Plant and Cell Physiology Advance Access published February 9, 2012

Running Title: Proteasomal degradation of selenoproteins

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Subject area:

Environmental and Stress Responses

Black and White figures: 7

Color figures: 1

Malformed Selenoproteins Are Removed By The Ubiquitin-Proteasome Pathway In *Stanleya pinnata*

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Abbreviations

Se, selenium; S, sulfur; DNP, dinitrophenylhydrazine; sHSP, small Heat Shock Protein; BiP2, binding protein2; AZC, azetidine-2-carboxylic acid, E1, ubiquitin activating enzyme, E2, ubiquitin conjugating enzyme

Abstract

Despite the widely accepted belief that selenium toxicity in plants is manifested by the misincorporation of selenocysteine into selenoproteins, there is a lack data suggesting selenoproteins are malformed or misfolded. Plant mechanisms to prevent the formation of selenoproteins are associated with increased selenium tolerance, yet there is no evidence to suggest that selenoproteins are malformed or potentially misfolded. We reasoned that if selenoproteins are malformed, then they might be degraded by the ubiquitin-proteasome pathway. The data demonstrate that selenate treatment induced the accumulation of both oxidized and ubiquitinated proteins, thus implicating both the 20S and 26S proteasome of *Stanleya pinnata*, a selenium-hyperaccumulating plant, in a selenate-response. Inhibition of the proteasome increases the amount of selenium incorporated into protein, but not other elements. Furthermore, a higher percentage of selenium was found in an ubiquitinated protein fraction compared to other elements, suggesting that malformed selenoproteins are preferentially ubiquitinated and removed by the proteasome. Additionally, levels of the 20S and 26S proteasome and two heat shock proteins increase upon selenate treatment. Arabidopsis mutants with defects in the 26S proteasome have decreased selenium tolerance, and further supports the hypothesis that the 26S proteasome likely prevents Se toxicity by removing selenoproteins.

Keywords

Selenoproteins, ubiquitin, proteasome, *Stanlyea pinnata*, selenium, oxidized proteins

Introduction

Selenocysteine is dubbed the 21st amino acid in organisms that require selenium (Se), such as humans, some protozoans, and prokaryotes. In contrast, vascular plants do not require Se. Still, interest in plant Se metabolism stems from two relevancies. First, plants with enhanced capacity to accumulate and tolerate selenium could be used for phytoremediation pursuits. Secondly, plants are the primary source of essential dietary intake of selenium (Zhu et al. 2009). Humans contain at least 25 selenoproteins (Kryukov et al., 2003), and a diet enriched in selenium may thwart some cancers (Amaral et al., 2010). Therefore, the development of crops with fortified levels of selenium may be welcomed in countries like Malawi, where low levels of dietary Se were recently linked to a low accumulation of Se in maize (Chilimba et al. 2011).

Although plants don't require Se, they can still accumulate it in their tissues, as already revealed. However, the accumulation of Se in most non-hyperaccumulating plants is considered toxic. Accumulation of selenium in plants is due in part to its chemical similarity with sulfur, an essential macronutrient (Terry et al. 2000). For example, it is well established that selenate and sulfate compete with sulfate transporters localized to the roots, such as SULTR1;2 (El Kassis et al. 2007). Selenate that enters the roots via sulfate transporters can then enter plastids where it can be assimilated into selenocysteine by presumably utilizing the sulfur reductive pathway (Zhu et al. 2009).

In plants, the reduction of selenate into selenocysteine is problematic as it can become misincorporated into protein, thereby replacing cysteine (Stadtman 1990). The amino acid cysteine plays an important role in protein function and structure. Cysteine is

often found at enzymes' active sites, and thus involved in catalysis. Furthermore, cysteine is essential for the formation of disulfide bridges, which act to maintain protein structure and can function in post-translational regulation of proteins, as is the case for thioredoxin (Schürmann 2000). Considering cysteine's role in proteins, it is intuitive to reason that replacing cysteine with selenocysteine could result in a malformed protein.

Minimizing the misincorporation of selenocysteine has been an effective strategy to increase selenium tolerance in plants. For example, Arabidopsis plants that overexpress a selenocysteine lyase (CpNifS) have enhanced selenate tolerance and selenium accumulation, yet a decrease in selenium in protein (Van Hoewyk, et al., 2005). The enhanced volatilization of selenium in some plants, which was recently extended to include broccoli (Zhou et al. 2009), is an additional mechanism to increase selenium tolerance by redirecting Se away from protein incorporation. The Se-hyperaccumulators *Stanleya pinnata* (Freeman et al. 2010) and *Astragalus bisulcatus* contain a selenocysteine methyltransferase, which methylates selenocysteine and prevents its incorporation into protein. Overexpression of an *A. bisulcatus* selenocysteine methyltransferase in Arabidopsis and *Brassica juncea* increased Se tolerance, accumulation, and volatilization(LeDuc et al. 2005). In summary, attempts to increase Se tolerance by preventing Se incorporation into protein have been successful, and suggests that selenoproteins impart toxicity because they are malformed.

Stress-induced accumulation of unfolded proteins can form aggregates that disrupt cellular processes if not promptly removed. Processes that ensure proper protein quality control and the removal of misfolded proteins are a ubiquitous feature in most eukaryotic compartments, and is particularly important in the endoplasmic reticulum

where disulfide bridges are formed (Liu and Howell 2010; Buchberger et al. 2010). Misfolded proteins are tagged with the small protein ubiquitin and delivered to the 26S proteasome for degradation. The 26S proteasome contains both the 19S regulatory particle that recognizes and binds to the polyubiqutinated proteins, and the catalytic 20S core that contains proteases to degrade the misfolded proteins (Smalle and Vierstra 2004). Arabidopsis mutants in the 19S regulatory particle have decreased 26S proteasome activity, and are less tolerant to stresses inducing protein misfolding. For example, these mutants (*rpn10-1, rpn12a-1,* and *rpt2a-2*) are sensitive to heat stress and the non-protein amino acids canavanine and azetidine-2-carboxylic acid, which can be replace arginine and proline, respectively, in proteins and induce protein misfolding (Kurepa et al. 2008; Peng et al. 2001).

