THESIS

SELENIUM UPTAKE, DIFFERENTIATION AND METABOLISM IN HYPERACCUMULATOR *STANLEYA PINNATA*

Submitted by

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ABSTRACT

SELENIUM UPTAKE, DIFFERENTIATION AND METABOLISM IN HYPERACCUMULATOR *STANLEYA PINNATA*

Selenium (Se) is a biologically essential element for many animals, some prokaryotes and algae. However, even in organisms that require Se, the range between sufficiency and toxicity for Se is narrow. Although there are no reports of a Se requirement or selenoproteins in higher plants, there are species that appear endemic to seleniferous soil and concentrate Se in their leaves to levels exceeding 1000 mg kg^{-1} dry weight. These plants are known as Se hyperaccumulators and have an exceptional ability to tolerate and enrich themselves with this toxic element. As a result of the Se concentrations in their tissues, Se hyperaccumulators are extremely toxic to most organisms. Studies have found that Se hyperaccumulation protects these plants from many herbivores and pathogens as an "elemental defense." Some of these hyperaccumulators have been studied for their use in phytoremediation of naturally occurring and anthropogenically contaminated seleniferous soils. Although the slow growth of most hyperaccumulators limits their direct application for phytoremediation, they can be utilized as a source of genes to genetically enhance Se accumulation and tolerance in popular phytoremediator species.

The goal of this study is to better characterize the uptake, metabolic fate and molecular mechanisms responsible for Se tolerance in *Stanleya pinnata*, a hyperaccumulator in the Brassicacae. Two main techniques were utilized: physiological experiments followed by elemental analysis to characterize Se uptake and interactions with the related element sulfur (S), and Illumina sequencing of the transcriptomes of *Stanleya pinnata* and related non-hyperaccumulator *Stanleya elata*.

The first chapter presents a literature review of Se hyperaccumulation: what is known about Se assimilation in higher plants, and some unique characteristics of hyperaccumulators. The metabolism of Se through the sulfate assimilation pathway is described, and known mechanisms of Se tolerance and accumulation in representative plants are reviewed. In addition, some of the previous work on *Stanleya* is

reviewed, including a number of studies that have shown ecological benefits of Se hyperaccumulation. Known beneficial genes for Se tolerance and accumulation are discussed in the context of phytoremediation.

In chapter 2, Se-specific uptake was tested in two ecotypes of *S. pinnata,* and contrasted with related non-hyperaccumulator *Brassica juncea.* To test for Se specificity of sulfate transporters, plants were supplied with varying concentrations of selenate and two concentrations of sulfate. The results showed that *S. pinnata* is able to take up large amounts of Se, even at exceedingly low supplied Se:S ratios. In addition, *S. pinnata* preferentially mobilized large amounts of Se to young leaves, without commensurate mobilization of S. These trends were not observed in the non-hyperaccumulator *B. juncea*, which showed dramatically reduced Se uptake under elevated sulfate supply. Moreover, there was no evidence of preferential allocation of Se to young tissues in *B. juncea.* Taken together, these findings support the hypothesis that *Stanleya* contains transporters with an increased specificity for Se, allowing it to take up preferentially and mobilize Se over S. Since previous work has shown that molybdate may be taken up in part by plant sulfate transporters, this element was also monitored. It was observed that increasing supply of selenate and sulfate significantly reduced the molybdenum (Mo) content of leaves in *S. pinnata*. In contrast, *B. juncea* showed an increase in Mo content with increases in supplied selenate.

In the experiment described in Chapter 3, Illumina sequencing was performed to compare the root and shoot transcriptomes of hyperaccumulator *S. pinnata* and non-hyperaccumulator *S. elata* in the presence or absence of selenate. An overview is presented of the overall transcriptome response patterns, followed by a more detailed analysis of transcripts involved in S/Se metabolism. In the presence of Se, 40 of the 56 S/Se-related genes were more highly expressed in *S. pinnata* than *S. elata*. Particularly promising findings include a vastly upregulated root sulfate/selenate transporter (Sultr1;2) and ATP sulfurylase (APS2).

Lastly, some preliminary findings are presented from several biochemical approaches used to further investigate *S. pinnata* hyperaccumulation mechanisms. Organic forms of Se were investigated in *S. pinnata* and *S. elata* using a newly developed liquid chromatography mass spectrometry (LC-MS) method. It was shown that *S. pinnata* accumulates significant amounts of selenocystathionine as well as methyl-selenocysteine. Moreover, activities of selenocysteine lyase (SL) and cysteine desulfurase (CysD) were investigated in *S. pinnata* and *S. elata*, which revealed strong SL activity in the hyperaccumulator. The possible role of this enzyme in Se hyperaccumulation remains to be elucidated. Finally, superoxide dismutase activities were compared between the two species in relation to Se supply.

Stanleya pinnata and other Se hyperaccumulators may be valuable resources for genes involved in Se tolerance and hyperaccumulation, to create genetically engineered plants for phytoremediation purposes. In addition to the potential environmental benefits, understanding potential biological roles for Se and its metabolism in these plants may have broad applications for human health. Many organic seleno-compounds have been studied for their anti-carcinogenic properties in multiple systems and types of cancer. Efficacy of these Se compounds appears to vary based on the form of Se. Plants capable of creating different forms of organic Se may become a valuable pharmaceutical resource.

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CHAPTER 1:

AN INTRODUCTION TO SELENIUM AND HYPERACCUMULATOR *STANLEYA PINNATA*

Selenium was first discovered in 1817 by Jöns Jakob Berzelius, who initially confused it with a tellurium compound with sulfur-like properties. It wasn't until a year later that he determined that his unusual finding was actually a new element. Berzelius named this new element selenium (Se), in reference to the moon, in honor of its chemical similarity to tellurium, which was named for the earth (Weeks, 1932). This initial confusion surrounding selenium's discovery is fitting given its frequently conflicted role in biology, where cells often mistake it for another of its sister chalcogens, sulfur (S) (Shrift, 1969; Terry and Zayed, 2000). Sometimes referred to as a "double-edged sword," Se serves as an essential micronutrient for many forms of life and also a potential toxin. Initially not recognized as an essential element, due to its exceptionally low biological requirements, selenium's essentiality was not known until 1957 (Schwarz and Foltz, 1957). It was then discovered that animals, many algae and some prokaryotes require Se for the formation of a number of selenoenzymes, including thioredoxin reductases and glutathione peroxidases (Ganther, 1999). The insertion of selenocysteine (SeCys) residues into these selenoenzymes requires a specific SeCys tRNA that recognizes a UGA codon in concert with a SeCys insertion sequence (SECIS) (Stadtman, 1996). The biological reasons for the use of SeCys rather than Cys are less well understood. Studies have found some *in vitro* reactions of selenols can occur much faster than sulfur analogs. In addition, the use of SeCys in proteins may be a safety mechanism, as the oxidized product, the selanyl radical is much less oxidizing than its cysteine-thiyl counterpart (Nauser et al., 2012). Selenoproteins and these SeCys tRNAs have not been found in higher plants (Terry and Zayed, 2000; Zhang and Gladyshev, 2009).

Selenium has gained attention in recent years for its importance in human health. Because selenoproteins are usually involved in redox activity, proper levels of Se in the diet have been implicated in the prevention and repair of DNA damage. Some biological forms of Se (i.e. methylselenocysteine or

selenomethionine) have been shown to have significant anti-carcinogenic effects (Whanger, 2002). One study found that both selenite and the overexpression of glutathione peroxidase (GPX-1), a selenoprotein, significantly decreased DNA damage in breast carcinoma cells and mouse fibroblasts (Baliga et al., 2007). Other studies have focused on selenium's effects on DNA methylation, since hypo- or hypermethylation frequently serves as a precursor to certain cancers. One study in rats showed Se deficient diets resulted in significant hypomethylation of DNA, which may be one underlying cause for increased rates of death from certain cancers in regions of the world that are Se deficient (Davis et al., 2000). Colorectal cancers have been shown to significantly down-regulate selenium-binding protein 1 (SBP1) through methylation, and SBP1 overexpressing cell lines showed much enhanced resistance to cancer migration and tumor growth (Pohl et al., 2009). In addition to helping prevent cancer formation, Se may help treat existing cancer, as many Se compounds have been shown to preferentially inhibit the growth of carcinogenic cells and increase apoptosis in certain cancers (Brozmanová et al., 2010). Aside from cancer prevention, proper dietary Se supply has been reported to be important for male fertility, as well as to reduce the impact of Keshan disease, and to slow the progression of HIV to AIDS (Rayman, 2000).

Despite the biological requirement for Se and its myriad potential health benefits, it quickly becomes toxic at relatively low doses. For example, while the USDA recommended daily allowance for humans is 0.06 mg/day, toxicity symptoms can begin to appear at intake amounts exceeding 0.4 mg/day (Goldhaber, 2003). This narrow window between sufficiency and toxicity has consequences that are evident worldwide, as Se is a limiting nutrient for humans and livestock in some parts of the world, while simultaneously costing millions of dollars in environmental clean-up in other areas such as the American west and parts of China. Some of these regions have such high levels of Se in the soil that crops grown on them can be toxic for humans and livestock (James, 1984).

Selenium is found in soils as selenate (SeO_4^{2-}) or more uncommonly as selenite (SeO_3^{2-}), selenide (Se^{2-}) or elemental Se. In much of the world, soil Se concentrations vary between 0.01-2.0 mg kg^{-1}

(Girling, 1984). However, soil Se levels have been reported in excess of 10 mg kg^{-1} in some regions of the United States, usually those with a prevalence of Cretaceous shale (James, 1984). Most plants non-specifically take up Se from the soil using sulfur transporters. The plant sulfate transporter gene family is a large one, with 4 groups and 15 putative sulfate transporters in *Arabidopsis thaliana* alone (Hawkesford, 2003). Although four groups have been classified based on their kinetic properties, and some have been localized, a full understanding of the functions of all sulfate transporters will require substantial future study. A model based on current understanding from Takahashi et al. (2011) is presented in figure 1. The sulfate transporters have 12 membrane spanning domains and a Sulfate Transporter and Anti Sigma factor antagonists domain (STAS) that may be involved in regulation, localization and/or function (Rouached et al., 2009). The two main high-affinity root transporters, Sultr1;1 and Sultr1;2 are symporters that use a proton motive force to drive transport. As would be expected, these transporters are primarily expressed in root epidermal cells such as root hairs (Takahashi et al., 2011). Although Sultr1;1 and 1;2 appear to be functionally redundant, Sultr 1;2 is much more highly expressed, and Sultr1;1 shows a much stronger response to S deprivation (Hawkesford, 2003). Interestingly, *A. thaliana* mutants for Sultr1;2 showed a strong selenate resistant phenotype and significantly lowered symplastic Se levels, suggesting that Sultr1;2 is the main transporter responsible for selenate transport into the root (Shibagaki et al., 2002; Ohno and Uraji, 2012). Sulfate transporter group 2 consists of lower affinity transporters that are expressed in roots and shoots and frequently show strong induction under S starvation (Hawkesford, 2003). Because of the strong induction under S deficient conditions and a large amount of expression in the central cylinder of the root, it is believed that this family may be involved in root to shoot translocation (Takahashi et al., 2011). Transporters in group 3 are poorly characterized, and possible functional roles and cellular localization vary widely within this group. Some of the transporters in this group may cofacilitate other transporters, play a role in the maintenance of symbiotic relationships or be involved in a pathogen response (Takahashi et al., 2011; Petre et al., 2012). Group 4 sulfate transporters appear to be primarily localized in the vacuole – with Sultr4;1 and 4;2 playing a role in the release of stored sulfate from the vacuole, especially under S limiting conditions (Kataoka, 2004).

Once inside the root, plants incorporate selenate and selenite into organic compounds through the S assimilation pathway (Fig. 2) (Terry and Zayed, 2000; Shibagaki et al., 2002). Most S (or Se) assimilation is believed to occur in the chloroplasts after sulfate (or selenate) is transported to the shoots (Ng and Anderson, 1979). However, significant amounts of organic Se in root vacuoles and in the xylem fluid of some hyperaccumulator plants suggest that Se may be assimilated in the roots of these species (Freeman et al., 2006b; Amos et al., 2012). Selenate is initially reduced to selenite via ATP sulfurylase and APS reductase, which is believed to be a rate-limiting step in Se assimilation, based on evidence from overexpression experiments and treatment of plants with selenate vs. selenite (Pilon-Smits et al., 1999). Selenite may be reduced to selenide by sulfite reductase, similar to sulfite, or may be reduced non-enzymatically by glutathione (Terry et al. 2000). Selenide is then combined with O-acetylserine (OAS) by O-acetylserine(thiol)lyase to form selenocysteine (SeCys) (White and Broadley, 2007). SeCys can be combined with O-phosphohomoserine (OPH) to form selenocystathionine. The formation of cystathionine or selenocystathionine is catalyzed by cystathionine-γ-synthase (CGS). Cystathionine may be broken down by cystathionine-β-lyase to form Se-homocysteine and further converted by methionine synthase to Se-methionine (SeMet). SeCys and SeMet are largely indistinguishable from their S analogues for most plants, resulting in non-targeted incorporation into proteins. It is believed that this unintended incorporation results in proteins misfolding or functioning improperly, which is likely part of the reason for selenium's toxicity (Brown and Shrift, 1981; Sabbagh and Van Hoewyk, 2012). Although Se-Met's relative hydrophobicity and lack of disulfide linkages makes it an unlikely candidate to induce protein misfolding, its potential conversion to S-adenosyl (Se) methionine (SAM) may result in effects on methyl donation for polyamines and ethylene synthesis (Brosnan and Brosnan, 2006). In addition, Se has been shown to damage cells through the formation of reactive oxygen species, especially selenite – through reactions with glutathione or organic selenides being converted to selenols in the presence of thiols. (Mezes et al. 2009, Freeman et al., 2010).

Not all plants suffer toxicity in the presence of high Se levels, and some even preferentially occur in seleniferous soil. Frequently, these "indicator plants" for seleniferous ecosystems are also Se hyperaccumulators. Hyperaccumulators are unique in their ability to concentrate Se in their tissues in excess of 1,000 mg of Se kg^{-1} DW, many orders of magnitude higher than their growth medium, with no negative effects (Beath et al., 1934). Hyperaccumulators may use different strategies for coping with Se, but four primary methods appear to be utilized to keep SeCys out of proteins: the breakdown of SeCys, methylation of SeCys, volatilization of Se compounds, or storage in other organic selenocompounds. SeCys can be broken down into elemental Se and alanine through the action of a selenocysteine lyase-like enzyme. The elemental Se form is expected to remain insoluble and biologically unavailable (Garifullina and Owen, 2003). Although several studies have shown that some cysteine desulfurases, like CpNifS, have selenocysteine lyase activity, there is only limited evidence showing that plants accumulate elemental Se in significant amounts (Hoewyk and Garifullina, 2005; Lindblom et al., 2011; Valdez Barillas et al., 2012). A second option, and the primary means by which hyperaccumulators of the *Astragalus* genus detoxify Se, is the methylation of selenocysteine to methylselenocysteine (MeSeCys) by selenocysteine methyl-transferase (SMT) (Shrift and Virupaksha, 1965). Since it is methylated, MeSeCys will not be incorporated into proteins and is much safer to accumulate. MeSeCys can also be processed further into dimethyldiselenide (DMDSe) and volatilized out of the leaf, the smell of which anyone who has walked in a field of *Astragalus* has experienced first-hand (Terry and Zayed, 2000). An alternate pathway for volatilization is through the conversion of selenomethionine to dimethylselenide (DMSe). The last method for keeping SeCys out of proteins is to tie it up organic Se in an intermediate metabolite, particularly selenocystathionine. While cystathionine rarely accumulates in measurable levels in plant cells, some hyperaccumulators such as *Stanleya pinnata* are able to accumulate this compound (Virupaksha and Shrift, 1963; Martin et al., 1971; Peterson and Butler, 1971; Dernovics et al., 2007). The mechanism for this accumulation is currently unknown

In addition to metabolizing most of the Se into organic forms, hyperaccumulators also appear to preferentially allocate Se into specific tissues (epidermal), cells (such as trichomes) and subcellular components like the vacuole (Freeman et al., 2006b; Valdez Barillas et al., 2012). In hyperaccumulators, organic Se has been found in xylem (guttation fluid), lending evidence to the theory that assimilation of Se may occur in the roots (Freeman et al., 2006b).

The reason hyperaccumulator plants actively accumulate such large amounts of Se is only partly understood. There is currently a great body of evidence supporting the "Elemental Defense Hypothesis," which suggests that Se serves as a deterrent and toxin to herbivores (both vertebrate and invertebrate) as well as some pathogens (Hanson et al., 2003; Freeman et al., 2007; Galeas et al., 2008; Quinn et al., 2008). Other studies have found evidence for Se hyperaccumulators' involvement in both positive and negative allelopathy (El Mehdawi et al., 2011; El Mehdawi et al., 2012). However, whether this effect is incidental or a form of targeted negative allelopathy against competitors will require more research.

Although the defense role of Se is well demonstrated, there may be more benefits of Se hyperaccumulation than herbivore protection. Some studies have suggested that hyperaccumulators may use Se as a form of elemental allelopathy to reduce competition by neighboring plants (El Mehdawi et al., 2011). In addition, and most intriguing, there is evidence of a beneficial effect of Se on plant growth, particularly of hyperaccumulator plants, even in the absence of herbivores (Broyer et al., 1972). This suggests a physiological role for Se, although there are still no known selenoproteins known in higher plants. When given in small amounts, Se has been reported to be a beneficial element for many plant species, including hyperaccumulators and nonaccumulators (Pilon-Smits et al., 2009). Plants treated with Se were found to contain higher levels of antioxidant compounds and enzymes, which may be a part of the underlying mechanisms of increased growth (Hartikainen, 2005).

Because of their ability to take up and store high levels of toxic elements, hyperaccumulators have been studied extensively for possible use in phytoremediation, a relatively inexpensive and

sustainable method for cleaning up contaminated sites (Zhu et al., 2009). Between Se-laden oil refinery waste and an estimated 160,000 square miles of land susceptible to irrigation-induced Se contamination, phytoremediation technology's importance for Se cannot be overstated (Seiler et al., 1999). However, this potential application has its challenges. Strong accumulators such as *Astragalus bisulcatus* frequently have a slow growth rate, while tolerant, large, and fast growing plants like *Brassica juncea* (Indian mustard) accumulate lower levels of the element.

There have been several attempts at genetically modifying plants to be better Se accumulators or more tolerant of high Se concentrations. Past studies have overexpressed various enzymes involved in S assimilation such as ATP sulfurylase (APS), cystathionine-ɣ-synthase (CgS), selenocysteine methyltransferase (SMT) and a selenocysteine lyase from mice in *B. juncea* (Pilon-Smits et al., 1999; Van Huysen et al., 2003; LeDuc et al., 2006; Pilon-Smits and LeDuc, 2009). ATP Sulfurylase (APS) is involved in the potentially rate limiting first step in S assimilation. When APS was overexpressed in *B. juncea*, those plants showed 2-3 fold higher accumulation in shoots, as well as an increased organic Se fraction (Pilon-Smits et al., 1999). When cystathionine-ɣ-synthase (CgS) was overexpressed in *B. juncea*, the plants showed a 2-3 fold increased Se volatilization rate, suggesting it may be rate-limiting for this process (Van Huysen et al., 2003). *B. juncea* plants expressing a selenocysteine methyltransferase from *Astragalus bisulcatus* or a selenocysteine lyase from *Mus musculus* both showed 1.7-1.8 fold higher Se concentrations in leaf tissue (Bañuelos et al., 2007). Taken together, these studies suggest that transgenic improvement of Se tolerance, accumulation and volatilization through the manipulation of S assimilation enzymes is an achievable goal. Surprisingly, there have been no reports of transgenic approaches to genetically modify or overexpress root transporters to increase Se uptake in non-accumulator plants (Shibagaki et al., 2002).

Some evidence suggests Se hyperaccumulator plants are able to selectively take up Se over S; compared to non-hyperaccumulators they have enhanced Se:S ratios in the leaves, even with extremely small Se:S ratios in the growth media (Sors et al., 2005b). However, despite numerous studies showing

this trend, there have been very few attempts to characterize and identify the root transporter(s) for Se in these hyperaccumulators. The study described in chapter 2 aims to further investigate the discrimination ability exhibited by hyperaccumulator plants with respect to Se and S analogues. The study focuses on two different ecotypes of Se hyperaccumulator *S. pinnata* as compared to *B. juncea,* a non-hyperaccumulator member of the Brassicaceae family.

Several approaches were used in this study to address the metabolic fate of Se in *S. pinnata,* and this plant's Se tolerance mechanisms. A new LC-MS method was developed to investigate the organic forms of Se accumulated in *S. pinnata* as compared to other members of the genus, and *A. bisulcatus.* Furthermore, using RNA sequencing, a first for this genus, several promising genes were identified that may play a role in Se tolerance and uptake. Together, the findings from these studies may help shed light on the evolution of hyperaccumulation in *S. pinnata,* as well as provide new possibilities for improving phytoremediation through transgenic methods.

FIGURES

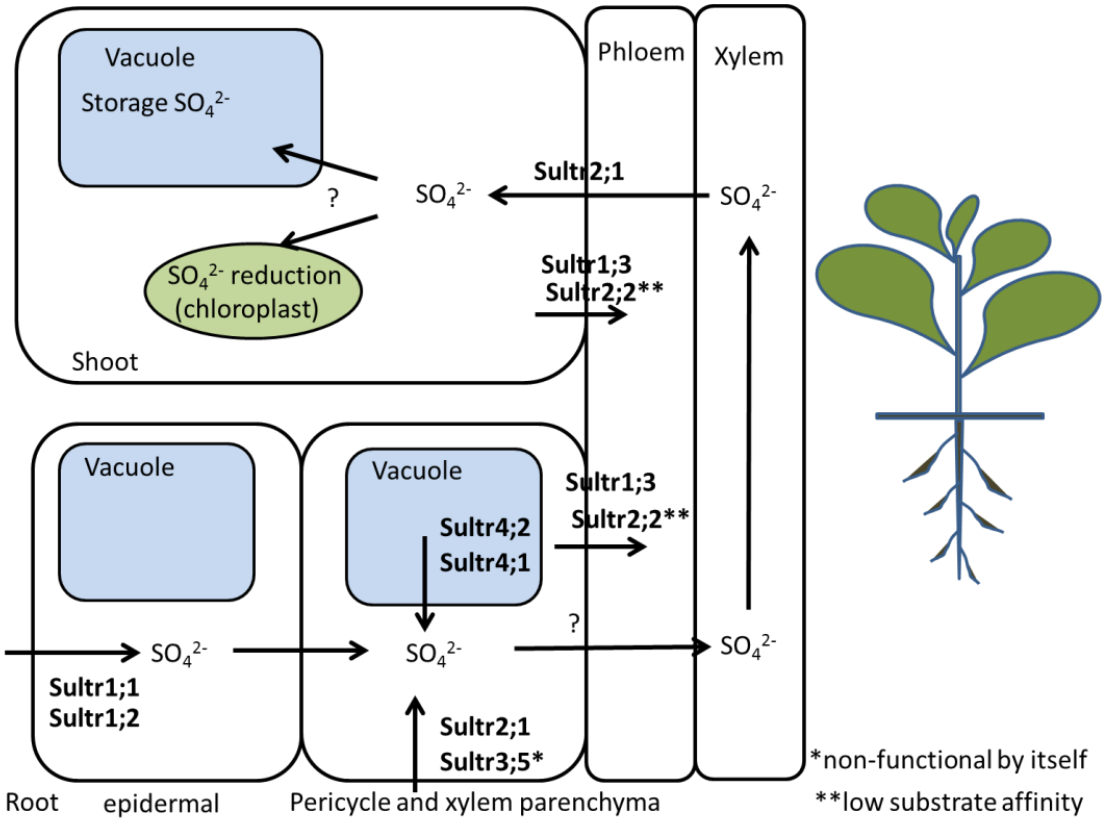

Figure 1. Model for the functional role and localization of several sulfate transporters. Adapted from Takahashi et al. 2011

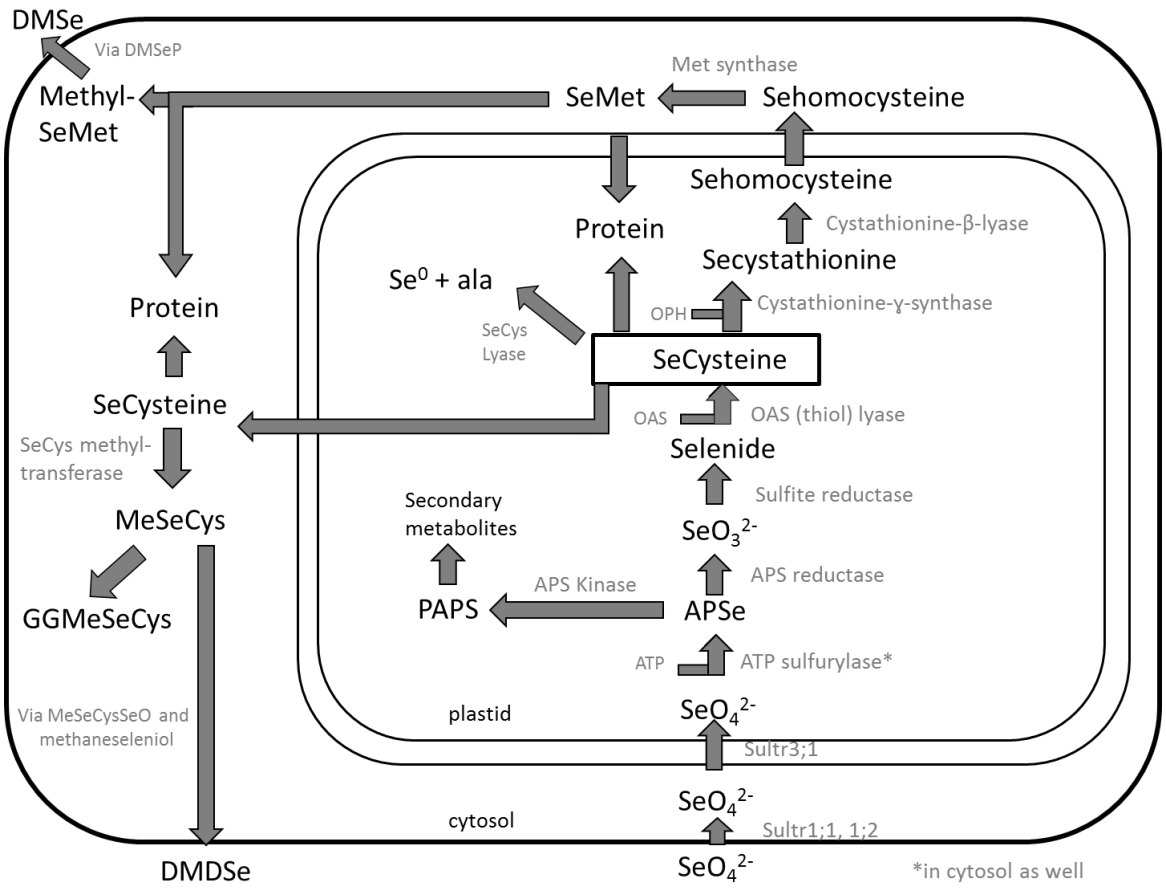

Figure 2. Proposed model for Se assimilation in plants. Enzymes are shown in grey.

