Proteomic analysis of affinity-purified 26S proteasomes identifies a suite of assembly chaperones in *Arabidopsis*

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ABSTRACT
The 26S proteasome is an essential protease that selectively eliminates dysfunctional and short-lived regulatory proteins in eukaryotes. To define the composition of this proteolytic machine in plants, we tagged either the core protease (CP) or regulatory particle (RP) sub-complexes in *Arabidopsis* to enable rapid affinity purification followed by mass spectrometric analysis. Studies on proteasomes enriched from whole seedlings, with or without ATP needed to maintain the holo-proteasome complex, identified all known proteasome subunits but failed to detect isoform preferences, suggesting that *Arabidopsis* does not construct distinct proteasomes sub-types. We also detected a suite of proteasome-interacting proteins, including likely orthologs of the yeast and mammalian chaperones Pba1, Pba2, Pba3, and Pba4 that assist in CP assembly; Ump1 that helps connect CP half-barrels; Nas2, Nas6, and Hsm3 that assist in RP assembly; and Ecm29 that promotes CP-RP association. Proteasomes from seedlings exposed to the proteasome inhibitor MG132 accumulated assembly intermediates, reflecting partially built proteasome sub-complexes associated with assembly chaperones, and the CP capped with the PA200/Blm10 regulator. Genetic analyses of *Arabidopsis* UMP1 revealed that, unlike in yeast, this chaperone is essential, with mutants lacking the major UMP1α and UMP1β isoforms displaying a strong gametophytic defect. Single *ump1* mutants were hypersensitive to conditions that induce proteotoxic, salt and osmotic stress, and also accumulated several proteasome assembly intermediates, consistent with its importance for CP construction. Insights into the chaperones reported here should enable study of the assembly events that generate the 26S holo-proteasome in *Arabidopsis* from the collection of 64 or more subunits.

INTRODUCTION
Protein quality control in eukaryotes is mediated by an intricate proteostasis network that helps maintain a healthy proteome and protects against
protein mis-folding and the accumulation of cytotoxic aggregates (1). Part of this network engages the ubiquitin-proteasome system (UPS) to remove short-lived or terminally aberrant proteins (2). The UPS involves tagging of substrates with chains of poly-ubiquitin via a highly polymorphic, ATP-dependent conjugation cascade, with specificity provided by a family of ubiquitin-protein ligases (or E3s) that can consist of hundreds or even thousands of unique members (3).

Protein breakdown by the UPS is ultimately catalyzed by the 26S proteasome, which recognizes poly-ubiquitylated targets, unfolds and deubiquitylates them, and then cleaves the linear polypeptides into short fragments for final release of the individual amino acids by other peptidases (4,5). 26S proteasomes are composed of two stable and functionally distinct sub-complexes; the 20S core protease (CP) that houses peptidase activities, capped at one or both ends by the 19S regulatory particle (RP) that captures and prepares appropriate substrates for degradation (6,7). The CP exists as a barrel generated by four stacked hetero-heptameric rings of seven \( \alpha \)- or \( \beta \)-subunits (known as PAA-PAG and PBA-PBG, respectively, in Arabidopsis) arranged in a C2 symmetric \( \alpha_1 \)-\( \beta_1 \)-\( \beta_1 \)-\( \alpha_1 \) configuration. It contains a central chamber housing six catalytic sites responsible for peptide bond cleavage, provided by the \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \) subunits (8). Substrates enter this chamber via two narrow opposing axial pores that are gated by N-terminal extensions from several \( \alpha \)-subunits (9). Through this distinctive architecture, the CP acts as a self-compartmentalized protease that can only degrade polypeptides that are deliberately recognized, unfolded, and imported.

The RP binds to either or both ends of the CP in the presence of ATP, where it provides the activities for substrate recruitment through recognition of the ubiquitin moieties, followed by deubiquitylation, unfolding, and translocation into the CP chamber (4,5). The RP can be separated into two sub-complexes in vitro, termed the base and the lid. The base directly contacts the CP and contains a hetero-hexameric ring of AAA-ATPases (RPT1-6) plus four non-ATPase (RPN) subunits (RPN1, RPN2, RPN10, and RPN13), whereas the lid is composed of an additional nine RPN subunits with varying functions. The RPN1, RPN10, RPN13, and SEM1/RPN15 subunits are known to bind ubiquitin (5,6,10), while the RPT ring couples ATP hydrolysis to substrate unfolding and translocation (7), and triggers re-positioning of CP \( \alpha \)-subunits extensions to permit substrate entry. RP-CP binding occurs by insertion of C-terminal \( \text{HbYX} \) motifs (where \( \text{Hb} \) represents a hydrophobic residue, \( \text{Y} \) is tyrosine, and \( X \) is any amino acid) from several RPT subunits into pockets formed at the interface between adjacent \( \alpha \)-subunits (11). Once assembled, the 26S particle can associate with a myriad of other factors, including additional receptors for ubiquitylated substrates, activators, inhibitors, enzymes that drive various post-translational modifications, E3s, and deubiquitylases that remove the ubiquitin moieties in an effort to rescue substrates from degradation and/or recycle ubiquitin (4,5,10).

Mammalian cells also exploit diversity within the \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \) subunit paralogs of the CP to generate immuno- and thymo-proteasomes with distinct peptidase activities (12).

The complexity and size of 26S proteasomes has made them an excellent model for understanding how intricate macromolecular structures are rapidly and faithfully built from dozens of components. Detailed studies on proteasome assembly in yeast, and then in archaea and mammalian cells, revealed that this process is mediated by a collection of dedicated chaperones that enhance assembly rate and fidelity (13,14). CP assembly begins with construction of the individual \( \alpha \)-rings, which is accelerated by two hetero-dimeric chaperones, termed Pba1-Pba2 and Pba3-Pba4 in yeast (15,16). Pba1 and Pba2 in particular possess C-terminal \( \text{HbYX} \) motifs that dock with the \( \alpha \)-subunit rings, and whose binding also prevents premature association of CP assembly intermediates with the RP or other activating factors (17,18). The Pba3-Pba4 heterodimer helps ensure integration of each \( \alpha \)-subunit in correct register (16,19-21). In the absence of Pba3 and Pba4, aberrant \( \alpha \)-rings accumulate that contain an \( \alpha_5 \)-\( \alpha_6 \)-\( \alpha_7 \)-\( \alpha_1 \) hetero-tetramer together with various permutations of \( \alpha_2 \), \( \alpha_3 \), and/or \( \alpha_4 \) (20).

Upon completion, the \( \alpha \)-ring provides a platform for assembling the \( \beta \)-ring, formation of which starts with \( \beta_2 \), followed by sequential integration of \( \beta_3 \), \( \beta_4 \), \( \beta_5 \), \( \beta_6 \), and \( \beta_1 \), via a process aided by binding of the Ump1 maturation factor (known as POMP in humans) at the \( \beta \)-ring center.
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(22,23). β7 is the last β-subunit to join (23,24), leading to a transient species called the 15S “half-proteasome” which is then capped and presumably stabilized by Blm10 (PA200 in mammals and plants (24)). Integration of Ump1 prevents premature dimerization of partially assembled CP precursors until a complete half-proteasome is formed, at which point the two halves assemble and Ump1 is degraded, thus becoming the first substrate of the complex (22).

Correct assembly of the RPT ring of the RP also occurs with the help of five dedicated chaperones, called Nas2, Nas6, Hsm3, Rpn14, and Adc17 in yeast (or p27, p28, S5b, and PAAF1, respectively, in mammals, which lack an Adc17 homolog (reviewed in (5,13,14)). These chaperones independently bind to the C-terminal region of specific RPT subunits, resulting in three distinct precursor assembly modules (13,14). These modules associate sequentially, followed by incorporation of RPN2, RPN13, and RPN10 to finalize RP base assembly and trigger chaperone eviction (13). As yet, no assembly chaperones have been identified for the RP lid, which is currently thought to combine spontaneously using the RPT ring as a scaffold, based on self-assembly studies in vitro and in Escherichia coli (13,25).

The final step in 26S proteasome assembly is association of the RP with the CP, which is driven by docking of the C-terminal HbYX motifs from several RPT ring subunits onto the α-subunit rings of the CP (11). This association also occurs spontaneously in vitro, is stabilized by ATP, and can be fully reversible (4,5). Here, Ecm29 appears to provide a critical quality control checkpoint by binding to structurally aberrant proteasomes and repressing both the ATPase activity of the RP and gate opening of the CP (26,27).

Our understanding of 26S proteasome composition in multiple species has been aided by the use of tagged subunits for its rapid affinity purification, which when followed by deep proteomic analysis allowed identification of all known CP and RP subunits and a large collection of proteasome-associated proteins (28,29). Using this strategy, we attempted to better define the composition of the Arabidopsis thaliana complex, with a focus on proteins that interact with the 26S particle and thus might be important for assembly and/or regulation. As most subunits of the Arabidopsis complex are encoded by gene pairs, we also tested the possibility that plants, like mammals, assemble multiple proteasome isoforms with potentially unique activities, which are created by the deliberate pairing of subunit paralogs. While our mass spectrometry (MS) results failed to find evidence for unique 26S proteasome isotypes, we did discover numerous factors that bind to the CP and/or RP subcomplexes. Included in this list were orthologs for a number of yeast and mammalian proteasome chaperones needed for CP and RP assembly, the CP effector PTRE1 (known as Fub1 and Pl31 in yeast and humans, respectively (30)), and the PA200/Blm10 regulator that caps the CP (29).

Genetic analysis of the Arabidopsis UMP1 maturation factor revealed that it promotes CP assembly and, unlike its yeast counterpart (22), is essential. Taken together, this study represents the first description of plant proteasome assembly chaperones, and provides a platform for future studies describing how this proteolytic machine is built in plants.

RESULTS

Generation of transgenic plants for RP-based proteasome purifications

Our prior proteomic studies on Arabidopsis 26S proteasomes relied on a transgenic line that genetically replaced the single gene encoding the CP α-subunit PAG1 (α7) with a FLAG-tagged variant (29). To complement this approach, we developed transgenic plants that replaced the paralogous RPT4a and RPT4b genes (At5g43010 and At1g45000, respectively (31)) encoding the RP base subunit RPT4 with 2XFLAG-tagged versions that would allow us to specifically isolate the RP sub-complex or the full 26S holo-proteasome enriched for these isoforms. The two 399-amino-acid RPT4 proteins (44.8 kDa each) share high sequence identity both to each other (97% identity between the two isoforms) and to orthologs from other plant, animal, and yeast species (Fig. S1, A, B, and C). Importantly, the cryo-electron microscopic structure of the yeast proteasome showed that the N-terminus of RPT4 is likely solvent-accessible in the Arabidopsis particle (32), thus enabling addition of the 2XFLAG sequence without perturbing RP assembly and activity.

For recipients of tagged RPT4, we identified a collection of T-DNA insertion mutants...
within the RPT4a or RPT4b loci (Fig. 1, A). Of use here were the rpt4a-1 and rpt4b-2 mutations, which when homozygous disrupted accumulation of the full-length transcripts as judged by RT-PCR analysis of total RNA, and thus were considered to be null alleles (Fig. 1, B). Surprisingly, homozygous rpt4a-1 and rpt4b-2 seedlings and mature plants, along with those harboring several other rpt4a or rpt4b alleles, were phenotypically indistinguishable to wild-type Col-0 plants when grown under nutrient-rich growth conditions and long- or short-day photoperiods (Fig. 1, C and D). This is in contrast to null mutants affecting several other RP subunit genes in Arabidopsis, which have been shown to display a range of phenotypic defects (33,34). However, the presence of at least one copy of RPT4 appeared to be essential to Arabidopsis, as we failed to identify double homozygous rpt4a-1 rpt4b-2 progeny from self-crosses of double heterozygous parents (Fig. 1, E). Even homozygous/heterozygous progeny (Aabb or aaBb) could not be found, indicating that both the male and female haploid gametes require at least one copy of a functional RPT4 gene (Fig. 1, E).

For the purification strategy used here, we complemented the rpt4a-1 and rpt4b-2 plants with FLAG-tagged versions of RPT4a and RPT4b, using a genomic fragment of each bearing the codons for a 2XFLAG sequence appended after the initiator methionine codon and preceded by a 600- or 2,000-bp region harboring the native RPT4a and RPT4b promoters, respectively. Plants homozygous for FLAG-RPT4a rpt4a-1 or FLAG-RPT4b rpt4b-2 were then identified in the F2 generation by genomic PCR and by the segregation ratio of BASTA tolerance in the F3 generation, which was introduced into the plants as a selectable marker along with the transgenes. Notably, the complemented plants were phenotypically indistinguishable from both the homozygous rpt4a-1 and rpt4b-2 parents and wild-type Col-0, suggesting that the tagged subunits were not detrimental to Arabidopsis growth and development (Fig. 1, F).

