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Studies on proteasome function in response to environmental stress in Arabidopsis

by

Huihui SUN

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Chapter 1

General Introduction

26S proteasome

26S proteasome is a multi-catalytic complex to selectively degrade damaged proteins, nonfunctional proteins and short life proteins to modulate organism's homeostasis and regulate adaption to various stress (Smalle and Vierstra 2004). As well as mammals and yeast, in Arabidopsis 26S proteasome is constructed by two sub-complexes 19S regulatory particle (19S RP) and 20S proteasome (also named as 20S core particle (20S CP)) (Voges *et al.,* 1999). 19S RP is capped to 20S CP to assist poly-ubiquitinated substrates to transmit into 20S proteasome catalytic center for hydrolysis. 19S RP can be divided into two parts, referred to as base and lid. The base contains three non-ATPase subunits (RPN1, RPN2 and RPN10) and six AAA-ATPase subunits (RPT1–RPT6); the lid contains at least nine additional RPN subunits (Fu *et al.,* 2001). Each 19S RP subunit is presumed to play particular function, for instance, RPN10, RPN13 and RPN1 were reported as receptors of poly-ubiquitinated proteins (Rosenzweig *et al.,* 2012; Husnjak *et al.,* 2008) and RPN1 can bind with ubiquitin-like (Ubl) domain shuttle proteins to help substrates recognition (Elsasser *et al.,* 2002). RPN11 showed a deubiquitylation activity to remove ubiquitin moieties from target proteins during their breakdown (Verma *et al.,* 2002). Base subunits ATPases provide major energy to unfold substrates, open 20S proteasome gate and translocate (Groll *et al.,* 2000; Rabl *et al.,* 2008). 20S proteasome is a cylinder chamber stacked by two outer α-rings and two inner β-rings, which are comprised by 7 α-subunits and 7 β-subunits, respectively. Three peptidase subunits β1, β2 and β5 are enclosed in the chamber center, which displays caspase-like, trypsin-like and chymotrypsin-like proteolysis, respectively (Unno *et al.,* 2002).

RPT2a

Different from mammals and yeast, most Arabidopsis proteasome subunits genes are paralogous. RPT2, regulatory particle triple-ATPase 2, is one RPT subunit of 26S proteasome. In Arabidopsis RPT2 contains two paralogous subunits RPT2a and RPT2b, which exhibit 99% identity (Book *et al.,* 2010) with only three amino acids differences (Sonoda *et al.,* 2009). However, RPT2a and RPT2b showed distinguished function in plants. One case was RPT2a specifically involved in the regulation of leaf size; *rpt2a* mutant displayed enlarged leaves and trichomes compared to wild type, whereas *rpt2b* mutant showed similar morphology with wild type (Sonoda *et al.,* 2009;

Sako *et al.,* 2010). More investigations demonstrated that increased cell size is in correlation to increased ploidy, which was produced by extended endoreduplication at an early stage of leaf development (Sako and Yamaguchi 2010). Chung *et al.* described RPT2a directly participated in uni-1D-induced signalling pathway. Cross *rpt2a* mutant with *uni-1D* mutant (a semi-dominant mutant showing constitutive increased level of *pathogenesis related-1* (*PR1*) and lethality in the early stage of true leaf formation) reduced *PR1* level and suppressed *uni-1D* lethality. However, RPT2b was not involved in this signalling pathway (Chung *et al.,* 2011). Kurepa *et al.* clarified 19S proteasome *rpt2a mut*ants were tolerant to oxidative stress, because existence ratio between 26S proteasome and 20S proteasome was changed in these mutants, decreased 26S proteasome level and increased 20S proteasome level leading to reduced proteolysis of ubiquitin-dependent substrates and promoted proteolysis of ubiquitin-independent substrates (Kurepa *et al.,* 2008). Among more than 30 proteasome subunits, Arabidopsis RPT2a subunit function has been broadly identified, which might be due to *rpt2a* mutant visibly distinguished phenotype under different research conditions. In my research RPT2a subunit was selected to elucidate the relationship between proteasome function and sugar stress.

Proteasome and immune response

During long terms of evolvement plants have developed efficient defense ability to adapt pathogen invasion, one adaptive immunity response strategy is called gene-for-gene interaction, the host plant produces a specific resistant gene product to perceive specific pathogen-encoded avirulence genes product (Ellis *et al.,* 2000). Another defense strategy is perception of mircobe-associated molecular patterns (MAMPs) via receptor molecules, for instance in Arabidopsis FLS2 (FLAGELLIN SENSITIVE 2), a plasma membrane associated receptor-like kinase, recognizes bacterial flagellum component flagellin to activate defense response (Chinchilla 2006). Flg22 is high conserved 22 amino acids in the N- terminal of flagellin in bacterial flagellum, the synthetic peptide derived from plant pathogen *Pseudomonas syringae* can be utilized as elicitor to mimic plant immunity response. After FLS2 perceives flg22 peptide, a series sequential events are triggered such as ethylene production (Mersmann *et al.,* 2010), activation of the MAPK cascade (Asai *et al.,* 2002), ROS (reactive oxygen species) burst (Legendre *et al.,* 1993) and enhancement of defense response genes like *PR1* (Gómez-Gómez

et al., 1999) and *GST1* (Asai *et al.,* 2002). Eventually both gene-for-gene interaction and perception general elicitor lead to activation of defense response.

Additionally proteasome itself plays a crucial role in immunity defense. In mammal interferon-γ-inducible β-subunits LMP2 (β1i), LMP10 (β2i) and LMP7 (β5i) are comprised into immunoproteasome, which produces antigenic peptide for generation of MHC class I ligands to initiate cell-mediated immunity (Tanaka *et al.,* 1998). In Arabidopsis, the paralogous gene' presence of proteasome subunit is intriguing, which assumes that high possibility of paralogous subunit exchange and replacement could be happened in response to different stress conditions. Hatsugai *et al.* clarified Arabidopsis proteasome subunit PBA1 showing caspase-3 associated activity, which was involved bacteria induced hypersensitive cell death. PBA1 RNAi knockdown mutants of decreased caspase-3 activity showed less cell death than WT response to infection of *Pseudomonas syringae pv. tomato DC3000 having the Avr gene avrRpm1 (Pst DC3000/avrRpm1)* because abolishment of fusion between large vacuole membrane and plasma membrane resulted in no vacuole antibacterial proteins discharge into the outside of cell. Eventually they presented a novel proteasome-dependent fusion of a large central vacuole with plasma membrane to discharge vacuole antibacterial protein to intercellular apace to prevent bacteria pathogen proliferation (Hatsugai *et al.,* 2009). Yao *et al.* uncovered RPN1a subunit was participated in innate immunity. Defense response assay showed *rpn1a* was increasingly susceptible to fungal pathogen *Golovinomyces cichoracearum* and bacterial *Pst* DC3000 strain. Simultaneously 19S proteasome subunits RPT2a and RPN8 were identified to participate in *edr2*-mediated disease response (Yao *et al.,* 2012). All in all proteasome subunits selves are involved in defense response. During my research flg22 was chosen as immunity elicitor to investigate the relationship between proteasome function and immune response.

In this dissertation

Although the importance of proteasome function as a protease has been broadly mentioned accompanying with E3 ligase founding, the deep exploration of proteasome subunit self-function is rare. Proteasome is not just as a protease for unnecessary protein degradation, more novel functions of proteasome subunits are imperative to investigate. I therefore tried to

clarify the function of proteasome in response to different circumstances. In Chapter 2, I attempted characterization of proteasome RPT2a subunit in response to abiotic sugar stress. Molecular biology detection and physiology assay demonstrated that RPT2a plays crucial and positive roles in sugar regulation. In Chapter 3, I attempted to investigate the proteasome function in response to biotic MAMP flg22 treatment. Specific proteasome subunit composition and proteasome activity were influenced by flg22, however mRNA expression of all peptidase genes, *PBA*, *PBB1/2*, *PBE1/2* and total proteasome amount were not changed by flg22. Consequently, I proposed that proteasome activity was regulated by putative post-translational modification. Finally, I have totally discussed about my investigations in this dissertation in Chapter 4. I expect that these studies could contribute to promote further elucidation and understanding of the relationship between proteasome function and environmental stress in Arabidopsis.

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Chapter 2

Sugar-inducible RPT2a, a subunit of 26S proteasome is associated to sugar response in Arabidopsis

Summary

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.

Introduction

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 participated in ABA signalling (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 signalling 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 signalling 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 signallings forms complex networks. Numerous genetic 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 (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 signalling. 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 S2-1. PCR amplification was performed at the optimum number of cycles for each gene using *Taq* DNA polymerase (New England BioLabs® Japan inc, Tokyo, Japan). *EF1*α 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 two 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 2-1A). 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 2-1B). 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 2-1B).

Similar morphological results were found under high glucose conditions (Figure S2-1), with marked germination arrest and pigmentation seen on 4% glucose compared to sucrose (Figure 2-1B). Furthermore, root elongation in *rpt2a-2* was drastically suppressed compared to the wild type, even on 0% sucrose (Figure S2-2). 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 S2-3), 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. (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.* (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 mutant 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 aloso 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.

Sugar regulation of RPT2a is independent of ABA signalling

Sugars affect post-germinative growth through a sugar-signalling 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 2-2). The wild type 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 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 2-1B and Figure S2-1). 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 2-3). 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 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 2-1B).

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 2-4); 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 signalling. 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 signalling by the degradation of ABI5 via the UPS. SUGAR-INSENSITIVE3 (SIS3) is also a 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.

Acknowledgments

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Figures and Legends

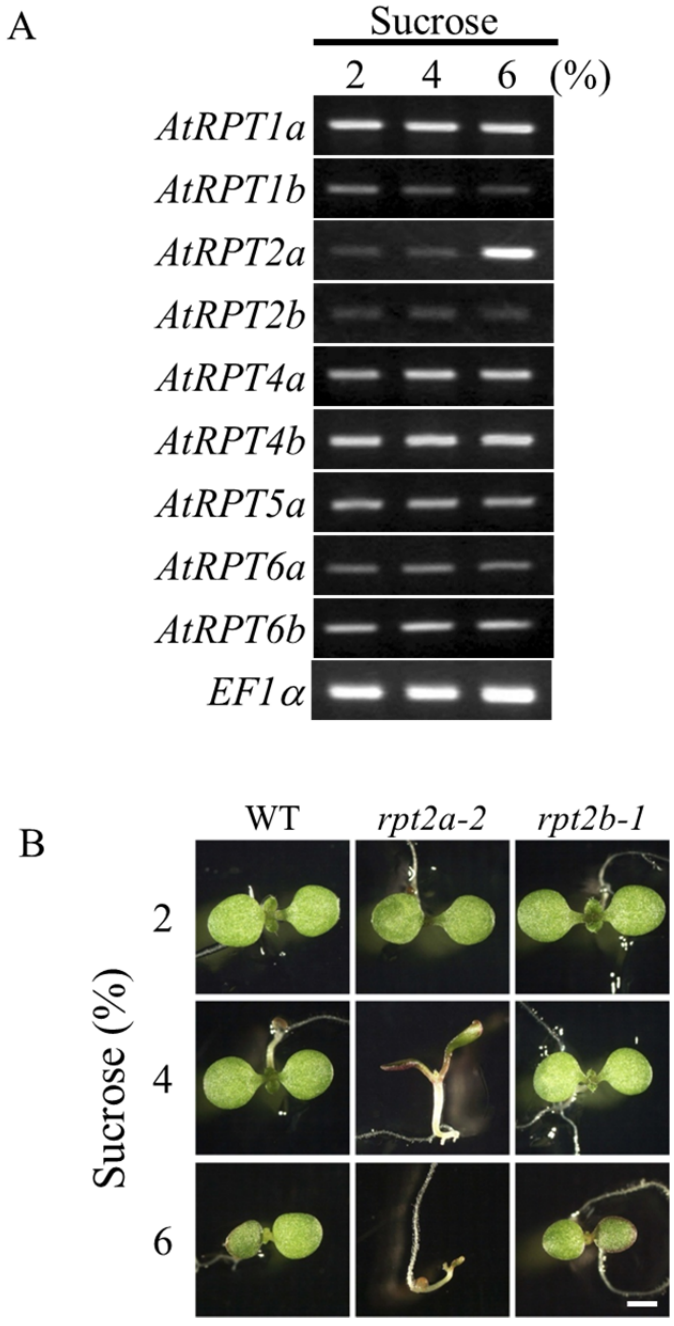

A
Sucrose
2
4
6
(%)
AtRPT1a
AtRPT1b
AtRPT2a
AtRPT2b
AtRPT4a
AtRPT4b
AtRPT5a
AtRPT6a
AtRPT6b
EF1α
B
WT
rpt2a-2
rpt2b-1
Sucrose (%)
2
4
6

Figure 2-1.

Figure 2-1. 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 one day. *EF1*α 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.

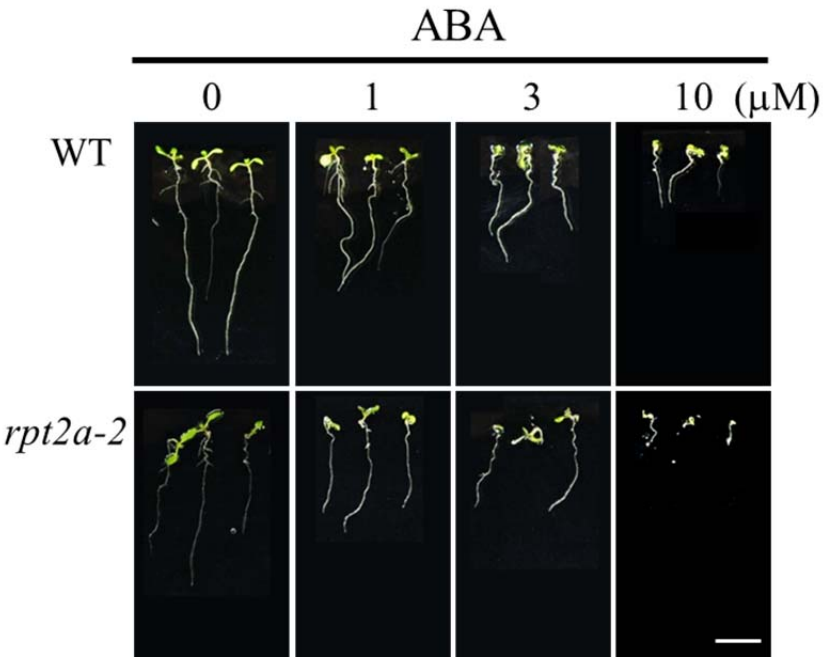


Figure 2-2.

Figure 2-2. 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 four days after germination. Plants were transferred to 2% sucrose MS medium containing 0, 1, 3 or 10 μM ABA. Scale bar: 1 cm.

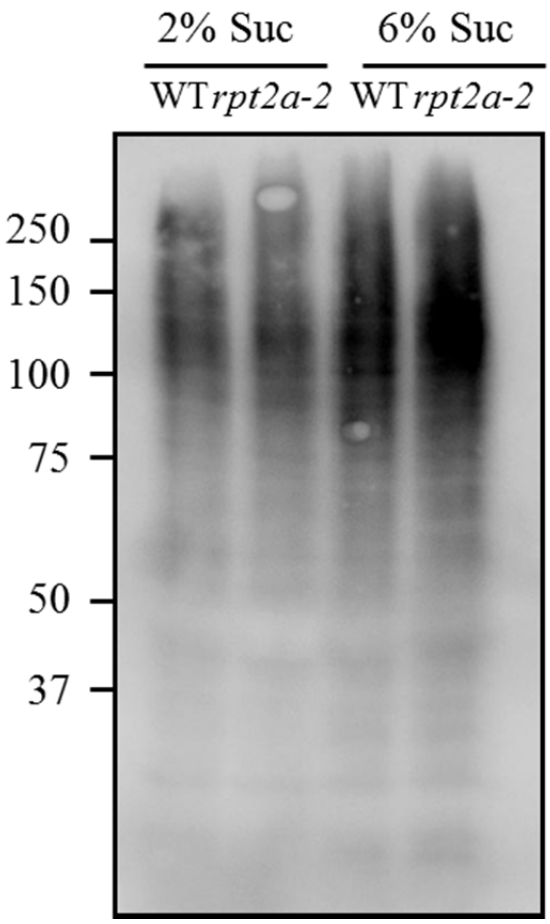


Figure 2-3.

