DISSERTATION

THE EVOLUTION OF SELENIUM HYPERACCUMULATION IN *STANLEYA* (BRASSICACEAE)

Submitted by

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ABSTRACT

THE EVOLUTION OF SELENIUM HYPERACCUMULATION IN *STANLEYA* (BRASSICACEAE)

Elemental hyperaccumulation is a fascinating trait found in at least 515 angiosperm species. Hyperaccumulation is the uptake of a metal/metalloid to concentrations 50-100x greater than surrounding vegetation. This equates to 0.01-1% dry weight (DW) depending on the element. Studies to date have identified 11 elements that are hyperaccumulated including arsenic, cadmium, cobalt, chromium, copper, lead, manganese, molybdenum, nickel, selenium (Se) and zinc. My research focuses on Se hyperaccumulation in the genus *Stanleya* (Brassicaceae). The threshold for Se hyperaccumulation is 1,000 mg Se kg^{-1} DW or 0.1% DW. *Stanleya* is a small genus comprised of seven species all endemic to the western United States. *Stanleya pinnata* is a Se hyperaccumulator and includes four varieties. I tested to what extent the species in *Stanleya* accumulate and tolerate Se both in the field and in a common-garden study. In the field collected samples only *S. pinnata* var. *pinnata* had Se levels >0.1% DW. Within *S. pinnata* var. *pinnata*, I found a geographic pattern related to Se hyperaccumulation where the highest accumulating populations are found on the eastern side of the Continental Divide. In the greenhouse *S. pinnata* var. *pinnata* accumulated the most Se within the genus, in both the young leaves and roots. I also discovered a polyploidy event within *S. pinnata*. All varieties of *S. pinnata* collected on the western slope of the Rocky Mountains were tetraploid and all but one population collected from the eastern slope of the Rocky Mountains were diploid. However, when tested, genome size did not correlate with Se hyperaccumulation capacity in *S. pinnata*. I isolated DNA from the field

collected leaves and conducted a phylogenetic analysis using four nuclear gene regions and fifteen morphological characters. Using the phylogeny, I conducted an ancestral-reconstruction analysis to predict the ancestral states for Se related traits in a parsimony framework. I infer from the results that tolerance preceded hyperaccumulation in the evolution of Se hyperaccumulation in *Stanleya* and that hyperaccumulation evolved in an ancestor of the *S. pinnata/bipinnata* clade. Lastly, I conducted a comparative transcriptomic analysis between *S. pinnata* var. *pinnata* and *S. elata*, a non-hyperaccumulator. I found higher transcript levels for many of the enzymes involved in sulfur (S) transport and assimilation in *S. pinnata* relative to *S. elata*. Surprisingly, I found high constitutive expression for many of the S assimilation enzymes in the roots of *S. pinnata*, particularly an isoform of ATP sulfurylase. I also found high constitutive expression for sulfate transporter 1;2 in the roots of *S. pinnata*. Based on these data I infer that *S. pinnata* assimilates Se in the root and that sulfate transporter 1;2 and ATP sulfurylase 2 may be key enzymes in Se hyperaccumulation in *S. pinnata*. Taken together these data, in conjunction with previous work, help provide a better understanding of the evolution of Se hyperaccumulation in *Stanleya* at the physiological, phylogenetic and transcriptional levels.

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Hyperaccumulation is the uptake of one or more metal/metalloids to concentrations greater than 50-100x those of the surrounding vegetation or 100-10,000 mg/kg dry weight depending on the element. Hyperaccumulation has been documented in at least 515 taxa of angiosperms. By mapping the occurrence of hyperaccumulators onto the angiosperm phylogeny we show hyperaccumulation has had multiple origins across the angiosperms. Even within a given order, family or genus there are typically multiple origins of hyperaccumulation, either for the same or different elements. We address which selective pressures may have led to the evolution of hyperaccumulation and whether there is evidence for co-evolution with ecological partners. Considerable evidence supports the elemental-defense hypothesis, which states that hyperaccumulated elements protect the plants from herbivores and pathogens. There is also evidence that hyperaccumulation can result in drought stress protection, allelopathic effects, or physiological benefits. In many instances ecological partners of hyperaccumulators have evolved resistance to the hyperaccumulated element, indicating co-evolution. Studies on the molecular evolution of hyperaccumulation have pinpointed gene duplication as a common cause of increased metal transporter abundance. Hypertolerance to the hyperaccumulated element often relies upon chelating agents, such as organic acids (e.g. malate, citrate) or peptide/protein chelators that can facilitate transport and sequestration. We conclude the review with a summary and suggested future directions for hyperaccumulator research.

Introduction

Over five hundred species of plants have been documented to hyperaccumulate one or more trace metal/metalloids, including arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co),

copper (Cu), lead (Pb), manganese (Mn), nickel (Ni), selenium (Se) and zinc (Zn). Most hyperaccumulators are flowering plants, but a few fern species have been documented to hyperaccumulate As (Reeves and Baker, 2000; Ma et al., 2001). In this review we focus on Angiosperm hyperaccumulators.

Plant hyperaccumulators are defined as having 50-100x higher concentrations of the element relative to that of the surrounding vegetation, or equivalent to 100-10,000 mg/kg dry weight (DW; Maestri et al., 2010; for a recent review on hyperaccumulation see also Van der Ent et al., 2013). Hyperaccumulators are predominantly found on soils that contain elevated levels of the hyperaccumulated element, which suggests that they have evolved in situ on these soils. Importantly, these soils are not just naturally occurring soils but also anthropogenically contaminated soil, which may indicate that hyperaccumulation can evolve rapidly. Alternatively, the hyperaccumulators may just have colonized the mine tailings from an original outcropping of surface-facing ore. Given the immobility of plants it is understandable that taxa inhabiting soils with high levels of toxic elements have evolved tolerance mechanisms. Indeed, hyperaccumulators are tolerant to the extreme soils they grow on. However, it is intriguing that hyperaccumulators appear to actively concentrate the toxic element(s). Hyperaccumulation is not necessary for survival on soils with elevated levels of the element, but rather requires extra evolutionary adaptations for hypertolerance of the high levels of the toxic metal/metalloid in their tissues. This review will address the following four evolutionary questions. First, how many times did hyperaccumulation evolve? Second, what selection pressures have driven the evolution of hyperaccumulation? Third, is there co-evolution with the ecological partners of hyperaccumulating plants? Fourth, what do we know about the molecular evolution of hyperaccumulation?

How many times did hyperaccumulation evolve?

In order to understand the evolutionary basis of hyperaccumulation we need to know how hyperaccumulation is distributed across the angiosperms. There have been reports of over 500 hyperaccumulators among the ~250,000 angiosperm species (Stevens, 2001 onwards), equating to ~ 0.2% of species (Suppl. Table 1.1). The majority of these 500 species are Ni hyperaccumulators. At the genus level, 181/13,000 genera = 1.4% hyperaccumulate, and 60/443 families = 13.5% of families contain hyperaccumulators. The following angiosperm families contain an unusually high percentage of the total hyperaccumulating taxa (Fig. 1.1): Brassicaceae, Euphorbiaceae and Asteraceae. Hyperaccumulation, even of a single element, appears to have evolved several times within vascular plants: it is distributed widely across the phylogeny. At multiple taxonomic levels (order, family, genus) we find several origins of hyperaccumulation. For instance, Krämer (2010) proposes that within the Brassicaceae there are at least six independent origins of Ni hyperaccumulation, three origins of Zn and Cd hyperaccumulation, two origins of As hyperaccumulation and one origin of Se hyperaccumulation. *Alyssum* (Brassicaceae) contains upwards of 50 nickel hyperaccumulating species without an obvious pattern of origin either at the phylogenetic or geographic level (Cecchi et al., 2010). Given the high number of hyperaccumulating taxa and the general lack of phylogenetic or geographic correlation of Ni hyperaccumulators, there may be a predisposition within the Alysseae to adapt to serpentine soils high in Ni.

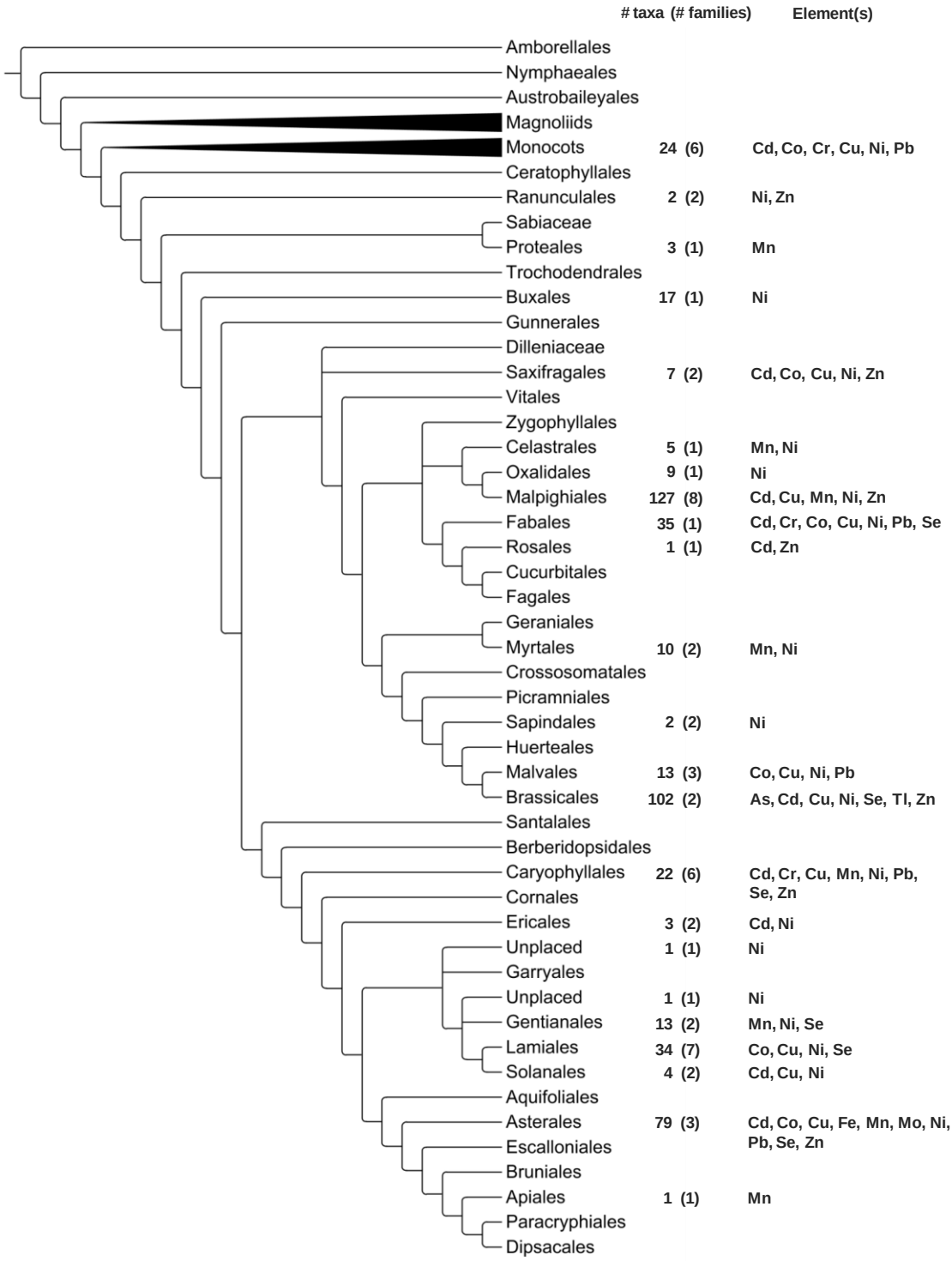

Figure 1.1. Occurrence of elemental hyperaccumulation in angiosperms. Phylogenetic tree modified from angiosperm phylogeny group (APG) III (Stevens 2001). For more detailed information about which taxa hyperaccumulate each element, see Supplemental Table 1.1.

Natural variation within species – Probably the two most studied hyperaccumulator species are *Arabidopsis halleri* and *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) (both in the Brassicaceae) which hyperaccumulate Zn and Cd, and Zn, Ni and Cd respectively. There is evidence that Zn hyperaccumulation is a constitutive trait in *A. halleri*, but that Cd hyperaccumulation is variable (Bert et al., 2002). A parallel situation exists in *N. caerulescens* (Lombi et al., 2000). Besnard et al. (2009) conducted a population genetic study on *N. caerulescens* using nuclear and plastid loci across multiple accessions from Switzerland. They found strong population structure (based on neutral markers) that was not associated with Cd or Zn levels in the soil. However, allelic richness of candidate loci, particularly metal transporters, was positively correlated with metal concentrations in the soil. In addition to variation in Cd uptake in *N. caerulescens* there is evidence for competition between the various metals (Cd, Ni and Zn), with Cd and Zn taken up in higher concentrations when competing with Ni—even from Ni hyperaccumulating populations (Assunção et al., 2008). In addition, *N. goesingense* populations were shown to accumulate high concentrations of Ni, Zn, Mn and Co regardless of whether the populations came from serpentine or non-serpentine soils (Reeves and Baker, 1984).

In contrast to hyperaccumulation being a constitutive trait, some species show strong ecotypic variation. Populations of *Stanleya pinnata* have been shown to vary in Se concentration depending on the population tested; these differences appear to be fixed based on common-garden experiments (Feist and Parker, 2001). Substantial population-level variation was also found for Ni accumulation in the Ni hyperaccumulator *Alyssum bertolonii* (Galardi et al., 2007). The Cd hyperaccumulator *Sedum alfredii* also has fixed ecotypic differences including differences in Cd sequestration (Tian et al., 2011) and translocation (Lu et al., 2008).

Phylogenetic conclusions – In conclusion, hyperaccumulation is broadly distributed over the plant phylogeny and must have evolved independently many times. Even within a family or genus there is rarely unequivocal evidence for one origin of hyperaccumulation when more than one species is a hyperaccumulator. If we take an even closer look at the species level, populations differ in their ability to accumulate and tolerate certain elements, and within a population individuals commonly differ in their tolerance and accumulation and these differences can be dependent on the element accumulated, e.g. Zn vs. Cd. Thus, hyperaccumulation is a trait constantly evolving and under selection.

Which selection pressures favored the evolution of hyperaccumulation?

Several hypotheses have been proposed for the functional significance of metal and metalloid hyperaccumulation by plants (Boyd and Martens, 1992). There is considerable evidence that accumulation of toxic elements can offer plants protection from herbivores and pathogens, which is in agreement with the elemental-defense hypothesis (Boyd and Martens, 1992). The protective effect against herbivores includes both deterrence and toxicity. High levels of Ni in plants have been shown to protect them from a variety of herbivores and pathogens (Boyd et al., 1994; Boyd and Moar, 1999; Martens and Boyd, 2002). Zinc and Cd can also protect plants from invertebrate herbivory (Pollard and Baker, 1997; Jhee et al., 1999). Similarly, Se can protect plants from a variety of herbivores and pathogens, including moth and butterfly larvae, aphids, thrips, spider mites, grasshoppers, prairie dogs, as well as fungal pathogens (Vickerman and Trumble, 1999; Hanson et al., 2004; Freeman et al., 2006b, 2007, 2009; Quinn et al., 2008, 2010). Arsenic has also been shown to protect plants from herbivores (Mathews et al., 2009). In further support of a defensive role, it appears that the hyperaccumulated element is often concentrated in organs and tissues that are most susceptible to herbivores and pathogens

(Freeman et al., 2006b; Tappero et al., 2008). The levels at which elements become protective against herbivores or pathogens depends on the element and chemical form the plant accumulates, as well as the sensitivity of the herbivore or pathogen in question. For example, Se in plants is effective aphids below 10 mg kg^{-1} DW (Hanson et al., 2004), whereas plants needed an order of magnitude more Se to be protected from Lepidoptera larvae or prairie dogs, and even higher levels to be protected from spider mites (Freeman et al., 2006b, 2009; Quinn et al., 2010). Different elements may also show synergistic interactions *in vivo* with each other and with organic defense compounds, reducing the level at which they become effective. This phenomenon has been termed the joint-effects hypothesis by Boyd (2012). These examples provide strong evidence that herbivores and pathogens can act as a selection pressure for the evolution of increasing elemental accumulation in plants. This selection pressure can be effective at tissue elemental concentrations well below hyperaccumulator level, and is expected to be incremental: the higher the elemental tissue concentration, the more effective and the broader the protection from a range of pests.

There is also some evidence that hyperaccumulators benefit from their accumulated element via allelopathy, i.e. negative effects on neighboring plants, in support of another hypothesis put forward by Boyd and Martens (1992). Soil around Se hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* was found to be significantly enriched with Se, and this soil significantly reduced germination and growth of the Se-sensitive plant species *Arabidopsis thaliana*, and enhanced its Se levels compared to soil collected from non-hyperaccumulators (El Mehdawi et al., 2011a). No clear allelopathic effects were found for the Ni hyperaccumulator *Alyssum murale* (Zhang et al., 2007). While Ni-amended soil reduced seed germination of eight herbaceous species, leaf litter from *A. murale* did not, perhaps due to low Ni

phytoavailability in leaf litter. More studies are needed, but it appears that for some hyperaccumulators allelopathic benefits may have contributed to the evolution of hyperaccumulation.

A third hypothesis for why plants hyperaccumulate is protection from drought resistance (Boyd and Martens, 1992). Whiting et al. (2003) found no evidence that Ni or Zn can protect plants from polyethylene glycol (PEG) induced osmotic stress. On the other hand, Bhatia et al. (2005) reported that Ni may play a role in osmotic adjustment in the hyperaccumulator *Stackhousia tryonii*, thereby protecting it from drought stress. While not a hyperaccumulator, it is interesting to note that Yao et al. (2012) found that Se treatment improved recovery of wheat seedlings from drought stress. Since the Se levels are low in this species, the mechanism may involve induction of some drought tolerance mechanism, rather than direct effect of the tissue Se on osmotic potential. In conclusion, there is evidence that hyperaccumulation may offer protection from drought stress in some species, but there is not as much evidence for protection from drought as protection from biotic stresses.

In some cases, enhanced accumulation of elements may have a positive physiological effect. This may be true for both essential and non-essential elements. For instance, the hyperaccumulator *Noccaea caerulescens* had a 75% increase in biomass when supplied with Cd after 31 days (Roosens et al., 2003). The element Se is not known to be essential for plants, but is beneficial for the growth of many plant species, especially Se hyperaccumulators (Virupaksha and Shrift, 1965; El Mehdawi et al., 2012).

It is also possible that hyperaccumulation resulted from inadvertent uptake if the accumulated elements are chemically similar to essential nutrients and plant populations evolve more efficient uptake and translocation systems for these nutrients. For instance, Boyd and

Martens (1998) suggested that Ni hyperaccumulation in *N. montanum* may be an inadvertent consequence of an efficient nutrient uptake system for another nutrient, perhaps for Zn or Ca. Similarly, it is feasible that hyperaccumulation of As, Cd and Se evolved as inadvertent side effects of enhanced phosphate, zinc/calcium and sulfate uptake, respectively.

Thus, several biotic and abiotic environmental factors as well as physiological processes may contribute to the evolution of elemental hyperaccumulation in a lineage. These various selection pressures may act simultaneously or individually, depending on the habitat, element and taxon.

Is there co-evolution of hyperaccumulators with ecological partners?

Plants have many ecological partners, including microbes, pollinators, herbivores, and other plants. Elemental hyperaccumulation has been shown to negatively affect these partners if they are sensitive to the element in question, as described above. Thus, the hyperaccumulator may exert selection pressure on its ecological partners, leading to their partners having increasing resistance to the toxic element over time. Such resistant ecological partners can then exclusively occupy the new niches offered by hyperaccumulator plants. There is indeed evidence that ecological partners of hyperaccumulators have evolved resistance to the hyperaccumulated element. For example, Boyd (2009) reported insects that feed almost exclusively on Ni hyperaccumulators. He identified 15 insect species across 3(sub)orders that can tolerate an internal Ni concentration of ≥ 500 mg/kg^{-1}. A few of these species appear to be specialists, since they chose to feed on a particular Ni hyperaccumulator even when given the choice of a different Ni hyperaccumulator or a non-hyperaccumulator. Similarly, several Se-resistant herbivorous moths, seed beetles and seed wasps were found to feed on Se hyperaccumulators *A. bisulcatus* and *S. pinnata* in their natural seleniferous habitat (Freeman et al., 2006b, 2012; Valdez-Barillas

et al., 2012). These situations appear to be classical examples of co-evolution, where plant and herbivore are in an "arms race", leading to ever increasing accumulation in the plant and resistance in the herbivore. There is also evidence of Se-tolerant microbial and micro-arthopod detrivores in seleniferous ecosystems that efficiently decompose hyperaccumulator litter (Quinn et al., 2011), as well as evidence of Se-tolerant endophytes and leaf pathogens that successfully occupy the niche provided by Se hyperaccumulators (Valdez-Barillas et al., 2012). Also, some Se-tolerant plant species were shown to benefit from close proximity to Se hyperaccumulators: they contained up to 10-fold higher Se levels and enjoyed less herbivory and enhanced growth (El Mehdawi et al., 2011b). As a result of their negative effects on sensitive ecological partners and positive effects on resistant ecological partners, hyperaccumulators may affect species composition in the community at multiple trophic levels. Hyperaccumulators may also affect cycling of the hyperaccumulated element through their local ecosystem, by affecting its soil distribution, its chemical form, and by forming a portal for its entry into higher trophic levels (El Mehdawi and Pilon-Smits, 2012).

How did hyperaccumulation evolve, at the molecular level?

Hyperaccumulators have evolved both enhanced capacity to sequester toxic elements, and enhanced tolerance to these elements. The underlying mechanisms may be hypothesized to include mutations in transporter protein genes that enhance expression levels or change the kinetic properties of the transporter. Hyperaccumulators may be expected to have higher expression levels of various membrane transporters responsible for uptake into the root symplast, loading into the root xylem, uptake into the leaf symplast and sequestration into the vacuole and in specific tissues such as the epidermis. To facilitate transport of the toxic elements in xylem and phloem, hyperaccumulators may also be expected to have enhanced levels of chelating

agents in either of these vascular tissues or in adjacent tissues. Such chelating agents may include organic acids (e.g. malate, citrate) or peptide/protein chelators (e.g. nicotianamine, glutathione, phytochelatins, histidine). Some of these same chelators may help the plants tolerate their extraordinarily high levels of these toxic elements. Hyperaccumulators may have enhanced levels of (other) antioxidant compounds and enzymes as additional tolerance mechanisms. In the case of Se, hyperaccumulators appear to have evolved mechanisms by which this element is converted to less toxic, organic forms (Neuhierl and Böck, 1996).