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CHAPTER 2:

EFFECTS OF VARYING SULFUR CONCENTRATIONS ON SELENIUM AND MOLYBDENUM ACCUMULATION AND TRANSPORT IN TWO ECOTYPES OF HYPERACCUMULATOR *STANLEYA PINNATA* AND NON-HYPERACCUMULATOR *BRASSICA JUNCEA* (BRASSICACEAE)

SUMMARY

Long-term sulfate, selenate and molybdate accumulation and translocation were investigated in two ecotypes of *Stanleya pinnata* and non-hyperaccumulator *Brassica juncea* under different levels of applied sulfate and selenate. Morphological differences were observed between the ecotypes of *S. pinnata*, but few differences in selenium (Se) and sulfur (S) accumulation were measured. Se to S ratios were nearly identical between the ecotypes under all treatments. When compared with *B. juncea*, several unique trends were observed in the hyperaccumulators. While both *S. pinnata* ecotypes showed no significant effect on Se content of young leaves when the supplied sulfate in the growth medium was increased 10 fold (from 0.5 to 5 mM), the Se levels in *B. juncea* decreased 4-12 fold with increased sulfate in the growth medium. Furthermore, *S. pinnata*'s S levels decreased slightly with high levels of supplied Se, suggesting competitive inhibition of uptake, while *B. juncea* showed higher S levels with increasing Se, possibly due to up-regulation of sulfate transporters. Both ecotypes of *S. pinnata* showed much larger Se concentrations in young leaves, while *B. juncea* showed slightly higher levels of Se in older leaves relative to young. Molybdenum (Mo) levels significantly decreased in *S. pinnata* with increasing sulfate and selenate in the medium; *B. juncea* did not show the same trends. These findings support the hypothesis that *S. pinnata* contains a modified sulfate transporter with a higher affinity for selenate.

INTRODUCTION

Some plants have the unusual ability to accumulate and tolerate one or more toxic elements to extremely high levels of 100-fold or more than surrounding vegetation (Baker and Brooks, 1989). Known

as hyperaccumulators, these plants are taxonomically diverse and vary in their tolerance and accumulation mechanisms. Many of these plants are able to metabolize their accumulated element into safe, organic forms, while others volatilize or store the element in a safe location such as the vacuole (Zayed et al., 1998). Because of their unique physiology, much of the research on hyperaccumulators has been focused on their possible use for phytoremediation of contaminated sites. Phytoremediation technology has promise as a cheap and environmentally friendly method to remove heavy metals or other pollutants from sites contaminated by industry (Peer et al., 2006). If the hyperaccumulated element is an essential nutrient, these plants may be added to livestock feed as a biofortification (Zhu et al., 2009). However, phytoremediation has its challenges. Strong accumulators of toxic elements frequently have slow growth rates, and can be difficult to germinate and grow. In an effort to overcome these shortcomings, there have been many attempts at genetically modifying fast-growing plants with traits from these hyperaccumulators(Pilon-Smits et al., 1999; Van Huysen et al., 2003; LeDuc et al., 2006; Pilon-Smits and LeDuc, 2009). In this regard, hyperaccumulators are a valuable genetic resource, as they can provide genes to improve accumulation and tolerance in faster growing and more economically valuable plants.

Selenium (Se) is common in the soil of some geographic regions, especially those with cretaceous chalk or shale. Selenium can also be anthropogenically introduced into the environment from industry, such as through oil refinery effluent or mining operations (Hansen et al., 1998). As an essential micronutrient for animals, many prokaryotes and some algae, Se enables the formation of many redox-active selenoproteins such as glutathione peroxidases and thioredoxin reductases (for a review see Ganther, 1999). The Se in these proteins is in the form of selenocysteine, which has been called the 21st amino acid (Stadtman, 1996). Despite the essential nature of Se, it quickly becomes toxic at relatively low levels; at 0.4 mg per day in humans some toxicity symptoms may begin to show (Birringer et al., 2002; Goldhaber, 2003). Selenium enters the food chain through plants, and seleniferous areas can contain plants with dangerous concentrations of Se. Hyperaccumulators of Se may contain up to 1.5% Se per kg dry weight (DW). To avoid Se toxicity, ranchers in the American West have learned to keep their

livestock away from soils containing this cretaceous shale and the potentially toxic Se hyperaccumulators that live there (Beath et al., 1934).

Currently, there is no known physiological role for Se or selenoproteins in higher plants. There is limited evidence to suggest increased growth or other beneficial effects of Se on non-accumulator plants, many of these experiments use very low doses or foliar applications (Hartikainen, 2005; Pilon-Smits et al., 2009). In hyperaccumulators, several studies have found over 2-fold enhanced growth in the presence of Se, which suggests a possible beneficial role for Se in the physiology of hyperaccumulators (Trelease and Trelease, 1938; El Mehdawi et al., 2012). However, no current study has provided a conclusive mechanism for beneficial physiological effects of Se.

Selenium hyperaccumulation may confer ecological benefits as an elemental defense (Trumble and Sorensen, 2008). Recent studies have shown that Se accumulation in plants deters both insect and mammalian herbivores, and reduces susceptibility to some pathogens (Galeas et al., 2008; Freeman et al., 2009; Quinn et al., 2010). The tendency of hyperaccumulator plants to preferentially partition Se to valuable organs such as young leaves, flowers and seeds further supports a role in defense (Freeman et al., 2006a; Quinn et al., 2011).

In higher plants, Se is taken up at the root level through the sulfate transporters. Previous studies have shown that *Arabidopsis* mutants for Sultr1;2 are highly selenate resistant, suggesting that this transporter, more than others in the large gene family, may play a large role in Se uptake (Shibagaki et al., 2002). After uptake, Se may remain in inorganic form, or get metabolized into selenocysteine or selenomethionine through the sulfate assimilation pathway (Terry and Zayed, 2000). Because of the chemical similarities between Se and sulfur (S), Se has the potential to affect S homeostasis, redox status and protein folding (De Kok and Kuiper, 1986). Selenoamino acids can be mistakenly incorporated into protein instead of cysteine and methionine; the resulting misfolding events may trigger ubiquitination and subsequent protein degradation (Sabbagh and Van Hoewyk, 2012).

Selenium hyperaccumulators are found in several taxonomic groups, but are best researched in the *Stanleya* and *Astragalus* genera (Shrift, 1969). Plants in both of these genera have evolved mechanisms for preventing organic Se from being incorporated into proteins. In particular, a Se-specific selenocysteine methyltransferase (SMT) is responsible for methylating selenocysteine in some hyperaccumulators and rendering it unavailable to be accidentally incorporated into protein (Neuhierl and Böck, 1996). In addition, hyperaccumulators have been hypothesized to have mechanisms for discriminating between Se and S, since they tend have a higher Se/S ratio compared to their growth substrate (Feist and Parker, 2001; White et al., 2007). Hyperaccumulators also show differential translocation of Se and S within the plant, between organs and different age leaves (Galeas et al., 2007).

In order to further characterize Se/S discrimination, root transport and translocation in a hyperaccumulator in comparison with a related non-hyperaccumulator, we attempted to find taxa that were genetically similar, but may have differing patterns of Se accumulation and tolerance. *Stanleya pinnata* offers an interesting candidate species for this, due to its wide geographic range across a number of habitats that vary in Se content in the soil. In a previous study, Feist and Parker (2001) sampled 15 populations of *S. pinnata* across the western United States and tested them for Se accumulation, tolerance, and Se-to-S ratios. They found a substantial amount of phenotypic diversity, with a 3-fold variation between populations in average shoot weights and leaf Se concentrations. We chose to further investigate two of the most diverse ecotypes from California and Colorado. In test conditions, the Colorado ecotype showed 50-100% more Se accumulation than California (Feist and Parker, 2001). In addition, we grew *Brassica juncea* (Indian mustard), a commonly studied plant for phytoremediation, which is tolerant to Se and a known "secondary-accumulator" that takes up reasonably large amounts of Se non-specifically due to its high rate of S uptake. Using these three plant types, Se and S uptake and accumulation was measured across a range of provided selenate concentrations and at two levels of sulfate supply, with the goal to elucidate differences in Se and S transport and competition in hyperaccumulators and non-hyperaccumulators. In addition to Se and S, molybdenum (Mo) uptake was measured, since other studies

have shown that molybdate can compete with sulfate and selenate for sulfate transporters (Schiavon et al., 2012).

METHODS

Selenium/sulfur interactions

Stanleya pinnata seeds from two accessions were grown on washed 2:1 Turface®/sand mixture in a grow room under fluorescent lights (even mix of Agrobrite© and AgroSun© tubes) with a 16/8 hour light/dark photoperiod. Seeds were germinated and thinned to 1 plant per 9 x 9 cm pot, and pots were rotated under the lights every week. The Colorado accession was obtained from Western Native Seed (Coaldale, Colorado). The California accession was generously provided by David Parker and harvested from the Mojave National Preserve in California. Replicates of five plants were watered with ¼ strength Hoagland's solution with 0, 10, 40 or 80 µM sodium selenate twice per week for five months. An additional four groups received the same treatment, but with 5mM additional sodium sulfate added to the existing 0.5 mM sulfate present in ¼ Hoagland's solution.

Brassica juncea (L.) Czern. (Cv. PI 426314) seeds were also grown on 2:1 Turface®/sand mixture in a greenhouse under a 16 hour light period for three months. Watering treatments were replicated as before, but with 0, 10, 20 and 40 µM sodium selenate, and eight replicates per treatment.

After three and five months, respectively, *B. juncea* and *S. pinnata* plants were washed, weighed and subdivided into young leaves (first and second set of leaves), mature leaves (lower than four sets of leaves down from youngest leaves) and root tissue. Plant material was dried at 50°C for 48 hours, after which dry weight was determined. Once tissue was dried, 100 mg of sample was digested with 1 mL of 70 % trace metal grade HNO on a heat block for 2 hours at 60° C and 6 hours at 120° C. Digested samples were diluted with ddH_2O to a volume of 10 mL. Elemental analysis was performed according to standard protocols for ICP-AES (Pilon-Smits et al. 1999).

Statistical analysis

Statistics were performed using JMP (Version 10. SAS Institute Inc., Cary, NC, 1989-2013). A Wilcoxon signed rank test was used to compare between treatments in a pair-wise fashion, due to non-parametric data.

RESULTS

The two ecotypes of *S. pinnata* from Colorado and California (CO and CA) showed some differences in growth as well as Se and S accumulation when grown at different Se/S ratios. For all Se concentrations except 40µM (0.5 mM sulfate), CA had significantly more root and shoot biomass than CO (Fig. 3A, $P < 0.05$). Both CO and CA growth was unaffected by Se concentrations up to 40 µM. At 80 µM selenate, both ecotypes' shoot biomass was significantly reduced (Fig. 3A, $P < 0.05$).

In the absence of Se, adding 10 fold higher sulfate levels (5 mM) had no effect on the biomass of the two ecotypes (Fig. 3A, B). However, additional sulfate ameliorated some of the negative effect of Se on growth in both ecotypes (Fig. 3B). For both CO and CA, the growth inhibition by Se was completely alleviated by additional sulfate, with no significant difference in biomass across all treatments.

Both of the ecotypes of *S. pinnata* showed hyperaccumulator levels of Se (>1,000 mg kg^{-1} DW) in young and mature leaves (Fig. 4A, B). Maximum leaf Se concentration was reached at the supplied 40 µM concentration (Fig. 4A, B). The Se levels in young leaves were ~25% higher in CO than in CA for the 40 and 80 µM Se treatments with 0.5 mM sulfate ($P < 0.05$ Fig. 4A). Mature leaves contained 6-12 fold lower levels of Se relative to young leaves for all Se treatments ($P < 0.05$, Fig. 4B). The Se levels in mature leaves were not significantly different between the ecotypes with 40 and 80 µM supplied selenate. Roots of both ecotypes showed similar levels of Se, with no significant differences. Both ecotypes showed a linear increase in root tissue concentration with increasing levels of supplied selenate, and contained Se levels similar to mature leaves (Fig. 4C).

In general, increasing the amount of provided S by 10 fold slightly decreased the amount of Se in both ecotypes across all organ types (Fig. 4). However, in young leaves, the only significant decrease occurred in CO at 80 µM ($P < 0.03$). In mature leaves and roots, there were some significant decreases at 40 and 80 µM for the two ecotypes, but mostly showed little effect of S on Se.

The CO ecotype contained significantly more S in young leaves than CA at 40 and 80 µM ($P < 0.05$), while there was no significant difference in mature leaves and roots under normal S conditions (Fig. 5). In young leaves, both ecotypes showed a similar decrease in S concentration with increasing Se provided, with significantly less S at 80µM selenate ($P < 0.05$, Fig. 5A). A similar interaction between leaf S and supplied Se was observed in the CO ecotype for mature leaves (Fig. 5B). In contrast, the CA ecotype S concentration in mature leaves was independent of external Se supply (Fig. 5B). In roots there was no clear interaction between S and Se observed in either ecotype (Fig. 5C).

Surprisingly, increasing the sulfate supply 10 fold did not result in a statistically significant increase of S concentration in young leaves (Fig. 5A, D). The CO ecotype accumulated significantly more S in its young leaves than ecotype CA under these high S conditions and at 0, 40 and 80 µM selenate ($P < 0.05$). In mature leaves, CO accumulated significantly more S than CA at 0 and 10 µM selenate ($P < 0.05$). Similar to what was observed at the lower S level, leaf S decreased with increasing supplied Se for both ecotypes in young leaves, but only for CO in mature leaves ($P < 0.05$, Fig. 3). The CA ecotype S concentration in mature leaves was independent of external Se supply even at the elevated S level (Fig. 5B, E). In the roots that received additional S, neither ecotype showed a significant interaction between S and Se (Fig. 5F).

Supplied Se had a strong inhibitory effect on Mo levels in young and mature leaves, particularly in the CO ecotype (Fig 6A, B). Trends in root tissue were not as clear (Fig. 6C). Molybdenum concentrations in mature leaves were higher in CO than CA at 0, 10 and 40 µM supplied selenate ($P < 0.05$), and both ecotypes had higher Mo levels in mature leaves than in young. The inhibitory effect of Se

on Mo uptake was less obvious with additional S, as 5 mM sulfate already dramatically reduced the levels of Mo in the leaves (Fig. 6E, F).

In contrast to *S. pinnata*, non-hyperaccumulator *B. juncea* showed no consistent biomass differences in shoot or root dry weight with increasing supplied selenate concentration (Fig. 7). The only significant difference was a decrease in root biomass from 0 to 10 µM selenate in the presence of 0.5 mM sulfate ($P < 0.05$).

Selenium concentrations in different tissues of *B. juncea* increased with Se supply in the presence of 0.5 mM sulfate (Fig. 8A-C, black circles). However, additional sulfate (5 mM) reduced the amount of Se accumulated 4-12 fold depending on supplied Se in all tissue types ($P < 0.05$, Fig. 8A-C, open circles). The Se concentration in mature and young leaves of *B. juncea* was similar for most treatments, except when supplied with 40 µM selenate, when there was ~60% more Se in mature leaves than in young leaves ($P < 0.001$, Fig. 8A, B). Sulfur concentrations were higher in all organs when plants were treated with 5 mM sulfate, but only in the absence of Se ($P < 0.05$, Fig. 8D-F). Interestingly, the presence of Se enhanced S levels in young and mature leaves of plants treated with 0.5 mM sulfate, but not in the high-sulfate plants. As a result, the 0.5 mM sulfate group accumulated more S than the 5 mM sulfate group in young leaves at 10, 20 and 40 µM selenate and in mature leaves at 20 and 40 µM selenate ($P < 0.05$, Fig. 8D, E).

There were few effects of increasing sulfate or selenate supply on Mo levels in the tissues of *B. juncea* (Fig. 9). There was a slight increase in Mo concentration in mature leaves at 20 and 40 µM Se ($P < 0.05$) but only in the 0.5 mM sulfate treatment (Fig. 9B).

High Se to S ratios in the leaves relative to the growth medium are frequently used as an indicator for hyperaccumulator species (White and Broadley, 2007). The two ecotypes of *S. pinnata* showed equally high Se/S ratios in young leaves, which were both significantly higher than in *B. juncea* for all treatments with Se (Fig. 10A, D, $P < 0.01$). Both *S. pinnata* ecotypes showed evidence of Se

enrichment in leaves at all levels of supplied selenate (Fig. 10A). This phenomenon is most striking for the high S treatment: at the 10 µM Se treatment (Se/S ratio = 0.002), the young leaves of both ecotypes contained a Se/S ratio of 0.21, 100 fold greater than the medium. *B. juncea* showed no evidence of Se enrichment: the tissue Se/S ratio was equal or smaller than the substrate Se/S ratio, and with increased sulfate this was reduced proportionally (Fig. 10). Both ecotypes of *S. pinnata* showed significantly lower Se/S ratios in mature leaves and in roots relative to young leaves (Fig. 10A – C). In *B. juncea* there were no significant differences in Se/S ratio between young and mature leaves. Increasing the provided sulfate in the medium did not significantly affect Se/S ratios in young leaves of *S. pinnata*, but *B. juncea* showed significantly lower Se/S ratios at 10, 20 and 40 µM supplied selenate ($P < 0.05$, Fig. 10). Mature leaves and roots of *B. juncea* had significantly lower Se/S ratios with increased sulfate supply at 10, 20 and 40 µM selenate ($P < 0.01$). The two ecotypes of *S. pinnata* showed decreased Se/S ratios with additional S at 40 µM Se ($P < 0.05$).

Selenium and S appear to show contrasting mobilization patterns in hyperaccumulators, both from mature to young leaves and from roots to young leaves (Figs. 11, 12). The Se levels were around 20 fold higher in young leaves of *S. pinnata* than in roots, while *B. juncea* showed only around 3-4 fold higher levels (Figs. 4, 8). Adding additional S to the media did not affect this pattern.

Sulfur levels in *S. pinnata* were similar in young and mature leaves (young leaves/mature leaves = 1), while the ratio of Se concentration in young and mature leaves ranged between 10-40 fold (Fig. 11A, B). The two ecotypes of *S. pinnata* showed similar responses in S distribution with increasing Se supply, with no significant differences (Fig. 8B). Surprisingly, increasing the amount of provided S increased the Se in young leaves relative to mature leaves in ecotype CA at 10 and 40 µM selenate ($P < 0.05$, Fig. 11A, C). The S ratio in young leaves relative to mature leaves was the same under regular and elevated S conditions (Fig. 11B, D).

There were no significant differences between the ecotypes of *S. pinnata* with respect to the ratio of Se and S in young leaves relative to roots (Fig. 12). However, the young leaf/root ratio of Se and S was significantly higher in both *S. pinnata* ecotypes than in *B. juncea* for all treatments except 10 µM Se with low sulfate ($P < 0.05$, Fig. 12). In addition, while there were few significant differences between S and Se in *B. juncea's* young leaf/root ratio, both ecotypes of *S. pinnata* showed significantly higher young leaf/root ratios for Se than for S at 10 and 40 µM selenate ($P < 0.05$, Fig. 12).

DISCUSSION

Comparison of S. pinnata and B. juncea

The results presented here show some pronounced differences between hyperaccumulator *S. pinnata* and related non-hyperaccumulator *B. juncea* with regard to Se, S and Mo uptake, accumulation and movement within the plant. Both *S. pinnata* ecotypes demonstrated significant Se enrichment relative to S in all organs tested, regardless of the ratio of Se/S provided in the growth medium; *B. juncea* did not show evidence of Se enrichment relative to S, but took up Se and S in the same ratio as supplied.

Brassica juncea accumulated substantial levels of Se (up to 2,000 mg kg^{-1} DW, Fig. 8A-C), likely due to its constitutively high uptake of S. *B. juncea* was tolerant to these tissue levels of Se concentrations, judged from the fact that it showed no differences in biomass or morphology between the Se treatments. *Brassica juncea*'s Se levels dropped 4-12 fold when the supply of sulfate was increased from 0.5 mM to 5 mM, which was probably due to competition for the same sulfate/selenate transporters (Fig. 8A-C , open circles). This inhibition of selenate uptake with increased supplied sulfate is similar to reports from previous studies on members of the *Brassica* genus. One study found as much as a 90% inhibition of selenate uptake from increased sulfate in the growth medium, but only a 33% decrease in selenite uptake (Zayed et al., 1998). The two *S. pinnata* ecotypes showed no significant decrease in Se concentration in young leaves when the supplied sulfate was increased 10 fold (Fig. 4). This suggests that *S. pinnata* has a root transporter with a much higher affinity for Se than S. A potential candidate to

mediate Se-specific transport into the root of the hyperaccumulator may be a homolog of the *Arabidopsis thaliana SULTR 1;2*. Knockout *A. thaliana* plants for this gene showed a dramatic increase in selenate tolerance which corresponded with reduced selenate uptake (Shibagaki et al., 2002).

In addition to root uptake, the mobilization and concentration of Se in the young leaves of *S. pinnata* were different from *B. juncea* (Figs 4, 11). *Stanleya pinnata* showed nearly 7 fold higher Se levels in young leaves than mature leaves, while *B. juncea* had a higher Se concentration in mature leaves than young leaves (Figure 8). In *S. pinnata* the preferential accumulation in young leaves was much more pronounced for Se than for its analog S (Figs 4, 5), suggesting preferential mobilization of Se over S. This is also visible in the different Se/S ratios in young vs. mature *S. pinnata* leaves (Fig. 10B). In *B. juncea* Se and S remobilization were more similar, and thus Se movement appears to be non-specific.

Comparison of S. pinnata CO and CA ecotypes

The two different ecotypes (Colorado and California) of *S. pinnata* were clearly different in their biomass, but only showed moderate differences in S and Se accumulation. The California ecotype grew significantly larger than Colorado in root and shoot biomass across nearly every treatment. The Colorado ecotype showed 25% higher levels of Se and S accumulation under some conditions (Fig 4, 5). This higher level of Se and S accumulation appears to be due to higher expression of S transporters, rather than changes in Se specificity, since the Se/S ratios were nearly identical in both ecotypes (Fig. 10). Our findings differed from that of Feist and Parker (2001), who found much larger differences between these ecotypes. In that study, supplied selenate and sulfate were recirculated continuously through the growth medium, resulting in a decreasing supply as the experiment progressed, while we supplied new solutions of Se and S bi-weekly. Our method resulted in significantly higher tissue concentrations of Se and S. The higher Se supply may have decreased the differences in uptake between the ecotypes. Based on our studies, both ecotypes hyperaccumulate Se.

Interactions between Se, S and Mo

The concentration of Mo in *S. pinnata* showed a strong negative response to increasing selenate and sulfate in the growth medium, likely due to competitive inhibition for root transport. Schiavon et al. (2012) showed that a molybdate is likely taken up through sulfate transporters (in addition to MOT1), due to significant evidence for competition with S and Se at the transporter level. This may explain why selenate and sulfate may have reduced the uptake of molybdate in our plants. It is interesting to note that relatively small concentrations of selenate in the medium (0-80 μM) had as large of an effect as adding 5 mM sulfate, suggesting selenate may outcompete molybdate for transport better than sulfate. It is also possible that interactions between Mo, S and Se happen at the level of various enzymes involved in sulfate assimilation, such as ATP sulfurylase which may act on molybdate and selenate in addition to sulfate (Schiavon et al., 2012). The higher levels of Mo in the Colorado ecotype as compared to CA under most conditions fits with the hypothesis that Mo finds its way into the root through sulfate transporters, and that CO has higher expression levels of sulfate transporters than CA.

Unlike in *S. pinnata,* there seemed to be little effect of Se on Mo content of the organs of *B. juncea.* These findings appear to contradict those of Schiavon et al. (2012); however, their study was over a much shorter period. Since our study was over several months, we believe the plants exposed to Se were increasing the abundance of sulfate transporters at the root level (as observed by increasing S levels with increasing Se, Fig. 8D-F) in response to a perceived S starvation in the presence of Se (also observed in *A. thaliana* by Van Hoewyk et al., 2008). If this is indeed the case, it may explain why at high Se levels *B. juncea* had significantly more Mo in roots and mature leaves than without Se (Fig 9B, C). *Stanleya pinnata* did not show this effect of Se supply on S and Mo levels, perhaps because their selenate/sulfate transport is permanently upregulated and not further induced by additional Se.

