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A quantitative method to monitor the efficacy of inhibitors against the chymotrypsin-like activity of the proteasome in tobacco leaf protoplasts

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ABSTRACT

Proteasome inhibitors are widely used to study the role of the ubiquitin proteasome system (UPS) in various cellular processes. These drugs have been shown to be highly effective in inhibiting the chymotrypsin-like activity of purified *Arabidopsis thaliana* proteasomes. However, the analysis of their efficacy *in vivo* is currently hampered by the absence of a simple method for the quantitative determination of proteasomal activity in plant cell extracts. Previous studies have shown that quantitative methods based on the use of fluorogenic peptides cannot be directly applied to plant homogenates, due to the presence of interfering proteases with cleavage specificities similar to that of the proteasome. To overcome this, we developed a simple and rapid fractionation procedure that efficiently separates most of the non-proteasomal chymotryptic enzymes, such that proteasome activity can be easily measured. We go on to demonstrate that *in vivo* treatment of tobacco protoplasts with high concentrations of three potent proteasome inhibitors can only partially suppress proteasomal chymotrypsin-like activity, resulting in the incomplete stabilisation of the protein toxin ricin A chain (RTA), a known endoplasmic reticulum-associated degradation (ERAD) substrate that normally undergoes extensive cytosolic degradation. We therefore conclude that negative results obtained using proteasome inhibitors in tobacco protoplasts and possibly other types of plant cells should be interpreted with a degree of caution.

Keywords: Ubiquitin-proteasome system, protein degradation, endoplasmic reticulum-associated degradation, proteasome inhibitors, ricin, tobacco protoplasts

INTRODUCTION

All cellular organisms, including plants, utilise a variety of methods to control the activity and abundance of their constituent proteins. One such method is selective proteolysis, a process largely mediated by the ubiquitin-proteasome system (UPS) which involves the attachment of multiple copies of the small protein tag, ubiquitin, to proteins destined for degradation (Kerscher et al. 2006). The appended ubiquitin moieties facilitate target recognition and subsequent breakdown by the 26S proteasome (Finley 2009). Such selective proteolysis by the UPS plays a pivotal role in virtually all aspects of plant biology, being responsible for modulating the levels of key short-lived regulatory proteins that are needed for cellular homeostasis, developmental regulation and environmental adaptation (Vierstra 2009), as well as ensuring the rapid removal of abnormal or non-functional polypeptides (Liu and Howell 2010). The importance of the UPS in plants is illustrated by its complexity, with almost 6% of *Arabidopsis thaliana* genes predicted to encode UPS components (Hua and Vierstra 2011), and over 1500 ubiquitylation targets recently being identified in the same plant (Kim et al. 2013)

The 26S proteasome itself is a ~2.4 MDa multi-subunit protease located in the cytosol and nucleus of eukaryotic cells. While most of our knowledge regarding this proteolytic complex is derived from studies on yeast and mammalian particles, it is now well established that complexes with similar biochemical and biophysical characteristics also exist in plants (Yang et al. 2004; Book et al. 2010). Eukaryotic proteasomes are composed of a 20S core protease capped at one or both ends by a 19S regulatory particle (Beck et al. 2012; Lander et al. 2012; Lasker et al. 2012). The core protease is a 670 kDa barrel-shaped structure composed of four stacked heteroheptameric rings (Groll et al. 1997; Unno et al. 2002), with the two outermost rings consisting of α -subunits (α_1 - α_7 , termed PAA-PAG in *Arabidopsis*) and the two central rings composed of β -subunits (β_1 - β_7 , termed PBA-PBG in *Arabidopsis*). Three subunits of each β -ring possess proteolytic activities that between them can cleave a broad range of peptide sequences (Arendt and Hochstrasser 1997; Heinemeyer et al. 1997; Dick et al. 1998). These differ in cleavage specificity, with the β_5 or PBE subunits cleaving after hydrophobic amino acids, the β_2 or PBB subunits cleaving after basic amino acids, and the β_1 or PBA subunits cleaving after acidic residues. The site specificities are thus generally classified as chymotrypsin-like for β_5 , trypsin-like for β_2 , and either caspase-like or post-acidic

for β_1 (Borissenko and Groll 2007). A potential fourth catalytic site, revealed by N-terminal processing of the β_7 or PBG subunits, has also been recently reported (Russell et al. 2013).

Protein degradation via this pathway can be inhibited both *in vitro* and *in vivo* using a variety of low molecular weight molecules that act on the active sites of the 20S core protease (Kisselev and Goldberg 2001; Kisselev et al. 2012). These inhibitors represent valuable tools for studying the regulatory role of the UPS and identifying its substrates (Di Cola et al. 2001; Ling et al. 2012; Salmon et al. 2008). Different proteasome inhibitors such as MG115, MG132, *clasto*-lactacystin β -lactone and epoxomicin have been shown to be active in inhibiting the chymotrypsin-like activity of purified plant proteasomes (Yang et al. 2004), but little is known about the *in vivo* efficacy of these compounds in plant cells.

Proteasome activity is routinely measured in mammalian and yeast cellular extracts using fluorogenic substrates specific for each of the three different catalytic specificities. This method has proven to be fast, accurate, reproducible, extremely sensitive and economical, and as such is considered the gold standard both for routinely screening proteasomal activity in many samples, and for dissecting in detail the relative contribution of each catalytic site in the hydrolysis of specific protein substrates (Kisselev and Goldberg 2005). This is evidenced by the large number of papers that are published each year adopting such methods. However, measurement of proteasomal activity by this approach in crude *Arabidopsis* extracts was shown to be hampered by the presence of other proteases able to hydrolyse the fluorogenic substrates (Yang et al. 2004).

Protoplasts have been extensively used for the transient expression of heterologous proteins and to study plant-virus interactions (Denecke et al. 2012). At the same time they represent a convenient system for assessing the effects of proteasome inhibitors on protein turnover. The use of protoplasts eliminates any possible problem associated with the diffusion of the inhibitor into intact tissues, and allows accurate monitoring of any effect on degradation kinetics using pulse-chase or cycloheximide chase experiments (Hamel et al. ; Ju et al. 2008; Di Cola et al. 2005). Here we report a rapid method for the determination of chymotrypsin-like proteasomal activity in tobacco protoplast extracts, and show that the inclusion of commonly used proteasome inhibitors in protoplast incubation medium does not result in the complete

inhibition of the proteasomal chymotrypsin-like activity. These findings raise the possibility that certain protein substrates can still be degraded by the proteasome even when protoplasts have been treated with high concentrations of these compounds.

MATERIALS AND METHODS

Isolation of tobacco leaf protoplasts and treatment with proteasome inhibitors

Mesophyll protoplasts were prepared from axenic leaves (4 to 7 cm long) of *Nicotiana tabacum* cv. Petit Havana SRI (Maliga et al. 1973). Protoplasts were resuspended in K3 medium (3.78 g/l Gamborg's B5 basal medium with minimal organics supplemented with 750 mg/l CaCl₂·2H₂O, 250 mg/l NH₄NO₃, 136.2 g/l sucrose, 250 mg/l xylose, 1 mg/l 6-benzylaminopurine, and 1 mg/l α -naphthaleneacetic acid, pH 5.5), before treatment with the indicated concentrations of *clasto*-lactacystin β -lactone, epoxomicin (all from Calbiochem, www.merck-chemicals.com; 20 mM stocks in DMSO) or bortezomib (Millennium Pharmaceuticals, www.millennium.com; 9 mM stock in water). At the desired time points W5 medium (9 g/l NaCl, 0.37 g/l KCl, 18.37 g/l CaCl₂·2H₂O, 0.9 g/l glucose) was added, and protoplasts were pelleted at 60 \times g for 10 minutes at 4 °C. Protoplasts were washed once in W5 medium and, unless subsequent fractionation was to be performed (see below), frozen in liquid N₂ and stored at -80 °C. When indicated, protoplasts were mock-transfected in the absence of plasmid DNA, using polyethylene glycol as previously described (Pedrazzini et al. 1994; Ceriotti et al. 2003), and incubated for 16 hours before treatment with proteasome inhibitors.