We then confirmed expression of the FLAG-tagged proteins by immunoblot analysis of crude extracts with anti-RPT4 antibodies, which were generated against the RPT4a isoform but equally recognize RPT4b, as judged by immunoblot analysis of recombinant proteins (Fig. 1, G). Accumulation of slower-migrating FLAG-RPT4a or FLAG-RPT4b proteins was detected in the complemented plants with both anti-RPT4a and anti-FLAG antibodies, but not in non-transformed plants (Fig. 1, H). In addition, bands representing untagged RPT4 were seen in the transformed rpt4a-1 and rpt4b-2 plants, which were likely derived from the wild-type RPT4a and RPT4a loci remaining in each line, respectively (Fig. 1, H). FLAG-RPT4a accumulation was consistently higher than that of FLAG-RPT4b across multiple independent transgenic lines, which was consistent with the normally higher levels of RPT4a versus RPT4b when comparing the levels of total RPT4 in the single rpt4a-1 and rpt4b-2 mutants (Fig. 1, H).

For use as an affinity reagent, FLAG-RPT4a and FLAG-RPT4b should readily integrate into 26S proteasomes. Indeed, when we analyzed the assembly of 26S particles by glycerol gradient centrifugation of total protein extracts from Arabidopsis seedlings, we found that both tagged versions associated with the 26S particle along with other RP and CP subunits, with little to no free FLAG-RPT4a or FLAG-RPT4b species evident at the top of the gradients (Fig. 2). In the absence of phenotypic rescue, this integration also indirectly confirmed that the FLAG-tagged versions of RPT4 remain functional.

Notably, like mutants impacting other 26S proteasome subunits (33-35), the rpt4a-1 and rpt4b-2 plants without the FLAG-RPT4a or FLAG-RPT4b transgenes accumulated an array of sub-species representing free CP and RP, which were not evident in wild type or the complemented lines (Fig. 2). The accumulation of these sub-species implied that the absence of RPT4a or RPT4b compromises efficient proteasome assembly in planta, possibly by an insufficient supply of total RPT4 either throughout the seedlings, or in specific cells due to variations in expression patterns between the paralogs.

**Affinity purification of Arabidopsis 26S proteasomes via the CP or RP**

Using these FLAG-RPT4a and FLAG-RPT4b lines together with the PAG1-FLAG line described previously (29), we exploited a rapid affinity method to purify proteasomes from clarified seedling extracts using a single anti-FLAG chromatography step followed by elution with FLAG peptide. As shown in Fig. 3, A, isolations
performed in the presence of ATP enabled strong enrichment of 26S particles containing both CP and RP sub-complexes, while those undertaken in the absence of ATP enabled strong enrichment of free CP or RP sub-complexes alone, with the characteristic array of lower molecular mass species (20-37 kDa) for the CP and higher molecular mass species (30-110 kDa) for the RP being visible. The identities of these complexes were then confirmed by immunoblot analyses of the samples with antibodies against subunits from the CP (PAC1 (α1), PAG1 (α2), PBA1 (β1), and PBF1 (β3)), the RP base (RPN1, RPT2, and RPT4), or the RP lid (RPN3, RPN5, and RPN12a; Fig. 3, B). As expected, the FLAG-RPT4a/b isoforms co-purified with the complete 26S complex with ATP and with the RP alone without ATP, while the PAG1-FLAG protein co-purified with the complete 26S complex with ATP and with the CP alone without ATP (Fig. 3, B).

To define the composition of 26S proteasomes purified via FLAG-RPT4a, FLAG-RPT4b, or PAG1-FLAG, we subjected trypsinized preparations isolated with or without ATP to deep protein sequencing by reverse-phase liquid chromatographic separation of the peptides followed by tandem MS (LC-MS/MS). The relative levels of each protein were then determined by label-free quantification (LFQ) based on the MS1 precursor ion intensities (36). These analyses resulted in the unequivocal identification of all 14 subunits of the CP, all 6 RPT ring subunits, and 12 out of 13 RPN subunits (the exception being RPN13), including SEM1/RPN15 that had previously escaped detection (Fig. 4, A; (29)). In most cases (the exceptions being PAC2 (α5), RPT1b, and RPN12b), polypeptides derived from both paralogs encoded within the Arabidopsis genome were detected. Previous studies suggested that these three predicted, but not detected, isoforms are encoded by pseudogenes (29,37). When the LFQ values for each protein were compared among the preparations generated with FLAG-RPT4a, FLAG-RPT4b, or PAG1-FLAG without ATP, an obvious enrichment was seen for CP subunits when using the PAG1-FLAG pag1-1 line, and for the RP subunits when using the FLAG-RPT4a rpt4a-1 or FLAG-RPT4b rpt4b-2 lines (Fig. 4, A). In fact, estimates based on LFQ values revealed a 23-fold enrichment for the CP over the RP when purified via PAG1-FLAG, and a 3.4-fold enrichment of the RP over the CP when purified via FLAG-RPT4a/b (Fig. 5).

Arabidopsis 26S proteasomes do not display isoform specificity with respect to RPT4

Given the possibility that Arabidopsis and other plants use diversity among paralogous genes to generate distinct proteasome subtypes with unique specificities (29,31,38-40), much like the exchange of mammalian β1, β2, and β5 subunit isoforms generate immuno- and thymo-proteasomes (12), we tested whether the RPT4a and RPT4b isoforms would selectively associate with isoforms from other proteasome subunits. In this case, we compared statistically the abundance of other isoforms in preparations obtained with FLAG-RPT4a or FLAG-RPT4b by volcano plots that displayed both fold change and p-values of significance between the two samples (Fig. 4, B). Based on the kernel density estimations (KDE) of their LFQ values, both samples displayed a strong overlap when comparing subunits isolated with either the entire 26S particle or with just the RP specifically (i.e., with and without ATP).

When comparing subunit isoforms individually, RPT4a and RPT4b were only modestly enriched relative to other subunits in the FLAG-RPT4a rpt4a-1 and FLAG-RPT4b rpt4b-2 samples obtained with ATP, respectively. Given that doubly-capped 26S proteasomes contain two RPs, each of which houses a single RPT4 polypeptide, this modest enrichment implied that 26S particles assemble with a mix of both RPT4a and RPT4b (Fig. 4, B). Enrichment of other proteasome subunits or isoforms was also not detected. However, when the RP was specifically enriched in samples obtained without ATP, and thus should contain only a single RPT4, the RPT4a and RPT4b polypeptides showed significant enrichment for their respective FLAG-tagged version (Fig. 4, B). Despite this, no other subunits, or subunit paralogs, were similarly enriched, indicating that RPT4a and RPT4b are not deliberately paired with other proteasome subunits or their isoforms. Based on this result with RPT4a/b, we found no evidence for the assembly of specific proteasomes types based on isoform preferences. The likely scenario is that Arabidopsis randomly assembles 26S proteasomes from the available pool of subunit isoforms in each.

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cell, although a final conclusion will require similar analyses with CP subunits, especially with respect to the PBB1/2 ($\beta_2$) and PBE1/2 ($\beta_5$) paralogs.

Identification of a suite of CP- and RP-specific proteasome-interacting proteins

Because our one-step protocol allowed rapid enrichment of 26S proteasomes from Arabidopsis tissues under non-denaturing conditions and without the need for stringent wash and elution steps, a number of proteasome-associated proteins remained bound and were then identified by LC-MS/MS (false discovery rate (FDR) < 0.01; Table 1; Figs. 4, C, and 5). Of particular note were proteins associated with proteasome sub-complexes purified in the absence of ATP, which could represent CP- or RP-specific interactors. Such specific associations were immediately evident in volcano plots of the preparations (performed with or without ATP), with the interacting proteins showing clear overlap based on KDEs associated with either the CP or RP (Fig. 5).

For the CP samples obtained using PAG1-FLAG, these interacting proteins included likely homologs of the proteasome maturation factor UMP1 (22), and the extrinsic ubiquitin-binding receptor DSK2 (41,42), plus REDUCED CHLOROPLAST COVERAGE 1 (REC1; (43)), and an F-Box protein of unknown function (Table 1). Importantly, four of the most highly enriched proteins were identified by position-specific iterative (PSI) and reciprocal Basic Local Alignment Search Tool (BLAST) searches as possible orthologs of the previously described yeast and mammalian CP assembly chaperones Pba1-4 and PAC1-4, respectively (13,14), despite low sequence identity (9-30%; see Figs. 6, A, and S2-S6). As these three letter annotations were already in use (29), we renamed these Arabidopsis proteins as PROTEASOME BIOGENESIS-ASSOCIATED CHAPERONE (PBAC)1-4. PBAC1 and PBAC2 were previously identified by PSI-BLAST searches with their yeast counterparts (16,17,29), and their identities were also supported by the presence of C-terminal HbYX or related HbF motifs, respectively, which are universally present in the yeast, archaeal, and mammalian members of these two chaperone families (Figs. S2, A, and S3, A). As described above, HbYX motifs are employed by assembly chaperones to bind and stabilize $\alpha$-subunits during initial $\alpha$-ring formation, as well as being used by other activators (including the RP itself) to mediate their binding to the CP and control $\alpha$-ring gate opening during substrate degradation (11). An additional PBAC-type protein with a HbYX motif was also found associated with the Arabidopsis CP (designated PBAC5), which likely represents a fifth member of the PBAC superfamily; its characterization is currently in progress (R.S. Marshall, D.C. Gemperline, and R.D. Vierstra, unpublished).

An additional list of interacting proteins was obtained for the RP enriched without ATP (Table 1; Figs. 4, C, and 5). Notably, three of the top interactors identified in both FLAG-RPT4a and FLAG-RPT4b samples were identified by PSI and reciprocal BLAST searches as possible orthologs of the previously described yeast and mammalian RP assembly chaperones NAS2/p27, NAS6/p28, and HSM3/S5b (13,14), again despite low sequence identity (10-36%; see Figs. 6, A, and S7-S9). As highlighted in Fig. 6, A, all the possible CP and RP chaperones have relatively high sequence identities within the plant kingdom, but this homology dropped considerably when compared to their yeast and human relatives. Additional high-confidence interactors that co-purified with both the CP and RP included homologs of the known alternate proteasome cap PA200/Blm10 (29), the proteasome assembly factor ECM29 (26,28), and the proteasome regulator PTRE1 ((30); Table 1; Fig. 5; Fig. S10). Also of interest were the AGAMOUS-LIKE 90 (AGL90) transcription factor and MULTI-DRUG RESISTANCE-ASSOCIATED PROTEIN 8 (MRP8), though the functional significance of these two interactions is currently unknown.

We considered it possible that some proteasome interactors could be direct targets being degraded by the complex. However, few if any matched known targets or the deep lists of ubiquitylation substrates from Arabidopsis (44, 45). In hindsight this absence was likely given the speed that proteasomes can process substrates (46) and the fact that the proteasomes were not purified in the presence of inhibitors that would block continued target hydrolysis after tissue extraction.
Proteasome assembly chaperones have distinct expression patterns in Arabidopsis

Under the assumption that proteasome assembly chaperones should be co-ordinately expressed to meet proteolytic demand, we attempted to provide support that the Arabidopsis collection (PBAC1-4, UMP1, NAS2, NAS6, HSM3, and ECM29) is indeed involved in proteasome assembly. However, examination of their expression patterns in 79 Arabidopsis tissues available within the Transcriptome Variation Analysis database (www.travadb.org (47)) failed to find a common theme, even among factors that should work together (e.g., PBAC1-4; Fig. 6, B). However, when seedlings were activated to express 26S proteasomes (and thus their assembly chaperones) by treatment with the protease inhibitor MG132 (48), or by the introduction of subunit mutations that compromise proteasome activity (33,37), several of these loci were found to be up-regulated along with the rest of the proteasome-stress regulon (Fig. 6, C). The extent of the up-regulation varied; whereas the transcript abundance for PBAC1, PBAC2, UMP1a, NAS6, HSM3, and ECM29 increased between 4 and 8 fold after 3 hr of MG132 treatment, those for PBAC3, PBAC4, and NAS2 were only modestly increased even after a 24-hr exposure (on average around 1.4 fold).

Assembly chaperones associate with proteasome assembly intermediates

As a second strategy to connect the proposed chaperones to proteasome assembly, we tested if they could be found linked to the various intermediates that should arise during holo-26S proteasome construction in planta (35,49). Here, the accumulation of these complexes was enhanced by pre-treating Arabidopsis seedlings with MG132 to encourage new proteasome synthesis (Fig. 6, C (48)), and the intermediates along with the CP, RP, and 26S particles were then affinity enriched via the PAG1-FLAG tag in the presence of ATP. While the profile of proteasome subunits was not dramatically altered by this treatment, (Fig. 7, A), the effects of MG132 on both proteasome assembly and capacity were evident by the increased ubiquitylation of the complex (35), increased association of the PA200/Blm10 regulator (29), and by the appearance of novel proteasome sub-particles in addition to the CP, RP, and holo-26S complex after separation by native PAGE (Fig. 7, A, B, and C).

