In vivo relevance of substrate recognition function of major Arabidopsis ubiquitin receptors

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Ubiquitylation marks proteins for destruction by the 26S proteasome. These signals are deciphered and targeted by distinct direct and indirect pathways involving a set of evolutionarily conserved ubiquitin receptors. Although biochemical and structural studies have revealed the mechanistic complexity of these substrate recognition pathways, conclusive evidence of the in vivo relevance of their substrate recognition function is currently not available. We recently showed that the structural elements involved in substrate recognition are not responsible for the important roles of the ubiquitin receptor RPN10 in vegetative and reproductive growth or for the abundance of the two-capped proteasomes (RP2-CP). Moreover, Arabidopsis plants subjected to severe knockdown or knockout any of the major ubiquitin receptors showed wild-type phenotypes. Our results clearly suggest a functional redundancy of the major Arabidopsis ubiquitin receptors, and this evolved multiplicity is probably used to secure the substrates delivery. Based on the reduced abundance of RP2-CP in rpn10-2 and a role of RPN10 in lid-base association, a structural role of RPN10 in 26S proteasome stability is likely to be more relevant in vivo. Further efforts using structural and functional analyses in higher-order mutants to identify the specific biological functions of substrate recognition for the major Arabidopsis ubiquitin receptors are described here.

Ubiquitylated Substrates Are Directly or Indirectly Recognized by the Proteasome through a Set of Conserved Ubiquitin Receptors

Due to their role in determining substrate specificity, biochemical and functional analyses of the enzymatic components involved in reversible ubiquitin modification have been the focus of numerous studies on the ubiquitin system.3,4 More recently, because of a potentially major role in deciphering the signals of ubiquitin chains attached to substrates and in targeting modified substrates for various cellular processes, intensive studies cover numerous ubiquitin binding proteins, including a set of evolutionarily conserved ubiquitin receptors involved in targeting ubiquitylated proteins to the 26S proteasome for destruction.5,6 Three major classes of ubiquitin receptors that are conserved among different species are involved in the recognition of ubiquitylated proteasome substrates.7 The first class includes the intrinsic 26S proteasome base subunits RPN10 and RPN13, which directly recognize ubiquitylated substrates. However, although RPN13 has been shown to be an integral proteasome subunit in mammals and yeast,8 its presence in affinity-purified Arabidopsis proteasomes was not detected.9 The association of RPN13 with proteasome in Arabidopsis likely is transient and mediated by RPN2 as its interaction with RPN13 was observed.10

Posttranslational modification by the reversible attachment of ubiquitin or ubiquitin chains of various linkages on cellular proteins plays a critical regulatory role in nearly all aspects of cellular processes, including DNA replication/repair, cell division, epigenetic regulation, transcription, RNA splicing and exporting, signal transduction, endocytosis and proteolysis.1 Reversible ubiquitin attachment can regulate the activity, half-life, subcellular compartmentalization, or protein-protein interactions of the modified proteins and their associated complexes and is a critical mechanistic and regulatory element of the cellular processes that involve the modified proteins. A large portion of eukaryotic genomes encodes components of the reversible ubiquitin modification system; for example, ~6% of the Arabidopsis genome encodes components of the ubiquitous proteasome.2 A growing body of evidence indicates that the modified proteins and their associated complexes and is a critical mechanistic and regulatory element of the cellular processes that involve the modified proteins. A large portion of eukaryotic genomes encodes components of the reversible ubiquitin modification system; for example, ~6% of the Arabidopsis genome encodes components of the ubiquitin/26S proteasome system.7,8 Its presence in affinity-purified Arabidopsis proteasomes was not detected.9 The association of RPN13 with proteasome in Arabidopsis likely is transient and mediated by RPN2 as its interaction with RPN13 was observed.
various ubiquitin receptors,5,15 that are assumed to be involved in receiving these shuttle factors.13 The third class of ubiquitin receptors has revealed their mechanistic complexity in addition to their role in the determination of substrate specificity.5,16 Although intensive structural and biochemical analyses of major ubiquitin receptors have shown that the association and targeting of ubiquitylated substrates. The ubiquitin-interacting motif (UIM), the pleckstrin-like receptor of the CDC48 complexes, including the NPL4-zinc finger (NZF) and UBA domains in NPL4 and p47, respectively, as well as the CDC48/p97 N-domain fold in CDC48 and UFD1. The major recognition pathways for ubiquitylated proteasome substrates appear to have diverged in different species with respect to substrate and proteasome binding and the associated interaction interfaces of the ubiquitin receptors that are involved, thereby suggesting a potential mechanistic and functional divergence.20,21 Although intensive structural and biochemical analyses of major ubiquitin receptors have shown that the association and targeting of ubiquitylated substrates for specific cellular processes remains scarce.