Implications for Phytoremediation

There have been multiple studies examining the phytoremediation potential of different ecotypes of *S. pinnata* (Parker et al., 2003; Freeman and Bañuelos, 2011). Some *S. pinnata* ecotypes were shown to be fairly fast-growing and tolerant to the challenges present at selenate-polluted environments (e.g. high B levels, salinity). Nevertheless, *Brassica* species like *B. juncea* or *B. napus* are still the most popular for reclamation of Se polluted areas, owing to their favorable agronomic properties and their economic value as crops. However, the results reported in this study clearly show that the selenate accumulation potential of *B. juncea* is severely inhibited by high sulfate levels. Of course our growth conditions, with a constant supply of nutrients, water and a 16 hour photoperiod, are not representative for many field conditions, but likely the same principle will be true in the field. Under field conditions that include high sulfate levels *S. pinnata* ecotype CA may be superior to *B. juncea* in terms of its Se phytoextraction capacity. Ecotype CO can accumulate even higher Se levels than CA but has a slower growth rate, making it overall less efficient. CO and CA did not differ in Se/S ratios, which may indicate that any functional changes in sulfate transporter selectivity for Se are similar between the two ecotypes and they only differ in their regulation and not their kinetic properties. For further studies it will be interesting to identify the responsible selenate transporter gene in *S. pinnata* and transfer it to high biomass species like *B. juncea*.

FIGURES

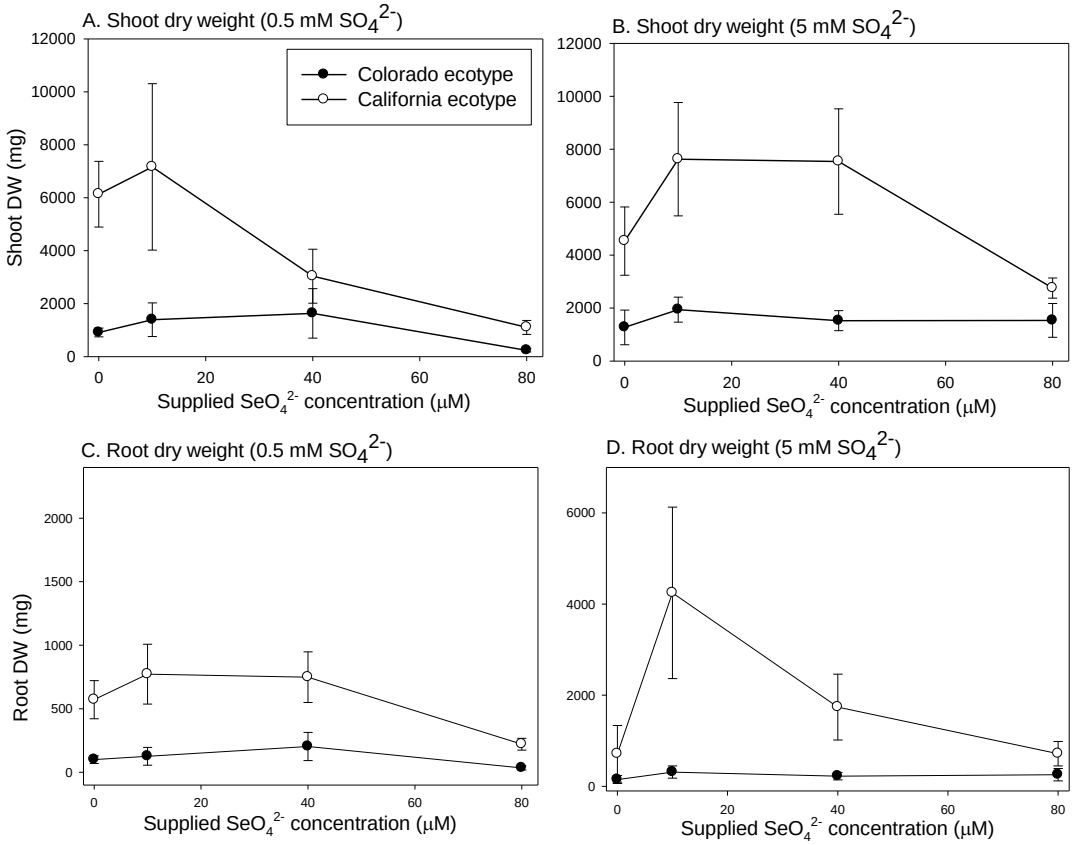

Figure 3. Shoot dry weights of two ecotypes of *Stanleya pinnata* treated with differing selenate and sulfate concentrations. Shown values represent mean with SEM of five biological replicates.

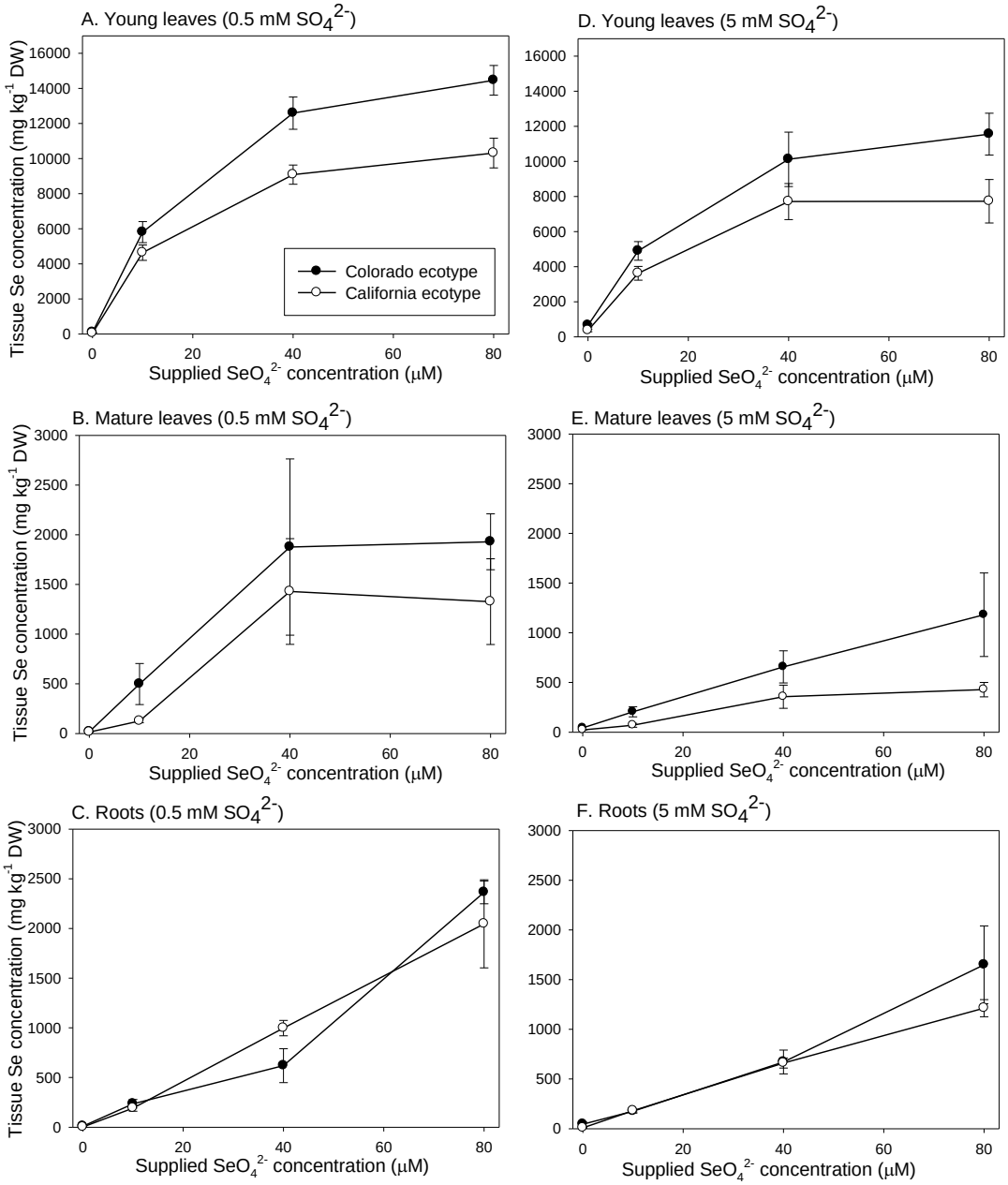

Figure 4. Selenium concentration in young leaves, mature leaves and roots of two ecotypes of *Stanleya pinnata* treated with differing selenate and sulfate concentrations. Shown values represent mean with SEM of five biological replicates.

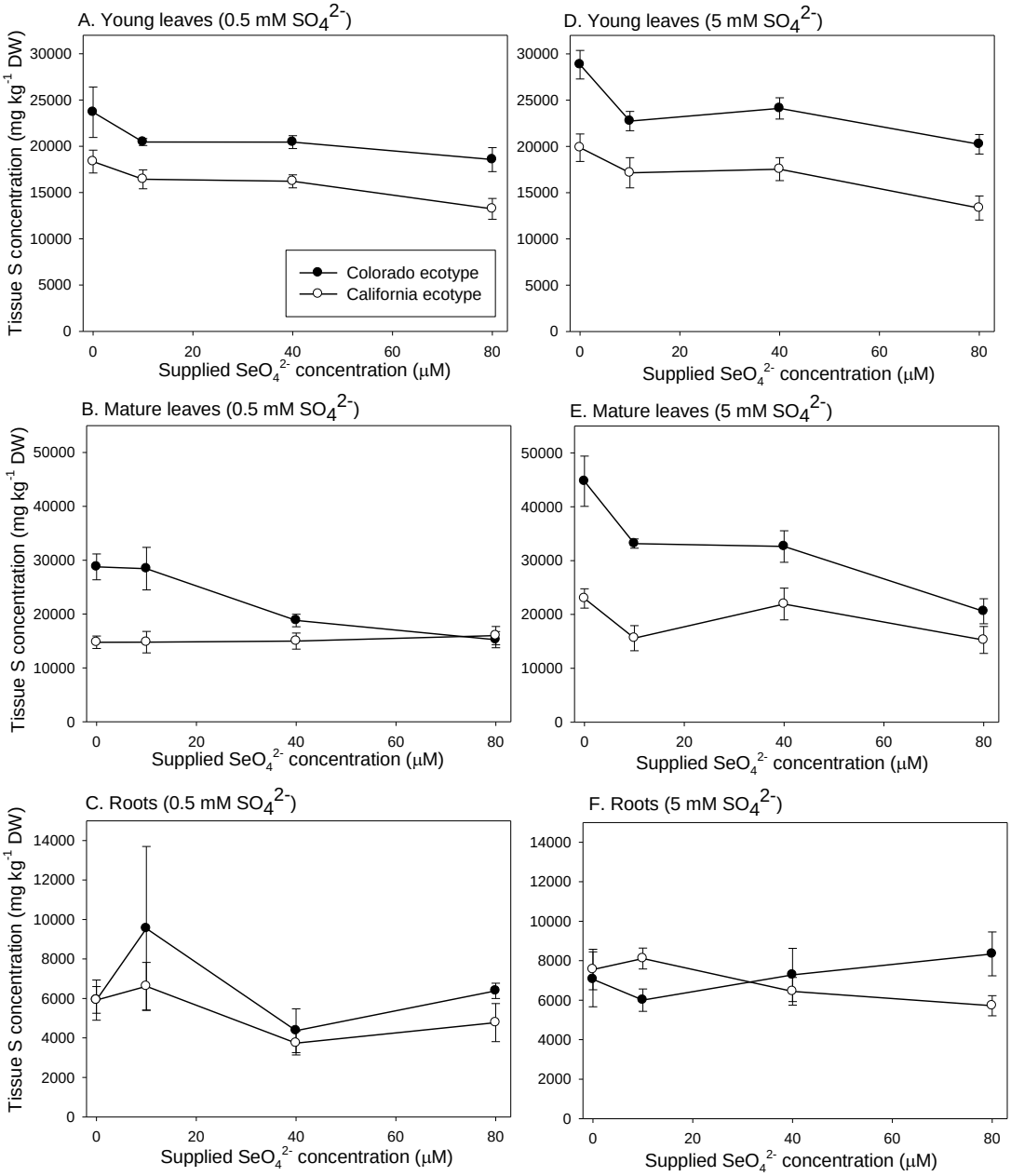

Figure 5. Sulfur concentration in young leaves, mature leaves and roots of two ecotypes of *Stanleya pinnata* treated with differing selenate and sulfate concentrations. Shown values represent mean with SEM of five biological replicates.

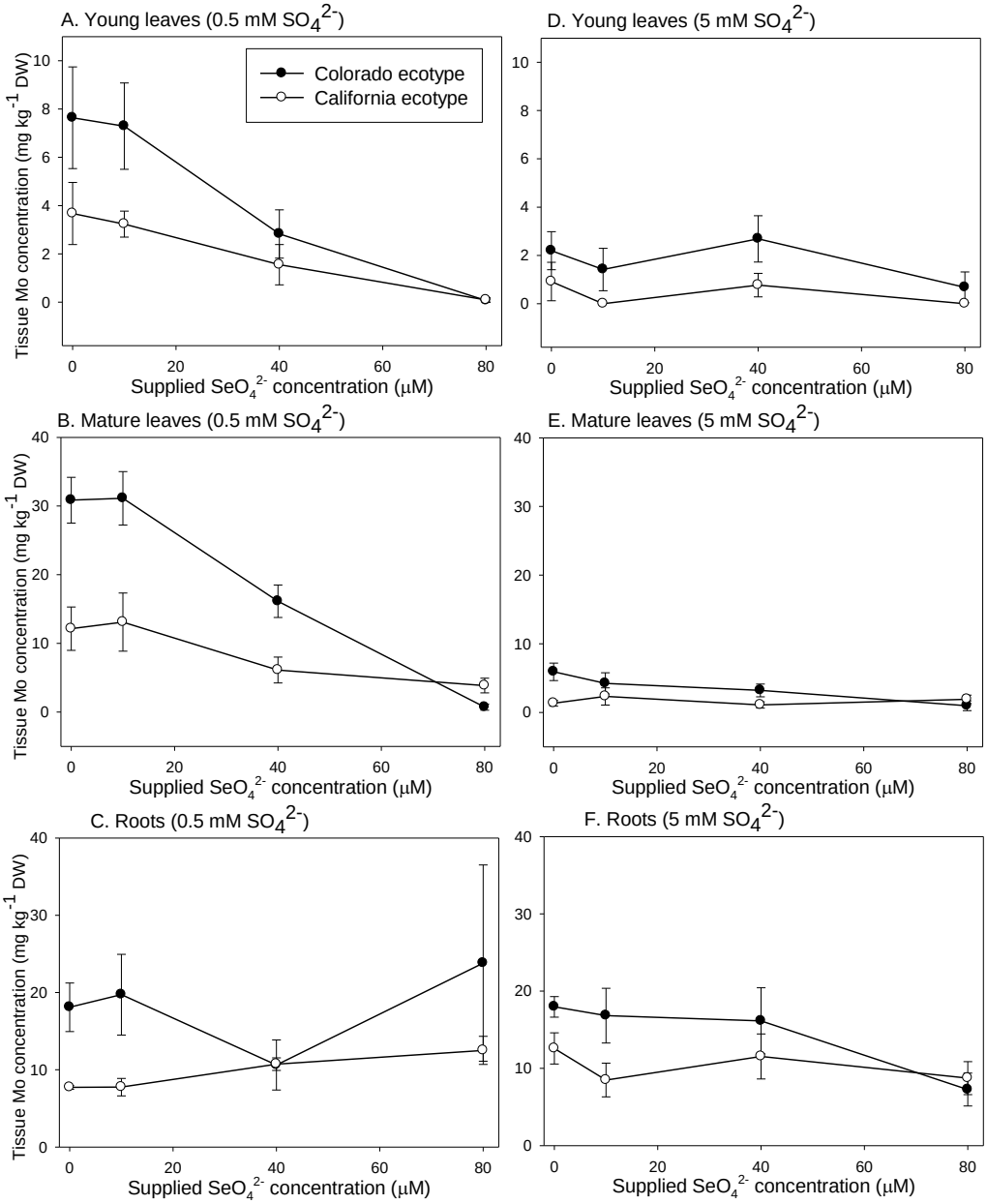

Figure 6. Molybdenum concentration in young leaves, mature leaves and roots of two ecotypes of *Stanleya pinnata* treated with differing selenate and sulfate concentrations. Shown values represent mean with SEM of five biological replicates.

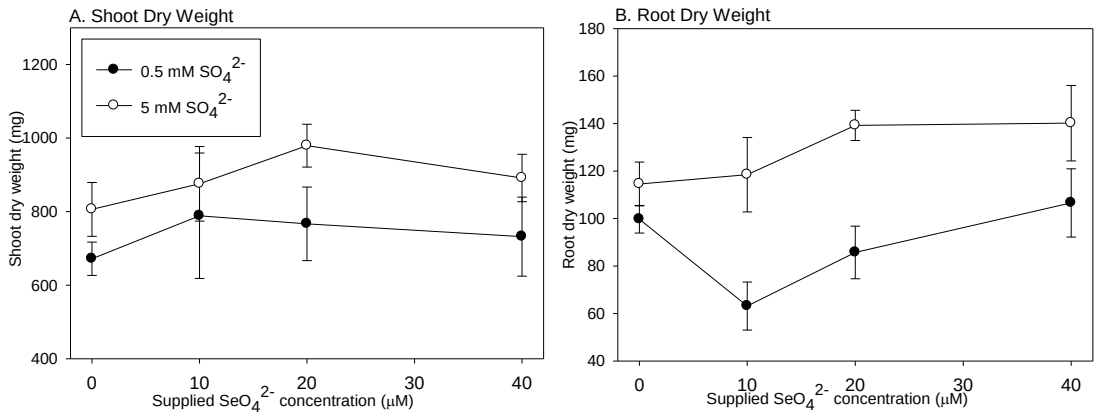

Figure 7. Shoot and root dry weights of *B. juncea* treated with differing selenate (0-40 μM) and sulfate concentrations (white and black circles). Shown values represent mean with SEM of eight biological replicates.

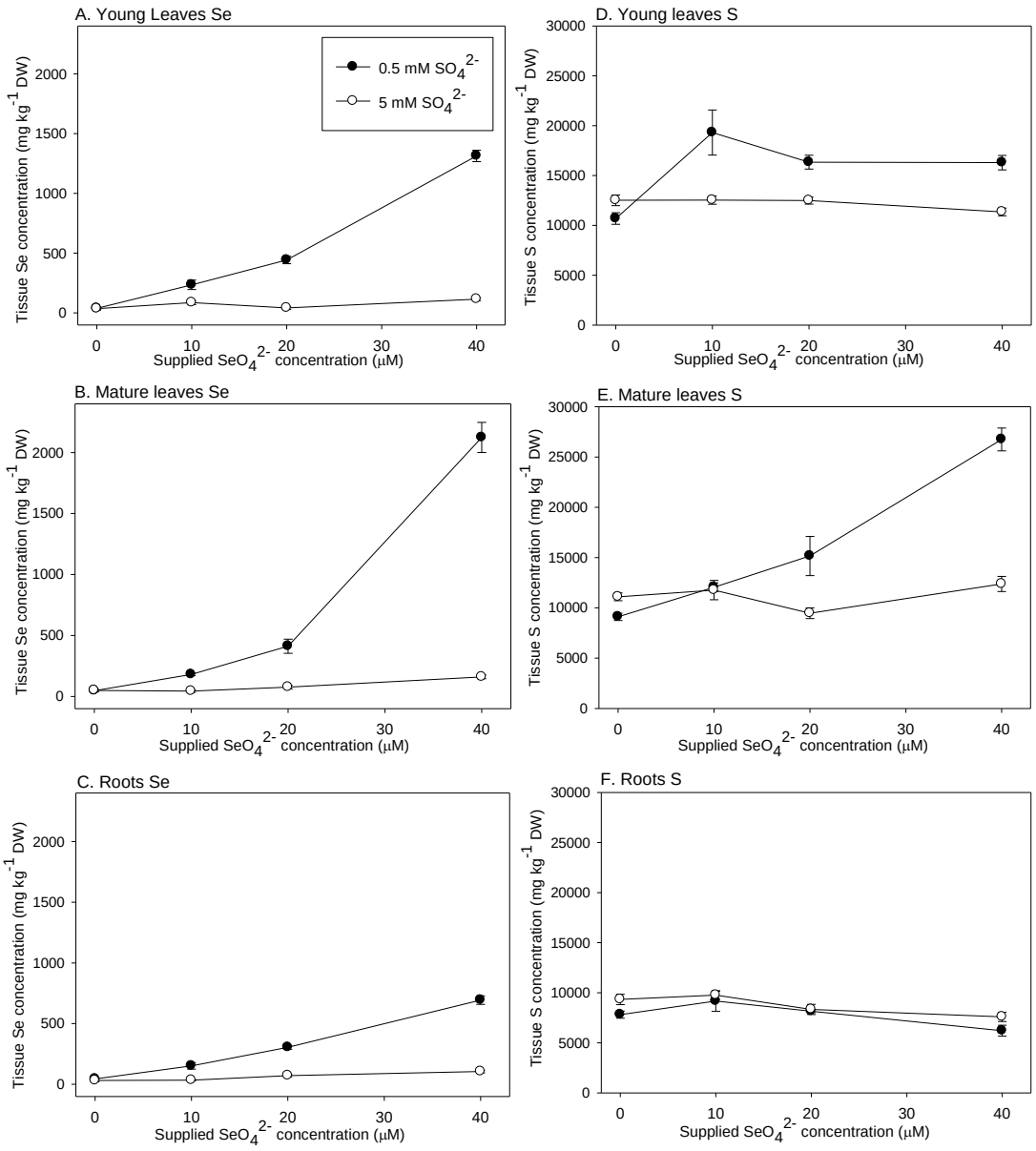

Figure 8. Selenium (A-C) and sulfur (D-F) concentrations in young leaves, mature leaves and roots of *B. juncea* treated with differing selenate and sulfate concentrations (white and black circles). Shown values represent mean with SEM of eight biological replicates.

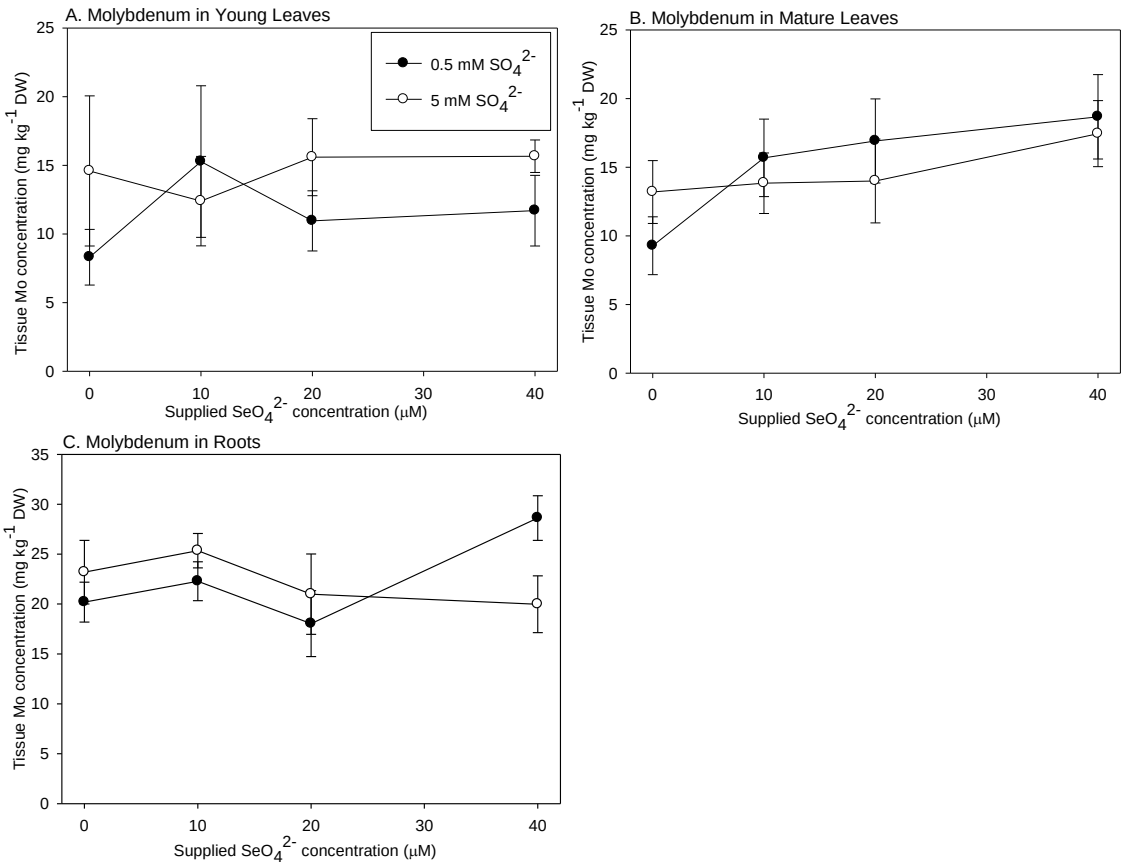

Figure 9. Molybdenum (A-C) concentrations in young leaves, mature leaves and roots of *B. juncea* treated with differing selenate and sulfate concentrations (white and black circles). Shown values represent mean with SEM of eight biological replicates.