To define the protein composition of each species in the native gels, the corresponding bands were excised and in-gel digested with trypsin, followed by LC-MS/MS analysis (Fig. 7, D). When the protein composition of each band was quantified by distributed Normalized Spectral Abundance Factor (dNSAF) values (50,51), several assembly intermediates were identified, along with stable CP and RP sub-complexes, that were consistent with PBAC1-4 and UMP1 being involved in CP assembly (Fig. 7, C and D). The smear of species detected at the bottom of the native gels, especially after MG132 treatment of the seedlings, was enriched in CP α-subunits, with low levels of β-subunits, together with the proposed CP assembly chaperones PBAC1-4, suggesting that these species mostly comprised partially or fully assembled α-rings (17). Indeed, PBAC3 and PBAC4 were found only in this region of the gels. In agreement with this smear containing partially assembled CPs, a low level of UMP1 was also detected in this region. The prominent band #1 labeled in Fig. 7, C contained only CP subunits (and not PBAC1-4), indicating that this species represented fully assembled CP, while the minor band #2 seen above upon MG132 treatment contained CP plus PBAC1-2, suggesting that it represents nearly assembled 15S half-barrels still associated with CP assembly chaperones (Fig. 7, C and D). Band #3 contained CP subunits together with PA200/Blm10 but little PBAC1-2, suggesting it contained CPs likely capped at one end by this CP regulator (Fig. 7, C and D). In MG132-treated samples, bands #4 and #5 appeared, whose compositions were consistent with them representing the CP capped with PA200/Blm10 and bound by the PBAC1-2 assembly chaperones, and the CP capped at both ends by PA200/Blm10, respectively. Bands #1-5 also contained ubiquitin, especially in samples from MG132-treated seedlings, which could reflect ubiquitylated CP or its intermediates now targeted for turnover by autophagy (35).

Moving further up the native gels, bands #6 and #7 were mostly enriched for RP subunits, with low levels of CP subunits, and thus represented mostly free RP sub-complexes (Fig. 7, C and D). As these RP species were isolated solely based on their association with CP harboring
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PAG1-FLAG, they should represent RPs that dissociated from the CP after isolation. Finally, bands #8, #9, and #10 appeared to contain equimolar amounts of subunits for both the CP and RP sub-complexes, and likely represented the fully assembled holo-26S particle containing either one or two RPs. Interestingly, only band #10 was found to contain the Arabidopsis ortholog of ECM29, albeit at low levels (Fig. 7, C and D), as might be expected if ECM29 helps tether the RP to the CP (26,27). The possible RP chaperones NAS2, NAS6, and HSM3 were not detected during this analysis, presumably because the proteasomes were enriched via the CP subunit PAG1, and thus should contain only fully assembled RP species.

PBAC3 and PBAC4 can rescue yeast mutants missing their orthologs

As a third strategy to confirm that these chaperones are involved in proteasome assembly, we attempted to rescue yeast mutants missing Pba3 and Pba4 with their likely Arabidopsis orthologs, PBAC3 and PBAC4, respectively. As shown in Fig. 8, A, yeast Δpba3 and Δpba4 strains are highly sensitive to proteotoxic stress, which can be seen by suppressed colony growth at low micromolar concentrations of the amino acid analog canavanine. Both strains were equally hypersensitive, consistent with Pba3 and Pba4 working as hetero-dimers (20,21). Strikingly, strong rescue of this growth defect on 5 μM canavanine was evident upon introduction of tagged versions of both Arabidopsis PBAC3 and PBAC4. Like yeast Pba3, HA-tagged PBAC3 rescued the defect in Δpba3 cells but not in Δpba4 cells, while HA-tagged PBAC4 rescued the defect in Δpba4 cells but not in Δpba3 cells (Fig. 8, A). This lack of cross-complementation, plus the failure of Δpba3 Δpba4 cells to be rescued by the two chaperones individually, further supported the non-redundant functions of PBAC3 and PBAC4, and implied that these Arabidopsis proteins also work as hetero-dimers.

UMP1 is a CP maturation factor essential in Arabidopsis

In a parallel fashion, we attempted to demonstrate that Arabidopsis UMP1 is orthologous to yeast Umpl, despite low sequence identity (18, 16, and 13% for Arabidopsis UMP1a, UMP1b and UMP1c, respectively), with our first approach being yeast complementation assays as above. As shown in Fig. 8, B, growth of yeast Δump1 cells is similarly hypersensitive to 5 μM canavanine, and also to heat stress at 37°C (22). Whereas HA-tagged yeast Ump1 rescued both of these growth defects, we were surprised to find that the three putative Arabidopsis orthologs all failed (Fig. 8, B), even though each was adequately expressed in the Δump1 background, based on immunoblots of total cell extracts with anti-HA antibodies (Fig. 8, C). Complementation of the canavanine-sensitivity phenotype was also not observed when combinations of two, or even all three, Arabidopsis UMP1 isoforms were expressed together, indicating that they do not function as hetero-dimers or -trimers (Fig. 8, B).

As an alternative, we tested whether Arabidopsis ump1 mutants harbor a similar hypersensitivity to proteotoxic stress induced by agents such as canavanine and MG132. As mentioned above, Arabidopsis potentially encodes three UMP1 paralogs, two of which (UMP1a (At1g67250) and UMP1b (At5g38650)) align reasonably well to yeast Ump1 and its mammalian counterpart POMP (Figs. 9, A and B, and S6). Only UMP1a is transcriptionally up-regulated by conditions that impair proteasome function, implying that it could be the dominant isoform (Fig. 6, C). The third paralog, UMP1c (At1g62920), shares homology within the central core region but is predicted to be missing 58 amino acids at the N-terminus and contain an additional 152 amino acids at the C-terminus, neither of which are well conserved among UMP1 proteins in general or among plant UMP1 proteins in particular (Figs. 9, A and B, and S6).

From a search of the Arabidopsis T-DNA insertion collections, we identified potentially useful mutants in all three Arabidopsis UMP1 genes, which were confirmed by genomic DNA sequencing around the insertion sites (Fig. 9, A and B). Transcript analyses by RT-PCR failed to detect corresponding full-length mRNAs in several of the alleles when homozygous (ump1a-2, ump1a-3, ump1b-2, ump1c-1, ump1c-2, and ump1c-5; Fig. 9, C). Given the positions of the T-DNAs, the ump1a-2, ump1b-2, and ump1c-2 alleles were studied in detail. For the ump1a-2 allele, no partial transcripts were amplified, while for the ump1b-2 and ump1c-2 alleles, only
transcripts generated upstream of the insertion site could be detected, suggesting that these mutants represent strong, if not null, alleles (Fig. 9, C). Under standard growth conditions with a long-day photoperiod, plants homozygous for each mutant appeared phenotypically normal and produced viable seeds, indicating that none of the three UMP1 loci are essential to Arabidopsis growth and development by themselves.

In an attempt to obtain Arabidopsis lines missing all three UMP1 isoforms, we began generating higher-order mutant combinations by introgressing the ump1a-2, ump1b-2, and ump1c-2 lines. While ump1a ump1c and ump1b ump1c double homozygous progeny could be easily identified with near normal Mendelian segregation ratios from a self-cross of double heterozygous parents, we surprisingly failed to find double ratios from a self-cross of double heterozygous identified with near normal Mendelian segregation one allele and homozygous for the other were plants that were heterozygous for ump1a ump1b. UMP1 is essential to double homozygous progeny could be easily lines. While

Under standard growth conditions with a long-day photoperiod, plants homozygous for each mutant were introgressing the generating higher-order mutant combinations by missing all three UMP1 isoforms, we began showing that UMP1c protein likely does not accumulate, despite evidence that the corresponding UMP1c locus is expressed (Fig. 6, B).

Arabidopsis UMP1 protects against proteotoxic stress by encouraging CP assembly

To test whether Arabidopsis ump1 mutants, like their yeast counterparts (22), are hypersensitive to proteotoxic stress, we measured the growth response of single homozygous ump1a, ump1b, or ump1c seedlings when exposed to various concentrations of canavanine and MG132, using the response of double mutant seedlings missing the NAC53 and NAC78 transcription factors (which are essential for activating the proteasome-stress regulon (48)) as a positive control. As shown in Fig. 10, A and B, wild-type seedlings grow reasonably well on sub-lethal doses of MG132 or canavanine beginning at germination, and became impacted only at higher doses (50 µM MG132 and 25 µM canavanine). By contrast, growth of the nac53-1 nac78-1 seedlings was strongly inhibited by even low concentrations of the drugs.

In agreement with UMP1c being a possible pseudogene, three different mutant alleles (ump1c-1, ump1c-2, and ump1c-5) were indistinguishable from wild type in their tolerance to long-term MG132 or canavanine exposure (Fig. 10, A and B). However, alleles impacting either UMP1a or UMP1b showed a strong hypersensitivity to both treatments. The growth inhibition of both UMP1a alleles (ump1a-1 and ump1a-2) was noticeably stronger than that for UMP1b (ump1b-2), suggesting again that UMP1a is the dominant locus (Fig. 10, A and B). This dominance could be caused by its higher overall expression level (90, 55 and 7 ESTs were found for UMP1a, UMP1b, and UMP1c, respectively, in the Arabidopsis Information Resource database (version 10.1)).

Recently, an Arabidopsis mutant in the proteasome subunit PBE1 (βs) was shown to have impaired CP assembly, resulting in a hypersensitivity to salt and drought stress, presumably due to insufficient levels of the CP or the entire 26S holocomplex (49). We therefore tested whether our panel of ump1 single mutants was similarly sensitive to such treatments. While we again found that the ump1c mutants grew equivalent to wild type, the growth of both the ump1a and ump1b mutants was strongly impaired on medium containing NaCl, mannitol, or the drought-responsive hormone abscisic acid (ABA) at concentrations known to only mildly suppress wild type Arabidopsis growth (Fig. 11, A and B). Notably, the consequences were similar but less pronounced than for the null pbe1-2 mutant inactivating the dominant locus encoding this catalytically active CP subunit (49). The ump1a
and ump1b seedlings thus display phenotypic defects similar to another proteasome mutant with dampened CP capacity (Fig. 11, A and B).

To definitively assess the importance of UMP1 to proteasome assembly, we monitored the integrity of the complex by glycerol gradient fractionation of total cell extracts from wild-type and ump1a-2 seedlings with or without a 16-hr pretreatment with 50 μM MG132. Consistent with prior results in Fig. 2, a single proteasome complex was detected in wild-type seedlings by immunoblot analysis of the fractions with a panel of proteasome subunit antibodies, which likely represented the 26S holo-complex (Fig. 10, C). Upon exposure to MG132, substantial amounts of free RP and CP subunits accumulated at the top of the gradient, as might be expected based on activation of the proteasome stress regulon by this inhibitor in an effort to increase 26S proteasome supply (48). In contrast, multiple additional species were seen within the gradient from untreated ump1a-2 seedling extracts besides free subunits and the 26S complex (Fig. 10, C). Based on sedimentation position, these likely reflected free RP, free CP β-subunits, and what appeared to be CP α-subunits organized into α-ring intermediates.

When we combined the ump1a-2 mutation with MG132 treatment, a host of proteasome species appeared that were consistent with up-regulated proteasome subunit synthesis combined with impaired CP (and thus holo-26S particle) assembly. Some of these intermediate species could also represent the CP singly or doubly capped with PA200 (Fig. 7, C; (29,35)). Remarkably, little of the fully assembled holo-26S proteasome complex was evident relative to the sub-complexes, thus explaining why ump1a-2 seedlings grew so poorly when exposed long-term to MG132 (Fig. 10, A and B). Taken together, our observations that mutants impacting Arabidopsis UMP1 accumulate a variety of intermediate proteasome complexes, generate the same hypersensitivity to proteotoxic stress as do yeast Δump1 strains, and display a similar hypersensitivity to salt, drought, and ABA stress as an Arabidopsis CP mutant with known assembly defects, provide strong support for UMP1 being crucial for Arabidopsis CP assembly.

DISCUSSION

The 26S proteasome plays a critical role in plants by degrading key regulatory proteins and eliminating mis-folded proteins that might otherwise become cytotoxic. Given the sophisticated architecture of this multi-subunit particle, it is unsurprising that numerous intrinsic and extrinsic factors combine to promote rapid and faithful assembly. However, while studies on proteasome assembly in mammals and yeast began almost two decades ago (13,14), understanding of this pathway in plants has remained sparse. Here, we described affinity methods to rapidly isolate the 26S particle from Arabidopsis based on the RP, which when combined with a prior method to isolate the CP (29), not only validated the composition of the plant particle but also identified a suite of CP- and RP-specific interacting proteins that presumably assist in particle assembly, activity, and/or regulation.

MS analyses of 26S proteasomes purified via PAG1, RPT4a, or RPT4b invariably identified the complete collection of CP and RP subunits, including all encoded isoforms except for PAC2, RPT1b and RPN12b, which are predicted to be pseudogenes (29). The use of the RPT4a and RPT4b paralogs for affinity enrichment also allowed us to test the hypothesis that Arabidopsis assembles distinct proteasome isotypes based on the selective incorporation of specific subunits or their isoforms. No differences in subunit or paralog compositions could be found between proteasomes purified via RPT4a or RPT4b, suggesting that, at least with respect to RPT4, proteasome subunit isoforms are assembled randomly. However, evidence has recently emerged that the expression of certain plant proteasome subunits increases in response to proteotoxic or salt stress, or upon elicitation of defense responses, suggesting that unique subunit expression patterns, rather than specific isoform incorporation, could play a role in diversifying proteasome function in plants (48,49,52,53). Whether these changes result in altered substrate processing properties, as observed with the immuno- or thymo-proteasomes found in humans, remains to be determined.