The Structural Elements Involved in the Recognition of Ubiquitylated Substrates in RPN10 Ortholog from Different Species Are Generally Dispensable In Vivo

to demonstrate conclusively in the in vivo importance of the ubiquitylated substrate recognition function of the major ubiquitin receptors, the necessity of their structural elements for specific cellular processes must be demonstrated. This necessity can be determined by complementation experiments in null mutants of the major ubiquitin receptors using a structural and functional correlation approach. The RPN10 ubiquitin receptor ortholog from several species have been extensively studied using this approach. The yeast (Saccharomyces cerevisiae) Rpn10 null mutant displayed only limited, mild phenotypes such as growth sensitivity to amino acid analog and reduced proteolysis of a specific UFD substrate, thereby indicating that Rpn10 has a nonessential role.19 In addition, the UIM site-substitution of the mutant 26S proteasomes appears to be less stable in vitro.19 However, none of the observed yeast Rpn10 null phenotypes was caused by the loss of substrate recognition, as all observed null mutant phenotypes were rescued by Rpn10 variants with a UIM that was site-substituted or deleted.20 In contrast, the N-terminal vWA domain of Rpn10 appears to be essential for the in vivo functions observed with the null mutant. The substitution of the Asp11 residue, which is predicted to be critical to the structural integrity of vWA, by Ala in an Rpn10 variant has been shown to affect the structural stability of the 26S proteasome in terms of the unstable lid-base association and is unable to complement all of the observed null phenotypes.21 These results clearly show that the primary in vivo function of yeast Rpn10 is related to its role in maintaining 26S proteasome structural integrity and not in ubiquitylated substrate recognition.

RPN10 appears to play more important roles in vivo in higher eukaryotes than in yeast. However, with the exception of one report in the mouse,22 there is no evidence to support the functional relevance of substrate recognition activity of RPN10 in vivo. Knockdown and knockout experiments revealed that RPN10 is involved in sex determination in Caenorhabditis elegans, mitotic cell division during larval development in the fly (Drosophila melanogaster), gametophore formation in moss (Physcomitrella patens), and embryo development in the mouse.22-24 The role of the substrate recognition function of RPN10 has not been examined in C. elegans and D. melanogaster. Moreover, the substrate recognition function of RPN10 in moss appears to be irrelevant, as the gametophore formation was restored when the RPN10 null mutant was complemented with a C-terminally (UIM) truncated RPN10 variant. However, the embryonic lethality associated with the RPN10 knockout mouse mutant could not be rescued when a C-terminal (UIM) truncated version was knocked in, thereby supporting the idea that the ubiquitin recognition activity of RPN10 plays an essential role in mouse embryonic development.25 However, although the C-terminally truncated RPN10 was incorporated into the 26S proteasome, the feedback regulation of the proteasome and the accumulation of ubiquitylated conjugates often associated with proteasome defects were observed. A potential structural defect similar to that of the yeast RPN10 null mutant may still be associated with the mouse 26S proteasomes that harbor the large C-terminally truncated RPN10. This possibility can be assessed by knocking a UIM site-substituted full-length RPN10 into the RPN10-null deleted mutant. If the substrate recognition function of RPN10 is essential for mouse embryo development, the reintroduced site-substituted variant should not be able to rescue the RPN10-null deleted mutant phenotype.

