Molecular Biology Reports **26:** 137–146, 1999.

Structure and functional analyses of the 26S proteasome subunits from plants

Plant 26S proteasome

Hongyong Fu[1,2], Pierre-Alain Girod[3], Jed H. Doelling[1], Steven van Nocker[1,4], Mark Hochstrasser[5], Daniel Finley[6] & Richard D. Vierstra[1,*]

[1]*Cellular and Molecular Biology Program and the Department of Horticulture, University of Wisconsin-Madison, Madison, WI 53706, USA;* [2]*Present address: Institute of Botany, Academia Sinica, 128, Sec. 2, Academy Rd. Nankang, Taipei, 11529, Taiwan, R.O.C.;* [3]*Laboratoire de Phytogénétique, Université de Lausanne 1015 Lausanne, Switzerland;* [4]*Present address: Department of Horticulture, Michigan State University, East Lansing, MI 48824, USA;* [5]*Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA;* [6]*Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA; *Author for correspondence*

Key words: Arabidopsis, *Physcomitrella patens*, proteasome, proteolysis, ubiquitin, yeast

Abstract

As initial steps to define how the 26S proteasome degrades ubiquitinated proteins in plants, we have characterized many of the subunits that comprise the proteolytic complex from *Arabidopsis thaliana*. A set of 23 Arabidopsis genes encoding the full complement of core particle (CP) subunits and a collection encoding 12 out of 18 known eukaryotic regulatory particle (RP) subunits, including six AAA-ATPase subunits, were identified. Several of these 26S proteasome genes could complement yeast strains missing the corresponding orthologs. Using this ability of plant subunits to functionally replace yeast counterparts, a parallel structure/function analysis was performed with the RP subunit RPN10/MCB1, a putative receptor for ubiquitin conjugates. RPN10 is not essential for yeast viability but is required for amino acid analog tolerance and degradation of proteins via the ubiquitin-fusion degradation pathway, a subpathway within the ubiquitin system. Surprisingly, we found that the C-terminal motif required for conjugate recognition by RPN10 is not essential for *in vivo* functions. Instead, a domain near the N-terminus is required. We have begun to exploit the moss *Physcomitrella patens* as a model to characterize the plant 26S proteasome using reverse genetics. By homologous recombination, we have successfully disrupted the *RPN10* gene. Unlike yeast *rpn10*Δ strains which grow normally, *Physcomitrella rpn10*Δ strains are developmentally arrested, being unable to initiate gametophorogenesis. Further analysis of these mutants revealed that RPN10 is likely required for a developmental program triggered by plant hormones.

Abbreviations: CP – core particle; 5-FOA – 5-fluoroorotic acid; RP– regulatory particle; Ub-Pro-β-Gal – ubiquitin-Pro-β-galactosidase.

Introduction

In yeast and animals, it is well established that the ubiquitin/26S proteasome proteolytic system participates in a number of essential cellular processes, primarily by controlling the half-life of critical, short-lived regulatory factors [1–4]. The system is composed of two major steps, both of which require energy in the form of ATP. In the first step, multiple ubiquitins are covalently attached to protein substrates. Conjugation is performed by the sequential action of three sets of enzymes, i.e., ubiquitin activation enzymes (E1s), ubiquitin conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) [5]. The last two, alone or in concert, recognize proteins destined for degradation and assemble a chain of ubiquitins (internally linked

via Lys 48) onto one or more free and accessible amino groups (N-terminal or lys ϵ-amino). In the next step, these multiubiquitinated proteins are recognized and degraded with the concomitant release of the ubiquitins in free forms. Breakdown is accomplished by the 26S proteasome, a multisubunit proteolytic complex. The 26S proteasome is composed of two subcomplexes: the 20S core particle (CP) which contains the proteolytic active sites and the 19S regulatory particle (RP) which imparts the ATP dependence and specificity for those proteins modified with ubiquitin chains [6, 7].

Substantial progress has been made in the past two decades with regard to defining many components of the ubiquitin/26S proteasome pathway, identifying its natural targets, and determining the functional consequences of target breakdown. However, there are many important questions that remain unanswered. For example, how does the system select particular substrates for ubiquitin conjugation? What are the conjugation signals within the substrates and how are they recognized by E2s and/or E3s? How does the 26S proteasome degrade the ubiquitinated substrates? And why does the 26S complex require ATP for proteolysis? For plants in particular, little is known about most steps of the ubiquitin pathway and how the pathway participates in plant development and response to the environment [8]. To date, only phytochrome A has been confirmed to be a target, implicating the pathway in photomorphogenesis [9, 10].