Figure 2-3. 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 2-1A. 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.

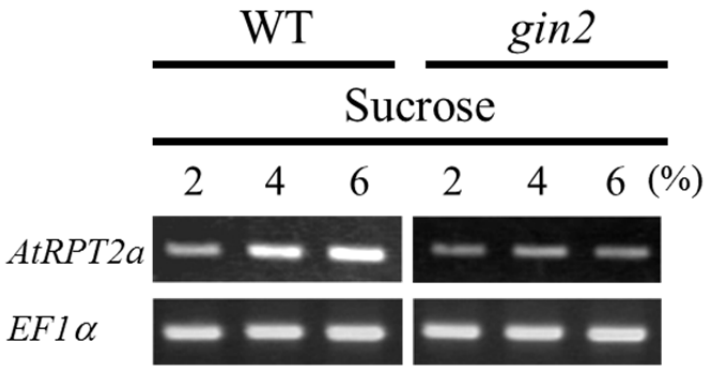


Figure 2-4.

Figure 2-4. 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 2-1A. *EF1*α was used as a control.

List of Supporting Information

Figure S2-1. Morphological observation of wild type (WT), *rpt2a-2* and *rpt2b-1* in response to glucose conditions.

Figure S2-2. Root elongation assay in the wide type and *rpt2a-2*.

Figure S2-3. Osmotic pressure effects check.

Table S2-1. Primer pairs for RT-PCR analysis.

Figures, Table and Legends of

Supporting Information

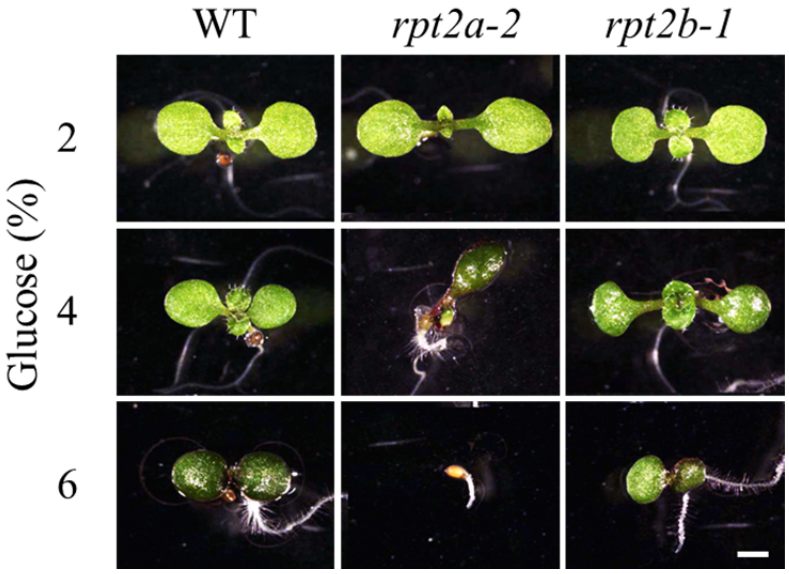


Figure S2-1.

Figure S2-1. Morphological observation of the wild type (WT) *rpt2a-2* and *rpt2b-1* in response to glucose conditions.
Seeds were sown on 1/2 MS medium containing 2%, 4% or 6% glucose, and were checked 7 DAS (days after sowing). Scale bar: 1 mm.

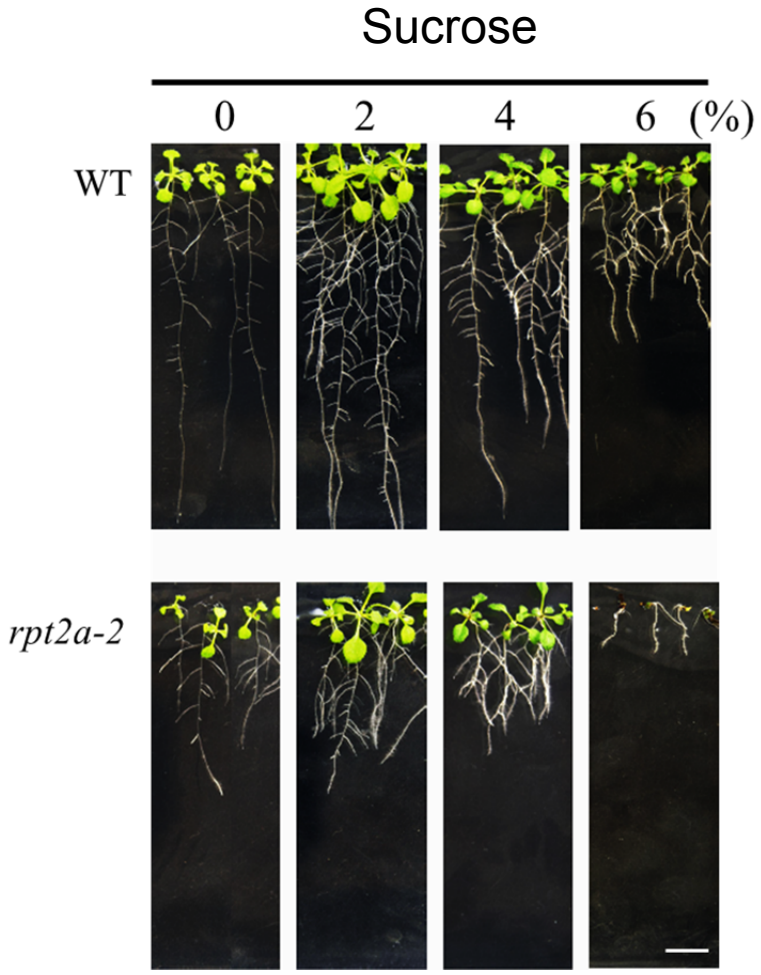


Figure S2-2.

Figure S2-2. Root elongation assay in the wide type and *rpt2a-2*.
The wild type (WT) and *rpt2a-2* were plated on 1/2 MS medium containing 2, 4 or 6% sucrose, and samples were checked after 14 days. Scale bar: 1 cm.

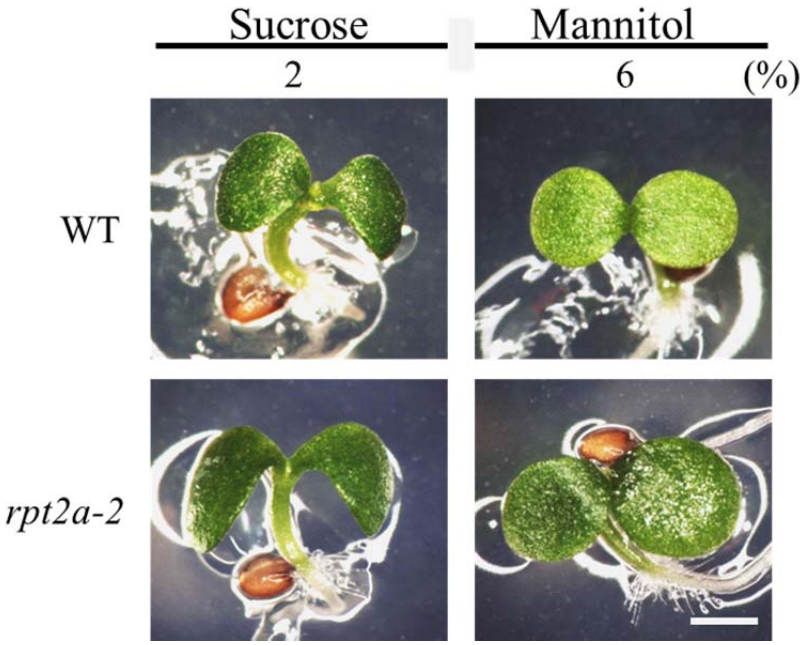


Figure S2-3.

Figure S2-3. Osmotic pressure effects check.
The wild type (WT) and *rpt2a-2* were sown on 1/2 MS medium containing 2% sucrose and 6% mannitol. Seven DAS plants were used to detect development status. Scale bar: 1 mm.

Gene	Primers	
AtRPT1a	5'-3' Forward:	GTGAAGGAGCTAGGATGGTT
	5'-3' Reverse:	CGAGTTACTGGAATAAGTTGTG
AtRPT1b	5'-3' Forward:	GTGAAGGAGCTAGGATGGTT
	5'-3' Reverse:	GAGTTGCTGAGTTTTGCATCA
AtRPT2a	5'-3' Forward:	GGAACTTCTGAACCAGCTTG
	5'-3' Reverse:	ATTGTTTTACCGGAGGAGAT
AtRPT2b	5'-3' Forward:	GAGATTGATGCTGTTGGCAC
	5'-3' Reverse:	TAAGCAGAGAGTGAAAACAG
AtRPT4a	5'-3' Forward:	GGAACTAGTGCTGATCGTGA
	5'-3' Reverse:	ACGTAGTAACTAAGCACACAC
AtRPT4b	5'-3' Forward:	GGAACTAGTGCTGATCGTGA
	5'-3' Reverse:	TAGGCCCTCTCTTCTTCTCT
AtRPT5a	5'-3' Forward:	AAGTCAGCGGAGACAGGGAA
	5'-3' Reverse:	TAGATGATCCTGATTCGAAAAC
AtRPT6a	5'-3' Forward:	AAATGGTGACAGTGAGGTGC
	5'-3' Reverse:	GAGTTTTGGGAATTTCCTTCC
AtRPT6b	5'-3' Forward:	AAATGGTGACAGTGAGGTGC
	5'-3' Reverse:	AAAGCTTAAAGCGCTAGCCG
EF1α	5'-3' Forward:	TCATACCAGTCTCAACACGTCC
	5'-3' Reverse:	GCTGTCCTTATCATTGACTCCACC

Table S2-1.

Table S2-1. Primer pairs for RT-PCR analysis.

Chapter 3

Proteomics analysis reveals a highly heterogenous proteasome composition and the post-translational regulation of peptidase activity under pathogen signaling in plants

Summary

The proteasome is a large multisubunit complex that plays a crucial role in the removal of damaged or selective ubiquitinated proteins, thereby allowing quality control of cellular proteins and restricted regulation of diverse cellular signaling in eukaryotic cells. Proteasome-dependent protein degradation is involved in almost all aspects of plant growth and responses to environmental stresses including pathogen resistance. Although the molecular mechanism for specifying targets by ubiquitin ligases is well understood, the detailed characterization of the plant proteasome complex remains unclear. One of the most important features of the plant proteasome is that most subunits are encoded by duplicate genes, suggesting the highly heterogenous composition of this proteasome. Here, we performed affinity purification and a combination of 2-dimensional electrophoresis and mass spectrometry, which identified the detailed composition of paralogous and modified proteins. Moreover, these proteomics approaches revealed that specific subunit composition and proteasome peptidase activity were affected by pathogen-derived MAMPs, flg22 treatment. Interestingly, flg22 treatment did not alter mRNA expression levels of the peptidase genes, *PBA*, *PBB1/2*, *PBE1/2* and total proteasome levels remained unchanged by flg22 as well. These results demonstrate the finely tuned mechanism that regulates proteasome function via putative post-translational modifications in response to environmental stress in plants.

Introduction

The 26S proteasome is a multicatalytic complex that selectively degrades damaged proteins, nonfunctional proteins, and short-lived proteins to modulate an organism's homeostasis, and to regulate activities of growth, development, and adaption to various stressors. As well as mammals and yeast (Coux and Goldberg, 1996), the plants 26S proteasome is comprised of two subcomplexes consisting of the 19S regulatory particle (19S RP) and the 20S core particle (20S CP, 20S proteasome) (Voges *et al.,* 1999). The 20S proteasome is capped at either end by the 19S RP, and this large multimeric complex assists the transmission of polyubiquitinated substrates into the 20S proteasome catalytic center for hydrolysis. The 19S RP can be divided into two parts, 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 at least nine additional RPN subunits (Fu *et al.,* 2001). Each 19S RP subunit is presumed to play a particular function; for instance, RPN1, RPN10 and RPN13 are reported to be receptors of polyubiquitinated proteins (Rosenzweig *et al.,* 2012; Husnjak *et al.,* 2008) and RPN1 can bind with ubiquitin-like domain shuttle proteins to help substrate recognition (Elsasser *et al.,* 2002). RPN11 has deubiquitinating activity that removes ubiquitin moieties from target proteins during their breakdown (Verma *et al.,* 2002). ATPase subunits provide most of the energy needed for substrate unfolding, gate opening of the 20S proteasome, and substrate translocation (Groll *et al.,* 2000; Rabl *et al.,* 2008). The 20S proteasome is a cylinder chamber stacked by two outer α-rings and two inner β-rings, which are comprised of 14 α-subunits and 14 β-subunits, respectively. Three peptidase subunits, β1, β2 and β5 are enclosed in the chamber center and display caspase-like, trypsin-like and chymotrypsin-like proteolysis, respectively (Unno *et al.,* 2002). Compared to the 19S RP, the biochemical function and regulatory mechanism of each 20S proteasome subunit is poorly understood.

Unlike mammals and yeast, most proteasome subunits in plants are encoded by two paralogous genes, which produce proteins with highly similar amino acid sequences. In Arabidopsis, 10 of 14 subunits in the 20S proteasome, and 12 of 17 subunits in the 19S RP have protein paralogs. For example RPT2a/b, RPN1a/b and PBE1/2 exhibit 99%, 90% and 97% identity, respectively (Book *et al.,* 2010). Even though each paralogous protein shares a highly conserved amino acid sequence, genetic analyses have

revealed that they do not have completely redundant physiological functions. RPT2a and RPT2b are RPT2 paralogous subunits that differ by only three amino acids (Sonoda *et al.,* 2009). Only the *rpt2a* loss-of function mutant showed a hypersensitive phenotype to sugar stress (Sun *et al.,* 2012) and exhibited enlarged leaves and trichomes compared to the wild-type plant and *rpt2b* mutant (Sonoda *et al.,* 2009; Sako and Yamaguchi, 2010; Lee *et al.,* 2011). In mammalian cells, comparison of constitutive type of proteasome, a special type of interferon-γ-inducible proteasome is produced, three interferon-γ-inducible β-subunits LMP2 (β1i), LMP10 (MECL1) (β2i) and LMP7 (β5i) are comprised into immunoproteasome, which produces antigenic peptide for generation of MHC class I ligands to initiate cell-mediated immunity (Tanaka and Kasahara,1998; Murata *et al.,* 2001). The thymus-specific subtype of proteasome, thymoproteasome, was also reported to consist of a thymus-specific β5 subunit called β5t, and is involved in $CD8^+$ T cell development (Murata *et al.,* 2007). In Arabidopsis, multiple isoforms of several proteasome subunits encoded by paralogous genes have been discovered, indicating that paralogous subunit exchange and replacement most likely occurs in response to developmental stage and different circumstances.

