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Original Paper

Sugar-inducible RPT2a, a subunit of 26S proteasome, participates in sugar response in *Arabidopsis*

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Abstract The ubiquitin/26S proteasome system (UPS) plays a central role in the degradation of short-lived regulatory proteins that control many cellular events. In this study, the *Arabidopsis* knockout mutant *rpt2a*, which contains a defect in the AtRPT2a subunit of the 26S proteasome regulatory particle, showed hypersensitivity to sugars as well as enlarged leaves. When the role of RPT2a in sugar response was examined in further detail it was found that putatively only the *AtRPT2a* gene of 19S proteasome was markedly transcriptionally promoted by sugar application. Notably, poly-ubiquitinated proteins degraded by the UPS accumulated significantly in *rpt2a* mutant under 6% sucrose conditions compared to wild type. In addition, the *AtRPT2a* gene in *gin2*, a glucose insensitive mutant with a defective glucose-sensing hexokinase, was not upregulated by sugar application, indicating that AtRPT2a is involved in hexokinase-dependent sugar response. Taken together, the above findings indicate that AtRPT2a plays an essential role in the maintenance of proteasome-dependent proteolysis activity in response to sugars.

Key words: Ubiquitin/26S proteasome, *AtRPT2a*, sugar response.

The 26S proteasome is a multisubunit ATP-dependent protease complex essential for regulating protein turnover in eukaryotes. Conjugation of ubiquitin to proteolytic substrates marks such substrates for degradation by the proteasome. The 26S proteasome is assembled from two particles: the 20S core particle (CP) and the 19S regulatory particle (RP) (Voges et al. 1999). The RP can be divided into two subcomplexes, referred to as the base and the lid. The base contains three non-ATPase subunits (RPN1, RPN2 and RPN10) and six AAA-ATPase subunits (RPT1–RPT6); the lid contains nine additional RPN subunits (Fu et al. 2001). Each proteasome subunit is presumed to have specific functions, but the roles of only a few subunits are known. Referring to current results, RPN10 probably participates in ABA signaling (Smalle et al. 2003), and RPT5 might have a role in recognizing poly-ubiquitinated proteins (Lam et al. 2002). The *Arabidopsis* (*Arabidopsis thaliana*) genome contains two genes, *AtRPT2a* and *AtRPT2b*, which are paralog RPT2 subunits with a difference of only three amino acids in the protein sequence (Sonoda et al. 2009). We recently discovered that the *rpt2a* mutant shows a specific phenotype of enlarged leaves caused by increased cell size in correlation to increased ploidy. Detailed analysis revealed that cell expansion increases in the *rpt2a* mutant by extended endoreduplication at an early stage of leaf development (Sako and Yamaguchi, 2010). Trichomes of the *rpt2a* mutant were also larger and had an increased branch number (Sako et al. 2010).

In plants, sugar has dual functions; in carbon and energy metabolism and as a signal molecule that coordinates many important development processes such as germination, cotyledon greening, root expansion, shoot growth and senescence (Koch 1996; Sheen et al. 1999; Smeekens 2000). Recent progress indicates that hexokinase (HXK), a bifunctional enzyme participating in metabolism and regulating activities, is a glucose sensor that integrates signal sense and transduction in response to environment stress (Moore et al. 2003). Mutant *gin2* (*glucose insensitive2*), which has loss-of-function of HXK1, displays cotyledon greening and expansion and hypocotyl and root elongation at an early stage of seedling development on MS medium containing high (6%) exogenous glucose levels whereas the wild type does not. Crosstalk between sugar sensing and hormone signals forms complex networks. Numerous genetic

Abbreviations: UPS, ubiquitin/26S proteasome system; ABA, abscisic acid.
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assays have uncovered connections between sugar and ABA, such as the fact that *gin1* (*glucose insensitive1*) is a novel *aba2* (*ABA-deficient mutant*) allele and that GIN5 (Glucose Insensitive 5) participates in the regulation of ABA level during glucose response (Arenas-Huertero et al. 2000). Moreover, the pathway of ABA-independent sugar response is broadly recognized; *sis5* (*sucrose insensitive 5*) and *sun6* (*sucrose uncoupled 6*) also show reduced sugar sensitivity and defective ABA response (Laby et al. 2000; Huijser et al. 2000; Ramon et al. 2008).

In this study, we analyzed transcription levels of most 19S *AtRPT* genes in wild type under various exogenous sugar conditions. In addition, plant phenotypes influenced by sugar stress were observed in the wild type, *rpt2a* and *rpt2b*, and root elongation of wild type and *rpt2a* were monitored under ABA stress. The influence of sucrose application on poly-ubiquitinated protein degradation and sugar response in *gin2* mutant background was examined. Our data indicate that AtRPT2a plays a specific role in sugar response and that regulation functions independently from ABA signaling. The data also suggest that proteasome-dependent proteolytic activity is associated to the hexokinase-dependent sugar response in plants.