To help understand how the 26S proteasome degrades ubiquitinated proteins, we have begun to characterize genes encoding many of the subunits from *Arabidopsis thaliana*. Using these plant genes, we have established a yeast complementation system to examine the structure/function of the encoded proteins in a simpler model organism. We also have begun to exploit *Physcomitrella patens* as a model system to determine the functional roles of the ubiquitin system *in planta*, using homologous recombination to create specific gene disruptions. As a first example, we successfully disrupted the 26S proteasome subunit gene *RPN10* (*MCB1*) and demonstrated that this knockout moss strain is developmentally impaired but can be partially rescued by auxin and cytokinin treatment.

Structural conservation of the Arabidopsis 20S core particle (CP)

Within the 26S proteasome, the CP is a highly stable subparticle that contains the proteolytic active sites [6, 7]. While it is ubiquitous among eukaryotes, less complex versions are also present in several species of eubacteria and archaebacteria [11]. The latter suggests that the complex evolved before the origin of the first eukaryote. Biochemical and structural studies of the CP from mammals, *Thermoplasma acidophilum*, and yeast (*Sarccharomyces cerevisiae*) show that the CP contains only two types of subunits (α and β) that together assemble into a barrel-shaped structure by the stacking of four rings [12, 13]. The two outer rings are each composed of seven α subunits and the two inner rings are each composed of seven β subunits; these β subunits contain the active sites for proteolysis. Assembly of all 28 subunits creates three connected internal chambers with the active sites for proteolysis located within the middle chamber. This unique architecture prevents the indiscriminate breakdown of cellular proteins by the 20S complex, because only those polypeptides that are deliberately directed into the central chamber are degraded. The *T. acidophilum* complex is relatively simple, containing only one type of α and one type of β subunit. It is more complex in eukaryotes. For example, each ring of the yeast CP contains seven distinct α or β subunits designated as α1-α7 and β1-β7 [13]. All the β subunits of *T. acidophilum* are presumably active, using a catalytic triad involving the N-terminal Thr, and proximal Glu and Lys residues to generate the catalytic site. However, in yeast, only three of the β subunits, β1, β2, and β5, have these spatially conserved residues and consequently are thought to be active [11].

From a survey of Arabidopsis EST and genomic database sequences, we identified and characterized a set of 23 genes encoding CP subunits [14]. Phylogenic analysis placed these sequences into 14 groups corresponding exactly to α1-α7 and β1-β7 subunits from yeast and other species (Table 1). Representatives of all 14 types of subunits were identified, indicating that this collection likely contains the full complement of subunits that is necessary to build the CP. The Arabidopsis subunits are designated as PAA-G and PBA-G (for *P*roteasome *A*lpha subunit *A*-*G* and *P*roteasome *B*eta subunits *A*-*G*) with numerical suffixes added to designate various members in each gene family. In all cases, each of the Arabidopsis subunits is more similar to the corresponding subunit from yeast

and mammals than to other subunits from Arabidopsis, indicating that these subunits arose prior to the divergence of the animal, fungal, and plant kingdoms.

In contrast to yeast where each subunit is encoded by a single gene, many of the Arabidopsis subunits (α1-α6 and β2, β3, and β4) are encoded by at least a pair of paralogs. Amino acid sequence identity among the paralogs is high (>90%) suggesting that the family members are functionally identical. The existence of these conserved paralogs in Arabidopsis may simply reflect the absolute requirement for expression of these gene products across a wide range of developmental states and environmental conditions. In support of this notion, possible differential expression of these paralogs is indicated by the highly diverged flanking sequences. This similarity is in contrast to that seen in mammals where the 3 catalytic β subunits (β1, β2, and β5) have relatives that are substantially more dissimilar (70% identity) in amino acid sequence and may be functionally different [6]. For each of the three subunits, one isoform is constitutively expressed and likely is required for general proteolysis by the 26S proteasome. The other isoform is inducible and appears to be involved in digesting foreign polypeptides during antigen presentation by the MHC class I immune system [6].

Many critical conserved structural motifs identified in the α and β subunits from other species are also present in their Arabidopsis counterparts. For the α subunits, these include a conserved N-terminal α-helix, an N-terminal nuclear localization signal, a Tyr residue important for contact among the α subunits within the ring, and several positional conserved Gly residues [14]. Residues that comprise the catalytic triad of the active site (Thr/Glu/Lys) are all positional conserved within the three Arabidopsis β subunits presumably required for proteolysis [β1 (PBA), β2 (PBB), and β5 (PBE)]. Similar to the yeast and human versions, five of the seven β subunits (β1, β2, and β5–β7) have the predicted propeptide cleavage site and thus are expected to be synthesized as larger precursors. Based on the existence of a similar set of phylogenically related subunits and conservation within critical structural motifs, we expect that the three dimensional structure of the Arabidopsis CP will be very similar, if not identical, to that determined for the yeast complex [13].