The ubiquitin-proteasome system is essential for the plant response to environmental stress, as it causes the regulated degradation of specific short-lived proteins and damaged proteins. Recent proteomic and genetic analyses have identified many ubiquitin ligases, as well as substrates for degradation involved in biotic and abiotic stress responses (Cho *et al.,* 2008; Sato *et al.,* 2011). In addition, the proteasome itself plays a crucial role in the plant response to pathogen attack. Hatsugai *et al.* demonstrated the direct involvement of the proteasome in pathogen resistance via activation of bacteria-induced hypersensitive cell death (Hatsugai *et al.,* 2009). RNA interference knockdown mutants of PBA1, which act as plant caspase-3 like enzymes, caused less cell death than wild-type PBA1 in response to infection of avirulent bacteria *Pst* DC3000 expressing avrRpm1, because abolishing the fusion between a large central vacuole with the plasma membrane prevented discharge of vacuolar antibacterial proteins to the outside of bacteria-infected cells, thereby preventing proliferation of bacterial pathogens. Suty *et al.* demonstrated that the mRNA expression of a specific defense-induced *PBA* gene paralog is stimulated by pathogen-derived microbe-associated molecular patterns (MAMPs) induced by cryptogein treatment, which inhibits the activity of r*espiratory burst oxidase homolog D* (RbohD) and regulates the generation

of reactive oxygen species during defense reactions (Suty *et al.,* 2003). These observations indicate that proteasome activity is directly associated with biotic stress resistance in plants; therefore, the regulation of proteasome activity is necessary for pathogen resistance and successful plant growth. However, the detailed characterization of the proteasome remains poorly understood, especially in plants.

The goal of this study was to establish a proteomic method that would allow identification of the subunit composition of the plant proteasome. A combination of 2-dimensional electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) after one-step affinity-purification revealed the detailed composition of the proteasome subunits in plant cells. In addition, the relationship between proteasome activity and composition was analyzed after treatment with flg22, a peptide derived from flagellin. Flg22 treatment affected specific peptidase activity, whereas mRNA expression and proteasome levels remained unchanged. Our 2-DE/MS analysis of the purified proteasome indicated that flg22 treatment altered the biochemical status of specific subunits, thereby demonstrating that an unknown mechanism regulates proteasome activity via post-translational modification in response to biotic stress in plants.

Materials and methods

Materials and growth condition

Suspension cultures of the *Arabidopsis thaliana* cell line MM2d were used and cultured as previously described (Menges *et al.,* 2002). Transgenic MM2d cells constitutively expressing *PBF1-FLAG* under the 35S promoter (*PBF1-FLAG*) were transformed according to the protocol by Hirano *et al.* (Figure S3-1 and S3-2 in the Supporting Information) (Hirano *et al.,* 2008). Cell cultures were grown in the dark at 27 °C with shaking at 120 rpm, and were subcultured every 7 days by transferring 3 ml of cultures into 300-ml flasks containing 50 ml LS medium (Menges *et al.,* 2002). Experiments were performed 4 days after subculture (DAS) of cell cultures.

Plasmid construction

Full-length *PBF1* cDNA (At3G60820) was amplified by PCR using the following primers: forward; 5'-CACCATGACTAAACAGCACGC-3', reverse; 5'-CTTGTAAAACTCAAAGTCCGTGT-3'. The purified cDNA products were introduced into the pENTR/D-TOPO vector (Invitrogen) to generate the pENTR-PBF1 plasmid. Full-length *PBF1* was then ligated into the pGWB11 T-DNA binary vector (Nakagawa *et al.,* 2007) under control of the 35S promoter according to the Gateway Instruction Manual, and tagged with FLAG at the C-terminus to generate PBF1-FLAG. For recombinant RPT3 preparation, the pENTR-RPT3 construct was produced with the following primers: forward; 5'-CACCATGGCTTCCGCGGCTG-3', reverse; 5'-CTTGTAAAACTCAAAGTCCGTG-3'. The construct was then ligated into the pDEST Trx-His vector (Tsunoda *et al.,* 2005) according to the Gateway Instruction Manual (Invitrogen) to generate a bacterial plasmid that expressed thioredoxin and a 6X His-tagged RPT3 fusion protein (Trx-His-RPT3). All PCR products and inserts were verified by DNA sequencing.

Antibodies

For immunoblot analyses, the following antibodies were used: anti-FLAG antibody (M2; Sigma), anti-ubiquitin antibody (FK2, Nippon Bio-Test Laboratories Inc.). Antibodies against RPT3 (At5g58290) and 20S CP were prepared from rabbit with injection of recombinant RPT3 protein and purified

spinach 20S CP proteins. Plasmid pDEST Trx-His-RPT3 was introduced into the *E. coli* strain BL21 (DE3) pLysS (Novagen) to produce the recombinant proteins Trx-His-RPT3, which was expressed and purified according to the manufacturer's instructions (Clontech). The 20S proteasome was purified from spinach by conventional column chromatography as described by Iwafune *et al*. (Iwafune *et al.,* 2002). Purified Trx-His-RPT3 and 20S CP were resolved by SDS–PAGE and stained with Coomassie brilliant blue. The antigens on the gel were cut out and injected into a rabbit (Figures S3-3, S3-4 and Table S3-1 in the Supporting Information).

Analysis of transcription levels

One milliliter of *PBF1-FLAG* MM2d cells stored in -80 °C was used for RNA isolation according to the Trizol reagent procedure (Invitrogen). DNase treatment and reverse transcription were completed with RQ1 RNase-Free DNase (Promega) and SuperScript™ II Reverse Transcriptase (Invitrogen), respectively. PCR conditions were performed as previously described (Yamamuro *et al.,* 2005). The primers information was described in Table S3-3 in Supporting Information. RT-PCR was performed using normalized cDNA samples, and 15–30 amplification cycles were conducted depending on the primer sets. PCR products were then electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Determination of protein amount

Crude extracts for immunoblot analyses of the proteasome subunits were prepared using proteasome affinity purification procedures. Anti-FLAG and anti-RPT3 antibodies were used to determine levels of the 26S proteasome subunits. Flamingo gel staining (Biorad) was performed for total protein staining.

Polyubiquitinated protein detection

Total proteins were isolated with 700 μl extraction buffer [50 mM Tris–HCl pH 7.5, 10% glycerol (v/v), 150 mM NaCl, 1% Triton X-100 (v/v), 1 mM EDTA, 10 μM MG132 (Sigma) and protease inhibitor cocktail (Roche)]. Clarified supernatants were collected after two rounds of centrifugation at 4 °C and 15,000 rpm for 5 min. Protein concentration was determined using the Biorad

protein assay, and proteins were denatured with the addition of 2X sample buffer [125 mM Tris–HCl, pH 6.8, 20% (v/v) glycerin, 4% (w/v) SDS, 100 mM DTT and 0.002% (m/v) BPB] at 95 °C for 5 min. Proteins were resolved on 10% SDS-PAGE gels. One gel underwent Flamingo gel staining, and proteins from the other gel were electrophoretically transferred to a PVDF membrane, followed by incubation with anti-ubiquitin (FK2, Nippon Bio-Test Laboratories) antibody.

Flg22 treatment

Four DAS *PBF1-FLAG* MM2d cells were treated with 100 nM flg22 peptide (Sigma Genosys), and autoclaved H_2O was used as a control. Flg22-treated cells (1 ml) were collected after 1, 6 and 24 hours, and the supernatant was removed after centrifugation at room temperature at 2000 rpm for 1 min. The cells were then snap frozen in liquid nitrogen, and stored at -80 °C.

Proteasome peptidase activity assay

Proteasome proteolysis activities of caspase-like, trypsin-like and chymotrypsin-like were measured with fluorogenic substrates Z-LLE-AMC, Boc-FSR-AMC and Suc-LLVY-AMC (Peptide Institute, Inc), respectively as previously described (Yanagawa *et al*., 1999) The crude proteins and each fluorogenic substrate was incubated in reaction buffer contained 100 mM Tris-HCl pH 8.0, 0.02% SDS with or without proteasome specific protease inhibitor MG132 for caspase-like and chymotrypsin-like activities or clasto-Lactacystin β-lactone for trypsin-like activity at a final concentration of 10 μM. SDS was removed from reaction buffer for trypsin-like activity analysis. After the reaction, the fluorescence intensity of free AMC was measured at an excitation of 380 nm and emission of 460 nm. The amount of digested substrate (pmol) was calculated using standard curves made with free AMC (Peptide Institute) under the same experimental conditions.

Affinity purification of proteasome

Frozen *PBF1-FLAG* cells were lysed at 4 °C in extraction buffer [50 mM Tris–HCl pH 7.5, 20% (v/v) glycerol, 5 mM $MgCl_2$, 2 mM ATP, 10 μM β-leupeptin and protease inhibitor cocktail without EDTA]. Cellular debris was removed by two rounds of centrifugation at 15000 rpm for 5 min at 4 °C.

Clarified lysate was then incubated with anti-FLAG M2 Affinity Gel (Sigma) for 3 hours at 4 °C. After incubation, beads were washed three times for 1 min with 800 μl wash buffer [50 mM Tris–HCl pH 7.5, 20% (v/v) glycerol, 5 mM $MgCl_2$ and 2 mM ATP], and FLAG-containing fractions were eluted using 3X FLAG peptide (100 μg/ml final concentration) or 800 mM NaCl (to disassociate 19S RP from the 20S proteasome). The quality of the purified 26S proteasome was checked by Flamingo gel staining after being resolved on a 12.5% SDS-PAGE gel. Subunits of the PBF1-FLAG, RPT3 and 20S proteasome were detected using anti-FLAG, anti-RPT3 and anti-20S CP antibodies, respectively.

Native-PAGE separation of proteasome complexes

For separation of proteasome complexes on native-PAGE gels, a previous protocol was used with some modifications (Dohmen *et al.,* 2005; *Leggett et al.,* 2005). Briefly, a 4% separating gel [90 mM Tris-borate pH 8.35, 5 mM $MgCl_2$, 0.5 mM EDTA, 4% acrylamide (w/v), 2 mM ATP, 1 mM DTT, 0.1% APS (w/v), 10 μl TEMED and H_2O to 10 ml], and a 2.5% stacking gel [50 mM Tris-HCl pH 6.8, 2.5% acrylamide (w/v), 0.05% APS (w/v), TEMED 10 μl and H_2O to 6 ml] were prepared. 5X native gel loading buffer [250 mM Tris-HCl pH 7.4, 50% glycerol (v/v) and 0.0625% bromophenol blue (w/v)] was added to each sample, and the samples were loaded on the gel. The gel was run at 100 V at 4 °C until the bromophenol blue dye migrated to the bottom of the gel. The proteins were electrophoretically transferred using the standard semi-dry transfer method, followed by blocking of the membrane in 5% milk at 4 °C overnight. The 26S proteasome complexes were visualized after incubation with anti-FLAG antibody.

2-DE and LC-MS/MS analysis

The affinity purified 20S proteasome was separated on a 18 cm 2-DE gel with a broad IPG strip pI [3-10] (GE Healthcare) for first dimension electrophoresis (1-DE; isoelectric focusing), and on a 12.5% SDS-PAGE gel for 2-DE. After Flamingo gel staining, each spot was excised for subsequent LC-MS/MS analysis. Peptides for LC-MS/MS analysis were prepared by in-gel digestion using sequencing-grade modified trypsin (Promega) (Fujiwara *et al.,* 2006) and LC-MS/MS analysis was performed using a LTQ-orbitrap XL-HTC-PAL system (Thermo Scientific). The MS/MS spectra were

compared against TAIR10, and MS scores were calculated using the MASCOT server as previously described (Fukao *et al.,* 2009) Analysis of the 20S proteasome composition in response to flg22 treatment was carried out by protein resolution on a 2-DE gel with a broad IPG strip pI [4-7] for 1-DE, and on a 12.5% SDS-PAGE gel for 2-DE.

Results

Affinity purification of the 26S proteasome from Arabidopsis cells

To evaluate the detailed composition of the plant proteasome, we established a high throughput method for purification of the proteasome complex from Arabidopsis. Transgenic Arabidopsis cultured MM2d cells constitutively expressing PBF1 subunit fused with FLAG-epitope tag (*PBF1-FLAG*) were established (Figure S3-1 and S3-2 in the Supporting Information), and used for affinity purification of the proteasome complex with anti-FLAG beads. Since the PBF subunit is encoded by a single gene (*PBF1*; At3g60820), it is expected that PBF1 would assemble in all proteasome complexes in the cell. The quality of the purified proteasome was determined by protein resolution on SDS-PAGE and native-PAGE gels, followed by Flamingo gel staining and immunoblot analyses. After elution with FLAG peptide, multiple specific protein bands were observed in these cells upon Flamingo gel staining, compared to wild-type cells not expressing *PBF1-FLAG* (Figure 3-1A). These data were in accordance with those resulting from other purification methods using conventional column chromatography and affinity purification from Arabidopsis (Book *et al.,* 2010; Yang *et al.,* 2004). Independent 20S CP and 19S RP complexes were isolated by eluting with a combination of FLAG peptide and concentrated sodium chloride (800 mM NaCl), which induces dissociation of 19S RP from 20S CP (Book *et al.,* 2010; Isono *et al.,* 2005). The putative proteasome-associated 200 (PA200) protein (Figure 3-1A) was estimated according to Book *et al.* To further confirm the purification of the 26S proteasome, immunoblot analysis was performed. As a result, in addition to the PBF1-FLAG protein, 20S proteasome subunits and the RPT3 subunit in 19S RP were specifically detected in purified samples from *PBF1-FLAG* MM2d cells (Figure 3-1B, left panel). On the other hand, native-PAGE analysis followed by immunoblotting with anti-FLAG showed purification of three subcomplexes of the proteasome; namely, the 20S proteasome capped with two 19S RP, the 20S proteasome with one 19S RP, and one 20S proteasome (Figure 3-1B, right panel). To confirm the successful enrichment of the intact 26S proteasome, peptidase activities were measured. Chymotrypsin-like peptidase activity was assayed using the Suc-LLVY-AMC substrate with crude extracts (input), and affinity-purified proteasome from wild-type and *PBF1-FLAG* overexpressing cells. There was little difference in the peptidase activity of input proteins in wild-type and

transgenic cells (Table 3-1); however, peptidase activity of the purified proteasome was enhanced about 600-fold in *PBF1-FLAG* compared to wild-type cells (Table 3-1), demonstrating the efficient purification of the functional 26S proteasome by one-step affinity purification from Arabidopsis cells.

Determination of the paralogous 20S proteasome subunit by 2-DE and MS analysis

Unlike in mammals and yeast, most Arabidopsis 26S proteasomes are a multisubunit protease complex encoded by two paralogous genes. Flamingo gel staining after SDS-PAGE could not separate the paralogous proteins (Figure 3-1A), and MS analysis of the excised gel could not detect the specific peptide of each paralog (data not shown). In order to obtain more detailed information about proteasome composition, 2-DE and LC-MS/MS analyses were performed with the affinity-purified proteasome. In addition, to determine the regulatory mechanism underlying peptidase activity, the 20S proteasome was selected for subsequent 2-DE/MS analysis. As a result of Flamingo gel staining after 2-DE, 45 spots were detected (Figure 3-2) and subsequent MS identified 20S proteasome proteins from 34 spots (Table 3-2 and Figure S3-5 in the Supporting Information). All 13 α-subunits and 11 β-subunits were detected, with the exception of PAC2 and PAD2; most paralogous proteins of the 20S proteasome could be specifically detected (Table 3-2 and Figure S3-5 in the Supporting Information). Interestingly, several proteins were detected from multiple different spots. For example, five spots with a high mass score of 1, 2, 3, 4 and 5 (Figure 3-2) were identified as α-ring subunit PAA1 proteins (Table 3-2 and Figure S3-5 in the Supporting Information). In the case of β-ring subunits, the peptidase subunits of PBB1/PBB2 and PBE1/PBE2 were also detected in multiple spots; for example, PBB1 was found in three spots numbered 17, 18 and 19, and PBE1 was found in six distinct spots numbered 26, 27, 28, 29, 30 and 31 (Figure 3-2 and Table 3-2 and Figure S3-5 in the Supporting Information). In the case of proteins with the same mass but altered pI, post-translational modifications were expected to result in varied patterns of these subunits.

