Plant Cell Rep (2010) 29:249–259
DOI 10.1007/s00299-010-0818-8

ORIGINAL PAPER

Arabidopsis sensitivity to protein synthesis inhibitors depends on 26S proteasome activity

Jasmina Kurepa · Consolée Karangwa · Liliana Sfichi Duke · Jan A. Smalle

Received: 24 December 2009 / Revised: 3 January 2010 / Accepted: 7 January 2010 / Published online: 20 January 2010

Abstract The 26S proteasome (26SP), the central protease of the ubiquitin-dependent proteolysis pathway, controls the regulated proteolysis of functional proteins and the removal of misfolded and damaged proteins. In Arabidopsis, cellular and stress response phenotypes of a number of mutants with partially impaired 26SP function have been reported. Here, we describe the responses of proteasome mutants to protein synthesis inhibitors. We show that the *rpt2a-3, rpn10-1* and *rpn12a-1* mutants are hypersensitive to the antibiotic hygromycin B, and tolerant to the translation inhibitor cycloheximide (CHX) and herbicide L-phosphinothricin (PPT). In addition to the novel mechanism for herbicide tolerance, our data suggests that the combination of hygromycin B, CHX and PPT growth-response assays could be used as a facile diagnostic tool to detect altered 26SP function in plant mutants and transgenic lines.

Keywords *Arabidopsis thaliana* · Cycloheximide · Herbicide tolerance · Hygromycin B · L-Phosphinothricin · Proteolysis

Introduction

The ubiquitin-proteasome system (UPS) regulates the degradation of most cellular proteins. It controls the proteolysis of key components of numerous signaling pathways, and thus regulates many—if not all—cellular processes (Smalle and Vierstra 2004; Vierstra 2009). The UPS is also essential for the removal of misfolded proteins that are generated either by mutations, translational errors or by the actions of cellular stressors (Goldberg 2003). These non-functional proteins need to be removed from the cell before they become proteotoxic, i.e., before they accumulate, aggregate and induce cell death. The main components of the UPS are ubiquitin (Ub), the enzymes that catalyze the attachment of Ub chains to target proteins, the enzymes that catalyze the hydrolysis of Ub chains from target proteins and finally, the 26S proteasome, a multi-subunit, multicatalytic protease that degrades the poly-ubiquitinated protein targets (Smalle and Vierstra 2004; Vierstra 2009). The 26S proteasome (26SP) is composed of two subcomplexes: the proteolytically active 20S proteasome (20SP) and the regulatory particle (RP) that recognizes, unfolds and channels most of the UPS targets into the 20SP for degradation (Kurepa and Smalle 2008).

Protein synthesis and protein degradation by the 26SP are the two main phases in the life cycle of many proteins, and they are networked at a number of levels (Chuang et al. 2005; Guerrero et al. 2008; Reits et al. 2000; Schubert et al. 2000). On the genomic level, we see a footprint of the link between translation and degradation in the structure of genes encoding Ub itself (Finley et al. 1989). In Arabidopsis for instance, from 14 Ub genes, five encode a single Ub peptide fused to a ribosomal subunit (Callis et al. 1995, 1990). Another link between translation and the UPS is inferred from the ubiquitination of many components of the translation machinery such as ribosomal subunits, ribosome associated proteins, and several translational initiation and elongation factors (Saracco et al. 2009; Strunk and Karbstein 2009). Proteasome-mediated co-translational proteolysis is

Communicated by J. Register.

J. Kurepa · C. Karangwa · L. S. Duke · J. A. Smalle (✉)
Plant Physiology, Biochemistry, Molecular Biology Program,
Department of Plant and Soil Sciences,
College of Agriculture, University of Kentucky,
1401 University Drive, Lexington, KY 40546-0236, USA
e-mail: jsmalle@uky.edu

another example of the complex cross-talk between translation and degradation. In co-translational proteolysis, proteins that are translated erroneously are labeled with Ub while emerging from the ribosomes, and are immediately degraded by the 26SP. This is a continuous process, and it has been estimated that it degrades up to 50% of newly synthesized proteins (Chuang et al. 2005; Guerrero et al. 2008; Reits et al. 2000; Schubert et al. 2000).

The first studies to reveal the link between translation and 26SP-dependent proteolysis at the organismal level described the isolation and mapping of *S. cerevisiae* cycloheximide resistant *crl* mutants (Gerlinger et al. 1997; McCusker and Haber 1988). The *crl* mutants, which are resistant to a minimum inhibitory concentration of the translation inhibitor cycloheximide (CHX) (McCusker and Haber 1988), carry mutations in 20SP and RP subunit genes (Gerlinger et al. 1997). The authors proposed that cells have a hypothetical protein-synthesis-rate sensor that is constitutively degraded by the proteasome. Protein synthesis inhibition would cause the level of this sensor to decrease below a critical threshold, resulting in growth arrest. In proteasome mutants, this critical threshold would be reached more slowly compared to the wild type, and that would lead to an improved CHX tolerance. Hanna et al. (2003) suggested that the hypothetical translation-rate sensor might be Ub. These authors showed that overexpression of Ub leads to an increase in CHX tolerance. Furthermore, treatment with a high dose of CHX, which was designed to maximally suppress protein synthesis, revealed a reduced depletion rate of the Ub monomer in a proteasome mutant background (Hanna et al. 2003). This study also showed that Ub overexpression leads to increased tolerance to the protein synthesis inhibitor hygromycin B (Hyg). Yeast proteasome mutants, on the other hand, have been shown to be Hyg hypersensitive (McCusker and Haber 1988). Hyg, similarly to other aminoglycoside antibiotics, induces misreading of aminoacyl-tRNA by distorting the ribosomal A site but also leads to the aberrant translocation of mRNAs (Cabañas et al. 1978a, b). Therefore, Hyg also increases the rate of translational misreading and thus, increases the production of misfolded proteins (Borovinskaya et al. 2008; McCusker and Haber 1988). A likely explanation for the hypersensitivity of *crl* mutants is that the potential positive effects of the protein stabilization caused by the reduced proteasome activity are counteracted by the reduced degradation rates of misfolded proteins in a proteasome mutant background.

To date, budding yeast is the only eukaryote in which the responses of proteasome mutants to protein synthesis inhibitors have been tested. To determine if the described responses are specific to budding yeast, we have analyzed the protein synthesis inhibitor tolerance of the Arabidopsis proteasome mutants *rpt2a-3*, *rpn10-1* and *rpn12a-1* that carry loss of function mutations in the genes encoding RP AAA ATPase subunit 2a (RPT2a), RP non-ATPase 10 (RPN10) and 12a (RPN12a), respectively (Kurepa et al. 2008, 2009; Smalle et al. 2002, 2003). Proteasome activity in these mutants is only partially suppressed, and the plants are viable and fertile and can be readily used in growth-response assays (Kurepa et al. 2008, 2009). An additional advantage of using this mutant set is that they carry defects in different subunits of the RP (Kurepa and Smalle 2008). Consequently, any phenotype that is in common to all three mutants can be safely associated with RP function in general. Similar to budding yeast, Arabidopsis RP mutants show increased resistance to the toxic effects of CHX and hypersensitivity to Hyg. In addition, these mutants have increased tolerance to L-phosphinothricin (PPT), the active compound of many non-selective systemic herbicides, which suppresses protein synthesis rates by inhibiting glutamine synthase (De Block et al. 1987; Vasil 1997).