In addition to misfolded proteins, accumulation of oxidized proteins during oxidative stress can also wreak cellular havoc if not removed efficiently. However, in contrast to the degradation of misfolded proteins, it is now well established that oxidized proteins are shipped to and destroyed by the free 20S proteasome in a ubiquitin and ATP non-dependent manner (Davies 2001; Bassett et al, 2002; Polge et al. 2000). Abiotic stress that induces protein oxidation includes heavy metals, such as arsenic, cadmium, cobalt, copper, mercury, and nickel (Santos et al., 2006 Pena 2008; Djebali 2008). Although selenium is a metalloid, selenium accumulation is known to decrease glutathione in plants, leading to oxidative stress and reduced tolerance (Van Hoewyk 2008, Hugouvieux et al, 2009). Recently it was proposed that an accumulation of selenate may be an additional mode of Se toxicity (Grant et al., 2011), yet there is no evidence to date suggesting the selenate oxidizes proteins.

Despite the widely accepted belief that plant selenoproteins are malformed, to our knowledge there is no experimental data suggesting that they are misfolded. The objective of this study was to provide evidence for the malformed plant selenoprotein hypothesis. We rationalized that if selenoproteins truly are malformed or potentially misfolded, selenate-treated plants would invoke the ubiquitin-proteasome pathway and might preferentially remove selenoproteins.

Results

Stanleya pinnata plants were treated with 0, 20, 40, and 80μM sodium selenate for twelve weeks. Selenium treatment did not affect chlorophyll levels (SI 1), and the plants did not visually appear stressed. To determine if selenate induces the accumulation of ubiquitinated proteins, leaf tissue from -/+ Se-treated plants were transferred to media with or without MG132, a proteasome inhibitor. In control media lacking MG132, a slight increase of ubiquitinated proteins is observed in plants treated with 40 and 80 μM selenate compared to plants fed 0 and 20 μM selenate. Inhibition of the proteasome greatly increased the amount of ubiquitinated proteins in plants fed selenate compared to plants treated with water (Fig 1). Ubiquitinated proteins accumulated the most in MG132-treated plants fed the highest concentration of selenate.

We sought to determine if the proteasome can specifically target selenoproteins. To help meet this challenge, proteins were precipitated from selenate-fed *S. pinnata* plants treated with or without the proteasome inhibitor MG132. An elemental analysis of

the precipitated proteins indicated that there was no significant difference in the amount of copper, iron, and sulfur in protein in MG132 treated plants compared to untreated plants. In contrast, inhibition of the proteasome increased the amount of selenium in protein by 60% in plants compared to the control group (Fig. 2).

To determine if ubiquitin tags selenoproteins for removal, the ubiquitinated and non-ubiquitinated protein fractions were isolated from selenate-fed plants treated with MG132. An ubiquitin antibody strongly reacted against the ubiquitinated protein fraction compared to the non-ubiquitinated fraction (Fig. 3A), and the free ubiquitin is absent in the ubiquitinated fraction. Still, it is possible that some ubiquitinated proteins remained in the non-ubiquitinated protein fraction. The separate protein fractions were then analyzed for their elemental composition; this approach allowed the calculation of the percentage of selenium found in the ubiquitinated protein fraction. Nearly a quarter of the selenium in protein was found in the ubiquitinated protein fraction (Fig. 3B). The percentage of selenium in the ubiquitinated fraction (22.3%) was higher compared iron (4.4%) and sulfur (12.7%). The amount of copper in the ubiquitinated fraction was below the detection limit and not reported.

Selenate is known to decrease levels of glutathione and ascorbic acid in *Stanleya pinatta* (Freeman et al., 2010), and induce oxidative stress in plants. Although various heavy metals- notably cadmium- are capable of oxidizing proteins, it has not been previously reported whether or not Se can have the same effect upon proteins. The accumulation of oxidized proteins was determined in +/- MG132-treated plants fed with or without 80 µM selenate. Plants fed selenate resulted in an accumulation of oxidized proteins compared to untreated plants. Inhibition of the proteasome prevents degradation

of oxidized proteins, resulting in an accumulation of oxidized proteins. In MG132-treated plants, the accumulation of oxidized proteins induced by selenate stress was greatly exacerbated compared to plants without selenate treatment (Fig. 4).

During abiotic stress, the 20S proteasome functions by removing oxidized proteins in a process not dependent upon ATP and ubiquitin. Selenate had the effect of increasing levels of both oxidized and ubiquitinated proteins in MG132-treated plants. To determine if the 20S and 26S proteasomal subparticles accumulate during selenate treatment, the abundance of these two proteasomes were estimated in the shoots of plants treated with or without 80 μM selenate. In nondenaturing gels lacking ATP, the abundance of the 20S proteasome (as detected by the 20S and Pba1 antibodies) increased when treated with selenate compared to untreated plants (Fig. 5A). Oxidative stress has been reported to decrease 26S proteasomal levels and activities (Reinheckel et al. 1998). Therefore, we thought it was imperative to determine if levels of the 26S proteasome decrease in selenate-treated plants. However, as judged from the levels of the 19S regulatory particle detected with RPT and RPN12 antibodies, it did not appear that the 26S proteasome levels diminished upon selenate-treatment; in fact, a slight increase in the 26S proteasome and associated subunits were observed in Se-treated plants as detected by SDS-PAGE (Fig. 5B).

Considering that selenate induced an accumulation of ubiquitinated proteins, we thought it was possible that other mechanisms to avoid or tolerate protein unfolding in the cytosol and ER might be involved. Selenate treatment has previously been reported to induce the transcription of genes encoding small heat shock proteins (Van Hoewyk et al. 2008, Freeman et al. 2010). In this study, at least two small heat shock proteins appear to

be induced by selenate (Fig 6). The ER is also susceptible to protein misfolding. The BiP2 protein is an endoplasmic reticulum chaperone that binds to native and misfolded proteins. BiP2 participates in the unfolded protein response, which prevents the aggregation of unfolded proteins by shipping them to the cytosolic 26S proteasome (Liu and Howell, 2010). However, protein levels of BiP2 were not affected by selenate treatment.