In Arabidopsis, the first characterized T-DNA insertion mutant rpn10-1 displayed pleiotropic phenotypes, including reduced germination, growth rate, stamen number, and fertility, as well as increased ABA accumulation and ABA sensitivity.26 Because the mutant has been shown to express at an extremely low level a C-terminal UIM-truncated RPN10 fused with the NPT-II marker, the substrate recognition of RPN10 is proposed to have
an important role in vivo. However, the majority of the 26S proteasome subunits were probably missing the truncated RPN10 fusion, due to its extremely low level of expression, which could account for the phenotypes. Our recent study using a second T-DNA-inserted null mutant rpn10-2 revealed that all pleiotropic vegetative and reproductive growth phenotypes, together with the reduced abundance of the double-capped proteasome, were rescued by a triple UME site-mutated RPN10 variant (designated u123), which is defective in both direct and indirect substrate recognition activities. This result suggests that the substrate recognition activity of RPN10 is not responsible for the various in vivo functions reflected by the phenotypes of the null or rpn10-1 mutants. Instead, the observation of the reduced abundance of double-capped proteasome in rpn10-2 suggests that similar to that observed in yeast, RPN10 functionality in the structural integrity or assembly of the 26S proteasome is likely to be more relevant to its in vivo roles.13

**Defective Nature of the RPN10-Deleted 26S Proteasomes**

Unique and overlapping structural defects of the 26S proteasome are likely to be associated with various subunit mutants. These structural defects could potentially affect the proteolysis of distinct and common proteasome substrates and often lead to partially overlapping phenotypes in plants expressing these subunit mutants.13 Based on the role of Rpn10 in stable proteasome lid-base association and the reduced abundance of double-capped proteasomes in rpn10-2 mutants, a similar structural defect associated with the 26S proteasomes missing RPN10 as observed in yeast is the most plausible cause of the various vegetative and reproductive growth phenotypes associated with rpn10-2. Interestingly, based on the deleterious effects, gametogenesis (especially male gamete) is particularly sensitive to the structural defect caused by RPN10 deletion.13 However, the exact nature of the defect in the Arabidopsis rpn10-2 proteasome has not been determined. It would be interesting to examine whether the abundance of free lid complexes is increased as compared with that in wild-type. Similarly, the question should be examined of whether an RPN10 variant with a disrupted vWA domain is unable to rescue various rpn10-2 phenotypes, including the proteasome defects. The potential readouts of the structural defects of the rpn10-2 proteasomes can also be scrutinized in vitro to determine whether various proteasome activities have been compromised, such as the proteolysis of synthetic peptides and conjugated substrates, gating, unfolding and deubiquitylation.

Proteasome subunit mutants examined in budding yeast often affect holocomplex assembly and accumulate various assembly intermediates.13,19 Assembly intermediates of the base subcomplex, and more recently those of the lid subcomplex, have been identified in yeast and mammals.25,59 Similar to core particle assembly, evolutionarily conserved chaperones are found to be involved in base assembly, and each of them is associated with distinct assembly intermediates. It would be interesting to examine whether RPN10 deletion affects RP assembly and whether Arabidopsis 26S proteasome assembly is conserved compared with that in yeast and mammals. Interestingly, all four base assembly chaperones (i.e., Hsc35/S5, Hsc2, Dsk2, and Nub1) are conserved in Arabidopsis.

**Functional Redundancy of Major Arabidopsis Ubiquitin Receptors**

Although protein-protein interaction analyses have suggested that Arabidopsis RPN10 plays a major role in both the direct and indirect recognition of ubiquitylated proteasome substrates, gating, unfolding and deubiquitylation. Due to this redundancy, it is necessary to establish higher order mutants as important resources for a continued effort to assess in vivo roles of the substrate recognition activities of the major Arabidopsis ubiquitin receptors. For example, we have established all combinations of single, triple and quadruple mutants for four RAD23 members.13 The phenotypes under normal and various treatments for all of these mutants will be examined to determine the relative importance of these four RAD23 members in the particular functions that were altered in the quadruple mutant. The question of whether the substrate recognition function of the RAD23 proteins is involved will be assessed by complementation using site-specific mutants. Similar approaches can be applied to other higher order mutants, such as the establishment of DSK2 RNAi lines in the DDI1 null background. Because the two DSK2 loci are closely related and juxtaposed, RNAi is the method of choice to knockdown both loci simultaneously.