Materials and methods

Plant materials, growth conditions and analyses of growth parameters

The *rpt2a-3* and the *rpn10-1* and *rpn12a-1* mutant lines in the Col-0 ecotype background were described previously (Kurepa et al. 2008, 2009; Sonoda et al. 2009). The transgenic lines expressing the *bar*-encoded PPT acetylase were generated using the pEarlyGate100 binary vector (Earley et al. 2006). pEarlyGate100 was introduced in the *Agrobacterium tumefaciens* strain C58C1Rif, and used to transform Col-0 plants using the floral dip method (Clough and Bent 1998). Primary transgenic plants were selected on MS/2 media (half-strength Murashige and Skoog (MS) basal salt mixture powder (Sigma), 1% sucrose, and 0.8% agar) containing 5 μg/ml PPT, and their progeny was tested for the number of T-DNA inserts. Homozygous T3 plants carrying one T-DNA insert were used in this study.

Cycloheximide (CHX), L-phosphinothricin (PPT) and hygromycin B (Hyg) were purchased from Sigma (St. Louis, MO, USA), Gold BioTechnology (St. Louis, MO, USA) and Roche Applied Science (Indianapolis, IN, USA), respectively. For tolerance assays, seeds were sown on control and CHX-, PPT- and Hyg-containing plates, kept in the dark at 4°C for 4 days, and then transferred to a controlled environment growth chamber (CU-36L4, Percival Scientific, Inc.) set at 22°C and continuous light of 140 μmol photons m^{-2} s^{-1}. Although the mechanism is still unknown, the previously described differences in growth rates of in vitro grown proteasome mutants (Kurepa et al. 2008, 2009) were less pronounced when seedlings were grown in continuous light of higher intensity.

Anthocyanin extraction was done for 16 h at 4°C in acid–methanol as previously described (Kubasek et al. 1992), and the anthocyanin content was presented as relative absorption at 530 nm per mg leaf fresh weight.

Plants were photographed with an Olympus SZX12 microscope equipped with a DP12 camera or with a Nikon D40 camera. For fresh weight measurements, pools of 5 or 10 seedlings have been analyzed because most of the treatments lead to a severe reduction of plant size. Descriptive statistics, plotting and hypothesis testing was done using Prism 5.0a software (GraphPad Software Inc., El Camino Real, CA, USA).

Protein extraction and immunoblotting analyses

Plants grown in liquid MS/2 media for 5 days were transferred to fresh liquid MS/2 medium containing either 100 μM CHX, 100 μM MG132 (Enzo Life Sciences, Plymouth Meeting, PA, USA), 100 μM Hyg or 100 μM PPT. The treatments were done for 14 h, and the protein extraction and immunoblotting analyses were done as previously described (Kurepa et al. 2008; Smalle et al. 2003). In brief, plants were weighed, frozen in liquid nitrogen, and ground with sand and two volumes of 2X SDS-PAGE loading buffer. Samples were boiled, and 20 μl was loaded per lane on a 10 cm × 10 cm-large SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes. Anti-Ub antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), anti-PBA1 antisera was obtained from Enzo Life Sciences, and the global glutamine synthase antibody that recognizes both cytoplasmatic (40 kDa) and chloroplastic (45 kDa) glutamine synthase (GS) was from Agrisera AB (Vännas, Sweden). Secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Analyses of signal intensities of immunoreactive proteins was done either by densitometric analyses using ImageJ software or by ChemiDoc XRS signal acquisition followed by analyses using Quantity One software (Bio-Rad, Hercules, CA, USA).

Results

Cycloheximide tolerance of RP mutants

It has been previously shown that high concentrations of CHX inhibit seed germination (Thomas et al. 2000). We tested a range of CHX doses that allow germination of the Col-0 wild type, and compared their effects on the growth of Col-0 and proteasome mutant seedlings (Fig. 1). After 10 days of growth on MS/2 media supplemented with CHX, the survival of plants of all tested lines (i.e., Col-0 wild type and RP mutants *rpt2a-3*, *rpn10-1*, and *rpn12a-1*) was quantified as the number of seedlings with expanded green or purple cotyledons. The sensitive seedlings were

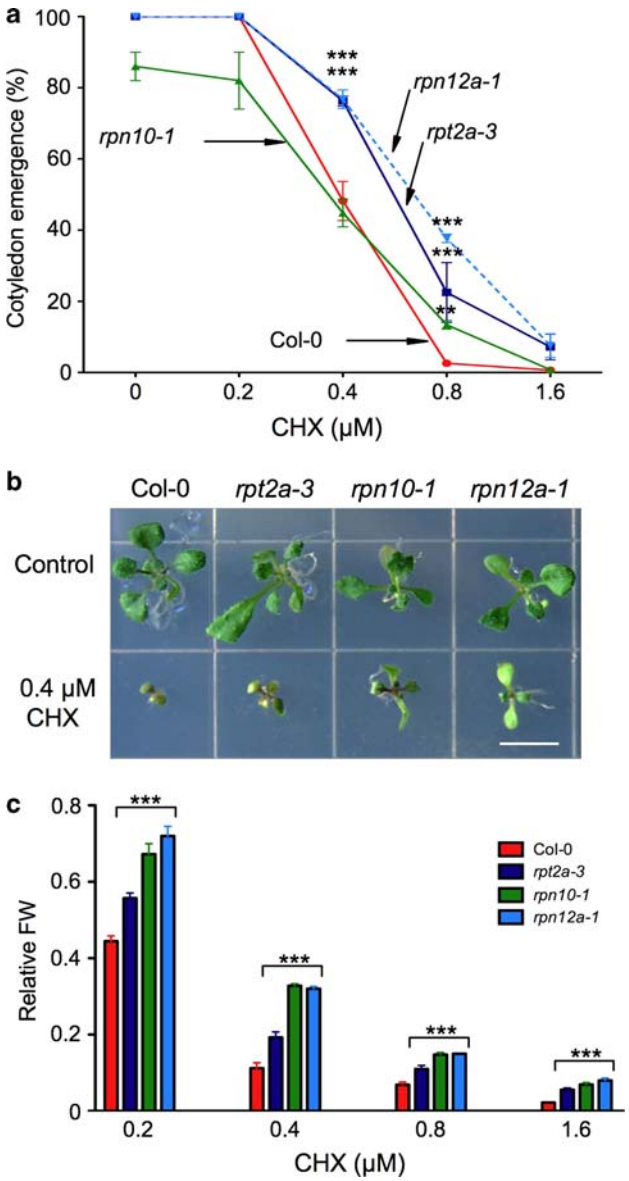

Fig. 1 Cycloheximide (*CHX*) tolerance of proteasome mutants. **a** The percentage of cotyledon emergence was calculated after 10 days of growth in continuous light at 22°C on MS/2 media supplemented with the denoted doses of CHX. Data are presented as mean ± SEM ($n \geq 50$). Significance was assessed by a two-way ANOVA with Tukey's post test. Stars represent the significance of the difference between Col-0 and the RP mutants within the same treatment category. ** $p < 0.01$, *** $p < 0.001$. **b** Seedlings were germinated and grown for 2 weeks on control medium or medium containing 0.4 μM CHX. *Scale bar* 6 mm. **c** Relative fresh weight of 2-week-old seedlings grown on CHX-supplemented media. Because the average fresh weights (*FW*) of the wild type and proteasome mutants grown on control media differ, the average FW of the untreated plants for each line was assigned a value of 1. Data are presented as mean ± SEM ($n \geq 6$ pools of 5 seedlings). Stars represent the significance of the difference between Col-0 and the RP mutants treated with the same CHX dose. *** $p < 0.001$

arrested, and had yellow or light-brown cotyledons. The *rpt2a-3* and *rpn12a-1* mutants were significantly more tolerant to CHX than Col-0 in this assay (Fig. 1a). Because the *rpn10-1* mutation has been shown to reduce seed germination (Smalle et al. 2003), the percentage of cotyledon emergence cannot be used to accurately quantify the CHX tolerance levels of this line. We therefore measured the fresh weight of seedlings grown for 2 weeks on MS/2 media with CHX. Indeed, those *rpn10-1* seeds that germinated on CHX media developed into seedlings that were larger than the wild type (Fig. 1b, c), confirming that all proteasome mutants were CHX tolerant.