Purification of proteasomes in the absence of ATP, which allows for CP-RP dissociation, permitted specific enrichment of the CP and RP sub-complexes, thus enabling identification of numerous CP- and RP-specific interacting
proteins. Importantly, several of these were homologous to known yeast and mammalian proteasome assembly chaperones. Included were relatives of yeast Pba1-4, Ump1, Nas2, Nas6, Hsm3, and Ecm29, along with the CP regulator PA200/Blm10. Amino acid sequence homology was surprisingly low, especially when comparing the plant sequences to their animal and fungal brethren (see Figs. S1-S10). For example, there was never greater than 18% amino acid identity between a plant and non-plant PBAC1 ortholog, and in the majority of cases the identities were less than 12%. Even within the plant kingdom, PBAC1 had over 70% identity among the four monocot species analyzed (Fig. S2, C), but dropped to 45% or less when compared to those in pineapple, Selaginella, and moss. Low sequence identity is also common outside of plants; the human PBAC1 ortholog PAC1 shared only 12%, 10% and 10% identity with Pba1 from three yeast species (Saccharomyces cerevisiae, Eremothecium gossypii and Kluyveromyces lactis), while the three yeast orthologs themselves also had less than 33% identity to each another (Fig. S2, C).

Likewise, the plant versions of PBAC2, PBAC3, PBAC4, UMP1, NAS2, NAS6, and HSM3 shared at most 30%, 25%, 25%, 30%, 35%, 36%, and 17% identity to their non-plant homologs, respectively.

Such low identity among the chaperones implies that secondary structure or clusters of amino acids are more important than the overall primary sequence. Indeed, crystal structures of hetero-dimers formed by the yeast and human assembly chaperones Pba3-Pba4 and PAC3-PAC4, respectively, showed that the two complexes adopt essentially identical three-dimensional folds despite little sequence conservation (21,54,55). While individual residues were not well conserved, structural features such as surface-charge complementarity were maintained. Especially notable was the presence of conserved HbYX-type motifs at the C-terminus of Arabidopsis PBAC1 and PBAC2, which likely help these CP assembly chaperones dock with the α-subunit ring of the CP.

To help validate that the predicted chaperones are indeed involved in proteasome assembly, we examined their expression patterns, tested for possible association with assembly intermediates of the CP, confirmed by complementation that some could replace their yeast relatives, and tested whether Arabidopsis mutants missing one of these chaperones might be hypersensitive to proteotoxic stress, as seen for comparable yeast mutants. While the expression patterns for the collection showed little developmental co-ordination, co-regulated expression upon proteotoxic stress induced by MG132 or proteasome mutations was evident for most (PBAC1, PBAC2, UMP1a, NAS6, HSM3, ECM29 and, to lesser extents, PBAC3, PBAC4 and NAS2), thus including these loci in the proteasome-stress regulon that ensures an adequate supply of 26S particles (48,56). MS analysis of CP assembly intermediates induced by treating PAG1-FLAG seedlings with MG132 further connected PBAC1-4, UMP1, and ECM29 with assembly of the CP and association of the CP with the RP.

Complementation studies using Arabidopsis PBAC3 and PBAC4 to rescue the hypersensitivity of yeast strains missing Pba3 and Pba4 to proteotoxic stress definitively showed that these two are assembly chaperones. However, similar studies with combinations of any of the three Arabidopsis UMP1 paralogs failed, suggesting that these proteins have sufficiently diverged to prevent cross complementation, as was also found to be the case with the mouse and human versions of Ump1 (known as POMP (57)). As an alternative, we generated a panel of mutants missing Arabidopsis UMP1a-c and showed that the lines missing UMP1a and UMP1b are hypersensitive to conditions that elicit proteotoxic stress, such as canavanine or MG132 treatments, as well as salt and drought stress, and displayed defects in proteasome assembly, much like their yeast Ump1 ortholog. Such assembly defects were particularly acute when combining the ump1a-2 mutation with MG132 exposure, with the Arabidopsis seedlings accumulating an array of sub-complexes and little of the 26S holo-complex. Surprisingly, while yeast strains missing Ump1 are viable, we failed to find Arabidopsis lines missing the dominant isoforms UMP1a and UMP1b, indicating that this chaperone is essential in plants.

During the preparation of this manuscript, the maize mutation defective kernel 40 (dek40) was described to encode a possible ortholog of Arabidopsis PBAC4 (58). The dek40 seeds displayed defective development, while the shoots
and roots showed slower growth after germination. Both overall proteasome activity and CP assembly were impaired in dek40 plants, which also hyper-accumulated ubiquitin conjugates presumably awaiting breakdown (58). Consistent with DEK40/PBAC4 working in CP assembly, its association with the maize ortholog of PBAC3 was also seen. However, despite defective proteasome assembly, the dek40 plants were still viable, suggesting that, unlike UMP1, the PBAC4 assembly chaperone is not essential in plants, as is also the case in yeast and mammals (16,19,20). Clearly, genetic analysis of the remaining plant chaperones is needed to determine which are essential for holo-26S proteasome assembly.

In addition to the chaperones described here, two other RP assembly chaperones have been identified in yeast, termed Rpn14 and Adc17 (13,59). However, no clear Arabidopsis relatives of Rpn14 or Adc17 were identified here by MS analysis of our RPT4-based affinity purifications, or by PSI-BLAST searches of the Arabidopsis genome using non-plant queries (Table 1; Fig. 5). While some homologs of Adc17 were found in chlorophyte and streptophyte algal species (e.g., Chlamydomonas, Chlorella, Klebsormidium, Micromonas, and Volvox spp.), none were confirmed to exist in any higher plant genome. We did find one annotated sequence related to Adc17 from cork oak (Quercus suber; Uniprot accession number A0A2P4GUY7), but this sequence is remarkably similar to fungal Adc17 sequences, and thus most likely represents genomic contamination.

Besides assembly chaperones, we also found a variety of proteasome-associated factors that might help regulate its integrity and activity, including PTRE1, PA200/Blm10, and the ubiquitin-binding shuttle factor DSK2. PTRE1 and its animal relative PI31 are of particular interest given their purported role in modulating proteasome activity in vivo (5) and their strong enrichment in Arabidopsis proteasome preparations (this report). Deletion of PTRE1 in Arabidopsis generates an auxin hyposensitive phenotype, with elevated levels of the AUX/IAA family of auxin-response repressors, suggesting that it promotes auxin signaling by controlling UPS-mediated AUX/IAA protein turnover (30). Mutants eliminating PA200 do not display defects in phenotype, ubiquitin conjugate accumulation, proteasome activity, or sensitivity to proteasome inhibitors (29), but a role for PA200 in proteasome regulation is likely given its enhanced binding to the CP under conditions that induce proteotoxic stress (Fig. 7, C; 29,35), and its observed role in chaperoning the CP into cytoplasmic proteasome storage granules during fixed-carbon starvation (60).

A number of plant proteins that are known orthologs of yeast or mammalian proteasome interactors were notably absent from our affinity-purifications. These include the ubiquitin-binding shuttle factors RAD23 and DDI1 (42), the E3 ligase UPL7/HUL5 (28), and the deubiquitylating enzyme UBP6 (28,61) that help control substrate degradation. It is possible that: (i) these proteins interact with Arabidopsis proteasomes at well below stoichiometric levels and thus evaded detection; (ii) the interactions are sensitive to even the mild wash and elution conditions employed here; (iii) the plant versions of these proteins do not interact with the proteasome under the conditions tested; and/or (iv) these proteins are not easily identified by MS. Clearly, additional studies will be required to understand why these proteins appear absent in Arabidopsis 26S proteasome preparations.

In summary, LC-MS/MS analysis of Arabidopsis proteasomes affinity-purified via either the CP or RP revealed a number of proteins interacting with a specific sub-complex, many of which are orthologs of known yeast and mammalian CP and RP assembly chaperones. These include PBAC1, PBAC2, PBAC3, and PBAC4 that likely promote CP assembly; UMP1 that is required for connecting CP half-barrels; NAS2, NAS6, and HSM3 that likely assist in RP assembly; and ECM29 that should provide a quality control checkpoint during CP-RP association. Subsequent mutational analysis of these chaperones should help confirm their roles in plant proteasome assembly and reveal how conserved the process is in plants compared to other organisms. For example, the existence of a possible fifth PBAC-type chaperone invites speculation of possible plant-specific proteasome assembly pathways. Using this list of chaperones, it should now be possible to define plant proteasome assembly through genetic and biochemical approaches in efforts to understand
how it might be manipulated for improved crop yield, stress protection, and nutrition.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions**

All *A. thaliana* plants were derived from the Columbia-0 (Col-0) ecotype, except the *ump1c-4* allele, which was from the Col-3 ecotype. Details of all T-DNA insertion mutants and stable transgenic lines are provided in Tables S1 and S2. All insertion mutants were confirmed by genomic PCR using 5' and 3' gene-specific primers (LP and RP, respectively) in conjunction with an appropriate T-DNA left border-specific primer (BP). Details of all oligonucleotide primers are provided in Table S3. Exact T-DNA insertion positions were determined by direct sequencing of appropriate PCR products. Before analysis, all mutants were backcrossed three times to the Col-0 parent and then selfed to obtain homozygous progeny, using growth on media containing 50 µg/ml kanamycin and/or 10 µg/ml BASTA for selection. Sterilized seeds (obtained via vapor-phase or liquid-phase sterilization) were vernalized at 4°C for 3 to 4 days and typically germinated on solid GM medium (3.2 g/l Gamborg’s B5 basal medium with minimal organics, 1% (w/v) sucrose, 0.05% (w/v) 2-(N-morpholino)-ethanesulfonic acid (MES; pH 5.7), 0.7% (w/v) agar) at 21 to 23°C under a long-day photoperiod (16-hr light/8-hr darkness), with a light intensity of 75 to 100 µmol/m²/sec and a relative humidity of 40 to 50%. Where indicated, agar plates were supplemented with the indicated concentrations of MG132 ((N-benzoxylcarbonyl)-leucinyl-leucinyl-leucinal), canavanine, NaCl, mannitol, or ABA. After 2 to 3 weeks, seedlings were transferred to soil (mixed in a 1:1 ratio with organic Coco Coir planting mixture, supplemented before use with 2 g/l Peters 20-20-20 fertilizer, 80 mg/l Ca(NO₃)₂, and 80 mg/l MgSO₄), and grown at 21 to 23°C under a long-day photoperiod.

To generate transgenic plants expressing FLAG-tagged RPT4a, the genomic region of *RPT4a* encompassing the full coding sequence plus 600-bp upstream of the ATG start codon, which includes the 5'- and 3'-untranslated regions (UTRs), was PCR-amplified from Col-0 genomic DNA and recombined into pDONR221 via the Gateway BP clonase II reaction (Thermo Fisher Scientific). Codons for the 2XFLAG tag (DYKDDDDK-DYKDDDDK) were then inserted by successive rounds of mutagenesis using the QuikChange II site-directed mutagenesis kit (Agilent Technologies). The sequence-confirmed *RPT4a::2XFLAG-RPT4a* clone was then recombined into the pMDC123 plant transformation vector (62) via the Gateway LR clonase II reaction (Thermo Fisher Scientific). To generate plants expressing FLAG-tagged RPT4b, the genomic region of *RPT4b* encompassing the full coding sequence plus 2,000-bp upstream of the ATG start codon, which again includes the 5'- and 3'-UTRs, was PCR-amplified from Col-0 genomic DNA using primer pairs encoding either the 2XFLAG tag plus *NcoI* and *SmaI* restriction enzyme sites, or *PsiI* and *NcoI* restriction enzyme sites, for the coding sequence and upstream region, respectively. The resulting PCR products and the recipient pCAMBIA3301 plant transformation vector (www.cambia.org) were digested with FastDigest *NcoI*, *SmaI*, and/or *PsiI* (Thermo Fisher Scientific), and ligated together using T4 DNA ligase (New England Biolabs) to generate the *RPT4b::2XFLAG-RPT4b* clone.

Sequence-confirmed *FLAG-RPT4a* and *FLAG-RPT4b* plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into homozygous *rpt4a-1* or *rpt4b-2* plants, respectively, by the *Agrobacterium*-mediated floral dip method (63). BASTA-resistant F₁ plants were selected and, after a self-cross, double homozygous plants were identified in the F₂ generation by PCR genotyping, immunoblot analysis (see below), and segregation of the F₃ generation on BASTA-containing medium.