Materials and methods

Plant materials and growth conditions

For germination of *Arabidopsis thaliana* (ecotype Columbia-0) wild type and mutants, seeds were surface-sterilized and placed on MS (Murashige and Skoog) medium supplemented with 2% sucrose (Germination inducible medium: GIM). After cold treatment for two days to synchronize germination, seeds were transferred to an environment of 22°C and 50% relative humidity under a 16/8 h light/dark cycle (this time point indicates 0 days after sowing: DAS). Seeds of knockout mutants of the *AtRPT2a* (*rpt2a-2*) and *AtRPT2b* (*rpt2b-1*) were obtained from the ABRC (The Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA; stock number: SALK_005596 and SALK_043450, respectively).

Transcription level analysis

Total RNA was extracted by the guanidine thiocyanate method (Chomczynski et al. 1987). Total RNA (0.6 μg RNA) was used as a template for the first strand cDNA synthesis using ReverTraAce-α-® reverse transcriptase (TOYOBO, Osaka, Japan). First strand cDNA (0.7 μl) was then assayed for gene-specific DNA fragments using primer pairs listed in Table S1. PCR amplification was performed at the optimum number of cycles for each gene using *Taq* DNA polymerase (New England BioLabs® Japan Inc., Tokyo, Japan). *EF1α* was used as an internal control. The amplified fragments were electrophoresed on 1.2% (w/v) agarose gels and visualized by ethidium bromide staining. Growth conditions of plants used for RT-PCR are clarified in the Figure legends.

Morphology assay

Seven DAS plants of wild type, *rpt2a-2* and *rpt2b-1* were observed using a stereomicroscope (LEICA CLS 150XD) to detect the morphological changes effected by sugar stress. The plants were cultivated on 1/2 MS medium with 2%, 4% or 6% sucrose. The experiment was repeated three times.

ABA sensitivity assay

Seeds of the wild type and *rpt2a-2* were sown on a 2% sucrose MS medium to germinate and grow, and then four DAS plants were transferred to 2% sucrose MS medium containing 0, 1, 3 or 10 μM ABA. The main root length of samples was counted four days after ABA treatment. The experiment was repeated three times.

Preparation of total protein and western blotting analysis

Total protein was prepared from the freshly harvested wild type and *rpt2a-2* plants. Growth conditions were kept the same for the transcription level assay. Tissue (200 mg fresh weight) was ground in 600 μl of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% [v/v] glycerin, 4% [w/v] SDS, and 10% [v/v] β-mercaptoethanol) and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was boiled at 100°C for 3 min and centrifuged again at 15,000 rpm for 5 min at 4°C. Then, analysis was carried out using SDS-PAGE followed by western blotting analysis with Monoclonal Antibody to Multiubiquitin Chains (FK2) (Nippon BioTest laboratories Inc.).

Results and discussion

RPT2a is involved in sugar response

Sugars play complex roles during plant development. The sugars that exert effects in the plant can be endogenous sugars produced by plants or exogenous sugars provided under experimental conditions. To exclude the effect of endogenous sugars, we arranged plant growth conditions as follows: first, plants grew for 10 days on 1/2 MS containing 2% sucrose, and were then transferred to 1/2 MS containing 0% sucrose to incubate for 2 days in darkness in order to exhaust endogenous sugars; finally, plants were transferred to 1/2 MS containing 2%, 4% or 6% sucrose to grow for one day in darkness to determine sugar effects precisely.

The *Arabidopsis* genome contains six *RPT* genes: *AtRPT1* to *AtRPT6*. These *AtRPT* genes are duplicated, except *RPT3*. For example, there are *AtRPT2a* (At4g29040) and *AtRPT2b* (At2g20140), which encode a paralog molecule of the 26S proteasome subunit RPT2. Expression analysis of *AtRPT* genes in the wild type indicated that only the *AtRPT2a* gene is transcriptionally promoted by increasing concentration of applied sucrose (Figure 1A). From this result, we predicted that the RPT2a plays a crucial role in sugar response. Morphology of the wild type, *rpt2a-2* and *rpt2b-1* were

observed to check post-germinative development effected by sucrose application. In the case of the 1/2 MS medium containing 4% sucrose, the wild type and *rpt2b-1* mutant showed normal development, but *rpt2a-2* exhibited purple pigmentation and small cotyledons (Figure 1B). The cotyledon development of *rpt2a-2* was completely arrested on 6% sucrose; however, the status of *rpt2b-1* was consistent with the wild type (Figure 1B).