Tobacco protoplast fractionation

Protoplasts were fractionated into a chloroplast-enriched and a chloroplast-depleted fraction using a modified version of a previously described protocol (Di Cola and Robinson 2005). Briefly, protoplast pellets (~1,000,000 cells) were resuspended in 500 μ l HS buffer (50 mM HEPES-KOH pH 8.0, 0.33 M sorbitol) supplemented immediately before use with 2 mM ATP and 5 mM MgCl₂. Cells were then homogenised on ice with 100 strokes of a Gilson-type micropipette through a 1000 μ l tip, and the lysate centrifuged at 1000 \times g for 8 minutes at 4 °C. The supernatant was removed, and both pellets and supernatants were frozen in liquid N₂ and stored at -80 °C.

Preparation of tobacco protoplast protein extracts for activity assays

Whole protoplasts or chloroplast-enriched pellets were sonicated in 500 μ l ice-cold buffer A (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA, 0.25 M sucrose) supplemented immediately before use with 2

mM ATP and 1 mM DTT. Whole protoplast and chloroplast-enriched or depleted extracts were then prepared by centrifugation at $10,000 \times g$ for 30 minutes at 4 °C. High molecular weight complexes (HMWC) were further pelleted by an additional ultracentrifugation at $100,000 \times g$ for 60 minutes at 4 °C in a Beckman L8-70M ultracentrifuge (Beckman Coulter, www.beckmancoulter.com). The pellet was then resuspended in 500 µl buffer A.

Proteasome activity assays and susceptibility to inhibitors

The chymotrypsin-like activity of the tobacco proteasome was assessed in cell extracts by monitoring the production of 7-amino-4-methylcoumarin (amc) from the fluorogenic peptide substrate succinyl-LLVY-amc (Bachem, www.bachem.com). The assay was performed essentially as previously described (Kisselev and Goldberg 2005; Cerruti et al. 2007). Briefly, succinyl-LLVY-amc was used at a final concentration of 100 µM in assay buffer (20 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 1 mM ATP, 0.2% (w/v) BSA). Reactions were started by adding an aliquot of cellular extract, and the fluorescence of released amc was monitored continuously at 37 °C with a Cary Eclipse fluorescence spectrophotometer (Varian Medical Systems, www.varian.com) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Assays were calibrated using standard solutions of the free fluorophore, and reaction velocities were calculated from the slopes of the initial linear portions of the reaction curves. Substrate consumption at the end of incubation never exceeded 1%. To evaluate the *in vitro* effect of different proteasome inhibitors, aliquots of total protoplast homogenate, or of fractions enriched or depleted of chloroplasts or HMWC, were pre-incubated at room temperature for 20 minutes in the presence or absence (vehicle alone) of the indicated concentrations of MG132 (Calbiochem, www.merck-chemicals.com; 20 mM stock in DMSO), clasto-lactacystin β-lactone, epoxomicin or bortezomib, and then immediately used for proteasome activity assays as described above.

Immunoblotting of tobacco protoplast proteins

Protoplasts were homogenised and fractionated into chloroplast-depleted (supernatant) or chloroplast-enriched (pellet) fractions as described above. Proteins contained in equivalent fractions of pellet and supernatant were separated by reducing SDS-PAGE and then electro-transferred to Odyssey nitrocellulose membrane (LI-COR Biosciences; www.licor.com). Proteins of interest were then detected using rabbit

polyclonal anti-histone H4 (Abcam; www.abcam.com), anti-tobacco BiP (Pedrazzini et al. 1997), anti-tobacco Rubisco (a kind gift from Spencer Whitney) or anti-spinach light harvesting complex II B1 (LHCB1) protein (Casazza et al. 2005) antibodies and the SuperSignal™ West Pico Chemiluminescent Substrate kit (Thermo Scientific; www.thermoscientific.com).

Transient transfection and *in vivo* radiolabelling of tobacco protoplasts

A DNA construct for transient expression of an active site mutant (E177D) of ricin A chain (RTA) in the CaMV 35S promoter-driven expression vector pDHA (Tabe et al. 1995) has been previously described (Frigerio et al. 1998). Polyethylene glycol-mediated plasmid transfection and radiolabelling with EasyTag™ EXPRESS ³⁵S protein labelling mix (PerkinElmer, www.perkinelmer.com) were performed as previously described (Pedrazzini et al. 1994; Ceriotti et al. 2003). In all cases, 40 µg of plasmid per 10⁶ protoplasts was used in each transfection. After transfection, protoplasts were incubated for 16 hours in K3 medium and then, where indicated, for a further 1 hour in the presence of the indicated concentration of proteasome inhibitor. Samples were then radiolabelled and chased for up to five hours in the presence of unlabelled methionine and cysteine, as previously described (Ceriotti et al. 2003). At desired time points, protoplast aliquots were removed and pelleted in W5 medium as described above. Samples were then frozen in liquid N₂ and stored at -80 °C.

Immunoprecipitation from tobacco protoplast protein extracts

Homogenisation of frozen protoplasts was performed by adding two volumes of protoplast homogenisation buffer (150 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM EDTA, 1.5% (w/v) Triton X-100) supplemented immediately before use with Complete™ protease inhibitor cocktail (Roche Applied Sciences, www.roche.com). Proteins were immunoselected from protoplast homogenates using rabbit anti-RTA polyclonal antisera followed by Protein G-Agarose (Invitrogen, www.invitrogen.com). Beads were then washed three times with NET-Gel buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 0.25% (w/v) gelatin, 0.02% (w/v) NaN₃). Immunoselected polypeptides were analysed by SDS-PAGE, and bands were detected using an FLA-9000 Image Scanner (Fujifilm, www.fujifilm.com). Band intensity was determined by densitometry using TotalLab™ software (Non-linear Dynamics, www.nonlinear.com).

Activity-based proteasome profiling

Activity-based proteasome profiling was performed as described by Gu and colleagues with minor modifications (Gu et al. 2010). Briefly, chloroplast-depleted extracts (3 μ g of proteins) were incubated for 4 hours at room temperature in the dark under agitation in the presence of the fluorescent vinyl sulfone probe MV151 (2 μ M, kindly provided by H.S. Overkleeft). Proteins were precipitated overnight with 10 volumes of ice-cold acetone, recovered by centrifugation at 16,000 x *g* for 10 minutes at 4 °C and separated by SDS-PAGE (13% acrylamide). Labeled proteins were visualized by in-gel fluorescence scanning using a Starion (FLA-9000) Image Scanner (λ_{ex} 532nm; BPG1 filter).

Statistical analysis of proteasome activity data

To compare average measurements of chymotrypsin-like proteasomal activity, a two-tailed Student's t-test was used, since the Shapiro-Wilk test has proven that spectrofluorimetric data are normally distributed (Shapiro and Wilk 1965). $P \leq 0.05$ was considered statistically significant, while $P \leq 0.01$ was termed highly significant.

