The current literature offers contradictory results regarding the role of the proteasome subunit RPN1a in Arabidopsis development. Here we show that plants lacking RPN1a are viable and have increased cell sizes, decreased heat shock tolerance, increased oxidative stress tolerance and other phenotypes characteristic for 26S proteasome subunit mutants. These results strengthen our contention that most of the phenotypes of 26S proteasome mutants in Arabidopsis described to date reflect a general impairment in 26S proteasome function rather than a specific defect of a single subunit, and suggest that the role of the RPN1a subunit during embryogenesis needs to be reconsidered.

**Keywords:** Arabidopsis thaliana • Cell size • Proteasome • Regulatory particle non-ATPase • Stress resistance.

**Abbreviations:** 20SP, 20S proteasome; 26SP, 26S proteasome; RP, regulatory particle; RPN, RP non-ATPase subunit; RPT, RP AAA ATPase subunit; RT–PCR, reverse transcription–PCR.

Protein degradation by the 26S proteasome (26SP) is an essential step of many—if not all—fundamental processes in eukaryotes (Smalle and Vierstra 2004, Kurepa and Smalle 2008a). The 26SP is a multisubunit, multicatalytic protease built out of a proteolytic 20S proteasome (20SP) core and two regulatory particles (RPs) which are composed of the base and lid subcomplexes. The RP base contains six different ATPase subunits that dock directly onto the 20SP and two non-ATPase subunits, RPN1 and RPN2, which form a functional unit that physically links the site of protein recruitment with the site of proteolysis (Rosenzweig et al. 2008).

In Arabidopsis, the RPN1 subunit is encoded by the more highly expressed RPN1a gene (At2g20580) and the lower but ubiquitously expressed RPN1b gene (At4g28470) (Yang et al. 2004). The role of the RPN1a isoform in plant development has been reported, but these studies yielded contradictory results (Brukhin et al. 2005, Huang et al. 2006). In one study, RPN1a has been shown to be essential for embryogenesis (Brukhin et al. 2005), while in another, a homozygous rpn1a allele has been obtained and used to determine that 26SP-dependent proteolysis has an important role in specifying leaf adaxial identity (Huang et al. 2006). In addition, it has been described that the removal of both the RPN1a and RPN1b isoforms does not affect gametogenesis (Brukhin et al. 2005), while on the other hand two independent studies have recently shown that the 26SP is essential for gametophyte development (Book et al. 2009, Gallois et al. 2009). These irreconcilable findings prompted us to re-evaluate the role of RPN1a in Arabidopsis development.

In contrast to the previous report (Brukhin et al. 2005), we were able to isolate the homozygous rpn1a-3 line that carries a T-DNA insertion 300 bp upstream of the RPN1a coding region (Fig. 1A and Supplementary Fig. S1). We also isolated homozygous rpn1a-4 and rpn1a-5 lines, which harbor a T-DNA insertion in the fifth intron and the 15th exon, respectively (Fig. 1A and Supplementary Fig. S1). While homozygous rpn1a-3 seedlings did not differ from the wild type, rpn1a-4 and rpn1a-5 seedlings had visibly increased anthocyanin content and a reduced rosette size (Fig. 1B, C). Siliques of the rpn1a-4 and rpn1a-5 plants contained areas with shrunk ovules, indicating reduced fertility, and siliques of the rpn1a-3 line contained white seeds (Fig. 1D). Since white seeds were observed in both the heterozygous (Brukhin et al. 2005) and homozygous version of the rpn1a-3
line (Fig. 1D), we concluded that this phenotype is not associated with RPN1a function. On the other hand, anthocyanin accumulation, reduced growth rate and reduced fertility are phenotypes that have been described for other RP mutants (Smalle et al. 2002, Kurepa et al. 2008, Book et al. 2009, Gallois et al. 2009). Therefore, the gross phenotypic analyses of the rpn1a alleles suggested that rpn1a-4 and rpn1a-5 carry a defect in 26SP function.

