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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 displayed 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.

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 ubiquitin/26S proteasome system.[2] A growing body of evidence indicates that ubiquitin modification is a critical regulatory element for nearly all aspects of plant growth and development, such as hormone responses, flower development, disease resistance, self-incompatibility, the circadian rhythm and photomorphogenesis.[2] Continued efforts to discover the functional roles and detailed mechanisms of the critical components of ubiquitin modification systems will increase our understanding of the plant functions that they are associated with and provide important information for future crop manipulation.

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.[5] 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,[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

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(R. Usharani and H. Fu, unpublished) similar to that in mammals and yeast.[8,10] The second class includes shuttle factors (e.g., RAD23, DSK2, DDI1 and NUB1) containing one N-terminal ubiquitin-like (UBL) domain and one to three C-terminal ubiquitin-associated (UBA) domains, which are involved in binding to the 26S proteasomes and the ubiquitylated substrates, respectively. Through these UBL-UBA shuttle factors, the 26S proteasome can indirectly recognize ubiquitylated substrates. Multiple docking sites for various UBL-UBA factors are located on the base subcomplex of the regulatory particle (RP), including sites on RPN1 and the ubiquitin receptors RPN10 and RPN13.[7,11,12] The relative importance of these docking sites for receiving shuttle factors appears to be divergent in different species. Whereas RPN1 is critical in yeast,[10,11] its role in receiving shuttle factors has not been detected in Arabidopsis or determined in mammals.[13] In contrast, Arabidopsis RPN10 plays a major role in receiving these shuttle factors.[13] The third class of ubiquitin receptors includes CDC48-based complexes, which are primarily involved in endoplasmic reticulum-associated degradation.[14]

Distinct ubiquitin-binding motifs or domains are used by the various ubiquitin receptors,[5,15] that are assumed to be involved in the association and targeting of ubiquitylated substrates. The ubiquitin-interacting motif (UIM), the pleckstrin-like receptor of ubiquitin (PRU) domain and the UBA domain are utilized by the 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 (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.[5,16] Although intensive structural and biochemical analyses of major ubiquitin receptors have revealed their mechanistic complexity in addition to their role in the determination of substrate specificity,[17] conclusive evidence to support the in vivo relevance of their roles in the recognition 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 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 analogs and reduced proteolysis of a specific UFD substrate, thereby indicating that Rpn10 has a nonessential role.[18] In addition, the lid-base association 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.[22-25] 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.[22] 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-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-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 ABI5 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 proteasomes of this mutant 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 UIM 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 variants.[13] Based on the role of Rpn10 in stable proteasome lid-base association and the reduced abundance of double-capped proteasomes in *rpn10-2*,[13,19,21] 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.[27,28] Assembly intermediates of the base subcomplex, and more recently those of the lid subcomplex, have been identified in yeast and mammals.[27-33] 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., Hsm3/S5b, Nas2/p27, Nas6/p28 and Rpn14/PAAF1) 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, the in vivo irrelevance of the structural elements of RPN10 that are responsible for substrate recognition clearly indicates that this function of RPN10 can probably be replaced by other ubiquitin receptors, such as RPN13 or UBL-UBA factors.[13] When individually investigated for Arabidopsis genes encoding other major ubiquitin receptors, including RPN13, RAD23a-d, and NUB1, near wild-type growth phenotypes were also associated with their T-DNA-inserted knockout mutants. Drastic RNAi knockdown mutant lines for both DSK2 members also displayed wild-type phenotypes. Moreover, a quadruple knockout mutant of all four RAD23 members displayed only limited growth phenotypes, such as partial ovule abortion, under normal growth conditions. Whether the phenotypes of the RAD23 quadruple mutant are caused by the loss of substrate recognition requires further examination using a structure-function correlation approach with complementation experiments in the RAD23 quadruple mutant. Taken together, our results suggest a functional redundancy of the major Arabidopsis ubiquitin receptors that are involved in the recognition of ubiquitylated proteasome substrates.