RESULTS

In this work we have used the prototypical fluorogenic peptide substrate succinyl-Leu-Leu-Val-Tyr-amido-4-methylcoumarin (succinyl-LLVY-amc) to monitor chymotrypsin-like activity in extracts of tobacco protoplasts. To assay activity, aliquots of cellular homogenate are incubated along with ATP and MgCl₂ in the presence of saturating concentrations of this small fluorogenic peptide substrate, which freely diffuses inside the proteasomal cavity without the need for prior ubiquitination. Consequently, the velocity of peptide hydrolysis, releasing fluorescent 7-amino-4-methylcoumarin, depends exclusively on the turnover rate of the chymotrypsin-like catalytic sites (Kisselev and Goldberg 2005; Cenci et al. 2012).

To investigate whether *in vivo* treatment of tobacco protoplasts with different irreversible/slow-dissociating proteasome inhibitors causes a reduction in succinyl-LLVY-amc-degrading activity, tobacco leaf protoplasts were incubated for 1 or 3.5 hours with high (80 μM) concentrations of *clasto*-lactacystin β-lactone (Fenteany et al. 1995; Dick et al. 1996), epoxomicin (Meng et al. 1999) or bortezomib (Adams et al. 1998; Richardson et al. 2006) - which is also known as PS-341 or Velcade. Both *clasto*-lactacystin β-lactone and epoxomicin have been shown to be highly effective at much lower concentrations in inhibiting the chymotrypsin-like activity of purified *Arabidopsis thaliana* proteasomes (Yang et al. 2004). Protoplasts were then homogenized and succinyl-LLVY-amc-degrading activity immediately assayed in crude extracts (Figure 1). In all cases, it was evident that substantial residual activity was present in extracts prepared from inhibitor-treated protoplasts. In fact, treatment with bortezomib only reduced total protease activity by about 10%, while *clasto*-lactacystin β-lactone and epoxomicin were only slightly more effective, such that after 3.5 hours of treatment, total protease activity was reduced to ~60% of the control value (Figure 1).

Crude *Arabidopsis* extracts have been shown to contain other peptidases that are able to degrade the succinyl-LLVY-amc substrate in addition to the chymotrypsin-like action of the proteasome (Yang et al. 2004). It was therefore essential to investigate whether the activity present in tobacco cellular extracts was of proteasomal origin, i.e. if it could be efficiently inhibited if proteasome inhibitors were directly included in the *in vitro* assays. The results presented in Table 1 clearly show that MG132, *clasto*-lactacystin β-lactone and epoxomicin caused only partial inhibition at concentrations known to effectively suppress the

chymotrypsin-like activity of purified plant proteasomes (Yang et al. 2004; Basset et al. 2002). Indeed, two digit micromolar concentrations of bortezomib and *clasto*-lactacystin β -lactone were necessary to cause substantial (though still incomplete) inhibition of succinyl-LLVY-amc hydrolysis in crude protoplast extracts (Table 1). Furthermore, not even a combination of high concentrations of both bortezomib and *clasto*-lactacystin β -lactone together was effective in completely eliminating the chymotryptic activity in tobacco homogenates. Since these agents are known to inhibit plant or mammalian proteasomes at nanomolar concentrations (Kisselev and Goldberg 2001), it is therefore likely that other non-proteasomal chymotrypsin-like proteases, which are not inhibited by high doses of these compounds, are also present in protoplast extracts.

Peptide aldehydes such as MG115 and MG132 are well known inhibitors of mammalian cysteine and serine proteases (Kisselev and Goldberg 2001), and MG132 has also been shown to inhibit papain-like cysteine proteases in plants (Gu et al. 2010). Additionally, while bortezomib and *clasto*-lactacystin β -lactone were initially described as specific inhibitors of the proteasome, subsequent studies have revealed that they too target several other cellular proteases (Kisselev et al. 2012). Despite this, it appears that tobacco protoplast extracts contain one or more chymotrypsin-like proteases that are capable of cleaving the succinyl-LLVY-amc substrate and which are not inhibited by the common proteasome inhibitors used in this study. This is confirmed by the results obtained using epoxomicin, the most specific and potent proteasome inhibitor currently known, with no other cellular targets having been identified to date (Kisselev et al. 2012). This inhibitor caused only a modest decrease (~30%) in activity at a concentration (2 μ M) reported to significantly suppress the chymotrypsin-like activity of purified *Arabidopsis* 26S proteasomes (Yang et al. 2004). Even at a concentration of 20 μ M, it reduced succinyl-LLVY-amc-degrading activity by only 40%, indicating that a substantial fraction (~60%) of chymotryptic activity in crude protoplast extracts is of extra-proteasomal origin. Clearly this significantly complicates any attempts (such as the one shown in Fig. 1) to interpret the effectiveness of proteasome inhibitors in tobacco protoplasts.

It was therefore necessary to separate proteasomal and non-proteasomal chymotrypsin-like activities to reliably monitor the extent of proteasome inhibition in control and treated protoplasts. Since our goal was

to quantitatively compare proteasome activity in different samples, we had to rely on simple purification procedures that minimised any variability in the recovery of proteolytic activity. We first attempted separation of the proteasomal and non-proteasomal activities through partial purification of proteasomes by ultracentrifugation, exploiting protocols widely used with mammalian cell extracts that allow the recovery of a fraction highly enriched in 26S proteasomes and virtually depleted of low-molecular-weight contaminating proteases (Kisselev and Goldberg 2005). Surprisingly however, succinyl-LLVY-amc-degrading activity present in the high-molecular-weight complex (HMWC)-enriched fraction could not be efficiently inhibited, even by the specific proteasome inhibitor epoxomicin (Figure 2a). Indeed, it is noticeable that for all the inhibitors tested (MG132, epoxomicin, *clasto*-lactacystin β -lactone and bortezomib), the profile showing the inhibition of activity in the HMWC fraction was generally similar to that observed when assays were performed on crude extracts (Figure 2a). This suggested that the protease(s) responsible for the non-proteasomal chymotrypsin-like activity were also high-molecular-weight complexes that co-fractionate with the proteasome.

While it was clearly impossible to discriminate between proteasomal (i.e. epoxomicin-sensitive) and non-proteasomal (i.e. epoxomicin-insensitive) activity using ultracentrifugation to enrich for HMWCs, we subsequently overcame this problem through a simple subcellular fractionation. The procedure we employed is routinely used as a first step in the preparation of intact chloroplasts and generates chloroplast-enriched (pellet) and chloroplast-depleted (supernatant) fractions. In this procedure, following homogenisation of protoplasts in sorbitol medium, intact and broken chloroplasts are sedimented by brief centrifugation. Analysis of the distribution of a light-harvesting complex II protein (Figure 2b) indicates that the bulk of thylakoid membranes were recovered in the chloroplast-enriched fraction (which was dark green, compared to the supernatant which was clear). While chloroplast membranes were clearly pelleted, we also observed that the chloroplast-depleted fraction was contaminated by stromal proteins, as judged by the distribution of ribulose biphosphate carboxylase oxygenase (Rubisco), an abundant stromal marker. Although Rubisco was clearly enriched in the pellet, a significant fraction was also present in the supernatant (Figure 2b), presumably released from broken chloroplasts. Nuclei also fractionated to the pellet, as shown by the distribution of histone H4, while an endoplasmic reticulum marker (immunoglobulin binding protein, BiP) was recovered in both fractions (Figure 2b).

Proteasome inhibitors were then tested for their ability to inhibit succinyl-LLVY-amc-degrading activity in the chloroplast-enriched (pellet) and chloroplast-depleted (supernatant) fractions. Activity in the pellet fraction showed the same profile as that observed in total extracts and HMWC-enriched fractions, being in large part insensitive to *clasto*-lactacystin β -lactone, epoxomicin, MG132 and low concentrations of bortezomib (Figure 2a). Strikingly this time, however, activity in the supernatant fraction could be reduced to ~25% of the control in the presence of even low concentrations of all inhibitors tested. In particular, the chymotrypsin-like activity present in the supernatant presented a sensitivity to the highly specific inhibitor epoxomicin that was comparable with that reported in the literature for purified *Arabidopsis* 26S proteasomes (Yang et al. 2004). This indicates that the bulk of succinyl-LLVY-amc-degrading activity present in this fraction is indeed of proteasomal origin. Incomplete removal of non-proteasomal proteases may be responsible for the small residual activity which is observed in the presence of 20 mM epoxomicin. Since this fraction is contaminated by stromal proteins that are most likely derived from broken chloroplasts (Figure 2b), we conclude that that the extra-proteasomal chymotryptic activities previously observed are unlikely to be of stromal origin.