Structural conservation of the Arabidopsis 19S regulatory particle (RP)

In yeast [15], the 19S RP is composed of ~18 subunits (Table 2). A consensus systematic nomenclature for these subunits was proposed recently [15, 16] and is used here. Six of the subunits [designated as RPT1-6 (for *R*egulatory *P*article *T*riple-A ATPase subunit] contain a conserved AAA cassette and thus have been included as members of the large AAA-ATPase superfamily [17]. Presumably, these subunits use ATP hydrolysis to assist in the recognition and/or hydrolysis of substrates by the 26S proteasome. Phylogenic analyses indicate that the RPT1-6 proteins form a separate branch from the 15 other AAA gene clades [17]. The rest of the yeast subunits are designated as RPN1-12 (for *R*egulatory *P*article *N*on-ATPase subunit). Except for RPN4 and RPN9, orthologs to all the yeast subunits have been identified in the RP from mammals [15].

A survey of existing databases identified numerous Arabidopsis genes that encode orthologs for many of the 18 yeast RP subunits (Table 2). These include all six of the AAA-ATPase subunits (RPT1-6) and six of the 12 non-ATPase subunits (RPN 1, 2, 6, 8, 10, and 11) (Table 2). Many of the genes have representative cDNAs in the EST databases indicating that they are actively transcribed. High sequence conservation of orthologs from plant and other species was apparent. For example, individual AAA-ATPase subunits from Arabidopsis are highly similar (65–75% identity) to the corresponding yeast proteins across the entire length of the peptides. Like many of the α and β subunits of the CP, at least two of the ATPase subunits, RPT4 and RPT5, have paralogs in Arabidopsis with high sequence identities (Table 2). Although the Arabidopsis EST databases are expected to contain most moderately to highly expressed genes, only one third of the genomic sequence is currently available. Consequently, we expect that additional RP subunits will be detected as sequencing of the Arabidopsis genome progresses and that more paralogs will be identified that are currently not present in the EST databases, presumably because their mRNAs are expressed at low levels.

Table 1. Subunits of the Arabidopsis 20S core particle

Systematic name[1]	Arabidopsis	Yeast[2]	Human
α-type subunits			
$\alpha 1$	PAA1	PRS2/C7/SCL1/Y8	IOTA/PROS27
	PAA2		
$\alpha 2$	PAB1	PRE8/Y7	C3
	PAB2		
$\alpha 3$	PAC1	PRE9/Y13	C9
	PAC2		
$\alpha 4$	PAD1	PRE6	XAPC7
	PAD2		
$\alpha 5$	PAE1	DOA5/PUP2	ZETA
	PAE2		
$\alpha 6$	PAF1	PRE5	C2/PROS30
	PAF2		
$\alpha 7$	PAG1	PRS1/PRE10/C1	C8
β-type subunits			
$\beta 1$	PBA1	PRE3	Y/Delta/LMP2[3]
$\beta 2$	PBB1	PUP1	Z/MECL1[3]
	PBB2		
$\beta 3$	PBC1	PUP3	C10
	PBC2		
$\beta 4$	PBD1	PRE1/C11	C7
	PBD2		
$\beta 5$	PBE1	DOA3/PRE2/PRG1	X/MB1//LMP7[3]
$\beta 6$	PBF1	PRS3/PRE7/C5	C5
$\beta 7$	PBG1	PRE4	N3/Beta

[1] Groll et al. [13].
[2] All yeast subunits are essential except $\alpha 3$/PRE9.
[3] γ-interferon inducible.

Many Arabidopsis 26S proteasome subunits can functionally replace corresponding orthologs in yeast

To further demonstrate that many of the Arabidopsis 26S proteasome subunits are functionally related to those from yeast, we tested whether the corresponding Arabidopsis genes could complement their corresponding yeast orthologs. Because most of the yeast genes are essential, complementation was performed by plasmid shuffle (e.g., [18]). In this technique, haploid yeast chromosomal deletion strains are created in which the chromosomal locus for the gene is disrupted and replaced with the equivalent wild-type gene expressed on a *URA3*-plasmid. The genes to be tested for complementation are introduced into these strains using a different selection plasmid. Cells containing both plasmids are grown on non-selective media to allow for spontaneous loss of either plasmid and then grown on media containing 5-fluoroorotic acid (5-FOA) to select against cells still containing the *URA3*-plasmid harboring the wild-type gene. Cells containing the heterologous test gene can form colonies only when its gene products can functionally replace the products of the wild-type essential gene.