**Immunological Techniques**

Frozen *Arabidopsis* seedlings were homogenized in 3 volumes of protein extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM MG132, 1X plant protease inhibitor cocktail) and clarified by centrifugation at 16,000 x g for 5 min at 4°C. The supernatant was then mixed with 0.25 volumes of 5X SDS-PAGE sample buffer (200 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue). Total protein extracts from yeast were obtained by re-suspending harvested cells (see
below) in 500 µl of lysis buffer (0.2 N NaOH, 1% (v/v) β-mercaptoethanol), followed by precipitation of proteins with 50 µl of 50% (w/v) trichloroacetic acid. Proteins were collected by centrifugation at 16,000 x g for 5 min at 4°C, washed once with 1 ml of ice-cold acetone, and re-suspended in 150 µl 2X SDS-PAGE sample buffer (80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 0.8% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.04% (w/v) bromophenol blue). Subsequent SDS-PAGE followed by silver staining was performed as previously described (64).

Native PAGE was also performed essentially as previously described (64), with minor modifications. Briefly, the resolving gel was composed of 4.5% (v/v) acrylamide, 0.12% (v/v) bis-acrylamide, 2.3% (v/v) sucrose, 1X TBE (i.e., 89 mM Tris-HCl (pH 8.4), 89 mM H3BO3, 2 mM Na2EDTA), 5 mM MgCl2, 1 mM ATP, 2.5% (v/v) Rhinohide (Thermo Fisher Scientific), whereas the stacking gel was composed of 2.5% (v/v) acrylamide and 0.62% (v/v) bis-acrylamide, with other components the same as for the resolving gel. Samples containing 10% glycerol were supplemented with xylene cyanol to 0.005% (w/v), and the gels were run for 16 or 36 hr at 4°C in 1X TBE supplemented with 0.5 mM ATP, with a constant current of 50 V. Proteins were visualized by staining the gels with silver using a protocol suitable for subsequent MS analyses (64).

For immunoblot analyses, proteins separated by SDS-PAGE were electrophoretically transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore) for 16 hr at 80 mA, and the membrane was blocked for at least 60 min with a 10% (w/v) non-fat dry milk solution in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). All incubations were performed at room temperature. The membrane was incubated with primary antibody solution (in 1% (w/v) non-fat dry milk solution in PBS) for 60 min, before being washed once with PBS, once with PBST (PBS containing 0.1% (v/v) Triton X-100), and once with PBS for 10 min each. The membrane was re-blocked with 10% (w/v) non-fat dry milk solution in PBS for 30 min, incubated for 60 min with secondary antibody solution (in 1% (w/v) non-fat dry milk solution in PBS), and then washed again as above. Blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate or the SuperSignal West Femto Maximum Sensitivity Substrate (both from Thermo Fisher Scientific).

Primary antibodies against RPN1 (40), RPN3, RPT4 (35), RPN5, RPN12a, RPT2, PAC1, PBA1, PBF1 (37), RPN10, ubiquitin (65), and PAG1 (29) were previously described, while antibodies against 6His, FLAG, HA, and histone H3 were purchased from Sigma-Aldrich (product numbers SAB4301134, F1804 and H6908) and AbCam (product number AB1791), respectively. Goat anti-rabbit secondary antibodies conjugated to horseradish peroxidise or alkaline phosphatase, or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase, were obtained from SeraCare (product numbers 5220-0336, 5220-0353, and 5220-0341, respectively).

To test antigenicity of the anti-RPT4a antibodies, the coding sequences of RPT4a and RPT4b were PCR-amplified from Col-0 cDNA (generated as described below) using primers encoding an Nhe restriction enzyme site at the 5' end and an Xho restriction enzyme site at the 3' end. The amplified PCR product was digested with FastDigest NheI and XhoI (Thermo Fisher Scientific), and ligated into pRSET-A (Thermo Fisher Scientific) cut with the same enzymes. The N-terminal 6His-tagged proteins were then expressed in E. coli strain BL21(DE3) pLysS (Promega). Cells were cultured at 37°C in 50 ml LB medium to an OD600 of between 0.6 and 0.8, followed by a 4-hr induction with 1 mM isopropyl-β-D-thiogalactopyranoside at 30°C. Cells were collected by centrifugation at 5,000 x g for 20 min at 4°C, frozen in liquid nitrogen, and lysed in 2 ml BugBuster Master Mix (EMD4 Biosciences). The tagged proteins were affinity-purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (QIAGEN), as previously described (35), and equal amounts of protein, as determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific), were subjected to SDS-PAGE and immunoblot analysis with anti-RPT4a and anti-6His antibodies.

Reverse Transcription-PCR Analysis
Total RNA was extracted from 50-100 mg of 10-d-old plate-grown or 7-d-old liquid-grown seedlings using the RNeasy plant mini kit (QIAGEN), as according to the manufacturer’s instructions. Following quantification with a NanoDrop 1000 spectrophotometer (Thermo
Fisher Scientific), 1 µg of total RNA was treated with DNase I (Thermo Fisher Scientific), and RNA integrity was assessed by a combination of OD\textsubscript{260}/OD\textsubscript{280} and OD\textsubscript{260}/OD\textsubscript{230} measurements and by running samples on denaturing formaldehyde agarose gels. RNA was converted into cDNA using the SuperScript III first-strand synthesis system (Thermo Fisher Scientific) and oligo(dT)\textsubscript{20} primers, again as according to the manufacturer’s instructions. Following first-strand synthesis the cDNA was diluted 1/30, and 5 µl was then amplified in a 20 µl reaction volume also containing 10 µl EconoTaq Plus Green master mix (Lucigen), 3 µl sterile H\textsubscript{2}O, and 1 µl each of 10 mM forward and reverse primers. Details of all oligonucleotide primers are provided in Table S3.

**Glycerol Gradient Centrifugation**

Glycerol gradient fractionation of 26S proteasome sub-complexes was performed essentially as previously described (35), with minor modifications. Seedlings were grown in 5 ml of liquid GM medium at 21 to 23°C under continuous light for 10 d with gentle shaking (90 rpm). Typically, ~30 mg of dry seeds were used per culture, resulting in ~2 g of fresh weight tissue. Frozen tissue was ground to a powder in a mortar and pestle at liquid nitrogen temperatures, and proteins were extracted on ice for 20 min in 1 volume of Buffer A (20 mM HEPES-KOH (pH 7.5), 5 mM MgCl\textsubscript{2}, 10% (v/v) glycerol, 2 mM ATP, 1 mM DTT, 2 mM PMSF, 6 µM chymostatin, 1X plant protease inhibitor cocktail) either containing or omitting 20 mM ATP. Extracts were filtered through two layers of Miracloth and clarified at 30,000 x g for 20 min at 4°C. The supernatant was immediately applied three times over a 12 ml PolyPrep chromatography column (Bio-Rad) containing 100 µl (equal to a 50 µl bead volume) of anti-FLAG M2 affinity resin (Sigma-Aldrich). The column was washed three times with 2 ml of Buffer B (containing or omitting 20 mM ATP as required), and remaining bound protein was eluted by incubating the beads for 30 min at 4°C with 250 µl of Buffer B containing 20 mM ATP and 500 ng/µl of the FLAG peptide (DYKDDDDK, synthesised by the University of Wisconsin Biotechnology Center Peptide Synthesis Facility). Samples of the crude extract, flow through (both diluted 1/10), third wash step, and/or elution were analyzed by native or SDS-PAGE (64), followed by silver staining or immunoblotting with antibodies against various proteasome subunits and ubiquitin (see above).

**Tandem Mass Spectrometry Sample Preparation**

For in-solution trypsin digestion, 200 µl of eluant from each proteasome affinity purification was vacuum dried to a final volume of approximately 25 µl, and denatured in 8 M urea, 25 mM (NH\textsubscript{4})HCO\textsubscript{3} in a total volume of 300 µl. Proteins were then reduced with 10 mM DTT at room
temperature for 1 hr, and alkylated in the dark in the presence of 50 mM 2-chloroacetamide at room temperature for a further 1 hr. Excess alkylating agent was quenched with 50 mM DTT for 5 min at room temperature, and the samples were diluted with 1.2 ml of 25 mM (NH₄)HCO₃ to reduce the urea concentration to below 1.5 M. Proteins were digested overnight at 37°C with 1 µg of sequencing grade modified porcine trypsin (Promega). Peptides were vacuum dried as above to a final volume of approximately 300 µl, acidified with 10% (v/v) trifluoroacetic acid (TFA) until the pH was less than 3.0, and desalted and concentrated on a 100 µl Bond Elut O MIX C18 pipette tip (Agilent Technologies), as according to the manufacturers instructions. Peptides were eluted in 50 µl of 75% (v/v) acetonitrile, 0.1% (v/v) acetic acid, then lyophilized and re-suspended in 30 µl of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid.

For in-gel trypsin digestion of samples after native PAGE, silver-stained protein bands were cut into 1 mm³ pieces, washed once with H₂O, and destained with a freshly prepared 1:1 mixture of 100 mM sodium thiosulfate (Na₂S₂O₃) and 30 mM potassium ferricyanide (K₃Fe(CN)₆) until colorless, followed by two washes with sterile distilled H₂O to remove excess silver. Gel pieces were then dehydrated with 50% (v/v) acetonitrile, vacuum dried as above, and then rehydrated with reducing solution (50 mM NH₄HCO₃, 25 mM DTT) for 20 min at 55°C. Gel pieces were next treated with alkylating solution (50 mM NH₄HCO₃, 55 mM iodoacetamide) in the dark for 20 min at room temperature, washed once with sterile distilled H₂O, and then dehydrated and vacuum dried as above. Proteins were digested overnight at 37°C with 1 µg of sequencing grade modified porcine trypsin (Promega) together with ProteaseMAX surfactant (66). Peptides were vacuum dried and acidified as above, desalted with self-packed C18 stage-tips using a spin protocol (67), then lyophilized and re-suspended in 30 µl of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid.

**Tandem Mass Spectrometry Instrument Parameters**

Nano-scale ultra-high performance liquid chromatographic (UHPLC) separation of tryptic peptides digested in solution was performed on a Dionex Ultimate 3000 Rapid Separation LC system (Thermo Fisher Scientific). The protein digests were loaded onto a 1 µl nano-Viper sample loop (Thermo Fisher Scientific), and separated on an analytical C18 column (Acclaim PepMap RSLC C18 column with a 2 µm particle size, 100 Å pore size, 75 µm x 15 cm (Thermo Fisher Scientific)) by the application of a 90 min linear gradient from 2% to 32% (v/v) acetonitrile in 0.1% (v/v) formic acid, with the column flow rate set to 300 nl/min. MS analysis of the eluted tryptic peptides was performed online using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) possessing a Nanospray Flex ion source (Thermo Fisher Scientific) fitted with a stainless steel nanobore emitter operated in positive electrospray ionisation (ESI) mode at a capillary voltage of 1.6 kV. Data-dependent acquisition of full MS scans within a mass range of 380-1500 m/z at a resolution of 70,000 was performed, with the automatic gain control (AGC) target set to 1 x 10⁶ ion intensity and the maximum fill time set to 100 msec. High energy collision-induced dissociation (HCD) fragmentation of the top 15 most intense peaks was performed with a normalized collision energy of 28, an intensity threshold of 1 x 10⁵ counts and an isolation window of 1.2 m/z, excluding precursors that had unassigned, +1, +7 or +8 charge states. MS/MS scans were acquired with a mass range of 200-2000 m/z at a resolution of 17,500, with an AGC target of 8 x 10³ counts and a maximum fill time of 80 msec. All peaks were recorded in profile mode. Dynamic exclusion was enabled with a repeat count of 2 and an exclusion duration of 10 sec, while the minimum MS ion count for triggering MS/MS was set to 8 x 10³ counts.

Peptide samples obtained from in-gel digestion were instead analyzed using a nanoAcquity UHPLC system (Waters Corporation) connected online to an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) operated in positive ESI mode. The protein digests were separated on a fused silica capillary C18 micro-column (Waters Corporation; 1.7 µm particle size, 130 Å pore size, 100 µm x 20 cm, with an emitter tip pulled to ~1 µm) by the application of a linear gradient from 2% to 30% (v/v) acetonitrile in 0.1% (v/v) formic acid, with the column flow rate set to 300 nl/min. Data-dependent acquisition of full MS scans within a
mass range of 300-1500 m/z at a resolution of 60,000 was performed, followed by HCD fragmentation of the top 10 most intense peaks, performed with a normalized collision energy of 42, an intensity threshold of 3 x 10^3 counts and an isolation window of 3 m/z, excluding precursors that had unassigned or +1 charge states. MS/MS scans were acquired with a mass starting at 100 m/z at a resolution of 7,500, with an AGC target of 1 x 10^5 and a maximum fill time of 500 msec. Dynamic exclusion was enabled with a repeat count of 2, an exclusion duration of 30 sec and an exclusion window of 2 min, while the minimum MS ion count for triggering MS/MS was set to 3 x 10^3 counts. Data were acquired in centroided mode.

