Arabidopsis thaliana 26S Proteasome Subunits RPT2a and RPT5a Are Crucial for Zinc Deficiency-Tolerance

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RPTs (regulatory particle triple-A-ATPase) are components of 26S proteasome. We found novel roles of RPT2a and RPT5a in Zn deficiency-tolerance. Arabidopsis thaliana mutants carrying T-DNA in RPT2a and RPT5a were more sensitive to Zn deficiency than the wild-type. In the rpt mutants, the shoot Zn contents were similar to those of the wild-type. Transcripts of Zn deficiency-inducible genes were highly accumulated in the rpt mutants, suggesting that the rpt mutants suffer from various Zn deficiency symptoms, although the Zn levels are not reduced. Lipid peroxidation levels, known to be increased under Zn deficiency, were higher in the rpt mutants than in the wild-type. Poly-ubiquitinated proteins were accumulated upon exposure to Zn deficiency, especially in the rpt mutants. Overall, this study indicates that RPT2a and RPT5a are involved in Zn deficiency-tolerance, possibly through alleviation of oxidative stresses and/or processing of poly-ubiquitinated proteins.

Key words: Arabidopsis thaliana; 26S proteasome; Zn deficiency; oxidative stress

The 26S proteasome (26SP) is an ATP-dependent protease complex catalyzing protein degradation in nucleus and cytoplasm. Proteins targeted by this proteolytic device are mostly modified by ubiquitin (Ub). Non-ubiquitinated proteins can also be targets.1–3) 26SP is composed of two functionally different sub-complexes, 20S proteasome (20SP) and 19S regulatory particle (RP). 20SP contains three types of proteolytic subunits mediating ATP- and Ub-independent peptidase activities. On the other hand, RP has roles in the recognition, promotion of unfolding, and translocation of target proteins.4)

There are several reports on the distinct functions of individual RP subunits in A. thaliana. RPN12a and RPN10 are involved in the responses to cytokinin and abscisic acid respectively.4,5) RPT2a is required for the maintenance of root apical meristems.6) In A. thaliana, most of the RP subunits were encoded by two genes, suggesting that various combinations of 26SP subunits are present in A. thaliana, this might contribute to functional diversity.7) It has also been found that the two paralogous genes of the A. thaliana RP subunits have similar, unique roles. RPT5a, RPT5b play a similar role in gametophyte development.7) Mutation in RPT2a causes increased ploidy levels and an enlargement of the leaf organ size, whereas these phenotypes were not observed in the RPT2b mutant.8,9) These functional differences are believed to arise from the specific association of individual RP subunits and its paralog with poly-ubiquitinated proteins.

Protein ubiquitination is mediated by the sequential action of the Ub-activating enzyme E1, the Ub-conjugating enzyme E2, and the Ub ligase E3. The E3 ligase largely determines substrate specificity. E3 ligases are classified into several classes, and are encoded by more than 1,300 genes in the A. thaliana genome.5) This implies that Ub-dependent proteolysis is involved in various cellular processes and that the nutritional response might be among them. In animal cells, glucose starvation decreased Ub-dependent 26SP proteolysis for

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Abbreviations: 26SP, 26S proteasome; 20SP, 20S proteasome; Ub, ubiquitin; RP, 19S regulatory particle; RPN, regulatory particle non-ATPase; RPT, regulatory particle triple-A-ATPase; TBA-rs, 2-thiobarbituric acid reactive substances; CT-L, chymotrypsin-like; SOD, super oxide dismutase; ROS, reactive oxygen species
several substrates through glycosylation of proteasome subunits, particularly RPT2, but, to our knowledge there is no information on the involvement of RP subunits in the nutritional response in plants.