The effect of flg22 on proteasome peptidase activity

To explore the possibility that proteasome activity is functionally regulated in

response to environmental stress, peptidase activities were analyzed in the presence of pathogen-derived MAMPs flg22 treatment. The 20S proteasome is a large protein complex with three active sites; namely, the β1 (PBA), β2 (PBB) and β5 (PBE) subunits (Borissenko and Groll, 2007). These three peptidase subunits have caspase-like, trypsin-like and chymotrypsin-like activities, respectively (Unno *et al.,* 2002). Z-LLE-AMC, Boc-FSR-AMC and Suc-LLVY-AMC substrates were used to measure the specific peptidase activities of the β1, β2 and β5 subunits. The results showed that β1 and β5 activities decreased about 0.17- and 0.58-fold 1 hour, and about 0.38- and 0.85-fold 6 hour after flg22 treatment, whereas β2 activity did not show significant changes compared to mock-treated cells (Figure 3-3). On the other hand 24 hours after flg22 treatment β1, β2 and β5 peptidase activities increased about 2.70-, 1.93- and 2.87-fold, respectively (Figure 3-3), suggesting the specific regulation of each peptidase under plant-defense signaling.

Effect of flg22 on polyubiquitinated protein degradation

In addition to analysis of peptidase activity, the relationship between flg22 treatment and physiological proteasome activity was evaluated by analyzing the accumulation of polyubiquitinated proteins. Immunoblot analysis with an anti-ubiquitin antibody indicated that the amount of polyubiquitinated proteins significantly increased about 1.43-fold 6 hours after flg22 treatment, whereas no obvious accumulation was present after 1 hour of treatment (Figure 3-4). On the other hand, the increased accumulation of ubiquitinated proteins recovered and slightly decreased 24 hours after flg22 treatment (Figure 3-4). The increased accumulation of polyubiquitinated proteins at 6 hours was consistent with decreased peptidase activities 1 hour and 6 hours after flg22 treatment (Figure 3-3). The recovery of accumulated protein at 24 hours might be associated with increased peptidase activity 24 hours after flg22 treatment. These results indicate that proteasome peptidase activity is physiologically affected in response to pathogen signaling in plant cells.

Total proteasome subunit levels are unaffected by flg22 treatment

In order to understand why 26S proteasome catalytic activity changes in response to flg22 treatment, the transcription level of 26S proteasome peptidase subunits were examined. Perception of flg22 by FLS2 receptor

(Robatzek *et al.,* 2006) activates various signaling transduction mechanisms such as MAP kinase activity (Asai *et al.,* 2002), calcium signaling *(Boudsocq et al.,* 2010) and production of reactive oxidative species (ROS) (Mersmann *et al.,* 2010), which finally results in global change of defense-related gene expressions (Asai *et al.,* 2002; Bethke *et al.,* 2009; Gómez-Gómez *et al.,* 1999). The transcription levels of the three peptidase subunits PBA1 (β1), PBB1/PBB2 (β2), PBE1/PBE2 (β5) and *GST1*, a marker gene for immune response (Asai *et al.,* 2002), were analyzed. *GST1* transcription levels were highly elevated 1 hour after flg22 treatment, which demonstrated the success of flg22 infection. However, the transcription levels of the peptidase subunits genes of *PBA1*, *PBB1/2* and *PBE1/2* remained constant 24 hours after flg22 treatment compared to mock-treated cells (Figure 3-5). These uniform transcription levels revealed that alterations in proteasome activity induced by flg22 were not due to changes in the mRNA expression of proteasome peptidase subunits. The protein levels of the proteasome subunits were also analyzed in the absence or presence of flg22 treatment. Immunoblot analysis with crude extract from *PBF1-FLAG* MM2d cells showed that protein levels of the 20S proteasome subunit PBF1-FLAG and the 19S RP subunit RPT3 were stable in flg22-treated and untreated cells (Figure 3-6A). The protein levels of the 26S proteasome subcomplex were analyzed by protein resolution on native-PAGE gels and immunoblotting using anti-FLAG antibody. The data showed that total 26S proteasome protein levels did not change after flg22 treatment (Figure 3-6B). Taken together, these data indicate that flg22-induced changes in proteasome peptidase activity is not due to changes in protein levels, but rather, may be due to proteasome regulation by post-translational modification of specific subunits.

Post-translational modifications occur on specific proteasome subunits after flg22 treatment

2-DE plus LC-MS/MS analyses of the affinity-purified proteasome revealed precise and abundant information regarding the proteasome subunit composition. To garner more detail with respect to the proteasome and changes in peptidase activity upon flg22 treatment, the 20S proteasome composition was analyzed by 2-DE/MS methodology. Affinity purification and 2-DE analyses were performed from *PBF1-FlAG* cells treated with water (mock) or flg22 for 24 hours. Although most spots showed similar staining patterns between mock and flg22-treated samples (Figure 3-7A,B),

interestingly, some spots (spot numbers 1-8) were shifted along the IEF dimension from acidic to basic pI after flg22 treatment (Figure 3-7C,D). For examples, spots 7 had a more basic pI in response to flg22 treatment compared to spot 2 in mock-treated cells (Figure 3-7C, D). The patterns of spots 3 and 4 in mock-treated cells and spot 8 in flg22-treated cells also differed (Figure 3-7C, D). To identify the subunit corresponding to spots 1-8, these eight spots were chosen for subsequent LC-MS/MS analysis. As a result, PBB1 was detected in spot 1, both PBB1 and PBB2 peptides in spot 2, and PBD2 in spots 3 and 4 in mock-treated cells (Table 3-3 and Figure S3-6 in the Supporting Information). On the other hand, PBB1 was detected from spot 5, both PBB1 and PBB2 were detected from spots 6 and 7, and both PBD1 and PBD2 were detected from spot 8 (Table 3-3 and Figure S3-6 in the Supporting Information). Compared to spots 1-2 and spots 5-7, the pI of the PBB1/2 subunit was shifted to a basic pI along the IEF dimension, while the molecular weight remained unchanged, suggesting the possibility of post-translational modifications of PBB1/2 in response to flg22 treatment. In the case of spots 3-4 and spot 8, PBD2 was also shifted to a basic pI along the IEF dimension similar to PBB1/2. Additionally, PBD1 was newly detected in flg22-treated MM2d cells. Since PBD1 was detected in a more acidic pI region in spot 23 (Figure 3-2 and Table 3-2), PBD1 was expected to be modified and detected in spot 8 after flg22 treatment. These results suggest that proteasome peptidase activity is affected by flg22 treatment via unknown post-translational modification of specific subunits.

Discussion

In this study, we used a one-step affinity purification with the FLAG-epitope tag, to isolate the 26S proteasome complex from Arabidopsis MM2d cells. Subsequent 2-DE and LC-MS/MS analyses of the purified 20S proteasome revealed detailed information about subunit composition, which indicated the existence of a highly heterogenous proteasome complex in plant cells due to unknown post-translational modifications. For instance, PBE1, which functions as a chymotrypsin–like peptidase, was detected from six different spots by 2-DE/MS analysis. Our 2-DE/MS data also revealed that various subunits displayed different protein levels between paralogous proteins. PBE1 was founds in six spots (26-31), and PBE2 was found in two spots (28 and 31). Spots 26 and 30 had relatively large protein content, while the other four spots had small amounts. Different amounts of paralogous subunits indicated that the proteasome complex might have different amounts of each paralogous subunit.

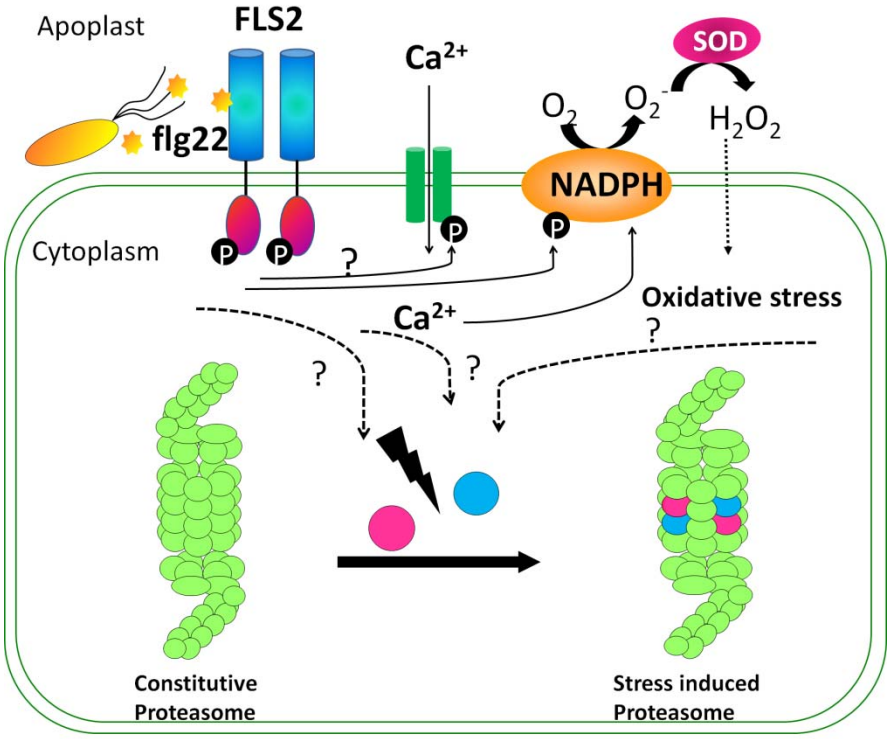
In addition to the peptidase subunit, the detection of PAC1 from five spots was also interesting. In yeast and mammals, PAC is demonstrated to be involved in the regulation of substrate entry into the 20S CP from the outside, since the N-terminal region of this subunit functions as a gate (Groll *et al.,* 2000). Kikuchi *et al*. revealed that many yeast proteasome subunits are phosphorylated on Ser/Thr residues by improved MS/MS analysis. Protein staining analysis with Pro-Q diamond, followed by Coomassie staining, indicated the same subunits containing phosphorylated and unphosphorylated forms at the same time (Kikuchi *et al.,* 2010). In the Arabidopsis plant, ubiquitination modification was identified on four kinds of alpha subunits, including PAC1. However, the authors were unable to detect Arabidopsis subunit phosphorylation using HPLC-ESI-MS/MS (high performance liquid chromatograph-electrospray ionization-MS/MS) with HCD (high energy collision dissociation) and ETC (electron transfer dissociation) methods (Book *et al.,* 2010). We also performed LC-MS/MS analyses to evaluate the phosphorylation of the Arabidopsis subunits of the purified proteasome; however, we did not succeed in finding any phosphorylated subunits (data not shown). The detection of phosphorylated Arabidopsis proteasome subunits needs more enriched phosphopeptide with metal affinity chromatography, and more sensitive MS/MS analysis. Another probable reason that one subunit had multiple spot patterns was due to splice variants. We collected splice

variants of the Arabidopsis 20S proteasome from the Arabidopsis genome database TAIR (Table S3-2 in the Supporting Information). The splice variants of the PAA2, PBD2 and PBE2 subunits showed large molecular weight differences (more than 2 kDa) compared to the splice variants of other subunits; some subunits, such as PBB1 and PBB2, exhibited similar molecular weights but marked differences in pI. Thus, we postulate splice variants as one putative reason of the diversified subunit pattern.

Next, we investigated changes in proteasome activity in response to environmental stress upon flg22 treatment. Interestingly, flg22 treatment induced fluctuations in peptidase activity 1 to 24 hours after treatment, especially in caspase-like (β1) and chymotrypsin-like (β5) activities. On the other hand, the mRNA expression and protein levels of proteasome subunits were unaffected by flg22 treatment. Finally, our proteomic analysis with 2-DE and MS suggested that post-translational modifications of specific subunits were induced by flg22 treatment, which might be involved in the regulation of proteasome activity. In this study, spots containing PBB1/2 and PBD1/2 shifted along the IEF dimension from acidic to basic pI without apparent changes in molecular weight. The PBB subunit has trypsin-like peptidase activity, which increased 24 hours after flg22 treatment, suggesting that peptidase activity may be directly regulated by post-translational modifications of specific subunits. Although the PBD subunit does not display peptidase activity, the involvement of PBD in pathogen resistance has been reported. Specifically, Arabidopsis cell suspension cultures were treated by fungal MAMPs from *Fusarium* for 24 hours, and PBD1 protein levels increased 1.84-fold compared to no treatment (Chivasa *et al.,* 2006). In our study, transcription levels of *PBD1* were not influenced by 24 hours of flg22 treatment, and *PBD2* transcription levels remained unaffected as well (Figure S3-7 in the Supporting information). It is possible that modified novel PBD subunits influenced 20S proteasome compositions and conformational structure, which accelerate proteasome activity, however future studies are needed to confirm this.

Although it is difficult to determine why proteasome activity changed in response to pathogen signaling, the quality control of damaged proteins during immune response may be involved. In Arabidopsis, FLS2 (Flagellin Sensitive 2), a plasma membrane associated receptor-like kinase (Shiu and Bleecker, 2001) recognizes bacterial flagellum component flagellin to activate the

defense response (Chinchilla *et al.,* 2006). After FLS2 perceives the flg22 peptide, a series of sequential events are triggered, such as activation of the MAPK cascade (Asai *et al.,* 2002), phytohormone production (Mersmann *et al.,* 2010), calcium influx (Jeworutzki *et al.,* 2010) and reactive oxygen species burst (Zhang *et al.,* 2007). While reactive oxygen species have essential roles in mediating immune signaling and inhibiting increases in bacteria (Torres and Dangl, 2005), they can also damage the plant cells themselves. Fungal MAMPs treatment of maize culture cells led to the generation of strong brown compounds, which suggested that H_2O_2 was induced and culture cell growth was disrupted (Chivasa *et al.,* 2005). In addition to the biotic stress response, the relationship between oxidative stress and plant proteasome activity was also demonstrated in sugar-starvation stress condition *(Basset et al.,* 2002). They showed the caspase-like and chymotrypsin-like peptidase activities were affected in sugar-starved maize root. Interestingly, they found the 20S proteasome was also oxidized under oxidative stress, suggesting that oxidized 20S proteasome could be associated with the degradation of oxidatively damaged proteins in carbon starvation situations. In yeast, H_2O_2 oxidative treatment disassociated the 26S proteasome into 19S RP and 20S proteasome (Wang *et al.,* 2010). Another group found that oxidation of the 20S proteasome subunit S-glutathionylation largely influenced chymotrypsin-like activity rather than trypsin-like activity (Demasi *et al.,* 2003). Taken together, it appears that oxidation is one reason for the multiple spots detected by our 2-DE/MS analysis, and it may be involved in the regulation of peptidase activity during pathogen stress response in plants.