In yeast, there was a strong positive correlation between CHX resistance levels and the reduction in proteasome-dependent degradation of test substrates (Gerlinger et al. 1997). The 26SP activity assays and the analyses of accumulated polyubiquitinated proteins have shown that the strongest Arabidopsis mutant in our test series was *rpn10-1* followed by *rpn12a-1*, and finally *rpt2a-3* (Kurepa et al. 2008). Fresh weight analyses of the CHX treated plants revealed that while *rpt2a-3* indeed had the lowest CHX tolerance level, the tolerance levels of the *rpn10-1* and *rpn12a-1* mutants were higher, but not statistically different from each other (Fig. 1c). Therefore, the CHX tolerance level in Arabidopsis cannot be used as an indicator of the strength of the 26SP defect. Nevertheless, because all RP mutants had increased CHX tolerance, this phenotype is the likely result of a general alteration in RP function.

The CHX tolerance of yeast proteasome mutants has been attributed to the reduced degradation of a protein that functions both as a sensor of protein synthesis rate and as a regulator of cellular growth arrest (Gerlinger et al. 1997). A later study suggested that the hypothetical translation-rate sensor might be Ub itself (Hanna et al. 2003). This study showed that when yeast cells were treated with a dose of CHX chosen to maximally suppress protein synthesis, the Ub depletion rate was reduced in a proteasome mutant background (Hanna et al. 2003). To test whether Ub is also involved in the CHX tolerance of Arabidopsis, we treated 5-day-old wild type and mutant plants with a high dose of CHX for 14 h, and analyzed the levels of polyubiquitinated proteins and free Ub using immunoblotting analyses (Fig. 2). We chose 100 μM CHX for these assays, because it has been shown previously that this concentration allows to monitor the decay rate of proteasome target proteins in Arabidopsis (Guo and Ecker 2003). As previously reported, polyubiquitinated proteins and free Ub accumulate in the *rpn10-1*, but not in the *rpt2a-3* mutant (Fig. 2a, b; Kurepa et al. 2008; Smalle et al. 2002, 2003). In older *rpn12a-1* seedlings, the polyubiquitinaed protein levels and free Ub levels were comparable to that of the

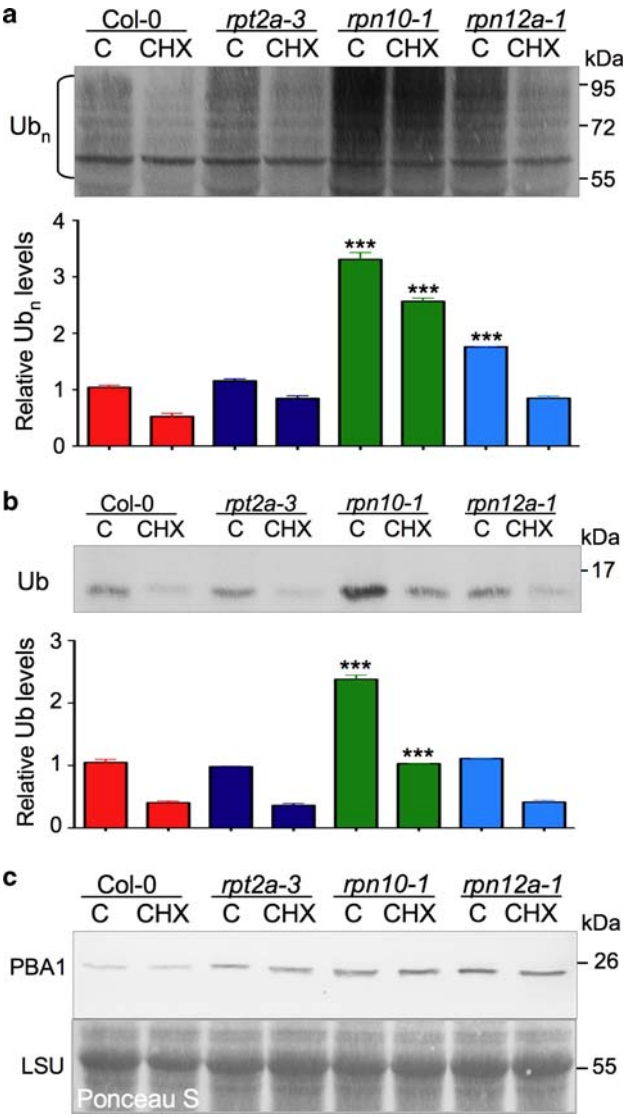

Fig. 2 CHX treatment reduces the abundance of polyubiquitinated proteins and the Ub monomer. **a** Plants germinated and grown in liquid MS/2 media for 5 days were treated with 100 μM CHX for 14 h. Total protein extracts were separated on 12% acrylamide gels, and transferred onto nitrocellulose membranes. After a 15 min-long treatment at 121°C, membranes were probed with anti-Ub antibodies. Representative immunoblot and a plots comparing the relative amounts of polyubiuqitinated proteins (Ub_n) with molecular weights from 55 to 95 kDa are shown. Relative Ub_n levels are compared to the amount of Ub_n in control (C) Col-0 plants which were assigned a value of 1. Values are means of two or more independent experiments, and the *error bars* represent the SEM. Significance was assessed by a two-way ANOVA with Tukey's post test. Stars represent the significance of the difference between Col-0 and the RP mutants within the same treatment category. $^{**}p < 0.01$, $^{***}p < 0.001$. **b** Representative immunoblot and relative signal intensities for the Ub monomer (Ub). Treatments, protein extracts, immunoblots and data analyses were done as outlined in **a**. **c** Immunoblot probed with antibodies against the proteasome *β* subunit 1 (PBA1) and a region of the Ponceau S-stained membrane encompasing the Rubisco Large Subunit (LSU) are shown as loading controls

wild type (Kurepa et al. 2008; Smalle et al. 2002). In young *rpn12a-1* seedlings however, we detected an increase in the accumulation of polyubiquitinated proteins, while the free Ub level was not significantly different from the wild type (Fig. 2a, b). The CHX treatment led to a marked decrease in Ub monomer, and consequently, a decrease in the abundance of polyubiquitinated proteins in all tested lines (Fig. 2a, b). In contrast, the CHX treatment did not suppress the accumulation of proteasome β subunit 1 (PBA1, Fig. 2c), which is known to be more abundant in the RP mutants due to a feedback up-regulation of the proteasome subunit gene set (Kurepa et al. 2008; Yang et al. 2004). The CHX also did not lead to a significant decrease in total protein levels per mg fresh weight as evidenced by the Ponceau S staining of the membranes used for immunoblotting analyses (Fig. 2c). Therefore, the Ub pool in plants, like the Ub pool in yeast, was a sensitive indicator of the CHX-induced translational inhibition, which should be the first characteristic of a protein-synthesis-rate sensor protein. However, the quantification of the signal strengths revealed that for all tested lines, CHX reduced the level of the Ub monomer to $\sim$60% (60 $\pm$ 7, 63 $\pm$ 10, 57 $\pm$ 10 and 59 $\pm$ 5% for Col-0, *rpt2a-2*, *rpn10-1* and *rpn12a-1*, respectively), and that, with the exception of the *rpn10-1* mutant, the free Ub and polyubiquitinated protein levels did not significantly differ between the CHX-treated wild type and CHX-treated mutants. Thus, Ub depletion in Arabidopsis may be one of the mediators of CHX toxicity, but by itself, it does not adequately explain the CHX tolerance of RP mutants. Either additional, unknown protein-synthesis-rate sensors are stabilized in the RP mutants or the CHX tolerance phenotype is the result of a general protein-sparing characteristic of mutants with reduced proteolysis rates.