From studies to date on different hyperaccumulator species, there appear to be several different cases by which hyperaccumulators have modified transporter abundance or activity. Some hyperaccumulators have increased the gene copy number of transporters via gene duplication events; examples include the HMA (heavy metal binding) transporter in *A. halleri* (Hanikenne et al., 2008) and *N. caerulescens* (Ueno et al., 2003; Craciun et al., 2012). In *A. halleri*, Hanikenne et al. (2008) sequenced bacterial artificial chromosomes and found three tandem copies of metal ATPase 4 (*HMA4*) relative to *A. thaliana*. In *N. caerulescens*, *HMA4* was again shown to have a variable copy number in populations that differ in Cd accumulation and tolerance. The population with the least efficient Cd translocation had the lowest expression level and the lowest copy number (Craciun et al., 2012). There can be up to four tandem copies of *HMA4* in *N. caerulescens* (Ó Lochlainn et al., 2011). In addition to *HMA4*, Heavy metal ATPase 3 (*HMA3*) was implicated as conferring tolerance by being a tonoplast located-transporter sequestering Cd into the leaf vacuoles. When higher and lower Cd accumulating ecotypes of *N. caerulescens* populations were compared, the authors found significantly higher *HMA3* expression in the more tolerant ecotype Ganges, which also had a greater number of gene copies (Ueno et al., 2003).

In hyperaccumulators of non-essential elements it appears that the hyperaccumulated element enters the plant via transporters for other, essential elements that are chemically similar to it. For instance, the non-essential elements Se, As and Cd are chemically similar to S, P, Zn, respectively, and enter the plant through the transporters for these elements. Sulfate transporter (Sultr)1;2 is apparently responsible for selenate import into the root in *Arabidopsis thaliana*, since lesions in *sultr1;2* confer selenate resistance and increased tolerance in *A. thaliana* by reducing symplastic accumulation of Se (Shibagaki et al., 2002; Ohno et al., 2012). Several sulfate transporters were found to be more highly expressed in hyperaccumulator *Astragalus* spp. at levels comparable to those in non-hyperaccumulator plants present under S starvation (Cabannes et al., 2011). Similarly, several genes encoding sulfate transporters are constitutively upregulated in the Se hyperaccumulator *S. pinnata* relative to the non-hyperaccumulator *S. albescens* (Freeman et al., 2010). Thus, constitutive expression of sulfate transporters may be one of the mechanisms of Se hyperaccumulation. In addition, there is evidence of at least one Se-specific transporter in the hyperaccumulator *S. pinnata*: in contrast to non-hyperaccumulator species, selenate uptake in *S. pinnata* is not significantly inhibited by high sulfate levels (Harris and Pilon-Smits, unpublished results). Similarly, it has been shown that arsenate most likely is taken up via a phosphate transporter; for a review of arsenic uptake and metabolism in plants see Zhao et al (2009).

The ZIP family (ZRT, IRT-like proteins) transporters are known to be involved in the movement of Fe, Zn, Mn and Cd, with 15 paralogs identified in *A. thaliana*. In Zn/Cd hyperaccumulators *A. halleri* and *N. caerulescens*, ZIP transporters have been shown to have a constitutively higher expression level relative to *A. thaliana* and *Thlaspi arvense* respectively (Assunção et al., 2001; Bechner et al., 2004; Weber et al., 2004). Furthermore, expression of two

copies of the ZIP transporter gene *IRT3*, *AtIRT3* and *AhIRT3* were able to rescue a Zn/Fe uptake-deficient *Saccharomyces cerevisiae* mutant (Lin et al., 2009). This work suggests that root transporters in the ZIP gene family are important factors in Zn hyperaccumulation. Beyond transporters, many metals/metalloids are complexed with chelators to allow for specific uptake, movement and sequestration within the different compartments of the plant body.

Many peptide/protein chelators have been implicated in providing a mechanism for (hyper)tolerance, including glutathione, histidine, nicotianamine and phytochelatins. Glutathione (GSH) may contribute to tolerance not only by binding toxic elements but also by scavenging free radicals. Levels of GSH were found to be constitutively enhanced in Ni hyperaccumulator *Noccaea goesingense* (Freeman et al., 2004), as well as in Se hyperaccumulator *Stanleya pinnata* (Freeman et al., 2010). In *Phytolacca americana*, a Cd hyperaccumulator, the tissue concentration of GSH was shown to increase when supplied with Cd (Zhao et al., 2011). Histidine has been shown to be an important factor in Ni hyperaccumulators *Alyssum lesbiacum* (Krämer et al., 1996) and *N. goesingense* (Krämer et al., 2000). Histidine has also been shown to be important in root-to-shoot translocation in *N. caerulescens* by preventing the accumulation of histidine-bound Ni in the root vacuoles (Richau et al., 2009). Nicotianamine is present at elevated levels in the hyperaccumulator species, *A. halleri* and *N. caerulescens* relative to closely related non-hyperaccumulator species (Deinlein et al., 2012). Furthermore, in *A. halleri* an RNAi knockout of nicotianamine synthase resulted in decreased translocation of Zn from the root to and shoot, and the knockout plants did not reach hyperaccumulator levels of Zn in the shoots (Deinlein et al., 2012). Lastly, phytochelatin levels have been to shown to significantly increase in *P. americana* with increasing Cd supply, potentially to help sequester Cd ions in the leaf vacuoles (Gao et al., 2013). However, phytochelatin concentrations are typically lower in

hyperaccumulator species than related non-hyperaccumulator species (Meyer et al., 2011) and therefore may not actually be an evolutionary mechanism in hyperaccumulator species. In addition to peptide/protein chelators, organic acids such as citrate and malate have also been implicated in tolerance and transport in hyperaccumulator species. It has been shown that citrate and malate bind to Zn and Ni to allow for loading and unloading into and out of xylem, phloem and across the tonoplast. In *S. alfredii*, Lu et al. (2013) showed increased levels of citrate in the xylem sap of plants from a hyperaccumulating population relative to a non-hyperaccumulating population and that the levels of citrate increased with increasing Zn concentration. Nickel hyperaccumulator *Alyssum murale* has increased citric acid and malic acid synthesis in the root mitochondria relative to non-hyperaccumulator *A. montanum* (Agrawal et al., 2013). In *N. goesingense* Ni has been shown to be bound to citrate (Krämer et al., 2000). In *N. caerulescens* Zn has been found to be stored chelated by malate and citrate in the vacuoles of epidermal cells. Elevated levels of these organic acids may help in Zn sequestration and tolerance of Zn in *N. caerulescens* (Schneider et al., 2012).

In addition to chelation, hyperaccumulators have in some cases evolved mechanisms by which elements are metabolized to less toxic forms. This is the case for Se. All plants can assimilate selenate to selenocysteine (SeCys) via the sulfate assimilation pathway. This SeCys may be non-specifically incorporated into protein, which is toxic. SeCys may also be further metabolized via selenocystathionine (SeCyst) to selenomethionine (SeMet) and other analogs of reduced S compounds. Plants can also form volatile dimethylselenide (DMSe) from SeMet (Terry et al., 2000). A transcriptome study showed that in the hyperaccumulator *Stanleya pinnata* many genes from the S assimilation pathway were upregulated, as compared to non-hyperaccumulator *S. albescens*, perhaps due to higher levels of the plant-growth regulators

jasmonate, salicylate and ethylene (Freeman et al., 2010). This may explain the hyperaccumulator's higher levels of Se and S. The forms of Se in hyperaccumulators and non-hyperaccumulators are also different: while many non-hyperaccumulators accumulate predominantly selenate when treated with selenate, hyperaccumulators tend to accumulate organic Se. In *S. pinnata*, for instance, a large fraction of the Se is present as SeCyst (Shrift and Virupaksha, 1965; Freeman et al., 2006b). These findings suggest that certain enzymes within the S assimilation pathway may be differentially expressed in hyperaccumulators and non-hyperaccumulators. In addition, Se hyperaccumulators often contain methyl-SeCys, which is produced by a Se-specific SeCys methyltransferase (Neuhierl and Böck, 1996; Sors et al., 2005; Freeman et al., 2006b). The accumulation of these non-protein amino acids SeCyst and methyl-SeCys prevent incorporation into protein, and thereby prevent toxicity to the plant (Brown and Shrift, 1981; Neuhierl and Böck, 1996). Hyperaccumulators may also volatilize Se at very high rates in the form of dimethyldiselenide (DMDSe), which is synthesized from methyl-SeCys (Freeman and Bañuelos, 2011). Therefore, Se hyperaccumulators in the genera *Stanleya* and *Astragalus* appear to have evolved a unique pathway that plays an important role in their Se tolerance.

Summary and Future directions

Many different hyperaccumulators have been studied, and from these studies several mechanisms responsible for hyperaccumulation have been identified. Often hyperaccumulators have enhanced levels of transporters (as a result of gene duplication) for uptake into the root and translocation within the plant. Hypertolerance mechanisms that have been identified include enhanced levels of chelators or of enzymes that convert the element to less toxic forms. Some interesting questions that may still be addressed include: is there a "master switch" gene (e.g.

some transcription factor) that upregulates all genes involved in hyperaccumulation and hypertolerance? Did hypertolerance evolve simultaneously with hyperaccumulation or did it evolve sequentially with hyperaccumulation? If the latter case is true then which came first? Does one commonly precede the other, and is one trait more difficult to evolve?

Studies on elemental hyperaccumulation so far generally have used relatively few approaches: molecular, physiological, genetic or ecological. The next challenge will be to integrate these diverse approaches within a selected taxon. Such a comprehensive study may start with a complete documentation of the variation in tolerance and accumulation in a group of species/populations, in concert with phylogenetics/population genetics of the taxa of interest. This may be followed up by a comparative study of selected taxa to obtain insight into underlying biochemical and genetic mechanisms, hopefully leading to identification of candidate alleles that contribute to hyperaccumulation. In parallel, the ecological effects of hyperaccumulation may be studied within natural ecosystems to obtain insight into selection pressures. Finally, candidate alleles may be studied in natural systems for evidence of selection, and at the molecular level for evidence of positive selection.

The results from such studies have intrinsic scientific value. Adaptation to selective pressures on populations from biotic or abiotic factors is a key process to study in evolutionary biology because it helps explain how interspecific differences arose. Rigorously studying local adaptation requires researchers to address questions at multiple organization levels of biology because only by synthesizing information from disciplines such as molecular biology, cell biology, physiology, ecology and phylogenetics can we thoroughly document local adaptation by linking cellular responses to the relationships of species over time, and correlate those with the ecological landscape. Hyperaccumulation offers a very promising model trait in this respect.

Beyond basic scientific value, results from such studies also may have broad applications in biofortification and phytoremediation. Identification of hyperaccumulation mechanisms and specific alleles allows for development of plants with enhanced tolerance to, and accumulation of, both nutrients and environmental pollutants.

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Chapter 2: Characterization of selenium and sulfur accumulation across the genus *Stanleya* (Brassicaceae). A field survey and common-garden experiment

Summary

Stanleya (Brassicaceae) is small genus comprised of seven species endemic to the western U.S. *Stanleya pinnata* is a hyperaccumulator of selenium (Se). We investigated to what extent the other taxa within *Stanleya* accumulate Se both in the field and a common-greenhouse setting on seleniferous soil. To our knowledge, this is the first study to examine a genus for Se hyperaccumulator properties both in the field and controlled conditions. We collected multiple populations of six of the seven species and all four varieties of *S. pinnata*. We tested leaves, fruit and soil for in situ Se and sulfur (S) concentrations. The seeds collected in the field were used for a common garden study in a greenhouse. We found that *S. pinnata* var. *pinnata* is the only hyperaccumulator of Se. Within *S. pinnata* var. *pinnata,* we found a geographic pattern related to Se hyperaccumulation where the highest accumulating populations are found on the eastern side of the continental divide. We also found differences in genome size within the *S. pinnata* species complex. The *S. pinnata* species complex has a range of physiological properties making it an attractive system to study the evolution of Se hyperaccumulation. Beyond the basic scientific value of understanding the evolution of this fascinating trait we can potentially use *S. pinnata* or its genes for environmental cleanup and/or nutrient-enhanced dietary material.

Introduction

Hyperaccumulation is the intriguing capacity exhibited by 0.2% of plant species to accumulate trace elements from the soil to concentrations greater than 100 – 1000 times those in the surrounding vegetation (Rascio and Navari-Izzo, 2011). The minimum concentration for a plant to be considered a hyperaccumulator varies depending on the element in question and is

0.01% of dry weight (DW) for cadmium (Cd), 0.1% for arsenic (As), copper (Cu), cobalt (Co), lead (Pb), nickel (Ni) and selenium (Se), and 1% for manganese (Mn) and zinc (Zn; Baker et al., 2000; Krämer, 2010). Hyperaccumulators have been found in at least 515 taxa from 60 different plant families (Cappa and Pilon-Smits, 2014 and references therein), suggesting hyperaccumulation evolved independently in different taxa. To date, there are relatively few studies that have examined the evolution of hyperaccumulation within a clade. This study addresses the evolution of Se hyperaccumulation within the genus *Stanleya* (Brassicaceae). Beyond the basic scientific value of understanding the evolution of this fascinating trait we can potentially use hyperaccumulators or their genes for phytoremediation (environmental cleanup using plants) and/or biofortification (nutrient-enhanced dietary material).

While different physiological mechanisms (transporters and/or chelators) are likely the basis for hyperaccumulation in different plant species, two traits are commonly associated with hyperaccumulation. Hyperaccumulating plants are typically hypertolerant to the high tissue levels of the accumulated element and tend to translocate a relatively high fraction of the element to the shoot. For example, the Zn hyperaccumulator *Noccaea caerulescens* (formerly known as *Thlaspi caerulescens)* exhibited 10 times greater root-to-shoot Zn translocation when compared to the non-hyperaccumulator *Thlaspi arvense* (Lasat et al., 1996). Similarly, a Cd hyperaccumulator ecotype of *Sedum alfredii* showed 10-fold greater translocation compared to a non-hyperaccumulator ecotype (Lu et al., 2008), and an As hyperaccumulator *Pteris vittata* showed higher As translocation to the shoot system when tested against *Nephrolepis exaltata*, a non-hyperaccumulator (Tu and Ma, 2005). Interestingly, hypertolerance and hyperaccumulation were shown to be controlled by different organs in the hyperaccumulator *N. caerulescens*: a

grafting experiment indicated that hyperaccumulation is controlled by the roots, whereas hypertolerance is controlled in the shoots (Guimarães et al., 2009).

A large proportion of hyperaccumulators is found in the Brassicaceae, which contain at least 80 Ni hyperaccumulators as well as hyperaccumulators of As, Cd, Se and Zn. Due to multiple independent origins of hyperaccumulation, these members constitute a polyphyletic group within the Brassicaceae (Krämer, 2010). The large amount of genetic information available for this family makes members of the Brassicaceae attractive models to study the complex evolution of elemental hyperaccumulation and tolerance. The Brassicaceae have high sulfur (S) levels and unique S compounds, and since reduced S compounds like glutathione and phytochelatins play a role in metal complexation and detoxification, this may contribute to their capacity to accumulate and tolerate toxic elements (Cobbett and Goldsbrough, 2002).

Considering the propensity of Brassicaceae to accumulate high S levels, and that Se is atomically similar to S, it is not surprising that the Brassicaceae also contain a genus that can hyperaccumulate Se: *Stanleya*. Previous studies have demonstrated substantial variation in Se accumulation within *Stanleya* from undetectable to >2000 mg kg^{-1} DW (Beath et al., 1939a, 1939b, 1940; Feist and Parker, 2001; Freeman and Bañuelos, 2011), making this group a potential model system to study the evolution of Se hyperaccumulation and hypertolerance. Selenium hyperaccumulation is defined as having minimal tissue concentrations 0.1%, or 1,000 mg Se kg^{-1} DW (Terry et al., 2000). Selenium hyperaccumulators, such as *Stanleya pinnata* (prince's plume), accumulate Se up to 0.5% of their dry weight while growing in their natural habitat on seleniferous soil. This is several orders of magnitude higher than the surrounding vegetation (Galeas et al., 2007).

Because of the similarity of Se and S, most plants non-specifically take up selenate from the environment by means of sulfate transporters and assimilate selenate into organic forms of Se via S metabolic pathways (Terry et al., 2000). A key to the extreme Se tolerance of Se hyperaccumulators is their capacity to incorporate Se into the non-protein organic acids, such as methyl-selenocysteine (Me-SeCys) and seleno-cystathionine, thereby preventing Se incorporation into proteins and its associated toxicity (Terry et al., 2000). Selenium hyperaccumulators also differ from most plants in that they tend to have higher shoot-to-root Se ratios and unusually high Se/S ratios in their shoot; therefore, they appear to preferentially take up Se over S (White et al., 2007). *Stanleya pinnata* has been recognized as a Se hyperaccumulator for over 70 years and exhibits all of the above physiological properties (Beath et al., 1939a; Feist and Parker, 2001; Freeman et al., 2006).

Stanleya is comprised of seven species, and one of these, *S. pinnata*, is divided into three varieties (Holgrem, 2005; Al-Shehbaz, 2010). Holgrem (2005) recognized var. *integrifolia*, var. *inyoensis* and var. *pinnata* in the *Intermountain Flora*, while Al-Shehbaz (2010) recognized var. *integrifolia*, var. *pinnata* and var. *texana* in *Flora North America*. Hence those two workers disagree as to whether or not var. *inyoensis* should be recognized as a distinct taxon. All seven *Stanleya* species are endemic to the western U.S.A. (Fig.2.1a,c). *Stanleya pinnata* occurs in most western states and has the largest range within the genus. Within *Stanleya pinnata*, var. *pinnata* has a large range, and the other varieties are sympatric with it, but not with each other. *Stanleya pinnata* var. *texana* is the only variety of *S. pinnata* that is allopatric relative to the rest of the genus; it occurs only in Brewster County in SW Texas, 500 km from the nearest population of other *Stanleya* taxa (Turner, 2004). The other six *Stanleya* species have narrower ranges; four are found in fewer than ten counties: *S. albescens, S. confertiflora, S. elata* and *S. tomentosa*.

To date, there has not been a comprehensive study as to which species in *Stanleya* hyperaccumulate Se and whether the varieties within *S. pinnata* share the same Se hyperaccumulation properties. This genus-level approach allows for the development of questions about the evolution of Se hyperaccumulation in a phylogenetic context and for future comparative studies concerning the physiology of Se hyperaccumulation in *Stanleya*. The questions we addressed in this study are: 1) To what degree do the different taxa in *Stanleya* accumulate Se, both in their natural habitat and in a common garden experiment? 2) Do the different *Stanleya* taxa differ in S uptake? 3) Which taxa exhibit characters commonly associated with Se hyperaccumulation, i.e. high Se/S ratios and high Se shoot/root ratios?

Material and Methods

Stanleya was sampled at the population level, with at least three populations, when possible, sampled for each taxon and a minimum of three individuals sampled from each population. For this study, all four varieties of *S. pinnata* were sampled and treated as distinct taxa. Fewer individuals were sampled for *S. albescens* (populations 2 and 3 were pooled at the time of collection due to the low amount of seed and leaf material available), *S. bipinnata* (only two populations were located) and *S. pinnata* var. *texana* (only one population was sampled due to an extensive drought in SW Texas). Collection sites were chosen based on information from herbaria (Harold M. Tucker Herbarium, Oregon State University Herbarium, Rocky Mountain Herbarium, and Sul Ross State University Herbarium). Leaf, seed, soil and herbarium voucher specimens were collected from each site, except *S. pinnata* var. *texana* where only soil and leaf samples were obtained because no plants were fruiting at the time of collection. Soil collections were 1 cm below the surface next to the stem after above-ground litter was removed. Leaves were preserved using silica crystals, and whole fruits were removed; both were stored at room

temperature. Voucher specimens were deposited in the Colorado State University Herbarium (Appendix 1). A total of 149 individuals were collected, representing all four varieties of *S. pinnata*, six of the seven species of *Stanleya*, and *Thelypodium laciniatum*, to use as an outgroup (the inferred sister genus of *Stanleya*, Ihsan Al-Shehbaz, pers. comm.). *Stanleya confertiflora*, a rare endemic (http://orbic.pdx.edu/rte-species.html) in Oregon and Idaho, was not sampled.

Ploidy level was determined for 79 individuals from 32 populations via flow cytometry analysis at Plant Cytometry Services (Schijndel, The Netherlands). Fresh material was used when available; otherwise silica dried leaves collected in the field were used.

Of the 149 plants sampled, in situ elemental concentrations were determined for 128 individuals' leaves and fruit as well as 129 plant-associated soil samples using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) as described below.

Brassica juncea (Indian mustard, accession no. 173874), was originally obtained from the North Central Regional Plant Introduction Station, Ames, IA.

For each taxon, seeds from the individual with the highest seed Se concentration in the field were used for a common-garden experiment on seleniferous soil in a greenhouse. This selection was made because *Stanleya* taxa are known to show substantial within-taxon variation in Se accumulation (Beath et al., 1940; Feist and Parker, 2001), and pilot experiments where we sampled from multiple populations resulted in very uneven variances between taxa (data not shown). The germination rates varied and, therefore, between 5 and 20 individuals were sampled from each taxon. Taxa with 20 individuals sampled include *Brassica juncea* (outgroup species), *S. elata* and *S. pinnata* var. *inyoensis.* Seventeen individuals were sampled for *S. viridiflora*, 16 for *S. pinnata* var. *pinnata*, 13 for *S. pinnata* var. *integrifolia*, 6 for *S. albescens* and 5 for both *S.*

tomentosa and *T. laciniatum*. No seeds of *S. bipinnata* germinated; therefore, this taxon was not included in the greenhouse study.