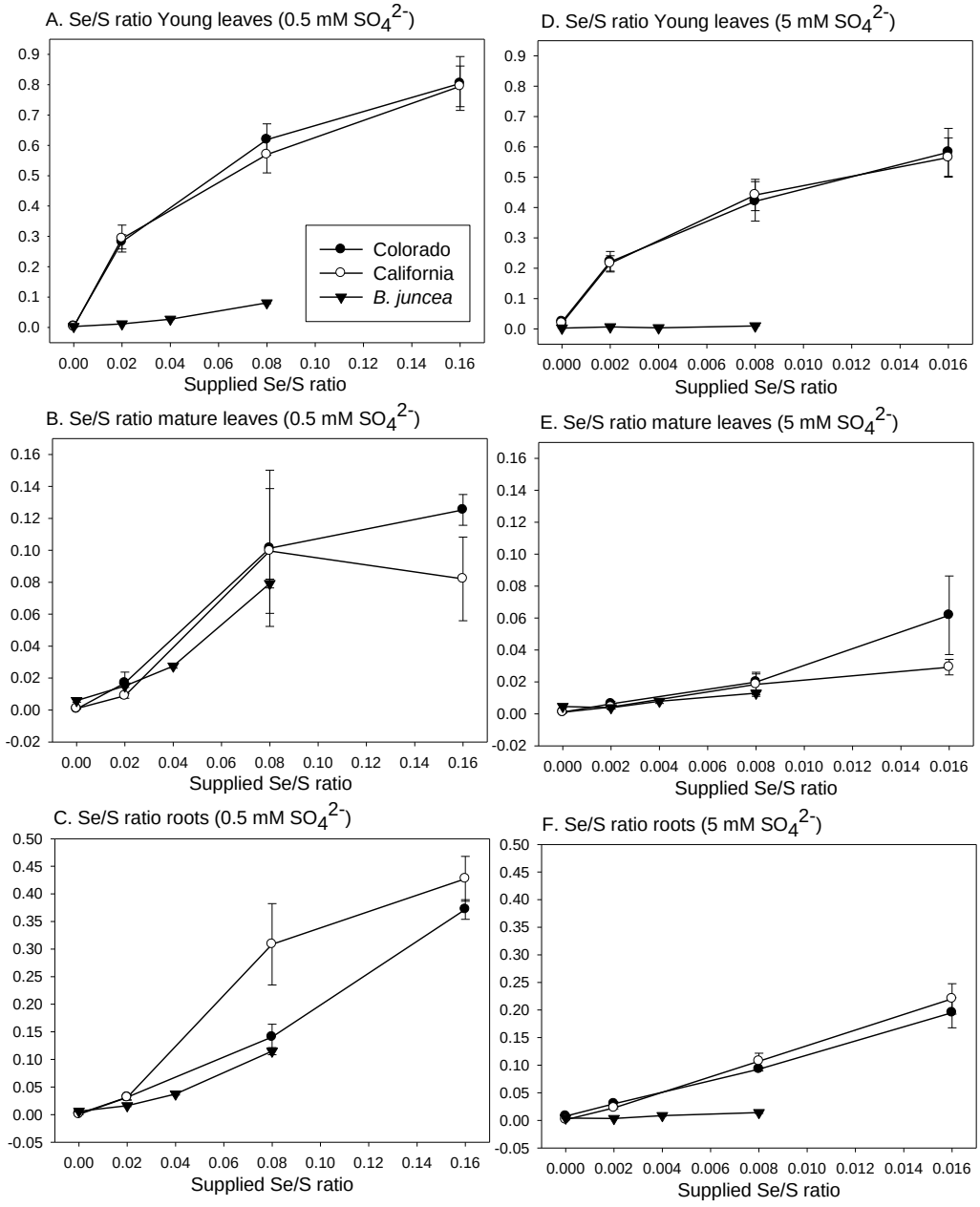

Figure 10. Selenium to sulfur ratios in young leaves, mature leaves and roots of two ecotypes of *Stanleya pinnata* and *B. juncea* treated with differing selenate and sulfate concentrations (supplied Se/S ratios shown on X-axis). Shown values represent mean with SEM of five biological replicates for *S. pinnata*, eight replicates for *B. juncea*.

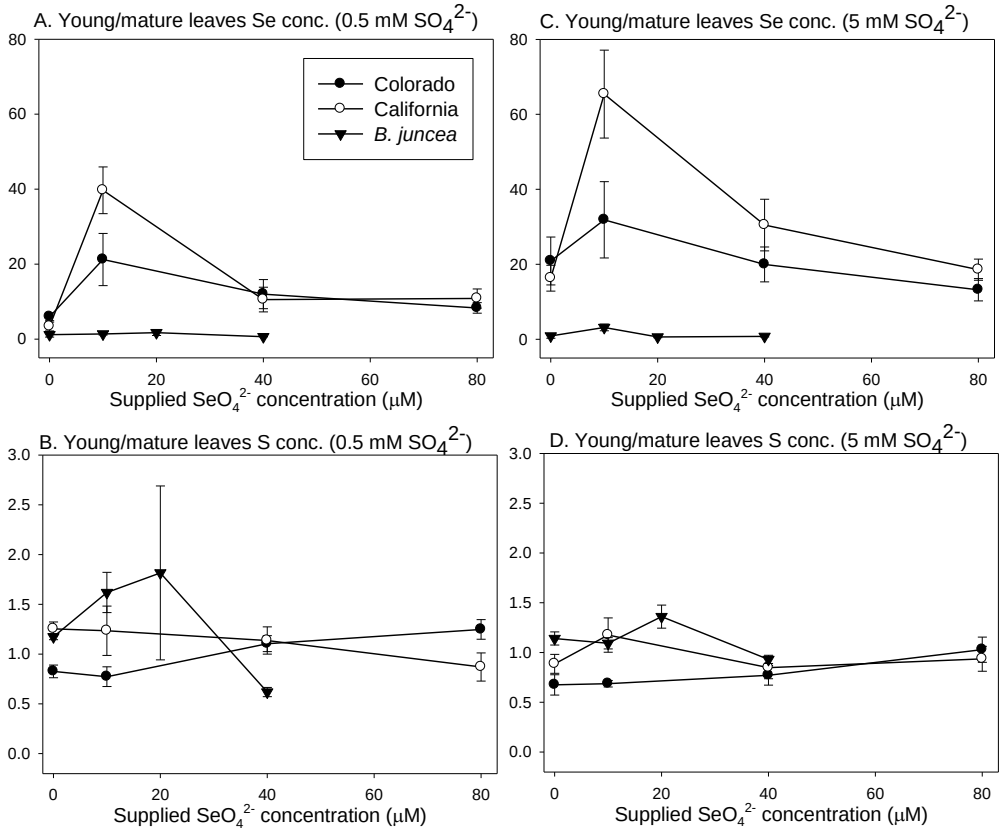

Figure 11. Ratio of selenium concentrations (A, C) and sulfur concentrations (B,D) in young leaves relative to mature leaves in two ecotypes of *S. pinnata* and *B. juncea* treated with differing selenate and sulfate concentrations. Shown values represent mean with SEM of five biological replicates for *S. pinnata*, and eight replicates for *B. juncea*.

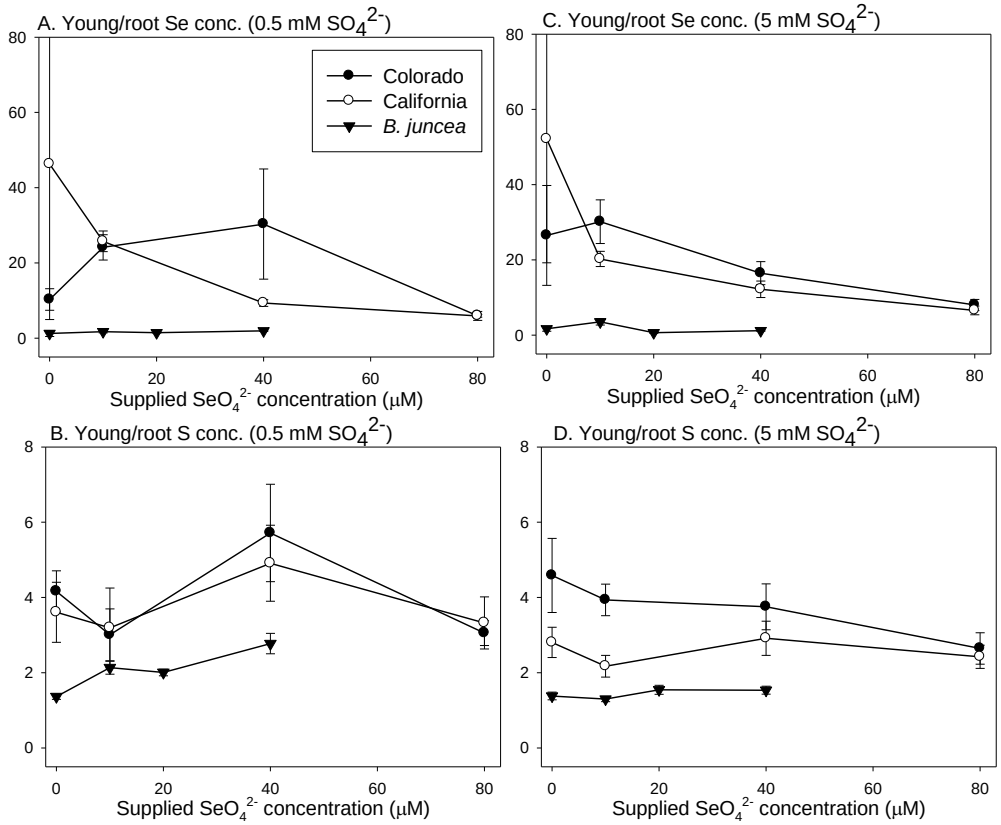

Figure 12. Ratio of selenium concentrations (A, C) and sulfur concentrations (B,D) in young leaves relative to roots in in two ecotypes of *S. pinnata* and *B. juncea* treated with differing selenate and sulfate concentrations. Shown values represent mean with SEM of five biological replicates for *S. pinnata*, and eight replicates for *B. juncea*.

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CHAPTER 3:

SELENIUM UPTAKE AND METABOLISM: INSIGHTS FROM THE TRANSCRIPTOME ANALYSIS OF *STANLEYA PINNATA* AND *STANLEYA ELATA*

SUMMARY

The transcriptomes of *Stanleya pinnata* and *Stanleya elata*, two species with contrasting patterns of selenium (Se) tolerance and accumulation were sequenced to gain insight into mechanisms of Se hyperaccumulation. The responses of the two species to 20 μM selenate were strikingly different. Non-hyperaccumulator *S. elata* showed many more up- and down-regulated genes at the root level, including many involved in electron transport, while hyperaccumulator *S. pinnata* showed much less response to Se in the roots. Conversely, *S. pinnata* exhibited more Se-induced gene regulation in the shoots, with many genes involved in light reactions, glycolysis and lipid synthesis showing 2-fold down regulation or more. Several sulfur-related genes were found to be highly abundant and constitutively expressed at much higher levels in *S. pinnata* than *S. elata* roots, particularly a sulfate transporter (*Sultr1;2),*ATP sulfurylase *(APS2),* cysteine synthase (*CYSD2)* and a cystathionine-β-lyase. These observations shed some light on the mechanisms responsible for Se hyperaccumulation in *S. pinnata*, and open avenues toward genetic engineering of enhanced plant Se accumulation for phytoremediation or biofortification.

INTRODUCTION

Selenium (Se) is both an essential micronutrient for many organisms and a potent toxin at relatively low concentrations. However, the essentiality of Se for higher plants has never been demonstrated (Ellis and Salt, 2003). Selenium is chemically similar to sulfur (S), and most plants are unable to differentiate between the two elements, resulting in Se sharing the S assimilation pathway (Terry and Zayed, 2000). When incorporated into proteins as selenocysteine (SeCys), Se may form S-Se or Se-Se bonds, which may result in protein misfolding, contributing to selenium's toxicity (Stadtman,

1996). Recent studies have found that Se-containing proteins are ubiquinated in plants and broken down through the proteasome (Sabbagh and Van Hoewyk, 2012).

Despite the potential toxicity of Se, some plants endemic to seleniferous soil are able to actively concentrate large amounts of Se in their tissues, frequently exceeding 1,000 mg kg^{-1} dry weight (DW) (Beath et al., 1934). These plants are known as Se hyperaccumulators for their exceptional ability to take up, accumulate, and tolerate large amounts of this toxic element. Selenium hyperaccumulators appear to be capable of enriching themselves with Se relative to S, leading to much higher Se/S ratios in the plant than in their growth medium (White et al., 2007). Contrary to non-hyperaccumulator plants, which are thought to take up Se non-specifically through sulfate transporters, Se hyperaccumulators may have evolved transporters with a higher, or even unique specificity for selenate, since increased expression of transporters alone cannot explain the observed Se/S discrimination (Cabannes et al., 2012, Chapter 2). In addition, while non-hyperaccumulators generally accumulate primarily inorganic selenate, hyperaccumulators process Se into non-protein organic forms such as methyl-SeCys and selenocystathionine (SeCyst) (Virupaksha and Shrift, 1963; Neuhierl and Böck, 1996). Accumulation of these forms of Se avoids non-specific incorporation of Se into proteins. Other unique characteristics of Se hyperaccumulators include the sequestration of Se in specialized tissues such as the leaf epidermis and leaf hairs, as well as preferential allocation to young leaves and reproductive organs (Galeas et al., 2007; Freeman et al., 2010). In light of this varied set of characteristics exhibited by Se hyperaccumlators, we can expect to find a suite of differentially expressed genes that enable them to accumulate, tolerate and metabolize large amounts of Se without detrimental effects.

The Brassicaceae contain many genera that hyperaccumulate toxic elements, including members of the genus *Stanleya*, which hyperaccumulate Se (Terry and Zayed, 2000). Hyperaccumulator *Stanleya pinnata* (Prince's Plume) occurs across a wide range of the western United States and is typically found growing in soils with high concentrations of Se (Feist and Parker, 2001). The related species *S. elata* has never been found to accumulate significant concentrations of Se in its native habitat of eastern CA and

western NV. The phenotypic diversity and genetic similarity between these two species, paired with the genetic tools inherent with working in the Brassicacea, make these *Stanleya* species an excellent system for genomic investigations into the mechanisms of Se hyperaccumulation.

Earlier studies have demonstrated the importance of genes involved in sulfate transport and assimilation for Se tolerance and accumulation. As mentioned, it is believed that plants take up selenate using sulfate transporters (White et al., 2004). The sulfate transporter gene family is a large one, with 4 groups and as many as 16 putative sulfate transporters in a single species (Hawkesford, 2003). Sulfur transporter group 1 contains the two main high-affinity root transporters, Sultr1;1 and Sultr1;2 which are primarily expressed in root epidermal cells (Takahashi et al., 2011). Although Sultr1;1 and 1;2 appear to be functionally redundant, Sultr 1;2 is constitutively expressed at much higher levels, while Sultr1;1 shows a stronger induction in low S conditions (Hawkesford, 2003). *Arabidopsis* mutants for Sultr1;2 showed a strong selenate resistant phenotype, suggesting that Sultr1;2 may be the primary transporter involved in Se accumulation (Shibagaki et al., 2002). Sulfur transporter group 2 consists of lower affinity transporters that are prevalent in the central cylinder of the root, show strong induction under S deficient conditions, and are likely involved in root-to-shoot translocation (Takahashi et al., 2011). Transporters in group 3 are poorly characterized. Possible functions for members of this family vary widely from maintenance of rhizosphere symbioses to involvement in a pathogen response (Takahashi et al., 2011; Petre et al., 2012). Sulfur transporters in group 4 appear to be primarily localized to the vacuole. Sultr4;1 and 4;2 have been shown to be responsible for the efflux of stored sulfate from the vacuole, especially under S limiting conditions (Kataoka, 2004).

Selenium is incorporated into organic compounds through the S assimilation pathway (Terry and Zayed, 2000; Shibagaki et al., 2002). Selenate is initially combined with ATP by ATP sulfurylase (APS) which results in the formation of adenosine phosphoselenate (APSe) and PPi. This is believed to be a rate-limiting step in Se assimilation, based on evidence from APS overexpression experiments and treatment of plants with selenate vs. selenite (Pilon-Smits et al., 1999). APS reductase (APR) converts

APSe to selenite. Selenite may be reduced to selenide by sulfite reductase, or non-enzymatically by glutathione (Terry et al. 2000). Selenide is then combined with O-acetylserine (OAS) by O-acetylserine(thiol)lyase (OASTL) to form selenocysteine (SeCys) (White and Broadley, 2007). OASTL is active in a complex with serine acetyl transferase (SAT), the enzyme that produces OAS. SeCys can be combined with O-phosphohomoserine (OPH) to form selenocystathionine. In plants, the formation of cystathionine or selenocystathionine is catalyzed by cystathionine-γ-synthase (CGS). Cystathionine can be broken down by cystathionine-β-lyase to form Se-homocysteine. SeMethionine (SeMet) is then formed from (Se-)homocysteine by methionine synthase. Although Met does not play as large a role as Cys in maintaining protein structure, it may be converted to S-adenosylmethionine, a methyl donor for polyamine synthesis, ethylene synthesis and many more plant compounds (Brosnan and Brosnan, 2006). In most plants sulfate and selenate are thought to be primarily reduced and assimilated in the shoots. However, some evidence suggests that in Se hyperaccumulators a significant fraction of selenate is reduced and assimilated in the roots, since large amounts of organic Se were found in root vacuoles, as well as significant levels of organic Se in guttation (xylem) fluid (Freeman et al., 2006b; Amos et al., 2012).

The fundamental mechanisms underlying Se hyperaccumulators' extreme Se tolerance and accumulation are not well known. In the *Astragalus* genus, there was found to be no correlation between Se hyperaccumulation and activities of the sulfate assimilation enzymes ATPS, APR, and SAT in shoot tissue. It was concluded that Se hyperaccumulation in *Astragalus* is not driven by an overall increase in the capacity of these enzymes (Sors et al., 2005a). An important enzyme conferring Se tolerance in this genus is SeCys methyltransferase (SMT), a modified homocysteine methyltransferase (HMT) which methylates SeCys and thereby renders Se unavailable for incorporation into proteins (Neuhierl and Böck, 1996). Different members of the *Astragalus* genus had small changes in the coding sequence for SMT, corresponding with significantly different levels of methylation of SeCys (Sors et al., 2009).

While there was not a significant link between the expression levels of sulfate assimilation genes and Se hyperaccumulation in *Astragalus*, there is evidence to the contrary in the genus *Stanleya*. In a macroarray study by Freeman et al (2010) comparing the expression of ~350 genes between Se hyperaccumulator *S. pinnata* and non-hyperaccumulator *S. albescens* it was found that many genes from the sulfate assimilation pathway were constitutively expressed at several-fold higher levels in *S. pinnata* than *S. albescens*.

In the current study, RNA-Sequencing was used to compare the transcriptome of Se hyperaccumulator *S. pinnata* and non-hyperaccumulator *S. elata* grown with and without Se to find constitutively more abundant genes in the hyperaccumulator, transcripts highly affected by Se, and the effects of provided selenate on S-related genes. This is the first application of next-generation sequencing for a Se hyperaccumulator in the Brassicaceae and can provide more insight into hyperaccumulation mechanisms as well as validation of some of the findings of the previous macroarray study using a different non-hyperaccumulator reference species. An overview is presented of genes up- and down-regulated by Se in roots and shoots of each *Stanleya* species, as well as an overview of Se-dependent transcriptome differences between the species, particularly with respect to sulfur-related genes.

METHODS

*Stanleya pinnata (*ordered from Western Native Seed, Coaldale, CO) and *Stanleya elata* (collected from near Las Vegas, NV 36°16'36"N 115°30'12"W) were grown under sterile conditions on ½ strength Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) for 2 months with a 16/8 L/D photoperiod. Three plants of each species were grown with 0 µM sodium selenate and three plants with 20 µM sodium selenate, for a total of 24 plant samples (12 roots and 12 shoots) to be sequenced. Plants were harvested at approximately the same time of day, around 2 PM. Each plant was washed for 1 minute in ddH_2O, then divided into root and shoot tissue, flash frozen in liquid nitrogen and shipped to the University of Missouri for sequencing using the Illumina Hi-Seq platform. RNA

extraction, sequencing and assembly were all performed by Patrick Edger at the Univeristy of Missouri DNA Core. Total RNA was extracted from root or shoot tissue using an Invitrogen PureLink RNA Mini Kit, and converted to an Illumina library with a TruSeq RNA Kit. Libraries were sequenced with 100 BP paired-end reads using a HiSeq-2000 instrument. Each paired end sample had an average of 62 million total reads (SD = 13.7 million). The paired-end 100 bp reads were quality filtered and trimmed using the NextGENe V2.17 (SoftGenetics, State College, PA, USA) software package and assembled using Trinity assembler for an average contig length of around 600 bp. Two additional biological replicates were sequenced with 50 bp single end reads and mapped on to the *de novo* assembly with an average of 43 million reads per sample (SD = 9.5 million). Statistically differently expressed contigs were identified by Patrick Edger using the software EdgeR (bioconductor.org) and DESeq (run through R, available at bioconductor.org).

Data were annotated to their closest *Arabidopsis thaliana* homolog using the Arabidopsis Information Database (TAIR – Arabidopsis.org). Contig expression was normalized between biological replicates and between species by "reads per kilobase of transcript per million mapped reads" (RPKM). For figure 16 and MapMan analysis, RPKM values for contigs mapping to the same locus were pooled. Transcripts were functionally grouped and visualized using MapMan (mapman.gabipd.org), with available maps and pathways for *Arabidopsis* used with no modifications.

RESULTS AND DISCUSSION

RNA sequencing and assembly yielded over 100,000 unique contigs for each species and organ with an average length of ~600 nucleotides (Fig. 13). In roots, the fraction of contigs regulated by Se was around twice as high in non-hyperaccumulator *S. elata* (40%) than in hyperaccumulator *S. pinnata* (20%). More contigs were down- than upregulated by Se in *S. elata* roots, while in *S. pinnata* equal numbers were up- and downregulated. In shoots, 25% of *S. pinnata* contigs was up- or downregulated, compared

to 18% of *S. elata* contigs (Fig. 13). Thus, Se appears to have affected gene expression more at the root level in the non-hyperaccumulator and slightly more at the shoot level in the hyperaccumulator.

Because there are no reference genomes available in *Stanleya*, contigs were annotated using closest match *Arabidopsis thaliana* homologs. Approximately 79,000 of the contigs matched to *A. thaliana* homologs out of a total of ~100,000 total. Transcript abundance of multiple contigs mapping to the same locus were pooled for comparison of expression levels between species, for a total of approximately 22,000 unique genes for each species. Figure 18 shows genes found in both *S. pinnata* and *S. elata* that were up or down-regulated at least 4-fold with Se treatment. Surprisingly, there were very few genes that showed the same response to Se in both species. Most of these were found to be up-regulated in the roots. Similar to the trends found in significantly regulated contigs, there were many more 4-fold or greater regulated genes in the roots of *S. elata* than in its shoots or in the roots of *S. pinnata.* Conversely, *S. pinnata* showed more 4-fold or greater down-regulated genes in its shoots than its roots, or than in the shoots of *S. elata*. In total, *S. elata* had 673 genes upregulated 4-fold or greater in roots and shoots combined, while *S. pinnata* only had 395. The finding that fewer transcripts respond to Se in *S. pinnata* than in its non-hyperaccumulator reference species correlates with a macroarray study by Freeman et al (2010), which found that more genes were up-regulated by Se in non-hyperaccumulator *S. albescens* than in the hyperaccumulator *S. pinnata*, due to constitutively higher levels of expression for many genes in *S. pinnata*. . It is important to note that a number of housekeeping genes (ACT1, UBQ11), and several genes shown to be stable under stress in *Arabidopsis* (AT5G12370, AT4G15415, AT1G79810, AT2G28390) (Mentzen and Wurtele 2008) were strongly down-regulated (2-3 fold) in the presence of Se in the roots of *S. elata* as well. This was not observed in *S. pinnata* or in the shoots of *S. elata.* In addition, the fresh weight of *S. elata* significantly decreased with the addition of selenate to the medium (Fig. 14). This suggests that the roots may have been unhealthy or dying and thus make strong comparisons between individual genes in the roots of the two species under Se stress difficult.

Table 2 lists annotated abundant contigs (with RPKM $\geq$ 10) that were most up- and down-regulated by Se in roots and shoots of *S. elata* and *S. pinnata*. In *S. elata*, (Fig. 15 A, B) 6 out of the 9 most up-regulated transcripts in the roots were also among the most up-regulated in shoots, suggesting a general, plant-wide response. Several of the up-regulated transcripts may have a regulatory function as transcription factors (LIL3:1) or involving small regulatory RNA (DUF6, tasiRNA involved in auxin response). Among the most down-regulated genes, there were two F-Box containing proteins in common between roots and shoots. The targets and exact role these regulatory elements may play in Se or general stress response is currently unknown.

The roots and shoots of *S. pinnata* had one highly expressed and Se-induced gene in common, a papain family protease (Figure 15 C, D). In the roots of *S. pinnata*, a cysteine-rich CAP protein was up-regulated ~2500 fold in the presence of Se. This same protein has been shown to be constitutively up-regulated in *Arabidopsis halleri*, a Zn and Cd accumulator related to *Arabidopsis thaliana*. In addition, a protein kinase, K^+ transporter and SKU5, a protein involved in copper ion binding were highly up-regulated in the roots of *S. pinnata.* In the shoots, up-regulated genes included a MADS-Box transcription factor and DOX1, a protein implicated in response to oxidative stress. A disease resistance protein (TIR-NBS-LRR class) involved in signal transduction, innate immune response and jasmonic acid (JA) production was highly down-regulated by Se in both roots and shoots of *S. pinnata*. It has been proposed that jasmonic and salicylic acid play an important role in selenium tolerance of *A. thaliana*, so it is not surprising that genes involved in this pathway may be affected (Tamaoki and Freeman 2008). In addition, several DNA regulatory elements were significantly down-regulated in the roots and shoots of *S. pinnata*, including Spt5, a transcription elongation factor that was predicted to be among the 20 most connected proteins in an *A. thaliana* interactome, suggesting its regulation may have far reaching effects (Geisler-Lee et al., 2007).