Having established a simple procedure to remove the bulk of non-proteasomal succinyl-LLVY-amc-degrading activity, we next determined the residual proteasomal chymotrypsin-like activity in supernatant fractions obtained from protoplasts that had been treated *in vivo* with 80 μ M of the different proteasome inhibitors. In parallel, we monitored the effect of each inhibitor on the degradation of RTA. This polypeptide, when synthesised in the absence of its partner ricin B chain (RTB), is retrotranslocated from its site of deposition in the endoplasmic reticulum lumen to the cytosol, where it is rapidly degraded (Di Cola et al. 2001; Di Cola et al. 2005; Marshall et al. 2008). Degradation is slowed down in the presence of MG132 and *clasto*-lactacystin β -lactone (Di Cola et al. 2001), and is modulated by the number of lysine residues present in the protein (Di Cola et al. 2005), indicating a role for the UPS in the turnover process.

Since expression of RTA is toxic to plant cells, we utilized an active site mutant (RTA_{E177D}) with reduced catalytic activity (Chaddock and Roberts 1993). Pulse-chase experiments with [³⁵S]-labelled cysteine and methionine confirmed that orphan RTA_{E177D} is an unstable protein with an estimated half-life of

approximately 2 hours, and that the *in vivo* treatment of protoplasts with 80 μ M of *clasto*-lactacystin β -lactone, epoxomicin or bortezomib resulted in only partial stabilisation of the protein (Figures 3a and 3b). None of the inhibitors were able to fully stabilise RTA_{E177D}, with 30-40% still being degraded over the 5 hour chase period. These degradation kinetics could be compatible with alternative proteolytic pathways contributing, together with the proteasome, to RTA_{E177D} turnover. However, parallel measurements of the residual proteasome activity in chloroplast-depleted protoplast extracts showed that, after 6 hours of treatment (corresponding to 5 hours of chase with excess non-radiolabelled amino acids), about 25% of the initial chymotrypsin-like activity was still present in protoplasts treated with *clasto*-lactacystin β -lactone and epoxomicin, while about 70% residual activity was present in protoplasts treated with bortezomib (Figure 3c). Our results do not allow us to determine which protease(s) is responsible for the continued turnover of RTA_{E177D} in the presence of proteasome inhibitors, but they clearly show that incomplete stabilization of this and any other substrate cannot be taken *per se* as evidence for the action of other non-proteasomal proteases. Partial inhibition of the proteasomal chymotrypsin-like activity was further confirmed in two additional independent experiments (Figure 4). Interestingly, the stabilizing effect of bortezomib, which proved the least effective compound against proteasome activity *in vivo*, was very similar to the one exerted by the two other proteasome inhibitors (Figures 3b and 3c).

The presence of residual proteasomal activity in tobacco protoplasts treated *in vivo* with high concentrations of inhibitors was further corroborated by proteasome activity profiling using MV151, a fluorescent vinyl sulfone probe that selectively labels the catalytic β -subunits in a mechanism-dependent manner (Gu et al. 2010; Kolodziejek and van der Hoorn 2010; Verdoes et al. 2006). Proteins in chloroplast-depleted extracts from control protoplasts, and from protoplasts that had been incubated for 1 or 6 h in the presence of proteasome inhibitors, were tagged with MV151 and separated by electrophoresis. Although the three different types of tobacco proteasome β -subunits were not resolved by SDS-PAGE, the results shown in Fig. 4b unambiguously reveal the persistence of significant proteasomal activity even in protoplasts that have been exposed for several hours to high concentrations of proteasome inhibitors. *Clasto*-lactacystin β -lactone, which proved to be the most effective inhibitor of the chymotryptic activity (Fig. 3c and 4a), was also the most effective in preventing the binding of MV151 to proteasomal active sites (Fig. 4b).

Taken together, our data show that inhibitors which are widely used to completely block proteasome activity in mammalian cells are not equivalently effective in inhibiting proteasomes in tobacco protoplasts. As such, experiments showing only partial protein stabilization when such inhibitors are employed in protoplasts should be treated with a corresponding degree of caution.

DISCUSSION

Since proteasome inhibitors have been extensively used to study the role of the UPS, it remains somewhat surprising that very little is known with regards to the efficacy of these inhibitors when administered to plant cells and tissues. We have therefore adapted a simple spectrofluorimetric method to determine chymotrypsin-like proteasome activity in tobacco leaf protoplast extracts and used it to monitor the *in vivo* efficacy of different proteasome inhibitors.

While other methods can be utilized to monitor proteasome activity in crude extracts, the use of fluorogenic substrates presents numerous advantages. Many different peptides have been recently developed (and are now commercially available) whose sequences were optimised to increase cleavage specificity by each of the three proteasomal active sites (Kisselev and Goldberg 2005). Of these, succinyl-LLVY-amc was shown to be hydrolysed by the proteasomal chymotryptic activity in different mammalian cell homogenates with very high (~95%) specificity (Kisselev and Goldberg 2005). Importantly, this peptide can be easily used to perform enzymological studies *in continuo* (both with a spectrofluorometer or with a plate reader), and if employed at a saturating concentration (i.e. 100 μ M, almost twice its K_M for the β_5 subunit) enables direct and reliable evaluation of the overall potential proteasomal chymotrypsin-like activity present in cellular extracts, as well as monitoring of the inhibitory effects caused by different doses of proteasome inhibitor (Kisselev and Goldberg 2005; Cenci et al. 2012).

To allow the quantitative determination of chymotrypsin-like proteasomal activity in protoplast extracts derived from plant cells, the method had to be adapted in order to eliminate interfering non-proteasomal activities. The adaptation relies on a simple fractionation, corresponding to the first steps of a commonly used chloroplast preparation procedure (Di Cola and Robinson 2005), and enables an efficient removal of the bulk of chymotrypsin-like activities present in crude protoplast extracts. Pellet and supernatant fractions are assayed using the fluorogenic peptide succinyl-LLVY-amc in a buffer containing ATP and $MgCl_2$, to prevent inactivation of 26S proteasomes and/or dissociation of the 19S regulatory particle from the 20S core protease (Smith et al. 2005; Liu et al. 2006). When hydrolysed, the fluorescent compound 7-amino-4-methylcoumarin is released and measured. The simplicity of the fractionation procedure minimises sample

loss and allows direct comparison of proteasome activity, as determined by spectrofluorimetric assay, in different samples.

While we have not characterised in detail the two subcellular fractions recovered after fractionation of protoplast homogenates (referred to as chloroplast-enriched and chloroplast-depleted fractions), it is clear that nuclei and chlorophyll-containing chloroplast membranes remain in the pellet, while the stromal protein Rubisco is found in both supernatant and pellet fractions. The Rubisco present in the supernatant fraction is likely to be derived from broken chloroplasts. The presence of nuclei in the pellet suggests that a fraction of cellular proteasomes are actually removed by our procedure, and implies that our conclusions apply to cytoplasmic proteasomes only. However it should be noted that work performed in mammalian cells has shown that measuring inhibition of cytosolic proteasomes provides an accurate estimate of proteasome inhibition in other cellular compartments (Kisselev et al. 2006). In any case, the fact that a fraction of cellular proteasomes is removed with the nuclei does not affect our conclusion that significant proteasomal activity is still present in inhibitor-treated protoplasts.