To further evaluate the role of the proteasome in preventing selenium toxicity, Arabidopsis plants with mutations in the 19S regulatory particle were obtained. These mutants (*rpn10-1, rpn12a-1,* and *rpt2a-2*) are reported to have decreased 26S proteasomal activity, and are more susceptible to stresses inducing protein misfolding; however, to compensate, 20S proteasome accumulates and provides the mutants with increased tolerance to oxidative stress (Kurepa et al., 2008). Thus, the mutants were ideal for testing if oxidative stress or protein misfolding was a bigger determinant of selenate toxicity in Arabidopsis plants. When grown on 40 µM selenate, the relative selenium tolerance decreased in all three mutants compared to wildtype plants (ecotype Columbia). The reduced selenate tolerance was most pronounced in *rpn10-1* plants, which decreased nearly 2-fold compared to wildtype plants (Fig. 7A).

To indirectly test the hypothesis that misincorporation of selenocysteine into proteins induces misfolding, *bip2* mutant Arabidopsis plants were studied. *bip2-1* plants have been reported to be susceptible to stresses that induces protein misfolding in the ER (Wang et al., 2005). On MS media grown with or without 40µM selenate, there is no difference in root length between wildtype and *bip2* mutants. However, when grown on 50 µM selenocysteine, *bip2-1* plants do not survive past germination (Fig. 7B).

Discussion

The misincorporation of selenocysteine into plant proteins replaces cysteine and is thought to be the cause of selenium toxicity in plants. Cysteine has a special role in protein structure, function, and regulation. Indeed, preventing Se incorporation into protein is associated with increased selenate tolerance in plants (Van Hoewyk et al., 2005). The objective of this study was to provide evidence that selenoproteins are malformed and therefore degraded by the ubiquitin-proteasome pathway, possibly hinting that such proteins have a tendency to misfold.

Stanleya pinnata, a Se-hyperaccumulating plant native to seleniferous soils in Western USA, was selected for this study due to its known ability to tolerate selenate and accumulate Se in protein. Inhibiting the proteasome of selenate-treated plants increased the amount of ubiquitinated proteins. This data suggest that selenate induces the formation of misfolded proteins, and implicates the 26S proteasome in a response to selenate. The 26S proteasome is known to remove misfolded proteins that accumulate in response to various abiotic stress, and in particular heat (Small and Vierstra, 2004). Additionally, the 26S proteasome removes misfolded proteins containing the non-protein amino acids canavanine and azetidine-2-carboxylic acid (AZC). Overexpression of RPN10, a subunit of the 19S regulatory particle, conferred elevated tolerance to canavanine in rice (Takase et al. 2004). Additionally, Arabidopsis mutants in the 19S regulatory particle (*rpn10-1, rpn12a-1,* and *rpt2a-2*) have decreased tolerance to both canavanine and AZC, which can replace arginine and proline, respectively. In analogy to the detrimental effects of canavanine and AZC, our data suggest that misincorporation of

selenocysteine into protein is likely to similarly induce misfolding. Interestingly, the 26S mutants also have increased 20S proteasomal activity and increased tolerance to oxidative stress. Thus, these mutants were very useful in deciphering which of the two modes of Se toxicity is more detrimental in a non-hyperaccumulator (e.g. selenate induced oxidative stress or malformed selenoproteins). The data suggest that selenium toxicity in Arabidopsis is more likely to be driven by malformed selenoproteins.

Although direct evidence is not presented to strongly indicate that selenoproteins are truly misfolded, data may indirectly suggest that malformed selenoproteins indeed have a tendency to misfold. Inhibiting the proteasome in selenate-treated plants increased the amount of selenium in protein, but had no effect on the amount of copper, iron, and sulfur in protein. Although this suggests that the proteasome preferentially removed Se-containing proteins, it couldn't fully implicate the 26S proteasome in the removal of selenoproteins, *i.e.* it is possible selenoproteins are oxidized and degraded by the 20S proteasome in a ubiquitin-independent process. However, the observation that the percentage of selenium in ubiquitinated proteins is greater than the amount of iron and sulfur gave credence to the malfolded selenoprotein hypothesis, and likely indicates that the 26S proteasome indeed removes misfolded selenoproteins.

Selenate accumulation is known to induce oxidative stress response in Arabidopsis (Van Hoewyk 2008) and *Stanleya pinnata* (Freeman et al, 2010). The observation that selenate oxidized proteins in *S. pinnata* coincides with previous reports indicating the Se induces oxidative stress. Thus, selenate induces two modes of toxicity, and a model is proposed depicting how both the 20S and 26S proteasomes may respond to selenium stress in plants (Fig 8). One of hand, selenate can becomes reduced into

organic molecules via the S assimilatory pathway, which can lead to malformed selenoproteins that are degraded by the 26S proteasome. The alternative to selenate reduction is probably not met without consequences. An APR2 Arabidopsis mutant, which inhibited the reduction of sulfate into sulfite and likely prevents the reduction of selenate into selenite, had decreased Se in protein and decreased tolerance to selenate. This phenotype was explained by the increase in selenate, which decreased the amount of glutathione and increased the amount of superoxide (Grant et al., 2011). Previously it was shown that selenate decreased ascorbic acid concentration in *S.pinatta* (Freeman et al., 2010), but not Arabidopsis (Van Hoewyk et al., 2007). Thus, it is possible that decreased levels of ascorbic acid might also contribute to the accumulation of oxidized proteins in *S. pinatta* during selenate stress.

The finding that oxidized proteins accumulate in selenate-fed plants is intriguing, because oxidative stress is much more likely to impair activity and the abundance of the 26S proteasome compared to the 20S proteasome (Rienheckel 1998). The data presented do not indicate that levels of the 26S proteasome have been inhibited by selenate. In fact, nondenaturing PAGE analysis suggests that 26S proteasome containing the 19S regulatory subunits appears to accumulate in Se-treated plants, although it must be noted that the antibodies against the Arabidopsis Pba1, RPT2, and RPN12 subunits did not react as robustly as the 20S antiserum in *S. pinnata*. Although the 20S and Pba1 antisera reacts against both the 20S and the 26S proteasome, in our hands we only observed one band in the approximate size range of the catalytic core (800 kDa) and not the 26S proteasome (SI 2) which is nearly 2,000 kDa. The structural integrity of the 26S proteasome is ATP dependent. Because the native gels lacked ATP, the single band

likely represents the catalytic core of both the 20S and 26S proteasome, and thus limits the ability to conclude decisively that the 20S proteasome was upregulated by selenate. Similarly, we report that the RPT2 and RPN12 antisera only produced one band on the nondenaturing gels. In the presence of ATP, the antisera should be immunoreactive against two forms of the 26S proteasome: a catalytic core containing one or two 19S regulatory lids, and thus producing two bands (Yang et al,. 2004). In the absence of ATP, the 19S regulatory lid dissociates from the catalytic core (Yang et al., 2004). Thus, it is possible that the single band reported in this study represents either one form of the 26S proteasome or the 19S regulatory particle lid.