When Se responses of the transcriptomes in *S. elata* and *S. pinnata* are visualized grouped by function, the large amount of regulation found in *S. elata* roots and *S. pinnata* shoots is again apparent

(Fig. 2). In *S. elata* roots (Fig. 19A), genes involved in glycolysis, the TCA cycle and amino acid metabolism were generally down-regulated. On the other hand, genes involved in mitochondrial electron transport, including many subunits of NADH dehydrogenase and cytochrome c oxidase were strongly up-regulated (> 8 fold) in the presence of Se. One possible explanation for this phenomenon may be damage to electron transport machinery due to free radicals associated with Se stress. Previous work has shown Se can significantly increase the production of free radicals in plant tissues (Freeman et al., 2010) and in animals, up-regulation of mitochondrial NADH dehydrogenases was observed in cells repeatedly subjected to H_2O_2 stress (Ghosh and Girigoswami, 2008). A similar mechanism may the cause of our observed trend. Alternatively, since NADH dehydrogenase subunits are particularly rich in iron-sulfur clusters, they may be particularly sensitive to possible disruption by Se of iron-sulfur cluster biogenesis through non-targeted incorporation. The observed down-regulation of glycolysis and TCA may be a result of slowed mitochondrial electron transport and an accumulation of NADH. Surprisingly for roots, many genes involved in chloroplast electron transport (light reactions) were also strongly (> 16 fold) up-regulated in the presence of Se in *S. elata*. As mentioned previously, it is difficult to analyze *S. elata's* Se-specific responses due to the strong effects observed on housekeeping genes. However, the strong effects observed on electron transport may be at least one underlying reason for the widespread, general response observed in *S.elata*. In the shoots of *S. elata*, relatively few genes appeared to be affected by Se, with no obvious patterns in entire functional groups (Fig. 19B).

In *S. pinnata* roots, relatively few genes responded to Se, and none of the trends seen in *S. elata* roots were observed (Fig. 19C). This may be due to observed rapid translocation and/or processing of Se from the observed strong up-regulation of S assimilatory enzymes in the roots of *S. pinnata*. In the shoots however, a large number of genes appeared to be down-regulated by Se, and several patterns of down-regulation were observed across functional groups (Fig. 19D). Many genes involved the light reactions, glycolysis, cell wall and phenylpropanoid metabolism (laccases) were down-regulated. This may suggest there is a metabolic cost to the high levels of translocated Se in the shoots, even for a hyperaccumulator.

However, since earlier work has shown that *S. pinnata* can tolerate levels of selenate exposure far greater than 20 µM with no abnormal phenotype (Feist and Parker 2001, Chapter 2). The physiological functions of these down-regulated genes are not known at this time.

Large differences were observed in the expression of sulfur-related genes between *S. pinnata* and *S. elata* in the roots (Figure 16A, 17). Fifty-six S-related genes were cross-referenced between species, and multiple contigs mapping to the same locus were summed for this table. In the absence of Se, 19 sulfur-related genes were $\geq$ 2 fold more highly expressed in the hyperaccumulator, and 9 were more highly expressed in the non-hyperaccumulator (Figure 15A, right column). In the presence of Se, 40 genes were > 2-fold more highly expressed in *S. pinnata* than in *S. elata*, and only two were more highly expressed in *S. elata*. However, these differences between +Se and –Se treatments were primarily due to the general down-regulation of most genes in *S. elata* rather than upregulation in *S. pinnata*.

Of the S-related genes that show constitutively higher expression in *S. pinnata*, many showed high levels of abundance (>100 RPKM) and more than 16-fold higher levels in *S. pinnata* than *S. elata.* ATP sulfurylase (APS2), a rate-limiting enzyme involved in the first step of sulfate and selenate reduction, was over 64-fold more highly constitutively expressed in *S. pinnata* than *S. elata* roots, even in the absence of Se. It is surprising that APS2 shows such a high expression level (RPKM > 6000 with summed contigs), particularly in the roots. APS2 is plastid- localized, and sulfate assimilation is thought to happen mostly in the shoot.

Interestingly, a homolog of sulfate transporter 1;2 (SULTR1;2), a high-affinity root transporter that has been implicated in selenate transport in *A. thaliana*, was also 30-fold more highly expressed in the roots of *S. pinnata* than *S. elata* and had an abundance in the hyperaccumulator of > 700 RPKM, placing it among the top 5% most abundant identifiable contigs in the roots. It is tempting to hypothesize that his transporter is a key mechanism for selenate uptake into the hyperaccumulator. Other genes that showed significantly higher expression in *S. pinnata* roots included several other sulfate transporters and

various other enzymes involved in sulfate assimilation: APS, serine acetyltransferases, cysteine synthases and cystathionine synthases and lyases. This suggests that *S. pinnata*'s sulfate uptake and assimilation pathway is overall up-regulated, both in the presence of Se and to a lesser extent in its absence. Synthesis of the organic S-compound glutathione (Glu-Cys-Gly) may also be upregulated in *S. pinnata* roots, judged from the ~10-fold higher transcript levels of glutathione synthetase (GSH2). Glutathione (GSH) may contribute to selenite reduction (Terry et al., 2000) and may help prevent Se-associated oxidative stress. *S. pinnata* was shown in an earlier study to have higher GSH levels than non-hyperaccumulator *S. albescens* (Freeman et al., 2010). The reason why *S. pinnata* up-regulates sulfate uptake and assimilation pathways may be that the plants perceive themselves to be S starved. Indeed, RESPONSE TO LOW SULFUR 4 (LSU4) was much more highly expressed in roots of *S. pinnata* than *S. elata*, both in the presence and absence of Se.

Another class of S-related genes upregulated in *S. pinnata* is the APS kinases, which produce phospho-adenosine phosphosulfate (PAPS), a substrate for various sulfation reactions. Also interesting to point out are the differences in expression for the homocysteine methyltransferase (HMT) genes: several HMTs were more highly expressed in *S. elata*, but one of the HMT1 alleles was up to 16-fold more highly expressed in *S. pinnata* than *S. elata*. In *Astragalus* hyperaccumulator species a modified HMT gene is responsible for this plant's SMT activity. SMT produces methyl-SeCys, which has been reported to be the main form of Se accumulated in *S. pinnata* as well (Freeman et al., 2006). More studies are needed to investigate whether any of the HMTs in *S. pinnata* may function as an SMT; the presence of MeSeCys in *S .pinnata* and other members of the Brassicaceae make this a likely possibility. Finally, an interesting class of genes more highly expressed in roots of the hyperaccumulator than the non-hyperaccumulator is that of the Se-binding proteins (SBP1-3). The function of these proteins is unknown, but an *A. thaliana* SBP was shown to be upregulated in response to selenate, and its overexpression has been shown to enhance selenate tolerance in *A. thaliana* (Hugouvieux et al. 2009).

In the shoots, many of the observed differences in the roots were not present (Figure 16B). The three ATP sulfurylase genes were expressed at lower levels in *S. pinnata* relative to *S. elata,*. *Sultr1;2,* being primarily a root transporter, showed no significant differences between the two species as its abundance was very low. In *S. elata,* several S-containing amino acid synthesis genes were > 4-fold more highly expressed than in *S. pinnata,* including cysteine synthetase D1 (CYSD1) and methionine synthases (MTO1, MS2). This response may be a compensatory mechanism for Se-containing amino acids getting mis-incorporated into proteins, as a plant-wide response to perceived low S-status is not observed. In addition, the three selenium binding proteins (SBP1-3) were all more highly expressed in the shoot of *S. elata* than that of *S. pinnata,* with SBP3's expression being especially pronounced at more than 500-fold greater than *S. pinnata.* On the other hand, several S-related genes were more abundant and more highly expressed in the shoots of *S. pinnata* than that of *S. elata,* including *Response to Low Sulfur 2* and 4, a Cystathionine-β-lyase and a Cystathionine-β-synthase family protein. Plants have never been shown to have cystathionine-β-synthase activity as mammals do, who use the enzyme to synthesize cystathionine from homocysteine. If the enzyme performs the same function in the hyperaccumulator it may explain its high levels of seleno-cystathionine. Other studies have shown that many genes in this family containing the CBS domain are involved in stress response and signaling (Kushwaha et al., 2009).

CONCLUSIONS

This preliminary summary of the transcriptomic response of *S. elata* and *S. pinnata* has identified many promising candidate genes for further validation and investigation. Some of these genes were identified previously in Freeman et al (2010), such as ATP sulfurylase 2 (APS2), but that study used a different non-hyperaccumulator (*S. albescens*) and looked only at a limited number of genes since it used a macroarray approach. Thus, it did not observe the breadth of responses that we did using the Illumina platform. In future studies it will be interesting to clone and sequence the APS2 and sulfate transporter 1;2 from *S. pinnata* and one or more non-hyperaccumulator *Stanleya* species. Sequence comparison may reveal specific mutations that may be responsible for the observed Se specificity in *S. pinnata.* Expression

of the cloned genes in yeast and in *A. thaliana* may yield further insight into functional differences between homologs from hyperaccumulator and non-hyperaccumulator *Stanleya* species, and may result in transgenics with enhanced Se specificity. Succesful introduction of Se-specific transporters and enzymes in non-hyperaccumulator crop species would have broad applications in phytoremediation and biofortification. Outside of the S assimilation pathway, there were many potentially interesting genes that showed very different expression profiles in non- and hyperaccumulator, including many transcription factors, the cysteine proteases, a cysteine-rich secretory protein and several genes involved in miRNA regulation. From this study, it is clear the Se hyperaccumulating phenotype in *Stanleya* is a result of more than a single enzyme, and instead involves a large suite of genes, including many S-related genes.

FIGURES

EdgeR Statistics	Unique contigs up-regulated with Se ($p \leq 0.01$)	Unique contigs with no significant difference with Se	Unique contigs down-regulated with Se($p \leq 0.01$)
S. pinnata root	12019	80749	12512
S. pinnata shoot	13532	78575	13173
S. elata root	17968	61759	22541
S. elata shoot	9026	83598	9644

Figure 13 – Overview of statistically significant RNA seq unique contigs regulated by Se treatment in *S. pinnata* and *S. elata*

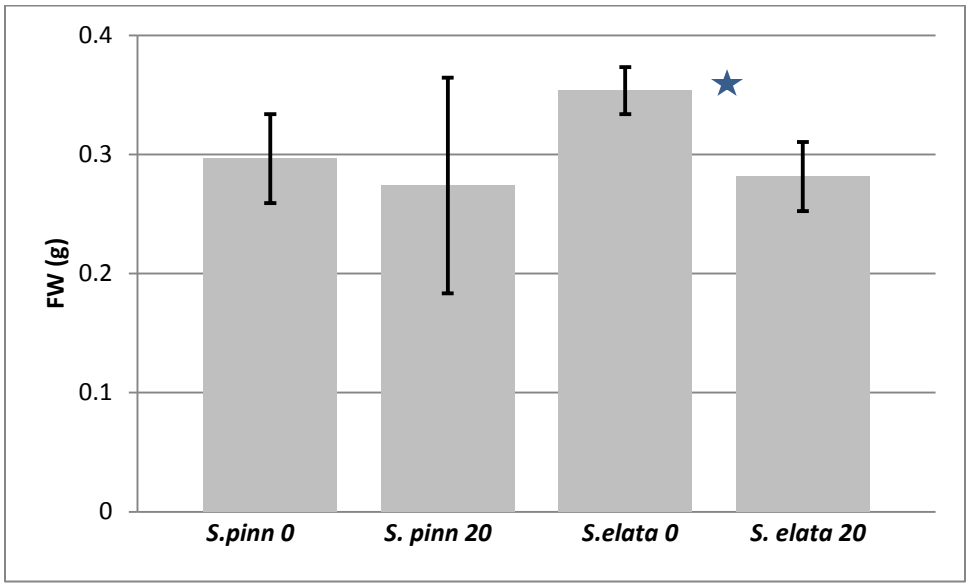

Figure 14 – fresh weights of samples used for sequencing. There was a significant decrease in *S. elata* between 0 and 20 µM selenate.

A.	**S. elata Roots**			
AT ID	Notes	**RPKM (-Se)**	**RPKM (+Se)**	**log2 (+Se/-Se)**
AT2G47230.2	(DUF6) RNA binding, miRNA involved	0.04	12.73	10.07
AT1G17170.1	glutathione S-transferase TAU 24 (GSTU24)	0.16	29.69	9.20
AT5G48320.1	Cysteine/Histidine-rich C1 domain family protein	0.11	18.67	8.99
AT5G25310.1	Exostosin family protein	0.07	9.25	8.79
AT4G36400.2	FAD-linked oxidases family protein	0.05	7.10	8.73
AT2G17430.1	MILDEW RESISTANCE LOCUS O 7 (MLO7)	0.14	12.32	8.07
AT1G29179.1	Cysteine/Histidine-rich C1 domain family protein	0.16	7.51	7.22
AT4G06477.1	gypsy-like retrotransposon family	0.34	12.80	6.89
AT3G17185.1	trans-acting siRNA regulating expression of auxin response	1.51	45.36	6.56
AT3G16440.1	myrosinase-binding protein-like protein-300B	34.64	0.03	-8.58
AT3G03040.1	F-box/RNI-like superfamily protein	21.14	0.12	-5.82
AT5G22720.2	F-box/RNI-like superfamily protein	51.28	0.37	-5.48
AT3G14270.1	FORMS APLOID AND BINUCLEATE CELLS 1B (FAB1B)	11.09	0.08	-5.47
AT3G16440.1	myrosinase-binding protein-like protein-300B (MLP-300B)	36.37	0.28	-5.41
AT3G21180.1	autoinhibited Ca(2+)-ATPase 9 (ACA9)	12.48	0.10	-5.26
AT2G42160.1	BRAP2 RING ZnF UBP domain-containing protein 1 (BRIZ1)	11.01	0.16	-4.44
AT4G31320.1	SAUR-like auxin-responsive protein family	10.91	0.17	-4.37
AT4G38660.1	Pathogenesis-related thaumatin superfamily protein	13.42	0.21	-4.36
AT5G17420.1	IRREGULAR XYLEM 3 (IRX3)	18.29	0.30	-4.31
B.	***S. elata*** **Shoots**			
AT ID	Notes	**RPKM (-Se)**	**RPKM (+Se)**	**log2 (+Se/-Se)**
AT2G17430.1	MILDEW RESISTANCE LOCUS O 7 (MLO7)	0.03	10.82	8.41
AT4G36400.2	FAD-linked oxidases family protein	0.08	21.11	7.94
AT1G29179.1	Cysteine/Histidine-rich C1 domain family protein	0.10	24.25	7.80
AT5G48320.1	Cysteine/Histidine-rich C1 domain family protein	0.17	28.92	7.33
AT3G49900.2	Phototropic-responsive NPH3 family protein	0.08	9.30	6.84
AT3G17185.1	trans-acting siRNA regulating expression of auxin response	1.41	126.70	6.44
AT5G43530.1	Helicase protein with RING/U-box domain	0.28	17.20	5.90
AT2G38230.1	pyridoxine biosynthesis 1.1 (PDX1.1)	0.32	17.92	5.75
AT4G20360.1	RAB GTPase homolog E1B (RABE1b)	0.59	29.94	5.60
AT4G17600.1	LIL3:1; FUNCTIONS IN: sDNA binding transcription factor	0.31	15.28	5.57
AT1G17170.1	glutathione S-transferase TAU 24 (GSTU24)	0.29	10.87	5.17
AT1G80780.3	Polynucleotidyl transferase, ribonuclease H superfamily protein	24.57	0.04	-9.31
AT3G03040.1	F-box/RNI-like superfamily protein	21.76	0.11	-7.62
AT2G17340.1	Uncharacterised conserved protein	8.93	0.05	-7.47
AT3G57570.2	ARM repeat superfamily protein	12.85	0.12	-6.75
AT5G22720.2	F-box/RNI-like superfamily protein	67.55	0.78	-6.48
AT1G31580.1	ECS1 (Cell wall protein)	17.75	0.35	-5.71
AT3G13510.1	Protein of Unknown Function	10.67	0.29	-5.27
AT2G40300.1	ferritin 4 (FER4); , ferric iron binding, binding	51.37	2.49	-4.41

Figure 15. Most up- and down-regulated annotated and abundant contigs (RPKM $\geq$ 10) in roots (A, B) and shoots (C, D) of *S. elata* (A, C) and *S. pinnata* (B, D) when grown in the presence of Se. Negative and positive $\log_2$ values indicate down and up-regulation respectively, in the presence of Se. All differences are statistically significant.

C.	***S. pinnata* Roots**			
AT ID	Notes	**RPKM (-Se)**	**RPKM (+Se)**	**log2 (+Se/-Se)**
AT4G33710.1	CAP (Cysteine-rich secretory proteins and Pathogenesis-related)	0.08	262.23	11.60
AT4G32500.1	K+ transporter 5 (KT5)	0.11	47.10	8.66
AT3G14470.1	NB-ARC domain-containing disease resistance protein	0.05	14.70	8.15
AT2G28590.1	Protein kinase superfamily protein	0.07	12.55	7.45
AT4G25100.5	Fe superoxide dismutase 1 (FSD1)	0.21	32.60	7.24
AT1G75790.1	SKU5 similar 18 (sks18)	0.10	14.59	7.20
AT1G62340.1	ABNORMAL LEAF-SHAPE 1 (ALE1)	0.18	25.82	7.08
AT1G03710.2	Cystatin/monellin superfamily protein	0.10	10.84	6.70
AT4G08078.1	gypsy-like retrotransposon family (Athila)	0.27	28.38	6.63
AT5G14680.1	Adenine nucleotide alpha hydrolases-like superfamily protein	0.22	19.70	6.40
AT4G11320.1	Papain family cysteine protease	7.79	341.11	5.38
AT4G36150.1	Disease resistance protein (TIR-NBS-LRR class) family	323.02	0.05	-12.67
AT5G37770.1	TOUCH 2 (TCH2); calcium ion binding	11.86	0.02	-9.20
AT3G03300.3	dicer-like 2 (DCL2)	18.05	0.04	-8.74
AT1G47950.1	copia-like retrotransposon family	25.07	0.06	-8.74
AT3G13220.1	WBC27; ABC-2 type transporter	11.74	0.06	-7.60
AT3G18750.3	with no lysine (K) kinase 6 (WNK6)	11.30	0.07	-7.32
AT1G63210.1	Transcription elongation factor Spt6	10.33	0.09	-6.95
AT3G21700.3	SGP2; GTP binding	19.95	0.18	-6.86
AT2G38380.1	Peroxidase superfamily protein	33.42	0.44	-6.31
AT2G24650.1	DNA binding;sequence-specific DNA binding transcription factors	9.81	0.18	-5.81
D.	***S. pinnata* shoots**			
AT ID	Notes	**RPKM (-Se)**	**RPKM (+Se)**	**log2 (+Se/-Se)**
AT1G03710.2	Cystatin/monellin superfamily protein	0.15	20.41	7.19
AT5G66250.4	kinectin-related	0.08	11.35	7.15
AT5G14680.1	Adenine nucleotide alpha hydrolases-like superfamily protein	0.12	15.18	7.13
AT5G26630.1	MADS-box transcription factor family protein	0.16	14.18	6.58
AT4G11320.1	Papain family cysteine protease	2.42	122.03	5.75
AT4G11310.1	Papain family cysteine protease	0.91	40.87	5.58
AT5G48790.1	Unknown chloroplastic protein	0.46	18.62	5.44
AT5G19650.1	ovate family protein 8 (OFP8)	0.99	30.25	5.03
AT3G01420.1	DOX1 ; lipoxygenase activity	3.26	87.51	4.84
AT3G55700.1	UDP-Glycosyltransferase superfamily protein	0.43	10.34	4.69
AT5G17890.1	DA1-related protein 4 (DAR4)	8.86	0.01	-9.21
AT5G37770.1	TOUCH 2 (TCH2); calcium ion binding	39.92	0.07	-8.98
AT4G36150.1	Disease resistance protein (TIR-NBS-LRR class) family	56.08	0.32	-7.38
AT4G21650.1	Subtilase family protein	14.70	0.10	-7.09
AT2G34210.1	Transcription elongation factor Spt5	247.32	0.06	-6.92
AT2G41280.1	M10, dessication tolerance	18.69	0.22	-6.30
AT4G37220.1	Cold acclimation protein WCOR413 family	10.62	0.18	-5.77
AT5G64341.1	conserved peptide upstream open reading frame 40	70.06	1.25	-5.71
AT2G24020.2	Uncharacterised BCR	20.58	0.40	-5.60

Figure 15 (Continued)

A. Root		*S. pinnata*	*S. pinnata*	*S. pinnata*	*S. elata*	*S. elata*	*S. elata*	***S. pin/S. ela***	***S. pin/S. ela***
	Sulfate Transporters	Se -	Se +	**Log2**	Se -	Se +	**Log2**	**log2 Se-**	**log2 Se+**
AT4G08620.1	sulfate transporter 1;1 (SULTR1;1)	2.57	3.75	**0.55**	2.64	0.91	**-1.53**	**-0.04**	**2.04**
AT1G78000.2	sulfate transporter 1;2 (SULTR1;2)	745.82	809.16	**0.12**	43.81	26.34	**-0.73**	**4.09**	**4.94**
AT5G10180.1	sulfate transporter 2;1 (SULTR2;1)	112.71	195.27	**0.79**	156.24	71.50	**-1.13**	**-0.47**	**1.45**
AT3G51895.1	sulfate transporter 3;1 (SULTR3;1)	19.12	27.30	**0.51**	102.58	15.46	**-2.73**	**-2.42**	**0.82**
AT4G02700.1	sulfate transporter 3;2 (SULTR3;2)	19.21	11.04	**-0.80**	12.97	2.62	**-2.31**	**0.57**	**2.08**
AT3G15990.1	sulfate transporter 3;4 (SULTR3;4)	82.01	73.69	**-0.15**	34.58	11.97	**-1.53**	**1.25**	**2.62**
AT5G19600.1	sulfate transporter 3;5 (SULTR3;5)	42.13	70.05	**0.73**	28.26	3.82	**-2.89**	**0.58**	**4.20**
AT5G13550.1	sulfate transporter 4.1 (SULTR4;1)	123.63	141.54	**0.20**	74.00	43.30	**-0.77**	**0.74**	**1.71**
AT3G12520.2	sulfate transporter 4;2 (SULTR4;2)	10.40	16.58	**0.67**	2.63	12.74	**2.28**	**1.99**	**0.38**
	Cysteine synthesis								
AT3G22890.1	ATP sulfurylase 1 (APS1)	170.11	217.57	**0.36**	627.25	221.57	**-1.50**	**-1.88**	**-0.03**
AT1G19920.1	ATP Sulfurylase 2 (APS2)	6636.83	6646.51	**0.00**	83.88	17.46	**-2.26**	**6.31**	**8.57**
AT4G14680.1	ATP Sulfurylase 3 (APS3)	209.06	220.75	**0.08**	170.54	48.38	**-1.82**	**0.29**	**2.19**
AT4G04610.1	APS Reductase 1 (APR1)	833.30	946.77	**0.18**	1725.13	409.10	**-2.08**	**-1.05**	**1.21**
AT1G62180.1	APS Reductase 2 (APR2)	72.44	125.41	**0.79**	64.97	49.06	**-0.41**	**0.16**	**1.35**
AT4G21990.1	APS Reductase 3 (APR3)	610.83	669.24	**0.13**	26.62	6.72	**-1.99**	**4.52**	**6.64**
AT4G21990.2	APS Reductase 3 (APR3)	178.26	229.70	**0.37**	190.71	*54.10*	**-1.82**	**-0.10**	**2.09**
AT5G04590.1	sulfite reductase (SIR)	572.40	668.73	**0.22**	658.00	142.14	**-2.21**	**-0.20**	**2.23**
AT3G59760.2	O-acetylserine (thiol) lyase isoform C (OASC)	43.82	51.70	**0.24**	57.39	5.44	**-3.40**	**-0.39**	**3.25**
AT4G14880.2	O-acetylserine(thiol)lyase (OAS-TL) isoform A1 (	41.55	50.01	**0.27**	18.82	1.67	**-3.50**	**1.14**	**4.91**
AT3G22460.1	O-acetylserine(thiol)lyase (OAS-TL) isoform A2 (	200.65	263.90	**0.40**	100.45	24.85	**-2.02**	**1.00**	**3.41**

Figure 16. Transcript abundance for sulfur-related genes in roots (A) and shoots (B) of *S. elata* and *S. pinnata*. Positive and negative Log_2 values in columns 5 and 8 reflect up- and down-regulation by Se, respectively, within a species. Log_2 values in columns 9 (-Se) and 10 (+Se) reflect differences in transcript abundance between species – positive values indicate higher transcript abundance in *S. pinnata* than *S. elata*.