The seeds were incubated at 4°C for 48 hours in water to promote germination before being directly sown on the soil. The soil was collected from the Pine Ridge Natural Area on the west side of Fort Collins, CO. This soil is naturally seleniferous (for soil properties see El Mehdawi et al. [2012]) and harbors two large populations of *S. pinnata* var. *pinnata*. To optimize drainage, the seleniferous soil was mixed with washed Turface® (Pennington, Madison, GA) in a ¾ soil ¼ Turface® mixture, and this brought the final pH to 6.5 (original pH was 7.5). The plants were grown (one plant per pot) for three months in the greenhouse. The plants were watered with ½ strength Hoagland's solution (Hoagland and Arnon, 1938) for the first two weeks and with plain tap water for the following 2 ½ months. At the time of harvest, samples were taken from directly around the apical meristem (young leaves) as well as 3-5 nodes below the meristem, i.e. the first fully expanded leaf (mature leaves). The mature leaves of *B. juncea* were senesced at the time of harvest, allowing us to only collect young leaves for this taxon. The roots were washed and excess Turface® removed before analysis.

Elemental analysis – Field samples tested for Se and S concentrations included mature silica-dried leaves, untreated whole fruits and sieved soil. The greenhouse leaves and roots were dried at 50°C for 48 hours. All samples were digested using nitric acid following Zarcinas et al. (1987). The digested tissues were analyzed via inductively coupled plasma atomic emission spectroscopy (ICP-AES) as described by Fassel (1978).

Statistical analysis – All field and greenhouse data had unequal variances that were not normally distributed. Log and square-root transformations did not solve either of these statistical issues, and therefore all data were analyzed via a non-parametric Kruskal-Wallis multiple-

comparisons test in R (version 2.15.1) in the package pgirmess (version 1.3.8; Giraudoux, 2006) with a Bonferroni adjustment. Field correlations were tested using Spearman's rank correlation. Graphs were produced in the package ggplot (version 0.9.0; Wickham, 2009). A Student t-test in Excel was used to test for differences between Se and S ratios within the same species. For some tissue samples collected in the field, the Se levels were below the reliable detection limit of 5 mg kg^{-1} DW (50 ppb in acid digest) and were changed to half the detection limit for statistical analysis.

Results

A total of 149 individual plants were collected from 41 populations (Fig. 2.1a,c). These individuals represent nine of the ten taxa of *Stanleya* and a closely related species, *T. laciniatum*. The exact population coordinates are given in Suppl. Table 2.1. As a first characterization of *Stanleya*, we tested for polyploidy. We found all species except *S. pinnata* to be diploid. Within *S. pinnata*, we found *S. pinnata* vars. *integrifolia* and *inyoensis* to be tetraploid, *S. pinnata* var. *texana* to be diploid, and *S. pinnata* var. *pinnata* composed of both diploids and tetraploids (Fig. 2.1b). Interestingly, within *S. pinnata*, we found the tetraploids on the western slope of the Rocky Mountains and the diploids on the eastern slope, separated by the continental divide, with the exception of one tetraploid population on the eastern slope, near the Great Divide Basin (Fig. 2.1b). The soil Se levels east of the continental divide, the area where the diploids occur, are overall higher relative to the soils west of the divide as judged by USGS Se soil data (Fig. 2.1d).

Selenium accumulation in the field – Our field-collected samples were significantly different among species for Se concentrations in both organs tested (leaves, Kruskal-Wallis chi-squared = 45.84, df = 9, p-value = <0.001; fruits, Kruskal-Wallis chi-squared = 89.02, df = 8, p-value = <0.001; Table 2.1). Only *S. pinnata* var. *pinnata* reached hyperaccumulator levels of Se,

while all other individuals tested were below the threshold of 1,000 mg kg^{-1} DW to be considered a Se hyperaccumulator. S*tanleya pinnata* var. *pinnata* had on average 12-fold higher Se in the leaves and 8-fold higher Se concentration in the fruits than the second highest Se accumulating taxon, *S. tomentosa* (Table 2.1). Within *S. pinnata* var. *pinnata* there was substantial variation in total Se levels in the tissues sampled, both between populations

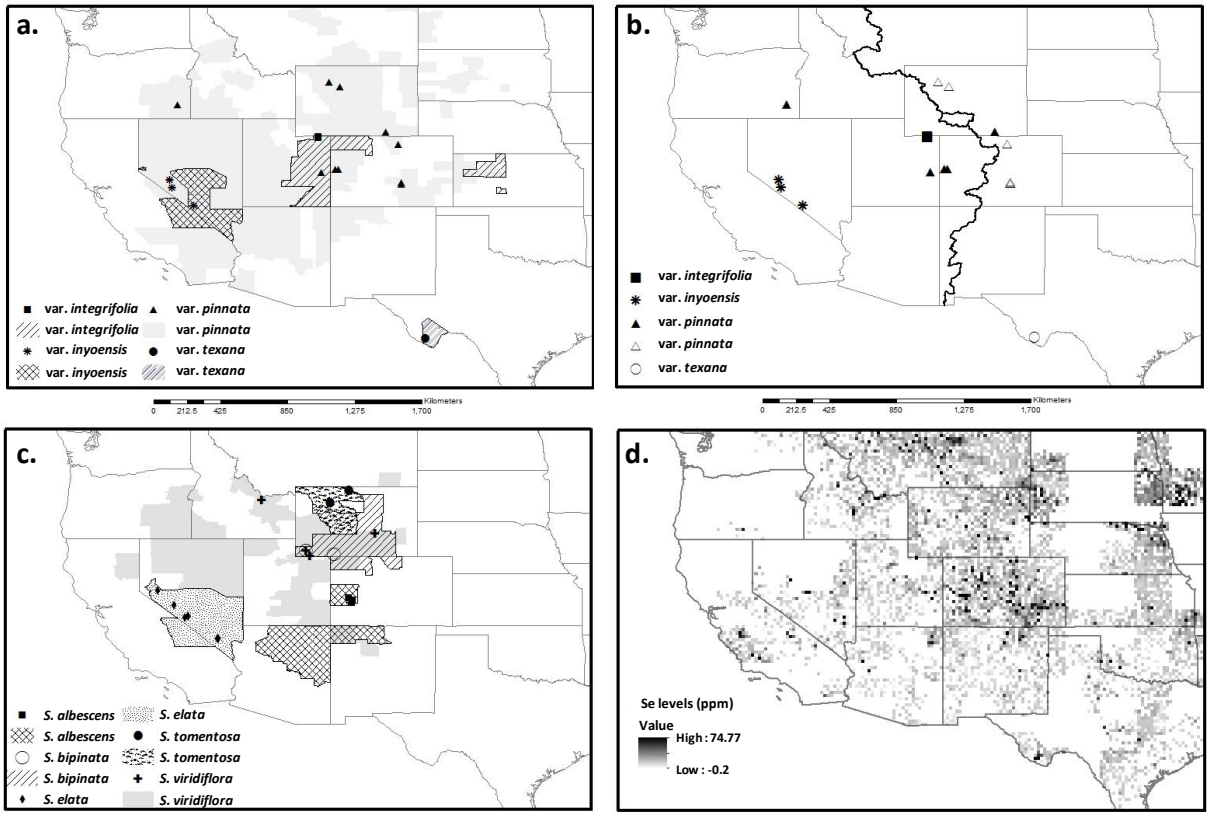

Figure 2.1. County-level taxa distributions (shaded regions) for all taxa of *Stanleya* (Brassicaceae) sampled. Taxon distributions were determined from the USDA Plants Database (http://plants.usda.gov/java/). Each point represents the collection site of a population for a given taxon determined from herbarium databases. Note: in some cases populations were sampled in such close proximity that sampling points on the map overlap. a. Distributions and collections for all four *S. pinnata* varieties. b. Ploidy levels within the *S. pinnata* species complex. Closed symbols represent tetraploids and open symbols represent diploids. The continental divide is shown. c. Distributions and collections for the remaining five species of *Stanleya* sampled. d. Soil Se concentrations as determined by the USGS (http://tin.er.usgs.gov/geochem/doc/averages/se/usa.html). The continental divide is shown.

and within populations, with populations collected on the eastern side of the continental divide having significantly higher Se concentrations in leaves (t-test, t= 2.04, df = 31, p-value = 0.049) and fruits (t-test, t= 3.02, df = 35, p-value = 0.005) compared to those from the western side. In contrast to Se, there were no significant differences in S levels of leaves or fruits between eastern and western populations of *S. pinnata* var. *pinnata*.

Sulfur accumulation in the field – Sulfur levels were significantly different across taxa for both organs tested (leaves, Kruskal-Wallis chi-squared = 27.68, df = 9, p-value = 0.001: fruits, Kruskal-Wallis chi-squared = 61.62, df = 8, p-value = <0.001). The differences in S concentrations were not as pronounced as those in Se: *Stanleya bipinnata* had the highest average leaf S level, which was only 2-fold higher than the lowest leaf S levels found in *S. viridiflora* Nutt. (Table 2.1). In the fruits, the highest S levels were found in *T. laciniatum* and were 4-fold higher than the lowest fruit S concentrations found in *S. albescens*. Interestingly, the translocation across organs was different for S and Se: there was more S in the leaves than the fruits in all taxa, while Se was higher in fruits than leaves. Because of this trend, the Se:S ratio was greater in fruits than leaves for all taxa except *S. elata* (Table 2.1).

Tissue selenium:sulfur ratios in the field – The leaf Se:S ratio in *S. pinnata* var. *pinnata* was significantly higher than three of the other taxa (Kruskal-Wallis chi-squared = 38.09, df = 9, p-value <0.001), and its fruit Se:S ratio was significantly higher than six other taxa (Kruskal-Wallis chi-squared = 91.29, df = 8, p-value <0.001; Table 2.1). The fruit:leaf Se concentration ratio (Fig. 2.2) was higher for *S. pinnata* var. *pinnata* and *S. pinnata* var. *integrifolia* than for *S. elata* (Kruskal-Wallis chi-squared = 37.98, df = 8, p-value <0.001). The opposite pattern was observed for the S ratio, which was higher in *S. elata* and *S. tomentosa* than in *S. pinnata* var. *pinnata*, *S. pinnata* var. *integrifolia* and *S. albescens* (Kruskal-Wallis chi-squared = 50.78, df =

8, p-value <0.001). Furthermore, four taxa; *S. pinnata* vars. *pinnata* and *integrifolia*, *S. viridiflora*, and *T. laciniatum*, had higher Se movement to the fruits relative to S movement (Table 2.2).

Finally, we tested whether the variation in tissue Se concentration within and between taxa was correlated with Se levels in the soil or with S levels in the same tissue (Suppl. Table 2.2). There was a significant positive correlation between Se levels in the leaves and Se concentration in the soil for *S. bipinnata, S. elata* and *S. pinnata* var. *inyoensis*. Furthermore, fruit and soil Se levels were positively correlated for *S. bipinnata* and negatively correlated for *S. pinnata* var. *inyoensis.* The same three taxa, *S. bipinnata, S. elata* and *S. pinnata* var. *inyoensis* had positive correlations in the leaf tissues for Se and S, while *S. pinnata* var. *pinnata* and *S. tomentosa* both had negatively correlated Se and S levels in the fruits.

Selenium accumulation in the greenhouse – To minimize environmental effects when comparing Se accumulation within *Stanleya*, a common-garden experiment was conducted on seleniferous soil under controlled greenhouse conditions. Seven *Stanleya* taxa, including three *S. pinnata* varieties, were compared to each other and to the outgroup species, *T. laciniatum* and *B. juncea*. The Se concentration was on average 4-5-fold higher in young leaves than in mature leaves and roots (Fig. 2.3a-c). The taxa differed significantly from each other with respect to Se levels in young leaves (Kruskal-Wallis; chi squared = 48.25, df = 8, p-value <0.001), mature leaves (Kruskal-Wallis chi-squared = 41.69, df = 7, p-value <0.001) as well as roots (Kruskal-Wallis; chi-squared = 37.99, df = 8, p-value <0.001). In young leaves (Fig. 2.3a), S*tanleya pinnata* var. *pinnata* had the highest concentration of Se, which was 13-fold greater than the lowest accumulator, *S. tomentosa*. Within the *S. pinnata* species complex, there was 2-3-fold

Table 2.1. Tissue Se and S concentrations (mg kg^{-1} DW) in *Stanleya* and *Thelypodium* field collected samples. Shown values are the means and standard error of the mean from all individuals for a given taxon. For individual values, population means and collection locales, see Appendix 2.1. A multiple comparison Kruskal-Wallis test was performed; the KW p-value indicates the significance across all taxa; taxa that are not connected by the same letter are significantly different $p \leq 0.05$.

Taxon	[Se] leaf	[S] leaf	Se:S leaf (x 10^{-4})	[Se] fruit	[S] fruit	Se:S fruit (x 10^{-4})
S. albescens	12.7 ± 6.5^{AB}	15880 ± 1721^{AB}	7.3 ± 3.1^{ABC}	58.4 ± 27.9^{AB}	3403 ± 709^{A}	188 ± 97^{AB}
S. bipinnata	16.5 ± 3.7^{AB}	24790 ± 4273^{AB}	6.2 ± 1^{ABC}	87.1 ± 39.7^{A}	11501 ± 968^{BC}	70.2 ± 30^{AC}
S. elata	5 ± 0.8^{A}	12316 ± 2410^{A}	7.9 ± 2.1^{AB}	4.4 ± 0.9^{A}	10133 ± 888^{BC}	4.7 ± 0.90^{C}
S. pinnata						
var. *integrifolia*	14.9 ± 5.3^{AB}	19292 ± 1752^{AB}	8.4 ± 2.8^{ABC}	55.3 ± 14.3^{AB}	6355 ± 452^{ABD}	92.5 ± 22^{AB}
var. *inyoensis*	8.4 ± 2.2^{A}	15640 ± 2064^{AB}	5 ± 0.8^{AB}	24.1 ± 14.3^{A}	7557 ± 1006^{ABCD}	36.1 ± 20^{AC}
var. *pinnata*	468 ± 151^{B}	18448 ± 1603^{AB}	1211 ± 697^{C}	1303 ± 258^{B}	6525 ± 448^{AD}	2875 ± 753^{B}
var. *texana*	12.7 ± 5.2^{AB}	13965 ± 3364^{AB}	8.7 ± 3.7^{ABC}	ND	ND	ND
S. tomentosa	36.5 ± 14.8^{AB}	14858 ± 2195^{AB}	26 ± 9.1^{AC}	150 ± 84^{AB}	10075 ± 650^{BCD}	203 ± 123^{AC}
S. viridiflora	21.4 ± 6.7^{AB}	10839 ± 1543^{A}	21.5 ± 8.1^{ABC}	52.8 ± 24.5^{A}	5662 ± 555^{AD}	96.9 ± 51^{AC}
T. laciniatum	2.8 ± 0.3^{A}	24097 ± 928^{B}	0.2 ± 0.06^{B}	7.6 ± 1.6^{A}	15109 ± 1696^{C}	5.7 ± 1.4^{AC}
KW p-value	<0.001	0.001	<0.001	<0.001	<0.001	<0.001

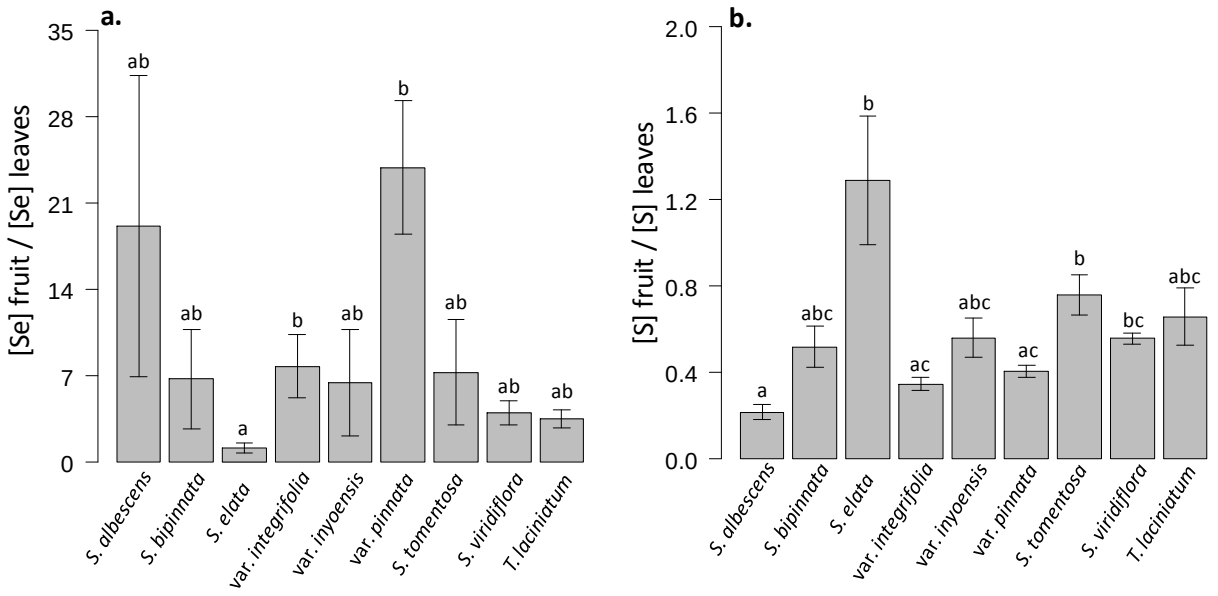

Figure 2.2. Ratio of elemental concentration in fruit relative to leaf tissue for a. Se and b. S in the *Stanleya* taxa collected in the field. *Stanleya pinnata* taxa are indicated only by their varieties. Significant differences ($p \leq 0.05$) were determined from a Kruskal-Wallis multiple comparisons test. Note the different Y-axis scales for Se and S.

variation in Se accumulation. In mature leaves (Fig. 2.3b), the differences in Se levels between the taxa were not the same as observed in young leaves. *Stanleya viridiflora* had the highest level of Se, which was 4-6-fold higher than those found in the three lowest accumulators. Root Se concentration showed significant differences not only between species but also within *S. pinnata* (Fig. 2.3c). *Stanleya pinnata* var. *pinnata* had the highest root Se concentration, which was 4-5-fold higher than that in the two lowest Se accumulators.

Sulfur accumulation in the greenhouse – Sulfur levels were approximately 3-fold lower in roots than leaves. In contrast to Se, S levels were similar in young and mature leaves (Fig. 2.3d-f). The S concentrations differed significantly between the taxa in young leaves (Kruskal-Wallis chi-squared = 43.33, df = 8, p-value <0.001), mature leaves (Kruskal-Wallis chi-squared

= 57.00, df = 7, p-value <0.001) as well as roots (Kruskal-Wallis chi-squared = 62.75, df = 8, p-value <0.001).

In young leaves, there was a 2-fold variation in S levels; thus the variation in S concentration was much smaller than the variation in Se. *Brassica juncea* had the highest S concentration (Fig. 2.3d). In the mature leaves (Fig. 2.3e), S levels were significantly different between taxa. Similar to Se, *S. viridiflora* had the highest S level in mature leaves. Within *S. pinnata*, there was also significant variation: var. *integrifolia* had a 2.6-fold higher S

Table 2.2. Comparison of Se and S distribution ratios between organs for each taxon (from Figs. 2 and 4). P-values are shown from within-taxa t-tests; if $p < 0.05$ then Se and S ratios are shown in bold. ND: not determined. Where Se and S ratios were different, Se movement was greater than S movement, except where indicated with an asterisk.

Taxa	Fruit/leaf Field	Young/mature leaf Greenhouse	Young leaf/root Greenhouse	Mature leaf/root Greenhouse
S. albescens	0.1534	0.2051	0.0925	**0.0013***
S. bipinnata	0.1423	ND	ND	ND
S. elata	0.7287	0.1178	0.0728	0.2761
S. pinnata				
var. *integrifolia*	**0.0097**	**0.0207**	**0.0182**	0.5017
var. *inyoensis*	0.1873	**0.0016**	**0.0011**	0.3085
var. *pinnata*	**<0.001**	**0.0019**	**<0.001**	**0.0383***
S. tomentosa	0.1478	0.5478	**0.0025***	0.2100
S. viridiflora	**0.0013**	**<0.001**	**0.0003**	0.7087
T. laciniatum	**0.0039**	0.7974	0.3629	0.3198
B. juncea	ND	ND	0.4526	ND

concentration than var. *inyoensis*. Root S levels varied 4-fold, with *B. juncea* showing a significantly lower level of S compared to all taxa except *S. viridiflora* and *S. tomentosa* (Fig. 2.3f).

Tissue selenium:sulfur ratios in the greenhouse – The ratio of Se to S is a trait commonly associated with Se hyperaccumulation (White et al., 2007). The Se/S ratios of the taxa are presented in Fig. 2.3g-i. In the young leaves, there were significant differences between species as well as within the *S. pinnata* complex (Kruskal-Wallis chi-squared = 53.58, df = 8, p-value <0.001). *Stanleya pinnata* var. *pinnata* had a 3-fold higher Se/S ratio than any of the other species (Fig. 2.3g). When comparing the Se/S ratio in the mature leaves (Fig. 2.3h), *S. elata* had the highest ratio (Kruskal-Wallis chi-squared = 40.83, df = 7, p-value <0.001). Within the *S. pinnata* complex, var. *pinnata* was significantly different from var. *integrifolia*. In roots (Fig. 2.3i), *S. pinnata* var. *pinnata* had the highest Se/S ratio and was significantly different from *S. pinnata* var. *integrifolia* (Kruskal-Wallis chi-squared = 23.93, df = 8, p-value = 0.002). Thus, *S. pinnata* var. *pinnata* clearly stands out as the only taxon consistently having a high Se/S ratio in all tissues sampled.