Due to this redundancy, it is necessary to establish higher order mutants as important resources for a continued effort to assess the 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.[5,13] Because the *RPN10* null mutant plants 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 *u123* lines

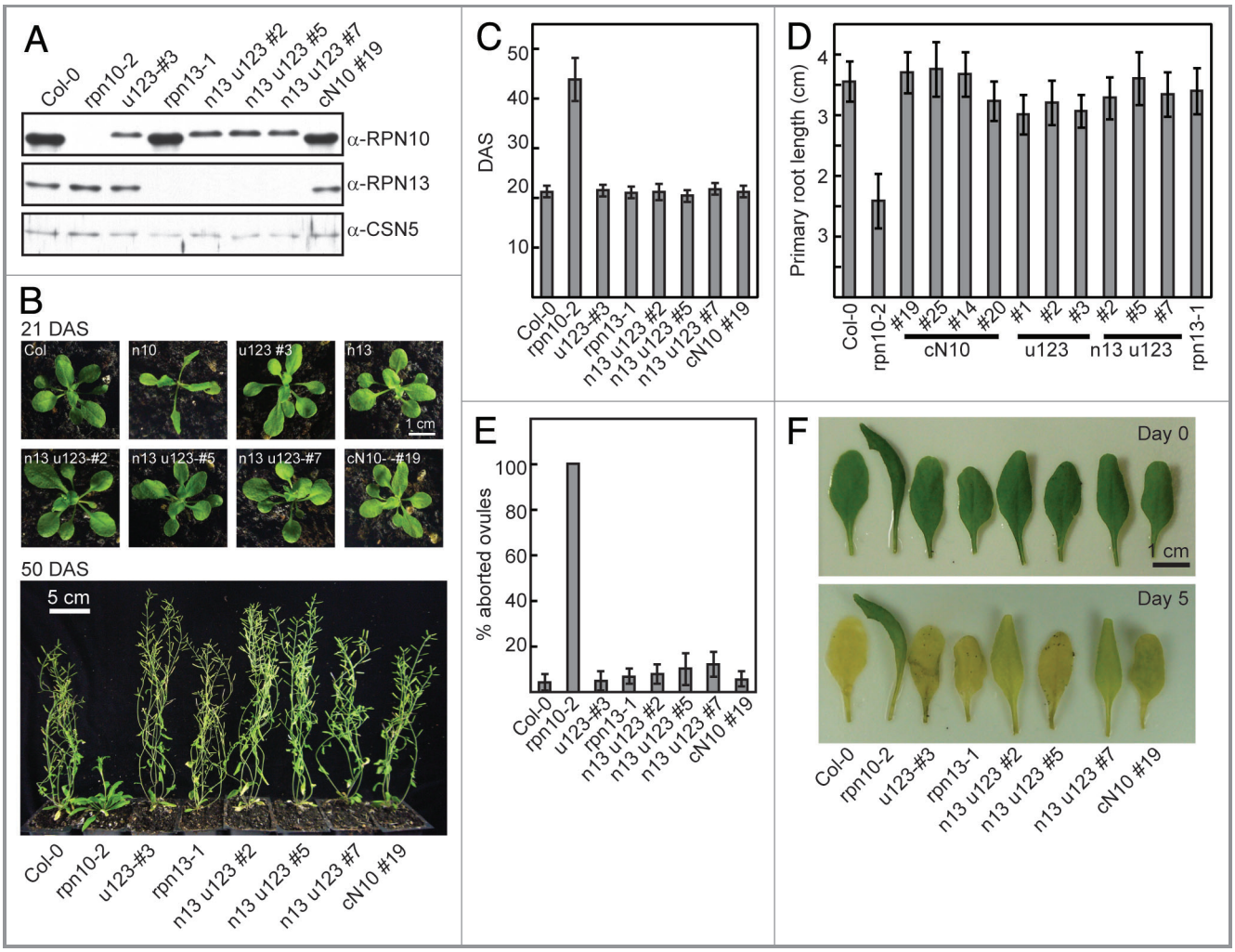

Figure 1. The combined function of RPN10 and RPN13 to recognize ubiquitylated substrates is dispensable in Arabidopsis. (A) The endogenous RPN13 and RPN10 proteins were not detected in the established *rpn13-1 u123* lines (*n13 u123*). Their expression in *rpn10-2*, *rpn13-1* and the *rpn10-2* complementation plants harboring wild-type (*cN10* #19) or substrate-recognition defective RPN10 (*u123* #3) was included for comparison. The obvious mobility shift of the triple-UIM RPN10 mutant is due to the UIM1 mutation as noted previously.[13,20] Crude extracts from 28-d-old rosette leaves of various genotypes were analyzed by immunoblotting using polyclonal antisera against Arabidopsis RPN10 and RPN13.[13] The expression of a COP9/signalosome subunit CSN5 was included to confirm approximately equal loading (α-CSN5). (B) Representative plants of different genotypes at 21 and 50 d after stratification treatment (DAS) are shown. Similar to *cN10* #19 and *u123* #3 plants displaying nearly wild-type growth rates and morphology, the introduction of an RPN13 null mutation into *u123* plants also resulted in similar wild-type phenotypes (*n13 u123* #2, #5 and #7). (C) The flowering time (DAS) for Arabidopsis plants of different genotypes. The flowering time was recorded when the floral stalk reached ~1 cm. Except for *rpn10-2* (n = 13), 24 plants were averaged for other genotypes. (D) The primary root lengths of 7-d-old Arabidopsis seedlings (n = 40) of various genotypes are averaged. (E) The average percentages of aborted ovules from Arabidopsis siliques (n = 21) of various genotypes. (F) Sensitivity to dark-induced senescence of Arabidopsis rosette leaves of various genotypes. Mature 32 DAS rosette leaves of various genotypes were shown before (day 0) and after 5-d (Day 5) incubation in the dark, as described previously.[13]

(**Fig. 1A**). Surprisingly, the derived plants (designated *rpn13 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 u123 was observed that is likely due to incomplete complementation of *rpn10-2* by the u123 variant expressed at low levels in these plants. These results indicate that the substrate recognition function performed by RPN10 and 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 RPN10 and either DSK2 or DDI1 could be similarly examined in the *u123* line.