Similar morphological results were found under high glucose conditions (Figure S1), with marked germination arrest and pigmentation seen on 4% glucose compared to sucrose (Figure 1B). Furthermore, root elongation in *rpt2a-2* was drastically suppressed compared to the wild type, even on 0% sucrose (Figure S2). To eliminate the effects of osmotic pressure, we used 6% mannitol as a negative control. The phenotype of the *rpt2a-2* exhibited a similar pattern to the wild type on 6% mannitol, and also showed the same growth status with 2% sucrose (Figure S3), indicating that the sugar effects in the r*pt2a* mutant are not due to osmotic pressure. These results indicate that the *rpt2a* mutant is hypersensitive to sugar compared to the *rpt2b* and wild type. Our research also revealed that the paralog genes of *AtRPT2a* and *AtRPT2b* have different roles in *Arabidopsis*; a sugar response role was seen in this study and an endoreduplication role in others (Sonoda et al. 2009).

Recently, Ueda et al. (2011) demonstrated that the RPT2b works in the root apical meristem, but is dispensable for it maintenance in the presence of the RPT2a. In contrast, the *rpt2a rpt2b* double mutant was lethal in male and female gametophytes, suggesting that the RPT2a and RPT2b are redundantly required for gametogenesis. Lee et al. (2011) reported that the *rpt2a* phenotype can be rescued by both RPT2a and RPT2b, indicative of functional redundancy, but not by RPT2a mutants altered in ATP binding/hydrolysis or missing the C-terminal hydrophobic sequence that docks onto the core protease (CP). They also suggested that RPT2 is important for plant nucleosome assembly. We previously demonstrated the 19S proteasome containing RPT2a (19S^{AtRPT2a}) regulated cell size in leaf organs, suggesting definition of the 19S^{AtRPT2a} as a cell size-specific proteasome. Thus, we hypothesize that not only 19S^{AtRPT2a} but also a specific combination of subunits would function as the cell size-specific proteasome (Sako and Yamaguchi 2010). Further studies would be needed to clarify the redundant functions between RPT2a and RPT2b in plant development.