Indeed, RNA gel blot analyses revealed that while in rpn1a-3 the RPN1a mRNA level was similar to that of the wild type, in rpn1a-4 and rpn1a-5 the RPN1a mRNA levels were altered (Supplementary Fig. S2A). The RPN1a
transcript was absent in rpn1a-4, suggesting that this is a null mutant. In rpn1a-5 plants, we detected a less abundant transcript using an antisense probe complementary to the 5′ region of the gene (Supplementary Fig. S2A). Based on the position of the T-DNA insertion in rpn1a-5, it is likely that this is a chimeric transcript that contains the RPN1a sequence upstream of the T-DNA insertion site fused to sequence from the T-DNA. Reverse transcription–PCR (RT–PCR) analyses with primers that span the T-DNA insertion site confirmed that rpn1a-5 does not produce a wild-type RPN1a mRNA (Supplementary Fig. S2C). Immunoblotting analyses confirmed that in rpn1a-4 and rpn1a-5, the RPN1a protein is missing (Fig. 1E). The RPN1a and RPN1b proteins share 90% identity at the amino acid level, and their predicted molecular masses are 98 and 85.5 kDa, respectively. Therefore, we suggest that a weaker cross-reacting band that has a lower molecular mass compared with RPN1a and which was clearly detected only in the total extracts of the rpn1a-4 and rpn1a-5 mutants represents the RPN1b isoform. The positions of the rpn1a-4 and rpn1a-5 insertion mutations combined with the fact that no truncated RPN1 versions were visible on the immunoblots suggested that both mutants represent null alleles of the RPN1a gene. Previous studies of different RP mutants have shown that the disruption of one RP subunit gene leads to the compensatory up-regulation of other 26SP subunit genes and to the concomitant increase in 26SP subunit abundance (Yang et al. 2004, Kurepa et al. 2008, Kurepa et al. 2009). In rpn1a-4 and rpn1a-5, we detected an increase in all 26SP subunits that were tested (Fig. 1E). Reductions in 26SP-dependent proteolysis often, but not always, lead to the accumulation of polyubiquitinated proteins in the cell (Kurepa and Smalle 2008a, Book et al. 2009). The current data suggest that the degree of accumulation of polyubiquitinated proteins reflects the strength of the defect in 26SP function (Smalle et al. 2002, Kurepa et al. 2008). Total levels of polyubiquitinated proteins in all rpn1a lines were similar to that of the wild type (Fig. 1E), suggesting that the proteasome defect in rpn1a-4 and rpn1a-5 lines is weak compared with, for example, the 26SP mutants rpn10-1 and rpn5a-1 that display a significant increase in ubiquitin conjugates (Smalle et al. 2003, Book et al. 2009, Kurepa et al. 2008). Another characteristic of RP mutants is their increased tolerance to the proteasome inhibitor MG132 (Kurepa et al. 2008, Kurepa et al. 2009). The dose–response assays show that rpn1a-4 and rpn1a-5, but not the rpn1a-3 line, have increased tolerance to MG132 (Fig. 1F, G). These results show that rpn1a-3 does not affect 26SP function as reported previously (Brukhin et al. 2005), and that the rpn1a-4 and rpn1a-5 mutations lead to a partial 26SP defect induced by the significant reduction in RPN1 subunit level.

Arabidopsis 26SP mutants defective in RP function are also characterized by altered stress responses (Kurepa et al. 2008). Misfolded proteins are recognized and labeled by the ubiquitination machinery, and are thus targets for 26SP-dependent proteolysis. Since RP mutants accumulate less 26SP, they have a decreased resistance to stresses (e.g. heat shock, drought and salt stress) and compounds (e.g. amino acid analogs) that lead to protein misfolding. In contrast, RP mutants are more tolerant to oxidative stress because they accumulate the free 20SP that is responsible for the degradation of oxidized proteins (Shringarpure et al. 2001, Shringarpure et al. 2003, Kurepa and Smalle 2008b). Germination assays for basal seed thermostolerance and halotolerance, and root growth assays testing sensitivity to the amino acid analog i-canavanine and the oxidative stress inducer methyl viologen showed that—as expected—the rpn1a-4 and rpn1a-5 mutant plants are more sensitive to protein misfolding stresses and more tolerant to oxidative stress compared with the wild type and the rpn1a-3 allele (Fig. 2). Furthermore, the salt hypersensitivity of the rpn1a mutants also confirms a recent discovery on the importance of optimal 26SP function for maintaining plant drought stress tolerance (Cho et al. 2008, Yee and Goring 2009).