Selenium and sulfur translocation ratios – As a proxy for translocation of Se and S in the plant, the ratios for each of these elements were calculated for young leaves relative to mature leaves, for young leaves relative to roots and for mature leaves relative to roots (Fig. 2.4). We analyzed all three ratios to differentiate between xylem and phloem mediated translocation. The resulting ratios were drastically different between Se and S, particularly within the shoot (young/mature leaf, Fig. 2.4a,d). For Se (Fig. 2.4a), there was a 30-fold variation between taxa (Kruskal-Wallis (Kruskal-Wallis chi-squared = 40.64, df = 7, p-value <0.001, Fig. 2.4d). The taxa that stood out for their high young/mature leaf Se ratio were the entire *S. pinnata* complex

as well as *S. albescens*. Note that *S. albescens* and *S. pinnata* var. *integrifolia* had extremely high variances; in each case, one plant had >100x more Se in the young leaves relative to the mature leaves, whereas S levels were comparable. The concentration ratio of young/mature leaf was

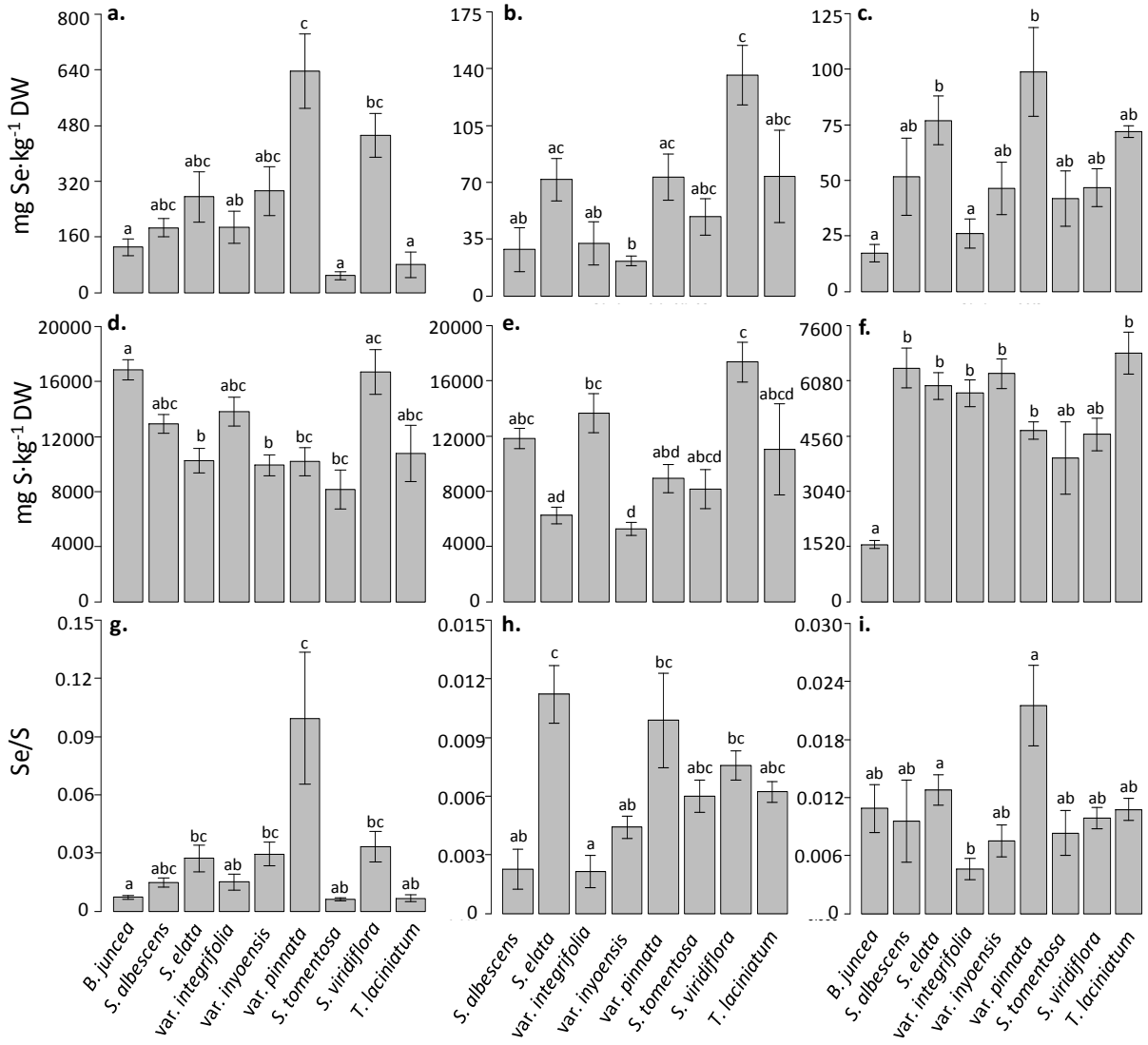

Figure 2.3. Tissue Se (a-c) and S (d-f) concentration and the Se/S concentration ratio (g-i) in young leaves (a, d, g), mature leaves (b, e, h) and roots (c, f, i) from a greenhouse study where plants were grown on seleniferous soil. Included in the study were seven of the nine field-collected taxa in *Stanleya* and two outgroup species, *Thelypodium laciniatum* and *Brassica juncea*. Significant differences ($p \leq 0.05$) were determined from a Kruskal-Wallis multiple comparisons test. Note the different Y-axis scales.

higher for Se than for S in all species except *S. tomentosa* and *T. laciniatum*. Four of the taxa showed significantly higher young/mature leaf ratios for Se than for S: the three *S. pinnata* varieties and *S. viridiflora* (for p-values see Table 2.2).

The shoot/root ratios differed less dramatically between Se and S than the within-shoot ratios (Fig. 2.4). The taxa differed with respect to Se ratio in young leaves/root (Kruskal-Wallis chi-squared = 31.68, df = 8, p-value <0.001, Fig. 2.4b), Se ratio in mature leaves/root (Kruskal-Wallis chi-squared = 21.75, df = 7, p-value = 0.002, Fig. 2.4c), S ratio in young leaves/root (Kruskal-Wallis chi-squared = 68.23, df = 8, p-value <0.001, Fig. 2.4e) and S ratio in mature leaves/root (Kruskal-Wallis chi-squared = 53.93, df = 7, p-value <0.001, Fig. 2.4f). The S ratio for young leaves/root was roughly similar for all members of the genus *Stanleya* and their closest relative, *T. laciniatum*, but *B. juncea* had a significantly (4-6 fold) higher ratio (Fig. 2.4e). *Brassica juncea* also had a high young leaf/root Se ratio, along with *S. pinnata* var. *pinnata* and var. *inyoensis*. Thus, *B. juncea* and *T. laciniatum* appear to have similar Se and S translocation rates, while all *Stanleya* species except for *S. tomentosa* translocated 5-6 times more Se than S (Fig. 2.4b,e). Indeed, when Se and S ratios were compared within individual taxa, all *Stanleya* species showed significant or near-significant ($p < 0.1$) differences between the two elements with respect to young leaf/root Se ratios, while *B. juncea* and *T. laciniatum* did not (Table 2.2). When comparing the movement of elements from the root system to the mature areas of the shoot (Fig. 2.4c,f), there were 5-10-fold differences between taxa, and overall the ratios were similar for Se and S. *Stanleya viridiflora* had the highest ratio for both Se and S. When Se and S ratios were compared within individual taxa (Table 2.2), *S. pinnata* var. *pinnata* and *S. albescens* showed significant differences between Se and S with respect to mature leaf/root ratios; in both cases, the S ratio was greater than the Se ratio.

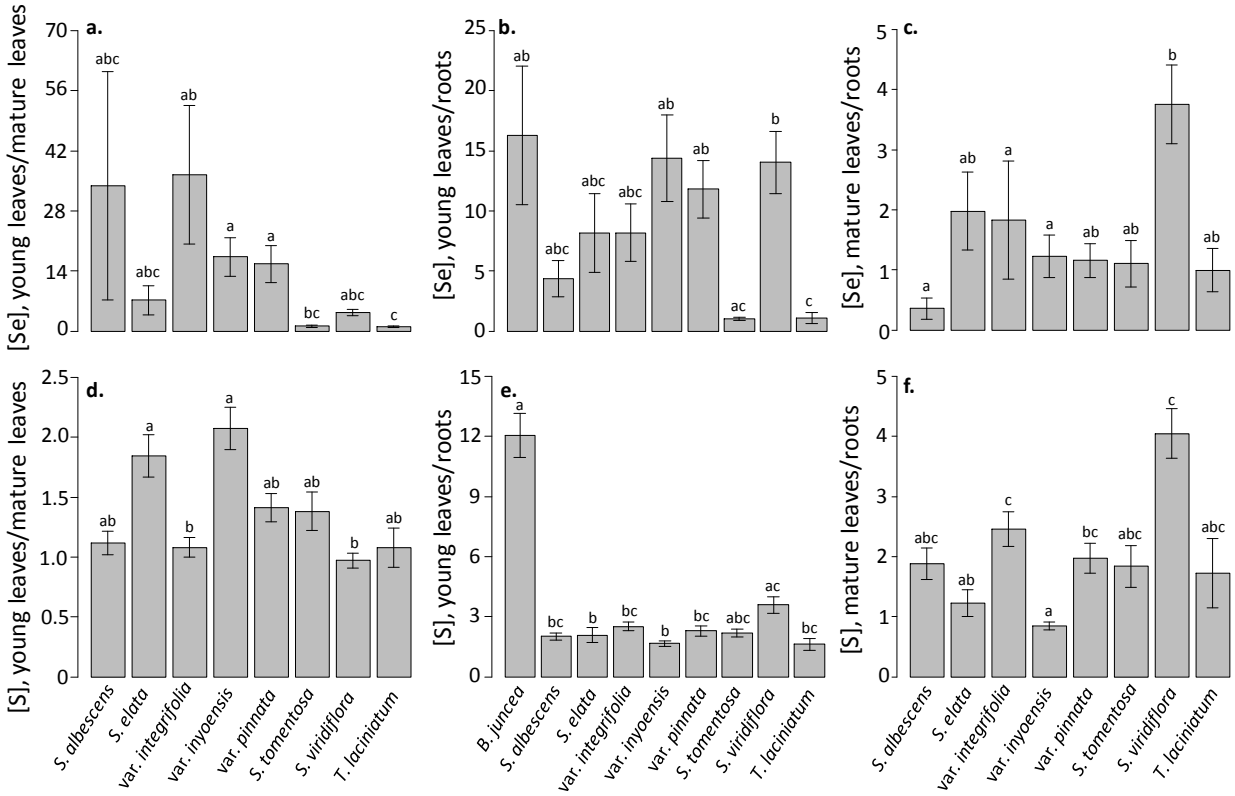

Figure 2.4. Ratio of elemental concentration between different organs and stages of development for Se (a-c) and S (d-f), comparing young leaves with mature leaves (a, d), young leaves with root (b, e) and mature leaves with root (c, f). *S. pinnata* taxa are indicated only by variety. The seven *Stanleya* taxa and two outgroup species, *Thelypodium laciniatum* and *Brassica juncea*, were grown on seleniferous soil in a greenhouse. Significant differences ($p \leq 0.05$) were determined from a Kruskal-Wallis multiple comparisons test. Note the different Y-axis scales.

Discussion

With the aim to investigate the occurrence of Se hyperaccumulation in *Stanleya*, the specific questions posed in this study were: (i) to what degree do the different taxa in *Stanleya* accumulate Se, both in their natural habitat and in a common-garden experiment; (ii) do the taxa differ in S levels; and (iii) which taxa exhibit the properties commonly associated with Se hyperaccumulation, i.e. preferential uptake of Se over S (high Se/S tissue ratios) and high Se shoot/root ratios. The findings of the study are that (i) the nine *Stanleya* taxa tested showed almost 100-fold variation in leaf Se accumulation and almost 300-fold variation in fruit Se

accumulation in the field. The taxa showed a 13-fold variation in Se levels in young leaves Se levels and 6-fold variation in mature leaves in the common-garden experiment; (ii) the taxa differed 2.5-3-fold in leaf and fruit S levels in the field and 2- to 2.5-fold in young and mature leaves in the greenhouse; (iii) *S. pinnata* var. *pinnata* was the only taxon that exhibited Se hyperaccumulation levels exceeding 1,000 mg kg^{-1} DW and all of the Se hyperaccumulation characteristics, i.e. high Se/S ratios, high Se shoot/root ratios. The other two varieties in the *S. pinnata* complex for which we have complete data did not meet the hyperaccumulator criterion. Thus, the intraspecific variation in Se/S related properties within the *S. pinnata* complex appears to be substantial.

These results are of significance because this is the first study to characterize Se accumulation across a genus in relationship with S both in the field and under controlled conditions, and, as such, form a framework for further studies into the evolution of Se hyperaccumulation. The observed intraspecific variation in Se hyperaccumulation in the *S. pinnata* complex also opens the possibility of genetic studies using crosses between Se hyperaccumulators and non-accumulators of the same species so as to identify quantitative trait loci for Se uptake, translocation and assimilation, which may further enhance our understanding of the mechanisms of Se hyperaccumulation. In addition to the intrinsic value, the knowledge and alleles discovered in *S. pinnata* may be applicable to other plant species for phytoremediation and biofortification.

Selenium mobilization to young leaves and reproductive tissues was particularly pronounced in the Se hyperaccumulator *S. pinnata* var. *pinnata*. (Fig. 2.2a, 2.4a). In an earlier field study, Se hyperaccumulators, *S. pinnata* and *Astragalus bisulcatus* A.Gray were also found to preferentially accumulate Se in young leaves and reproductive tissues (Galeas et al. 2007).

The translocation of Se to the most valuable organs, young leaves and reproductive organs is in agreement with the elemental-defense hypothesis for hyperaccumulation, which states that hyperaccumulation serves to protect plants from herbivores (Boyd and Martens, 1992).

The hyperaccumulator, *S. pinnata* var. *pinnata*, not only showed evidence of preferential uptake of Se over S (as evidenced by its extremely high leaf Se/S ratio), but also of independent movement of Se and S in the vascular tissue. The hyperaccumulator showed a more pronounced tendency than other taxa to store Se (but not S) in young leaves and reproductive organs. This may suggest a difference in source-to-sink Se movement between hyperaccumulators and non-hyperaccumulators as well as between Se and S movement in hyperaccumulators. While the mature leaf/root concentration ratios were similar for Se and S, there were large differences between Se and S between the young leaf/root and young leaf/mature leaf ratios (Fig. 2.4), which indicate preferential movement of Se over S to sink tissues. However, this trend is only found in *Stanleya*. *Brassica juncea* showed identical Se and S translocation ratios in young leaf/root while *Stanleya* translocated 5-6 times more Se than S. *Thelypodium laciniatum* showed a similar pattern to *B. juncea* with similar Se and S translocation ratios, albeit much lower, for both the young leaf/root ratio and the young leaf/mature leaf ratio (1-fold). This stark difference between *Stanleya* and its closely related members of the Brassicaceae could be caused by Se-specific transporters of Se and S isologs. It is also possible that Se and S are translocated in different forms in *Stanleya*. The apparent propensity in *Stanleya* species to differentiate between Se and S may be an evolutionary precursor or even a prerequisite for Se hyperaccumulation.

Based on Se concentrations of field-collected tissue (average Se concentration in the fruit), there appear to be roughly four groups: (i) *S. pinnata* var. *pinnata* (>1,000 mg Se kg^{-1} DW); (ii) *S. tomentosa* and (to a lesser extent) *S. bipinnata* (>80 mg Se kg^{-1} DW); (iii) *S.*

pinnata var. *integrifolia, S. albescens, S. viridiflora* (>50 mg Se kg $^{-1}$ DW); and (iv) *S. pinnata* var. *inyoensis, S. elata* and the outgroup *T. laciniatum* (<25 mg Se kg $^{-1}$ DW). Thus, *S. pinnata* shows the entire range of Se-related physiology that we observed. Although *S. pinnata* var. *pinnata* stands out to some extent, there are no clearly distinguished groups in the common-garden experiment.

Given the differences in Se-accumulation patterns between field and greenhouse, there appears to be a strong environmental component to Se (hyper)accumulation. Of course, soil Se levels were different for the field-collected individuals, but this can only partially explain the observed variation in plant-tissue Se levels, since soil and tissue Se levels were correlated for only three of the nine taxa. Naturally seleniferous soil, including its microbial community, was used in the common-garden experiment. It may be that some of these microbes facilitated Se uptake across all species. The chemical species of Se and S may also differ between taxa in the field, but these chemical species were assumed to be uniform in the soil for all taxa in the greenhouse experiment. Another important difference between the greenhouse and field samples was that the greenhouse plants were grown for only three months, whereas the plants in the field were likely several years old based on their size and woodiness. It may take years for plants to reach Se hyperaccumulator levels when growing on seleniferous soil, which would explain why *S. pinnata* var. *pinnata* did not accumulate >1,000 mg kg^{-1} DW in the greenhouse.

Our finding that *S. pinnata* var. *pinnata* had leaf Se levels >1,000 mg kg^{-1} DW is in agreement with earlier reports from Beath et al. (1939a; 1939b; 1940). However, while Beath and coworkers also reported *S. bipinnata* to have leaf Se levels >1,000 mg kg^{-1} DW in the field, this taxon did not stand out as a Se hyperaccumulator in our study. A possible explanation could be that this species has a large intraspecific variation in Se-accumulation properties, similar to *S.*

pinnata, and that the populations sampled here are not the highest accumulators. We know of only five populations of *S. bipinnata* that have been reported in herbarium records, and three of these have since disappeared due to development, leaving only the two sampled here. With the loss of these three potential ecotypes, the hyperaccumulation capacity may have been lost from the species.

There appears to be a large intraspecific variation within the *S. pinnata* clade, with *S. pinnata* var. *pinnata* being the only taxon that showed true hyperaccumulation; *S. pinnata* var. *integrifolia* showed lower Se accumulation and *S. pinnata* var. *inyoensis* even lower. The latter two varieties were both collected on the western side of the continental divide, in Utah/Wyoming and Nevada, respectively. *Stanleya pinnata* var. *pinnata* showed the highest levels of Se accumulation but also the largest variation in Se accumulation of all taxa examined. It also has the widest range (Fig. 2.1a). Interestingly, the populations sampled west of the continental divide (Suppl. Table 2.1, populations 3-6) showed significantly ($p < 0.05$, t-test) lower leaf (24-fold) and fruit (6-fold) levels of Se (but not S) than those collected east of the divide (populations 1,2, 7-10). Based on these data, only the *S. pinnata* var. *pinnata* populations east of the divide are true Se hyperaccumulators.

Our finding that the only true Se hyperaccumulating populations are *S. pinnata* var. *pinnata* east of the continental divide is in agreement with the earlier report by Feist and Parker (2001), wherein 16 ecotypes of *S. pinnata* were compared, and the highest four Se accumulators (CO4, MT1, WY2, WY3, with 140-1200 mg kg^{-1} DW) all originated from east of the continental divide. The remaining 12 populations had substantially lower Se levels (0.3-82 mg kg^{-1} DW), with the lowest levels found in CA and NV, which is in agreement with our results for *S. pinnata* var. *pinnata* near the NV-OR border, and *S. pinnata* var. *inyoensis* from NV (Suppl. Table 2.1).

Taken together, these results suggest that Se hyperaccumulation may be restricted to the east side of the continental divide, perhaps because hyperaccumulation evolved within this variety there and the Rocky Mountains formed a barrier for gene flow to the west, or because there is a greater cost to hyperaccumulation in the west (e.g. metabolic cost, toxicity to mutualist symbionts). In addition to this geographic barrier, there appears to be a reproductive barrier between eastern and western populations due to a ploidy difference with the diploids being found on the eastern side of the divide. We were able to successfully cross individuals from the eastern and western slopes (var. *pinnata* × var. *inyoensis*); the resulting offspring were triploid (data not shown) and did not produce any seed. It is interesting to note that one population of S. *pinnata* var. *pinnata* found near the eastern edge of the Great Divide Basin is tetraploid (Fig. 2.1b). This geographic region is a likely area for migration across the continental divide in *Stanleya.* South Pass, just north of the Great Divide Basin, has the lowest elevation along the continental divide, between the Central Rocky Mountains and Southern Rocky Mountains. Further studies are needed to investigate whether differential selective pressures (e.g. Se soil levels) or other evolutionary processes (e.g. genetic drift or bottleneck effect) may have driven the proliferation of only tetraploid *S. pinnata* lineages in the west and diploids east of the divide.

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Chapter 3: Evolution of selenium hyperaccumulation in *Stanleya* (Brassicaceae) as inferred from phylogeny, physiology and X-ray microprobe analysis

Summary

Past studies have identified herbivory as a likely selection pressure for the evolution of hyperaccumulation, but few have tested the origin(s) of hyperaccumulation in a phylogenetic context. We focused on the evolutionary history of selenium (Se) hyperaccumulation in *Stanleya* (Brassicaceae). Multiple accessions were collected for all *Stanleya* taxa and two outgroup species. We sequenced four nuclear gene regions and performed a phylogenetic analysis. Ancestral reconstruction was used to predict the states for Se related traits in a parsimony framework. Furthermore, we tested the taxa for Se localization and speciation using X-ray microprobe analyses. True hyperaccumulation was found in three taxa within the *S. pinnata/bipinnata* clade. Tolerance to hyperaccumulator Se levels was found in several taxa across the phylogeny, including the hyperaccumulators. X-ray analysis revealed two distinct patterns of leaf Se localization across the genus: marginal and vascular. All taxa accumulated predominantly (65-96%) organic Se with the C-Se-C configuration. These results give insight into the evolution of Se hyperaccumulation in *Stanleya* and suggest that Se tolerance and the capacity to produce organic Se are likely prerequisites for Se hyperaccumulation in *Stanleya*.

Introduction

Adaptation to selective pressures on populations from biotic or abiotic factors, is a key process to study in evolutionary biology because it helps explain how interspecific differences arose. The seminal work on Darwin's finches provided empirical evidence for the link between phenotype and genotype (Boag and Grant, 1978; Boag, 1983). More recent studies of adaptation include the enzyme *Adh* in *Drosophila,* which provided a two-allele model to study adaptive change in a novel environment (Chambers, 1991), life-history evolution in guppies due to predation (Reznick et al.,

1996), and floral evolution due to interaction with pollinators (Galen, 1996). In order to study complex phenotypic responses to environmental pressures we must use interdisciplinary approaches to study evolution from molecular to ecological changes. One such interesting complex trait that has not been studied in great detail outside of a physiological and/or ecological context is elemental hyperaccumulation in plants.