Our data show that tobacco protoplasts treated with high doses of the three proteasome inhibitors still contain substantial chymotrypsin-like activity. In general, all inhibitors proved less effective than in mammalian cells, where a 1 hour exposure to 0.5 μM bortezomib, 2 μM epoxomicin, or 10 μM *clasto*-lactacystin β -lactone (doses that are considerably lower than the 80 μM used here) reduces chymotrypsin-like activity by 92.3, 97.5, and 99.5%, respectively (Kisselev et al. 2006). This unexpected finding was consistent with the results obtained by proteasome activity-based profiling. This assay clearly demonstrated the persistence of a non-negligible fraction of active β -sites after prolonged *in vivo* treatment with high concentrations of inhibitors. Although standard electrophoretic procedures were unable to resolve the three catalytic β -subunits of tobacco proteasomes, the persistence of an intense activity-dependent signal following treatment with concentrations of proteasome inhibitors that are significantly higher than those usually used to suppress at the same time all three proteasome activities in mammalian cells (Crawford et al. 2006; Kisselev et al. 2006; Schubert et al. 2000) confirms the reduced *in vivo* efficacy of these compounds in tobacco protoplasts. The mechanisms hampering efficient proteasome inhibition in tobacco protoplasts remain to be determined, but limited permeability of the plasma membrane and/or the presence

of detoxifying mechanisms are possible explanations. A precedent for this exists in yeast where a defect in the synthesis of ergosterol, the principal membrane sterol in this organism, is required to make cells permeable and thus sensitive to MG132 and *clasto*-lactacystin β -lactone (Lee and Goldberg 1996). Otherwise, yeast cells are insensitive to these inhibitors.

While commonly used proteasome inhibitors target the chymotrypsin-like site, they are generally much less active on the other two proteolytic sites. It is therefore reasonable to expect that the degree of inhibition of the two other sites that can be obtained with these proteasome inhibitors in plant cells is lower than for the chymotrypsin-like site. It will now be of interest to discover whether our procedure is useful to measure the trypsin-like and caspase-like activities in tobacco protoplasts.

Our findings have important implications regarding the interpretation of experiments based on the use of proteasome inhibitors in plant research. Widely used inhibitors, such as MG132 (Rock et al. 1994), act primarily on the chymotrypsin-like active sites of proteasomes (Kisselev et al. 1999), and though this activity is generally considered rate-limiting for protein breakdown *in vivo*, this view has recently been challenged. In fact, studies performed on mammalian proteasomes indicate that the relative importance of the different proteolytic sites varies with protein substrates (Kisselev et al. 2006). This raises the possibility that certain substrates may be difficult to stabilise using the common proteasome inhibitors that mainly target the chymotryptic sites, especially when, as in the case of our experiments with tobacco protoplasts, inhibition of the chymotrypsin-like activity is far from being complete. This issue should be carefully considered when interpreting the results of experiments that make use of proteasome inhibitors in plant cells.

However, it should be stressed that a relatively small inhibition of the chymotrypsin-like site can lead to a clear effect on protein degradation, as in the case of RTA. In addition, our results indicate that relatively large differences in the extent of inhibition do not necessarily result in corresponding differences in the extent of substrate stabilization (Fig. 3). It is therefore clear that, although unable to completely ablate the chymotrypsin-like proteasomal activity *in vivo*, proteasome inhibitors remain a primary tool to investigate the involvement of the proteasomal system in protein turnover.

As in the case of RTA, incubation of protoplasts with proteasome inhibitors often results in partial, rather than total, stabilization of a protein substrate. The presence of substantial chymotrypsin-like proteasome activity indicates that residual degradation in the presence of proteasome inhibitors cannot be taken as conclusive evidence for the action of non-proteasomal proteases. Different approaches, including the use of mutants defective in specific proteolytic activities, or of inhibitors that are not effective on the proteasome active sites, should be used to assess the involvement of other proteases.

CONCLUSIONS

Our work has identified a simple fractionation procedure that allows the spectrofluorimetric determination of chymotrypsin-like proteasomal activity in tobacco protoplasts. Using this method, we found that substantial levels of chymotrypsin-like proteasomal activity can be detected in tobacco protoplasts treated with high concentrations of commonly used proteasome inhibitors. This finding was consistent with the results of activity-based proteasome profiling. This partial inhibition, may be insufficient to detectably stabilize certain protein substrates, and we therefore suggest that negative results obtained using proteasome inhibitors in tobacco protoplasts should be taken with a degree of caution.

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REFERENCES

- Adams J, Behnke M, Chen S, Cruickshank AA, Dick LR, Grenier L, Klunder JM, Ma YT, Plamondon L, Stein RL (1998) Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids. *Bioorg Med Chem Lett* 8:333-338
- Arendt CS, Hochstrasser M (1997) Identification of the yeast 20S proteasome catalytic centers and subunit interactions required for active-site formation. *Proc Natl Acad Sci USA* 94:7156-7161
- Basset G, Raymond P, Malek L, Brouquisse R (2002) Changes in the expression and enzymic properties of the 20S proteasome in sugar-starved maize roots: evidence for an *in vivo* oxidation of the proteasome. *Plant Physiol* 128:1149-1162
- Beck F, Unverdorben P, Bohn S, Schweitzer A, Pfeifer G, Sakata E, Nickell S, Plitzko JM, Villa E, Baumeister W, Forster F (2012) Near-atomic resolution structural model of the yeast 26S proteasome. *Proc Natl Acad Sci USA* 109:14870-14875
- Book AJ, Gladman NP, Lee SS, Scalf M, Smith LM, Vierstra RD (2010) Affinity purification of the *Arabidopsis* 26S proteasome reveals a diverse array of plant proteolytic complexes. *J Biol Chem* 285:25554-25569
- Borissenko L, Groll M (2007) The 20S proteasome and its inhibitors: crystallographic knowledge for drug development. *Chem Rev* 107:687-717
- Casazza AP, Rossini S, Rosso MG, Soave C (2005) Mutational and expression analysis of ELIP1 and ELIP2 in *Arabidopsis thaliana*. *Plant Mol Biol* 58 (1):41-51
- Cenci S, Oliva L, Cerruti F, Milan E, Bianchi G, Raule M, Mezghrani A, Pasqualetto E, Sitia R, Cascio P (2012) Protein synthesis modulates responsiveness of differentiating and malignant plasma cells to proteasome inhibitors. *J Leukoc Biol* 92:921-931
- Ceriotti A, Vitale A, Paris N, Frigerio L, Neuhaus JM, Hillmer S, Robinson DG (2003) Plant cell biology. In: Davey J, Lord JM (eds) *Essential Cell Biology, Volume 1: Cell Structure*. Oxford University Press, New York, pp 133-161
- Cerruti F, Martano M, Petterino C, Bollo E, Morello E, Bruno R, Buracco P, Cascio P (2007) Enhanced expression of interferon- γ -induced antigen-processing machinery components in a spontaneously occurring cancer. *Neoplasia* 9:960-969
- Chaddock JA, Roberts LM (1993) Mutagenesis and kinetic analysis of the active site Glu177 of ricin A-chain. *Protein Eng* 6 (4):425-431
- Crawford LJ, Walker B, Ovaa H, Chauhan D, Anderson KC, Morris TC, Irvine AE (2006) Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132. *Cancer Res* 66 (12):6379-6386
- Denecke J, Aniento F, Frigerio L, Hawes C, Hwang I, Mathur J, Neuhaus JM, Robinson DG (2012) Secretory pathway research: the more experimental systems the better. *Plant Cell* 24 (4):1316-1326
- Di Cola A, Frigerio L, Lord JM, Ceriotti A, Roberts LM (2001) Ricin A chain without its partner B chain is degraded after retrotranslocation from the endoplasmic reticulum to the cytosol in plant cells. *Proc Natl Acad Sci USA* 98:14726-14731
- Di Cola A, Frigerio L, Lord JM, Roberts LM, Ceriotti A (2005) Endoplasmic reticulum-associated degradation of ricin A chain has unique and plant-specific features. *Plant Physiol* 137:287-296