Glutathione is involved in maintaining a constant redox state in plants and is the most abundant form of nonprotein thiols. Selenate is known to mimic sulfur deficiency and decrease levels of reduced glutathione in Arabidopsis (Grant et al. 2011, Hugouvieux et al. 2008) and *Stanleya pinnata* (Freeman 2010). Previously, it was speculated that glutathione concentrations decrease during selenate-treatment in an attempt to recycle cysteine for more important sulfur compounds, such as proteins (Van Hoewyk et al. 2008). However, given that selenate induces the oxidation of protein, it is also possible to speculate that glutathione is consumed as it thiolyates oxidized proteins (Shenton et al. 2003). Thiolyation of proteins is known to occur during oxidative stress in order to protect oxidized residues of proteins, namely cysteine and methionine. In a proteomic study to identify thiolyated proteins under oxidative stress, it was noted that subunits of the 20S proteasome were thiolyated (Dixon et al. 2005). This observation might suggest why the 20S proteasome is more resistant to oxidative stress compared to the 26S proteasome. It is also possible that if Se decreases glutathione to dangerously low levels

in some crops, the 20S proteasome subunits cannot be thiolyated properly, which could exacerbate oxidative stress. Whether or not selenate-treated plants have decrease glutathione levels due to the thiolyation of oxidized proteins has yet to be experimentally determined.

Levels of glutathione have also been reported to modulate the 26S proteasome activity. For example, decreased glutathione concentration can inhibit ubiquitin conjugation activity of E1 and E2 enzymes, which helps explain why oxidative stress impairs 26S proteasome activity (Jahngen-Hodge 1997; Obin et al., 1998). Previously, it was reported that levels of glutathione in *S. pinnata* were higher after selenate-treatment compared to *Stanley albenscens*, a closely related species that is not a Se-hyperaccumulator. Thus, it could be speculated that the higher levels of glutathione in *S. pinnata* may help thwart the susceptibility of the ubiquitin-proteasome pathways during selenate-induced oxidative stress, and explain why levels of the 26S proteasome did not decrease.

Although this is the first study to provide evidence that selenate-treatment induced the accumulation of ubiquitinated proteins, other studies have suggested that selenate regulates transcripts whose protein products are involved in the proteasome pathway. For example, the CHIP protein in Arabidopsis is an E3 ligase, whose expression at the transcript level was upregulated by selenate and heat (Yan et al. 2003). Furthermore, a transcriptome study in Arabidopsis indicated transcripts involved in ubiquitin-proteasome pathways are significantly overrepresented as a group in response to selenate-treatment (Van Hoewyk et al. 2008). Analysis of the microarray data also indicated that selenate increased the transcripts levels of heat shock proteins in the roots

and shoots. Small heat shock proteins bind to unfolded proteins and help prevent nonnative protein aggregation in the cytosol (Mogt, 2003). Therefore, the observation that selenate induced the expression of two small heat shock proteins in this study concurs with other data suggesting that selenate induces protein misfolding. Also consistent with the idea that selenoproteins are malformed, Arabidopsis *bip2* mutants do not survive past germination when grown on selenocysteine, suggesting errors in ER protein folding or quality control. Impaired growth of *bip2-1* on selenocysteine could be do to the formation of diselenide or sulfide-selenide bonds, however this has not been experimentally proven. However, the extent of selenocysteine's impact on the impaired growth of *bip2-1* remains in question. It was necessary to reduce selenocystine to selenocysteine with DTT, and it should be noted that high concentrations of DTT can also reduce disulfide bonds and denature proteins, particularly in the ER. Agents that induce protein misfolding, including DTT and tunicamycin, increase the expression of *Bip2* (Wang et al., 2010). Indeed, the *bip2-1* mutants have increased sensitivity to tunicamycin as previously reported (Wang et al., 2005) and in this study DTT; these phenotypes in *bip2-1* are explained by the accumulation of unfolded proteins in the ER.

Whether or not plants differ in proteasomal processes is response to selenate is not known, however poplar plants treated with selenate also caused an increase in both oxidized proteins and ubiquitinated proteins (SI 3). Selenium hyperaccumulating plants such as *S. pinnata* are noted for their relatively slow growth rate. Protein synthesis and the removal of misfolded proteins are energetically costly for the cell. The proteasome in selenate-treated *Stanleya pinnata* plants removes ubiquitinated selenoproteins and oxidized proteins, but we propose that this comes at a tradeoff. We believe that it is

likely that the proteasome serves to protect *Stanleya pinnata* against misfolded selenoproteins, but on the other hand may limit their growth rate if large amounts of cellular resources are consumed to remove malformed selenoproteins and initiate protein synthesis.

Materials and Methods

Stanleya pinnata growth conditions and MG132 treatments

Stanleya pinnata seeds were germinated on Metromix potting soil for twelve weeks. Plants were treated with 0, 20, 40 and 80 µM selenate under controlled conditions (light intensity of 100 µmol m^{-2} s^{-1}; 16-h-light/8-h-dark cycle at 25°C). Prior to experimental setup, chlorophyll concentration was estimated using a SPAD-502 chlorophyll meter (Konica Minolta; Tokyo, Japan). The proteasome was inhibited by transferring 2 g of leaf discs from mature leaves into Hoagland's Solution (+/- Se) containing 0.1% DMSO +/- 100 µM MG132; this treatment was performed at 25°C in the light for 16 h.

Immunoblotting

Accumulation of ubiquitinated proteins was tested in plants grown with 0, 20, 40 and 80 µM selenate (-/+ MG132 treatment). Briefly, 50 µg of protein were separated on an 8% SDS-PAGE gel and transferred to nitrocellulose by electroblotting. Ubiquitinated proteins were detected using ubiquitin antiserum (Santa Cruz Biotechnology; Santa Cruz, CA, USA). The large subunit of rubisco was also detected with Ponceau stain to ensure equal loading of protein between lanes. The immunoreactive proteins were detected

using alkaline phosphatase. Unless mentioned otherwise, immunoblotting for all other experiments used protein extracts from plants treated with or without 80 µM selenate.