Hypothesis and model of proteasome function in response to pathogen signaling

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Perspectives

Here, we found that proteasomal activity changes in response to flg22, and post-translational modification of the proteasome subunits was proposed as a putative reason for this phenomenon. The proteasome is a functional multisubunit complex that selectively catalyzes abnormal proteins and short-lived regulator proteins (Vierstra, 2009; Finley, 2009). The proteasome catalytic capacity is strictly regulated by subunit composition, complex conformational structure, proteasome interactor proteins (PiPs), subunit post-translational modifications and other mechanisms (Tanaka and Kasahara, 1998; Wang and Huang, 2008; Guo *et al.,* 2011). Comparison of proteasome research progress in mammals and yeast, and deep exploration of proteasome function in plants has become crucial and urgent. In particular, research has been directed towards identifying novel PiPs and unknown proteasome post-translational modifications. From an applied perspective, basic studies of the proteasome in *Arabidopsis thaliana* may provide more useful knowledge and techniques to apply in agriculture cultivation. Since oxidative stress is induced in various stress conditions, sufficient removal of oxidatively damaged protein is required to adapt to environmental condition. In addition, the proteasome is one of the direct targets of bacterial effector (Groll *et al.,* 2008). When *Pseudomonas syringe* pv. *syringae* (*Pss*) attack the plant, effector compound Sylinglin A (SylA) is secreted, which inhibits all of proteasome peptidase activity via binding to catalytic subunit and decrease the plant resistance to pathogen. Actually, SylA-nagative mutant in *Pss* strain was markedly less virulent on its host, *Phaseolus vulgaris* (bean). *Potato virus Y* (*PVY*) also interferes the proteasome activity with HC-Pro protein via direct protein-protein interaction with all three PBA, PBB and PBE peptidase subunits (Jin *et al.,* 2007). If the specific modifications that protect the proteasome activity from these effectors could be identified, it would contribute to the enhanced resistance to pathogen attack. The appropriate modulation of proteasome activity and function might contribute to the improvement of agricultural crops yield and qualities.

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Figures, Table and Legends

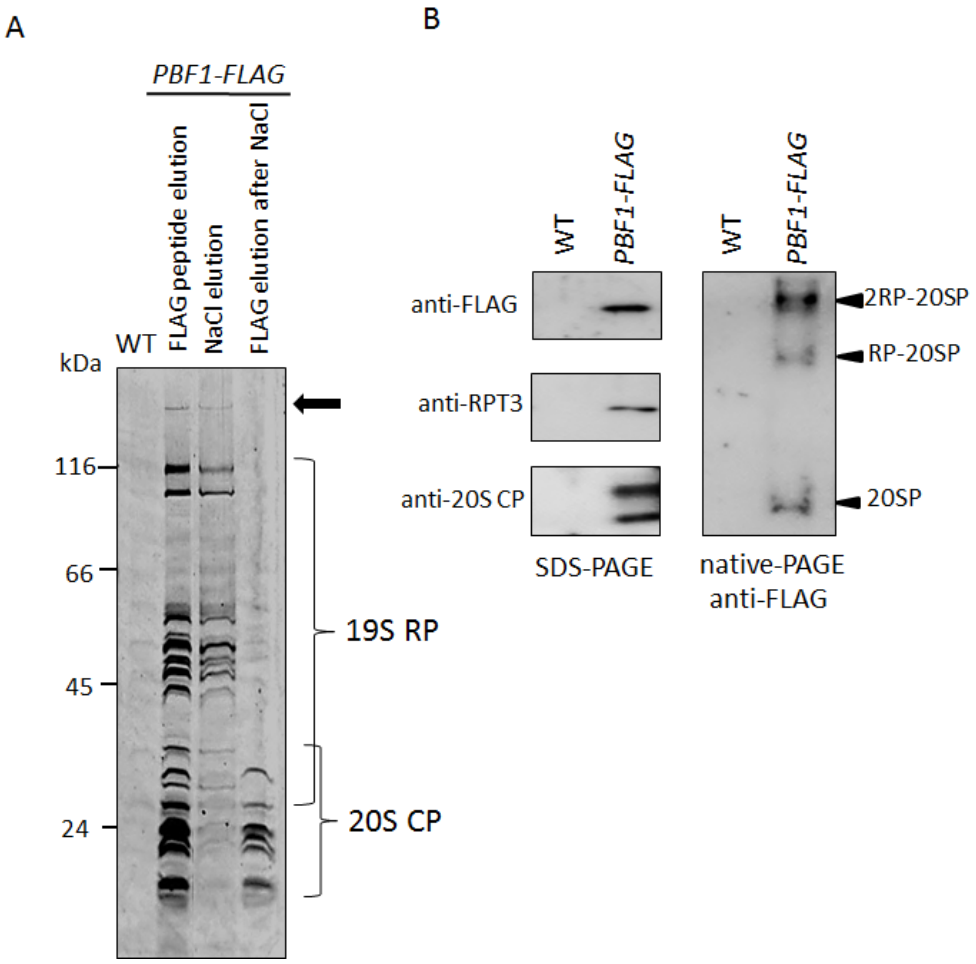
A
PBF1-FLAG
WT
FLAG peptide elution
NaCl elution
FLAG elution after NaCl
kDa
116
66
45
24
19S RP
20S CP
B
WT
PBF1-FLAG
anti-FLAG
anti-RPT3
anti-20S CP
SDS-PAGE
WT
PBF1-FLAG
2RP-20SP
RP-20SP
20SP
native-PAGE
anti-FLAG

Figure 3-1.

Figure 3-1. Affinity purification of the 26S proteasome complex from Arabidopsis cells.
(A) SDS-PAGE and Flamingo gel staining of the affinity purified 26S proteasome complex. Wild-type (WT) and *PBF1-FLAG* MM2d cells were used for purification with anti-FLAG beads. After incubation and washing, precipitated beads were incubated with 3X FLAG peptide, 800 mM NaCl, or 3X FLAG peptide after NaCl treatment. Brackets indicate the 19S RP and 20S CP subunits. Arrows indicate the expected band of PA200;
(B) Immunoblot analysis of proteasome subunits. Purified 26S proteasome subunits were separated on SDS-PAGE (left panels) or native-PAGE (right panels) gels, and each protein was detected by immunoblot analysis with anti-FLAG, anti-RPT3 or anti-20S CP antibodies.

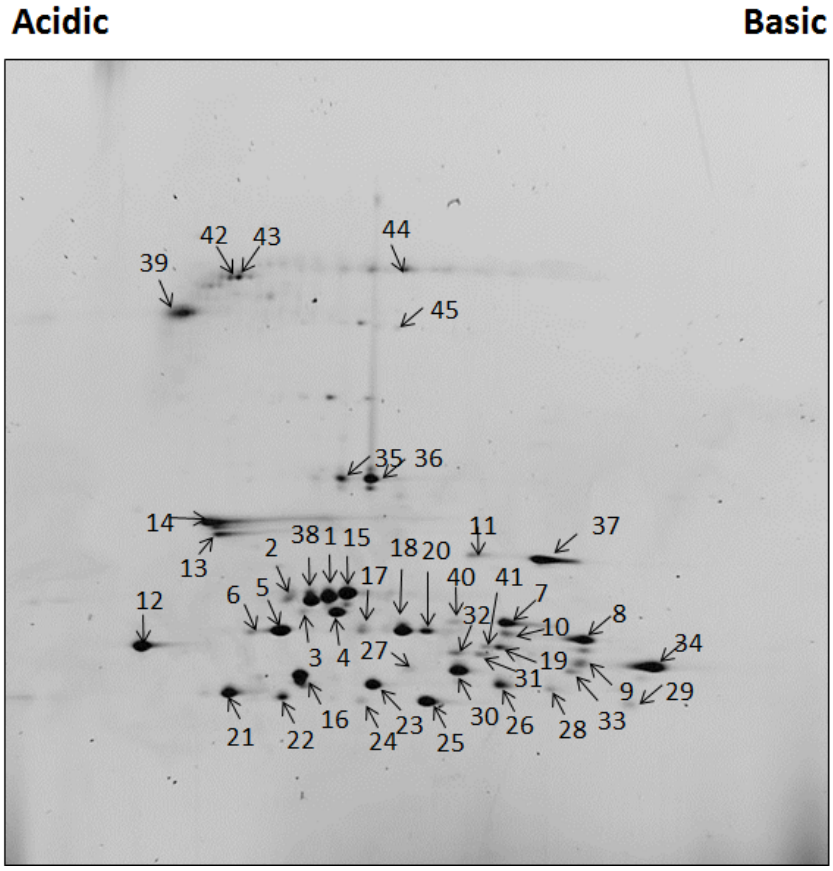
Acidic
Basic
42
43
44
39
45
35
36
14
38
1
15
11
37
2
18
20
13
17
40
41
7
6
5
12
32
8
10
34
3
4
27
19
31
9
29
16
23
30
26
28
33
21
22
24
25

Figure 3-2.

Figure 3-2. 2-DE and Flamingo gel staining of the purified 20S proteasome. Arabidopsis-purified 20S proteasome subunits were separated on a 18 cm 2-DE gel with a broad IPG strip pI [3-10] for 1-DE, and on a 12.5% SDS-PAGE gel for 2-DE. Separated proteins were detected by Flamingo gel staining. Forty-five spots were observed and excised for subsequent MS analysis. The figure represented pI [4.5-8] region.

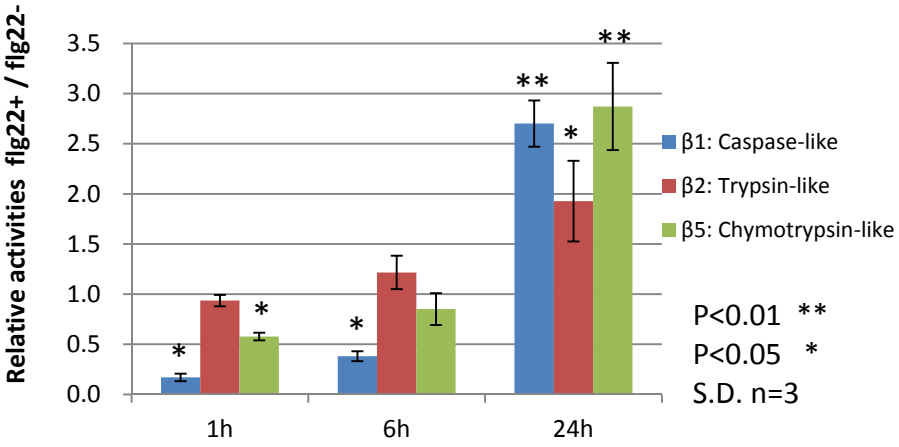
Relative activities flg22+ / flg22-
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1h
6h
24h
β1: Caspase-like
β2: Trypsin-like
β5: Chymotrypsin-like
P<0.01 **
P<0.05 *
S.D. n=3

Figure 3-3.

Figure 3-3. Measurement of proteasome peptidase activity in response to flg22 treatment.

Caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) proteasome activities were analyzed using 50 μM of the substrates Z-LLE-AMC, Boc-FSR-AMC and Suc-LLVY-AMC, respectively. Total proteins isolated from *PBF1-FLAG* MM2d cells treated with 100 nM flg22 for 1, 6 and 24 hours; water was used as a control. Error bars represent SD, n=3. Student's *t*-test was carried out to determine if there was a statistically significant difference in proteasome activity between flg22-treated and control cells. *, $P<0.05$; **, $P<0.01$.

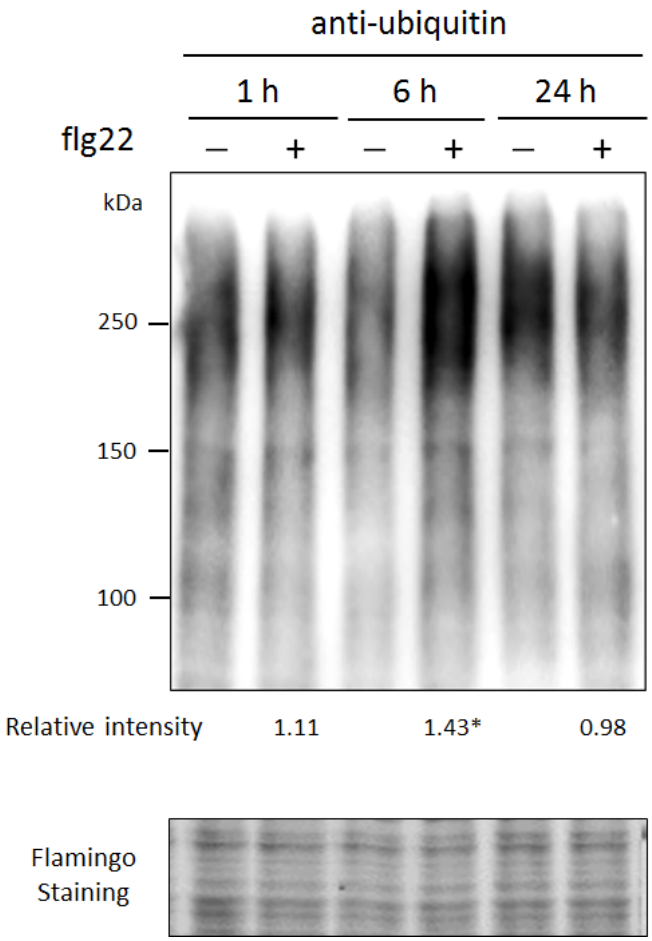
anti-ubiquitin
1 h
6 h
24 h
flg22
– + – + – +
kDa
250
150
100
Relative intensity
1.11
1.43*
0.98
Flamingo
Staining

Figure 3-4.

Figure 3-4. Accumulation of polyubiquitinated proteins in response to flg22 treatment.
Polyubiquitinated proteins in crude extracts were detected with an anti-ubiquitin antibody from *PBF1-FLAG* MM2 cells treated with 100 nM flg22 for 1, 6 and 24 hours (upper panel). Equal protein loading was confirmed by Flamingo gel staining (lower panel). The relative intensity of polyubiquitinated protein bands after flg22 treatment compared to mock treatment (water) sample was designated as 1.00. The signal intensity of ubiqutinated protein was normalized to total protein. Student's *t*-test was carried out to determine if there was a statistically significant difference in proteasome activity between flg22-treated and mock-treated samples. *, $P<0.05$.

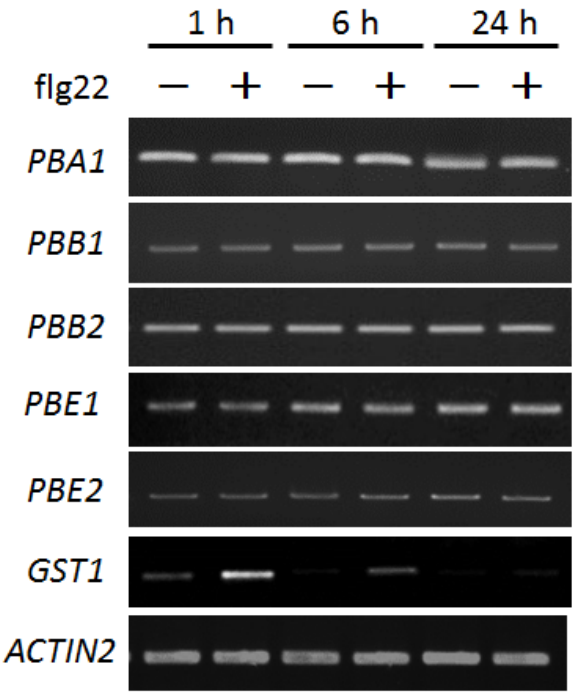

Figure 3-5.

Figure 3-5. Transcript level of each peptidase subunit genes in response to flg22.
Total RNA extracted from *PBF1-FLAG* MM2d cells 1, 6 and 24 hours after treatment with 100 nM flg22 was purified, followed by RT-PCR analysis of each mRNA transcript. The transcript levels of the proteasome peptidase subunits genes of *PBA1*, *PBB1/2*, and *PBE1/2*, were checked with the appropriate primer set and PCR condition (Table S3-3 in the Supporting Information). *GST1* was chosen as a marker of flg22 treatment. *ACTIN2* was used as an internal control gene.