By this strategy, Arabidopsis genes encoding 26S proteasome subunits $\alpha 5$, $\beta 1$–7, and RPT1-6 were tested for their ability to rescue the corresponding yeast deletions. Among the CP subunits, only the Arabidopsis genes *PAE*1 ($\alpha 5$) and *PBC*2 ($\beta 3$) were effective, but the complemented strains grew more slowly than those complemented with the yeast counterparts, suggesting that the Arabidopsis $\alpha 5$ and $\beta 3$ subunits were only partially effective. Results were better for the six AAA subunits (*RPT1-6*); in this case,

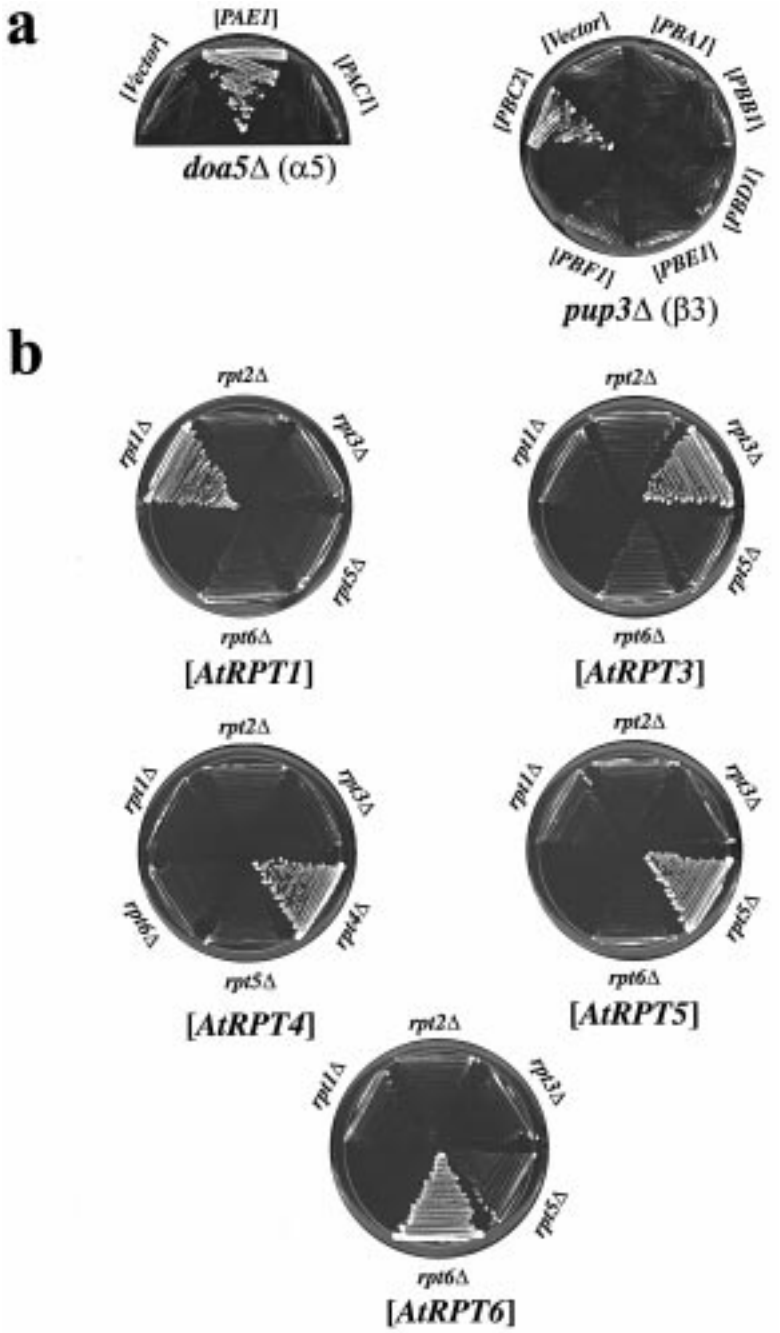

Figure 1. Functional complementation of yeast 26S proteasome subunit genes with orthologs from *A. thaliana*. (a) Two Arabidopsis 20S core particle subunit genes *PAE1* and *PBC2*, but not others, can specifically replace corresponding yeast orthologous genes *DOA5* (α5) and *PUP3* (β3). (b) Five Arabidopsis AAA-ATPase subunit genes, *AtRPT1* and *AtRPT3-6*, of 19S regulatory particle can specifically replace their corresponding orthologous genes in yeast. Functional complementations were performed by plasmid shuffle experiments [18] on media containing 5-FOA at 1 g/L.