Root (Cont.)		*S. pinnata*	*S. pinnata*	*S. pinnata*	*S. elata*	*S. elata*	*S. elata*	***S. pin/S. ela***	***S. pin/S. ela***
	Cysteine synthesis (Cont.)	Se -	Se +	**Log2**	Se -	Se +	**Log2**	**log2 Se-**	**log2 Se+**
AT5G56760.1	serine acetyltransferase 1;1 (SERAT1;1)	185.98	169.49	**-0.13**	344.60	88.11	**-1.97**	**-0.89**	**0.94**
AT2G17640.1	serine acetyltransferase 3;2 (SERAT3;1)	156.57	178.53	**0.19**	59.19	19.61	**-1.59**	**1.40**	**3.19**
AT4G35640.1	serine acetyltransferase 3;2 (SERAT3;2)	2.58	5.63	**1.13**	4.00	3.59	**-0.16**	**-0.63**	**0.65**
AT3G03630.1	cysteine synthase 26 (CS26)	8.64	10.88	**0.33**	10.15	2.22	**-2.20**	**-0.23**	**2.30**
AT3G61440.3	cysteine synthase C1 (CYSC1)	89.63	66.84	**-0.42**	28.47	2.74	**-3.38**	**1.65**	**4.61**
AT3G04940.1	cysteine synthase D1 (CYSD1)	51.82	67.51	**0.38**	355.74	91.67	**-1.96**	**-2.78**	**-0.44**
AT5G28020.6	cysteine synthase D2 (CYSD2)	467.64	500.18	**0.10**	73.92	19.44	**-1.93**	**2.66**	**4.69**
	Methionine related								
AT3G01120.1	METHIONINE OVERACCUMULATION 1 (MTO1)	429.66	378.27	**-0.18**	728.14	201.09	**-1.86**	**-0.76**	**0.91**
AT1G65320.1	Cystathionine beta-synthase (CBS) family protei	66.27	71.63	**0.11**	31.85	8.73	**-1.87**	**1.06**	**3.04**
AT5G10860.1	Cystathionine beta-synthase (CBS) family protei	682.47	760.35	**0.16**	252.23	70.52	**-1.84**	**1.44**	**3.43**
AT4G27460.1	Cystathionine beta-synthase (CBS) family protei	9.66	3.83	**-1.33**	4.88	0.25	**-4.31**	**0.98**	**3.96**
AT3G57050.2	cystathionine beta-lyase (CBL)	238.27	261.58	**0.13**	119.86	31.66	**-1.92**	**0.99**	**3.05**
AT3G57050.3	cystathionine beta-lyase (CBL)	139.68	165.40	**0.24**	14.27	2.09	**-2.77**	**3.29**	**6.31**
AT5G17920.2	Cobalamin-Ind. Methionine cynthase (ATCIMS)	2801.92	2253.16	**-0.31**	2138.05	349.76	**-2.61**	**0.39**	**2.69**
AT3G03780.3	methionine synthase 2 (MS2)	881.25	643.95	**-0.45**	3359.17	615.54	**-2.45**	**-1.93**	**0.07**
AT5G20980.2	methionine synthase 3 (MS3)	27.42	26.89	**-0.03**	21.40	8.72	**-1.30**	**0.36**	**1.63**
	Other S-related								
AT1G33320.1	(PLP)-dependent transferase protein	60.31	36.59	**-0.72**	109.95	36.72	**-1.58**	**-0.87**	**-0.01**
AT1G16540.1	ABA DEFICIENT 3 (ABA3)	54.30	47.42	**-0.20**	26.47	14.21	**-0.90**	**1.04**	**1.74**
AT3G03900.1	adenosine-5'-phosphosulfate (APS) kinase 3 (AP	4.00	6.53	**0.71**	13.87	2.42	**-2.52**	**-1.79**	**1.43**
AT5G67520.1	adenosine-5'-phosphosulfate (APS) kinase 4 (AP	26.98	41.00	**0.60**	7.95	4.26	**-0.90**	**1.76**	**3.27**
AT2G14750.1	APS kinase (APK)	105.77	132.86	**0.33**	193.17	31.85	**-2.60**	**-0.87**	**2.06**
AT4G39940.1	APS-kinase 2 (AKN2)	45.68	49.09	**0.10**	88.69	14.20	**-2.64**	**-0.96**	**1.79**
AT1G08490.1	chloroplastic NIFS-like cysteine desulfurase (CPI	25.01	25.30	**0.02**	39.66	11.65	**-1.77**	**-0.67**	**1.12**
AT5G27380.1	glutathione synthetase 2 (GSH2)	222.80	279.27	**0.33**	110.17	24.05	**-2.20**	**1.02**	**3.54**
AT3G25900.1	homocysteine methyltransferase (HMT1)	19.36	22.58	**0.22**	6.98	1.28	**-2.45**	**1.47**	**4.14**
AT3G25900.3	homocysteine methyltransferase (HMT1)	0.70	0.50	**-0.49**	16.49	4.84	**-1.77**	**-4.55**	**-3.28**
AT3G63250.1	homocysteine methyltransferase 2 (HMT2)	2.92	3.24	**0.15**	63.15	15.30	**-2.04**	**-4.43**	**-2.24**
AT3G22740.1	homocysteine S-methyltransferase 3 (HMT3)	7.61	23.18	**1.61**	55.12	21.92	**-1.33**	**-2.86**	**0.08**
AT5G65720.1	nitrogen fixation S (NIFS)-like 1 (NFS1)	9.68	11.68	**0.27**	8.49	0.90	**-3.24**	**0.19**	**3.70**
AT5G24660.1	RESPONSE TO LOW SULFUR 2 (LSU2)	184.69	367.55	**0.99**	239.66	44.44	**-2.43**	**-0.38**	**3.05**
AT3G49570.1	RESPONSE TO LOW SULFUR 3 (LSU3)	31.49	44.98	**0.51**	99.16	49.10	**-1.01**	**-1.65**	**-0.13**
AT5G24655.1	RESPONSE TO LOW SULFUR 4 (LSU4)	255.75	329.12	**0.36**	45.11	14.92	**-1.60**	**2.50**	**4.46**

Figure 16 (continued)

Root (Cont.)		*S. pinnata*	*S. pinnata*	*S. pinnata*	*S. elata*	*S. elata*	*S. elata*	***S. pin/S. ela***	***S. pin/S. ela***
	Selenium specific	Se -	Se +	**Log2**	Se -	Se +	**Log2**	**log2 Se-**	**log2 Se+**
AT4G31360.1	selenium binding protein	102.29	101.06	**-0.02**	108.47	24.80	**-2.13**	**-0.08**	**2.03**
AT2G24440.1	selenium binding protein	34.78	41.63	**0.26**	4.10	1.46	**-1.49**	**3.08**	**4.84**
AT4G14030.2	selenium-binding protein 1 (SBP1)	246.35	397.84	**0.69**	203.46	84.13	**-1.27**	**0.28**	**2.24**
AT4G14040.1	selenium-binding protein 2 (SBP2)	160.49	251.76	**0.65**	79.69	12.21	**-2.71**	**1.01**	**4.37**
AT3G23800.1	selenium-binding protein 3 (SBP3)	50.13	47.95	**-0.06**	56.55	10.56	**-2.42**	**-0.17**	**2.18**
AT5G58640.2	Selenoprotein, Rdx type	52.93	58.84	**0.15**	53.77	28.76	**-0.90**	**-0.02**	**1.03**

B. Shoot		*S. pinnata*	*S. pinnata*	*S. pinnata*	*S. elata*	*S. elata*	*S. elata*	***S. pin/S. ela***	***S. pin/S. ela***
	Sulfate Transporters	Se -	Se +	**Log2**	Se -	Se +	**Log2**	**log2 Se-**	**log2 Se+**
AT4G08620.1	sulphate transporter 1;1 (SULTR1;1)	0.05	0.02	**-1.26**	0.12	0.10	**-0.28**	**-1.45**	**-2.43**
AT1G78000.2	sulfate transporter 1;2 (SULTR1;2)	19.05	13.58	**-0.49**	13.48	11.38	**-0.24**	**0.50**	**0.26**
AT5G10180.1	slufate transporter 2;1 (SULTR2;1)	164.35	80.93	**-1.02**	50.98	122.94	**1.27**	**1.69**	**-0.60**
AT3G51895.1	sulfate transporter 3;1 (SULTR3;1)	297.71	189.45	**-0.65**	158.44	140.69	**-0.17**	**0.91**	**0.43**
AT4G02700.1	sulfate transporter 3;2 (SULTR3;2)	7.52	7.31	**-0.04**	2.89	3.04	**0.08**	**1.38**	**1.26**
AT3G15990.1	sulfate transporter 3;4 (SULTR3;4)	26.52	20.70	**-0.36**	24.37	26.70	**0.13**	**0.12**	**-0.37**
AT5G19600.1	sulfate transporter 3;5 (SULTR3;5)	35.22	41.14	**0.22**	18.15	15.76	**-0.20**	**0.96**	**1.38**
AT5G13550.1	sulfate transporter 4.1 (SULTR4;1)	66.52	52.12	**-0.35**	70.35	63.63	**-0.14**	**-0.08**	**-0.29**
AT3G12520.2	sulfate transporter 4;2 (SULTR4;2)	5.16	2.82	**-0.87**	1.50	3.03	**1.02**	**1.78**	**-0.11**
	Cysteine synthesis								
AT3G22890.1	ATP sulfurylase 1 (APS1)	338.70	272.45	**-0.31**	905.88	664.85	**-0.45**	**-1.42**	**-1.29**
AT1G19920.1	ATP Sulfurylase 2 (APS2)	393.75	176.66	**-1.16**	139.79	157.13	**0.17**	**1.49**	**0.17**
AT4G14680.1	ATP Sulfurylase 3 (APS3)	329.20	273.28	**-0.27**	142.37	131.05	**-0.12**	**1.21**	**1.06**
AT4G04610.1	APS Reductase 1 (APR1)	906.27	965.24	**0.09**	1096.48	743.31	**-0.56**	**-0.27**	**0.38**
AT1G62180.1	APS Reductase 2 (APR2)	127.22	125.90	**-0.02**	66.31	44.47	**-0.58**	**0.94**	**1.50**
AT4G21990.1	APS Reductase 3 (APR3)	589.10	750.74	**0.35**	19.30	16.62	**-0.22**	**4.93**	**5.50**
AT4G21990.2	APS Reductase 3 (APR3)	153.54	221.66	**0.53**	232.32	126.22	**-0.88**	**-0.60**	**0.81**
AT5G04590.1	sulfite reductase (SIR)	648.15	401.37	**-0.69**	599.87	762.13	**0.35**	**0.11**	**-0.93**
AT4G14880.2	O-acetylserine (thiol) lyase (OAS-TL) isoform A1 (OA	44.54	40.74	**-0.13**	20.73	19.89	**-0.06**	**1.10**	**1.03**
AT3G22460.1	O-acetylserine (thiol) lyase (OAS-TL) isoform A2 (OA	101.00	140.57	**0.48**	120.62	62.94	**-0.94**	**-0.26**	**1.16**
AT3G59760.2	O-acetylserine (thiol) lyase isoform C (OASC)	43.97	19.04	**-1.21**	33.14	38.72	**0.22**	**0.41**	**-1.02**

Figure 16 (continued)

Shoot (Cont.)		*S. pinnata*	*S. pinnata*	*S. pinnata*	*S. elata*	*S. elata*	*S. elata*	***S. pin/S. ela***	***S. pin/S. ela***
	Cysteine synthesis (Cont.)	Se -	Se +	**Log2**	Se -	Se +	**Log2**	**log2 Se-**	**log2 Se+**
AT5G56760.1	serine acetyltransferase 1;1 (SERAT1;1)	228.86	227.55	**-0.01**	306.90	273.02	**-0.17**	**-0.42**	**-0.26**
AT2G17640.1	ATSERAT3;1	76.53	67.97	**-0.17**	71.25	60.65	**-0.23**	**0.10**	**0.16**
AT4G35640.1	serine acetyltransferase 3;2 (SERAT3;2)	0.93	1.32	**0.51**	5.61	2.39	**-1.23**	**-2.60**	**-0.85**
AT3G03630.1	cysteine synthase 26 (CS26)	22.21	21.18	**-0.07**	12.05	12.88	**0.10**	**0.88**	**0.72**
AT3G61440.3	cysteine synthase C1 (CYSC1)	115.21	128.55	**0.16**	29.98	32.29	**0.11**	**1.94**	**1.99**
AT3G04940.1	cysteine synthase D1 (CYSD1)	14.34	16.60	**0.21**	438.76	383.69	**-0.19**	**-4.93**	**-4.53**
AT5G28020.6	cysteine synthase D2 (CYSD2)	146.72	280.13	**0.93**	60.06	51.29	**-0.23**	**1.29**	**2.45**
	Methionine related								
AT3G01120.1	METHIONINE OVERACCUMULATION 1 (MTO1)	254.93	153.22	**-0.73**	713.47	982.02	**0.46**	**-1.48**	**-2.68**
AT4G27460.1	Cystathionine beta-synthase (CBS) family protein	0.77	0.68	**-0.17**	0.67	0.70	**0.07**	**0.21**	**-0.03**
AT1G65320.1	Cystathionine beta-synthase (CBS) family protein	90.17	74.17	**-0.28**	45.29	47.00	**0.05**	**0.99**	**0.66**
AT5G10860.1	Cystathionine beta-synthase (CBS) family protein	554.28	664.20	**0.26**	287.21	285.33	**-0.01**	**0.95**	**1.22**
AT3G57050.2	cystathionine beta-lyase (CBL)	111.25	63.85	**-0.80**	109.97	116.55	**0.08**	**0.02**	**-0.87**
AT3G57050.3	cystathionine beta-lyase (CBL)	92.23	38.11	**-1.28**	14.25	16.64	**0.22**	**2.69**	**1.20**
AT5G17920.2	COBALAMIN-INDEPENDENT METHIONINE SYNTHASE	2239.66	1614.57	**-0.47**	1533.71	1257.07	**-0.29**	**0.55**	**0.36**
AT3G03780.3	methionine synthase 2 (MS2)	1407.93	504.65	**-1.48**	2957.40	2556.77	**-0.21**	**-1.07**	**-2.34**
AT5G20980.2	methionine synthase 3 (MS3)	38.95	43.67	**0.16**	20.41	22.58	**0.15**	**0.93**	**0.95**
	Other S-related								
AT1G33320.1	(PLP)-dependent transferase protein	6.70	3.28	**-1.03**	4.86	4.28	**-0.18**	**0.46**	**-0.38**
AT1G16540.1	ABA DEFICIENT 3 (ABA3)	32.59	28.88	**-0.17**	22.84	24.42	**0.10**	**0.51**	**0.24**
AT3G03900.1	adenosine-5'-phosphosulfate (APS) kinase 3 (APK3)	8.76	11.87	**0.44**	15.11	5.05	**-1.58**	**-0.79**	**1.23**
AT5G67520.1	adenosine-5'-phosphosulfate (APS) kinase 4 (APK4)	30.55	38.04	**0.32**	4.03	7.94	**0.98**	**2.92**	**2.26**
AT2G14750.1	APS kinase (APK)	235.99	78.95	**-1.58**	204.13	174.49	**-0.23**	**0.21**	**-1.14**
AT4G39940.1	APS-kinase 2 (AKN2)	97.80	27.81	**-1.81**	86.46	67.32	**-0.36**	**0.18**	**-1.28**
AT1G08490.1	chloroplastic NIFS-like cysteine desulfurase (CPNIFS	38.94	31.86	**-0.29**	50.52	45.69	**-0.14**	**-0.38**	**-0.52**
AT5G27380.1	glutathione synthetase 2 (GSH2)	321.55	282.26	**-0.19**	171.66	190.66	**0.15**	**0.91**	**0.57**
AT3G25900.1	homocysteine methyltransferase (HMT1)	6.93	7.61	**0.14**	4.65	3.43	**-0.44**	**0.58**	**1.15**
AT3G25900.3	homocysteine methyltransferase (HMT1)	0.03	0.09	**1.60**	8.08	7.08	**-0.19**	**-8.14**	**-6.35**
AT3G63250.1	homocysteine methyltransferase 2 (HMT2)	1.71	1.39	**-0.30**	57.49	37.41	**-0.62**	**-5.07**	**-4.75**
AT3G22740.1	homocysteine S-methyltransferase 3 (HMT3)	31.26	14.90	**-1.07**	70.46	52.75	**-0.42**	**-1.17**	**-1.82**
AT5G65720.1	nitrogen fixation S (NIFS)-like 1 (NFS1)	10.76	10.94	**0.02**	7.11	10.32	**0.54**	**0.60**	**0.08**
AT5G24660.1	RESPONSE TO LOW SULFUR 2 (LSU2)	250.33	442.12	**0.82**	282.10	78.64	**-1.84**	**-0.17**	**2.49**
AT3G49570.1	RESPONSE TO LOW SULFUR 3 (LSU3)	49.50	49.83	**0.01**	48.33	25.26	**-0.94**	**0.03**	**0.98**
AT5G24655.1	RESPONSE TO LOW SULFUR 4 (LSU4)	140.85	144.94	**0.04**	26.18	18.21	**-0.52**	**2.43**	**2.99**

Figure 16 (continued)

Shoot (Cont.)		*S. pinnata*	*S. pinnata*	*S. pinnata*	*S. elata*	*S. elata*	*S. elata*	***S. pin/S. ela***	***S. pin/S. ela***
	Selenium specific	Se -	Se +	**Log2**	Se -	Se +	**Log2**	**log2 Se-**	**log2 Se+**
AT4G31360.1	selenium binding protein	98.52	139.06	**0.50**	62.39	54.33	**-0.20**	**0.66**	**1.36**
AT2G24440.1	selenium binding protein	35.81	67.42	**0.91**	4.50	3.59	**-0.33**	**2.99**	**4.23**
AT4G14030.2	selenium-binding protein 1 (SBP1)	97.98	103.12	**0.07**	293.08	231.35	**-0.34**	**-1.58**	**-1.17**
AT4G14040.1	selenium-binding protein 2 (SBP2)	163.50	72.47	**-1.17**	176.37	167.00	**-0.08**	**-0.11**	**-1.20**
AT3G23800.1	selenium-binding protein 3 (SBP3)	0.87	0.47	**-0.89**	485.05	275.06	**-0.82**	**-9.13**	**-9.20**
AT5G58640.2	Selenoprotein, Rdx type	34.58	40.95	**0.24**	51.10	47.41	**-0.11**	**-0.56**	**-0.21**

Figure 16 (continued)

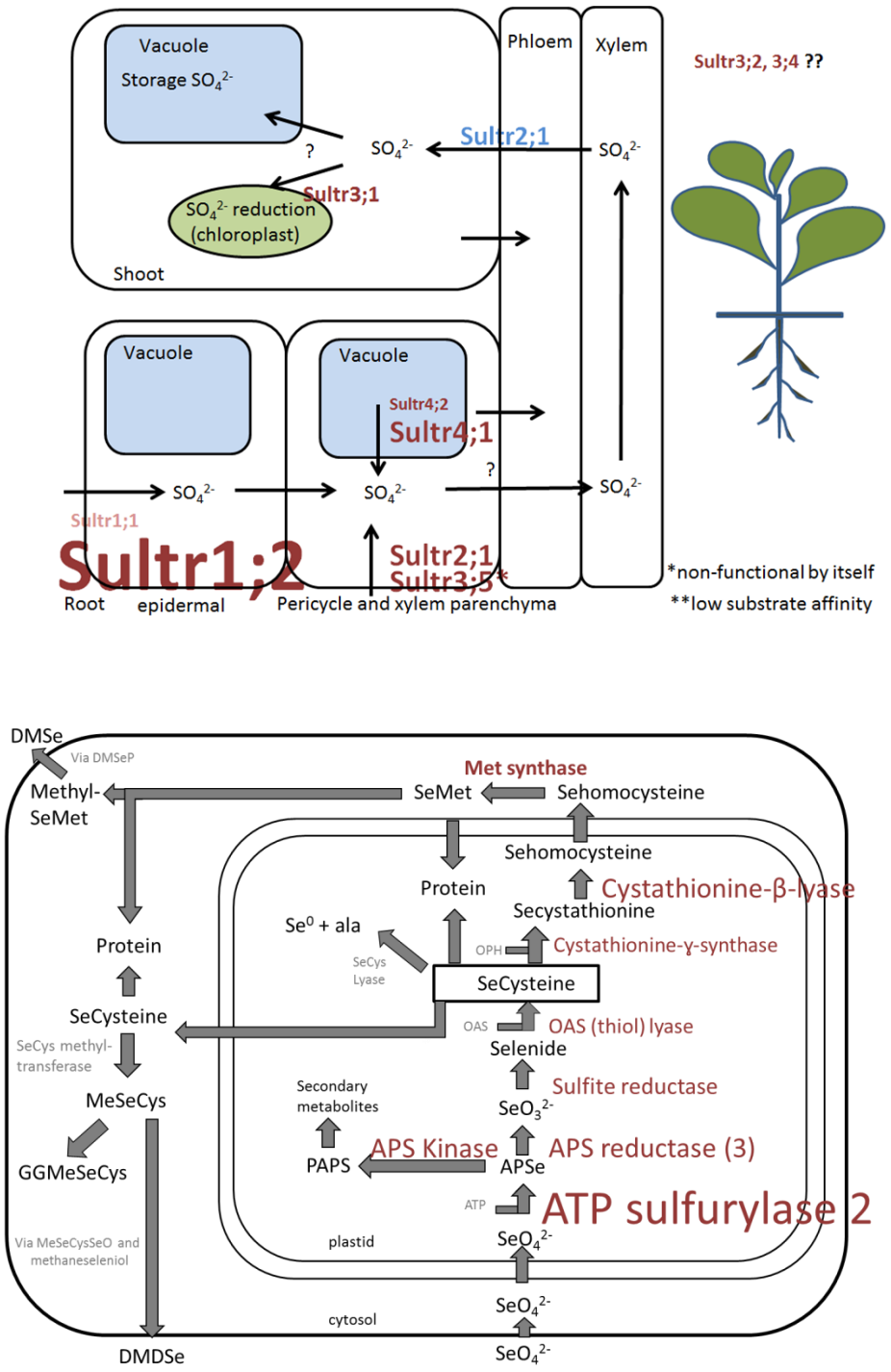

Figure 17- Proposed model of sulfate transporters (top- adapted from Takahashi et al. 2011) and sulfate assimilation pathway (bottom). Enzymes shown with relative abundance in *S. pinnata* relative to *S. elata* with larger, red colored enzymes being much more highly expressed in *S. pinnata* roots in the presence of Se.

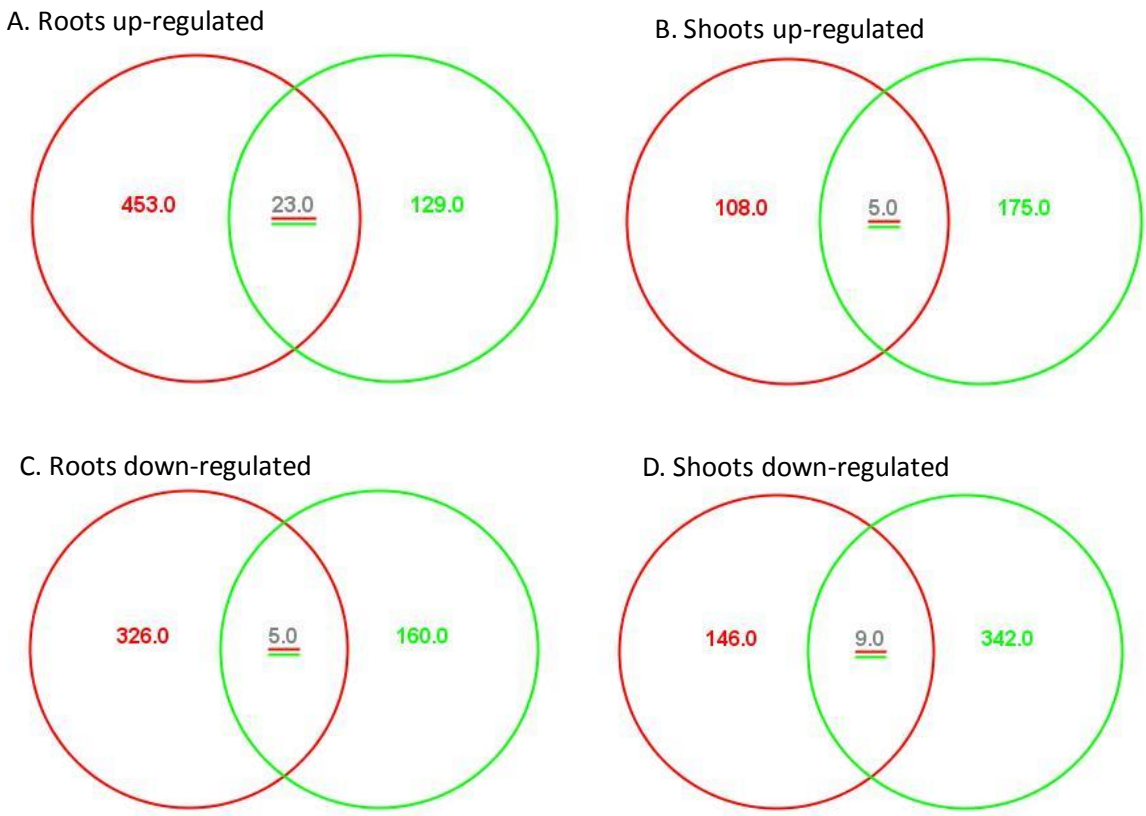

Figure 18– Venn diagrams (MapMan) showing the number of genes present in both species that are 4 fold or greater up-(A, B) or down-regulated (C, D) by Se. Red circles represent *S. elata* and green circles represent *S. pinnata*. Roots are shown on the left, shoots on the right

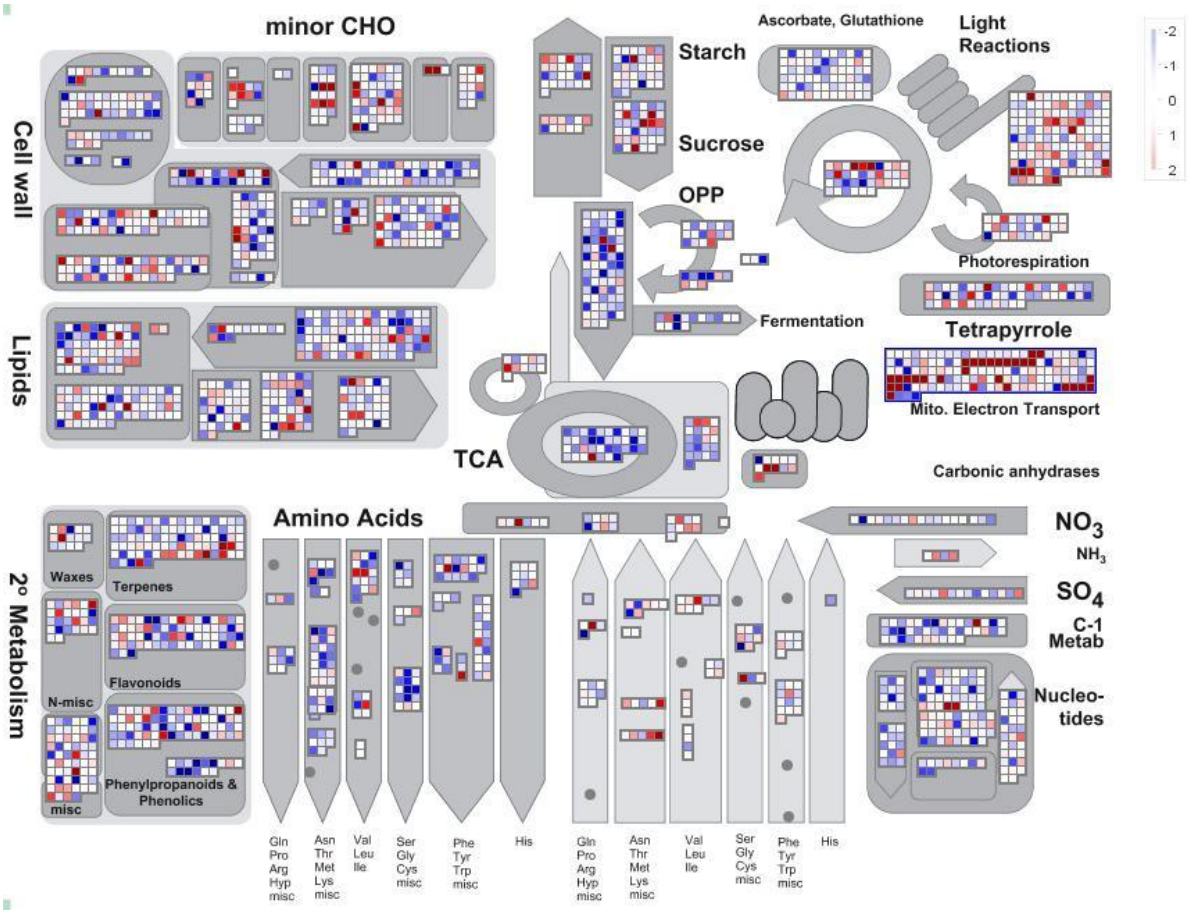

Figure 19(A) – MapMan metabolism overview diagrams showing functional groups of genes differentially expressed in roots (A, C) and shoots (B, D) of *S. elata* (A, B) and *S. pinnata* (C, D) when grown in the presence of Se. Genes shown in red are up-regulated, blue are down-regulated. White boxes indicated a $\log_2$ change of near 0.