Hygromycin B hypersensitivity of RP mutants

Hyg, an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus*, inhibits the translocation of mRNA and tRNAs on the ribosome in both bacteria and eukaryotes and affects decoding fidelity (Borovinskaya et al. 2008). Therefore, treatments with sublethal Hyg doses will not only reduce protein synthesis rates in plants, but those nuclear, mitochondrial and chloroplastic proteins that are synthesized are likely to be mistranslated and thus misfolded. Because the action of Hyg combines protein synthesis inhibition with protein misfolding stress, the effects of Hyg on the growth of RP mutants are expected to be different from the effects of CHX and may be similar to those of amino acid analogues such as L-canavanine to which all Arabidopsis RP mutants have been shown to be hypersensitive (Kurepa et al. 2008; Wang et al. 2009). The growth-response assays revealed that all tested RP mutants were indeed hypersensitive to Hyg (Fig. 3a). For example, treatment with 1.25 μg/ml Hyg reduced fresh weight to $\sim$60 and $\sim$40% of the untreated controls in Col-0 and RP mutants, respectively (Fig. 3a). Anthocyanin biosynthesis has been shown to be up-regulated by many stresses (Winkel-Shirley 2002). To test whether Hyg treatments also induce anthocyanin accumulation and whether the Hyg hypersensitivity of RP mutants can be detected using this assay, we isolated anthocyanins from 2-week-old seedlings. Indeed, the anthocyanin levels were increased in all Hyg-treated plants, and the RP mutant seedlings germinated and grown on Hyg-supplemented media accumulated more anthocyanin than the wild type (Fig. 3b). Prolonged growth on Hyg-containing media, which retarded growth of the wild type, caused seedling death in all RP mutants (Fig. 3c). Therefore, similar to the CHX responses of RP mutants, the Hyg hypersensitivity can be considered the result of a general defect in 26SP function making the Hyg growth assay a useful diagnostic tool for detecting 26SP defects in Arabidopsis mutants and transgenic lines.

Next, we analyzed the free Ub and polyubiquitinated protein levels in Hyg-treated plants (Fig. 4). In contrast to CHX, and similarly to other stresses that lead to protein misfolding, Hyg treatments induced a statistically significant increase in polyubiquitinated protein levels in all tested lines (Fig. 4a). Furthermore, and again in contrast to CHX, Hyg treatments did not significantly affect the abundance of the Ub monomer (Fig. 4b). Because the effects of Hyg were opposite to the CHX effects, and based on the previously reported role of Hyg in promoting the formation of misfolded proteins, we propose that the Hyg hypersensitivity of RP mutants is caused by their impaired ability to remove misfolded proteins, which accumulate and cause proteotoxicity.

Phosphinothricin tolerance of RP mutants

To test the tolerance of RP mutants to a protein synthesis inhibitor that does not cause protein misfolding and that is chemically and functionally different than CHX, we tested the tolerance of the RP mutant set to PPT. PPT is a tripeptide of L-glutamic acid and two alanine residues, and it inhibits glutamine synthase (GS) (Vasil 1997). GS, which catalyzes the condensation of L-glutamate and ammonia to form L-glutamine, plays a key role in amino acid synthesis and in nitrogen utilization, and its inhibition causes the depletion of amino acid pools, leading to a reduction in protein synthesis rates (Bernard and Habash 2009; D'Halluin et al. 1992; De Block et al. 1987).

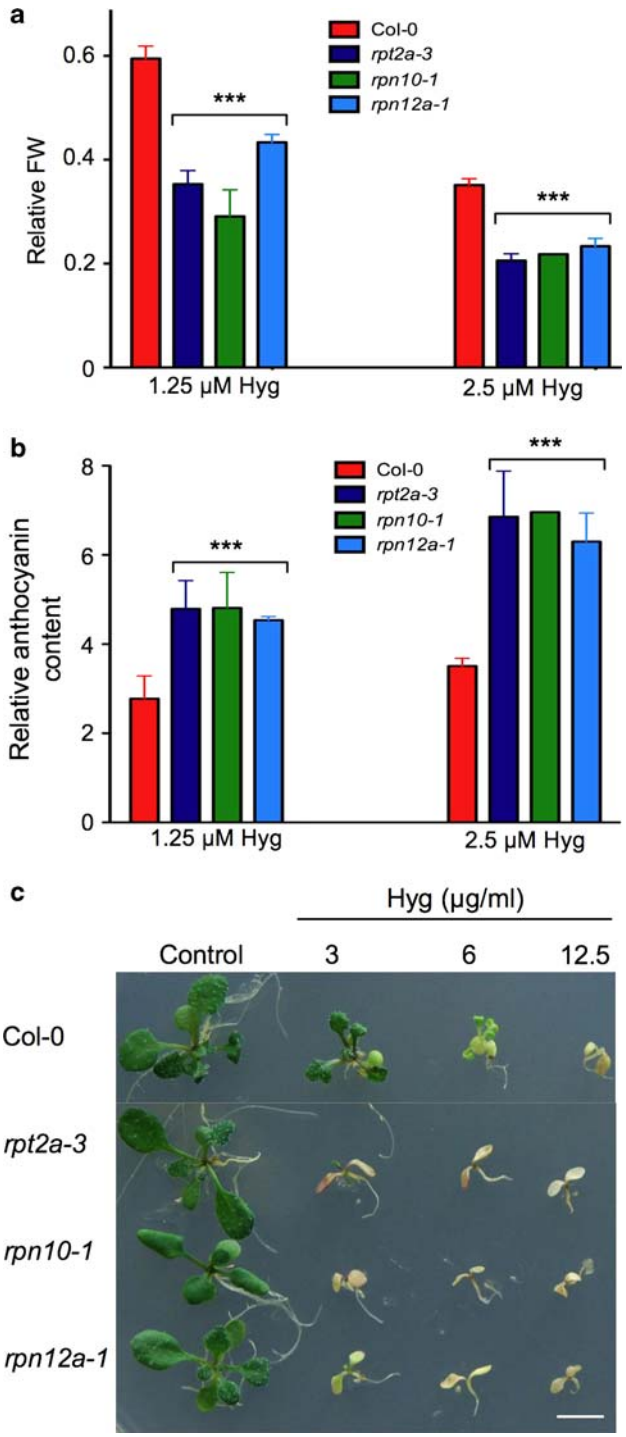

Fig. 3 Proteasome mutants are hypersensitive to hygromycin B (*Hyg*). **a** Relative fresh weight (*FW*) of seedlings germinated and grown for 2 weeks on control medium or medium containing the denoted doses of Hyg. FW was measured for pools of 5 seedlings, and the average FW ± SEM ($n \geq 10$) for the control treatment of each line was assigned the value of 1. Significance was assessed with Tukey's multiple comparison test, and the stars represent the significance of the difference between the treated Col-0 and the treated RP mutant plants. ***$p < 0.001$. **b** Relative anthocyanin content was calculated from the average A530 of acid–methanol extracts of seedling pools ($n \geq 2$, 5 plants per pool). The anthocyanin content of untreated seedlings for each line was assigned the value of 1. Data is presented as mean ± SEM, and the data was analyzed as in **a**. **c** Representative seedlings grown on media with denoted doses of Hyg for 18 days. *Scale bar* 5 mm