Table 2. Identified subunits of the 19S regulatory particle from Arabidopsis

New nomenclature[1]	Identification status[3]	Previous nomenclature		
		Yeast[4]	Bovine human	Others
RPN1	+	Hrd2/Nas1$^+$	S2/Trap-2/p97	Mts4
RPN2	+	Sen3$^+$	S1/p112	
RPN3	–	Sun2$^+$	S3/p58	
RPN4	–	Son1/Ufd5$^-$		
RPT1	+	Cim5/Yta3$^+$	S7/Mss1	
RPT2	+	Yta5$^+$	S4/p56	Mts2
RPT3	+	Yta2/Ynt1$^+$	S6/Tbp7/p48	MS73
RPT4[2]	+	Crl3/Sug2/Pcs1$^+$	S10b/p42	CADp44
RPT5[2]	+	Yta1$^+$	S6′/Tbp1/p50	
RPN5	–	Nas5$^+$	p55	
RPN6	+	Nas4$^+$	S9/p44.5	
RPN7	–	ORF u32445	S10a/p44	
RPT6	+	Sug1/Cim3/Crl3$^+$	S8/Trip1/p45	m56
RPN8	+	Nas3	S12/p40	Mov-34
RPN9	–	ORF u33007$^-$		
RPN10	+	Mcb1/Sun1$^-$	S5a	Mbp1/p54
RPN11	+	Mpr1$^+$	Poh1	pad1
RPN12	–	Nin1$^+$	S14/p31	mts3

[1]Glickman et al. [15]. [2]paralog genes identified in Arabidopsis with high sequence identity (>95% at amino acid sequence level).
[3]+ and – indicate corresponding subunit has been identified or not been identified, respectively, in Arabidopsis.
[4]+ and – indicate corresponding subunit is essential or nonessential, respectively, in yeast.

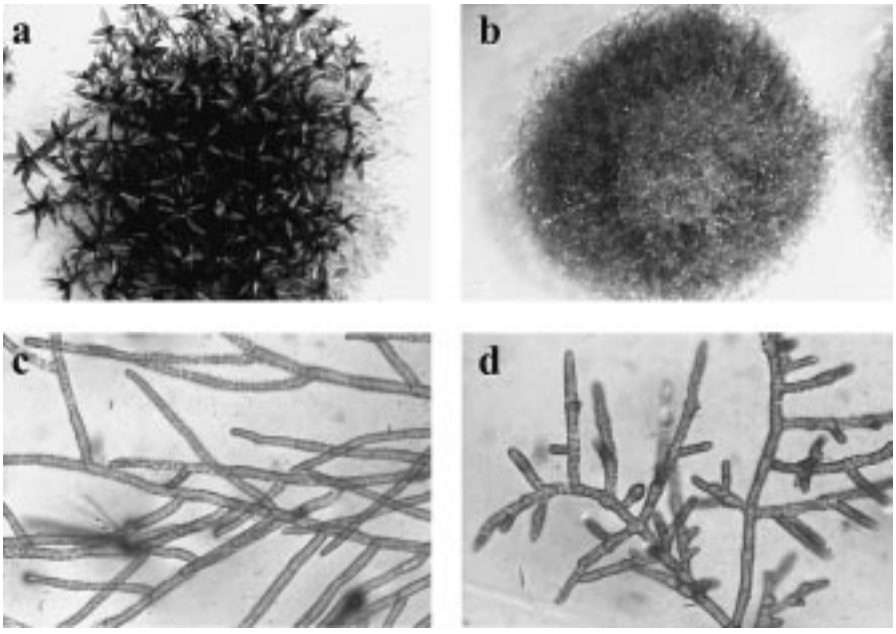

Figure 2. Disruption of RPN10 inhibits developmental progression in *P. patens*. Wild-type (a and c) and *rpn10*Δ (b and d) *P. patens* strains were grown in 16 h light/8 h dark photoperiods on minimal media (minus NH_4) for one and three months, respectively. Overall colony growth (a and b) and enlarged protonema (c and d) are shown separately.

all but Arabidopsis *RPT2* could functionally replace its corresponding yeast ortholog (Figure 1).

With respect to the non-essential genes, only the RP subunit RPN10 and the CP subunit α3 (PAC1) have been tested. In both of these cases, complementation involved expression of the Arabidopsis gene from a selection plasmid directly in the corresponding yeast deletion strain (*rpn10*Δ and *pre9*Δ, respectively). Both Arabidopsis subunits effectively rescued the respective yeast defects. Arabidopsis *PAC1* restored amino acid analog tolerance to yeast *pre9*Δ strains, whereas Arabidopsis *RPN10* restored amino acid analog tolerance and the ability to degrade a ubiquitin-fusion degradation pathway substrate to a yeast *rpn10*Δ strain [14, 19].