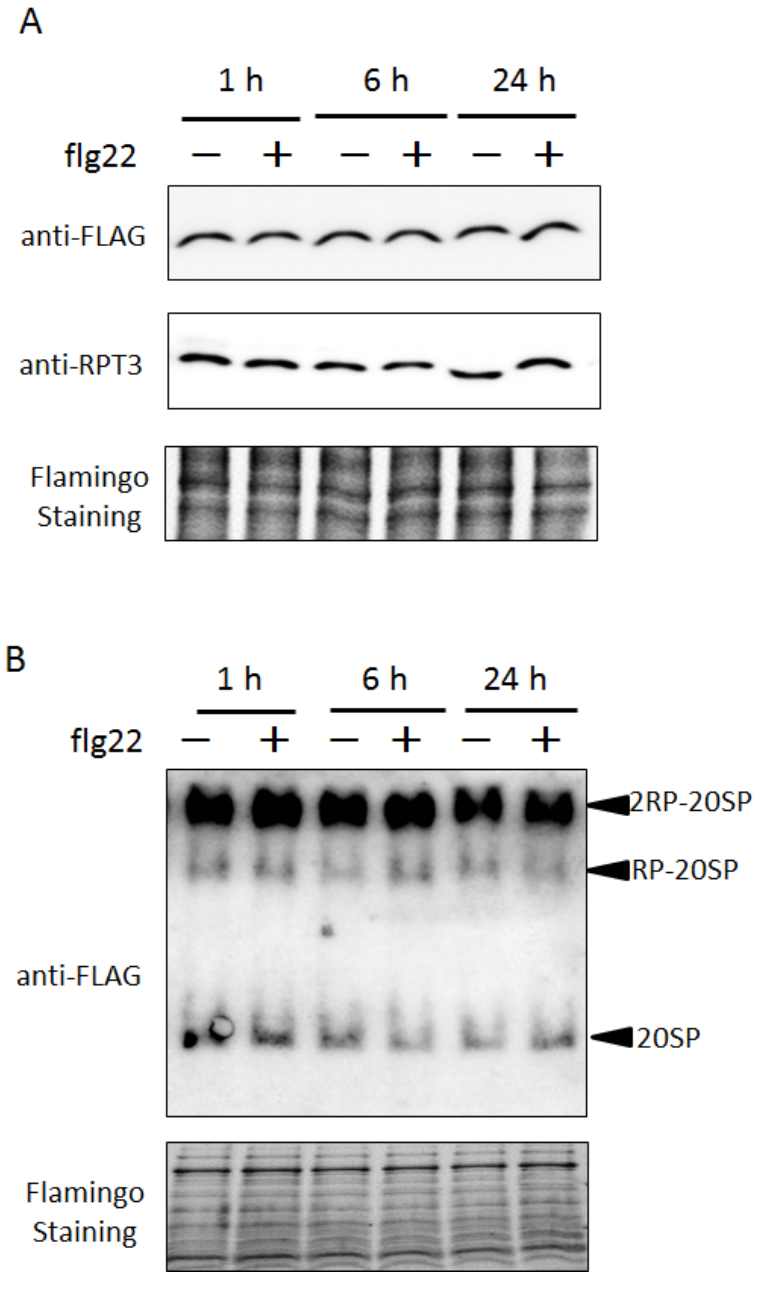
A
1 h
6 h
24 h
flg22
– + – + – +
anti-FLAG
anti-RPT3
Flamingo Staining
B
1 h
6 h
24 h
flg22
– + – + – +
2RP-20SP
RP-20SP
20SP
anti-FLAG
Flamingo Staining

Figure 3-6.

Figure 3-6. Proteasome protein levels in response to flg22 treatment.
(A) Proteasome subunit proteins in crude extracts were detected with anti-FLAG and anti-RPT3 antibodies in *PBF1-FLAG* MM2d cells treated with 100 nM flg22 for 1, 6 and 24 hours. Flamingo gel staining revealed equal protein loading.
(B) Total proteasome levels were detected by resolution on native-PAGE gels, followed by immunoblot analysis of the purified proteasome complex. Anti-FLAG antibody was used for immunoblotting (upper panel). Flamingo gel staining indicate equal amount of input proteins for purification (lower panel).

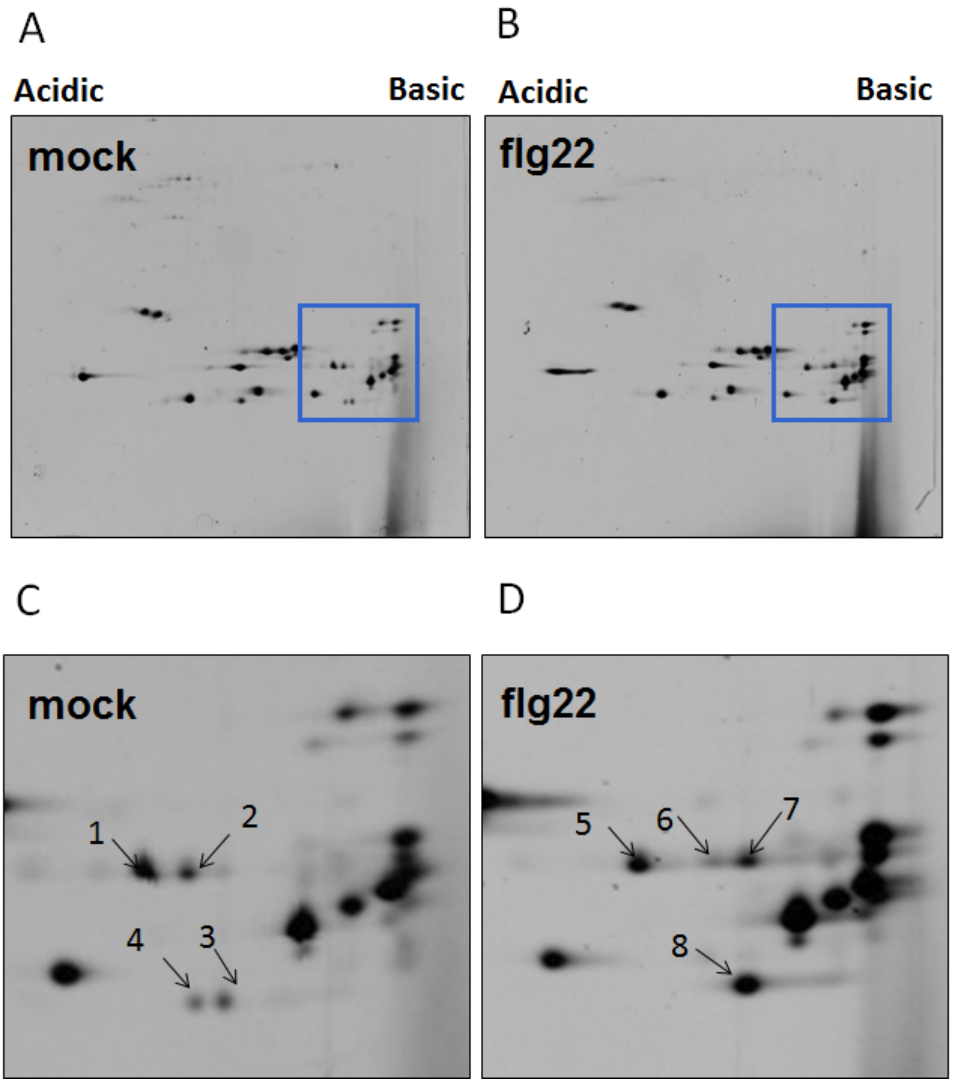
A
Acidic
Basic
mock
B
Acidic
Basic
flg22
C
mock
1
2
4
3
D
flg22
5
6
7
8

Figure 3-7.

Figure 3-7. 2-DE and Flamingo gel staining of the purified 20S proteasome after flg22 treatment.
(A)-(B) 20S proteasome complex was purified from *PBF1-FLAG* MM2d cells treated with water (mock) or 100 nM flg22 for 24 hours. Arabidopsis-purified 20S proteasome subunits were separated on a 13 cm 2-DE gel with a broad IPG strip pI [4-7] for 1-DE, and on a 12.5% SDS-PAGE gel for 2-DE. Separated proteins were detected by Flamingo gel staining (A: mock, B: flg22). (C)-(D) Photo of blue frame in (A) and (B) are shown in (C) and (D), respectively. Spots 1-8 were excised for subsequent MS analysis.

	Sample	Activity (pmol/mg/h)	Efficiency [a]
Input	WT	847.60±173.16	--
	PBF1-FLAG	774.34 ± 62.61	1.00
Purified	WT	n.d.	--
	PBF1-FLAG	515773.45 ± 83509.22	666.08**

Table 3-1.

Table 3-1. Chymotrypsin-like peptidase activity of the purified proteasome
Results are presented as the standard deviation (SD) of three independent experiments. n.d. means not detected.
a, Efficiency indicates relative peptidase activity of the purified proteasome compared to the crude extract (Input) in *PBF1-FLAG* MM2d cells. **, P<0.01

Subunit	AGI_ID [a]	Protein name	Spot No.[b]	Score [c]	MW, Da [d]
α-type					
α1	At5g35590	PAA1	1	1383	27,294
			2	243	27,294
			3	115	27,294
			4	198	27,294
			5	196	27,294
	At2g05840	PAA2	3	240	27,350
			4	437	27,350
			5	152	27,350
			17	46	27,350
α2	At1g16470	PAB1	5	432	25,701
			6	208	25,701
	At1g79210	PAB2	5	467	25,733
			6	163	25,733
α3	At3g22110	PAC1	7	357	27,475
			8	505	27,475
			9	391	27,475
			10	514	27,475
			33	36	27,475
	At4g15160	PAC2	n.d.		
α4	At3g51260	PAD1	11	458	27,337
	At5g66140	PAD2	n.d.		
α5	At1g53850	PAE1	12	555	25,947
	At3g14290	PAE2	12	508	25,977
α6	At5g42790	PAF1	13	313	30,476
			14	400	30,476
	At1g47250	PAF2	14	282	30,410
α7	At2g27020	PAG1	1	365	27,377
			2	73	27,377
			15	623	27,377
β-type					
β1	At4g31300	PBA1	16	486	25,151 (23,997)
β2	At3g27430	PBB1	17	102	28,817 (25,342)
			18	358	28,817 (25,342)
			19	112	28,817 (25,342)
	At5g40580	PBB2	19	134	29,617 (25,404)
			20	78	29,617 (25,404)

β3	At1g21720	PBC1	21	323	22,798
	At1g77440	PBC2	21	323	22,759
			22	181	22,759
β4	At3g22630	PBD1	23	378	22,541
	At4g14800	PBD2	24	27	21,984
			25	376	21,984
β5	At1g13060	PBE1	26	149	29,667 (26,059)
			27	143	29,667 (26,059)
			28	106	29,667 (26,059)
			29	166	29,667 (26,059)
			30	248	29,667 (26,059)
			31	156	29,667 (26,059)
	At3g26340	PBE2	28	137	29,485 (23,387)
			31	210	29,485 (23,387)
β6	At3g60820	PBF1	2	303	24,644
			32	191	24,644
β7	At1g56450	PBG1	9	88	27,651
			33	297	27,651
			34	527	27,651

Table 3-2.

Table 3-2. LC-MS/MS analysis of each 20S proteasome subunit separated by 2-DE after affinity purification.

a, Accession number of Arabidopsis genes.

b, Spots signed in Figure 3-2. n.d. means not detected.

c, Score of MASCOT software assigned to each identified protein after database searching.

d, Predicted molecular weight of detected proteins. The molecular weights of matured PBA1, PBB1/2 and PBE1/2 after N terminal proteolytic processing were represented in the brackets.

Spot No. [a]	AGI_ID [b]	Protein name	Score [c]	Peptide No. [d]	MW, Da [e]
1	AT3G27430	PBB1	161	2	28,817 (25,342)
2	AT5G40580	PBB2	77	1	29,617 25,404)
	AT3G27430	PBB1	76	1	28,817 (25,342)
3	AT4G14800	PBD2	75	3	21,984
4	AT4G14800	PBD2	74	4	21,984
5	AT3G27430	PBB1	178	2	28,817 (25,342)
6	AT3G27430	PBB1	106	2	28,817 (25,342)
	AT5G40580	PBB2	74	1	29,617 (25,404)
7	AT5G40580	PBB2	212	2	29,617 (25,404)
	AT3G27430	PBB1	105	2	28,817 (25,342)
8	AT4G14800	PBD2	1337	14	21,984
	AT3G22630	PBD1	726	8	22,541

Table 3-3.

Table 3-3. LC-MS/MS analysis of the 20S proteasome subunit showing specific alterations in response to flg22 treatment.

a, Spot number signed in Figure 3-7.

b, Accession number of Arabidopsis genes.

c, Score of MASCOT software assigned to each identified protein after database searching.

d, Number of detected peptide which has unique amino acid sequence between the paralogous subunits.

e, Predicted molecular weight of detected proteins. The molecular weights of matured PBB1/2 after N terminal proteolytic processing were represented in the brackets.

List of Supporting Information

Figure S3-1. *PBF1-FLAG* mRNA expression in transgenic MM2d cells

Figure S3-2. Protein amounts of proteasome subunits.

Figure S3-3. Preparation of recombinant RPT3 protein and anti-RPT3 antibody.

Figure S3-4. Preparation and detection of anti-20S CP antibody.

Figure S3-5. 2-DE/MS analysis of purified 20S CP proteasome subunits from Arabidopsis cells..

Figure S3-6. 2-DE/MS analysis of purified 20S CP proteasome subunits from Arabidopsis cells after flg22 treatment.

Figure S3-7. Transcripts level of *PBD1* and *PBD2* subunit genes in response to flg22 treatment.

Table S3-1. Arabidopsis chymotrypsin-like peptidase activity during proteasome affinity-purification..

Table S3-2. Arabidopsis 20S proteasome subunits splice variants.

Table S3-3. Transcription level check primer list.

Figures, Table and Legends of

Supporting Information

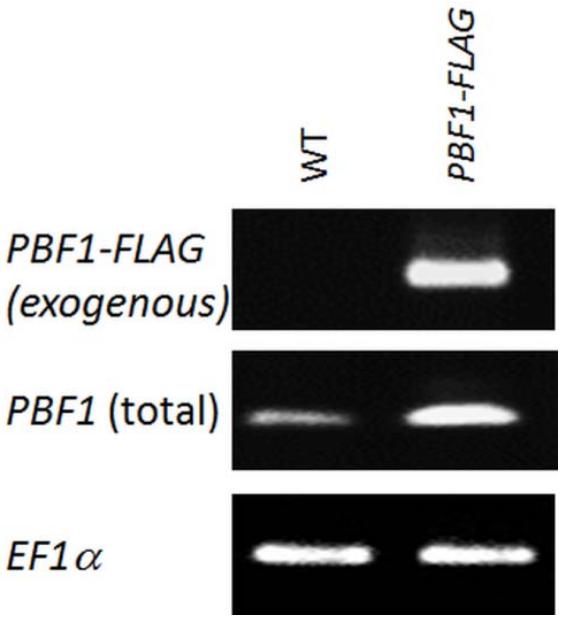

Figure S3-1.

Figure S3-1. *PBF1-FLAG* mRNA expression in transgenic MM2d cells
Total RNA was isolated from WT and *PBF1-FLAG* MM2d cells. Expression of exogenous *PBF1-FLAG* and total *PBF1* mRNA were analyzed with primers of *PBF1* forward and *FLAG* reverse; *PBF1* forward and *PBF1* reverse, respectively (Table S3-3 in the Supporting Information). *EF1*α was used as the internal control.