Dose-response assays revealed that all RP mutants have increased tolerance to PPT (Fig. 5). However, in contrast to the CHX tolerance which was the highest in the *rpn10-1* and *rpn12a-1* mutants (Fig. 1), PPT tolerance was the highest in *rpt2a-3* (Fig. 5a, b). The increased tolerance of *rpt2a-3* was especially apparent at 2.5 µg/ml PTT, a dose which caused chlorosis and growth inhibition in the other lines (Fig. 5). The RP mutants can, however, not be considered PPT resistant: doses higher than 2.5 µg/ml clearly inhibited the growth of all mutants, while the growth of transgenic plants expressing the *bar* gene, which encodes a PPT detoxifying enzyme (D'Halluin et al. 1992; De Block et al. 1987), was not affected even by 20 µg/ml PPT (Fig. 5c).

To investigate the molecular bases of the PPT tolerance, we first tested the levels of Ub and polyubiquitinated proteins in PPT-treated Col-0 plants and in the strongest PPT tolerant mutants, *rpt2a-3* and *rpn12a-1*. In contrast to CHX, treatment with a high PPT dose did not lead to a significant decrease in polyubiquitinated proteins and free Ub levels (Fig. 6a, b). Curiously, the overall level of high molecular weight Ub conjugates was even increased in PPT-treated *rpt2a-3* seedlings (Fig. 6a). Because *rpt2a-3* tolerance levels were significantly higher than those of the other RP mutants (Fig. 5b), the accumulation of polyubiquitinated proteins in this line suggests the existence of a specific PPT-tolerance mechanism. It has been shown that increased PPT tolerance could be acquired by elevating the abundance of cytosolic GS (Donn et al. 1984; Eckes et al. 1989). To test whether the unexpectedly high tolerance of the *rpt2a-3* line was due to a specific stabilization of cytosolic GS, we tested whether GS is a 26SP target and whether it is more abundant in *rpt2a-3* plants (Fig. 7). Immunoblotting analyses showed that a 14 h-long treatment with 100 µM CHX did not affect the abundance of either cytosolic or chloroplastic GS in either Col-0 plants or in any of the RP mutants (Fig. 7a, b). Treatments with the proteasome inhibitor MG132 and analyses of the GS levels in RP mutants showed that GS was not degraded by the 26SP and was not stabilized in the *rpt2a-3* mutant (Fig. 7a, b). Furthermore, a 14 h-long PPT treatment also did not lead to any changes in GS levels in either the wild type or the RP mutants indicating that the inhibition of this enzyme did not affect its synthesis or degradation rates (Fig. 7c). Although, the molecular mechanism of PPT tolerance has

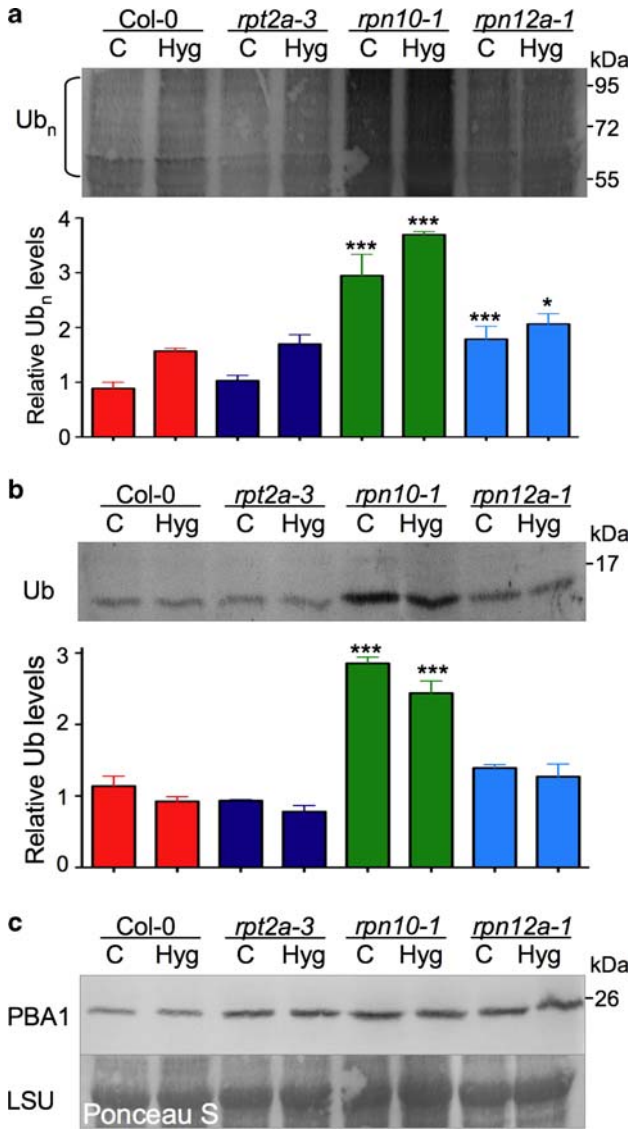

Fig. 4 Hyg treatment increases polyubiquitinated protein levels. **a** Plants were germinated and grown in liquid MS/2 media for 5 days and then treated for 14 h with 100 μM Hyg. Representative immunoblot and a plot comparing the relative amounts of polyubiuqitinated proteins (Ub_n) with molecular weights from 55 to 95 kDa are shown. Relative Ub_n levels are compared to the amount of Ub_n in control (C) Col-0 plants which were assigned a value of 1. Values are means of two or more independent experiments, and the error bars represent the SEM. Significance was assessed by a two-way ANOVA with Tukey's post test. *Stars* represent the significance of the difference between Col-0 and the RP mutants within the same treatment category. $^{**}p < 0.01$, $^{***}p < 0.001$. **b** Representative immunoblot and a plot of relative signal intensities for the Ub monomer (Ub). Relative Ub levels are compared to the amount of Ub in Col-0 control plants which were assigned a value of 1. Data analyses were done as outlined in **a**. **c** Immunoblot probed with antibodies PBA1 and a region of the Ponceau S-stained membrane encompasing the Rubisco Large Subunit (*LSU*) are shown as loading controls

not been resolved, the finding that all tested RP mutants were PPT tolerant implies that the increased tolerance reflects a general decrease in 26SP function.