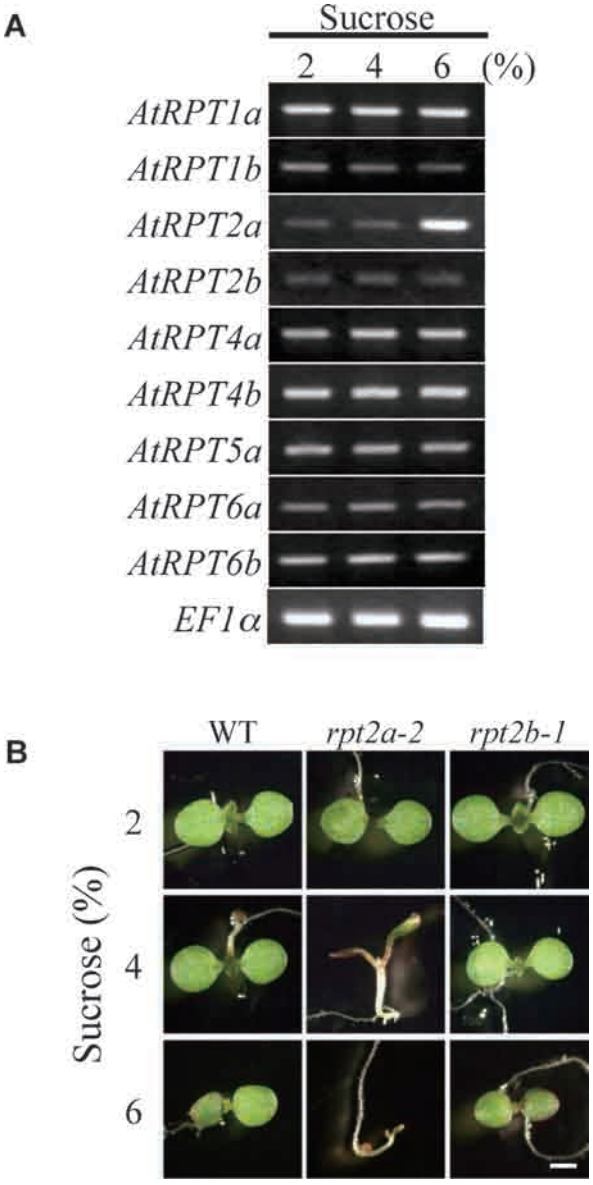

Figure 1. Transcriptional level assay for *AtRPT* genes and morphological observation. (A) Transcriptional levels of 26S proteasome *AtRPT* genes, including *AtRPT1a*/*b*, *AtRPT2a*/*b*, *AtRPT4a*/*b*, *AtRPT5a* and *AtRPT6a*/*b*, were analyzed under different sucrose stresses. Wild type plants were plated on 2% sucrose 1/2 MS medium to grow for 10 days and then transferred to 0% sucrose 1/2 MS medium in dark conditions to metabolize plants' internal sugar for 2 days, and finally plants were transferred to 1/2 MS medium containing 2%, 4% or 6% sucrose in dark conditions to incubate for 1 day. *EF1α* was used as an internal control. (B) Morphological observation of the wild type (WT), *rpt2a-2* and *rpt2b-1* response to sucrose conditions. Seeds were sown on 1/2 MS medium containing 2, 4 or 6% sucrose, and were observed at 7 DAS (days after sowing). Scale bar: 1 mm.

Sugar regulation of RPT2a is independent of ABA signaling

Sugars affect post-germinative growth through a sugar-signaling pathway that is partially controlled by ABA (Lopez et al. 2001). Higher concentrations of ABA inhibit post-germinative growth, and most mutants that are insensitive to high concentrations of sugars also show resistance to ABA at the post-germination stage; *sun6* (*sucrose uncoupled-6*) is insensitive to sucrose and the *SUN6* gene is identical to *ABI4* (*ABSCISIC ACID INSENSITIVE-4*) (Huijser et al. 2000). As *rpt2a* also exhibited an elevated sensitivity to sugars, we examined the response of *rpt2a-2* to ABA (Figure 2). The wild type

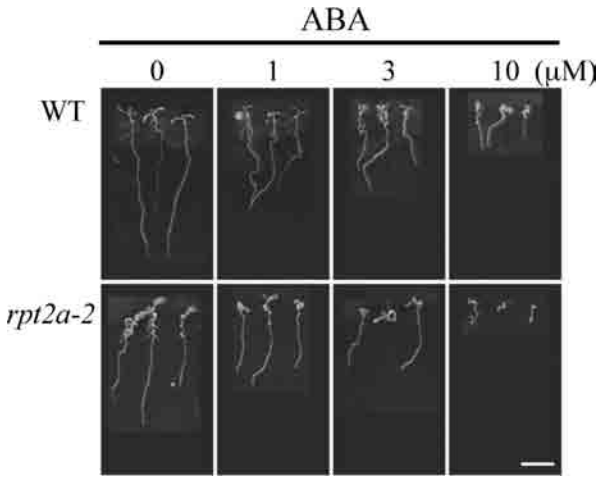

Figure 2. ABA sensitivity assay in the wild type and *rpt2a-2*. The wild type (WT) plants and *rpt2a-2* were grown on 2% sucrose MS medium for 4 days after germination. Plants were transferred to 2% sucrose MS medium containing 0, 1, 3 or 10 μM ABA. Scale bar: 1 cm.

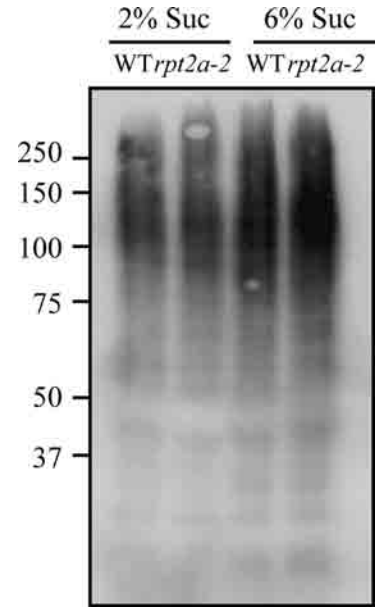

Figure 3. Analysis of poly-ubiquitinated protein pattern influenced by sucrose stress. The wild type (WT) and *rpt2a-2* were grown under the same conditions as in Figure 1A. Total protein was extracted with the SDS sample buffer. Equal quantities of total protein were subjected to complete the Western blot experiment and immunoblot analysis was conducted with an anti-multiubiquitin chain antibody.

and *rpt2a-2* were plated on MS medium containing up to 10 μM ABA. The root lengths of the *rpt2a-2*, growing on the media containing 0, 1 and 3 μM ABA, nearly showed insignificant differences compared to the wild type. Further root elongation was severely inhibited in both the *rpt2a-2* and the wild type under 10 μM conditions. These results suggest that RPT2a is coordinated by an ABA-independent sugar response pathway. We previously reported that UPS mediated by ubiquitin ligase ATL31 is associated to the carbon/nitrogen balance through the ABA-independent processes (Sato et al. 2009). The results in this study might be consistent with the previous reports.