**Tandem Mass Spectrometry Data Processing**

Raw MS/MS spectral files were processed with MaxQuant version 1.5.3.30 (68), set up to use the Andromeda search engine to interrogate the Arabidopsis proteome file TAIR10_pep_20101214_updated.fasta, obtained from the Arabidopsis Information Resource (TAIR) database version 10.1, (www.arabidopsis.org), which contained 35,518 entries (Table S4). Search parameters were set to assume trypsin digestion with a maximum of 2 missed cleavages, precursor mass tolerances of 20 ppm, and fragment mass tolerances of 10 ppm. Carbamidomethylation of cysteine was specified as a static modification, while oxidation of methionine, N-terminal acetylation, and Gly-Gly footprints (i.e., the ubiquitylation remnant on a lysine residue after cleavage with trypsin) were specified as dynamic modifications. The target FDR of 0.01 was used as validation for peptide-spectral matches (PSMs) and peptides. Ten proteins were marked as likely contaminants, including nitrilases, cruciferins, and seed storage albumins (Table S5).

LFQ analyses were performed using the MaxLFQ algorithm in MaxQuant (36) with default settings, except that only unique peptides were used for quantification so that protein isoforms could be discriminated. Matching was set to “match from and to samples” to increase the number of quantifiable proteins per run, using the “match between runs” feature in MaxQuant. The resulting proteingroups.txt files (Table S6) were processed using Perseus (www.perseus-framework.org (69)). MaxLFQ values were averaged for both technical replicates, and missing MaxLFQ quantification values were imputed with values representing a normal distribution around the detection limit of the mass spectrometer, i.e., the mean and standard deviation for the distribution of the real intensities were determined, then a new distribution with a downshift of 1.8 standard deviations and a width of 0.3 standard deviations was created. The total matrix was then imputed using these values, enabling statistical analysis. Volcano plots comparing samples were generated in Perseus, with parameters set to use a two-sided t-test, an FDR of 0.01, an S0 value of 2, and 250 permutations, and then plotted using the Seaborn Python graphing library (http://doi.org/10.5281/zenodo.824567). KDEs were also calculated in Seaborn, while the median and Kolmogorov-Smirnov (KS) tests for enrichment were performed using SciPy Statistical Functions version 1.0 (www.docs.scipy.org/scipy/references/stats). For the bar graphs in Fig. 3, missing values were imputed with a Log2(MaxLFQ value) of 15 so that a “zero value” could be more easily viewed along the Y-axis.

For analysis of native PAGE gel slices, MS/MS spectra were analysed using the Morpheus search engine (70), set up to search the same proteome database as above. Search parameters assumed digestion with trypsin, a maximum of 2 missed cleavages, a minimum peptide length of 6, precursor mass tolerances of 2.10 Da, and fragment mass tolerances of 0.01 Da. Static and dynamic modifications were specified as above, and the FDR of 0.01 was used as validation for peptide-spectral matches (PSMs) and peptides. LFQ was performed with Morpheus Spectral Counter (51) based on dNSAF values (50; Tables S7 and S8). The data were plotted in R (www.cran.r-project.org) using the heatmap.2 function, with centroided hierarchical clustering based on Pearson’s correlation used as the distance function.

**Gene Expression Analysis**

Raw transcript abundance data (excluding UMP1b, for which no data was available) were downloaded from the Transcriptome Variation Analysis database (www.travadb.org (47)) and plotted in R using the heatmap.2 function as
above. All read counts were normalized to values ranging from 0 to 1 by the “median-of-ratios” method as in DESeq2 (71), and then divided by the maximum expression for each gene. Data for gene expression in response to MG132 treatment or in the rpn10-1 and rpn12a-1 mutants were obtained from a previously published RNA-seq dataset (GSE81668 (48)), and the heat map was plotted with Perseus (69).

**Yeast Complementation Assays**

Unless otherwise noted, all yeast manipulations were performed according to standard protocols. Details of all strains used in this study are given in Table S9, with strains BY4741 or BY4742 used as the wild-type controls (72). Deletion strains (Δpba3, Δpba4, and Δump1) were obtained from the yeast knockout collection (Dharmacon) and cultured on YPDA medium containing 200 mg/ml Geneticin. All genomic deletions were confirmed by PCR genotyping, using primer pairs A + B, A + KanB, C + D, KanC + D, and A + D for each deletion collection strain, sequences of which are provided in Table S3.

To clone required coding sequences, total RNA was first extracted from Arabidopsis seedlings or yeast cells and then converted into cDNA. Arabidopsis RNA was extracted from 50 to 100 mg of 7-d-old liquid-grown seedlings as described above. To obtain total yeast RNA, the cell walls of ~2 x 10^7 freshly harvested BY4742 cells (taken from a 15 ml culture grown in YPDA) were digested with 100 U of lyticase (from Arthrobacter luteus) in 100 ml buffer Y1 (1 M sorbitol, 100 mM EDTA, 0.1% (v/v) β-mercaptoethanol (pH 7.4)) for 1 hr at 30°C, and RNA was then extracted using the RNeasy mini kit (QIAGEN). Total RNA was then converted into first strand cDNA as described above. Coding sequences amplified by PCR were first recombined into pDONR221 via the Gateway BP clonase II reaction (Thermo Fisher Scientific), with the sequence for an N-terminal FLAG or HA tag incorporated into the appropriate PCR amplification primers. Sequence-confirmed genes were then recombined into the pAG423-GPD-ccdB, pAG425-GPD-ccdB or pAG426-GPD-ccdB vectors (Addgene, product numbers 14150, 14154 and 14156, respectively) via the Gateway LR clonase II reaction (Thermo Fisher Scientific), and resulting plasmids (or the empty vectors as controls) were transformed into the indicated yeast strains using the standard lithium acetate procedure. Transformed cells were cultured on synthetic dropout medium lacking histidine and/or leucine as appropriate.

For growth assays, cells were grown in 10 ml synthetic dropout medium lacking histidine, leucine and/or uracil. Following 12-hr overnight growth, cultures were diluted to an OD_{600} of 1.0 in the same medium then subjected to a series of 5-fold dilutions, before 5 µl of each dilution was spotted onto the same medium containing or lacking 5 µM canavanine. Cells were then grown for 36 hr at 30°C or 37°C prior to imaging.

**Sequence Alignments and Phylogenetic Analyses**

The predicted full-length nucleotide and protein sequences of A. thaliana PBAC1, PBAC2, PBAC3, PBAC4, UMP1a, HSM3, NAS2, NAS6, and ECM29 (obtained from the Arabidopsis Information Resource database version 10.1 (www.arabidopsis.org)) were used as queries in BLAST searches for related loci in other plant genomes available in the Joint Genome Initiative’s Phytozone database (www.phytozone.net). Related animal and yeast sequences were identified as the top hits in each respective proteome resulting from position-specific iterative (PSI)-BLAST searches using the BLOSUM62 substitution matrix, provided that the default expectation value (E-value) was above the threshold of 0.005 (73). Domain positions were predicted by the SMART (www.smart.embl-heidelberg.de) and PFAM (www.pfam.sanger.ac.uk) databases.

Progressive alignments of the predicted full-length amino acid sequences were performed using Clustal Omega (www.clustal.org/omega) with the default settings. Following minor manual editing, the final alignments were displayed with BoxShade version 3.2.3 (www.ch.embnet.org/software/BOX_form.html).

Species abbreviations used are: Aa, Aedes aegypti; Ac, Ananas comosus; Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Bd, Brachypodium distachyon; Br, Brassica rapa; Ce, Caenorhabditis elegans; Cr, Chlamydomonas reinhardtii; Cs, Citrus sinensis; Dm, Drosophila melanogaster; Dr, Danio rerio; Ego, Eremothecium gossypii; Egr, Eucalyptus grandis; Gm, Glycine max; Hs, Homo sapiens; Kl, Kluyveromyces lactis; Mm,
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Mus musculus; Os, Oryza sativa; Pp, Physcomitrella patens; Pt, Populus trichocarpa; Sh, Sorghum bicolor; Sc, Saccharomyces cerevisiae; Sl, Solanum lycopersicum; Sm, Selaginella moellendorffii; Zm, Zea mays. Sequence identities and similarities were calculated in Clustal Omega and displayed using the Seaborn Python graphing library.

Bayesian phylogenetic analyses were performed with MrBayes version 3.2.2 (74), using the General Time Reversible evolutionary model with the mixed amino acid model and γ-distributed rate variation with a proportion of invariable sites. The software was run for 1,000,000 generations, with a sampling frequency of every 1,000 generations. The first 250,000 generations (25%) were discarded as “burn-in” after checking that the log likelihood values had plateaued and that the potential scale reduction factor was close to 1.000. The resulting consensus trees were displayed using FigTree version 1.4.2 (www.tree.bio.ed.ac.uk/software/figtree).

Statistical Analyses
Details of MS-based statistical analyses are provided above. All other datasets were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc tests to identify significantly different data points. At least three biological replicates were performed in all cases, unless otherwise indicated in the Figure Legend.

Accession Numbers
All accession numbers for genes and proteins used in this study are given in Table S10. The MS-based proteomics data have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD014610. The sample to which each .raw file corresponds is given in Table S11.
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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

AUTHOR CONTRIBUTIONS
D.C.G., K.H.L., and Q.Z. identified the different T-DNA insertion mutants; D.C.G. and K.H.L. generated the transgenic lines; R.S.M. performed the glycerol gradient fractionations, RT-PCR, native PAGE, yeast complementation and Arabidopsis growth studies; D.C.G. and R.S.M. performed the proteasome affinity purifications and assembled sequence alignments; D.C.G. performed all the MS data analyses; F. M., M.S., and L.M.S. helped generate the MS datasets; W.H. performed the gene expression analyses; D.C.G., R.S.M., and R.D.V. designed the research, analyzed the data, and wrote the paper with input from all other authors.
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**FOOTNOTES**
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This article contains Figs. S1-S10 and Tables S1-S11.

¹The abbreviations used are: BLAST, basic local alignment search tool; CP, core protease; dNSAF, distributed normalized spectral abundance factor; FDR, false discovery rate; KDE, kernel density estimation; KS, Kolmogorov-Smirnov; LC, liquid chromatography; LFQ, label-free quantification; MS, mass spectrometry; PAGE, poly-acrylamide gel electrophoresis; PBAC, proteasome biogenesis-associated chaperone; PCR, polymerase chain reaction; RP, regulatory particle; RPN, regulatory particle non-ATPase; RPT, regulatory particle AAA-ATPase; S, Svedberg unit; SAM, significant analysis of microarray; UPS, ubiquitin-proteasome system.
**FIGURE LEGENDS**

**Figure 1.** Description of *Arabidopsis* plants expressing FLAG-tagged RPT4 isoforms. *A*, Diagrams of the RPT4a and RPT4b genes. The boxes represent coding regions (colored) or predicted untranslated regions (white), while lines represent introns. Green boxes identify the AAA-ATPase domain. Positions of the T-DNA insertions are indicated by the red triangles; sites of amino acid sequence disruption are shown in Fig. S1. Positions of oligonucleotide primers used for reverse transcription (RT)-PCR in panel (B) are indicated by the half arrowheads. 

**B**, RT-PCR analysis of the RPT4a and RPT4b transcripts in the indicated T-DNA insertion mutants. Total RNA was extracted from 7-d-old wild-type or homozygous mutant seedlings, converted into cDNA, and subjected to RT-PCR using the indicated primer pairs as shown in (A). Numbers on the right represent size markers (base pairs). 

**C** and **D**, The rpt4a and rpt4b mutants described in (A) and (B), are phenotypically indistinguishable to wild type. Shown are plants grown for 10 d in a long-day photoperiod (C), or for 42 d in a short-day photoperiod (D). E, RPT4 is essential in *Arabidopsis*. The genotypes of 230 progeny from a selfed double heterozygous *rpt4a-1/+ rpt4b-2/+* plant were determined by genomic PCR, and the number and percentage of each genotype observed are indicated, together with the expected percentages if all genotypes were viable. No double homozygous plants were identified. F, *Arabidopsis* rpt4a-1 and rpt4b-2 complemented with the FLAG-RPT4a or FLAG-RPT4b transgenes are phenotypically indistinguishable to wild type or their respective homozygous mutants. Shown are plants grown for 7 d on GM medium followed by 14 d on soil. G, The anti-RPT4a antibodies equally recognize both RPT4a and RPT4b. Recombinant RPT4a and RPT4b bearing N-terminal 6His tags were diluted in 5-fold increments, separated by SDS-PAGE, and immunoblotted with anti-RPT4a antibodies, using anti-6His antibodies to confirm near equal protein loading. Numbers on the right indicate molecular mass markers. 

**H**, Immunoblot analysis of total protein extracts from 7-d-old *Arabidopsis* seedlings with anti-RPT4 and anti-FLAG antibodies confirmed expression of the FLAG-RPT4a and FLAG-RPT4b proteins. Open and closed arrowheads locate tagged and untagged RPT4a/b, respectively. Numbers on the left indicate molecular mass markers. Immuno-detection of histone H3 was used to confirm near equal protein loading.