Oxidized proteins are substrates of the Ub-independent proteolysis of 20SP. Oxidation of 20SP elevates the proteolytic activity of 20SP. It is also known that under carbon starvation, the activity of 20SP is elevated together with an accumulation of oxidatively damaged proteins. Zinc (Zn) deficiency induces oxidative stress as in other forms of nutrient starvations, and under these conditions, it is likely that protein oxidation is accelerated. Recently, Kurepa et al. reported that mutations in RPT2a, RPT5a, and RPN12a caused repression of Ub-dependent proteolytic activity but increases in Ub-independent 20SP activity. This suggests the importance of RP subunits in maintaining 20SP activity.

In the present study, we found that RPT2a and RPT5a, subunits of RP, are involved in Zn deficiency-tolerance in A. thaliana. With reference to characterization of the RPT2a and RPT5a mutants, the possible roles of RPT2a and RPT5a in Zn deficiency-tolerance through Ub-independent and Ub-dependent proteolysis are discussed.

Materials and Methods

Plant materials and growth conditions. All the A. thaliana (ecotype Col-0) mutants used in this study were obtained from the ABRC and carried T-DNA in the corresponding genes. The mutants used were rpt2a-2 (SALK_005596), rpt2b-1 (SALK_0043540), rpt5a-4 (SALK_046321),17) and rpt5b-3 (SAIL_293_H08).18) Lines carrying T-DNA in the homozygote were established, and the presence of T-DNA was determined by PCR using the following primer sets: T-DNA specific primers LBb1 5'-GGATGATCCTGATTCGAAAAC-3' and RBb2 5'-TCGGAAAGGGTGCCATGAG-3'. (F) and 5'-GCGTGGACCGCTTGCTGCAACT-3' (R) for ZIP4; 5'-CATCATTCCCGTACGG-3' (F) and 5'-GATATGAGCCTGGATCGAG-3' (R) and 5'-GCCCAACACAGAAGATACAC-3' (R) for CSD1; 5'-TCCGAAAGGGTGCCATGAG-3' (F) and 5'-GCTTAGCCTTGATTAGGGC-3' (R) for CSD2; 5'-GCAACAGCCATGGGAGAC-3' (F) and 5'-GTCTTGATACACCCGACG-3' (R) for CCS. Actin8 was used for normalization, and the primer sequences were the same as the primers used for semi-quantitative RT-PCR, as explained above.

Results

Sensitivity of 26S proteasome mutants rpt2a-2 and rpt5a-4 to Zn deficiency

For T-DNA insertion lines, rpt2a-2 and rpt5a-4, we established homozygotes for the T-DNA insertion and used them in subsequent experiments (Fig. 1A). In the shoots of these mutants, the transcripts of corresponding genes were below the detection limit on semi-quantitative RT-PCR (Fig. 1B). rpt2a-2 and rpt5a-4 showed shoot growth similar to Col-0 under the control condition (1 μM Zn), whereas under the Zn-deficient condition (without Zn supplement), the shoot growth of these mutants was severely inhibited, at approximately 50% of that of Col-0 (Fig. 1C and D). The leaves turned purple in both rpt2a-2 and rpt5a-4 only under the Zn-deficient condition, whereas the leaves of Col-0 stayed green under that condition (Fig. 1C).

We also examined the possible roles of paralogs of RPT2a and RPT5a in the Zn response. RPT2b and
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RPT5b are paralogous genes having more than 90% similarity at the amino acid sequence level to RPT2a and RPT5a (data not shown). T-DNA inserted mutants of RPT2b and RPT5b, rpt2b-1 and rpt5b-3, were established. In the shoots of these mutants, the transcripts were not detected (Supplemental Fig. 1A and B; see Biosci. Biotechnol. Biochem. Web site). In contrast to rpt2a-2 and rpt5a-4, there was no significant difference in shoot growth between the Col-0 and T-DNA inserted mutants, rpt2b-1 and rpt5b-3 under the control and the Zn-deficient conditions (Fig. 1C and D). No obvious difference was observed in leaf color either. These results suggest that both RPT2a and RPT5a are required for Zn deficiency-tolerance while their paralogs are not.