Detection of oxidized proteins was performed using the OxyBlot Protein Oxidation Detection Kit (Millipore Company; Bellerica, MA, USA). Briefly, 10 µg of protein were derivitized with 2,4-dinitrophenylhydrazine (DNP), and separated on 10% SDS-PAGE. Proteins were detected with rabbit anti-DNP primary antibody. On a second gel, 10 µg of protein were separated on a 10% SDS gel, and the large subunit of Rubisco was detected with a primary antibody raised in rabbit (Agrisera; Vännäs, Sweden).

The 20S and 26S proteasome and associated subunits were detected in leaves of plants treated with or without selenate. Proteins were extracted without DTT, and separated using 12% SDS-PAGE or 6% nondenaturing-PAGE. DTT (1mM) was added to protein extracts that were loaded onto denaturing gels, and ATP (5mM) was added to protein extracts intended for native gels. SDS gels and native-gels contained 20 µg of heat-treated protein and 50 µg of native protein, respectively. The polyclonal 20S proteasome antibody was used as previously described (Polge et al. 2009). The Pba1 antisera used in this study reacts against the 20S catalytic core. RPT2 and RPN12 are 19S regulatory particle subunit antisera, both of which reacted specifically against the 26S proteasome that contains 19S particle subunits. Pba1, RPT2, and RPN12 antibodies were used according to the manufacturer's instructions (Santa Cruz Biotechnology; Santa Cruz, CA, USA). Gels were transferred to nitrocellulose, and allowed to react against an anti-rabbit secondary antibody (1:10,000). Immunoreactive proteins were detected using alkaline phosphatase.

For the detection of small Heat Shock Proteins (sHSP) and binding protein-2 (BiP2), 20 µg of protein were separated on a 12% and 10% gel, respectively. SHSP and BiP polypeptides were detected using antiserum previously described. (Heckathorn et al. 1999; Burén et al. 2011).

Elemental analysis of proteins

Incorporation of selenium in protein was determined from selenium-grown plants treated with or without MG132. The MG132 treatment was performed as described, except that 10 g of leaf material from five-separately pooled samples were used. After MG132 treatment, a non-denaturing lysis buffer was used to isolate a protein extract. To determine the effect of proteasome inhibition on the amount of Se in protein, 10 mg of protein were precipitated with tri-chloroacetic acid (TCA), acid digested, and analyzed by means of ICP-AES (inductively coupled plasma -atomic emission spectrometry) on a Perkin Elmer Optima 7300 analyzer.

An ubiquitinated protein fraction and non-ubiquitinated protein fraction were isolated and from four of the remaining protein extracts collected from selenium-grown plants treated with MG132. The ubiquitinated and non-ubiquitinated protein fractions were separated and collected using an Ubiquitin Protein Enrichment Kit according to the manufacturer's protocol (EMD Chemicals; Gibbstown, NJ, USA). Briefly, 5 mg of protein extract were incubated with 150 µl suspension of polyubiquitin affinity beads for 3 hours on a rocking platform at 4^{o}C. Beads conjugated to the ubiquitinated proteins were centrifuged (1000 xG) for 5 s and washed 4 times. The supernatant containing the non-ubiquitinated protein fraction was also collected, and the proteins were TCA

precipitated for further analysis. A 2 µl aliquot of the ubiquitinated fraction and 20 µg of protein from the non-ubiquitinated fraction were subsequently subjected to SDS-PAGE analysis, and allowed to react against ubiquitin antiserum as described. This step was performed to ensure that a large majority of the ubiquitinated proteins was found in the ubiquitin-enriched protein fraction. Elemental analysis of the ubiquitinated and non-ubiquitinated protein fractions was determined by means of ICP-AES. The amount of copper, iron, sulfur, and selenium in these two fractions was estimated by subtracting values from control samples containing polyubiquitin affinity beads that were not allowed to incubate with the protein extract.

Analysis of selenium tolerance in Arabidopsis mutant plants

Arabidopsis mutant plants (*rpn10-1*, *rpn12a-1*, and *rpt2a-2*) with decreased 26S proteasomal accumulation and activity were obtained (Kurepa et al., 2008) to determine if the 26S proteasome helps prevent selenium toxicity. These plants had mutations in the subunits that compose the 19S regulatory particle, which binds to and unfolds proteins sent to the 26S proteasome. Wildtype seeds (Columbia ecotype) and the mutants were grown on vertical plates containing half-MS media with 1% sucrose for 7 days, and then transferred to plates -/+ 40 µM selenate. After an additional 7 days, the root lengths were measured. Because these plants have different root lengths on control media, relative selenate tolerance was determined by dividing the root length of plants grown on selenate by the mean root length of plants grown on control media.

Arabidopsis plants with a mutation in *BiP2* (binding protein-2) were also analyzed for their tolerance to selenate and selenocysteine. Wildtype and *bip2-1* plants

were grown on vertical plates containing half-MS media with 1% sucrose and -/+ 40 µM selenate for 10 days, after which root lengths were measured. Additionally, the plants were also grown on selenocysteine. Selenocystine was reduced to selenocysteine by incubating it with 0.2mM DTT for 4 h at 37°C. Because higher concentrations of DTT can induce protein unfolding and potentially decrease the growth of *bip2-1*, plants were grown on vertical plates containing half-MS media supplemented with 1% sucrose and 0.2mM DTT, with or without 50 µM selenocysteine for 10 days, after which root lengths were measured.

Statistical analyses included student T-test and ANOVA, were performed in the KaleidaGraph software program (Synergy Software).

Funding

This work was supported by the National Science Foundation [grant number MCB-0950648] awarded to D.V.H

Acknowledgements

DVH thanks Goran Samuellson and Scott Heckathorn for kindly providing antibodies against BiP and Small Heat Shock Proteins, respectively. 26S Arabidopsis mutant seeds and the *bip2-1* seeds were generously provided by Jan Smalle and Rebecca Boston, respectively. We thank Bryan Brattin (Center for Environmental Medicine at Colorado State University, USA) for helping perform the elemental analysis of proteins. Lastly, we also acknowledge the help of colleagues at Colorado State University, including Jonathon Harris for providing plant material, and Karl Ravet and Marinus Pilon for

helpful discussions during this project.

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Legends

Figure 1.