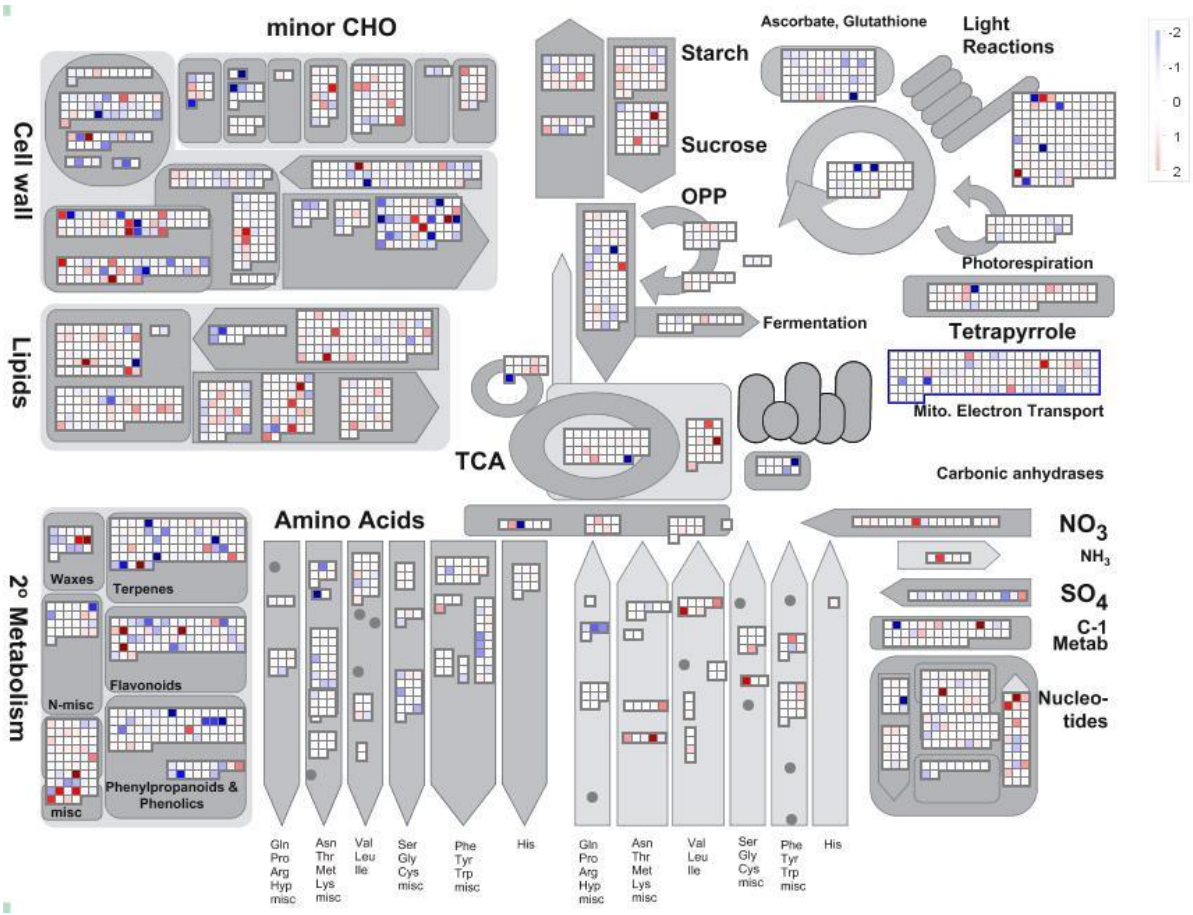

Figure 19B (continued)

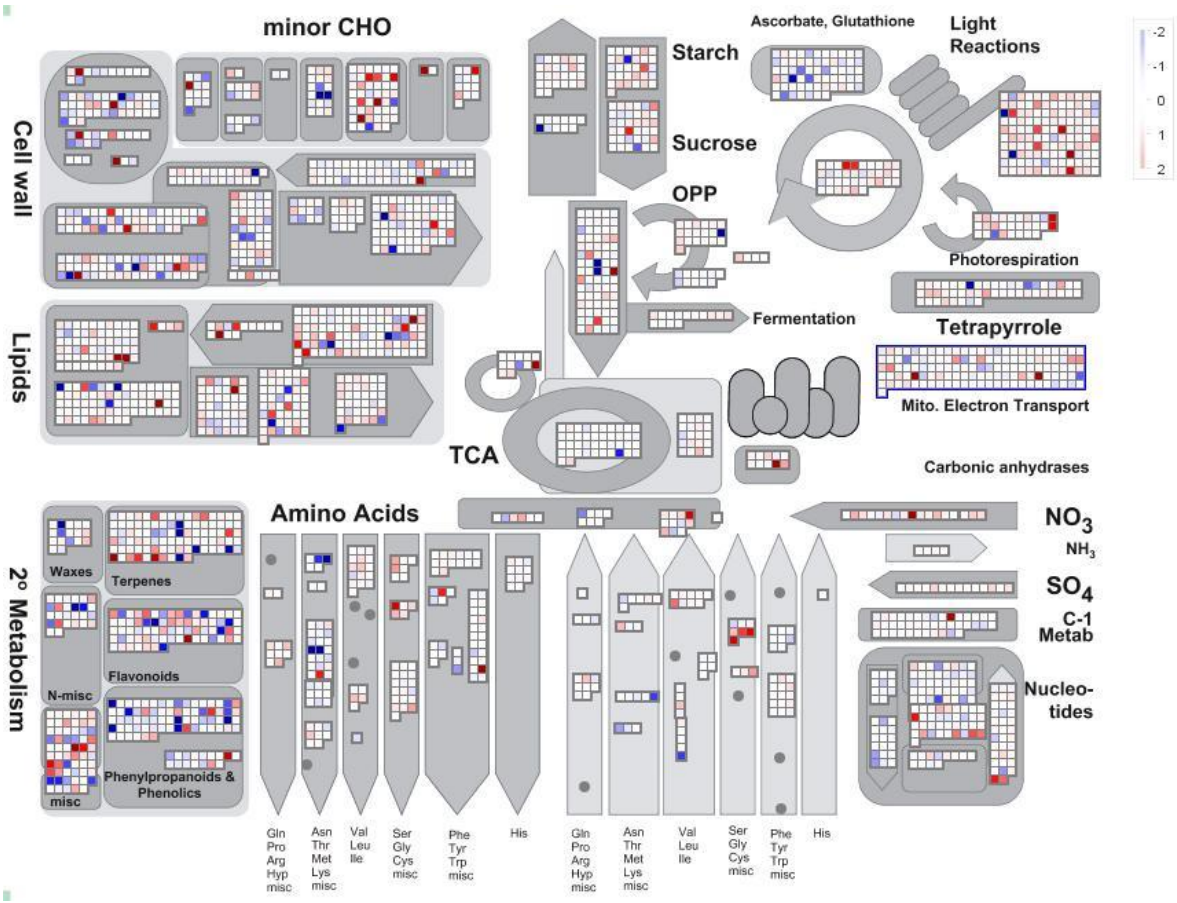

Figure 19C (continued)

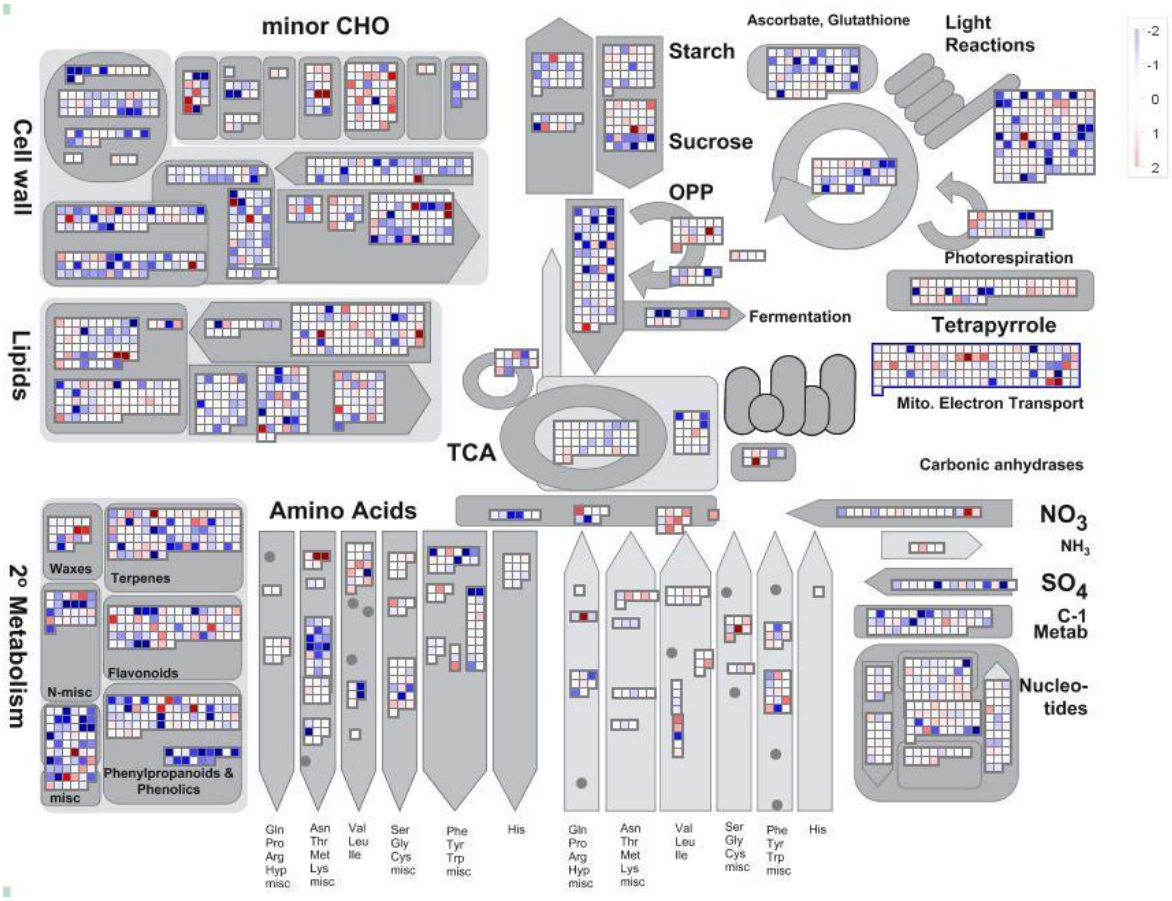

Figure 19 D (continued)

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APPENDIX: ADDITIONAL OBSERVATIONS

INTRODUCTION

In addition to the physiological and RNA Seq studies described in the previous chapters, several follow-up biochemical tests were performed in an attempt to shed some light on the mechanisms of tolerance in *S. pinnata.* The results are preliminary but included here so that they may serve as a starting point for further studies. First, I describe the development of a new method for organic selenium (Se) extraction, identification and quantification using liquid chromatography – mass spectrometry (LC-MS), and some early results using this method (A). Then, biochemical studies are described that aimed to investigate potential contributions of superoxide dismutase (B) and selenocysteine lyase (C) activities to Se hyperaccumulation and tolerance.

A) ORGANIC SELENOCOMPOUNDS IN HYPERACCUMULATORS

Introduction

One characteristic of Se hyperaccumulators is their ability to store Se in organic, non-protein bound forms, primarily as either methyl-selenocysteine or selenocystathionine. Selenocysteine is processed to methylselenocysteine (MeSeCys) by selenocysteine methyl-transferase (SMT) (Shrift and Virupaksha 1965). This represents the primary mechanism by which hyperaccumulating members of the *Astragalus* genus are able to tolerate Se. Since it is methylated, MeSeCys will not be incorporated into proteins, and is much safer to accumulate. Alternatively, SeCys can be accumulated as the organic compound selenocystathionine, an intermediate in the pathway from selenocysteine to selenomethionine in plants. Although cystathionine rarely accumulates in significant amounts in most plants, several Se hyperaccumulators have been found to contain significant amounts of selenocystathionine. The discovery of selenocystathionine in hyperaccumulators was first reported by Virupaksha and Shrift in 1963, and has since been found in other Se accumulators, such as *Morina reticulata, Astragalus pectinatus, Neptunia*

amplexicaulis and *Lecythis minor* (Monkeypot nuts) (Dernovics et al. 2007; Peterson and Butler 1971; Peterson and Butler 1967).

The mechanism for the production and accumulation of selenocystathionine by these hyperaccumulators is not well known. However, spinach extracts incorporated selenocysteine into selenocystathionine quickly, with cystathionine-ɣ-synthase showing a higher affinity for selenocysteine than cysteine (Dawson and Anderson 1988). The accumulation of selenocystathionine in *Astragalus* could not be accounted for by specificity of cystathionine-γ-synthase, as it showed rates of incorporation of selenocysteine and cysteine to be similar (Dawson and Anderson 1989). In some organisms, cystathionine lyases have shown stronger affinity for selenocystathionine when compared to cystathionine; it is feasible that the inverse is true in hyperaccumulators, resulting in selenocystathionine remaining in the cells, unable to be broken down (Birringer et al. 2002).

The function of cystathionine-γ-synthase is not clearly understood, even in *Arabidopsis.* When *Arabidopsis* plants were engineered with antisense DNA for cystathionine-γ-synthase, they showed 20-fold reduction of enzyme activity, but only a 35% reduction of methionine pool. However, the plants also showed a 7-fold decrease in S-methylmethionine (SMM) which is involved in sulfur transport (Gakière et al. 2000). Overexpression of a cystathionine-γ-synthase from *Arabidopsis* in *B. juncea* resulted in 2-3 fold increased Se volatilization rate, but only when plants were given selenite, not selenate (Van Huysen et al. 2003).

Methods: LC-MS Sample preparation

Plant samples were ground in liquid nitrogen and free amino acids were extracted overnight at 4° C in LC-MS grade water adjusted to pH 2.5 with HCl, at a ratio of 1 g fresh weight (FW) to 2 mL extraction solution. Typically 1 g of plant material was extracted with 2 mL of solution. Samples were centrifugated at 3000 x *g* in a swing-out rotor, and supernatant was removed.

The subsequent sample preparation method for LC-MS is still under development, and has gone through several iterations so far. Originally we used C18 Sep-Pak columns (Waters Corp.), according to their established protocol for amino acid extraction. However, after issues with organic Se compounds precipitating after a short time, even when frozen or refrigerated, we changed to Phenomenex Strata X-C sample preparation columns. 1 mL of supernatant was added to a Phenomenex Strata X-C (33u polymeric strong cation) SPE column that had previously been conditioned with 2 mL of methanol and 2 mL of LC-MS grade water (method calls for "acidified" water). Columns were then washed with 1 mL of 0.1 N HCl in water, and 1 mL of 0.1 N HCl in LC-MS grade methanol, with fractions collected for analysis. Columns were dried under a vacuum for 5 minutes and samples were eluted using 7.5% ammonium hydroxide in methanol. It should be noted that the exact amount of washing with HCl and MeOH has yet to be determined. MeSeCys concentrations decreased sharply in samples when the wash step was increased to 4 mL in an attempt to remove more chlorophyll from the sample. This procedure still needs to be optimized further.

Methods LC-MS

Extracted samples were separated on an Agilent 1200 series HPLC using an Atlantis T3 3 µm 2.1 x 150mm column (Waters corp.). The column was kept at room temperature, and 2 µL of sample was injected with a flow rate of 0.3 mL/min, starting with water and 0.1% Formic acid for 2 minutes, followed by ramping up to 50% methanol for the remaining 18 minutes. Columns were run for a post time of 10 minutes. The current method is saved as (EPS_Se_Atl_1.m). Mass spectra were obtained using an Agilent 6220 TOF MS.

Due to difficulties obtaining a reliable secystathionine standard, the sulfur analogues of cystathionine and methylcysteine as well as methylselenocysteine were used as a standard. Extracted peaks of each standard were similar in count (as described below, Fig. 21, 22).

Findings and discussion

Although previous reports have found around 20% of organic Se to be present as Se-cystathionine in *S. pinnata*, with the rest being methyl selenocysteine (Freeman et al. 2010; Virupaksha and Shrift 1963), our new method shows a significantly higher ($P < 0.05$) fraction of organic Se to be seleno-cystathionine (Fig. 20). In addition, a very preliminary experiment (sample size of 1) found substantially (~4 fold) more SeCyst in the roots of *S. pinnata* than in the shoots. Once the protocol is optimized, this measurement can be repeated with more replicates for both *S. pinnata* and *S. elata.*

B) SELENIUM AND SUPEROXIDE DISMUTASE ABUNDANCE/ACTIVITY IN *STANLEYA PINNATA*

Selenium has been shown to increase free radical production in plants, especially non-accumulator species (Freeman et al. 2010). Thus, the capacity to prevent or alleviate oxidative stress may contribute to plant Se hyperaccumulation. In the study described in Chapter 2, the Colorado ecotype of *S. pinnata* showed a negative correlation between selenium and copper content of leaves (Fig. 23). This prompted us to study levels of Cu/Zn, Fe, and Mn Superoxide Dismutases (SOD) in relation to plant Se status, since SODs have been implicated in plant resistance to oxidative stress. Using in-gel activity assays, as well as Western blots, regulation of superoxide dismutase by Se in hyperaccumulator *S. pinnata* was investigated.

Methods - Sample preparation – native protein extraction for activity/western blots

Samples ($\geq$ 0.2 g FW) were ground in liquid nitrogen. Extraction buffer (50 mL solution – 2 mL of 1M K_2HPO_4, 495 µL of 1M KH_2PO_4, 50 mg Ascorbate, 25µL β-mercaptoethanol, 100 µL Triton X-100) was added 2:1 volume/weight and samples were thoroughly vortexed while kept below 4° C. Samples were then centrifugated at 14,000 rpm for 10 minutes at 4° C, upon which the supernatant was

collected and the pellet discarded. The centrifugation step was then repeated, and final supernatant was quantified for protein content using a Bradford assay.

SOD activity

A native, 12.5% polyacrylamide gel was prepared, and samples were loaded at 20 µg of total protein per well. Gels were run at 80 volts at 4° C for approximately 2.5 hours. Gels were soaked in 20 mL of 1 mg/mL nitro-blue tetrazolium (NBT) in ddH_2O in the dark on an orbital shaker for 20 minutes. 45 mL of stain was prepared with 1.47 mL 1 M K_2HPO_4, 148 µL 1M KH_2PO_4, 450 µL of 1mg/mL riboflavin (2.8mM), 145 µL TEMED and water to volume. The gels were soaked in this stain for an additional 20 min in the dark on the shaker, then stain was removed, gels were rinsed in deionized water and exposed to bright light for 1-4 hours to develop.

Findings and discussion

As Se supply increased, the protein abundance (Fig. 24) and activity (Fig. 26) of the Cu/Zn SODs in *S. pinnata* decreased. This may be at least partly explained by the decreasing Cu concentrations in the leaves. Previous work has shown that Cu/Zn SODs are quickly down-regulated in conditions which the plant may perceive as Cu deficient (Yamasaki et al. 2007). A possible mechanism may be that as supplied Se amount increases, reduced selenide binds to Cu in the roots and/or shoots, leading to insoluble CuSe and rendering Cu unavailable for further translocation or for insertion into Cu proteins. Also, as seen in figure 25, FeSOD increased in amount and activity as Cu/Zn SOD activity decreased, possibly as a compensatory mechanism. These results do not suggest SODs play an important role in alleviating oxidative stress caused by Se, as total SOD levels do not appear to increase in the presence of Se.

C) ENZYME WITH ABUNDANT SELENOCYSTEINE LYASE ACTIVITY FOUND IN *STANLEYA PINNATA*

SeCysteine, a toxic amino acid for plants due to its potential to be mis-incorporated into protein, can be broken down into elemental Se and alanine through the action of selenocysteine lyase enzymes. Elemental Se is believed to be insoluble and therefore biologically unavailable and relatively non-toxic (Garifullina and Owen 2003). Although several studies have shown that some cysteine desulfurases (CysD), like CpNifS, have selenocysteine lyase activity, there is only limited evidence showing that plants accumulate elemental Se in significant amounts (Hoewyk and Garifullina 2005; Lindblom et al. 2011; Valdez Barillas et al. 2012). Previous work with overexpression of a mouse selenocysteine lyase demonstrated the potential of SL activity in preventing Se toxicity in plants: (over)expression of mouse- or plant SL enzymes in plants led to reduced incorporation of Se into proteins, and enhanced Se tolerance and accumulation (Garifullina and Owen 2003; Pilon et al. 2003; Van Hoewyk et al., 2005). In this study we investigate the presence and localization of potential SL enzymes in hyperaccumulator *S. pinnata* and several other hyperaccumulator and non-hyperaccumulator reference species.

In-gel activity assay for cysteine desulfurase and selenocysteine lyase activity –

A native, 10-12.5% polyacrylamide gel was prepared, and samples were loaded with 20 µg of protein per well for cysteine desulfurase activity, and 100 µg for selenocysteine lyase activity. Gels were run at 80 volts at 4 ° C for approximately 2.5 hours.

Substrate was prepared 1 hour prior to the development of the gel. This consisted of 100mM Tris/HCl at a pH of 10, 25 mM DTT, and 10 mM of L-cysteine or 5 mM of L-selenocystine depending on assay performed. Immediately before adding to gel, 10 mM $AgNO_3$ was added to the solution, mixed thoroughly, then poured onto the gel (appx. 10-20 mL per gel). Gels were shaken gently under vacuum, then remained under a vacuum for 1-6 hours to allow bands to form (Cysteine desulfurase bands generally appear much sooner than selenocysteine lyase bands).

Chloroplast isolation for SL assay

Young leaves were collected from *S. pinnata* and *S. elata* and cut into 1mm thin strips with a razor blade. The midvein of each leaf was not cut. The leaves were then dipped in a cellulose enzyme solution (20 mM MES, pH 5.7, 400 mM mannitol, 20 mM KCl, 1.5% (w/v) cellulose, 0.4% (w/v) macerozyme, 10 mM $CaCl_2$, 0.1% (w/v) BSA). 10 mL of enzyme solution was added to a petri dish, and enough leaves were cut to fill the dish with one layer of leaf tissue. Enzyme solution and leaves were placed under vacuum, in the dark, at 4° C overnight with gentle shaking. Protoplasts were separated the following day by adding 10 mL of W5 solution (20 mM MES/KOH pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl) and swirling gently. This solution was pipetted through a pre-wetted (with W5) 215 µm nylon mesh to remove large debris. Samples were kept in the dark and on ice whenever possible. Protoplasts were checked for integrity using a microscope and 200x magnification. Protoplasts were pelleted using a swing out rotor at 200g for 2 min at 4° C. Supernatant was removed and pellets washed with "half and half" (10 mM MES/KOH pH 5.7, 77 mM NaCl, 63 mM $CaCl_2$, 2 mM KCl, 200 mM mannitol). After resuspension , protoplasts were centrifuged as before and resuspended in ~1 mL Chloroplast isolation buffer (CIB) (EGTA, 5mM EDTA, 10mM $NaHCO_3$, 0.1% BSA). These protoplasts were transferred to a 10 mL syringe with a 18 µm mesh attached, and protoplasts were forced through into pre-cooled 2 mL low bind centrifuge tubes. Lysed protolasts were centrifuged at 1125 x g for 2 min at 4°. The supernatant was removed and the pellet was resuspended in CIB without any BSA.. Chlorophyll content was standardized to 1µg/µl for intact protoplasts and chloroplasts. Samples were stored at -80° C.