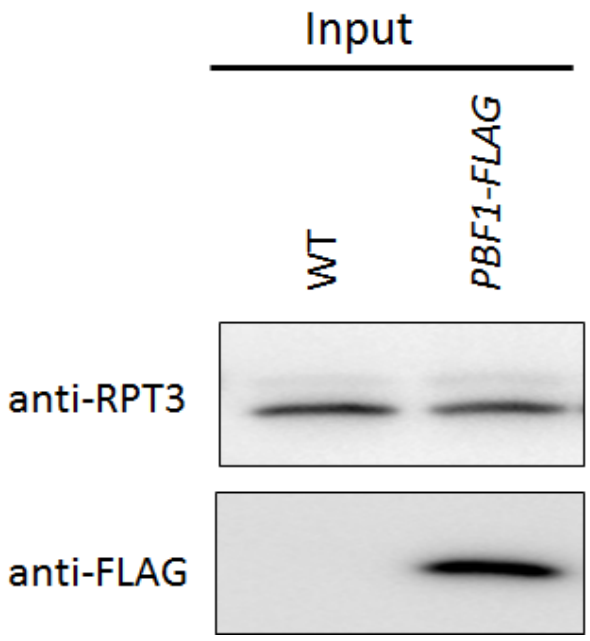

Figure S3-2.

Figure S3-2. Protein amounts of proteasome subunits.
Total input proteins were extracted from WT and *PBF1-FLAG* MM2d cells. Check protein concentration with Biorad protein assay kit, same amount input proteins were applied for immunoblotting. RPT3 and PBF1-FLAG protein amount of WT and *PBF1-FLAG* MM2d cells were detected with anti-RPT3 antibody and anti-FLAG antibody.

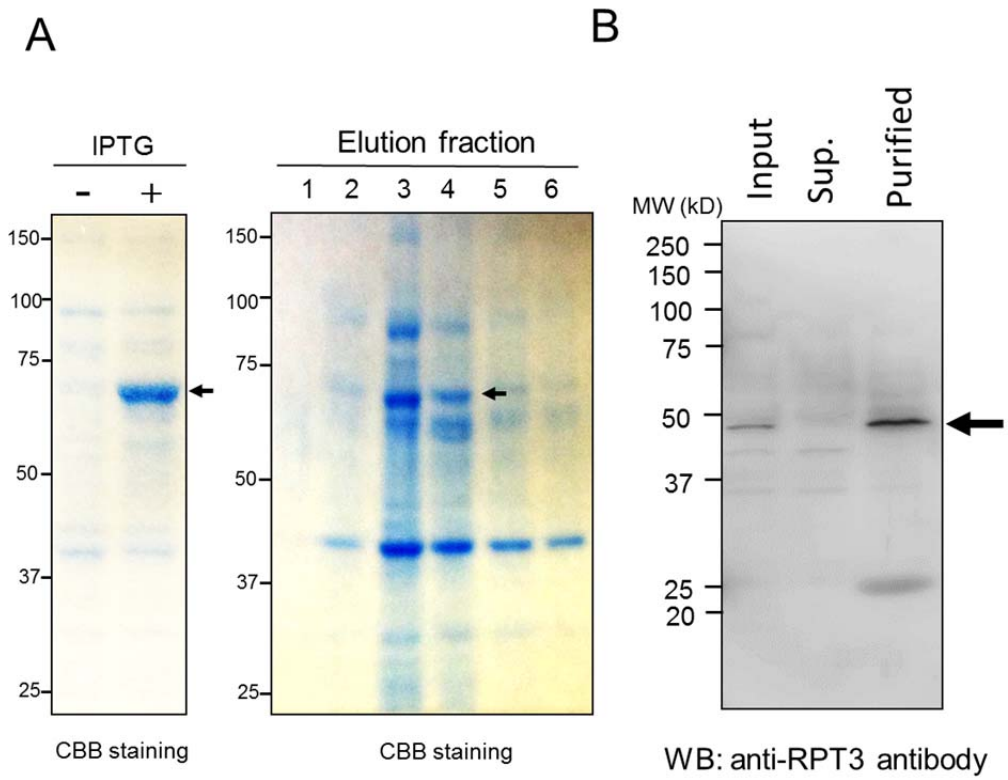
A
IPTG
-
+
150
100
75
50
37
25
CBB staining
Elution fraction
1 2 3 4 5 6
150
100
75
50
37
25
CBB staining
B
MW (kD)
Input
Sup.
Purified
250
150
100
75
50
37
25
20
WB: anti-RPT3 antibody

Figure S3-3.

Figure S3-3. Preparation of recombinant RPT3 protein and anti-RPT3 antibody.

(A) CBB staining of crude extract protein from IPTG-inducted *E. coli strain BL21 (DE3) pLysS* (Left panel) and purified Txr-His-RPT3 in each eluted fraction (right panel). Arrow indicates the position of Trx-His-RPT3.

(B) Immunoblotting by anti-RPT3 antibody with crude extract and purified proteasome from Arabidopsis cells. Arrow indicates the position of intact RPT3 protein.

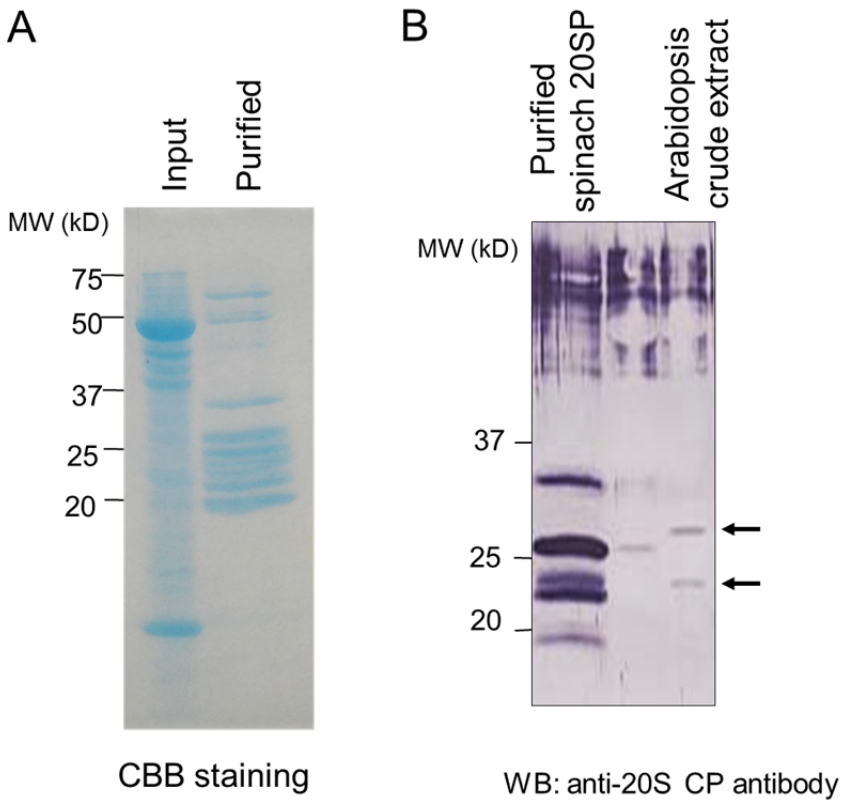
A
Input
Purified
MW (kD)
75
50
37
25
20
CBB staining
B
Purified spinach 20SP
Arabidopsis crude extract
MW (kD)
37
25
20
WB: anti-20S CP antibody

Figure S3-4.

Figure S3-4. Preparation and detection of anti-20S CP antibody.
CBB staining of input protein and purified proteasome from spinach leaves.
(A) CBB staining of input and purified spinach 20S CP proteasome.
(B) Immunoblotting by anti-20S CP antibody with purified spinach 20S CP proteasome and crude extract from Arabidopsis cells. Arrow indicates the position of intact Arabidopsis 20S CP proteins.

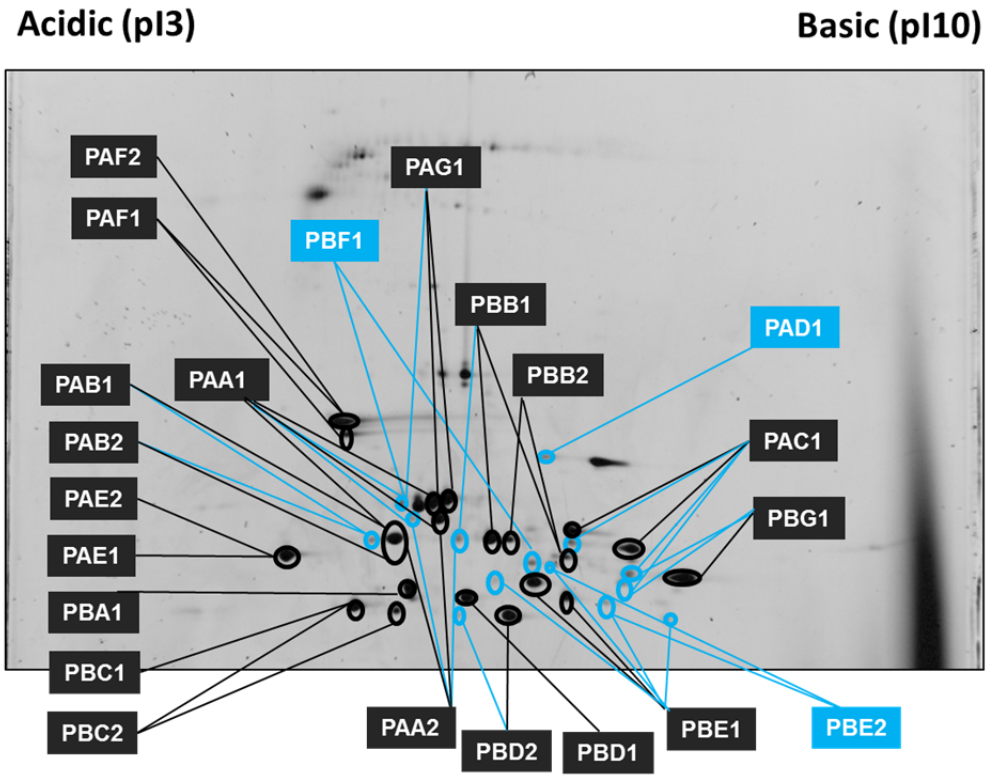
Acidic (pI3)
Basic (pI10)
PAF2
PAF1
PAG1
PBF1
PBB1
PAD1
PAB1
PAA1
PBB2
PAB2
PAC1
PAE2
PBG1
PAE1
PBA1
PBC1
PBC2
PAA2
PBD2
PBD1
PBE1
PBE2

Figure S3-5.

Figure S3-5. 2-DE/MS analysis of purified 20S CP proteasome subunits from Arabidopsis cells.
The description of each spot was consistent with Figure 3-2 and Table 3-2. Each spot was pointed with Arabidopsis 20S proteasome subunit name, not detected spots were not included in this figure. Relatively large amount subunits were pointed with black line and circle; small amount subunits were marked with blue line and circle. 2-DE was conducted using a broad IPG strip pI [3-10] for 1-DE, and on a 12.5% SDS-PAGE gel for 2-DE.

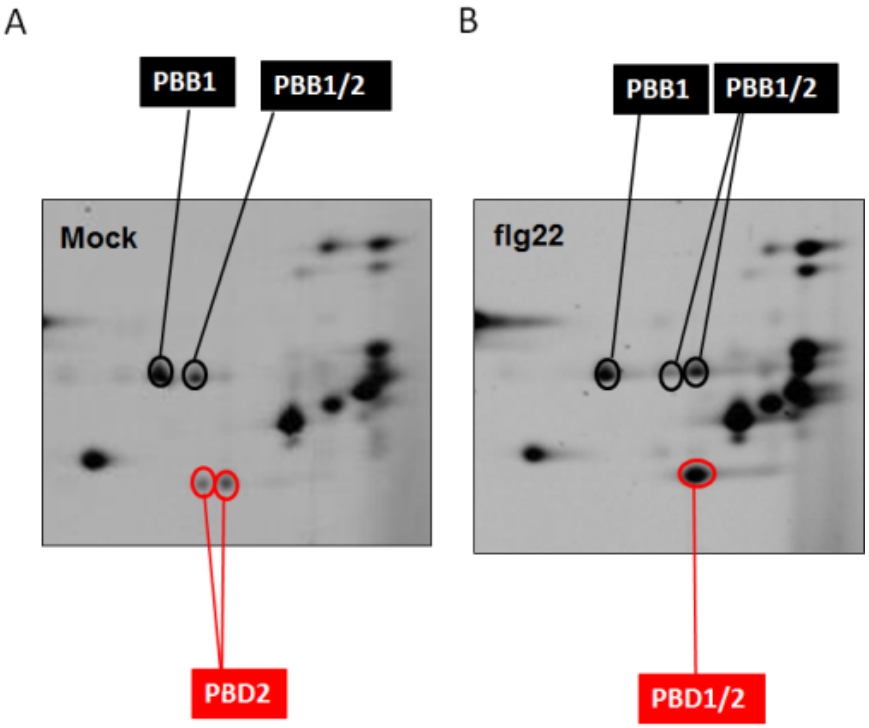

Figure S3-6.

Figure S3-6. 2-DE/MS analysis of purified 20S CP proteasome subunits from Arabidopsis cells after flg22 treatment.
The description of each spot was consistent with Figure 3-7 C, D and Table 3-3.
Each spot was pointed with Arabidopsis 20S proteasome subunit name.
2-DE was conducted using a broad IPG strip pI [4-7] for 1-DE, and on a 12.5% SDS-PAGE gel for 2-DE.

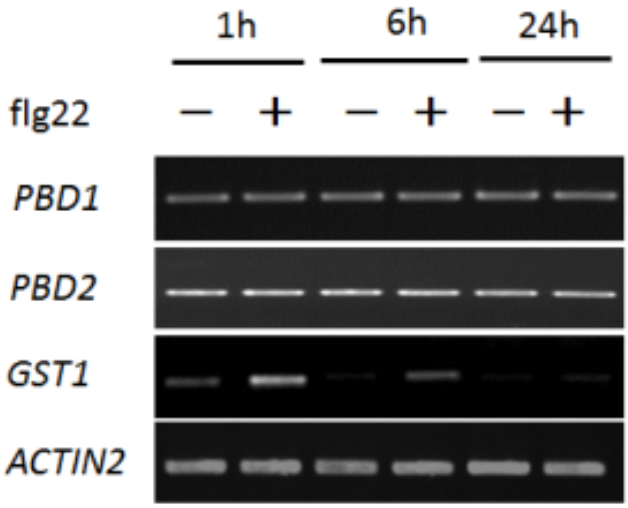

Figure S3-7.

Figure S3-7. Transcripts level of *PBD1* and *PBD2* subunit genes in response to flg22 treatment.
Total RNA from *PBF1-FLAG* MM2d at 1, 6 and 24 hours after treatment by 100 nM flg22 was purified and RT-PCR analysis of each mRNA transcript was performed. Proteasome subunits genes *PBD1* and *PBD2* transcription levels were checked with appropriate primer set and PCR condition (Table S3-3 in the Supporting Information). *GST1* was chosen as the marker of flg22 responsible gene. *ACTIN2* was an internal control gene.

Step [a]	Concentration (mg/ml)	Vol. (ml)	Total protein (mg)	AMC (pmol) [b]	Specific peptidase activity (pmol/liter/min/μg)	Purification (fold) [c]
Crude extract	4.315	290	1251	49.169	0.019	1.000
Ammonium sulfate prep.	18.960	11	208	10.885	0.026	1.368
DEAE	4.598	35	160	27.854	0.086	4.526
Hydroxyapatite	0.458	15	6.870	21.280	1.548	81.474
Poros HQ/L	0.141	4	0.564	4.415	3.900	205.263

Table S3-1.