Elemental hyperaccumulation is an intriguing trait that has been documented in over 500 plant species (Krämer, 2010; Cappa and Pilon-Smits, 2014). The criterion for a species to be a hyperaccumulator depends on the element in question and ranges from 0.01% - 1% dry weight (DW; Krämer, 2010). This criterion has been applied to several toxic elements including arsenic (As), cadmium (Cd), cobalt (Co), lead (Pb), nickel (Ni), selenium (Se) and zinc (Zn) (Krämer, 2010). There is convincing evidence for several adaptive advantages of elemental hyperaccumulation, including protection from herbivores (Pollard and Baker, 1997; Boyd and Moar, 1999; Jhee et al., 1999; Freeman et al., 2007, 2009; Quinn et al., 2008, 2010), protection from pathogens (Boyd et al., 1994; Hanson et al., 2003) and elemental allelopathy to neighboring plants (El Mehdawi et al., 2011). Given these elements are toxic in high enough concentrations to both plants and their associated herbivores and pathogens, it is reasonable to hypothesize that only those genotypes that can survive with high internal concentrations of these toxic elements or exclude them altogether, will have a selective advantage in these environments.

Relatively few studies have tested the number of origins of hyperaccumulation in a phylogenetic context. From mapping the occurrence of hyperaccumulators on the angiosperm phylogeny (Cappa and Pilon-Smits, 2014) it is clear that hyperaccumulators constitute a polyphyletic group across flowering plants. Interestingly, over half of hyperaccumulators are found in three orders; Malpighiales (8 families and 127 taxa), Brassicales (2 families and 102

taxa), and Asterales (3 families and 79 taxa). The Brassicaceae constitute the largest fraction of known hyperaccumulators for any family, with >100 taxa. Krämer (2010) proposed at least 13 independent origins of hyperaccumulation within the Brassicaceae. Even within a genus there can be multiple origins of tolerance or hyperaccumulation. For example, Cecchi et al. (2011) suggested at least six origins of obligate endemics to serpentine soils in the genus *Onosma* (Boraginaceae) with non-serpentine endemics representing the ancestral phenotype. *Alyssum* (Brassicaceae) has been shown to also have multiple origins of Ni hyperaccumulation, with multiple events of local adaptation and selection across southern European serpentine soils (Cecchi et al., 2010).

We are particularly interested in the evolution and origin of Se tolerance and hyperaccumulation in *Stanleya* Nutt. (Brassicaceae; tribe Thelypodieae). *Stanleya* is comprised of seven species and one of these, *S. pinnata,* is divided into three varieties. Treatment of these varieties differs depending on the flora used: Holgrem et al. (2005) recognized var. *integrifolia*, var. *inyoensis* and var. *pinnata* while Al-Shehbaz (2010) recognized var. *integrifolia*, var. *pinnata* and var. *texana*. All seven species occur only in the western U.S.; for ranges see Cappa et al. (2014). *Stanleya pinnata* var. *pinnata* is well-documented as a Se hyperaccumulator (Beath et al., 1939ab, 1940, 1941; Feist and Parker, 2001; Galeas et al., 2007; Freeman et al., 2010) and occurs in most western states. *Stanleya bipinnata* has also been reported to be a Se hyperaccumulator (Beath et al., 1940).

Selenium hyperaccumulation is particularly interesting because, while Se is an essential micronutrient for many animals, prokaryotes and algae, it has not been shown to be essential for vascular plants (Ellis and Salt, 2003; Sors et al., 2005; Zhang and Gladyshev, 2008). Most plants likely take up Se inadvertently because it is atomically similar to sulfur (S). For a plant to be

considered a Se hyperaccumulator it must accumulate Se to >1,000 mg kg^{-1} or 0.1% of dry weight (DW). *Stanleya pinnata* var. *pinnata* can accumulate Se up to 0.5% DW and preferentially takes up Se over S (White et al., 2007; Parker et al., 2003; Harris et al., 2014). Below the threshold of Se hyperaccumulation, two other tiers of Se accumulation can be distinguished: Se accumulators/secondary Se accumulators accumulate 0.01-0.1% Se in the field (100-1,000 mg kg^{-1} DW), while non-Se accumulators accumulate less than 0.01% (100 mg kg^{-1} DW) (El Mehdawi and Pilon-Smits, 2012).

In trying to understand the evolution of Se hyperaccumulation, key questions to address are: why do plants hyperaccumulate this non-essential, toxic element, (i.e. which selection pressures may have led to Se hyperaccumulation)? As mentioned above, protection from herbivores and pathogens may have been a selection pressure for Se hyperaccumulation, in addition to benefits from elemental allelopathy. A second question is: how do plants hyperaccumulate Se? Finally, which genetic and metabolic changes have occurred that led to the evolution of Se hyperaccumulation? Increased, constitutive expression of sulfate transporters may be one of the mechanisms of Se hyperaccumulation. Freeman et al. (2010) showed that several transcripts for sulfate transporters were constitutively upregulated in Se hyperaccumulator *S. pinnata* relative to non-hyperaccumulator *S. albescens*. There is also evidence for the presence of sulfate-transporter homologues with enhanced selenate specificity (Harris et al., 2014). The currently hypothesized tolerance mechanism of Se hyperaccumulation is the production of the organic selenocompounds selenocystathionine (SeCyst) and methyl-SeCys. All plants can assimilate inorganic selenate into selenocysteine (SeCys) via the sulfate assimilation pathway (Terry et al., 2000). This SeCys is toxic when it is non-specifically incorporated into proteins. This toxicity can be prevented if the SeCys is further metabolized to

selenocystathionine (SeCyst), selenomethionine (SeMet) or methyl-SeCys (Neuhierl and Böck, 1996; Sors et al., 2005; Freeman et al., 2006b).

The primary question addressed in this study is: What is the evolutionary history of Se hyperaccumulation in *Stanleya*? To address this question we used a combination of physiological, molecular and biophysical approaches. We determined Se tolerance across *Stanleya* taxa in a sterile common-garden environment. We also determined Se distribution and chemical speciation in vegetative and reproductive tissues, using X-ray microprobe analyses. Furthermore, we used a combination of molecular and morphological traits to resolve the phylogenetic relationships of *Stanleya*. Finally, we mapped Se tolerance and Se accumulation properties onto the inferred phylogenetic relationships of *Stanleya*. By mapping these Se-related traits onto the *Stanleya* phylogeny we show hyperaccumulation evolved in the *S. bipinnata/pinnata* clade and hypothesize tolerance likely preceded hyperaccumulation in *Stanleya*.

Materials and methods

Sampling – All taxa included in this study were collected from field sites determined from herbaria databases. When possible, we collected a minimum of three populations and a minimum of three individuals from those populations for each taxon (for details see Cappa et al., 2014). At each site, leaves, seeds and soil were collected. The leaves were silica-dried (Chase and Hills, 1991) and later used for DNA extraction and molecular phylogenetic analyses (as described below). Seeds were used for the common garden experiment and XAS analyses (as described below).

Plant growth – Seeds were surface sterilized; rinsed in 70% ethanol for 30 seconds, placed on a rocker for 20 minutes in 15% bleach and rinsed five times with sterile distilled

deionized H_2O. The seeds were placed at 4°C for 48 hours before being transferred to sterile petri dishes with filter paper. Once cotyledons emerged the seedlings were transferred to ½ Murashige and Skoog (1962) agar containing 0, 20 µM, 53 µM, 80 µM or 160 µM sodium selenate ($NaSeO_4$). The plants were grown for 30 days at 23°C in a growth chamber at a light intensity of 150 µE with a 16/8 light period. The 53 µM treated plants were used for XAS analysis. Due to low germination rates of *S. bipinnata* and *S. tomentosa* they were only grown in the 0, 53 µM and 80 µM $NaSeO_4$ treatments.

Tolerance and accumulation analysis – Whole plants were harvested and roots rinsed in deionized H_2O to remove any external Se. Plants were dried at 50°C for 72 hours before being weighed and nitric-acid digested according to Zarcinas et al. (1987). The digest was analyzed via inductively coupled plasma atomic emission spectroscopy (ICP-AES) according to Fassel (1978). Pearson's correlation was used to test for significant correlations between internal Se concentration and biomass, as a measure of tolerance. ANOVA followed by Tukey-Kramer post hoc analyses were carried out to test for significant differences in Se accumulation between species. Both analyses were conducted in R (version 2.15.1). Graphs were produced in SigmaPlot ver. 11.

X-ray microprobe analysis – Frozen intact leaves and field collected seeds were analyzed via µx-ray fluorescence (XRF) for chemical mapping of Se, Ca and Fe. Micro x-ray near edge absorption spectrometry (XANES) was used to determine the chemical speciation of Se using standard selenocompounds XANES spectra (Quinn et al., 2011). The spectra were fitted using a linear least squares combination where the quality of the fit was measured as the sum of squares.

DNA extraction and amplification – Total genomic DNA was isolated from silica-dried tissue using a Qiagen DNeasy Kit according to manufacturer's instructions or the protocol

described by Alexander et al. (2006). The following four nuclear markers were used: chalcone synthase (CHS; Koch et al., 2000), luminidependens (LD; Slotte et al., 2006), ITS (Blattner, 1999) and SAT (SATF - 5' AGATGTTTTCTTGGAAATATTATCAG 3', SATR - 5' TTAATGRTCAAGAATATTAGATCAAAC 3', developed for this study). At least three individuals per taxon (when possible) were sequenced for all four gene regions. All four gene regions were amplified on a thermocycler according to the following temperature regime: 96°C for 3 min (initial denaturation) followed by 10 cycles of 96°C for 45 s (denaturation), 50°C for 30 s (annealing) and 72°C for 2 min (extension), then 25 cycles of 96°C for 20 s, 50°C for 30 s and 72°C for 2 min. PCR products were purified with a Qiagen PCR Purification Kit and the resulting purified products were sequenced by the University of Chicago Cancer Research Center DNA Sequencing Facility via ABI DNA Analyzers. *Arabidopsis thaliana* and *Brassica rapa* sequences were obtained from TAIR and GenBank, respectively. Leaf samples from herbarium specimens were generously donated by four herbaria (MO, NY, OSU, RM) for two *S. bipinnata*, two *S. pinnata* var. *texana*, one *T. laciniatum*, one *Th. ambigua* and all four *S. confertiflora* samples (Appendix 3.1). Primers used for amplification were also used for sequencing. All sequences were deposited in GenBank (Appendix 3.1).

Phylogenetic analyses – Preliminary nucleotide alignments were obtained independently for each gene region using MAFFT ver. 6.5 (Katoh and Toh, 2008). G-INS-i, the most accurate MAFFT algorithm for aligning gene regions other than rDNA, was used for all four gene regions. The 1PAM nucleotide scoring matrix and the default gap opening penalty (1.53) were applied. Manual adjustments to the MAFFT alignments were performed in MacClade ver. 4.08 (Maddison and Maddison, 2001) using the procedure outlined by Simmons (2004) following Zurawski and Clegg (1987). Two ambiguously-aligned regions were eliminated from the

analysis (LD: 271-281, SAT: 457-467). Ambiguously-aligned nucleotides of individual sequences in regions that could not be unambiguously aligned with the remaining sequences were re-scored as ambiguous ("?").

Gap characters, whose inclusion often affects the inferred tree topology and increase branch-support values (Simmons et al., 2001), were manually scored using modified complex indel coding (Simmons and Ochoterena, 2000; Müller, 2006). A total of 19 parsimony-informative gap characters were scored from unambiguously aligned regions (ITS: 4, LD: 7, Sat: 8).

A total of 15 vegetative and reproductive morphological characters (Suppl. Table 3.1), were included in the simultaneous analysis. These characters were chosen based on the Flora of North America (Al-Shehbaz, 2010) dichotomous key.

Equally weighted parsimony tree searches were conducted using TNT ver. 1.1 January 2013 (Goloboff et al., 2008). Branches with a minimum possible optimized length of zero were collapsed to improve efficiency of tree searches and help minimize artifacts caused by missing data (Kitching et al., 1998; Davis et al., 2005). Up to 50 trees were held (Davis *et al*., 2005) within each of 10,000 random-addition-sequence (RAS) tree-bisection-reconnection (TBR) searches. Parsimony jackknife (Farris et al., 1996) analyses were conducted with the removal probability set to approximately e^{-1} (0.37). One-thousand parsimony jackknife replicates were performed with 100 RAS TBR searches (each with a maximum of 50 trees held) per replicate.

jModeltest ver. 2.1.4 (Posada, 2008) was used to select the best-fit likelihood model for each data matrix using the Akaike Information Criterion (AIC; Akaike, 1974) without considering invariant-site models following Yang (2006). The models selected all incorporate

the gamma distribution (Yang, 1993). The Q-matrices selected are HKY (Sat), SYM (ITS), TIM2 (CHS), TPM1uf (LD), and GTR (all four gene regions together).

PartitionFinder ver. 1.1.1 (Lanfear et al., 2012) was used to determine the number of partitions to use for the all-four-gene-regions analysis by using the AIC and the defaults for all other settings. PartitionFinder selected a different partition for each of the four gene regions. This partitioning scheme was implemented in GARLI ver. 2.01.1067 (Zwickl, 2006) by allowing different model parameters and different subset rates between the four partitions (linkmodels = 0; subsetspecificrates = 1) with the GTR + Γ model for each partition.

Maximum likelihood analyses (Felsenstein, 1973) were performed with GARLI. Because the TIM, and TPM Q-matrices are not implemented in GARLI, the GTR model was applied to CHS and LD instead. ITS was analyzed using the SYM model by setting all nucleotides to equal frequencies and SAT was analyzed using the HKY model. Following the recommended setting in GARLI, branches with a length of 1×10^{-8} (i.e., effectively zero; Zwickl, 2012) were collapsed. The GARLI analyses were performed by using the least rigorous settings for an intensive search recommended by Zwickl (2009; streefname = stepwise; attachmentspertaxon = 50, genthreshfortopoterm = 20,000, numberofprecreductions = 20, treerejectionthreshold = 100) for both optimal-tree searches (1,000 search replicates) and the bootstrap (BS; Felsenstein 1985; 1,000 replicates, each with 10 searches).

Ancestral reconstruction – All weakly supported branches, with <50% jackknife and bootstrap support, were collapsed in TreeGraph2 ver. 2.0.44 (Stöver and Müller, 2010). The resulting tree was imported into Mesquite ver. 2.75 (Maddison and Maddison, 2011) where a categorical character matrix was created for accumulation, tolerance and survival. Accumulation was designated as follows: non-hyperaccumulator <1000 and hyperaccumulator >1000 mg Se

kg^{-1} DW. For these designations, we used the highest Se concentration recorded for a given taxon published from field surveys (Beath et al., 1939b, 1940, 1941; Cappa et al., 2014). Tolerance was scored from the scatterplots as the 50% inhibition point calculated from biomass production as a function of internal Se concentration using the linear equation produced (Fig. 3.1). Tolerance was categorically ranked as follows: non-tolerant <1000 and tolerant >1000 mg Se kg^{-1} DW. *Brassica rapa* and *A. thaliana* were scored as Se tolerant (Garifullina et al., 2003; Van Huysen et al., 2003) and Se sensitive (Zhang et al., 2007), respectively. For survival, the number of individuals that survived the 30 day treatment was averaged across all three Se treatments. Those taxa with an average >50% across all Se treatments were scored as high survival while those with <50% were scored as low survival. The accumulation, tolerance and survival characters were mapped onto the tree using Fitch (1971) optimization in Mesquite.

Results

Tolerance and accumulation – As a measure of tolerance we plotted the internal Se concentration attained in the common-garden agar experiment (Suppl. Fig. 3.1) against the total biomass for each individual across all treatments; 0, 20 µM, 80 µM and 160 µM (Fig. 3.1). *Stanleya pinnata* var. *pinnata* reached the highest maximum tissue Se levels (close to 3,000 mg Se kg^{-1} DW) followed by *S. pinnata* var. *integrifolia* and *S. bipinnata*. *Thelypodium laciniatum* and *S. viridiflora* had the lowest Se accumulation levels (Fig. 3.1). *Stanleya bipinnata* is the only species that had a trend for increasing biomass production with increasing tissue Se concentration, but due to low germination there were too few plants to show a significant effect. In *S. albescens* and *T. laciniatum* internal Se concentration and biomass were not significantly correlated; the same was true for *S. pinnata* var. *pinnata*, although the p-value (0.052) was close to significance. *Stanleya tomentosa* had a marginally significant negative response to Se

concentration, and *S. elata*, *S. pinnata* vars. *integrifolia* and *inyoensis* and *S. viridiflora* had a significantly negative response to increasing internal Se concentration ($p < 0.01$).

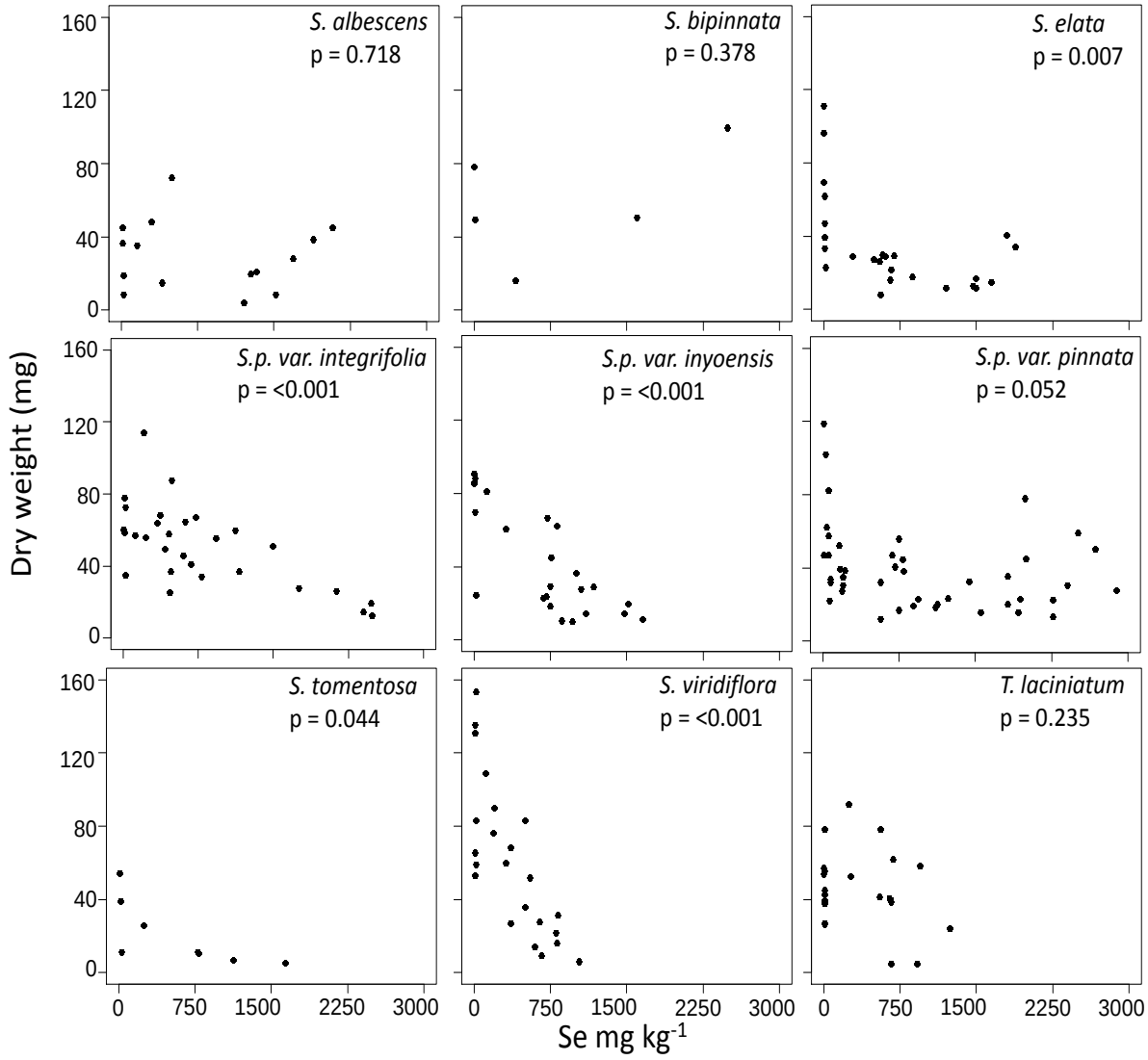

Figure 3.1. Selenium tolerance of different *Stanleya* and *Thelypodium* species, as judged from their dry weight production as a function of their internal Se concentration when grown on agar medium supplemented with 0 µM, 20 µM, 80 µM or 160 µM of sodium selenate (pooled data). A p-value < 0.05 indicates a significant positive or negative correlation between internal Se concentration and growth (Pearson's correlation analysis).

XRF – Two distinct patterns of Se localization were found in *Stanleya*. Four of the eight species (*S. bipinnata, S. pinnata* var. *integrifolia, S. pinnata* var. *pinnata* and *S. tomentosa*) all had Se localized in patches in the margin of the leaf (Fig. 3.2). In contrast; *S. elata, S. pinnata* var. *inyoensis, S. viridiflora* and *T. laciniatum* all had Se localized in the vascular tissue (Fig. 3.3). All species had the same Se distribution in the seeds. The Se was localized in the embryos (Fig. 3.4). Only *S. pinnata* var. *pinnata* had a slight Se signal in the seed coat. Selenium was not found in the endosperm of any species.

XANES – All species sampled had >50% organic Se in their tissues, mainly modeled as C-Se-C compounds (Table 3.1). Note that the XANES spectra for selenomethione, methyl-selenocysteine and selenocystathionine are indistinguishable and thus the C-Se-C Se in these leaves and seeds can be any combination of the three compounds. The species with the marginal Se localization pattern generally also had a greater percentage of organic Se. The species with the marginal distribution (*S. bipinnata, S. pinnata* var. *integrifolia, S. pinnata* var. *pinnata* and *S. tomentosa*) had on average 96%, 92%, 96% and 87% organic Se, respectively. The species with the vascular Se localization (*S. elata, S. pinnata* var. *inyoensis, S. viridiflora* and *T. laciniatum*) had 80%, 83%, 100% and 67% organic Se, respectively. The (inorganic) remainder of the leaf Se was selenate, selenite and elemental Se (Table 3.1). Regardless of plant species or the leaf localization and Se speciation we found the seeds to have almost exclusively organic Se, again mainly modeled as C-Se-C compounds; the remainder (inorganic) Se was modeled as elemental Se (Table 3.2).