Based on protein-protein interaction analyses, RPN10 and RPN13 play a major and minor role, respectively, in both direct and indirect substrate recognition.19 Because the RPN10 null mutants expressing the RPN10 variant u123 that was defective in direct and indirect substrate recognition behaved like wild-type plants (designated the u123 lines), the combined in vivo functional roles of substrate recognition contributed by both RPN10 and RPN13 could be examined by introducing a T-DNA-inserted RPN13 null mutant (rpn13-1) into a u123 lines...
Fig. 1A. Surprisingly, the derived plants (designated \( \text{rpn13}\) \( \text{u123} \)) also behaved generally like the wild-type for the examined vegetative and reproductive phenotypes, including the overall morphology, growth rate, flowering time, primary root length of the seedling, fertility and induced leaf senescence (Fig. 1B–E). Increased final inflorescence height of plants harboring \( \text{u123} \) was observed that is likely due to incomplete complementation of \( \text{rpn10-2} \) by the \( \text{u123} \) variant expressed at low levels in these plants. These results indicate that the substrate recognition function performed by \( \text{RPN10} \) and \( \text{RPN13} \) in combination is generally dispensable, which further strengthens the functional redundancy of ubiquitylated substrate recognition provided by the major ubiquitin receptors in Arabidopsis. The combined substrate recognition roles for \( \text{RPN10} \) and either \( \text{DSK2} \) or \( \text{DDI1} \) could be similarly examined in the \( \text{u123} \) line.

Interestingly, although \(~2\%\) homozygous \( \text{RPN10} \) null plants (\( \text{rpn10-2} \)) also behaved generally like the wild-type for the examined vegetative and reproductive phenotypes, including the overall morphology, growth rate, flowering time, primary root length of the seedling, fertility and induced leaf senescence (Fig. 1B–E). Increased final inflorescence height of plants harboring \( \text{u123} \) was observed that is likely due to incomplete complementation of \( \text{rpn10-2} \) by the \( \text{u123} \) variant expressed at low levels in these plants. These results indicate that the substrate recognition function performed by \( \text{RPN10} \) and \( \text{RPN13} \) in combination is generally dispensable, which further strengthens the functional redundancy of ubiquitylated substrate recognition provided by the major ubiquitin receptors in Arabidopsis. The combined substrate recognition roles for \( \text{RPN10} \) and either \( \text{DSK2} \) or \( \text{DDI1} \) could be similarly examined in the \( \text{u123} \) line.

Interestingly, although \(~2\%\) homozygous \( \text{RPN10} \) null plants (\( \text{rpn10-2} \)) could be obtained by the segregation of the heterozygous \( \text{rpn10-2} \) plants,\(^\text{15}\) no homozygous \( \text{rpn10-2} \) \( \text{rpn13-1} \) plant could be detected after segregating the double heterozygous plants (\( n = 4746 \)) or plants homozygous for \( \text{rpn13-1} \) and heterozygous...
of double heterozygous plants, in which the genotype ratio for mutant plants were further reduced to ~0.9% by the segregation of double heterozygous plants, in which the genotype ratio for mutant plants were further reduced to ~0.9% by the segregation of double heterozygous plants. This result suggests that the substrate recognition function of RPN13 is involved in the process of double heterozygous plants, in which the genotype ratio for mutant plants were further reduced to ~0.9% by the segregation of double heterozygous plants. Thus, the substrate recognition function of RPN13 may be important for the segregation of double heterozygous plants.

The results presented in this study support the hypothesis that ubiquitin receptors, are highly evolutionarily conserved in all eukaryotes. It appears that additional studies, such as the examples provided above using structural and functional analyses in higher-order mutants, are required to determine the functional roles of the ubiquitin receptors in the Arabidopsis proteasome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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