Table S3-1. Arabidopsis chymotrypsin-like peptidase activity during proteasome affinity-purification.

a, Steps of Arabidopsis 20S proteasome purification refer to (Iwafune *et al*., 2002).

b, Catalyzed Suc-LLVY-AMC substrate amount (pmol) by each protein fraction during purification.

c, Proteasome purification efficiency of each step; the efficiency of crude extract was set as 1.000, other efficiency was calculated by specific peptidase activity of each step dividing specific peptidase activity of crude extract.

Protein	AGI_ID [a]	Length (aa) [b]	MW (Da) [c]	Isoelectric point
PAA1	AT5G35590.1	246	27,294	5.66
PAA2	AT2G05840.1	246	27,350	5.97
	AT2G05840.2	220	24,456	6.68
PAB1	AT1G16470.1	235	25,701	5.39
	AT1G16470.2	235	25,701	5.39
PAB2	AT1G79210.1	235	25,733	5.39
	AT1G79210.2	235	25,733	5.39
	AT1G79210.3	235	25,733	5.39
PAC1	AT3G22110.1	250	27,475	7.14
PAC2	AT4G15160.1	451	48,139	9.05
PAD1	AT3G51260.1	250	27,337	7.50
	AT3G51260.2	243	26,569	7.50
PAD2	AT5G66140.1	250	27,324	8.96
PAE1	AT1G53850.1	237	25,947	4.41
	AT1G53850.2	237	25,947	4.41
PAE2	AT3G14290.1	237	25,977	4.41
PAF1	AT5G42790.1	278	30,476	4.73
PAF2	AT1G47250.1	277	30,410	4.72
PAG1	AT2G27020.1	249	27,377	6.26
PBA1	AT4G31300.1	233	25,151	5.24
	AT4G31300.2	234	25,279	5.24
	AT4G31300.3	233	25,151	5.24
PBB1	AT3G27430.1	267	28,812	8.95
	AT3G27430.2	273	29,555	7.15
	AT3G27430.3	267	28,897	6.86
PBB2	AT5G40580.1	274	29,617	7.20
	AT5G40580.2	274	29,617	7.20
	AT5G40580.3	268	28,959	6.93
PBC1	AT1G21720.1	204	22,798	5.10
PBC2	AT1G77440.1	204	22,759	5.70
	AT1G77440.2	204	22,759	5.70
PBD1	AT3G22630.1	204	22,540	6.36

PBD2	AT4G14800.1	199	21,984	6.68
	AT4G14800.2	215	23,827	6.02
PBE1	AT1G13060.1	274	29,667	6.40
	AT1G13060.2	298	32,344	6.72
PBE2	AT3G26340.1	273	29,485	6.16
PBF1	AT3G60820.1	223	24,644	7.49
	AT3G60820.2	223	24,685	7.95
	AT3G60820.3	223	24,644	7.49
PBG1	AT1G56450.1	246	27,651	6.52

Table S3-2.

Table S3-2. Arabidopsis 20S proteasome subunits splice variants.
Data collected from Arabidopsis genome database TAIR.
a, The accession number of Arabidopsis genes.
b, Amino acid number of each protein.
c, Predicted molecular weight of each proteins.

Gene	Primers	
PBA1	5’-3’ Forward:	TAGTCGAGCAACCGTTTGCCA
	5’-3’ Reverse:	CATGGCCATTGGTTCAGGA
PBB1	5’-3’ Forward:	CACTGGTCCACATCTGCATAC
	5’-3’ Reverse:	TTCCTCCATAGCTTCACCAACT
PBB2	5’-3’ Forward:	TACCGGGCCTCATCTTCACA
	5’-3’ Reverse:	TAGTATCTGTCTCTCACACG
PBD1	5’-3’ Forward:	TGGTGACCGGGTTCAGTTTAC
	5’-3’ Reverse:	AGTGTCAAAACACTAACTCCCGAC
PBD2	5’-3’ Forward:	TGGTGACCGGGTTCAGTTTAC
	5’-3’ Reverse:	CAGAGAATATAAATGTGAGCCGAG
PBE1	5’-3’ Forward:	AAAGACATGTTGAAGCATGCAAAA
	5’-3’ Reverse:	GCAACAACAAGAAAGGGAGT
PBE2	5’-3’ Forward:	CGTCTACATGAGCTGGCAAAC
	5’-3’ Reverse:	TTCGGCAGCCTCTTCCATCA
PBF1	5’-3’ Forward:	GCTCATACGAGAGAGTTGGATA
	5’-3’ Reverse:	GTCTTTCCTCAGGTCCATGAG
FLAG	5’-3’ Reverse:	CTTGTCATCGTCATCCTTGTAGT
GST1	5’-3’ Forward:	CTCAGACAAAGGAAACAACCTT
	5’-3’ Reverse:	AAGAACCTTCTGAGCAGAAGG
ACTIN2	5’-3’ Forward:	AAACCTCAAAGACCAGCTCTTC
	5’-3’ Reverse:	AACGATTCCTGGACCTGCCT
EF1α	5’-3’ Forward:	GCACAGTCATTGATGCCCCA
	5’-3’ Reverse:	CCTCAAGAAGAGTTGGTCCCT

Table S3-3.

Table S3-3. Transcription level check primer list.

Chapter 4

General Discussion

Studies on proteasome RPT2a subunit function and sugar stress

To elucidate proteasome function in response to abiotic sugar stress, 19S RP RPT2a subunit was selected. RPT2a function has been characterized in the aspects of gametogenesis, leaf size regulation and innate immunity; whereas RPT2a function of sugar response has not been well known.

In Chapter 2, I clarified RPT2a function participating in sugar response. Firstly, *RPT2a* gene transcription level was significantly increased compared with the other *RPT* genes under gradient sucrose concentration. Moreover comparison with WT and *rpt2b* mutant, *rpt2a* mutant showed hypersensitivity to high sucrose stress, such as suppression of cotyledon expansion and root elongation. In order to exclude chemical specificity, glucose was applied instead of sucrose and the results were consistent with sucrose data. Additionally 2% and 6% mannitol were used to observe growth status of WT and *rpt2a* mutant, which uncovered that growth arrest of *rpt2a* mutant, was caused by high sugar substrates but not because of osmotic stress. Sugar is a bifunctional compound as metabolism substrate and signalling molecular (Sheen *et al.,* 1999; Smeekens 2000); sugar and phytohormone are closely connected to regulate plant growth and development, for example sugar can affect abscisic acid (ABA) biosynthesis and signalling through HXK (hexokinase) (Rolland *et al.,* 2006). ABA sensitivity assay indicated that RPT2a participates in an ABA-independent sugar response pathway. However, in the case of other plant growth hormones like cytokinine and auxin, further investigation are needed to elucidate “how does RPT2a regulate sugar response?” and “which signalling pathway is involved in RPT2a function of sugar regulation?”. In addition, poly-ubiquitinated proteins were slightly accumulated in *rpt2a* mutant under 6% sucrose compared with WT, which could be explained by the existence of paralogous subunit RPT2b. RPT2b might complement to construct 26S proteasome complex to maintain proteolysis capacity. Taken together, the above findings indicate that RPT2a plays an essential role in response to sugar stress.

Studies on proteasome function and immune response

Proteasome immune function was well characterized accompanying the study of E3 ligases; however, proteasome subunit self-function in response to immune response was poorly understood.

In Chapter 3, I elucidated proteasome function in response to biotic pathogen derived MAMP flg22 stress. Firstly, *PBF1-FLAG* MM2d transgenic cells were produced as experiment material, which was demonstrated to be an appropriate sample to purify proteasome efficiently without influence on proteasome activity. Secondly, 20S proteasome subunit composition was detected with affinity purification and combination of 2-DE/MS analyses. Interestingly most 20S proteasome subunits exhibit multiply spots pattern, for example proteasome peptidase PBE1 subunit contained six spots, which indicated largely heterogenous proteasome were existed in Arabidopsis cells. In yeast proteasome subunits staining with Pro-Q diamond and followed with CBB were conducted, the results indicated same subunits containing phosphorylated form and un-phosphorylated form at the same time (Kikuchi *et al.,* 2010). Consider of above results, I proposed some unknown post-translational modification were happened in Arabidopsis proteasome subunits to lead diversiform presence patterns. Another amazing founding was paralogous subunits showed distinguished protein level, for instance, PBE1 amount was much larger than PBE2, which suggested that there were more PBE1-associated proteasome complex than PBE2-associated proteasome, and also indicate that PBE1 might play major function in Arabidopsis proteasome. Moreover, proteasome caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) activities were affected by flg22 treatment. To look for the cause, I checked mRNA level of proteasome peptidase subunits genes *PBA*, *PBB1/2* and *PBE1/2,* but their transcription level didn't change. At the same time proteasome amount detection also showed no influence. Using 2-DE/MS analyses several intriguing shifted spots were determinated after flg22 treatment, one was peptidase subunit PBB (trypsin-like activity) and the other was subunit PBD. Above results indicate some unknown post-translational modifications were induced by flg22 signalling pathway. Taken together, proteasome function and activity are regulated by specific subunit composition and putative post-translational modification in response to flg22 biotic stress.

Perspectives

In this dissertation proteasome function in response to abiotic sugar stress and biotic pathogen-derived MAMP flg22 stress were elucidated, whereas several subjects remain to be revealed.

During sugar stress study at the first, one crucial question need be clarified

is “why *RPT2a* gene was transcriptionally specific increase rather than *RPT2b* gene respond to high sugar stress?” Arabidopsis paralogous subunits RPT2a and RPT2b just have three amino acids difference (Sonoda *et al.,* 2009), but they play different functions. For instance, comparison of *rpt2b* mutant, *rpt2a* mutant is not only hypersensitive to high sugar stress but also exhibits enlarged leave size (Sonoda *et al.,* 2009; Sako *et al.,* 2010). The phenomenon of *RPT2a* gene as transcriptional promotion aroused two doubts: is this because of different promoter sequence between *RPT2a* gene and *RPT2b* gene? Or is this due to sugar-related transcription factor, which specifically controls *RPT2a* gene mRNA transcription? To answer these questions studies of RPT2a promoter and RPT2a-related sugar inducible transcription factor might be required.

In parallel, another intriguing question need to be revealed is “what is the presence status of RPT2a in response to sugar stress?” There are two hypotheses of RPT2a existence status under high sugar stress: one is that RPT2a appears as a free subunit, the other is that the RPT2a exists as proteasome-associated complex. Preliminary experiment showed that RPT2a mainly presents in proteasome complex using immunoblot with sucrose density gradient centrifugation fractions under normal growing conditions. But I cannot confirm that the RPT2a also appears as associated form under high sugar growing conditions. In the case of RPT2a appearance as proteasome complex, further understanding of proteasome assembly mechanisms is suggested, which chaperon is induced by sugar to be involved in the assembly of RPT2a-associated proteasome complex. In the case of free RPT2a form, I expect some phytohormone signaling pathway is involved in RPT2a to be regulated by sugar response. Further work is necessary to investigate hormone-related signalings.

During proteasome and flg22 study, putative post-translational modification was proposed to regulate Arabidopsis proteasome subunit composition and activity in response to flg22 biotic stress. In yeast and mammals, proteasome post-translational modifications were largely identified, such as phosphorylation (kikuchi *et al.,* 2010), oxidation (Ishii *et al.,* 2005), ubiquitination (Peng *et al.,* 2003), acylation (Kikuchi J *et al.,* 2010) and others. Above modifications were demonstrated to affect proteasome activity. Guo *et al,* 2011 uncovered the first proteasome-specific phosphatase UBLCP1 in yeast, which clearly uncovered phosphorylation was involved in regulation of proteasome activity in yeast. In Arabidopsis proteasome post-translational

modifications like oxidation and ubiquitination were determinated (Book *et al.,* 2010) although the functions were not well known. Book *et al*. 2010 tried but failed to detect phosphorylation modification using HPLC-ESI-MS/MS (high performance liquid chromatograph-electrospray ionization-MS/MS) with HCD (high energy collision dissociation) and ETC (electron transfer dissociation) methods; I also performed Arabidopsis proteasome subunits phosphorylation detection with 30min flg22 treated cells using LC-MS/MS. However, I did not succeed to find any phosphorylated subunits (data not show). All in all Arabidopsis proteasome subunits phosphorylation detection need more sensitive methods and phosphopeptide enrichment with metal affinity chromatography usages of Phos-Tag Agarose or Titansphere Phos-TiO kit were suggested.

As the other question, the mechanism of proteasome activity affected by oxidative stress induced by flg22 is still unclear. flg22 can activate ROS burst through plasma membrane-associated NADPH oxidases, especially Rboh D and Rboh F (respiratory burst oxidase homologs D, F) are found essential for ROS production (Torres *et al*., 2002 and 2005). In this dissertation *GST1* was chosen as flg22 response marker, at the same time GST1 is an antioxidant to clear ROS toxics (Mhamdi *et al.,* 2012), so the transcription level of *GST1* is also a good symbol of oxidative stress. During flg22 treatment *GST1* transcription level was promoted at 1 h and 6 h and abolished at 24 h. I proposed oxidative stress was existent at early 6 h flg22 treatment and was not apparent at late 24 h flg22 treatment. In mammals and yeast oxidation of proteasome decrease proteasome activity are reported. In human Rpt3 was observed to contain large carbonyl levels after oxidative treatment, and 19S proteasome ATPase activity was reduced because of oxidized Rpt3 (Ishii *et al.,* 2005). Carrard *et al*. reported oxidation is related to the inhibition of protease activity of the mammal proteasome (Carrard *et al.,* 2002). In my result proteasome activity (caspase-like and chymotrypsin-like) were decreased before 6h flg22 treatment and activities of (caspase-like, trypsin-like and chymotrypsin-like) were increased at 24h flg22 treatment. The peptidase activity change pattern was consistent with the change of *GST1* transcription level. The results putatively suggested oxidation stress could decrease proteasome activity in Arabidopsis suspension cells. But more experiments are required to detect oxidative stress induced by flg22, such as H_2O_2 amount, oxidized protein accumulation, GSH/GSSG (reduced glutathione / oxidized glutathione) ratio and others.

Finally, it would be expected that further experiments can reveal

mechanisms of proteasome function and activity regulation through subunit composition and putative post-translational modification in response to environmental stress.

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List of Publications

(1) Huihui Sun, Kaori Sako, Yuya Suzuki, Shugo Maekawa, Shigetaka Yasuda, Yukako Chiba, Takeo Sato, Junji Yamaguchi. (2012) Sugar-inducible RPT2a, a subunit of 26S proteasome, participates in sugar response in Arabidopsis. *Plant Biotechnology*, **29**, 279–284.

(2) Hui H. Sun, Yoichiro Fukao, Sakiko Ishida, Hiroko Yamamoto, Shugo Maekawa, Masayuki Fujiwara, Takeo Sato and Junji Yamaguchi. 2-DE and LC-MS/MS analysis revealed highly heterogenous proteasome composition and post-translational regulation of peptidase activity under pathogen signal in plants. **Revising.**

List of Publications (Appendix)

(1) Liping Hu, Huihui Sun, Ruifu Li, Lingyun Zhang, Shaohui Wang, Xiaolei Sui, Zhenxian Zhang (2011) Phloem unloading follows an extensive apoplasmic pathway in cucumber (Cucumis sativus L.) fruit from anthesis to marketable maturing stage. *Plant Cell Environ.* **34**, pp.1835–1848.