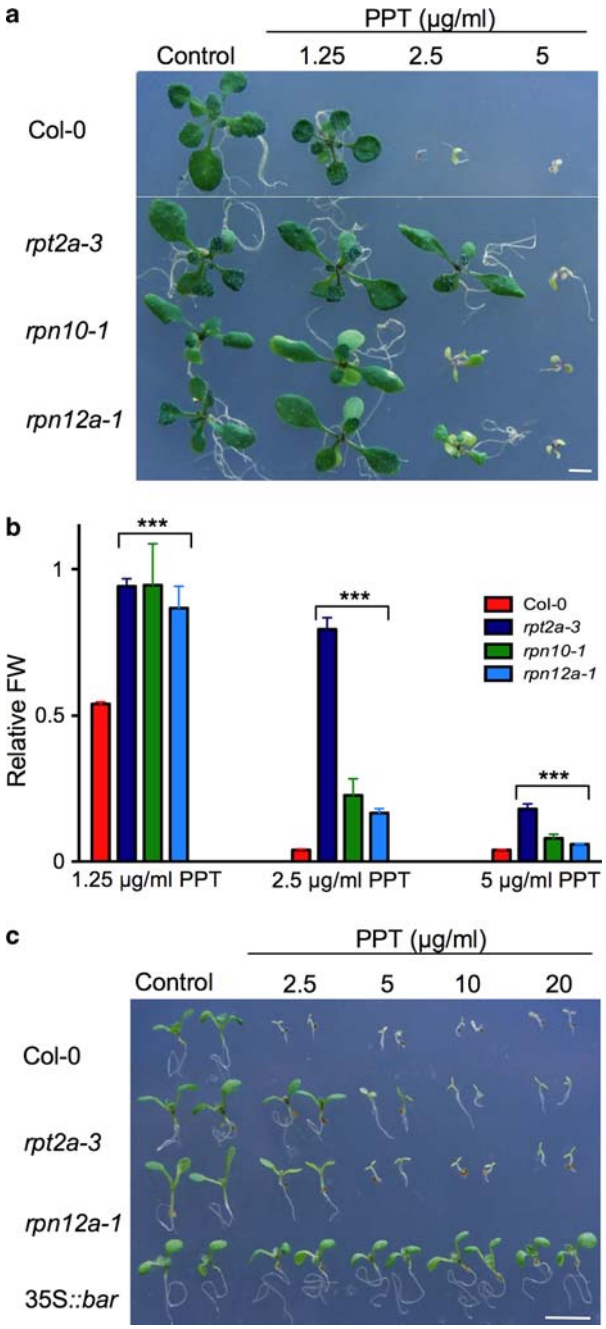

Fig. 5 Proteasome mutants have increased tolerance to L-phosphinothricin (PPT). **a** Two-week-old seedlings grown on low PPT doses. Except for 5 μg/ml, the denoted range of PPT doses are below those that are traditionally used for the selection of transgenic plants transformed with the 35S::*bar* transgene. *Scale bar* 5 mm. **b** Fresh weight (*FW*) of 2-week-old seedlings grown on media with the denoted doses of PPT was determined for pools of 5 seedlings. The average FW of the untreated control for each line was assigned a value of 1, and the data are presented as mean ± SEM ($n \geq 6$). The stars represent the significance of the difference between the Col-0 and RP mutants treated with the same PPT dose. $^{***}p < 0.001$. **c** Representative ten-day-old seedlings grown on a range of PPT doses commonly used for the selection of plants transformed with the 35S::*bar* transgene (5 μg/ml). *Scale bar* 5 mm

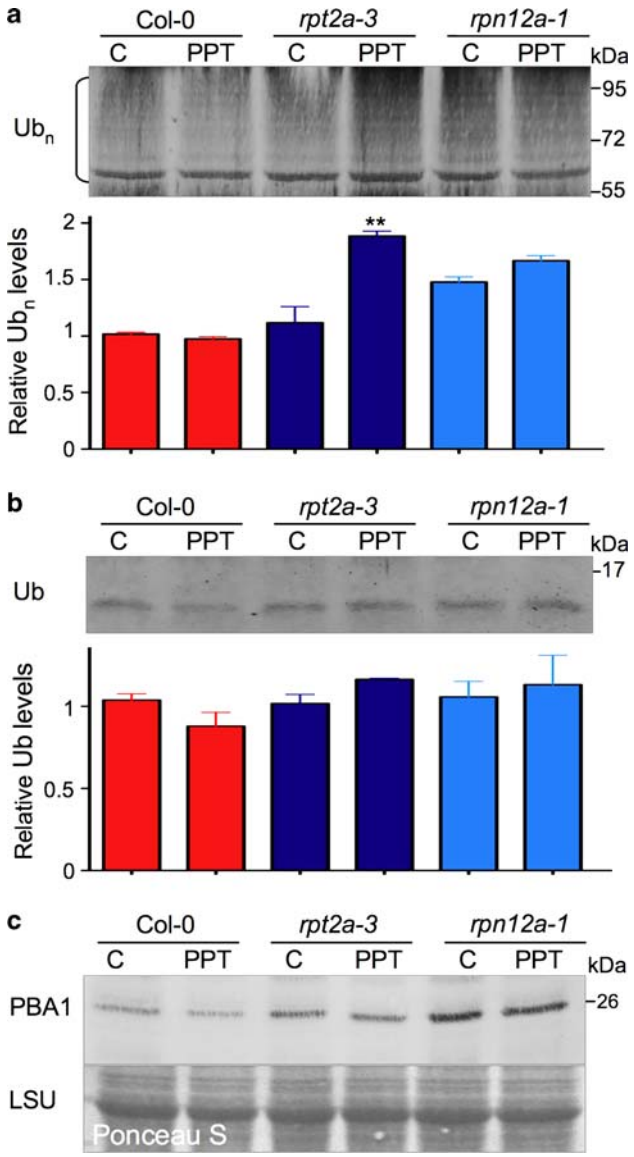

Fig. 6 Effects of PPT treatments on the levels of Ub and of polyubiquitinated proteins. **a** and **b** Immunoblotting analyses of total protein extracts of 5-day-old plants treated for 14 h with 100 µM PPT. The polyubiquitinated protein (Ub_n) and mono-Ub (Ub) levels are shown on panels A and B respectively. Bar graphs illustrate the relative amounts of Ub_n with molecular masses from 55 to 95 kDa (**a**) and of the Ub monomer (**b**). The relative intensities were calculated from densitometric scans of two or more independent immunoblots, and the absolute signal intensity measured for the untreated Col-0 samples was assigned a value of 1. *Stars* represent the significance of the difference between the control (C) and the PPT-treated plants within the same line. $^{**}p < 0.01$. **c** The same protein extracts shown in **a** and **b** were tested with anti-PBA1 antibodies. Region of a Ponceau S-stained membrane with the LSU is shown as a loading control