- Di Cola A, Robinson C (2005) Large-scale translocation reversal within the thylakoid Tat system *in vivo*. J Cell Biol 171:281-289
- Dick LR, Cruikshank AA, Grenier L, Melandri FD, Nunes SL, Stein RL (1996) Mechanistic studies on the inactivation of the proteasome by lactacystin: a central role for *clasto*-lactacystin β -lactone. J Biol Chem 271:7273-7276
- Dick TP, Nussbaum AK, Deeg M, Heinemeyer W, Groll M, Schirle M, Keilholz W, Stevanovic S, Wolf DH, Huber R, Rammensee HG, Schild H (1998) Contribution of proteasomal β -subunits to the cleavage of peptide substrates analysed with yeast mutants. J Biol Chem 273:25637-25646
- Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 268:726-731
- Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 78:477-513
- Frigerio L, Vitale A, Lord JM, Ceriotti A, Roberts LM (1998) Free ricin A chain, proricin, and native toxin have different cellular fates when expressed in tobacco protoplasts. J Biol Chem 273:14194-14199
- Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature 386:463-471
- Gu C, Kolodziejek I, Misas-Villamil J, Shindo T, Colby T, Verdoes M, Richau KH, Schmidt J, Overkleeft HS, van der Hoorn RA (2010) Proteasome activity profiling: a simple, robust and versatile method revealing subunit-selective inhibitors and cytoplasmic, defense-induced proteasome activities. Plant J 62:160-170
- Hamel LP, Benchabane M, Nicole MC, Major IT, Morency MJ, Pelletier G, Beaudoin N, Sheen J, Seguin A (2011) Stress-responsive mitogen-activated protein kinases interact with the EAR motif of a poplar zinc finger protein and mediate its degradation through the 26S proteasome. Plant Physiol 157 (3):1379-1393
- Heinemeyer W, Fischer M, Krimmer T, Stachon U, Wolf DH (1997) The active sites of the eukaryotic 20S proteasome and their involvement in subunit precursor processing. J Biol Chem 272:25200-25209
- Hua Z, Vierstra RD (2011) The cullin-RING ubiquitin-protein ligases. Annu Rev Plant Biol 62:299-334
- Ju HJ, Ye CM, Verchot-Lubicz J (2008) Mutational analysis of PVX TGBp3 links subcellular accumulation and protein turnover. Virology 375 (1):103-117
- Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol 22:159-180
- Kim DY, Scalf M, Smith LM, Vierstra RD (2013) Advanced proteomic analyses yield a deep catalog of ubiquitylation targets in Arabidopsis. Plant Cell 25 (5):1523-1540
- Kisselev AF, Akopian TN, Woo KM, Goldberg AL (1999) The sizes of peptides generated from protein by mammalian 20 and 26S proteasomes: implications for understanding the degradative mechanism and antigen presentation. J Biol Chem 274:3363-3371
- Kisselev AF, Callard A, Goldberg AL (2006) The importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with protein substrate. J Biol Chem 281:8582-8590
- Kisselev AF, Goldberg AL (2001) Proteasome inhibitors: from research tools to drug candidates. Chem Biol 8:739-758

- Kisselev AF, Goldberg AL (2005) Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. *Methods Enzymol* 398:364-378
- Kisselev AF, van der Linden WA, Overkleeft HS (2012) Proteasome inhibitors: an expanding army attacking a unique target. *Chem Biol* 19:99-115
- Kolodziejek I, van der Hoorn RA (2010) Mining the active proteome in plant science and biotechnology. *Curr Opin Biotechnol* 21 (2):225-233
- Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, Martin A (2012) Complete subunit architecture of the proteasome regulatory particle. *Nature* 482:186-191
- Lasker K, Forster F, Bohn S, Walzthoeni T, Villa E, Unverdorben P, Beck F, Aebersold R, Sali A, Baumeister W (2012) Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. *Proc Natl Acad Sci USA* 109:1380-1387
- Lee DH, Goldberg AL (1996) Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. *J Biol Chem* 271:27280-27284
- Ling Q, Huang W, Baldwin A, Jarvis P (2012) Chloroplast biogenesis is regulated by direct action of the ubiquitin-proteasome system. *Science* 338 (6107):655-659
- Liu CW, Li X, Thompson D, Wooding K, Chang TL, Tang Z, Yu H, Thomas PJ, DeMartino GN (2006) ATP binding and ATP hydrolysis play distinct roles in the function of the 26S proteasome. *Mol Cell* 24:39-50
- Liu JX, Howell SH (2010) Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell* 22:2930-2942
- Maliga P, Sz-Breznovits A, Marton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. *Nat New Biol* 244:29-30
- Marshall RS, Jolliffe NA, Ceriotti A, Snowden CJ, Lord JM, Frigerio L, Roberts LM (2008) The role of CDC48 in the retrotranslocation of non-ubiquitinated toxin substrates in plant cells. *J Biol Chem* 283:15869-15877
- Meng L, Mohan R, Kwok BH, Elofsson M, Sin N, Crews CM (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* anti-inflammatory activity. *Proc Natl Acad Sci USA* 96:10403-10408
- Pedrazzini E, Giovinazzo G, Bielli A, de Virgilio M, Frigerio L, Pesca M, Faoro F, Bollini R, Ceriotti A, Vitale A (1997) Protein quality control along the route to the plant vacuole. *Plant Cell* 9:1869-1880
- Pedrazzini E, Giovinazzo G, Bollini R, Ceriotti A, Vitale A (1994) Binding of BiP to an assembly-defective protein in plant cells. *Plant J* 5:103-110
- Richardson PG, Mitsiades C, Hideshima T, Anderson KC (2006) Bortezomib: proteasome inhibition as an effective anticancer therapy. *Annu Rev Med* 57:33-47
- Rock KL, Gramm C, Rothstein L, Clark K, Stein RL, Dick LR, Hwang D, Goldberg AL (1994) Inhibitors of the proteasome block the degradation of most cellular proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78:761-771
- Russell JD, Scalf M, Book AJ, Ladror DT, Vierstra RD, Smith LM, Coon JJ (2013) Characterization and quantification of intact 26S proteasome proteins by real-time measurement of intrinsic fluorescence prior to top-down mass spectrometry. *PLoS One* 8 (3):e58157

- Salmon J, Ramos J, Callis J (2008) Degradation of the auxin response factor ARF1. *Plant J* 54:118-128
- Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR (2000) Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404 (6779):770-774
- Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* 52:591-611
- Smith DM, Kafri G, Cheng Y, Ng D, Walz T, Goldberg AL (2005) ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins. *Mol Cell* 20:687-698
- Tabe LM, Wardley-Richardson T, Ceriotti A, Aryan A, McNabb W, Moore A, Higgins TJV (1995) A biotechnological approach to improving the nutritive value of alfalfa. *J Anim Sci* 73:2752-2759
- Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T (2002) The structure of the mammalian 20S proteasome at 2.75 Å resolution. *Structure* 10:609-618
- Verdoes M, Florea BI, Menendez-Benito V, Maynard CJ, Witte MD, van der Linden WA, van den Nieuwendijk AM, Hofmann T, Berkers CR, van Leeuwen FW, Groothuis TA, Leeuwenburgh MA, Ovaa H, Neefjes JJ, Filippov DV, van der Marel GA, Dantuma NP, Overkleeft HS (2006) A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem Biol* 13 (11):1217-1226
- Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat Rev Mol Cell Biol* 10:385-397
- Yang P, Fu H, Walker JM, 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

FIGURE LEGENDS

Figure 1: Residual chymotrypsin-like activity in cellular extracts of tobacco protoplasts treated *in vivo* with proteasome inhibitors.