The effect of selenate on ubiquitinated (Ub) proteins in leaf tissue from plants treated with or without MG132. Immunoblot of ubiquitinated proteins extracted from *Stanleya pinnata* plants grown on 0, 20, 40, and 80 μM selenate for twelve weeks, and then treated with or without the proteasomal inhibitor MG-132 for 16 hours. Shown below are levels of the large subunit of rubisco detected by Ponceau stain. Fifty micrograms of total protein was separated by 8% SDS-PAGE. The immunoblot is representative of three replicate gels from two experimental replicates.

Figure 2

Elemental analysis of protein from Se-grown plants treated with or without MG132. Proteins (10mg) were precipitated from plants grown on 80 µM selenate for twelve weeks, and then treated with or without MG132. Shown are the mean and standard deviation (n = 5 precipitated protein samples from separately pooled leaf tissue). Different lowercase letters above bars represent a significant difference between treatments ($p < 0.05$).

Figure 3

Elemental analysis of ubiquitinated and non-ubiquitinated proteins in Se-grown plants treated with MG132. Five mg of protein extract from plants grown on 80 µM selenate and treated with 0.1mM Mg132 were allowed to incubate with resin containing polyubiquitin affinity beads. The ubiquitinated (white) and non-ubiquitinated (gray) protein fractions were collected and the ubiquitinated proteins were (A) identified and separated on SDS-PAGE and (B) analyzed for their elemental composition. Results show the mean standard deviation for the percentage of iron, sulfur, and selenium in the ubiquitinated protein fraction and non-ubiquitinated fraction (n=4). Note that levels of copper in the ubiquitinated fraction were below detection limits, and thus omitted. Different lowercase letters represent a significant difference in the percentage of the element in the ubiquitinated fraction ($p < 0.05$).

Figure 4

The effect of selenate on oxidized proteins in plants treated with or without MG132. Immunoblot of protein carbonyl group in *S. pinnata* grown on 80 µM selenate for eight weeks and treated with or without MG132. Ten mg of protein extract were derivitized with 2,4-dinitrophenyl-hydrazone (DNP) and separated on a 10% gel. Oxidized proteins were detected with DNP antibodies. The large subunit of rubisco (Rbc-L) was detected on a second 10% SDS gel and identified with antiserum against Rubisco-L. The immunoblot is representative of three other gels from two experimental replicates.

Figure 5

The effect of selenate on the levels of the 20S and 26S proteasome and proteasomal subunits in plants treated with 80 µM selenate for eight weeks. (A) Complexes of the 20S and 26S were detected by separating 50 µg of protein on 6% nondenaturing gels lacking ATP (top). Rubisco was detected with Ponceau stain to ensure equal loading (below). (B) Individual subunits of the 20S and 26S proteasome were detected by separating 20 µg of protein on 12% SDS-PAGE. Antiserum reacting against the 20S proteasome include a polyclonal antibody against the 20S maize proteasome and Pba1 (β subunit A1). Antiserum reacting against the 26S proteasome containing the 19S regulatory complex include RPR2 (subunit S4) and RPN12 (subunit S14). A coomasie gel is also shown to ensure equal loading of proteins between lanes (C). Images are representative of two technical experiments.

Figure 6

The effect of selenate on the levels small heat shock proteins and binding protein (BiP2) in *S. pinnata*. Immunoblot of small heat shock proteins (sHSP), binding protein (BiP), and the large subunit of rubisco (Rbc-L). Twenty µg of protein was loaded per lane. The immunoblot is representative of two additional experiments.

Figure 7

Selenate tolerance in Arabidopsis 26S proteasome mutants and BiP2 mutants. (A) Relative selenate tolerance (root length on selenate/root length on control medium) in Arabidopsis wildtype (WT) and 26S mutant (*rpn10-1, rpn12a-1,* and *rpt2a-2*) plants. Plants were grown for seven days on half-MS media and then transferred to plates with or without 40 µM selenate for an additional seven days. (B) Selenium tolerance as determined by measuring root length of Arabidopsis wildtype (WT) plants and a Binding Protein-2 mutant (*bip2-1*). Plants were grown for ten days on half-MS media containing 0 or 40 µM selenate; plants were also grown on 0.2mM DTT with or without 50 µM of selenocysteine. Shown are the means (n = 30) and standard deviation. Different lowercase letters above bars denote significant differences ($p < 0.05$).

Figure 8

Proposed model explaining how the 20S and 26S proteasome alleviate two different modes of selenium toxicity.