Responses of RPT2a and RPT5a genes to Zn deficiency

To determine whether expression of RPT2a and RPT5a genes responds to Zn deficiency, the transcript accumulation of these genes was analyzed by semi-quantitative RT-PCR. It revealed that the accumulation of the RPT2a and RPT5a transcripts was higher under the Zn-deficient condition than the control condition (Fig. 1B). On the other hand, the transcript accumulation of RPT2b and RPT5b was similar among the distinct Zn conditions (Fig. 1B). RPT2a expression in rpt5a-4 and RPT5a expression in rpt2a-2 were drastically induced under the Zn-deficient condition as compared to Col-0, but not RPT2b and RPT5b (Fig. 1B). This suggests that expression of RPT2a and RPT5a genes is induced by Zn deficiency, and that the transcript accumulation of RPT2a and that of RPT5a influence each other.

Measurement of the metal concentrations in rpt2a-2 and rpt5a-4 under the Zn-deficient condition

To determine whether the Zn deficiency-sensitive phenotypes of rpt2a-2 and rpt5a-4 are associated with reduced accumulation of Zn, the contents of several metals in the shoots of Col-0 and the rpt mutants were determined (Fig. 2). When the plants were grown under the control condition, there was no significant difference in Zn contents between Col-0 and the rpt mutants (Fig. 2A). The shoot Zn contents were also similar among Col-0 and the rpt mutants under the Zn-deficient condition (Fig. 2A). No significant difference in Fe contents between Col-0 and the rpt mutants was detected irrespective of Zn conditions (Fig. 2B). The Cu contents in the rpt mutants were approximately 75% of that in Col-0 under the control condition, but were not different from Col-0 under the Zn-deficient condition (Fig. 2C). On the other hand, the Mn contents under the Zn-deficient condition were lower in the rpt mutants than in Col-0 (Fig. 2D). It was also found that Zn deficiency enhanced Mn accumulation in Col-0 and rpt2a-2, but not in rpt5a-4 (Fig. 2D). The Mn contents in the rpt mutants were approximately 60% of that in Col-0 under the control condition (Fig. 2D). Taken together, these results suggest that RPT2a and RPT5a are involved in the regulation of Mn accumulation but not in Zn accumulation in shoots in response to Zn deficiency.
Expression patterns of Zn deficiency-responsive genes in rpt2a-2 and rpt5a-4 under the Zn-deficient condition

To determine whether the transcriptional response to Zn deficiency in Zn deficiency-sensitive rpt mutants is altered, next we analyzed the expression patterns of Zn transporters and super oxide dismutase (SOD)-related genes in rpt2a-2 and rpt5a-4 under the Zn-deficient condition. The accumulation of transcripts of ZIP4 and ZIP9, Zn transporter genes, is elevated in response to Zn deficiency. The accumulation of the ZIP4 and ZIP9 transcripts was higher in the rpt mutants than in Col-0 under the Zn-deficient condition. The differences in ZIP4 and ZIP9 transcript accumulation between Col-0 and the rpt mutants were less evident under the control condition (Fig. 3A). Expression of CSD1, CSD2, and CCS genes is known to be reduced in response to Zn deficiency. These genes encode proteins for SOD synthesis and assembly. Expression of the CCS, CSD1, and CSD2 genes was lower under the Zn-deficient condition than under the control condition in both Col-0 and the rpt mutants (Fig. 3B). There was no clear difference in the expression of CCS, CSD1, and CSD2 between Col-0 and the rpt mutants. These studies indicate that part of the expression of the Zn-responsive genes was affected in the rpt mutants.

Evaluation of oxidative stress in rpt2a-2 and rpt5a-4 under the Zn-deficient condition

It has been suggested that oxidative damage to critical cell components caused by reactive oxygen species (ROS) is the basis of plant growth inhibition under Zn-deficient conditions. We speculated that the Zn deficiency-sensitive rpt mutants suffer strongly from oxidative stress under such conditions. To investigate this possibility, we determined the lipid peroxidation levels under the Zn-deficient condition (Fig. 4A). Expression of CSD1, CSD2, and CCS genes was lower under the Zn-deficient condition than under the control condition in both Col-0 and the rpt mutants. This suggests that the shoot growth phenotype of the rpt mutants under the Zn-deficient condition is not caused by increases in oxidized protein accumulation.