Tobacco mesophyll protoplasts were incubated for the indicated time in the presence or absence of 80 μM bortezomib, *clasto*-lactacystin β -lactone or epoxomicin. Succinyl-LLVY-amc-degrading activity was assessed in cellular extracts and indicated as a percentage of the control. The average of three independent experiments (\pm SD) is shown.

Figure 2: Effect of proteasome inhibitors on the chymotrypsin-like activity in crude and fractionated extracts.

a: Succinyl-LLVY-amc-degrading activity was assessed in total tobacco protoplast homogenates (protoplasts), in a high-molecular-weight complex-enriched fraction (HMWC), and in a chloroplast-enriched (pellet) or chloroplast-depleted (supernatant) fractions (see Materials and Methods for details), either in the presence or absence of the indicated concentrations of proteasome inhibitor (MG132, epoxomicin, *clasto*-lactacystin β -lactone or bortezomib). Activity is indicated as a percentage of the control, and data represent the average of three independent experiments (\pm SD). **b:** Protoplasts were homogenised and then fractionated into chloroplast-enriched (P) and chloroplast-depleted (S) fractions. Equivalent amounts of the two fractions were separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose and probed with antibodies against markers for the chloroplast stroma (Rubisco), the thylakoids (LHCB1), nuclei (histone H4), and endoplasmic reticulum (BiP). The positions of molecular mass markers (kDa) and of the Rubisco large (LSU) and small (SSU) subunits are indicated.

Figure 3: *In vivo* treatment with proteasome inhibitors fails to completely inhibit plant proteasome activity and to fully block the degradation of RTA.

Tobacco mesophyll protoplasts were mock-transfected or transfected with a DNA construct encoding the E177D catalytic site mutant of RTA, radiolabelled for 1 hour with [^{35}S]-cysteine and methionine, and chased for the indicated times. Where indicated, *clasto*-lactacystin β -lactone, epoxomicin or bortezomib (80 μM final concentration) was added to the samples 1 hour before radiolabelling. **a:** RTA_{E177D} was

immunoprecipitated from protoplast homogenates and analyzed by reducing SDS-PAGE and fluorography.

b: Densitometric quantification of RTA_{E177D} from total protoplast homogenates in the presence or absence of proteasome inhibitors, expressed as a percentage of total RTA_{E177D} present at the end of the pulse. Data represent the average of the experiment shown in A and of two additional independent experiments (\pm SD).

c: Mock-transfected protoplasts were incubated for 1 or 6 hours with the indicated proteasome inhibitor (80 μ M), fractionated into chloroplast-enriched and depleted fractions, and chymotrypsin-like activity was assayed in the chloroplast-depleted fraction. For each time point, data are expressed as percentage residual activity with respect to the corresponding untreated control.

Figure 4: Residual proteasomal chymotrypsin-like activity after *in vivo* treatment of tobacco protoplasts with proteasome inhibitors.

a: Tobacco mesophyll protoplasts were treated as in Figure 3c, and chymotrypsin-like activity was assayed in the chloroplast-depleted fraction. Graphs show the average of the data obtained from the experiment shown in Figure 3c and from two analogous experiments (\pm SD). Statistical significance was calculated by a two-tailed Student's t-test. **b:** Tobacco mesophyll protoplasts were treated as in Figure 3c, and an aliquot of the chloroplast-depleted fraction was labelled for 4 hours with 2 μ M MV151. Proteins were separated by SDS-PAGE and detected by fluorescence scanning of the gel. The arrowhead indicates MV151-labelled proteasome catalytic subunits. The positions of molecular mass markers (kDa) are indicated on the left.