Figure 1

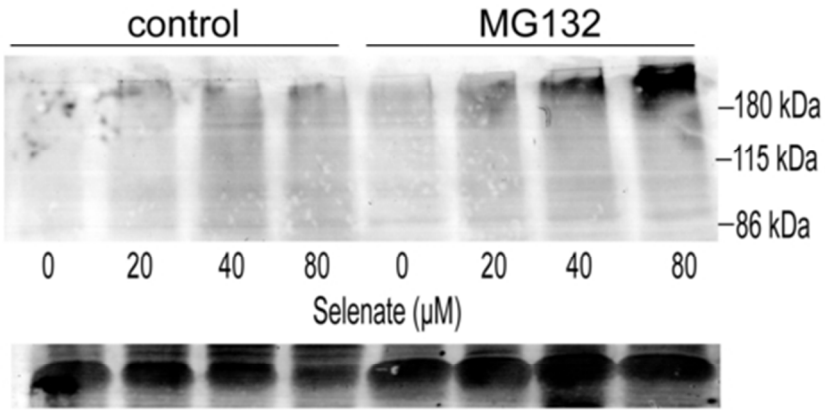

Figure 1
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Figure 2

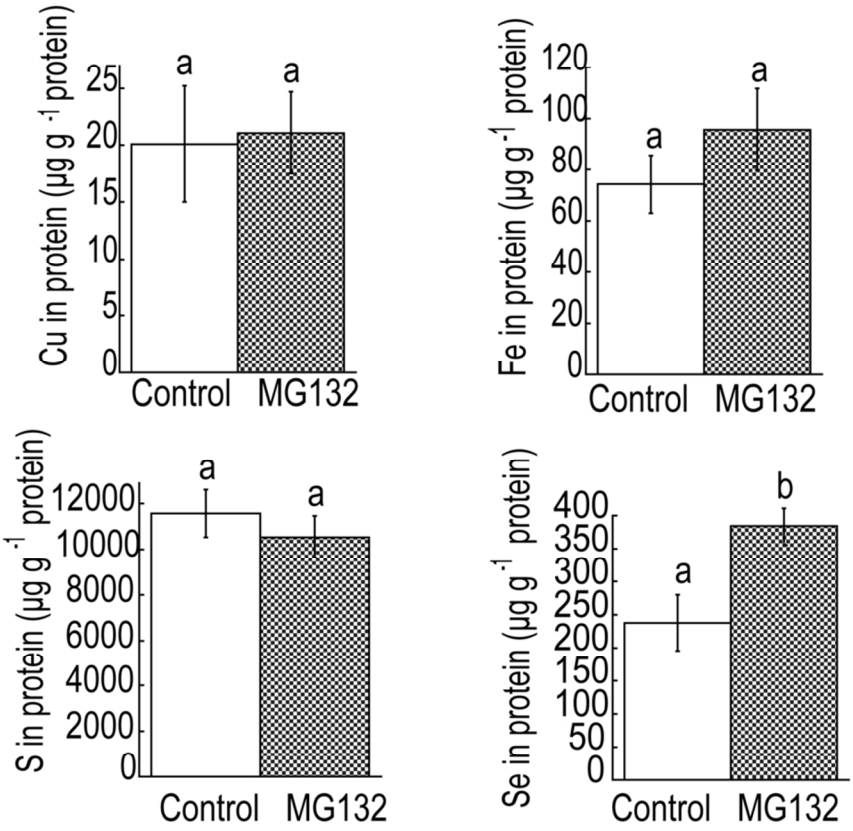

Fig2
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Figure 3

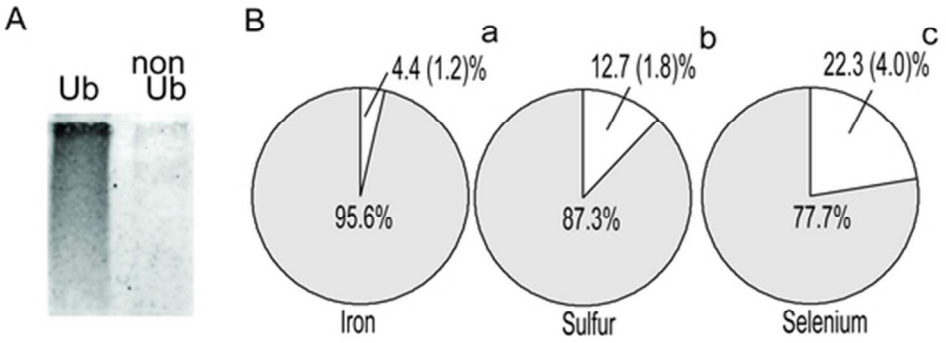

Fig 3
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Figure 4

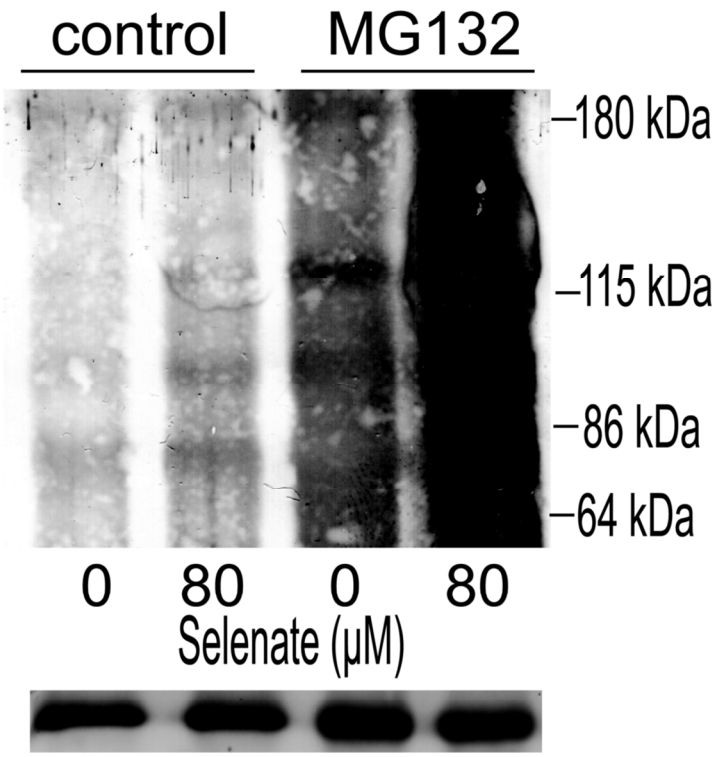

Fig 4
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Figure 5