Discussion

The main function of the 26SP in eukaryotes is to degrade proteins that have been covalently modified with a polyubiquitin chain. Consequently, strong defects in 26SP function lead to the accumulation of Ub–protein conjugates that can be visualized by immunoblotting with anti-Ub antisera. To date, a number of Arabidopsis proteasome mutants have been isolated, and most of them have altered growth and development, implying that the 26SP activities in these lines are suboptimal (Book et al. 2009; Brenner et al. 2009; Gallois et al. 2009; Huang et al. 2006; Smalle et al. 2002; Smalle et al. 2003; Sonoda et al. 2009; Sung et al. 2009; Ueda et al. 2004; Wang et al. 2009). However, only two partial loss-of-function mutants (namely, *rpn10* and *rpn5a*) were shown to accumulate high molecular weight Ub–protein conjugates at all developmental stages (Book et al. 2009; Smalle et al. 2003). This finding led to the characterization of other 26SP mutants as weak (Book et al. 2009; Brenner et al. 2009; Gallois et al. 2009; Huang et al. 2006; Kurepa and Smalle 2008; Smalle et al. 2002, 2003; Sung et al. 2009; Ueda et al. 2004; Wang et al. 2009). Here we show that even these weak mutants carry a sufficient defect in 26SP function to have increased tolerance to the translation inhibitor CHX. CHX treatment of Arabidopsis, similar to yeast, leads to depletion of the Ub pool, indicating that plant cells also consume a substantial fraction of the free Ub during 26SP-dependent proteolysis. From the three tested Arabidopsis RP mutants, only *rpn10-1* contained more Ub monomer both before and after CHX treatment. No statistically significant differences in Ub levels were observed between the wild type and the *rpn12a-1* and *rpt2a-3* mutants, although we cannot exclude the possibility that the single-time point CHX treatment might not have revealed possible differences in steady-state Ub levels resulting from mutant-specific Ub degradation rates. In contrast to the yeast studies, we did not find a strong correlation between the strength of the proteasome defect and the CHX tolerance level, suggesting that the increase in CHX tolerance cannot be used as an accurate quantitative measure of the loss of 26SP activity in plants. For example, while *rpt2a-3,* the weakest of the three RP mutants, indeed had the lowest tolerance level, the CHX tolerance of the *rpn12a-1* and *rpn10-1* mutants did not correlate with the strength of their proteasome defects. A similar observation was made for the RP mutants' responses to Hyg: while all three mutants were clearly hypersensitive, there was no clear correlation between the degree in Hyg hypersensitivity and the strength of the 26SP defect. Therefore, we propose that in plants, similar to *S. cerevisiae*, CHX and Hyg can be used as diagnostic tools for the detection of 26SP defects. However, no quantitative conclusions about the 26SP activity levels should be drawn from the changes in tolerance levels to these translation inhibitors.

We also investigated the responses of RP mutants to PPT that impacts protein synthesis indirectly by inhibiting

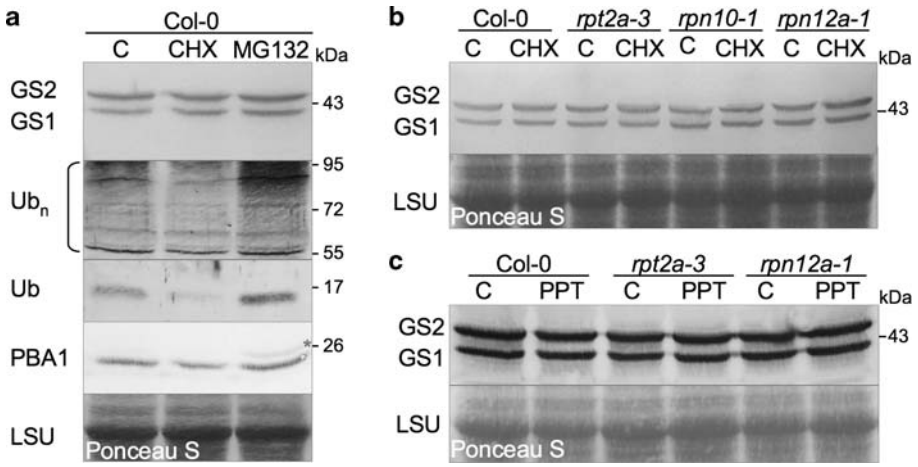

Fig. 7 Glutamine synthase (*GS*) levels were not affected by CHX, proteasome activity inhibition and PPT treatments. **a** Immunoblotting analyses of total protein extracts from 5-day-old seedlings treated with 100 μM CHX or 100 μM of the proteasome inhibitor MG132 for 14 h. The global anti-GS antisera recognizes both the cytosolic (GS1) and chloroplastic (GS2) isoforms of GS. The *asterisk* above the PBA1 band in MG132 treated seedlings indicates the pro-PBA1 peptide (Yang et al. 2004). Ponceau S-stained membrane is shown as a loading control. **b** Extracts of CHX-treated seedlings (Fig. 2) were separated on a 10% acrylamide gel, blotted and probed with the global GS antibody. **c** Extracts of PPT-treated seedlings (Fig. 6) were used for analyses with the global GS antibody

GS. Cytosolic GS plays a key role in the assimilation of ammonium either taken up from soil or supplied by internal nitrogen recycling, and therefore the inhibition of this enzyme leads to a reduction of protein and nucleic acids synthesis rates (Bernard and Habash 2009). Although the Arabidopsis RP mutants were tolerant to PPT, the yet unidentified mechanisms involved are likely to differ from those leading to the CHX tolerance. On the organismal level, this was reflected by the ranking of proteasome mutants according to their degree in tolerance. CHX tolerance was the strongest in the *rpn12a-1* and *rpn10-1* mutants that carry more severe defects in 26SP-dependent proteolysis compared to *rpt2a-3*. In contrast, PPT tolerance was the strongest in *rpt2a-3,* followed by *rpn12a-1*, *rpn10-1*. This suggests that PPT tolerance mechanism(s) do not simply depend on the stabilization of a particular protein, because this would lead to a stronger tolerance phenotype in the stronger proteasome mutants (*rpn10-1* and *rpn12a-1*). Indeed, the most likely candidate protein whose stabilization would lead to PPT tolerance, the cytosolic GS, did not accumulate in the RP mutants. The tolerance mechanism is probably not caused by any advantages associated with a decrease in global 26SP-dependent proteolysis rates (i. e., protein sparing), because this would also lead to a stronger tolerance in the *rpn10-1* and *rpn12a-1* mutants. The unexpectedly strong tolerance of *rpt2a-3* to PPT also suggests a qualitative difference between the tested RP mutants. Thus, in addition to a general defect in proteasome activity, loss of RPT2a function may influence particular processes that are not affected by the *rpn12a-1* and *rpn10-1* mutations.

In conclusion, we have shown that partial loss of 26SP function leads to changes in Arabidopsis sensitivity to CHX, Hyg and PPT. While the molecular basis of the PPT tolerance remains unknown, we suggest that the molecular mechanisms underlying the CHX tolerance and Hyg hypersensitivity are based on a general protein sparing effect and proteasome overload with misfolded proteins respectively. Our findings have three applied implications. First, PPT is the active ingredient of a number of widely used herbicides. To date, three types of mechanisms have been identified that can lead to increased PPT tolerance: (1) mutations in GS that lower the sensitivity of the enzyme to the herbicide, (2) increased capacity to detoxify PPT, and (3) decreased PPT uptake. Here, we show that PPT tolerance can also be a result of reduced 26SP activity. Second, both PPT and Hyg are often used for the selection of transgenic plants. Our data suggest that Hyg is a better selection marker for the isolation of transgenic lines in proteasome mutant backgrounds. If binary vectors with PPT resistance genes are used for the transformation of proteasome mutants without a prior determination of the mutant PPT tolerance levels, the percentage of false positives can be significant (data not shown). Finally, because the structure and function of the 26SP is highly conserved, we can postulate that the CHX and PPT tolerance as well as the Hyg hypersensitivity will characterize proteasome mutants of any plant species. Therefore, the combination of CHX, Hyg and PPT tolerance assays may develop into a valuable diagnostic tool for the detection of altered 26SP activity in mutants, transgenic lines, cultivars and germplasms.

Acknowledgments We are grateful to Dr. Songhu Wang and Yan Li for critical reading of the manuscript. This work was supported by grants from the Kentucky Tobacco Research and Development Center, the USDA Cooperative State Research, Education and Extension Service (Grant number 20053530416043) and the Kentucky Science and Engineering Foundation (Grant number 14850206189).