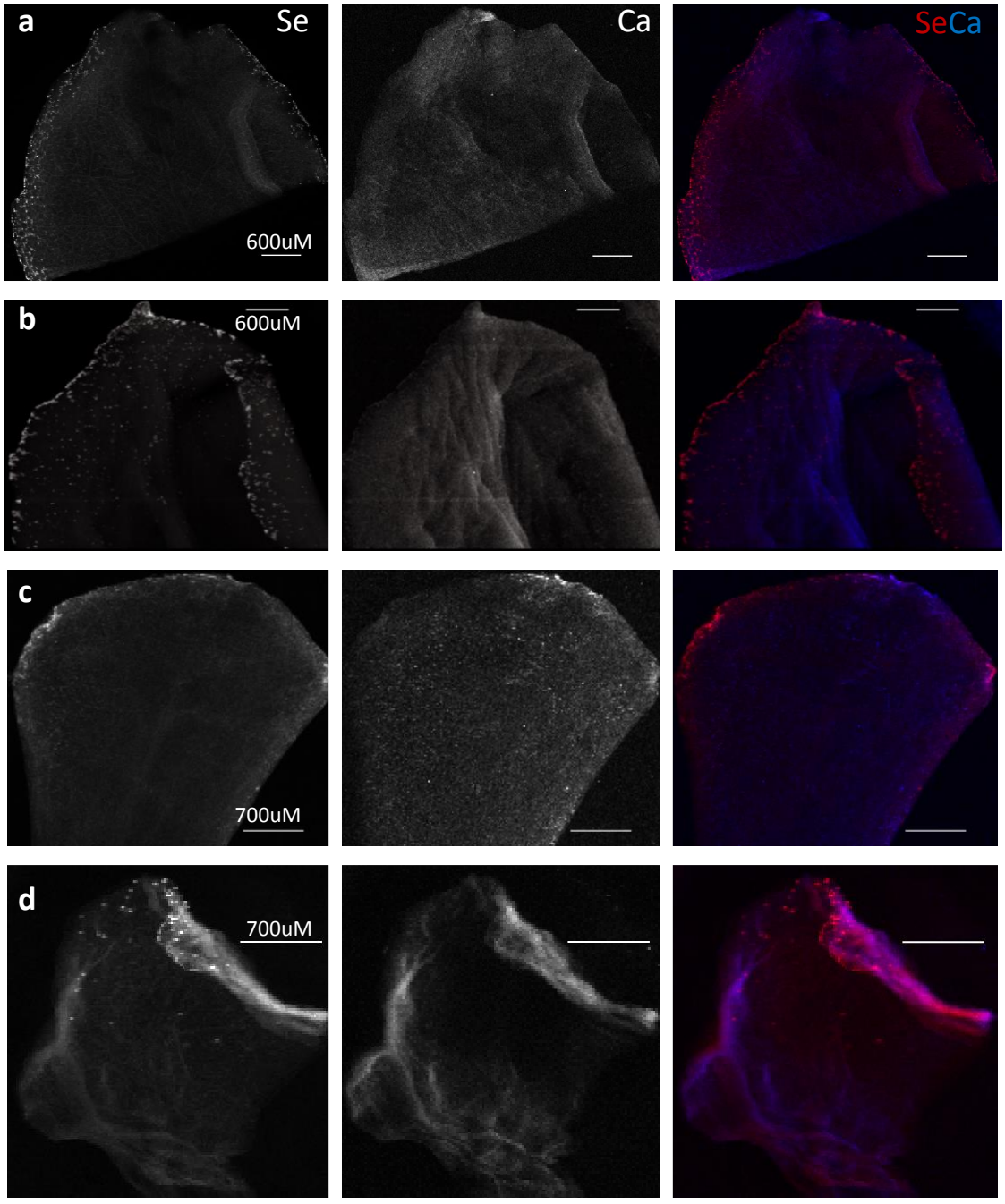

Figure 3.2. X-ray-fluorescence elemental mapping of leaves of different *Stanleya* species grown on agar medium supplemented with 53 µM sodium selenate. a. *S. pinnata* var. *integrifolia*; b. *S. pinnata* var. *pinnata*; c. *S. bipinnata*; d. *S. tomentosa*. Left column: Se (in white); middle column: Ca (in white); right column: Se (red) and Ca (blue) overlay.

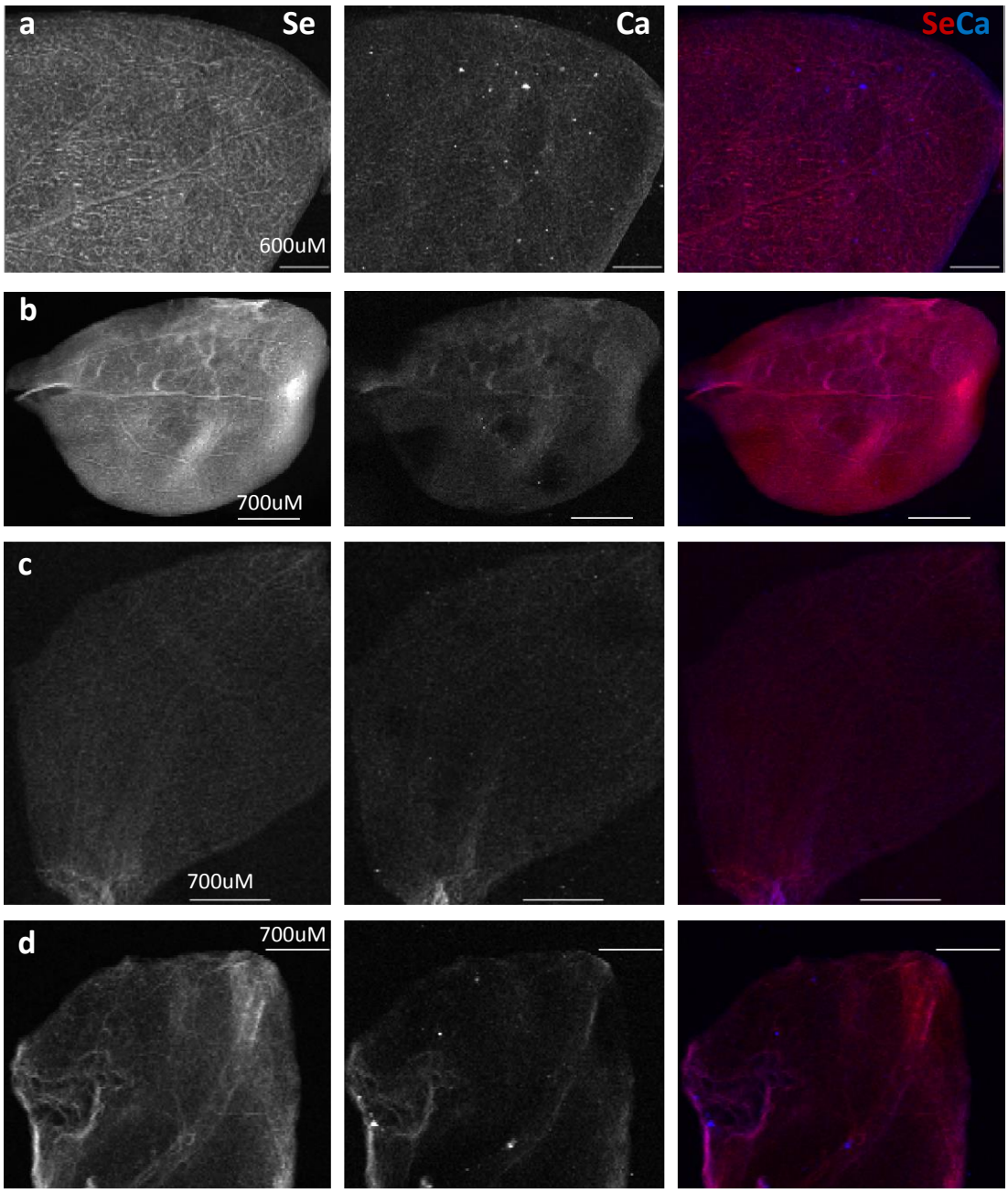

Figure 3.3. X-ray-fluorescence elemental mapping of leaves of different *Stanleya* and *Thelypodium* species grown on agar medium supplemented with 53 µM sodium selenate. a. *S. pinnata* var. *inyoensis*; b. *S. viridiflora*; c. *S. elata*; d. *T. laciniatum.* Left column: Se (in white); middle column: Ca (in white); right column: Se (red) and Ca (blue) overlay.

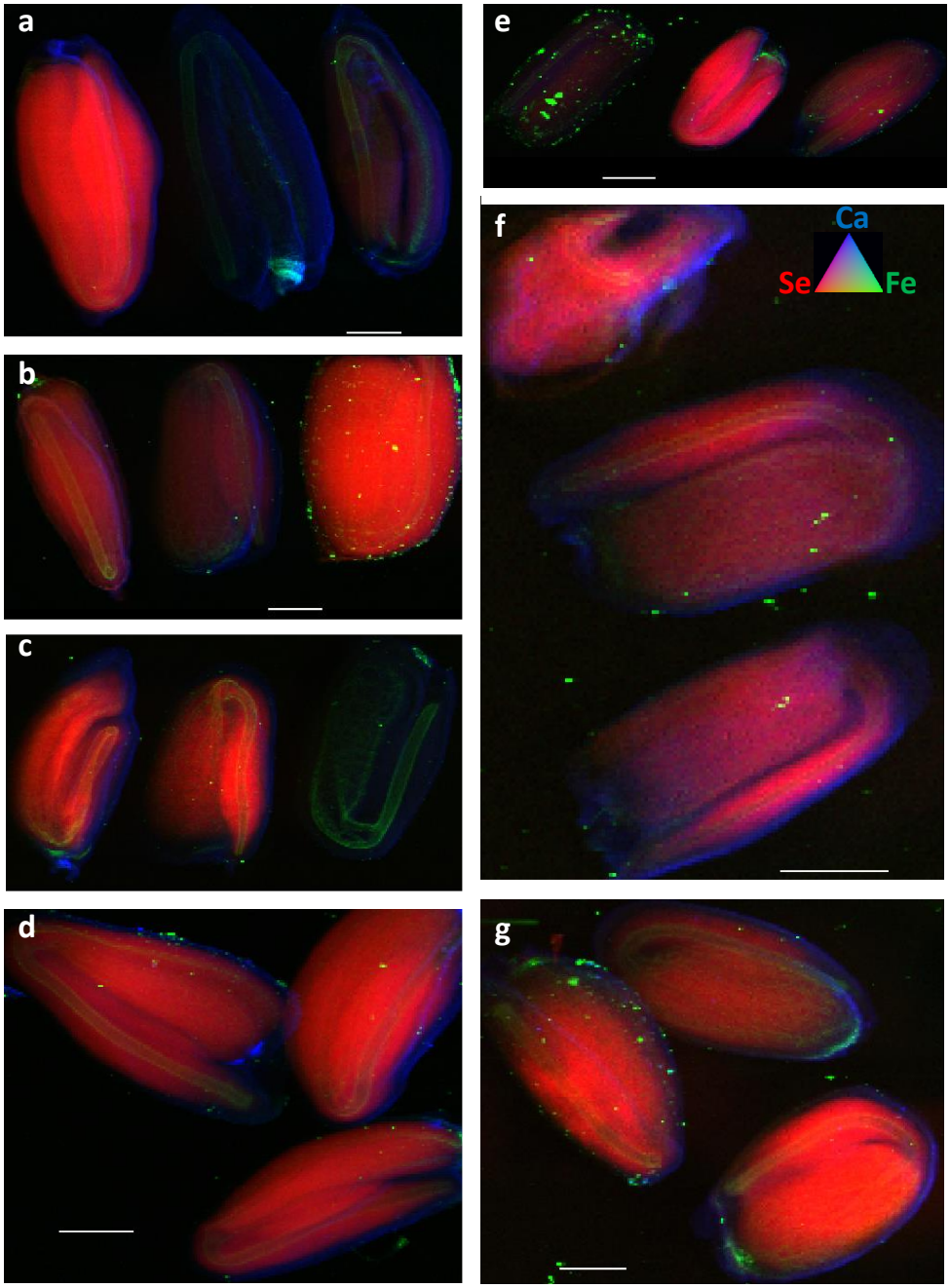

Figure 3.4. X-ray-fluorescence elemental mapping of seeds of different *Stanleya* species collected in the field. a. *S. pinnata* var. *integrifolia*; b. *S. pinnata* var. *pinnata*; c. *S. pinnata* var. *inyoensis*; d. *S. bipinnata; e. S. albescens;* f. *S. viridiflora; g. S. tomentosa*. Se distribution is shown in red, Ca in blue and Fe in green. All scale bars are 600µm.

Table 3.1. Selenium speciation in leaves of different *Stanleya* and *Thelypodium* species grown on agar medium supplemented with 53 µM sodium selenate. Results from least squares linear combination fitting of experimental XANES spectra with standard selenocompounds. C-Se-C: methyl-selenocysteine/Se-Methionine (same spectra). Se^0: red or gray elemental Se. NSS: normal sum of squares (quality of fit; 0 = perfect fit); nd: compound not detected. Selenocystine was not detected in any sample. Na: not applicable. Numbers following plant species names denote biological replicates. Spectra with identical numbers were collected at different positions on the sample.

sample ID	NSS (10^{-4})	SeO_4	SeO_3	SeGSH2	SeCys	C-Se-C	Se^0
S. bipinnata	6.47	nd	9	nd	nd	91	nd
S. bipinnata	3.59	nd	0	nd	nd	99	nd
S. bipinnata	4.42	nd	nd	nd	nd	99	nd
average		nd	5	nd	nd	96	nd
standard deviation		nd	na	nd	nd	5	nd
S. elata 1	3.83	nd	2	nd	nd	96	nd
S. elata 1	4.82	nd	8	nd	nd	71	20
S. elata 1	2.92	nd	6	nd	nd	80	12
S. elata 2	8.07	7	11	nd	nd	79	nd
S. elata 2	7.72	9	11	nd	nd	78	nd
average		8	8	nd	nd	80	16
standard deviation		na	4	nd	nd	9	na
S. pinnata var. *integrifolia*	2.14	nd	5	nd	nd	82	14
S. pinnata var. *integrifolia*	6.5	nd	nd	14	nd	91	nd
S. pinnata var. *integrifolia*	7.41	nd	nd	nd	nd	96	nd
S. pinnata var. *integrifolia*	7.24	nd	nd	nd	nd	95	nd
S. pinnata var. *integrifolia*	5.78	2	nd	nd	nd	82	11
S. pinnata var. *integrifolia*	3.1	nd	nd	nd	nd	101	nd
S. pinnata var. *integrifolia*	4.8	nd	3	nd	nd	99	nd
average		na	4	na	nd	92	12
standard deviation		na	na	na	nd	8	na
S. pinnata var. *inyoensis* 1	6.33	nd	nd	nd	nd	97	nd
S. pinnata var. *inyoensis* 1	6.05	nd	nd	nd	nd	98	nd
S. pinnata var. *inyoensis* 1	4.26	nd	nd	nd	nd	98	nd
S. pinnata var. *inyoensis* 2	16.8	55	8	nd	nd	31	nd
S. pinnata var. *inyoensis* 3	3.73	nd	0	nd	nd	100	nd
S. pinnata var. *inyoensis* 3	8.39	nd	1	nd	37	59	nd
S. pinnata var. *inyoensis* 3	4.01	0	nd	nd	nd	99	nd
S. pinnata var. *inyoensis* 3	6.86	nd	nd	15	nd	82	nd
average		na	3	na	na	83	nd
standard deviation		na	3	na	na	25	nd

sample ID	NSS (10^{-4})	SeO_4	SeO_3	SeGSH2	SeCys	C-Se-C	Se^0
S. pinnata var. *pinnata* 1	4.18	nd	nd	nd	nd	101	nd
S. pinnata var. *pinnata* 1	2.87	nd	nd	nd	nd	100	nd
S. pinnata var. *pinnata* 2	5.45	nd	nd	nd	nd	84	10
S. pinnata var. *pinnata* 2	2.09	nd	4	nd	nd	95	nd
average		nd	na	nd	nd	96	nd
standard deviation		nd	na	nd	nd	7	nd
S. tomentosa 1	6.18	nd	6	nd	nd	69	24
S. tomentosa 1	5.63	nd	nd	nd	nd	85	14
S. tomentosa 1	8.9	nd	nd	nd	nd	98	nd
S. tomentosa 2	9.29	nd	nd	nd	nd	95	nd
average		nd	na	nd	nd	87	19
standard deviation		nd	na	nd	nd	13	na
S. viridiflora	4.39	nd	nd	nd	nd	101	nd
S. viridiflora	14.2	nd	nd	nd	nd	102	nd
average		nd	nd	nd	nd	101	nd
standard deviation		nd	nd	nd	nd	na	nd
T. laciniatum	12.8	nd	nd	nd	nd	97	nd
T. laciniatum	23.1	nd	46	nd	nd	52	nd
T. laciniatum	18.9	nd	25	nd	nd	72	nd
T. laciniatum	16.8	nd	21	nd	nd	75	nd
average		nd	31	nd	nd	67	nd
standard deviation		nd	13	nd	nd	19	nd

Table 3.2. Selenium speciation in seeds of different *Stanleya* taxa collected from the field. Results from least squares linear combination fitting of experimental XANES spectra with standard selenocompounds. C-Se-C: methyl-selenocysteine/Se-Methionine (same spectra). Se^0: red or gray elemental Se. NSS: normal sum of squares (quality of fit; 0 = perfect fit); nd: compound not detected. Additional standard compounds not detected in any sample: selenocystine, selenocysteine, selenoglutathione. Na: not applicable. Numbers following plant-species names denote biological replicates. Spectra with identical numbers were collected at different positions on the sample.

sample ID	NSS (10^{-4})	SeO_4	SeO_3	C-Se-C	Se^0
S. albescens	2.46	<1	2	87	12
S. albescens	2.79	2	nd	88	11
S. albescens	4.76	nd	nd	100	nd
S. albescens	4.23	nd	1	100	nd
average		1	2	94	12
standard deviation		na	na	7	na
S. bipinnata	1.63	nd	nd	101	nd
S. bipinnata	1.52	nd	nd	93	8
average		nd	nd	97	na
standard deviation		nd	nd	na	na
S. pinnata var. *integrifolia* 1	4.43	1	nd	100	nd
S. pinnata var. *integrifolia* 1	3.59	nd	2	90	9
S. pinnata var. *integrifolia 1*	3.74	nd	<1	101	nd
S. pinnata var. *integrifolia 1*	2.44	nd	nd	100	nd
S. pinnata var. *integrifolia* 2	2.44	nd	nd	100	nd
average		na	1	98	na
standard deviation		na	na	4	na
S. pinnata var. *inyoensis 1*	5.03	1	nd	99	nd
S. pinnata var. *inyoensis 1*	4.88	nd	nd	101	nd
S. pinnata var. *inyoensis* 1	5.71	nd	nd	100	nd
S. pinnata var. *inyoensis* 2	2.2	nd	nd	102	nd
S. pinnata var. *inyoensis* 2	2.9	nd	nd	100	nd
average		na	nd	100	nd
standard deviation		na	nd	1	nd
S. pinnata var. *pinnata* 1	2.8	nd	nd	100	nd
S. pinnata var. *pinnata* 1	3.78	nd	<1	69	26
S. pinnata var. *pinnata* 2	2.86	nd	2	95	4
S. pinnata var. *pinnata* 2	3.56	1	nd	100	nd
S. pinnata var. *pinnata* 2	2.58	nd	<1	101	nd
S. pinnata var. *pinnata* 2	4.89	nd	nd	100	nd
S. pinnata var. *pinnata* 2	3.28	9	4	81	14
average		5	2	92	15
standard deviation		na	2	13	11
S. tomentosa	3.24	nd	nd	101	nd
S. viridiflora	3.29	nd	nd	101	nd

Phylogeny – Stanleya was not supported as monophyletic, as currently circumscribed. *Stanleya confertiflora* was resolved as sister to the clade of *T. laciniatum* and *Th. ambigua* (Fig. 3.5; Suppl. Fig. 3.2). This result was recovered in the simultaneous analysis (Kluge, 1989) in both parsimony and likelihood (Suppl. Fig. 3.2) as well as in two gene trees (LD and SAT; Suppl. Fig. 3.3). The rest of *Stanleya* form a clade with 100% jackknife and 99% bootstrap support. Two main subclades were resolved within *Stanleya*. *Stanleya pinnata* (all varieties) and *S. bipinnata* constitute one highly supported subclade (99% jackknife, 93% bootstrap) while all other species were resolved as a separate subclade (< 50% jackknife, 91% bootstrap). *Stanleya tomentosa* and *S. viridiflora* were resolved as sister species in all analyses (Fig. 3.5, Suppl. Figs. 3.2, 3.3). Within the *S. pinnata* clade, vars. *integrifolia* and *texana* were resolved as exclusive lineages, each with >50% jackknife support. In the simultaneous analysis, *Stanleya bipinnata* was resolved as most closely related to two diploid *S. pinnata* var. *pinnata* accessions (both collected from the eastern slope of the Continental Divide). *Stanleya pinnata* var. *inyoensis* and one tetraploid *S. pinnata* var. *pinnata* (collected from the western slope of the Continental Divide) constitute another weakly supported clade. The remaining two *S. pinnata* var. *inyoensis* accessions also constitute a clade. In contrast, in the exclusively molecular phylogeny, *Stanleya bipinnata* is resolved as a clade with two diploid and one tetraploid accession of *S. pinnata* var. *pinnata* (all collected from the eastern slope of the Continental Divide).