Fig. 2. Metal Concentrations in rpt2a-2 and rpt5a-4 under the Zn-Deficient Condition.

Zn (A), Fe (B), Cu (C), and Mn (D) concentrations in shoots of Col-0, rpt2a-2 and rpt5a-4 grown on media with and without Zn were determined by ICP-MS. Values are shown as mean ± standard error, n ≥ 5. Means sharing the same letter within a column for each species are not significantly different at 5% level of probability by Tukey’s multiple-range test.
Fig. 4. Oxidative Stress Levels in rpt2a-2 and rpt5a-4 under the Zn-Deficient Condition.

Oxidative stress levels in shoots of 14-d-old seedlings of Col-0, rpt2a-2, and rpt5a-4 grown on media with and without Zn. Quantitative real-time RT-PCR was performed. Transcriptional responses of Zn transporter, ZIP4, and ZIP9 (A), Cu/Zn-SOD encoding genes (CSD1 and CSD2), and copper chaperon (CCS) (B), in shoots of Col-0, rpt2a-2, and rpt5a-4. Gene expression was normalized with Actin8 expression. Values are shown as mean ± standard error, n = 3. Means sharing the same letter within a column for each species are not significantly different at 5% level of probability by Tukey’s multiple-range test.

Discussion

We found that 26SP subunits RPT2a and RPT5a are involved in Zn deficiency-tolerance in A. thaliana (Fig. 1B and C). To our knowledge, this is the first report on the function of specific subunits of RP in Zn homeostasis in plants.

In A. thaliana, similar and unique functions among RPT paralogs have been reported.7-9 We found that the T-DNA insertion mutants of RPT2b and RPT5b were not Zn deficiency-sensitive (Fig. 1D). This suggests that as condition than under the control condition in both Col-0 and the rpt mutants (Fig. 5A). The rpt mutants exhibited higher CT-L activities than Col-0 irrespective of Zn conditions (Fig. 5A). This suggests that Ub-independent 20SP activity responds to Zn deficiency-stress, and that mutations in RPT2a and RPT5a promote this response. Ub-independent proteolysis by 20SP is known to be one of the degradation pathways of oxidized proteins,1,16) and this might explain the fact that accumulation of oxidized proteins did not increase as a result of Zn deficiency (Fig. 2B).

To investigate further the effects of Zn deficiency on Ub-dependent proteolysis through 26SP, we compared the profiles of Ub-conjugated proteins among Col-0, rpt2a-2, and rpt5a-4. Western analysis indicated that poly-ubiquitinated proteins were highly accumulated under Zn deficiency treatment in the shoots of both Col-0 and the rpt mutants, and that more poly-ubiquitinated proteins accumulated in the rpt mutants as compared with Col-0 under the Zn-deficient condition (Fig. 5B). This suggests that Ub-dependent 26SP activity was inhibited under the Zn-deficient condition, especially in the rpt mutants.
to Zn deficiency tolerance, RPT2a and RPT5a act differently from the corresponding paralogs RPT2b and RPT5b respectively. Based on the hypothesis that plants assemble multiple 26SP forms with different compositions of subunits, it is possible that 26SPs containing RPT2a or RPT5a function in Zn deficiency-tolerance.

It is plausible to speculate that Zn deficiency-sensitive rpt mutants are defective in Zn transport or its regulation, which results in lower Zn accumulation in shoots and subsequent suppression of shoot growth. Our results indicate that Zn contents under both the control and the Zn-deficient conditions were not different between the wild-type and the rpt mutants (Fig. 2). This suggests that changes in Zn accumulation are not a cause of the phenotype, and that RPT2a and RPT5a are not likely to be involved in Zn transport to the shoots, but misregulation of cellular Zn distribution in the rpt mutants remains a possibility.

