Supplemental Figure 5. The Arabidopsis Base Subunit RPN10 Contains Separate Interfaces for the Recognition of RAD23 and DSK2 Members.

Supplemental Figure 6. Sequence Alignment of Arabidopsis Ubiquitin and the UBL Domains of RAD23a-d, DSK2a-b, and DDI1.

Supplemental Figure 7. The UBL Domains of Arabidopsis RAD23 and DSK2 Members Are Critical for RPN10 Association.

Supplemental Figure 8. Polyploidy Analyses of Petals and Leaves of Arabidopsis Plants.

Supplemental Figure 9. Detection of Two T-DNA Insertions on Arabidopsis RPN10.

Supplemental Table 1. Isolated Arabidopsis cDNAs Encoding Potential Ubiquitin Receptors.

Supplemental Table 2. Constructs Used for GST Pull-Down Assays.

Supplemental Table 3. Arabidopsis T-DNA Insertion Lines for Major Ubiquitin Receptors.

Supplemental Table 4. Organ Number Distribution in Different Whorls of Flowers from Arabidopsis rpn10-2 and from Complemented Lines.

Supplemental Table 5. Primer Pairs.

Supplemental References.

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AUTHOR CONTRIBUTIONS

H.F. and Y.-L.L. designed the research, analyzed data, and wrote the article. Y.-L.L., S.-C.S., H.-L.T., T.-T.Y., R.R., R.U., A.S.F., H.-Y.L., and Y.-Y.W. performed the research.

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The Defective Proteasome but Not Substrate Recognition Function Is Responsible for the Null Phenotypes of the Arabidopsis Proteasome Subunit RPN10

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