Functional domains of the RP subunit RPN10

RPN10 (previously designated as MBP1, MCB1, S5a, and μ54) was first identified as a human RP subunit that could bind Lys_{48}-linked multiubiquitin chains *in vitro*, especially those containing four or more ubiquitins [20]. This binding specificity and preference for longer chains led to a proposal that the subunit may function within the 26S proteasome as a recognition factor for ubiquitinated substrates. The gene encoding RPN10 was subsequently isolated from a wide range of species including Arabidopsis [21], human [22], yeast [23], Drosophila [24], and *Physcomitrella patens* [25]. Because the proposed role for RPN10 was central to the ubiquitin pathway, we expected that yeast strains missing this subunit would be inviable. Surprisingly, yeast *rpn10*Δ strains were phenotypically normal and grew at wild-type rates on rich media [23, 26]. This non-essential nature suggested that other recognition factors besides RPN10 must exist within the RP, but as yet no other candidate binding protein has been identified. However, interesting conditional phenotypes were observed with the yeast *rpn10*Δ strains. These included an increased sensitivity to growth on media containing canavanine and p-fluorophenylalanine (analogs of arginine and phenylalanine, respectively) and a stabilization of the ubiquitin substrate Ub-Pro-β-Gal [23]. Normally, this protein is degraded rapidly (half-life of 5–10 min) by the ubiquitin-fusion degradation pathway, a subpathway within the ubiquitin system in yeast. In the *rpn10*Δ strain, Ub-Pro-β-Gal was slowly turned over with a half-life of $>$2 h [23].

To test whether the conditional phenotypes of the yeast *rpn10*Δ strains are related to the ability of the protein to bind multiubiquitin chains, a structure/function analysis was performed by expressing mutated versions of the Arabidopsis and yeast proteins in the yeast *rpn10*Δ strain [19]. *In vitro* chain binding assays with deletion and site-directed RPN10 mutants uncovered an LAL/MALRV sequence motif near the C-terminus of the proteins from both species that is critical for ubiquitin recognition. This motif is highly hydrophobic and is located within a conserved domain present within all RPN10 isologs. Whereas most RPN10 orthologs only contain one of these hydrophobic domains, two are present in the Arabidopsis version [21]. Despite its involvement in multiubiquitin binding, the LAL/MALRV motif was not necessary for the *in vivo* functions of RPN10. Mutants in which the LAL/MALRV was completely deleted or mutated to contain less hydrophobic residues (e.g., LAL/MALRV conversion to NNNNNRV) could still rescue the *rpn10*Δ phenotypes (i.e., conferring amino acid analog resistance and restoring the degradation of Ub-Pro-β-Gal) [19]. Therefore, RPN10 must have another more critical *in vivo* function in the RP in addition to its ability to bind multiubiquitinated proteins.

Further mutagenesis of both yeast and Arabidopsis RPN10 revealed that the first 60 amino acids at the N-terminus are critical for its phenotypic functions. The N-terminal deletion (Δ1–60) not only failed to complement the *rpn10*Δ phenotypes, but actually increased the sensitivity of the *rpn10*Δ strain to amino acid analogs, suggesting it was toxic in the absence of wild-type protein [19]. More recent detailed mutagenesis analyses of this 60 amino acid-region with yeast RPN10 has narrowed down the essential residues to a 9-amino acid domain just proximal to the N-terminus (residues 7–15). Several residues within this domain are highly conserved, being present in all RPN10 isologs (H. Fu and R. D. Vierstra, unpubl.). The functions of these residues are unknown. One possibility is that they promote important contacts among the neighboring subunits of the RP. In support, Glickman *et al.* [27] have recently shown that yeast 26S complexes isolated from *rpn10*Δ strains are less stable than those from wild-type yeast.

RPN10 is required for developmental progression in the moss *Physcomitrella patens*

In contrast to yeast and animals, we know very little about the functional roles and *in vivo* substrates of the ubiquitin/26 proteasome pathway in plants [8]. One of the major obstacles has been the lack of informative mutants defective in components of the pathway. Forward genetic approaches have been limited in plants due to a lack of predictable phenotypes and the presence of paralogs and isologs with overlapping functions. Reverse genetics has been hampered in higher plants by the lack of efficient methods to disrupt genes by homologous recombination, an approach that has been used with great success in yeast. Recently however, this barrier has been overcome in the haploid moss *Physcomitrella patens*, with the development by Schaefer and Zyrd of an efficient method for targeted gene disruption [28]. We have begun to exploit this model seedless plant to develop specific gene knockouts in the ubiquitin/26S proteasome pathway. Through such mutants we hope to determine the roles of this proteolytic pathway in plant growth and development.