Ubiquitinated proteins accumulate in the sugar response

Germination on media containing a high concentration of sugars is a stressful event for plants, inducing strong purple pigmentation in the wild type under 6% sugar (Figure 1B and Figure S1). Moreover, proteolysis catalyzed by UPS is required for survival of plants under extensive stress conditions such as drought (Cho et al. 2008) and extreme high carbon/low nitrogen stress (Sato et al. 2009; Sato et al. 2011a; Sato et al. 2011b). Accumulation of ubiquitinated-protein conjugates were determined by detection with specific antibody (Figure 3). There was no obvious change in the pattern of poly-ubiquitinated proteins between the *rpt2a-2* and the wild type on mild sugar conditions at 2% sucrose, whereas a marked accumulation on 6% sucrose was observed in both the wild type and *rpt2a-2*, indicating that UPS-dependent protease activities are clearly suppressed by sugar stress. Additionally, on 6% sucrose application, poly-ubiquitinated proteins accumulated dramatically in the *rpt2a-2* mutant compared to the wild type. The overaccumulation of poly-ubiquitinated proteins in

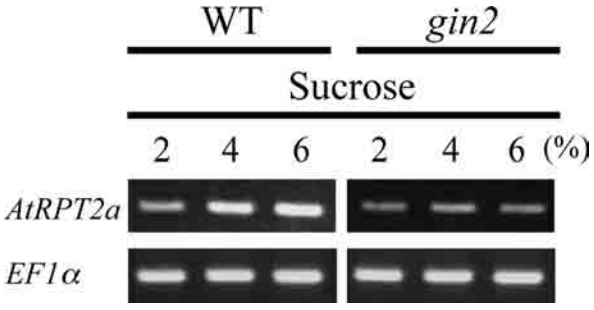

Figure 4. Transcriptional level assay for *AtRPT2a* in the wild type and *gin2*. The wild type (WT) and *gin2* mutant were treated under the same growth conditions as in Figure 1A. *EF1α* was used as a control.

the *rpt2a* mutant might be caused by abundance of AtRPT2a-interacting substrates, which are unable to undergo degradation by the AtRPT2a-deficient proteasome. Therefore, arrest of post-germinative growth in the *rpt2a* mutant under high sugar status probably results in accumulation and aggregation of the proteins to be degraded by the UPS (Figure 1B).

Expression of AtRPT2a gene is not promoted by sugar in gin2 mutant

To determine the function of RPT2a in the sugar response, we evaluated the expression level of the AtRPT2a gene in *gin2 (glucose insensitive2)*, a loss-of-function hexokinase mutant. The development of the *gin2* mutant was not arrested under potentially harmful 6% glucose stress (Rolland et al. 2002). Promotion

of *AtRPT2a* gene following sucrose concentration was not observed in *gin2* (Figure 4); nevertheless, enhanced expression of the gene was detected in the wild type. These results indicate that sugar-induction of the *AtRPT2a* gene occurs in a hexokinase-dependent manner.

Ubiquitin/26S proteasome pathway and sugar responses

We demonstrated in this study that AtRPT2a, a subunit of 19S proteasome, is associated with hexokinase-dependent sugar responses in *Arabidopsis*, which implies that the key proteins in sugar responses are degraded by an AtRPT2a-specific UPS. Indeed, a number of ubiquitin ligases have been reported to be involved in sugar signaling. Loss-of-function mutant of KEEP ON GOING (KEG), which is a RING-HCa type ubiquitin ligase, shows hypersensitivity to sugars; furthermore, post-germinative growth in such mutants is arrested by sugar application (Stone et al. 2006). The *keg* mutant shows a hypersensitive phenotype to ABA, and KEG directly interacts with the ABI5 protein, indicating that the KEG regulates ABA signaling by the degradation of ABI5 via the UPS. SUGAR-INSENSITIVE3 (SIS3) is also an ubiquitin ligase containing a RING domain (RING-H2 type) and putative transmembrane domains (Huang et al. 2010). SIS3 loss-of function leads to a phenotype insensitive to excessive sugars in the medium. On the other hand, the ABA response of *sis3* mutant is similar to that of the wild-type plant, suggesting that the SIS3 regulates sugar response via an ABA-independent pathway. The ubiquitination target of SIS3 has not been reported. The ubiquitinated protein catalyzed by these ubiquitin ligases would be degraded by AtRPT2a-specific proteasome. Further experiments will be needed to clarify the various processes involved.

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