Ancestral reconstruction – Three *Stanleya* taxa (*S. bipinnata*, *S. pinnata* vars. *integrifolia* and *pinnata*) have been documented as having Se concentrations of (or close to) >1,000 mg Se kg^1 DW in the field (Fig. 3.6a). Since these three taxa constitute a clade with *S. pinnata* var. *inyoensis* (Fig. 3.5), the origin of hyperaccumulation is ambiguously optimized for the *S. bipinnata/pinnata* clade after collapsing clades with < 50% jackknife and bootstrap support (Fig.

3.6a). If *S. pinnata* var. *texana* is indeed the sister group of the remaining members of *S. pinnata* and *S. bipinnata* (Fig. 3.5), then the most parsimonious inference is that selenium hyperaccumulation evolved within the clade after the divergence of *S. pinnata* var. *texana*. In contrast, the clade comprising of the rest of *Stanleya* is not inferred to have a hyperaccumulator ancestor (Fig. 3.6a).

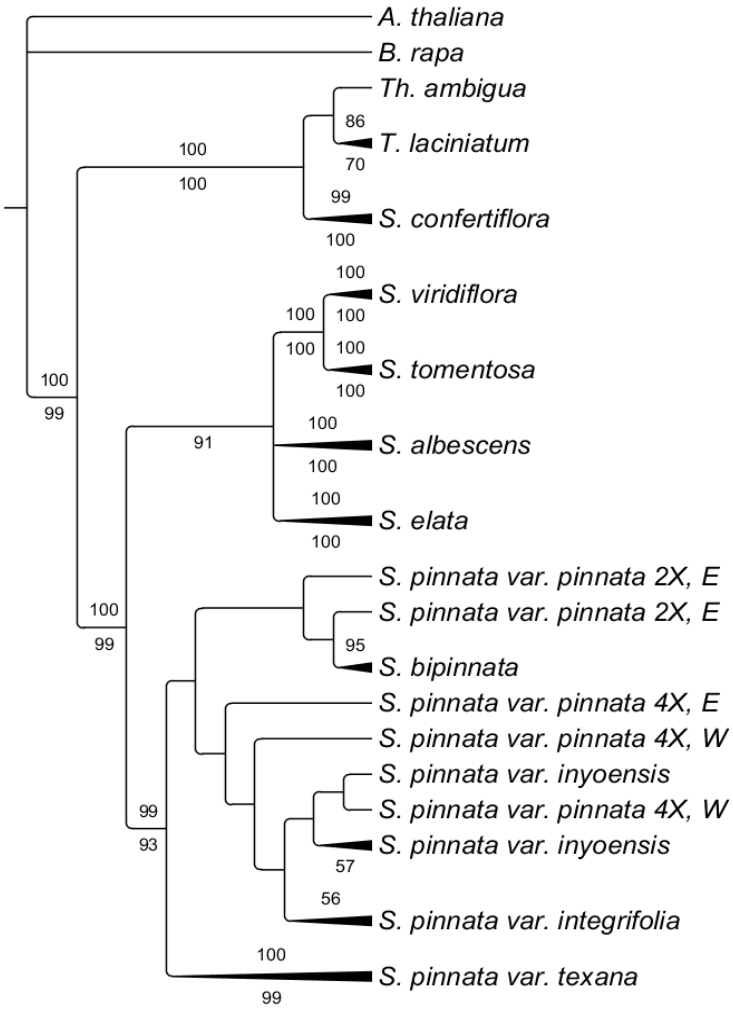

Figure 3.5. Strict consensus of three most parsimonious trees for the simultaneous analysis of both molecular and morphological data. Clade symbols represent 2-4 individuals per taxon. Values above and below the branches represent parsimony jackknife and likelihood bootstrap support values ≥ 50%, respectively. Values next to *S. pinnata* var. *pinnata* accessions indicate diploid (2X) or tetraploid (4X) and collection site east slope (E) or west slope (W).

Table 3.3. Data-matrix and tree statistics for each of the phylogenetic analyses. "CI" = ensemble consistency index (Kluge and Farris, 1969) on the most parsimonious tree(s) for the parsimony-informative characters. "RI" = ensemble retention index (Farris, 1989).

Matrix	# terminals	# characters analyzed	# of parsimony informative characters	% missing / inapplicable	Most parsimonious tree length	# of most parsimonious trees	# of jackknife / bootstrap clades ≥ 50%	Average jackknife / bootstrap support (%)	CI	RI
CHS	35	824	46	3.5	163	3296	8/9	77/55	0.97	0.98
ITS	39	713	62	7.6	211	21202	9/11	94/58	0.71	0.87
LD	32	626	76	12.1	173	9	12/9	82/79	0.91	0.96
SAT	33	621	40	11.6	131	56	14/11	78/60	0.88	0.95
all molecular	39	2784	224	17.7	696	297	15/17	75/74	0.8	0.9
morphological	14	15	14	0.1	42	6	1	57	0.73	0.71
simultaneous	39	2799	238	17.6	745	3	17	64	0.78	0.9

Based on the results from our agar experiment, most of the taxa we sampled are tolerant to hyperaccumulator Se levels (>1,000 mg kg^{-1} DW; Fig. 3.6b); Se sensitive exceptions include the most distant outgroup (*A. thaliana*) as well as the *S. viridiflora/S. tomentosa* clade and *S. pinnata* var. *inyoensis.* Three taxa showed no significant growth reduction by Se, at tissue Se levels upwards of 2,000 mg kg^{-1} DW. These include the Se hyperaccumulators *S. pinnata* var. *pinnata* and *S. bipinnata* as well as *S. albescens.* Thus, Se tolerance at the ≥ 1,000 mg kg^{-1} DW level is unambiguously optimized as the ancestral state for the entire *Stanleya/Thelypodium/Brassica* clade. There appears to be an additional level of hypertolerance at the 2,000 mg kg^{-1} DW level in the two most extreme Se hyperaccumulator taxa (*S. bipinnata* and *S. pinnata* var. *pinnata*). High survivability was found in all *Stanleya* taxa (Fig. 3.6c). But was not found in *T. laciniatum*. From these combined ancestral reconstructions of Se tolerance/survivability and accumulation we infer that Se tolerance and high survivability in Se environments evolved prior to Se accumulation in *Stanleya*.

Discussion

The primary question addressed in this study is: what is the evolutionary history of Se hyperaccumulation in *Stanleya*? This includes: how many times has hyperaccumulation evolved and been lost, and did hyperaccumulation and tolerance evolve simultaneously in *Stanleya* or did one precede the other? Our results show that Se hyperaccumulation (>1,000 mg Se kg^{-1} DW *in situ*) is restricted to the *S. bipinnata/pinnata* clade (Fig. 3.6a). Based on the collapsed tree shown in Fig. 3.6a, there are two alternative parsimony reconstructions, each with three steps. Either hyperaccumulation evolved on the branch leading to the *S. bipinnata/pinnata* clade and was lost independently in *S. pinnata* vars. *inyoensis* and *texana*, or there were three independent origins of hyperaccumulation in *S. bipinnata* and *S. pinnata* vars. *integrifolia* and *pinnata.* To fully

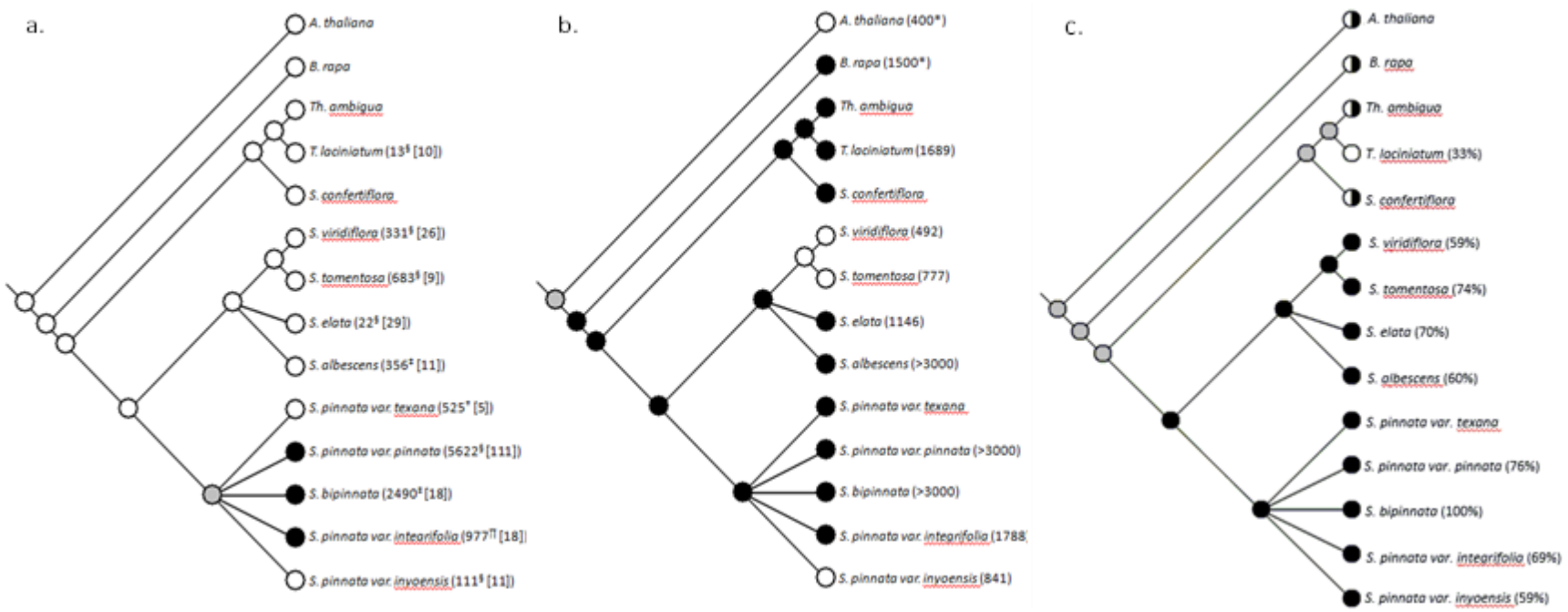

Figure 3.6. Se-related traits mapped onto the collapsed, simultaneous-analysis phylogeny using parsimony. a. accumulation (maximum field Se level [n]) b. tolerance (50% inhibition as mg Se kg^{-1} DW) c. Survival (percent survival averaged across all three Se treatments). Light gray = ambiguous; white = unknown, non-hyperaccumulator, sensitive and <50% survival; black = hyperaccumulator, tolerant and >50% survival; bicolor = unknown and not predicted. *Estimated from Garifullina et al., 2003; Van Huysen et al., 2003; Zhang et al., 2007, § Cappa et al., 2014, † Beath et al., 1939b, ‡ Beath et al., 1940, ∏ Beath et al., 1941.

answer this question the relationships within the *S. bipinnata/pinnata* clade need to be fully resolved. Based on the relationships within that clade shown in Fig. 3.5, wherein *S. pinnata* var. *texana* is sister to the remaining members of this clade, hyperaccumulation is most parsimoniously inferred to have a single origin within this clade and was followed by one loss in *S. pinnata* var. *inyoensis*. Among the non-Se hyperaccumulator taxa, all but one are classified as secondary Se accumulators based on the maximal field Se levels reported (>100 mg kg^{-1} DW, Fig. 3.6a). *Stanleya elata* is classified as a non-Se accumulator, together with outgroup *T. laciniatum*.

Tolerance to Se occurs broadly in the *Stanleya/Thelypodium/Brassica* clade (Fig. 3.6b). While high survivability occurs within *Stanleya* (Fig. 3.6c). The ancestral reconstruction of tolerance, as scored here, unambiguously indicates that tolerance evolved before hyperaccumulation in *Stanleya*. Based on our results, tolerance to tissue Se levels above 1,000 mg kg^{-1} DW is more prevalent in *Stanleya* than the capacity to actually attain these hyperaccumulator levels in the field. While Se tolerance may be a prerequisite for hyperaccumulation, it is not always predictive of it. Based on this reconstruction, we infer that the *S. viridiflora/tomentosa* clade lost tolerance to high Se concentrations. Interestingly, in both of these species the maximum field Se level recorded was just below their 50% inhibition concentration.

Our inferences about Se tolerance levels are based on the agar study presented here (Fig. 3.1), which currently is the only common-garden experiment where the growth of most *Stanleya* taxa (excluding *S. confertiflora* and *S. pinnata* var. *texana*) were compared in the presence and absence of Se. While agar experiments are commonly used to assess the tolerance index to toxic elements, it is possible that alternative experimental systems (e.g. on hydroponics or soil) or

testing more mature plants may give different results. For instance, in one of our earlier studies *S. albescens* was significantly less tolerant to Se than *S. pinnata* var. *pinnata* (Freeman *et al*., 2010). Moreover, growth of *S. elata* has been shown in our earlier studies to be 50% inhibited at tissue Se concentrations well below 1,000 mg kg^{-1} DW, which would classify it as Se sensitive in this study (El Mehdawi et al., 2012; Lindblom et al., 2014).

The main tolerance mechanism in Se hyperaccumulators is to store Se in the form of non-protein amino acids, as reviewed in the introduction. Indeed, the Se hyperaccumulator taxa all stored Se in the form of organic C-Se-C compounds (Tables 3.1 and 3.2). The outgroups, *A. thaliana* and *B. juncea*, were shown in earlier studies to accumulate predominantly inorganic selenate when supplied with selenate (de Souza et al., 1998; Van Hoewyk et al., 2005; Freeman et al., 2006). A close relative of *Stanleya*, *T. laciniatum,* had the lowest percentage of organic Se (65%) for all species tested here. All *Stanleya* taxa tested accumulated at least 80% of Se in the form of C-Se-C compounds. This suggests that *Stanleya* has evolved an increased capacity to convert selenate to organic C-Se-C, as compared to related genera. Despite the finding that all tested *Stanleya* taxa contained predominantly C-Se-C compounds, they showed variation in Se tolerance. The reason for this variation could be that they contained different C-Se-C compounds. In *S. albescens*, for instance, the C-Se-C was found to be selenocystathionine, while in *S. pinnata* var. *pinnata* it was methyl-selenocysteine (Freeman et al., 2006, 2010). Overall, the results from these XAS studies indicate that production of organic Se compounds is not necessarily predictive of tolerance or hyperaccumulation, but it may be a step in the evolution of Se hyperaccumulation, and perhaps a prerequisite.

In the ancestral reconstruction of Se hyperaccumulation and tolerance discussed above, *S. pinnata* var. *pinnata* was treated as a single lineage, since there was <50% support for resolution

of the different accessions (Fig. 3.5). As described earlier (Cappa et al., 2014), *S. pinnata* var. *pinnata* contains both diploid and tetraploid accessions, with all diploids occurring east of the Continental Divide and all but one tetraploid accessions occurring west of the Continental Divide. Hyperaccumulation was found exclusively in diploid accessions, with one exception: a tetraploid found close to the lowest point of the Continental Divide (Great Divide Basin, Wyoming). It is intriguing that there are two ploidy levels in *S. pinnata*, and tempting to hypothesize that ploidy levels correlate with hyperaccumulation capacity. However, when tested under controlled conditions, *S. pinnata* var. *pinnata* can reach hyperaccumulator levels regardless of its ploidy level or geographic origin (Harris et al., 2014; Suppl. Fig. 3.4). In addition, all *S. pinnata* varieties tested, appear to have hyperaccumulation capacity when provided with selenate in a controlled environment (Suppl. Fig. 3.4), but whether these taxa actually hyperaccumulate in the field appears to depend not only on this innate capacity but also on the environment. A similar division may be the case for *S. bipinnata*. Beath et al. (1940) reported *S. bipinnata* with tissue Se concentrations of up to 2,490 mg Se kg^{-1} DW in a population outside Laramie, WY, on the eastern side of the Continental Divide. Unfortunately the population does not exist anymore due to development, and could not be resampled for our study. All of the *S. bipinnata* collected by Cappa et al. (2014) were west of the continental divide, and none reached hyperaccumulator concentrations *in situ*. But these accessions did have hyperaccumulation capacity when provided with selenate in a controlled environment (Fig. 3.1).

The uncollapsed phylogeny of *Stanleya* (Fig. 3.5) resolves *S. bipinnata* nested within *S. pinnata*, and most closely related to the Se hyperaccumulating diploids of *S. pinnata* var. *pinnata.* Thus, *S. bipinnata* may actually be a variety of *S. pinnata* as asserted by Rollins (1939). If this clade continues to be supported in a more extensive phylogenetic analysis, with increased

character sampling, we hypothesize that Se hyperaccumulation evolved within this clade, after the divergence of *S. pinnata* var. *texana* (which is highly supported as distinct from all other member of *S. pinnata*). The other subclade within the *S. pinnata/bipinnata* clade (Fig. 3.5) is composed of all *S. pinnata* tetraploid accessions, including the tetraploid lineages of *S. pinnata* var. *pinnata*, and varieties *integrifolia* and *inyoensis*. The 2X and 4X *S. pinnata* var. *pinnata* accessions are separated from each other into two sister clades in the simultaneous analysis (Fig. 3.5). However, the tetraploid population from the Eastern Slope was resolved as most closely related to the diploid accessions from the eastern slope in the molecular only analysis (Suppl. Fig. 3.2), albeit with low support. Based on these conflicting results the *S. pinnata* clade needs further study. Another taxonomic consideration from this study is that *S. confertiflora* is clearly not a member *Stanleya sensu stricto*. Based on our sampling, it cannot be determined which other genus it should classified as; this will require more study. Cacho et al. (2014) showed two species of *Stanleya* (*S. pinnata* and *S. elata*) to be nested within *Streptanthus* and sister to species of *Caulanthus*. The monophyly of *Stanleya*, as resolved here, needs to be more rigorously tested, with greater taxonomic sampling, across the Thelypodieae.

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Chapter 4: Comparative transcriptomics reveal key genes involved in selenium hyperaccumulation in *Stanleya pinnata* (Brassicaceae)

Summary

Selenium (Se) hyperaccumulation convergently evolved in six different flowering plant families. It is characterized by extraordinary levels of tolerance to, and accumulation of, Se, which it sequesters in organic forms. The genetic basis of selenium (Se) hyperaccumulation is still poorly understood in any clade, and may involve modification of sulfur (S) assimilation, as Se and S are chemically similar. To obtain better insight into the importance of S assimilation processes in Se hyperaccumulation in *Stanleya pinnata* (Brassicaceae), its transcriptome was compared to closely related non-hyperaccumulator *Stanleya elata*. Transcriptome libraries were created and sequenced in triplicate from roots and shoots of plants grown in the presence or absence of 20 μM selenate. Relative to *S. elata*, *S. pinnata* showed higher transcript levels for many of the enzymes involved in sulfate/selenate transport and assimilation, particularly in roots of Se-treated plants. Two genes stood out as most highly and most differentially expressed between the species. The first, a homolog of a high-affinity root plasma membrane sulfate transporter *ASsultr1;2*, was present at up to 30-fold higher transcript levels in *S. pinnata* than *S. elata*. The second is a homolog of ATP sulfurylase 2 (*APS2*), the key enzyme of the sulfate assimilation pathway, which was expressed at up to 300-fold higher levels in the hyperaccumulator. These results suggest that *S. pinnata* assimilates Se predominantly in its root and that extraordinarily high *sultr1;2* and *APS2* root expression levels are key genetic mechanisms underlying the trait of Se hyperaccumulation in this species.

Introduction

The central dogma of biology states that DNA is transcribed into RNA, which is translated into protein. In trying to understand and catalogue changes that occur at these various levels of organization we can gain insight into the molecular reactions that species have when their environment changes. Recent advances in sequencing technology have allowed for new frontiers in biology via RNASeq because it is more sensitive than macroarrays (Cloonan and Grimmond, 2008) and more cost effective than traditional Sanger sequencing. This method has been applied to many questions including nutritional differences in catfish (Li et al. 2014), embryogenesis under different temperatures in spruce (Yakovlev et al. 2014), and the affect of potassium starvation in watermelon (Fan et al. 2014) by looking at how different environments effect the RNA pool. We applied this method to better understand how *Stanleya pinnata* (Brassicaceae) hyperaccumulates and tolerates high levels of selenium (Se).

Elemental hyperaccumulation is an interesting trait found in at least 515 flowering plant species. These species can concentrate one or more toxic elements to levels two orders of magnitude higher (0.01-1%) than most other plant species. Hyperaccumulated elements include arsenic, cadmium, cobalt, lead, nickel, Se and zinc (Krämer, 2010). Several studies have addressed the question of why plants hyperaccumulate and reported convincing evidence in support of the elemental-defense hypothesis, which states that hyperaccumulated elements serve as protection for the plant against herbivores and pathogens (Boyd and Martens, 1992). Thus, herbivory may have served as the primary selective pressure for elemental hyperaccumulation. Other studies (e.g. Hanikenne et al., 2008; Cracuin et al., 2012) have investigated the mechanisms of elemental hyperaccumulation, which in several cases provided evidence for increased metal/metalloid transporter activity or abundance.

Increased gene-copy numbers were found for heavy metal binding (HMA) transporters in several hyperaccumulators (Ueno et al., 2003; Hanikenne et al., 2008; Craciun et al., 2012). In the Zn and Cd hyperaccumulator *Arabidopsis halleri* (Brassicaceae), there are three tandem copies of metal ATPase 4 (*HMA4*) relative to the non-accumulator *A. thaliana* (Hanikenne et al., 2008). In the Zn, Ni and Cd hyperaccumulator *Noccaea caerulescens* (formerly *Thlaspi caerulescens*; Brassicaceae) both *HMA4* (Craciun et al., 2012) and *HMA3* (Ueno et al., 2003) have been shown to have variable copy numbers. In populations that differ in Cd accumulation and tolerance, copy number positively correlated with higher accumulation and tolerance (Craciun et al., 2012). In addition to *HMA* transporters, the *ZIP* family (*ZRT, IRT*-like proteins) transporters, involved in the transport of Fe, Zn, Mn and Cd, have been shown to be more highly expressed in *A. halleri* and *N. caerulescens* than in *A. thaliana* and *Thlaspi arvense*, respectively (Assunção et al., 2001; Bechner et al., 2004; Weber et al., 2004).