As a first attempt, we chose to disrupt the *P. patens RPN10* gene because preliminary DNA gel blot analyses showed that only one *RPN10* locus is present in the genome. Using Arabidopsis *RPN10* as a probe [21], we isolated a cDNA encoding RPN10 from *P. patens*. Binding studies of the encoded protein expressed in *E. coli* revealed that it, like its animal and Arabidopsis counterparts, binds multiubiquitin chains using the LALALRV hydrophobic patch (see above). A knockout construction was created in which a selectable marker was inserted into the middle of the *PpRPN10* cDNA coding region. *P. patens rpn10*Δ strains were generated by transforming moss protoplasts with the construction and then screening for targeted colonies that grew on selection medium. DNA blot analyses revealed that the *RPN10* locus was disrupted in several of the resistant transgenic lines. As expected, this disruption eliminated protein expression of *RPN10* in *rpn10*Δ strains as determined by immunoblot analysis using Arabidopsis RPN10 antibodies [25].

Like yeast *rpn10*Δ strains, *P. patens rpn10*Δ strains had elevated level of ubiquitin conjugates and were hypersensitive to amino acid analogs. In contrast to yeast *rpn10*Δ strains which grow normally and are not morphologically altered [23, 26], we found that the *P. patens rpn10*Δ strains were developmentally impaired (Figure 2). In the normal life cycle of *P. patens* (for review see [29]), haploid spores germinate and grow initially as filaments of two cell types: chloronema and caulonema. Chloronema cells develop first followed by caulonema cells. As the colony expands, buds originate from the caulonema which eventually develop into the large leafy gametophores. These structures predominate the life cycle and ultimately bear the antheridia and archegonia (male and female gametophytes, respectively) that produce the gametes for sexual reproduction. Although the *rpn10*Δ strains produced near normal chloronema, they failed to produce normal caulonema and never generated buds and gametophores. As a result, while wild-type *P. patens* colonies expanded and eventually became decorated with gametophores after a few weeks of growth, the *rpn10*Δ strains remained indefinitely as solid clumps of chloronema and aberrant caulonema.

For wild-type *P. patens*, formation of buds and subsequent gametophores is hormonally induced and can be accelerated by the addition of auxin and cytokinin to the medium [30]. We observed a similar promotive effect when the wild-type strain was grown on the auxin – IAA and the cytokinin – isopentenyladenine (IPA). For the *rpn10*Δ strains, this treatment restored the formation of caulonema cells and stimulated the development of buds and gametophores. When exposed to as little as 50 nM IAA and IPA, buds arose which eventually progressed to form gametophores. However, these gametophore structures did not expand normally and did not develop beyond the four-to-six leaf stage (It was not determined if antheridia or archegonia were formed). Although each hormone alone triggered some differentiation, both hormones in combination proved to be most effective.

One of the *P. patens rpn10*Δ strains was complemented with various mutants of RPN10 to identify which region is important for gametophorogenesis. Results indicated the LALALRV motif essential for multiubiquitin chain binding *in vitro* is not required for proper development. RPN10 mutants that have this structural motif deleted or substituted with nonconservative residues could still complement the *rpn10*Δ strain and restore, at least partially, gametophore development.

Taken together, the results show that RPN10 has an expanded role in plant development as compared to its limited role in yeast. However, like yeast RPN10, its functions in *P. patens* do not appear to require the ability to bind multiubiquitin chains. In both species, successful complementation was observed for mutant

RPN10 proteins that could no longer bind ubiquitin chains *in vitro*. The developmental defects in *P. patens* could be partially reversed by treatment with the plant hormones, auxin and cytokinin, suggesting that response to these hormones may require degradation of one or more short-lived regulators by the 26S proteasome. One intriguing possibility is that these regulators act as repressors that prevent development in the absence of appropriate hormonal signals. When a sufficient concentration of hormones is reached, these repressors are removed by degradation and/or overcome by activators, thus allowing development to proceed. However, when these short-lived proteins are stabilized (as may be the case in the *rpn10*Δ mutants), progression is blocked unless supra optimal levels of hormone are achieved.

Certainly the ability to target the disruption of specific genes, as well as methods that allow for successful complementation, make *Physcomitrella patens* a useful model to study plant growth and development. With respect to the ubiquitin/26S proteasome pathway, also important was the finding that many components identified in Arabidopsis have closely related orthlogs in this moss. In addition to RPN10, these include ubiquitin, CP subunits, and proteins involved in ubiquitin conjugation (E2s and E3s) (data not shown). Thus, studies with *Physcomitrella* should provide useful information for all higher plants. The more complex phenotype elicited by disruption of *RPN10* in *Physcomitrella* as compared to yeast also highlights the more expanded role of the ubiquitin/26S proteasome pathway in multicellular organisms and thus the need for parallel studies in more complex model systems.