References

Bernard SM, Habash DZ (2009) The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. New Phytol 182:608–620

Book AJ, Smalle J, Lee KH, Yang P, Walker JM, Casper S, Holmes JH, Russo LA, Buzzinotti ZW, Jenik PD, Vierstra RD (2009) The RPN5 subunit of the 26S proteasome is essential for gametogenesis, sporophyte development, and complex assembly in Arabidopsis. Plant Cell 21:460–478

Borovinskaya MA, Shoji S, Fredrick K, Cate JHD (2008) Structural basis for hygromycin B inhibition of protein biosynthesis. RNA 14:1590–1599

Brenner ED, Feinberg P, Runko S, Coruzzi GM (2009) A mutation in theproteosomal regulatory particle AAA-ATPase-3 in Arabidopsis impairs the light-specific hypocotyl elongation response elicited by a glutamate receptor agonist, BMAA. Plant Mol Biol 70:523–533

Cabañas MJ, Vazquez D, Modolell J (1978a) Inhibition of ribosomal translocation by aminoglycoside antibiotics. Biochem Biophys Res Commun 83:991–997

Cabañas MJ, Vázquez D, Modolell J (1978b) Dual interference of hygromycin B with ribosomal translocation and with aminoacyl-tRNA recognition. Eur J Biochem 87:21–27

Callis J, Raasch JA, Vierstra RD (1990) Ubiquitin extension proteins of *Arabidopsis thaliana*. Structure, localization, and expression of their promoters in transgenic tobacco. J Biol Chem 265:12486–12493

Callis J, Carpenter T, Sun CW, Vierstra RD (1995) Structure and evolution of genes encoding polyubiquitin and ubiquitin-like proteins in *Arabidopsis thaliana* ecotype Columbia. Genetics 139:921–939

Chuang S-M, Chen L, Lambertson D, Anand M, Kinzy TG, Madura K (2005) Proteasome-mediated degradation of cotranslationally damaged proteins involves translation elongation factor 1A. Mol Cell Biol 25:403–413

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

D'Halluin K, De Block M, Denecke J, Janssens J, Leemans J, Reynaerts A, Botterman J (1992) The *bar* gene as selectable and screenable marker in plant engineering. Methods Enzymol 216:415–426

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gosselé V, Movva NR, Thompson C, Montagu MV, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513–2518

Donn G, Tischer E, Smith JA, Goodman HM (1984) Herbicide-resistant alfalfa cells: an example of gene amplification in plants. J Mol Appl Genet 2:621–635

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45:616–629

Eckes P, Schmitt P, Daub W, Wengenmayer F (1989) Overproduction of alfalfa glutamine synthetase in transgenic tobacco plants. Mol Gen Genet 217:263–268

Finley D, Bartel B, Varshavsky A (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature 338:394–401

Gallois J-L, Guyon-Debast A, Lécureuil A, Vezon D, Carpentier V, Bonhomme S, Guerche P (2009) The Arabidopsis proteasome RPT5 subunits are essential for gametophyte development and show accession-dependent redundancy. Plant Cell 21:442–459

Gerlinger UM, Gückel R, Hoffmann M, Wolf DH, Hilt W (1997) Yeast cycloheximide-resistant *crl* mutants are proteasome mutants defective in protein degradation. Mol Biol Cell 8:2487–2499

Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426:895–899

Guerrero C, Milenković T, Pržulj N, Kaiser P, Huang L (2008) Characterization of the proteasome interaction network using a QTAX-based tag-team strategy and protein interaction network analysis. Proc Natl Acad Sci USA 105:13333–13338

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by $SCF^{EBF1/EBF2}$-dependent proteolysis of EIN3 transcription factor. Cell 115:667–677

Hanna J, Leggett DS, Finley D (2003) Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Mol Cell Biol 23:9251–9261

Huang W, Pi L, Liang W, Xu B, Wang H, Cai R, Huang H (2006) The proteolytic function of the Arabidopsis 26S proteasome is required for specifying leaf adaxial identity. Plant Cell 18:2479–2492

Kubasek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell 4:1229–1236

Kurepa J, Smalle J (2008) Structure, function and regulation of plant proteasomes. Biochimie 90:324–335

Kurepa J, Toh-e A, Smalle J (2008) 26S proteasome regulatory particle mutants have increased oxidative stress tolerance. Plant J 53:102–114

Kurepa J, Wang S, Li Y, Zaitlin D, Pierce AJ, Smalle JA (2009) Loss of 26S proteasome function leads to increased cell size and decreased cell number in Arabidopsis shoot organs. Plant Physiol 150:178–189

McCusker JH, Haber JE (1988) *crl* mutants of *Saccharomyces cerevisiae* resemble both mutants affecting general control of amino acid biosynthesis and omnipotent translational suppressor mutants. Genetics 119:317–327

Reits EAJ, Vos JC, Grommé M, Neefjes J (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. Nature 404:774–778

Saracco SA, Hansson M, Scalf M, Walker JM, Smith LM, Vierstra RD (2009) Tandem affinity purification and mass spectrometric analysis of ubiquitylated proteins in Arabidopsis. Plant J 59:344–358

Schubert U, Antón L, Gibbs J, Norbury C, Yewdell J, Bennink J (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404:770–774

Smalle J, Vierstra RD (2004) The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol 55:555–590

Smalle J, Kurepa J, Yang P, Babiychuk E, Kushnir S, Durski A, Vierstra RD (2002) Cytokinin growth responses in Arabidopsis involve the 26S proteasome subunit RPN12. Plant Cell 14:17–32

Smalle J, Kurepa J, Yang P, Emborg TJ, Babiychuk E, Kushnir S, Vierstra RD (2003) The pleiotropic role of the 26S proteasome subunit RPN10 in Arabidopsis growth and development supports a substrate-specific function in abscisic acid signaling. Plant Cell 15:965–980

Sonoda Y, Sako K, Maki Y, Yamazaki N, Yamamoto H, Ikeda A, Yamaguchi J (2009) Regulation of leaf organ size by the Arabidopsis RPT2a 19S proteasome subunit. Plant J 60:68–78

Strunk BS, Karbstein K (2009) Powering through ribosome assembly. RNA 15:2083–2104

Sung D-Y, Kim T-H, Komives EA, Mendoza-Cózatl DG, Schroeder JI (2009) ARS5 is a component of the 26S proteasome complex and negatively regulates thiol biosynthesis and arsenic tolerance in Arabidopsis. Plant J 59:802–813

Thomas C, Rajagopal A, Windsor B, Dudler R, Lloyd A, Roux SJ (2000) A role for ectophosphatase in xenobiotic resistance. Plant Cell 12:519–533

Ueda M, Matsui K, Ishiguro S, Sano R, Wada T, Paponov I, Palme K, Okada K (2004) The *HALTED ROOT* gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of *Arabidopsis* meristems. Development 131:2101–2111

Vasil IK (1997) Phosphinotricin-resistant crops. In: Duke SO (ed) Herbicide-resistant crops: agricultural, environmental, economic, regulatory, and technical aspects, pp 85–89

Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol 10:385–397

Wang S, Kurepa J, Smalle JA (2009) The Arabidopsis 26S proteasome subunit RPN1a is required for optimal plant growth and stress responses. Plant Cell Physiol 50:1721–1725

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218–223

Yang P, Fu H, Walker J, Papa CM, Smalle J, Ju YM, Vierstra RD (2004) Purification of the Arabidopsis 26S proteasome: biochemical and molecular analyses revealed the presence of multiple isoforms. J Biol Chem 279:6401–6413