**Figure 2.** Incorporation of FLAG-tagged RPT4 into *Arabidopsis* 26S proteasomes. Total protein extracts from 10-d-old wild type (WT), rpt4a-1, FLAG-RPT4a rpt4a-1, rpt4b-2, and FLAG-RPT4b rpt4b-2 seedlings were subjected to glycerol gradient fractionation, and samples from each fraction were analyzed by SDS-PAGE followed by immunoblot with antibodies against the indicated proteasome subunits. The expected positions of free CP, free RP, and the holo-26S proteasome are indicated by the horizontal brackets. The location of each protein within the 26S complex is indicated for the WT gels. Numbers on the left indicate molecular mass markers. The FLAG-RPT4a and FLAG-RPT4b proteins are observed in the same fractions as all other proteasome subunits, indicating that they assemble into the 26S complex.

**Figure 3.** Affinity purification of *Arabidopsis* 26S proteasomes via FLAG-RPT4. *A*, Affinity purification of 26S proteasomes from *Arabidopsis* seedlings expressing either PAG1-FLAG, FLAG-RPT4a, or FLAG-RPT4b in their corresponding pagl-1, rpt4a-1, or rpt4b-2 mutant backgrounds. Total protein extracts from 10-d-old seedlings were incubated with anti-FLAG beads in the presence or absence of 20 mM ATP, washed, and bound proteins were eluted with FLAG peptide. Preparations were separated by SDS-PAGE and then stained for protein with silver. The position of the alternate capping particle PA200 is indicated by an arrowhead, while the positions of other CP and RP subunits are indicated by the brackets. B, Proteasomes affinity purified as in (A) were analyzed by SDS-PAGE followed by immunoblot with antibodies against the indicated proteasome CP and RP subunits, and the FLAG epitope. Open and closed arrowheads locate FLAG-tagged and endogenous (i.e., non-tagged) proteasome subunits, respectively.

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Figure 4. Label-free quantification of 26S proteasomes affinity purified via the CP or RP in the presence or absence of ATP. A, LFQ-MS analysis of 26S proteasomes affinity purified via the PAG1-FLAG, FLAG-RPT4a, or FLAG-RPT4b subunits in the presence or absence of ATP. Values represent the mean (±SD) from three independent biological replicates. B, Volcano plots comparing proteasomes affinity purified via the FLAG-RPT4a or FLAG-RPT4b subunits in the presence (left) or absence (right) of ATP. The dashed lines demarcate proteins that were differentially represented in the enriched samples relative to their control, as judged by a SAM test using an FDR of 0.01. Two-dimensional KDEs are shown for CP subunits (red) and RP subunits (blue). Individual CP and RP subunits are indicated as red and blue squares, respectively. Other proteins that did not reach SAM significance are shown as grey circles. The distribution of CP and RP subunits is shown below each volcano plot. The X-axis shows the difference in CP (red) or RP (blue) protein levels between samples affinity purified via FLAG-RPT4a or FLAG-RPT4b, with the area of each density defined as 1. Solid vertical lines represent the median values of either the CP (blue) or RP (red) subunits. Cryo-electron microscopic structures of the proteasome sub-complexes enriched in each sample are shown (adapted from (32)), with the CP in red, the RP base in blue, and the RP lid in gold. C, LFQ-MS analysis of proteasome-associated proteins that were enriched via the PAG1-FLAG, FLAG-RPT4a, or FLAG-RPT4b subunits in the presence or absence of ATP. Values represent the mean (±SD) from three independent biological replicates. The color code for the bars is the same as in (A).

Figure 5. Enrichment of 26S proteasome sub-complexes upon affinity purification via the CP or RP in the absence of ATP. Proteasomes were affinity purified via the PAG1-FLAG (left), FLAG-RPT4a (middle), or FLAG-RPT4b (right) subunits in the presence (bottom) or absence (top) of ATP, as in Fig. 2, A, and subjected to tandem MS. The dashed lines demarcate proteins that were differentially represented in the enriched samples relative to their control, as judged by a significant analysis of microarray (SAM) test using an FDR of 0.01. Two-dimensional KDEs are shown for CP subunits (red) and RP subunits (blue). Individual CP and RP subunits are indicated as red and blue squares, respectively. Proteins likely involved in CP assembly are shown as green squares, while proteins likely involved in RP assembly are shown as yellow squares. Proteasome-associated proteins with unknown or possible regulatory functions are shown as grey and black squares, respectively. Other proteins that did not reach SAM significance are shown as grey circles. The distribution of CP and RP subunits is shown below each volcano plot. The X-axis shows the difference in CP (red) or RP (blue) protein levels between affinity-purified samples and their respective controls, with the area of each density defined as 1. Solid vertical lines represent the median values of either the CP (blue) or RP (red) subunits. Fold changes (FC) for CP abundance compared to the RP (for PAG1-FLAG samples), or for RP abundance compared to the CP (for FLAG-RPT4a/b samples) were calculated based on the median of each distribution. p-values for median and KS tests are shown. Cryo-electron microscopic structures of the proteasome sub-complexes likely to be enriched in each sample are shown (adapted from (32)), with the CP in red, the RP base in blue, and the RP lid in gold.

Figure 6. Arabidopsis proteasome assembly chaperones have weak sequence homology and distinct expression patterns. A, A heat map displaying the percentage amino acid sequence identity of 9 predicted Arabidopsis proteasome assembly chaperones identified in Fig. 4, compared to those for orthologs from poplar, maize, moss, yeast, and humans. The percentage sequence identity between each chaperone from the above species was calculated in Clustal Omega. B, A heat map displaying the relative transcript abundance for the predicted Arabidopsis proteasome assembly chaperones shown in panel (A), plus UMP1c, in 32 manually curated tissues. Expression data for UMP1b was not available. mRNA levels were obtained from the Transcriptome Variation Analysis database and hierarchically clustered by both tissue distribution and expression patterns. All read counts for each gene were normalized by the “median-of-ratios” method, with the maximum value set at 1. C, A heat map displaying the expression levels of transcripts encoding the predicted Arabidopsis proteasome assembly chaperones in Arabidopsis
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Figure 7. Proteasome inhibition induces the accumulation of assembly intermediates. A, Composition of affinity-purified preparations of Arabidopsis 26S proteasomes upon inhibition. Total protein extracts from 10-d-old wild-type (WT) or Pag1-FLAG pag1-1 seedlings treated with or without 50 µM MG132 were incubated with anti-FLAG beads, washed, and bound proteins were eluted with FLAG peptide. Preparations were separated by SDS-PAGE and then stained for protein with silver (left) or immunoblotted with anti-ubiquitin (Ub) antibodies (right). The position of the alternate capping particle PA200 is indicated by an arrowhead, while the positions of other CP and RP subunits are indicated by the brackets. B and C, Separation of the proteasome preparations from (A) by native PAGE for 16 (B) or 36 (C) hr. The gels were then stained for total protein with silver. Migration positions of various complexes, including free CP, CP capped with PA200, free RP, singly- and doubly-capped holoproteasomes, CP complexes containing predicted assembly chaperones, and free α-rings are indicated based on tandem MS analysis of the samples, as shown in (D). The dashed boxes represent gel sections that were excised, trypsinized, and subjected to tandem MS analysis in (D) The asterisk represents an unknown assembly intermediate. D, LFQ-MS analysis of the protein compositions of the various proteasome species affinity purified following inhibition. Shown is a heat map displaying dNSAF values for specific proteins in the gel slices shown in (B) and (C), which were calculated using Morpheus Spectral Counter and then hierarchically clustered by Pearson’s correlation. Smear represents the diffuse species detected at the bottom of the native gel shown in (B).

Figure 8. Arabidopsis PBAC3 and PBAC4, but not UMP1, can replace their yeast counterparts. A, Wild-type (WT), Δpha3, Δpha4, or Δpha3 Δpha4 yeast cells were transformed with plasmids encoding HA- or FLAG-tagged variants of yeast Pba3 or Pba4, or Arabidopsis PBAC3 and/or PBAC4 (or the empty vectors as a control) and grown to mid exponential phase in synthetic dropout medium lacking histidine and/or leucine as required. Near equal numbers of cells were spotted in 5-fold serial dilutions onto solid synthetic dropout medium lacking histidine and/or leucine as above and containing or lacking 5 µM canavanine (Can), and then grown at 30°C for 36 hr. B, WT or Δump1 yeast cells were transformed with plasmids encoding HA-tagged yeast Ump1 or the indicated combinations of the three Arabidopsis UMP1a-c isoforms (or the empty vectors as a control), grown and spotted as in (A), and then incubated at 30°C or 37°C for 36 hr. C, The Arabidopsis UMP1 isoforms are all expressed in yeast. Shown is an immunoblot of total protein extracts from cells grown as in (B) with anti-HA antibodies. The dashed line indicates where different portions of the blot were joined together. Immunodetection of histone H3 was used to confirm near equal protein loading.

Figure 9. The 26S proteasome maturation factor UMP1 is essential in Arabidopsis. A, Diagram of the Arabidopsis UMP1a, UMP1b, and UMP1c genes. The boxes represent coding regions (dark green) or predicted untranslated regions (light green), while lines represent introns. Positions of the T-DNA insertions are indicated by the red triangles; sites of amino acid sequence disruption are shown in Fig. S6. Positions of oligonucleotide primers used for reverse transcription (RT)-PCR in (C) are indicated by the half arrowheads. B, Sequence alignment of UMP1/POMP proteins from Arabidopsis, yeast, and humans. Identical (50% threshold value) and similar amino acids are shown with black and grey backgrounds, respectively. Positions of the ump1a-2, ump1b-2 and ump1c-2 T-DNA insertions are indicated by the red arrowheads; positions of other T-DNA insertions are shown in Fig. S6. Amino acid numbers are given on the right. Species abbreviations are: At, Arabidopsis thaliana; Sc, Saccharomyces cerevisiae; Hs, Homo sapiens. C, RT-PCR analysis of the UMP1a, UMP1b, and UMP1c transcripts in the indicated T-DNA mutants. Total RNA was extracted from 7-d-old wild type or homozygous mutant seedlings, converted into cDNA, and subjected to RT-PCR using the indicated primer pairs shown in (A). Numbers on the right represent size markers (base pairs). Amplification of ACT2 was included to confirm analysis of near equal amounts of cDNA. D, Homozygous double mutants affecting UMP1a and UMP1b

seedlings treated with MG132, or in the Arabidopsis rpn10-1 or rpn12a-1 mutants with impaired proteasome function. Data was obtained from a previously published RNA-seq dataset (GSE81668 (45)).
undergo seed abortion. Shown are regions of a silique from a self-fertilized wild type (WT; top) or double heterozygous ump1a-2/+ ump1b-2/+ (middle) flower along with a higher magnification of an aborted seed (bottom). E, F, and G, The UMP1a and UMP1b isoforms are essential in Arabidopsis. The indicated double heterozygous plants were self-fertilized and the genotypes of the resulting 230 or 210 progeny were determined by genomic PCR. The number and percentage of each genotype observed is shown, together with the expected percentages if all genotypes were viable. No double homozygous ump1a ump1b plants were identified. Asterisks indicate genotypes that were also significantly under-represented in the population ($\chi^2$ test, $p = 6.33 \times 10^{-6}$).

**Figure 10.** Arabidopsis ump1a and ump1b mutants are hypersensitive to proteotoxic stress. A, Plants of the indicated genotypes were grown for 10 d on solid GM medium containing either DMSO (control), 25 μM MG132, or 5 μM canavanine (Can). The nac53-1 nac78-1 double mutant was used as a positive control. B, Quantification of seedling sensitivity to various concentrations of MG132 or Can. Shown is the fresh weight of 10 seedlings of the indicated genotype grown as in (A). Bars represent the mean (±SD) from three independent biological replicates. Different letters represent values that are statistically significantly different from one another and the control, as determined by one-way ANOVA followed by Tukey’s post-hoc test. C, The ump1a-2 mutant accumulates proteasome assembly intermediates. Total protein extracts from 10-d-old wild type (WT) or ump1a-2 seedlings treated with or without 50 μM MG132 for 16 hr were subjected to glycerol gradient fractionation, and samples from each fraction were analyzed by SDS-PAGE followed by immunoblot with antibodies against the indicated proteasome subunits. Numbers on the left indicate molecular mass markers. The location of each protein within the 26S complex is indicated for the right panel. The predicted positions of free proteasome subunits (free), assembly intermediates (interm.), free CP, free RP, and the holo-26S proteasome are indicated by the horizontal brackets.