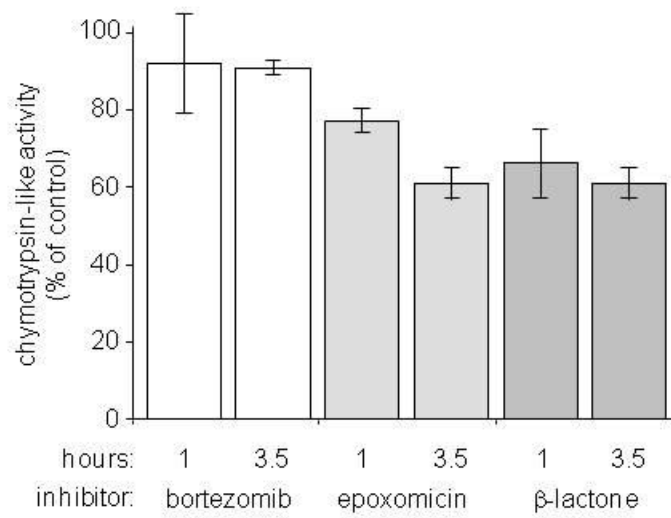


Fig. 1

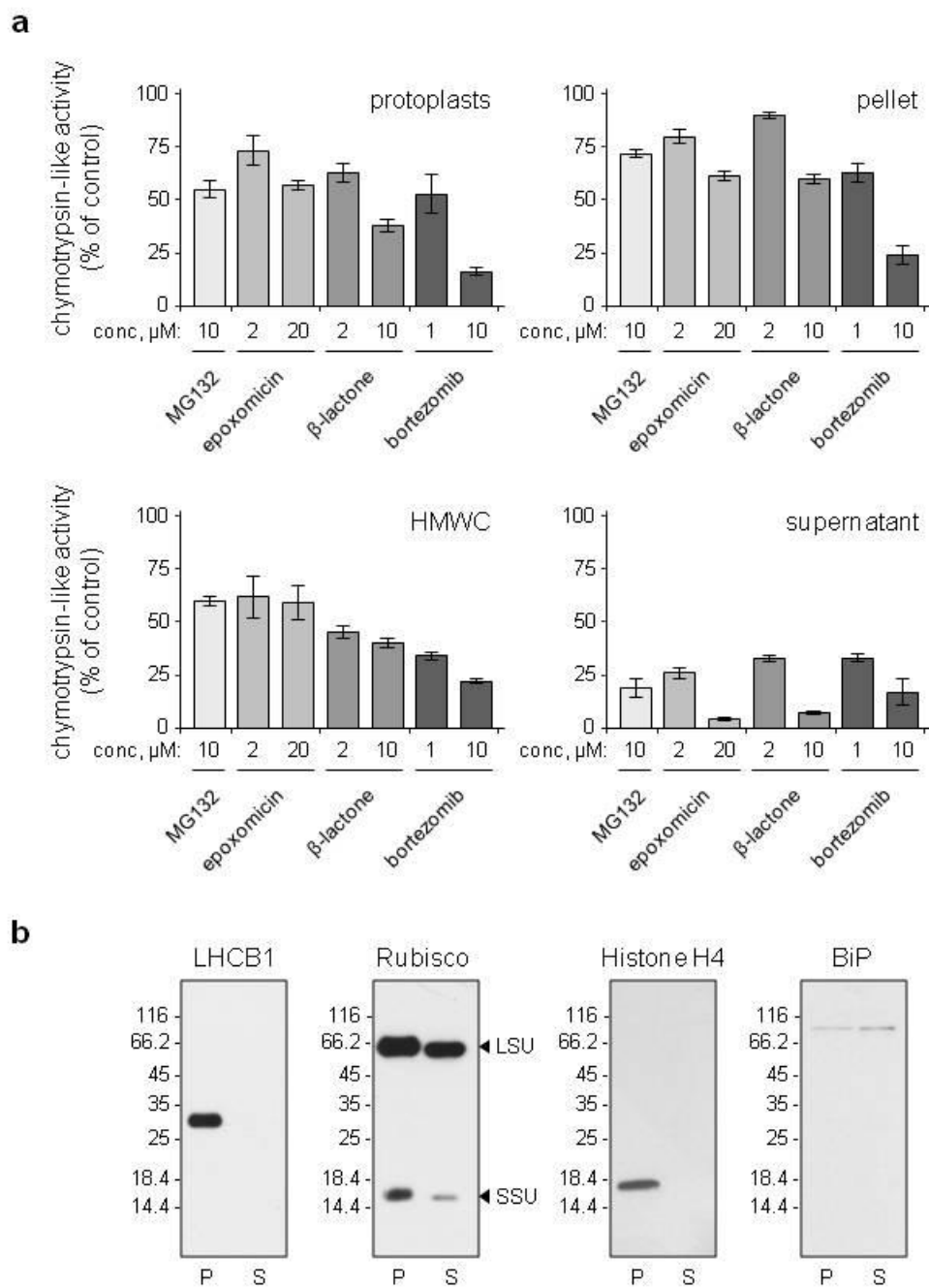


Fig. 2

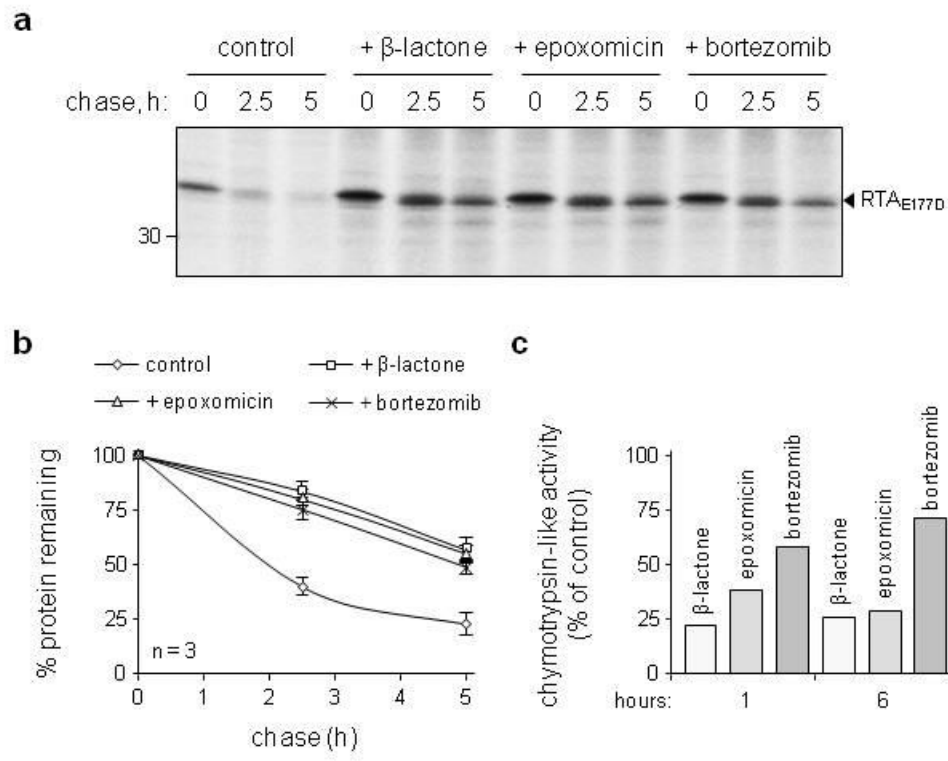
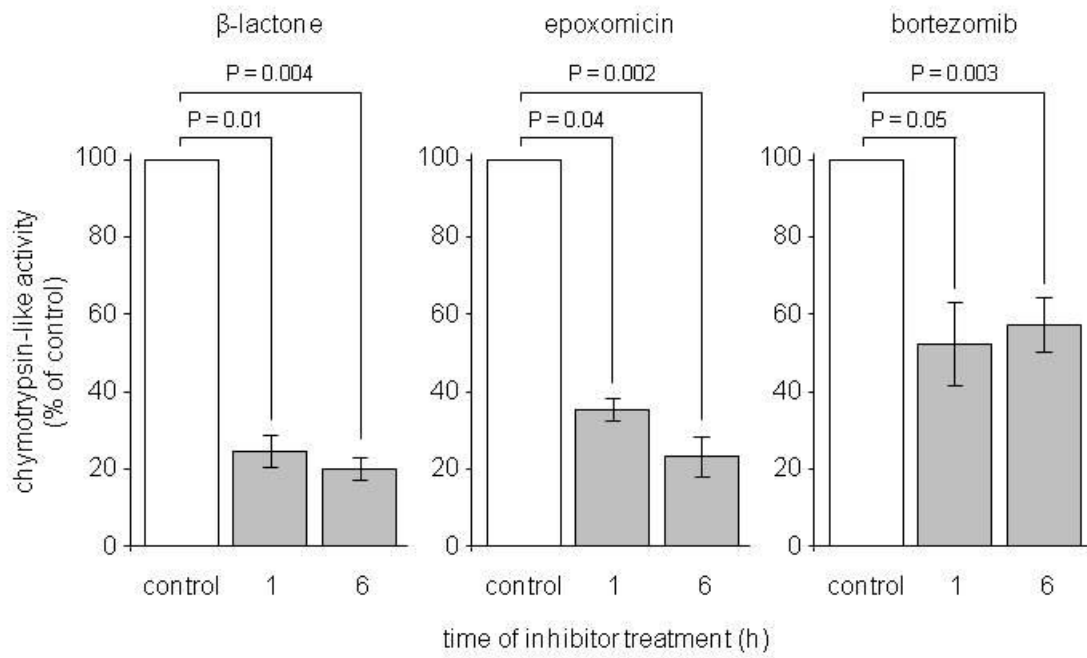


Fig. 3

a



a

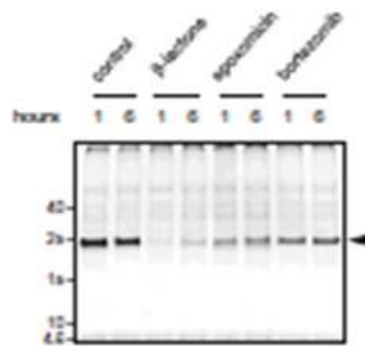


Fig. 4

Table 1. Effect of different proteasome inhibitors on the chymotryptic activity present in tobacco cellular extracts.

Inhibitor	Concentration	Chymotrypsin-like activity (% of control)
None		100
MG132	10 μ M	54.8 \pm 3.9
	20 μ M	53.6 \pm 7.0
β -lactone	2 μ M	63.4 \pm 4.0
	10 μ M	37.9 \pm 3.4
	20 μ M	32.5 \pm 3.7
Epoxomicin	0.5 μ M	76.0 \pm 1.6
	2 μ M	72.6 \pm 7.7
	10 μ M	55.5 \pm 0.7
	20 μ M	57.3 \pm 2.8
Bortezomib	1 μ M	52.6 \pm 9.1
	10 μ M	16.0 \pm 2.5
	30 μ M	15.3 \pm 4.1
Bortezomib	30 μ M	8.4 \pm 1.8
β -lactone	20 μ M	

Values are means of three independent experiments \pm S.D.