Perspectives

Structural information has provided a wealth of information with regard to understanding how the 26S proteasome functions mechanistically. However, information is still lacking about how the complex degrades proteins, releases peptide fragments and amino acids, and how it selects targets for degradation and funnels the unfolded polypeptides into the lumen of the CP. Besides direct advanced physical methods, e.g., NMR and crystal structure analyses, approaches *via* classical molecular genetics can provide critical information about important protein-protein interactions and about residues involved in catalysis. Both the structural conservation of the 26S complex and the rapid progress in various genomic sequencing efforts allowed us to quickly establish systems to directly study the plant 26S proteasome. As exemplified by our analysis of the regulatory particle subunit RPN10, we should be able to use the established yeast complementation systems to dissect critical structural motifs of 26S proteasome subunits from either endogenous or heterologous sources. Further characterization of this subunit should shed light on the organization of the regulatory particle and important interactions that impart its stability. The observed phenotype of the *P. patens rpn10*Δ strain demonstrates the usefulness of this moss for functional study of the ubiquitin system in plants. Further characterization of the *rpn10*Δ strain may eventually lead to identification of the substrate(s) whose half-lives are controlled by the ubiquitin/26S proteasome pathway.

References

1. Hershko A & Ciechanover A (1998) Annu. Rev. Bioch. 67: 425–479
2. Ciechanover A (1994) Cell 79: 13–21
3. Hochstrasser M (1995) Curr. Opin. Cell Biol. 7: 215–223
4. Hochstrasser M (1996) Annu. Rev. Genet. 30: 405–439
5. Hershko A & Ciechanover A (1992) Annu. Rev. Biochem. 61: 761–807
6. Coux O, Tanaka K & Goldberg AL (1996) Anu. Rev. Biochem. 65: 801–847
7. Baumeister W, Walz J, Zuhl F & Seemuler E (1998) Cell 92: 367–380
8. Vierstra RD (1996) Plant Mol. Biol. 32: 275–302
9. Jabben M, Shanklin J & Vierstra RD (1989) J. Biol. Chem. 264: 4998–5005
10. Clough RC & Vierstra RD (1997) Plant Cell Environ. 20: 713–721
11. Lupas A & Baumeister W (1998) In: Peters JM, Harris JR & Finley D (ed.) Ubiquitin and the Biology of the Cell, Plenum Press, New York, pp. 127–146
12. Löwe J, Stock D, Jap F, Zwickl P & Baumeister W (1995) Science 268: 533–539
13. Groll M, Ditzel L, Löwe J, Stock D, Bochtler M, Bartunik HD & Huber R (1997) Nature 386: 463–471
14. Fu H, Doelling JH, Arendt CS, Hochstrasser M & Vierstra RD (1998) Genetics 149: 677–692
15. Glickman MH, Rubin DM, Fried VA & Finley D (1998) Mol. Cell. Biol. 18: 3149–3162
16. Finley D, Tanaka K, Mann C, Feldmann H, Hochstrasser M, *et al.* (1998) Trends Biochem. Sci. 23: 244–245
17. Beyer A (1997) Protein Sci. 6: 2043–2058
18. Arendt CS & Hochstrasser M (1997) Proc. Natl. Acad. Sci. USA 94: 7156–7161
19. Fu H, Sadis S, Rubin DM, Glickman MH, van Nocker S, Finley D & Vierstra, RD (1998). J. Biol. Chem. 273: 1970–1989
20. Deveraux Q, Ustrell V, Pickart C & Rechsteiner M (1994) J. Biol. Chem. 269: 7059–7061
21. van Nocker S, Deveraux Q, Rechsteiner M & Vierstra RD (1996) Proc. Natl. Acad. Sci. U. S. A. 93: 856–860

22. Ferrell K, Deveraux Q, van Nocker S & Rechsteiner M (1996) FEBS Lett. 381: 143–148
23. van Nocker S, Sadis S, Rubin DM, Glickman MH, Fu H, Coux O, Wefes I, Finley D & Vierstra RD (1996) Mol. Cell. Biol. 11: 6020–6028
24. Haracska L & Udvardy A (1995) Eur. J. Biochem. 231: 710–725
25. Girod PA, Fu H, Zyrd JP & Vierstra RD (1999) (submitted)
26. Kominami K, Okura N, Kawamura M, DeMartino GN, Slaughter CA, Shimbara N, Chung CH, Fujimuro M, Yokosawa H, Shimizu Y, Tanahashi N, Tanaka K & Toh-e A (1997) Mol. Biol. Cell 8: 171–187
27. Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, Baumeister W, Fried VA & Finley D (1998) Cell 94:615–623
28. Schaefer DG & Zryd J (1997) Plant J. 11: 1195–1206
29. Cove D J & Knight CD (1993) Plant Cell 5: 1483–1488
30. Ashton NW, Grimsley NH & Cove DJ (1979) Mol. Gen. Genetics 154: 87–95