**Figure 11.** Arabidopsis ump1a and ump1b mutants are hypersensitive to salt and drought stress. A, Plants of the indicated genotypes were grown for 10 d on solid GM medium containing either methanol (control), 50 mM NaCl, 100 mM mannitol, or 0.25 μM abscisic acid (ABA). The pbe1-2 mutant was used as a positive control. B, Quantification of seedling sensitivity to various concentrations of NaCl, mannitol or ABA. Shown is the fresh weight of 10 seedlings of the indicated genotype grown as in (A). Bars represent the mean (±SD) from three independent biological replicates. Different letters represent values that are statistically significantly different from one another and the control, as determined by one-way ANOVA followed by Tukey’s post-hoc test.
Table 1. 26S Proteasome-Associated Proteins

| Gene ID    | Protein Name | Avg MS/MS Count | % Coverage | Fold Enrich | p-Value  |
|------------|--------------|-----------------|------------|-------------|----------|
| **PAG1-FLAG (–ATP)** | | | | | |
| At3g18940.1 | PBAC2        | 41.5            | 56.2       | 2734        | 1.2e-06  |
| At3g07640.1 | PBAC5        | 49.8            | 59.9       | 2462        | 5.1e-06  |
| At3g25545.1 | PBAC1        | 58.2            | 68.4       | 2319        | 2.7e-07  |
| At5g13330.1 | PA200        | 54.3            | 30.1       | 847         | 3.0e-05  |
| At5g38650.1 | UMP1b        | 11.5            | 67.4       | 279         | 1.3e-05  |
| At1g67250.1 | UMP1a        | 8.7             | 56.0       | 277         | 2.0e-06  |
| At3g53970.1 | PTRE1        | 6.3             | 29.4       | 241         | 1.0e-05  |
| At1g23520.1 | Unknown      | 8.7             | 3.0        | 165         | 1.0e-03  |
| At1g01320.2 | REC1         | 2.0             | 1.7        | 156         | 4.3e-07  |
| At1g48170.1 | PBAC4        | 19.8            | 79.5       | 138         | 5.3e-02  |
| At5g14710.1 | PBAC3        | 7.8             | 39.4       | 126         | 5.8e-02  |
| At3g13090.1 | ABCC6/MRP8   | 0.0             | 0.5        | 87          | 1.0e-01  |
| At2g47360.1 | Unknown      | 3.2             | 2.6        | 65          | 3.4e-03  |
| At2g26780.1 | ECM29        | 3.5             | 9.7        | 36          | 1.9e-02  |
| At4g17100.1 | Poly(U) endoribonuclease | 0.2 | 4.4 | 25 | 6.6e-05 |
| At4g27980.1 | AGAMOUS-like 90 | 0.2 | 6.3 | 24 | 2.7e-02 |
| At1g21530.1 | AMP-dependent synthetase | 0.2 | 3.1 | 22 | 1.2e-05 |
| At1g23780.1 | F-Box protein | 2.0 | 8.6 | 15 | 1.6e-04 |
| At3g20020.1 | PP2A B subunit | 0.8 | 2.7 | 14 | 2.5e-04 |
| At2g17200.1 | DSK2b        | 2.5             | 9.4        | 13          | 1.1e-02  |

| **FLAG-RPT4a (–ATP)** | | | | | |
| At2g26780.1 | ECM29        | 153.7           | 55.3       | 499         | 2.7e-03  |
| At2g03430.1 | NAS6         | 22.8            | 68.5       | 155         | 1.7e-03  |
| At3g13090.1 | ABCC6/MRP8   | 0.0             | 1.3        | 136         | 4.8e-06  |
| At4g15180.1 | HSM3         | 54.5            | 51.8       | 110         | 4.3e-06  |
| At4g27980.1 | AGAMOUS-like 90 | 1.0 | 7.5 | 94 | 6.4e-03 |
| At5g57950.1 | NAS2         | 2.8             | 32.9       | 82          | 5.9e-06  |
| At5g53970.1 | PTRE1        | 1.8             | 11.1       | 23          | 9.0e-05  |
| At2g28070.1 | ABCG3        | 0.0             | 0.7        | 19          | 1.0e-04  |
| At5g49650.1 | BLOC1 subunit 2 | 0.7 | 10.5 | 17 | 5.1e-04 |
| At2g10410.1 | F-Box protein | 0.5 | 2.6 | 15 | 7.8e-05 |
| At5g13330.1 | PA200        | 0.0             | 0.7        | 14          | 2.9e-03  |
| At4g13450.1 | Adenine α-hydrolase | 0.7 | 7.5 | 9 | 1.4e-03 |

| **FLAG-RPT4b (–ATP)** | | | | | |
| At2g26780.1 | ECM29        | 126.3           | 52.1       | 325         | 3.5e-03  |
| At2g03430.1 | NAS6         | 28.7            | 68.5       | 177         | 1.6e-03  |
| At5g13090.1 | ABCC6/MRP8   | 0.0             | 1.3        | 105         | 3.3e-06  |
| At4g15180.1 | HSM3         | 51.8            | 52.0       | 82          | 5.4e-06  |
| At4g27980.1 | AGAMOUS-like 90 | 0.5 | 7.5 | 54 | 1.0e-02 |
| At5g57950.1 | NAS2         | 1.7             | 20.2       | 34          | 6.9e-05  |
Figure 1. Gemperline, Marshall et al.

A

B

C

D

E

F

G

H

Genotypes of progeny from selfed rpt4a-1/+ rpt4b-2/+ | Genotype | Number | % | Expected \%
--- | --- | --- | ---
AABB | 30 | 14.3 | 6.25
AaBB | 46 | 21.9 | 12.5
AABB | 36 | 17.1 | 12.5
aabB | 40 | 19.0 | 25
aabB | 28 | 13.3 | 6.25
AAbb | 30 | 14.3 | 6.25
aabB | 0 | 0.0 | 12.5
aabB | 0 | 0.0 | 12.5
aabB | 0 | 0.0 | 6.25

*Total individuals genotyped = 230
*Expected genotypes if all combinations were viable
Figure 2. Gemperline, Marshall et al.
Figure 3. Gemperline, Marshall et al.
Figure 4. Gemperline, Marshall et al.

A

![Bar charts showing Log2(LFQ) for CP α-Ring, CP β-Ring, RPT subunits, and RPN subunits.](image)

B

![Distribution plots for RPT4a vs RPT4b (+ ATP) and RPT4a vs RPT4b (- ATP).](image)

C

![Bar charts for associated proteins with and without ATP.](image)
Figure 5. Gemperline, Marshall et al.

**Legend:**
- CP Density
- RP Density
- SAM Signif.
- Unknown PAP
- CP-Chap
- RP-Chap
- Known PAP
- Contaminant?

**Graphs:**
1. **PAG1-FLAG (~ ATP)**
2. **FLAG-RPT4a (~ ATP)**
3. **FLAG-RPT4b (~ ATP)**
4. **PAG1-FLAG (~ ATP)**
5. **FLAG-RPT4a (~ ATP)**
6. **FLAG-RPT4b (~ ATP)**

**Statistics:**
- FC values:
  - PAG1-FLAG (~ ATP): FC = 23.61
  - FLAG-RPT4a (~ ATP): FC = 3.43
  - FLAG-RPT4b (~ ATP): FC = 3.41
  - PAG1-FLAG (~ ATP): FC = 1.56
  - FLAG-RPT4a (~ ATP): FC = 2.06
  - FLAG-RPT4b (~ ATP): FC = 1.89

- KS p-values:
  - PAG1-FLAG (~ ATP): KS p = 1.74E-10
  - FLAG-RPT4a (~ ATP): KS p = 3.03E-5
  - FLAG-RPT4b (~ ATP): KS p = 5.55E-5
  - PAG1-FLAG (~ ATP): KS p = 0.0491
  - FLAG-RPT4a (~ ATP): KS p = 0.017
  - FLAG-RPT4b (~ ATP): KS p = 0.068

- Median p-values:
  - PAG1-FLAG (~ ATP): Median p = 4.30E-10
  - FLAG-RPT4a (~ ATP): Median p = 7.11E-5
  - FLAG-RPT4b (~ ATP): Median p = 7.11E-5
  - PAG1-FLAG (~ ATP): Median p = 0.256
  - FLAG-RPT4a (~ ATP): Median p = 0.265
  - FLAG-RPT4b (~ ATP): Median p = 0.023
Figure 6. Gemperline, Marshall et al.

A

|      | PBAC1 | PBAC2 | PBAC3 | PBAC4 | NAS2 | NAS6 | HSM3 | ECM29 |
|------|-------|-------|-------|-------|------|------|------|-------|
| Poplar | 64    | 53    | 66    | 64    | 63   | 58   | 67   | 66    |
| Maize  | 53    | 41    | 60    | 53    | 52   | 40   | 60   | 55    |
| Moss   | 39    | 31    | 56    | 47    | 51   | 39   | 51   | 41    |
| Yeast  | 15    | 12    | 28    | 15    | 18   | 11   | 13   | 16    |
| Human  | 22    | 14    | 34    | 23    | 24   | 23   | 25   | 13    |

B

Germinating seeds (1 DAS)
Shoot meristem (7 DAG)
Shoot meristem (11 DAG)
Seedling meristem
Dry seeds
Seeds of yellow silique
Senescent silique (stage 2)
Root apex
Seedling root
Seeds (stage 3)
Inflorescence meristem (13 DAG)
Young anthers
Inflorescence axis
Ovules (6th & 7th flowers)
Cotyledons
Pedicel
Young sepals
Young seeds (stage 1)
Flower (stage 1)
Internode
Stamen filament
Stigma
Carpels before pollination
Silique (stage 2)
Senescent internode
Mature petals
Mature sepals
Hypocotyl
Mature leaf
Senescent silique pod (stage 1)
Mature anthers (before opening)
Opened anthers

C

WT + MG132
0 hrs
3 hrs
24 hrs
rpn10-1
rpn12a-1

Log Fold Expression

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Figure 7. Gemperline, Marshall et al.
Figure 8. Gemperline, Marshall et al.
Figure 9. Gemperline, Marshall et al.

- **Expected genotypes if all combinations were viable**
- **Total individuals genotyped = 230**

### Genotypes of progeny from selfed *ump1a-2/+ ump1c-2/+*  

| Genotype      | Number | %     | Expected | %     |
|---------------|--------|-------|----------|-------|
| AABB          | 24     | 10.4  | 6.25     |       |
| AAbb          | 37     | 16.1  | 12.5     |       |
| AAbb          | 38     | 16.5  | 12.5     |       |
| AAbb          | 78     | 33.9  | 25       |       |
| AbBB          | 24     | 10.4  | 6.25     |       |
| ABB           | 22     | 9.6   | 6.25     |       |
| ABB           | 4      | 1.7   | 12.5*    |       |
| aAbb          | 3      | 1.3   | 12.5*    |       |
| aabb          | 0      | 0.0   | 6.25     |       |

- **Total individuals genotyped = 230**
- **Expected genotypes if all combinations were viable**

### Genotypes of progeny from selfed *ump1b-2/+ ump1c-2/+*  

| Genotype      | Number | %     | Expected | %     |
|---------------|--------|-------|----------|-------|
| AABB          | 26     | 11.4  | 12.5     |       |
| AABB          | 53     | 22.5  | 25       |       |
| aAbb          | 12     | 5.7   | 6.25     |       |
| aAcc          | 15     | 7.1   | 6.25     |       |
| Acc           | 28     | 13.3  | 12.5     |       |
| aCC           | 26     | 12.4  | 12.5     |       |
| acc           | 12     | 5.7   | 6.25     |       |

- **Total individuals genotyped = 230**
- **Expected genotypes if all combinations were viable**

### Genotypes of progeny from selfed *ump1b-1 ump1b-2/+*  

| Genotype      | Number | %     | Expected | %     |
|---------------|--------|-------|----------|-------|
| AABB          | 12     | 5.7   | 6.25     |       |
| BBCC          | 27     | 12.9  | 12.5     |       |
| BBCC          | 26     | 12.4  | 12.5     |       |
| BbCc          | 55     | 26.2  | 25       |       |
| Bbcc          | 14     | 6.7   | 6.25     |       |
| BBCC          | 14     | 6.7   | 6.25     |       |
| Bbcc          | 24     | 11.4  | 12.5     |       |
| bbcc          | 25     | 11.9  | 12.5     |       |
| bbcc          | 13     | 6.2   | 6.25     |       |

- **Total individuals genotyped = 210**
- **Expected genotypes if all combinations were viable**
Figure 10. Gemperline, Marshall et al.
Figure 11. Gemperline, Marshall et al.

A

Control
WT
ump1a-1
ump1a-2
pbe1-2
ump1b-2
ump1c-1
ump1c-2
ump1c-5

50 mM NaCl
WT
ump1a-1
ump1a-2
pbe1-2
ump1b-2
ump1c-1
ump1c-2
ump1c-5

100 mM mannitol
WT
ump1a-1
ump1a-2
pbe1-2
ump1b-2
ump1c-1
ump1c-2
ump1c-5

0.25 µM ABA
WT
ump1a-1
ump1a-2
pbe1-2
ump1b-2
ump1c-1
ump1c-2
ump1c-5

B

Fresh weight (mg/10 seedlings)

WT
ump1a-1
ump1a-2
ump1b-2
ump1c-1
pbe1-2

Control
NaCl
mann
ABA

[µM]

[µM]
Proteomic analysis of affinity-purified 26S proteasomes identifies a suite of assembly chaperones in Arabidopsis

David C. Gemperline, Richard S. Marshall, Kwang-Hee Lee, Qingzhen Zhao, Weiming Hu, Fionn McLoughlin, Mark Scalf, Lloyd M. Smith and Richard D. Vierstra

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