Interestingly, although ~2% homozygous *RPN10* null plants (*rpn10-2*) could be obtained by the segregation of the heterozygous *rpn10-2* plants,[13] no homozygous *rpn10-2 rpn13-1* plant could be detected after segregating the double heterozygous plants (n = 4746) or plants homozygous for *rpn13-1* and heterozygous

for *rpn10-2* (n = 8867). The obtained homozygous *RPN10* null mutant plants were further reduced to ~0.9% by the segregation of double heterozygous plants, in which the genotype ratio for homozygous wild-type RPN13 and heterozygous *rpn13-1* is approximately 0.95:1.05. The complete synthetic lethality between *rpn10-2* and *rpn13-1* provides a window to assess whether the substrate recognition function of RPN13 is involved by complementation experiments using RPN13 variants that are defective in this function. The involvement of other RPN13 activities, such as interactions with the proteasome and UCH37 (UCH1/2 are the plant homologs) that are observed in mammals, can also be examined.[8,34] The functional importance of any of these RPN13 activities, including ubiquitin binding, should correlate with the inability to segregate homozygous *rpn10-2 rpn13-1* progeny from plants heterozygous for *rpn10-2* and homozygous for *rpn13-1* that are harboring the corresponding RPN13 variant defective in that particular activity. However, our preliminary experiments suggest that the loss of the substrate recognition activity of RPN13 does not account for the combined complete lethality (R. Usharani and H. Fu, unpublished).

One potential reason for the presence of such functional redundancy among the major recognition pathways involving conserved ubiquitin receptors for ubiquitylated substrates in Arabidopsis is to secure their delivery to the proteasome. Alternatively, ubiquitylated substrate recognition routing to the proteasome may not be a rate-limiting step. It is difficult to believe that the latter scenario is possible because the ubiquitin signal, its conjugation machinery components, and post-ubiquitylation processing components, including the above-described 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 identify the specific biological functions associated with the ubiquitin recognition activity of the major Arabidopsis ubiquitin receptors involved in targeting proteasome substrates.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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