Another phenotype associated with RP mutants is an increased cell size in aerial organs (Kurepa et al. 2009). To test whether this cellular phenotype is induced by the rpn1a-4 and rpn1a-5 mutations, we analyzed trichome branch numbers and parenchymatic cells of mature cotyledons (Fig. 3). As predicted, parenchymatic cells of the rpn1a-4 and rpn1a-5 lines were larger than cells of the wild type and the rpn1a-3 line (Fig. 3A). In addition, trichomes of these two mutant lines also have an increased number of branches (Fig. 3B).

The 26SP is essential for a myriad of fundamental processes in all eukaryotes. Thus, it can be expected that mutations causing a severe loss of 26SP function result in embryonic lethality. However, our data suggest that we should reconsider the role of the RPN1 subunit in embryogenesis. Brukhin et al. (2005) described the embryonic lethality of the rpn1a-1, rpn1a-2 and rpn1a-3 lines. Huang et al. (2006) could not confirm the phenotype of the rpn1a-2 mutant line (SALK_129604), and we were unable to confirm the embryonic lethality in rpn1a-3 plants. In addition, we isolated two new rpn1a T-DNA mutants that are viable and have all the phenotypes that have been described for other weak 26SP mutants (Kurepa et al. 2008, Kurepa et al. 2009). Therefore, we propose that RPN1a—comparably with other RP subunits—is required for all developmental processes regulated by the 26SP, and it does not have a specific function in embryo development.

Materials and Methods

The rpn1a-3, rpn1a-4, and rpn1a-5 alleles in the Col-0 background were obtained from the Arabidopsis Biological
Resource Center (SALK lines _030503, _027970 and _127430C, respectively). Growth conditions and stress treatments were as previously described (Kurepa et al. 2008). Protein extraction and immunoblotting analyses were done as previously described (Smalle et al. 2003, Kurepa et al. 2008). All sera except anti-RPN1a were purchased from Biomol (Plymouth Meeting, PA, USA). The recombinant RPN1a was produced in Escherichia coli as described (Yang et al. 2004), and was used to produce rabbit antisera (Cocalico Biologicals, Inc., Reamstown, PA, USA).

For microscopic analyses of cotyledon palisade cells, cotyledons were immersed in Fluoro-Gel (Electron Microscopy Col-0 rpn1a-4 rpn1a-3 rpn1a-5

Fig. 2 Stress tolerance of rpn1a mutant lines. (A) Thermotolerance of mutant lines. Vernalized seeds were exposed to either 22°C (control) or 40°C (heat shock) for 3 h, and then grown in the dark at 22°C for 3 d. Hypocotyl lengths were measured from the photographs, and the data are presented as percentage inhibition calculated from the means (n ≥ 11). ANOVA with Bonferroni post-tests (P < 0.05) was used to determine the statistical significance of the heat shock effect (**P < 0.001). (B) Oxidative stress tolerance and sensitivity to the amino acid analogs was tested by transferring 3-day-old seedlings (n ≥ 20) to plates containing the denoted concentrations of L-canavanine (CAN) or methyl viologen (MV). Plates were positioned vertically, and the root length was measured at the moment of transfer (l₀) and then after 3 d of treatment (l₁). Data represent the means ± SEM of l₁/l₀. ***P < 0.001. (C) Salt stress sensitivity of rpn1a lines was tested by germinating and growing plants on MS/2 media containing 100 mM NaCl. Plants were grown for 8 d before they were photographed.

Fig. 3 Cellular phenotypes of rpn1a mutants. (A) Palisade cells in fully expanded cotyledons are enlarged in rpn1a-4 and rpn1a-5. Scale bar: 200 µm. (B) Trichome branch numbers are increased in rpn1a-4 and rpn1a-5 mutants. The graph illustrates the frequency of classes of trichomes with the indicated number of branches. Data are presented as the mean ± SD of two independent experiments, and the total number of mature leaf trichomes counted was 243, 149, 293 and 189 for Col-0, rpn1a-3, rpn1a-4 and rpn1a-5, respectively.
Supplementary data

Supplementary data are available at PCP online.

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References


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