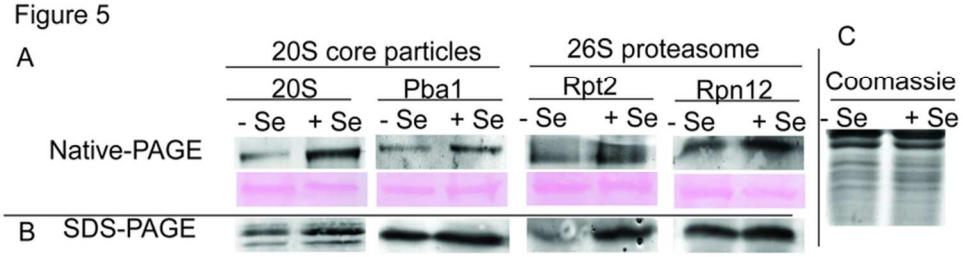

Figure 5
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Figure 6

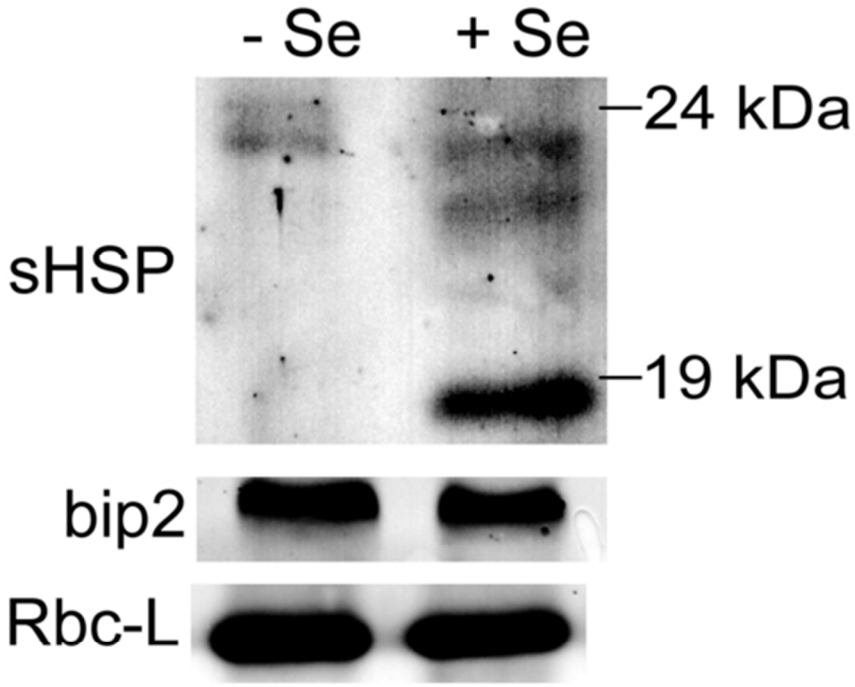

Figure6
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Figure 7

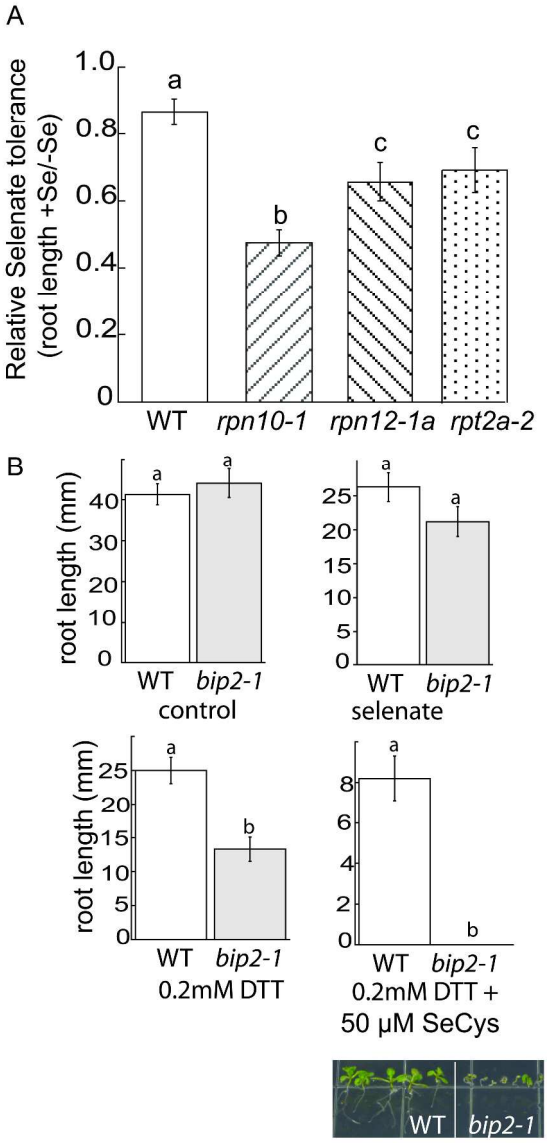

Fig 7
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Figure 8

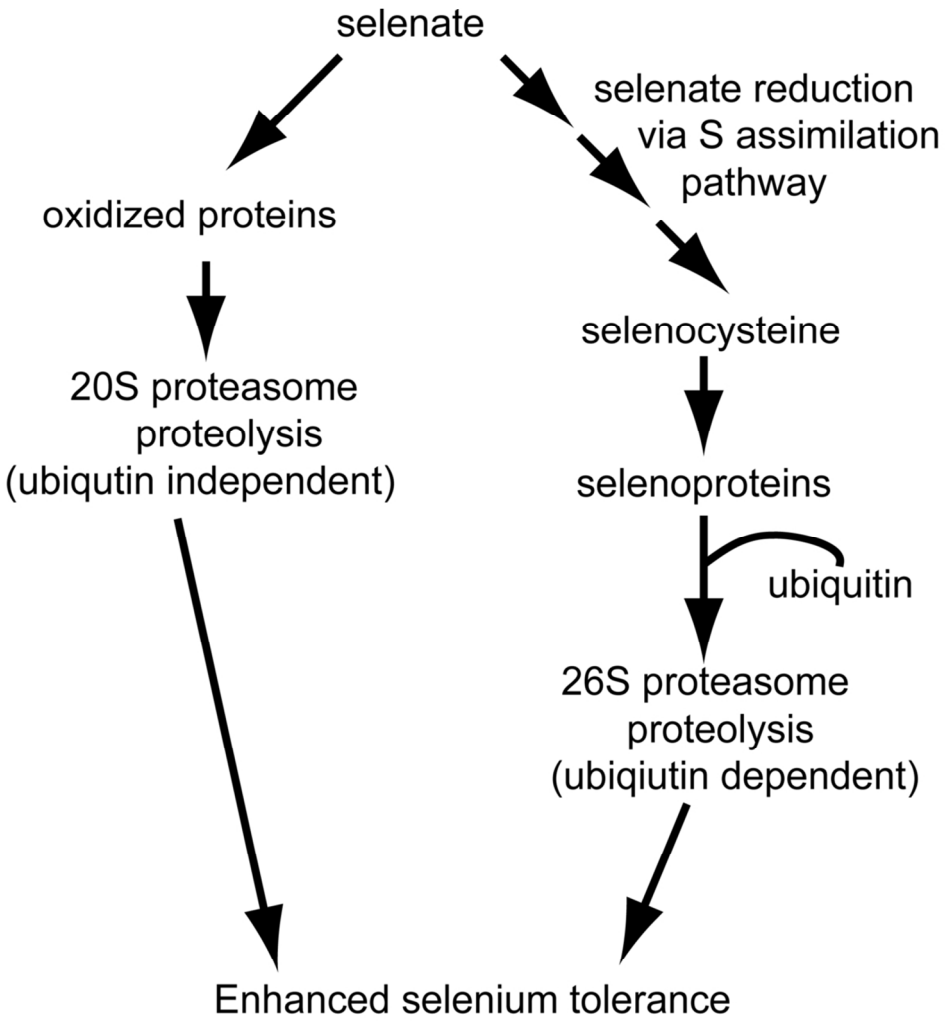

Fig 8
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