Immunoblot –

For detection of housekeeping genes and SODs, 10 µg of total protein was added to each well of a 10-15% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane at 15 V overnight (0.2 µm, Bio-Rad). Membranes were then incubated for 1 hour in 3% milk and TBS-T (50 mM Tris/HCl,

pH 7.5, 150 mM NaCl, 0.1% Tween-20). Primary antibody was added to TBS-T and used at a dilution of 1:3000 in 1% milk and incubated with gentle shaking for 2 hours at room temperature. After primary antibody is removed, membranes were washed for 1 hour with TBS-T with buffer changing occurring twice. Secondary antibody in TBS-T (dilution 1:10,000) was added for 1 hour, followed by another wash step as before. Protein bands were visualized through the activity of alkaline phosphatase conjugated to the secondary antibody in the presence of 5-Bromo-4-chloro-3-indolyl phosphate BCIP and NBT.

Findings and discussion

Stanleya pinnata contained a large enzyme or enzyme complex (exact size is difficult to determine with native gels) with both cysteine desulfurase activity and a large amount of selenocysteine lyase (SL) activity. This SL activity was much greater than any found in A*rabidopsis thaliana* or *Astragalus bisulcatus* (Fig. 27) . The activity of this enzyme was dramatically reduced with leaf age and with exposure to Se (Fig. 28). Through chloroplast isolation, it was shown that this enzyme was not localized to the chloroplast (Fig 29, 30). Thus, it is likely not cpNifS, the chloroplastic protein involved in iron-sulfur cluster biosynthesis. Two additional NifS-like proteins are known to exist in plants: one is in the mitochondrion and involved in iron-sulfur cluster biosynthesis there (mtNifS), and one is in the cytosol (ABA3) and involved in molybdenum cofactor (MoCo) modification. The observed SL in S. pinnata could be either of these other two proteins, or a yet undescribed enzyme. Whether the SL activity of this enzyme is relevant *in vivo*, and whether any elemental Se is formed in *S. pinnata* is at this point not clear. So far, very little elemental Se has been found in leaf tissue in XAS studies. It is intriguing that this SL activity is so high in *S. pinnata*. The observation that its activity is highest in the absence of Se may suggest a role in Se acquisition, particularly in young tissues, rather than in Se detoxification. Further studies are needed to investigate the functions of this enzyme.

FIGURES

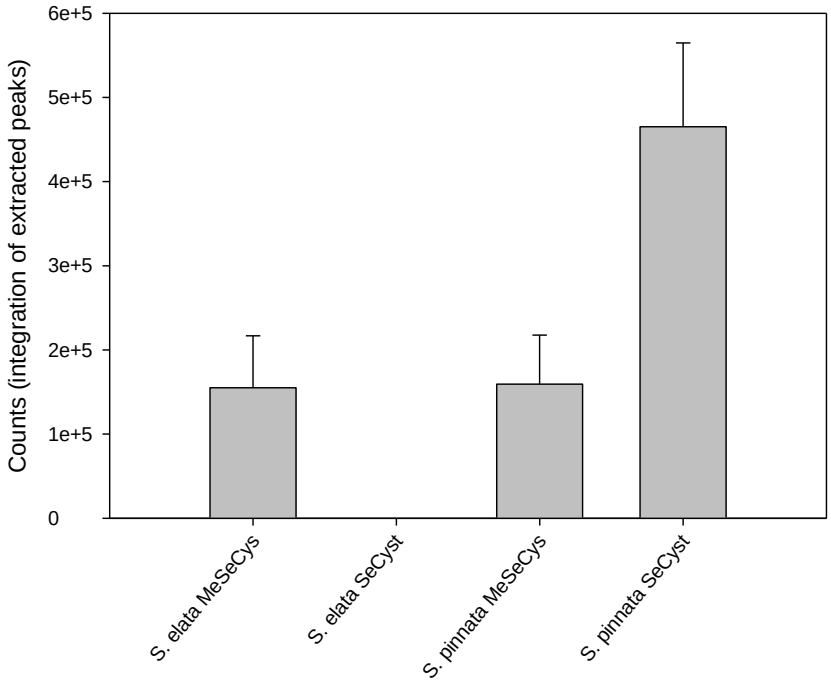

Figure 20–Relative abundance of methyl-selenocysteine (MeSeCys) and Seleno-Cystathionine (SeCyst) in shoots of *S. pinnata* and *S. elata*. *Note: A. bisulcatus* is not shown here. Using adifferent LC-MS protocol it was found to contain 100% MeSeCys.

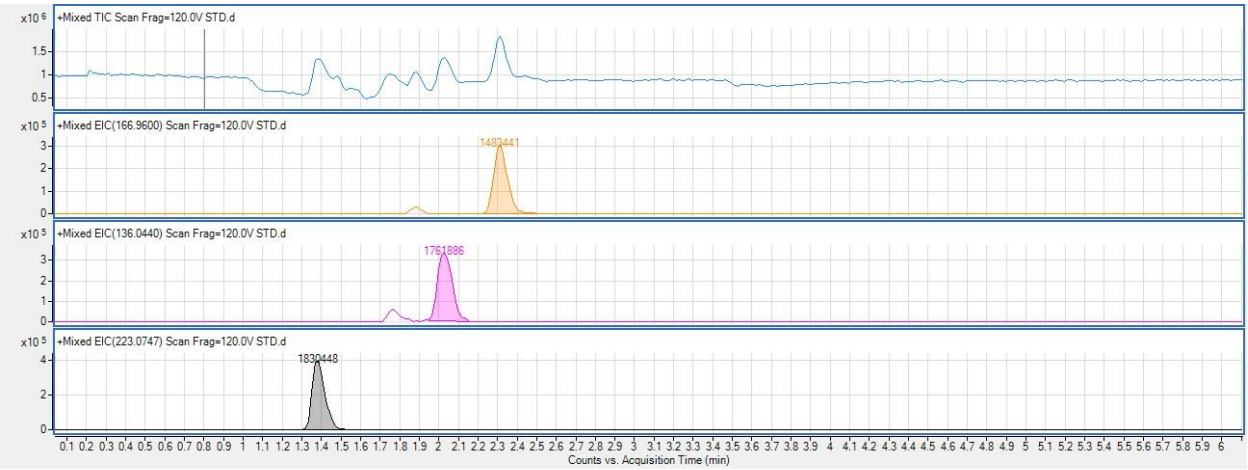

Figure 21 – Total spectra for standard (top), followed by extracted peaks for methyl-selenocysteine, methyl-cysteine and cystathionine, respectively. Numbers above peaks represent extracted counts, and show a similar correlation between concentration and peak size.

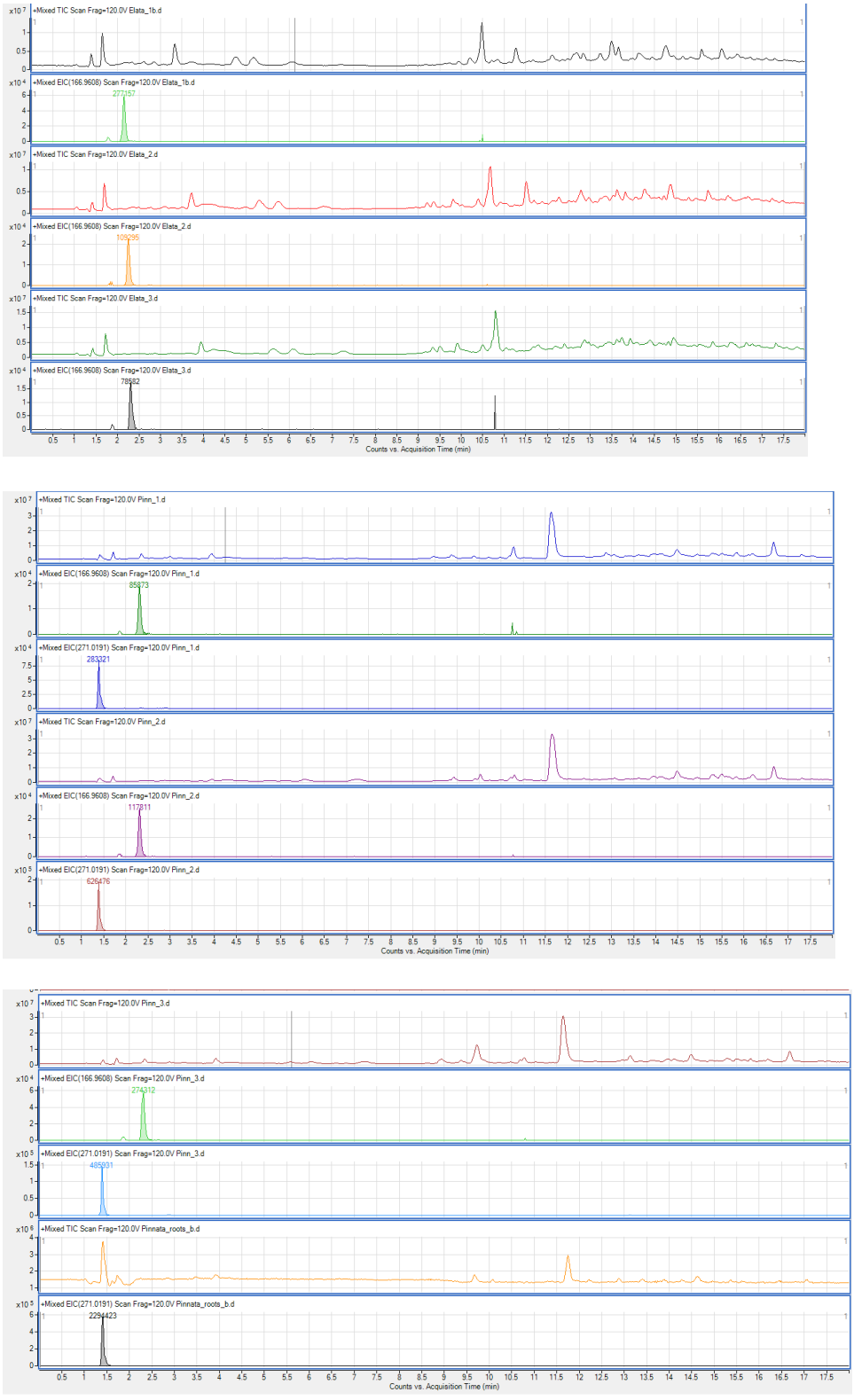

Figure 22 – LC-MS spectra from three biological replicates of *S. pinnata* and *S. elata* leaves, with extracted peaks of selenocompounds.

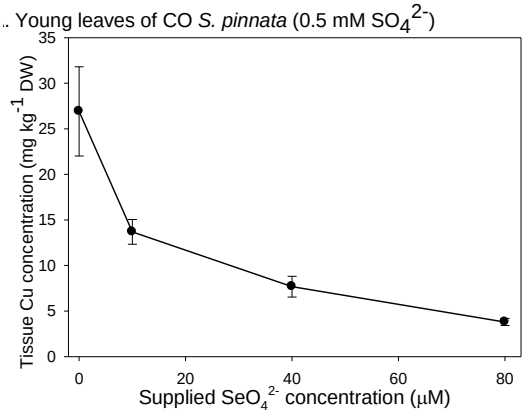

Figure 23 – Copper concentrations in young leaves of the CO ecotype of *S. pinnata* with low (0.5 mM) sulfate treatment.

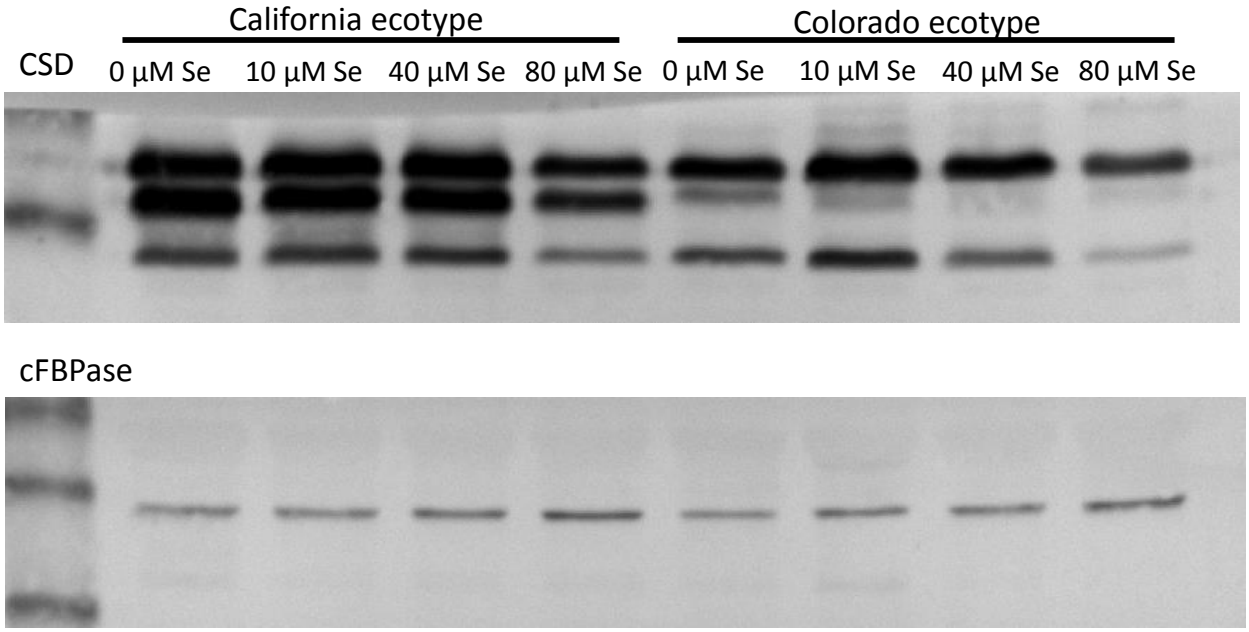

Figure 24 – (Top) Western blot for Cu/Zn superoxide dismutase (SOD) (3 different bands visible) in the Colorado and California ecotypes of *S. pinnata* under different Se concentrations. Bottom blot shows cFBPase loading control for the same samples.

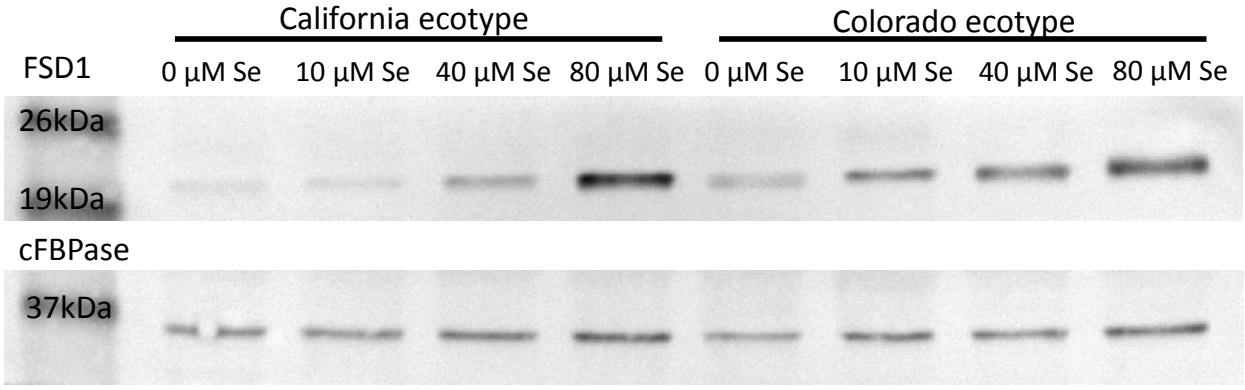

Figure 25 – (Top) Western blot for Fe SOD in the Colorado and California ecotype of *S. pinnata* under different Se concentrations. Bottom blot shows cFBPase loading control for the same samples.

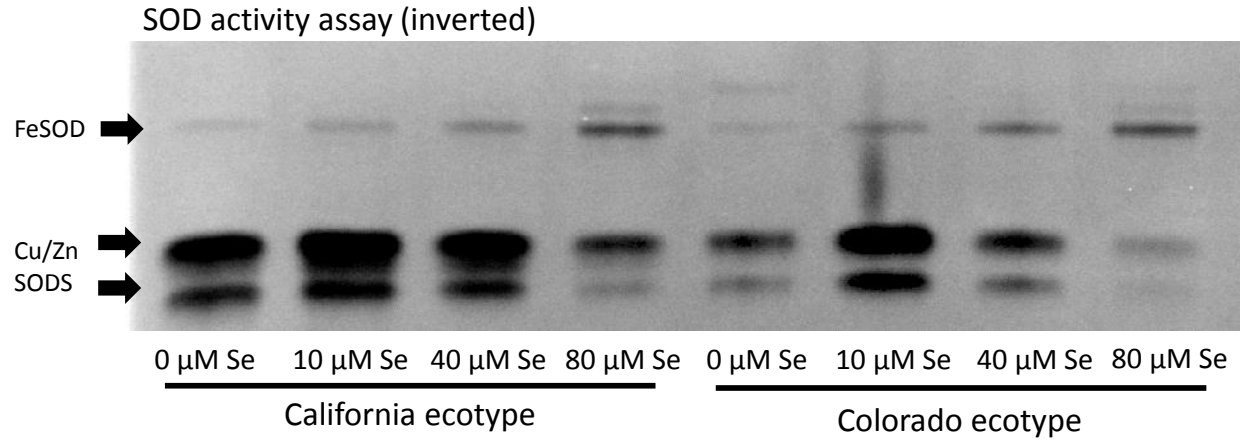

Figure 26 – In-gel activity assay for SOD in the Colorado and California ecotype of *S. pinnata* under different Se concentrations (color is inverted for clarity).

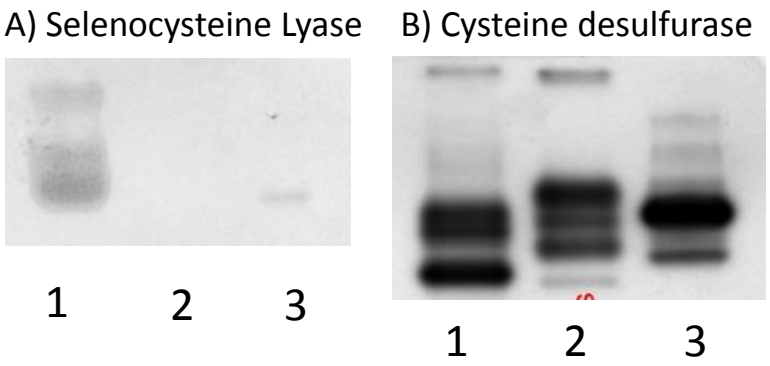

Figure 27 – In-gel assay showing selenocysteine lyase (A) and cysteine desulfurase (B) activities from young leaves of *Stanleya pinnata* (1), *Astragalus bisulcatus*(2) and the Columbia ecotype of *Arabidopsis thaliana* (3).

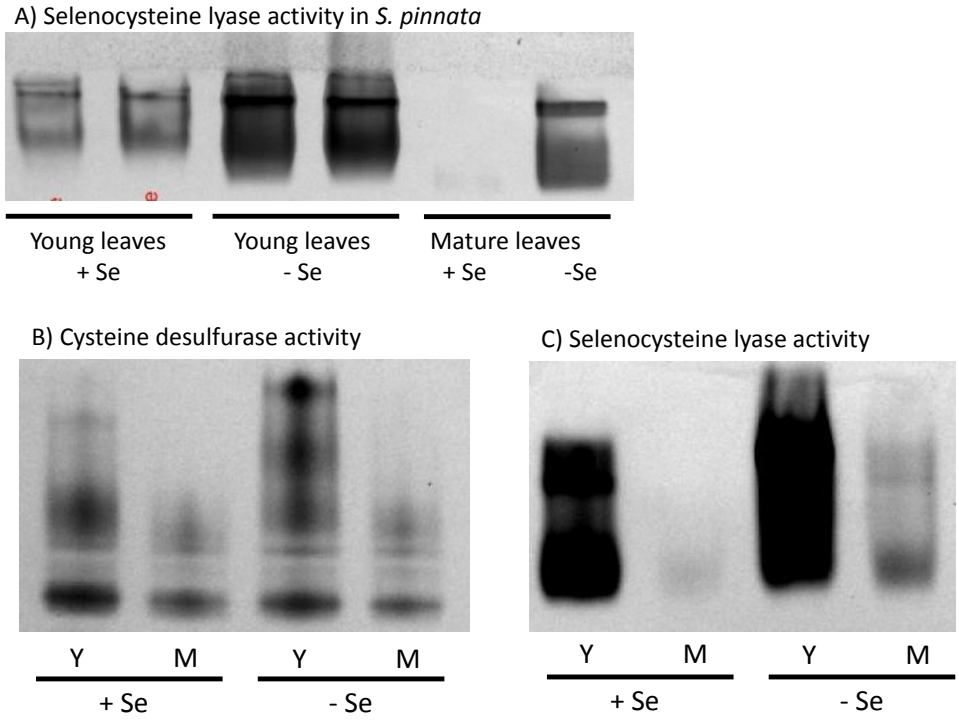

Figure 28 – A) In-gel assays showing selenocysteine lyase activity in young and mature leaves of *S. pinnata* growth with or without 40 µM selenate (two biological replicates); B) Cysteine desulfurase

activity in young or mature leaves of *S. pinnata* grown with or without 40 µM selenate; C) Selenocysteine lyase activity in young or mature leaves of *S. pinnata* grown with or without 40 µM selenate.

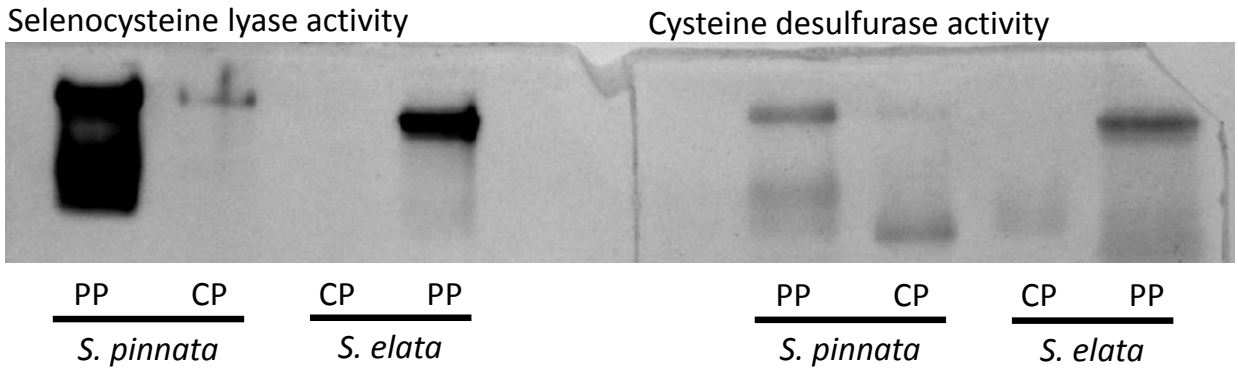

Figure 29 – In-gel assays showing selenocysteine lyase and cysteine desulfurase activities in purified protoplasts (PP) and chloroplasts (CP) of *S. pinnata* and *S. elata*.

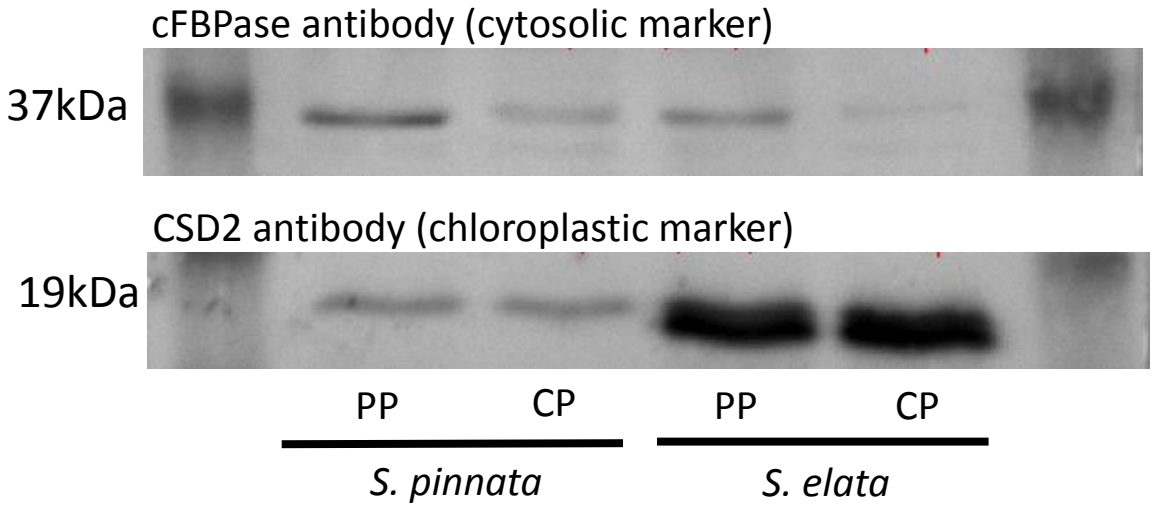

Figure 30 – Top: Western blot for cFBPase to show cytosolic presence in purified protoplasts (PP) and purified chloroplasts (CP). Bottom: CSD2 Western blot to show equal loading of chloroplasts in CP and PP fractions of the same species.

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