The Zn deficiency-responsive Zn transporter genes ZIP4 and ZIP9(25) were highly expressed in the rpt mutants as compared to the wild-type, especially under the Zn-deficient condition (Fig. 3A). It is possible that higher expression of ZIP4 and ZIP9 genes responds to Zn concentrations in a certain compartment in the cell, and that both RPT2a and RPT5a are involved in the regulation of Zn concentrations in the compartment, but not in the overall concentration of Zn. On the other hand, the rpt mutants showed reduced Cu and Mn contents under the control condition, suggesting that RPT2a and RPT5a are associated with the regulation of Cu and Mn transport to the shoots. Considering the Mn increment due to Zn deficiency in the wild-type, Mn regulation might affect tolerance of Zn deficiency in plants.

It is thought that a breakdown in the defense systems against oxidative stress is an early effect of Zn deficiency.(14) We found Zn-deficiency reduced expression of Cu/Zn-SOD genes (Fig. 3B) and elevated lipid peroxidation levels in the shoots in both the wild-type and the rpt mutants (Fig. 4A). The increments of lipid peroxidation due to Zn deficiency treatment were much higher in rpt2a-2 and rpt5a-4 than that in wild-type (Fig. 4A). ROS causes lipid peroxidation and SODs can eliminate ROS. It is possible that RPT2a and RPT5a function in anti-oxidative stress by suppressing ROS production under the Zn-deficient condition.

ROS also causes protein oxidation. Accumulation of oxidized proteins was not increased by Zn deficiency in the wild-type or the rpt mutants (Fig. 4B). Oxidized proteins are known to be a substrate for Ub-independent 20SP proteolysis.(1,11,16) In our study, 20SP activity was elevated under the Zn-deficient condition both in the wild-type and in the rpt mutants, and the increase in activity was more evident in the rpt mutant (Fig. 5A). It is possible that highly activated 20SP reduces the contents of oxidized proteins to normal levels under the Zn-deficient condition. If this is the case, it indicates that the high 20SP activity in the rpt mutants reflects high ROS production in these mutants under the Zn-deficient condition.

It has been found that Ub-independent 20SP activity increases when Ub-dependent 26SP activity is inhibited.(16) The profiles of poly-Ub conjugated proteins in the total soluble extracts indicated that Ub-dependent 26SP activity was inhibited in rpt mutants (Fig. 5B). It has been found that up-regulation of 26SP subunit genes reflects decreases in Ub-dependent 26SP activity in plants.(16) Zn deficiency induced the expression of both RPT2a and RPT5a genes, and the extents of induction of these genes was much higher in rpt2a-2 and rpt5a-4 (Fig. 1B), confirming our hypothesis.

The fact that Ub-dependent 20SP activity is inhibited by Zn deficiency indicates the possible existence of specific unknown substrates degraded through Ub-dependent proteolytic pathway in response to Zn deficiency. In animal cells, RPT2 is known to be glycosylated in response to glucose starvation, resulting in decreases in Ub-dependent 26SP activities to several substrates.(10) It is therefore tempting to speculate that the unknown substrates specifically processed through RPT2a and RPT5a are associated with Zn deficiency-tolerance.

In our results, the transcripts of RPT2a and RPT5a were Zn-deficiency responsive, but their paralogs RPT2b and RPT5b were not (Fig. 1B). Thus it is possible that the induction of a certain subunit gene by a certain stress indicates its involvement in tolerance to the stress. The published transcriptome data using A. thaliana(25) indicate that the transcripts of six RPTs are both similarly and distinctively induced by various types of stresses,
such as cold, osmotic, salinity, genotoxic, and heat shock stress. This suggests that the respective subunits of RP including RPT2a and RPT5a have common, unique functions in a variety of stresses. More specifically, both RPT2a and RPT5a were induced under osmotic, salinity, and UV-B conditions that are known to induce ROS.\textsuperscript{25–27} It is possible that both RPT2a and RPT5a are also crucial to tolerance of these stresses as in the case of Zn-deficiency. Their responses to ROS might have a role in stress tolerance.

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