The element of particular interest for this study is selenium (Se), which is hyperaccumulated to levels upwards of 0.1% in *S. pinnata* and several *Astragalus* (Fabaceae) species. Selenium is different from the metals already discussed, because of its similarity to sulfur (S). Because of this similarity, Se can be assimilated into organic forms, including amino acids and proteins. Selenium is especially interesting because it is not an essential nutrient for vascular plants, but it is an essential micronutrient for animals, some prokaryotes and algae (Terry et al., 2000). In phyla that require Se, it is used in the form of selenocysteine (SeCys) and found in the active site of a small group of proteins that have redox functions. Selenocysteine is considered the 21st protein amino acid. It is encoded by the UGA stop codon and inserted via a 3' UTR insertion sequence (Shen et al., 1993; Fu et al., 2002). Because of the close similarity between Se and S, the study of Se hyperaccumulation is commonly associated with S

metabolism, and Se hyperaccumulation mechanisms likely involve modification of S transporters and S metabolic processes.

Sulfur assimilation is thought to primarily happen in the chloroplasts in the leaves of vascular plants. Sulfate transporter (sultr)1;2 has been shown to be the main transporter responsible for selenate import into the roots of the model species *Arabidopsis thaliana*. Damage to *sultr1;2* conferred selenate resistance in *A. thaliana* by reducing symplastic accumulation of Se (Shibagaki et al., 2002; Ohno et al., 2012). After root uptake, sulfate is thought to be transported to the shoot by means of additional sulfate transporters where, through a series of enzymatic reactions, the sulfate is reduced to sulfide in the chloroplast and added to O-acetylserine (OAS) to produce cysteine. Cysteine can be converted to methionine or incorporated into various other reduced S compounds such as glutathione (for review see Takahashi et al., 2011). The current paradigm is that Se is inadvertently taken up by most plants species and that these plants assimilate the Se via the S pathway into SeCys (Terry et al., 2000). This SeCys is toxic if it is mis-incorporated into protein in place of cysteine (Terry et al., 2000). The selenium hyperaccumulator *Astragalus bisulcatus* has been shown to have a selenocysteine methyltransferase (SMT) enzyme that can methylate SeCys to methyl-SeCys, preventing said toxicity (Virupaksha and Shrift, 1965; Neuhierl and Böck, 1996). A similar mechanism may be used by *S. pinnata*: it was found to contain predominantly organic Se, of which 80% was methyl-SeCys and 20% Se-cystathionine (Freeman et al., 2006). Indeed, Freeman et al. (2010) used anti-SMT antibodies and found a protein band to be more highly expressed in *S. pinnata* than in non-hyperaccumulator relative *Stanleya albescens*. As another tolerance mechanism, Se hyperaccumulators may use tissue-specific sequestration in epidermal tissue: the Se in *A.*

bisulcatus was found mainly in the leaf hairs, while in *S. pinnata* it was located along the leaf margins, in vacuolar compartments in the epidermis (Freeman et al., 2006, 2010).

The mechanisms responsible for the extreme Se hyperaccumulation trait remain to be elucidated. In view of the similarity of selenate to sulfate, likely candidates responsible for the enhanced Se uptake in Se hyperaccumulators are the sulfate transporters. Freeman et al. (2010) indeed demonstrated several homologs of *Arabidopsis* sulfate transporters to be constitutively upregulated in Se hyperaccumulator *S. pinnata* relative to non-hyperaccumulator *S. albescens*, in a macroarray experiment. In addition to enhanced expression levels of sulfate/selenate transporters, Se hyperaccumulators may have evolved transporters with different kinetic properties with respect to sulfate and selenate. Selenium hyperaccumulators appear to preferentially take up Se over S, since there typically is a higher Se/S ratio in their tissues than in their growth medium (Parker et al., 2003; White et al., 2007). Moreover, selenate uptake was not significantly inhibited by high sulfate concentrations in *S. pinnata*, in contrast to non-hyperaccumulator relatives, which indicates that *S. pinnata* has a selenate-specific transporter (Harris et al., 2014).

In this study, we tested *S. pinnata* against the less tolerant, non-hyperaccumulating close relative *S. elata*. We generated 24 transcriptome libraries. These libraries were constructed in triplicate from root and shoot organs of plants given plus and minus selenate treatments. The main question addressed in this study is: how is the hyperaccumulator species *S. pinnata* different from its close relative and non-hyperaccumulator species, *S. elata* at the transcriptional level? As a first step in analyzing the transcriptome data we focused our attention on the S assimilation pathway and sulfate transporters, to explore to what extent changes in S metabolism may explain Se hyperaccumulation and tolerance in *S. pinnata*.

Materials and methods

Plant growth – Seeds of *S. pinnata* (Western Native Seed, Coaldale, CO) and *S. elata* (NV, 36°16'36"N 115°30'12"W) were surface-sterilized followed by a 4°C treatment for 48 hours before being transferred to sterile petri dishes. Once seeds germinated they were transferred to ½ strength Murashige and Skoog (1962) agar on 0 or 20 µM sodium selenate. Plants were grown for 30 days at a light intensity of 150 µE with a 16/8 light period at 23ºC in a growth chamber. For each treatment we had a total of nine plants—three closed containers each with three plants per container.

One plant per container was harvested and its roots rinsed to remove any external Se. These plants were separated into root and shoot and flash frozen for transcriptome analysis (as described below). The remaining two plants from each container were harvested, the roots rinsed and dried at 50°C for 72 hours before being weighed and nitric-acid digested following Zarcinas et al. (1987). The digest was analyzed via inductively coupled plasma atomic emission spectroscopy (ICP-AES; Fassel 1978). ANOVA with Tukey-Kramer post hoc analyses were carried out to test for significant differences between species in R (version 2.15.1).

RNA sequencing – Frozen plants were shipped to the University of Missouri where total RNA was extracted using an Invitrogen RNA Mini Kit. The resulting RNA was transformed into an Illumina library via a TruSeq RNA Kit. The resulting libraries were sequenced on an Illumina HiSeq-2000 at the DNA Core facility at the University of Missouri. Eight initial libraries were 100 bp paired-end sequenced: one library for each of our treatments (two species, two Se treatments, two organs) using one lane. The remaining 16 libraries were 50 bp single-end sequenced using three additional lanes.

The initial paired-end 100 bp libraries were trimmed and quality filtered in NextGENe ver. 2.17 (SoftGenetics, State College, PA, USA) and *de novo* assembled in Trinity (Grabherr et al. 2011) using default parameters. After filtering and assembly any sequence less than 40 bp in length was removed. The additional 50 bp reads were aligned to the *de novo* assembly using a criterion of 95% sequence similarity. Statistical analyses to test for significant differences between Se treatments within species were conducted in R using EdgeR ver. 3.2.4 and DESeq ver. 1.12.1. Both analyses used the negative binomial distribution to test for significant differences in expression. EdgeR uses an empirical Bayes estimate while DESeq chooses a model based on the mean and variance of the data (Anders and Huber, 2010). Assembled reads were annotated using BLASTn against the *Arabidopsis thaliana* cDNA transcript list and assigned putative homologs with an e-value cutoff of 0.00005.

RNA-seq analysis – Annotated transcripts were first filtered for low transcript abundance (reads per kilobase per million; RPKM) values. Any transcript with <0.1 RPKM for both treatments was removed from the analysis. For comparisons between species, each transcript was aligned to the *A. thaliana* homolog and (when available) the *Brassica* homolog in MAFFT ver. 7 (Kotah & Toh 2008) using E-INS-I and the 1PAM nucleotide scoring matrix and the default gap opening penalty (1.53). This was to verify that we were comparing homologous genes between the *Stanleya* species; poorly aligned sequences, having multiple gaps and low sequence similarity, were removed from the analysis. Typically there were multiple (3-4 on average) transcript contiguous sequences (contigs) that were annotated as the same gene. These were compared for expression patterns. If contig sequences differed in expression patterns (e.g. one contig was upregulated and one contig was downregulated) then they were treated separately. Contig sequences annotated to the same gene and showing similar expression patterns were

pooled by summing the RPKM values. To test for significant differences between species, Student's t-test was applied to the summed RPKM values of homologous transcripts, one treatment at a time.

Results

Se accumulation – After one month of growth on selenate-containing agar medium, *S. pinnata* had accumulated 3-fold higher shoot Se levels than *S. elata* (Fig. 4.1). The concentration of Se in the 0 μM Se treatment for *S. pinnata* is similar to the 20 μM treatment of *S. elata*, indicating that the (wild-collected) seeds of *S. pinnata* contained Se. There was also a generally higher sulfur (S) concentration in *S. pinnata* relative to *S. elata*, which was only significant between Se-treated *S. pinnata* and non-Se treated *S. elata* (Fig. 4.1).

Transcriptome – A total of 205,543 transcripts were assembled. After transcripts less than 300 bp were removed, the dataset included 101,875 transcripts, totaling 60,467,644 bp. Most transcripts greater than 4 kb were mitochondrial or plastid contamination. These include our 393 longest assembled transcripts. Of our 101,875 transcripts excluding the >4 kb contaminants, our average sequence length was 594 bp. To ensure the datasets between species were comparable we graphed all reads and determined the average number of transcripts across replicates, organs and treatments (Suppl. Figs. 4.1, 4.2). In non-Se treated roots the average number of reads across bioreplicates was 12.4 in *S. pinnata* and 12.8 in *S. elata*. However, in Se-treated roots the average number of reads was 12.7 in *S. pinnata* and 5 in *S. elata*. In the shoots, the averages were similar between species for both treatments. The average number of reads in *S. pinnata* was 13.9 and 13.8 and in *S. elata* 14.8 and 14.9 in non-Se and Se treated shoots, respectively.

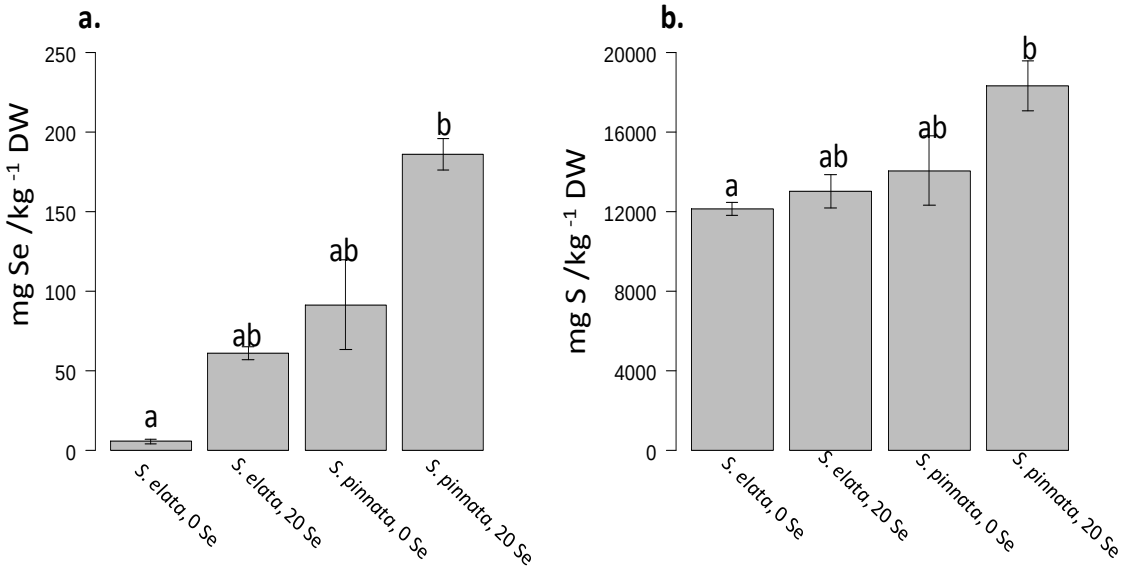

Figure 4.1. Selenium and sulfur accumulation in *Stanleya pinnata* and *Stanleya elata*. Plants were grown for one month in ½ Murashige and Skoog agar medium containing either 0 or 20 µM sodium selenate. Letters above bars (SEM) indicate significant differences between treatments (n = 5) via an ANOVA with a Tukey-Kramer post hoc analysis. a. Selenium accumulation b. Sulfur accumulation

Sulfate/selenate transporter genes – The homolog of *A. thaliana* sulfate transporter *sultr1;2*, responsible for uptake of sulfate/selenate into the root (Takahashi et al., 2011), was shown to have much higher transcript abundance in *S. pinnata* relative to *S. elata* (Table 4.1, Fig. 4.2). When grown in the absence of Se there was a 14-fold higher *sultr1;2* abundance in the roots of *S. pinnata* than *S. elata* (Fig. 4.3), and in the presence of Se the difference in expression was 30-fold (Fig. 4.4). Selenium did not significantly affect *sultr1;2* RPKM levels in either species. With RPKM values around 650, the *S. pinnata sultr1;2* was by far the most highly expressed sulfate transporter in either species (Table 4.1). In shoots, *sultr1;2* transcript levels were much lower than in roots for both species; as in roots, *S. pinnata* contained higher *sultr1;2* levels than *S. elata*, both with and without Se (Table 4.1).

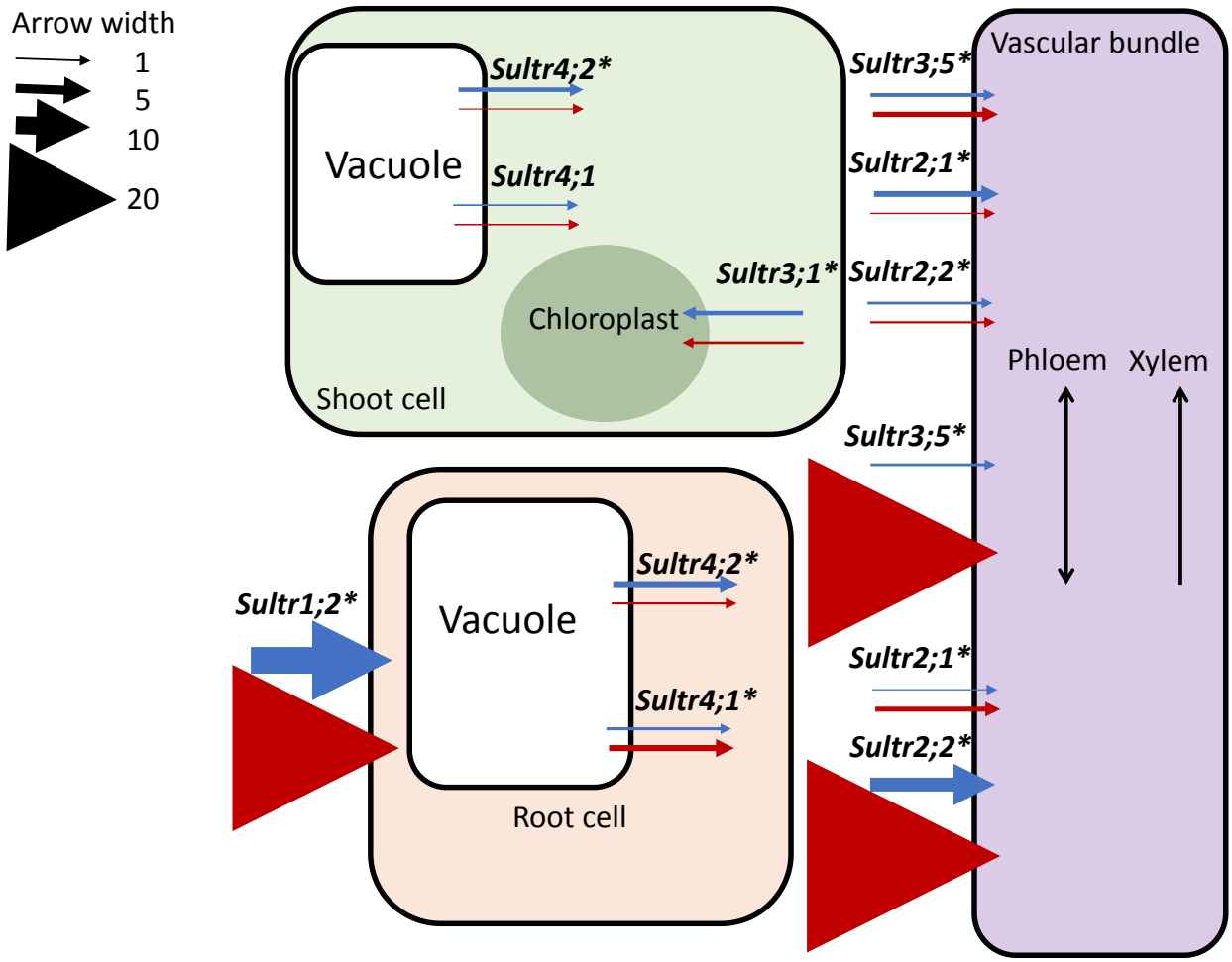

Figure 4.2. Inferred transport across membranes of sulfate/selenate throughout the plant. Width of arrows is based on the ratio of *S. pinnata* RPKM over *S. elata* RPKM for same gene transcript, treatment, and organ. Blue arrows are for plants grown without Se and red arrows for plants grown with 20 μM SeO_4^{2-}. Stars indicate significant differences between species (t-test, $p<0.05$).

The sulfate transporter homolog with the greatest transcript abundance in the shoot of both *S. pinnata* and *S. elata* was *sultr3;1*, i.e. the chloroplastic sulfate importer (Cao et al., 2013). The shoot expression level of *sultr3;1* was higher in *S. pinnata* than in *S. elata*, both in the absence and presence of Se (Table 4.1). In both species the shoot *Sultr3;1* transcript levels were lower in the presence of Se than in its absence (Table 4.1).

Table 4.1. Transcript levels for homologues of sulfate/selenate transporters in roots and shoots of hyperaccumulator *S. pinnata* and non-accumulator *S. elata* grown the presence or absence of 20 μM sodium selenate. The values are mean RPKM ± Standard error of mean (SEM) (n = 3) for species rows. The ratio is *S. pinnata* RPKM over *S. elata* RPKM. Stars indicate significant differences between species (t-test, $p<0.05$).

	sultr1;2	*sultr2;1*	*sultr2;2*	*sultr3;1*	*sultr3;2*	*sultr3;3*	*sultr3;4*	*sultr3;5*	*sultr4;1*	*sultr4;2*
Roots 0μM Se										
S. pinnata	619 ± 9	112 ± 2	23 ± 1	19 ± 1	19 ± 0.3	3 ± 0.5	70 ± 2	42 ± 1	124 ± 1	10 ± 1
S. elata	42 ± 2	156 ± 2	3 ± 0.3	102 ± 1	13 ± 1	6 ± 0.5	35 ± 0.7	27 ± 0.4	74 ± 3	3 ± 0.4
ratio	14.7*	0.7*	7.7*	0.2*	1.5*	0.5*	2*	1.6*	1.7*	3.3*
Roots 20μM Se										
S. pinnata	676 ± 15	195 ± 4	32 ± 0.3	27 ± 1	11 ±0.6	5 ± 0.2	57 ± 0.5	70 ± 1	142 ± 1	16 ± 0.4
S. elata	22 ± 1	71 ± 1	0.9 ± 0.3	15 ± 0.3	3 ± 0.2	2 ± 0.3	11 ± 1	2 ± 0.2	43 ± 2	13 ± 1
ratio	30.7*	2.7*	35.6*	1.8*	3.7*	2.5*	5.2*	35*	3.3*	1.2*
Shoots 0μM Se										
S. pinnata	16 ± 1	164 ± 1	39 ± 2	298 ± 6	8 ± 0.1	28 ± 0.6	17 ± 0.1	35 ± 1	67 ± 3	5 ± 1
S. elata	11 ± 1	51 ± 1	26 ± 1	158 ± 2	3 ± 0.3	86 ± 2	24 ± 1	16 ± 1	70 ± 3	2 ± 0.1
ratio	1.5*	3.2*	1.5*	1.9*	2.7*	0.3*	0.7*	2.2*	1.0	2.5*
Shoots 20μM Se										
S. pinnata	10 ± 1	80 ± 1	34 ± 1	189 ± 2	7 ± 0.1	21 ± 0.6	7 ± 0.3	41 ± 0.5	52 ± 1	3 ± 0.1
S. elata	8 ± 0.5	122 ± 3	28 ± 1	140 ± 2	3 ± 0.1	57 ± 1	27 ± 0.5	14 ± 1	64 ± 1	5 ± 0.1
ratio	1.3*	0.7*	1.2*	1.4*	2.3*	0.4*	0.3*	2.9*	0.8*	0.6*

Sulfate transporters 2;1, 2;2 and 3;5 co-facilitate the loading of xylem and phloem in root and/or shoot, and thus are involved in root-to-shoot translocation and source-to-sink remobilization. When grown without Se, *S. pinnata* had lower root and higher shoot RPKM levels for *sultr2;1* than *S. elata*, whereas in the presence of Se this pattern was reversed (Table 4.1). The two species showed opposite *sultr2;1* responses to Se: in *S. pinnata sultr2;1* was up-regulated in the root and down-regulated in the shoot in the presence of Se, whereas *S. elata* showed the opposite pattern. *Sultr2;2* was expressed more highly in *S. pinnata* than *S. elata*, particularly in roots of Se-treated plants, where the difference was 35-fold (Table 4.1). *Sultr3;5* was also present at significantly higher transcript levels in *S. pinnata* compared to *S. elata*, in root and shoot, both with and without Se. Here, too, the maximal difference between the species was 35-fold in roots of Se-treated plants, where *sultr3;5* was upregulated in *S. pinnata*, but downregulated in *S. elata*.

The two group 4 sulfate transporters (*sultr4;1*, *sultr4;2*), responsible for sulfate/selenate efflux from the vacuole, were present at significantly higher RPKM values in the roots of *S. pinnata* compared to *S. elata* in both Se treatment groups. In the shoots there were no substantial differences in this respect.

Transcripts of other transporters from group 3, whose functions are not known, were also found to be present at different levels in *S. pinnata* and *S. elata*. First, *Sultr3;2* was present at higher RPKM levels in *S. pinnata* than *S. elata* for all treatments and organs. Furthermore, *S. pinnata sultr3;3* and *sultr3;4* RPKM levels were higher in root but lower in shoot than *S. elata*, regardless of Se treatment (Table 4.1).

Sulfate/selenate assimilation genes – The first enzyme in the pathway from sulfate to cysteine, ATP sulfurylase (APS), is responsible for the activation of sulfate/selenate by coupling

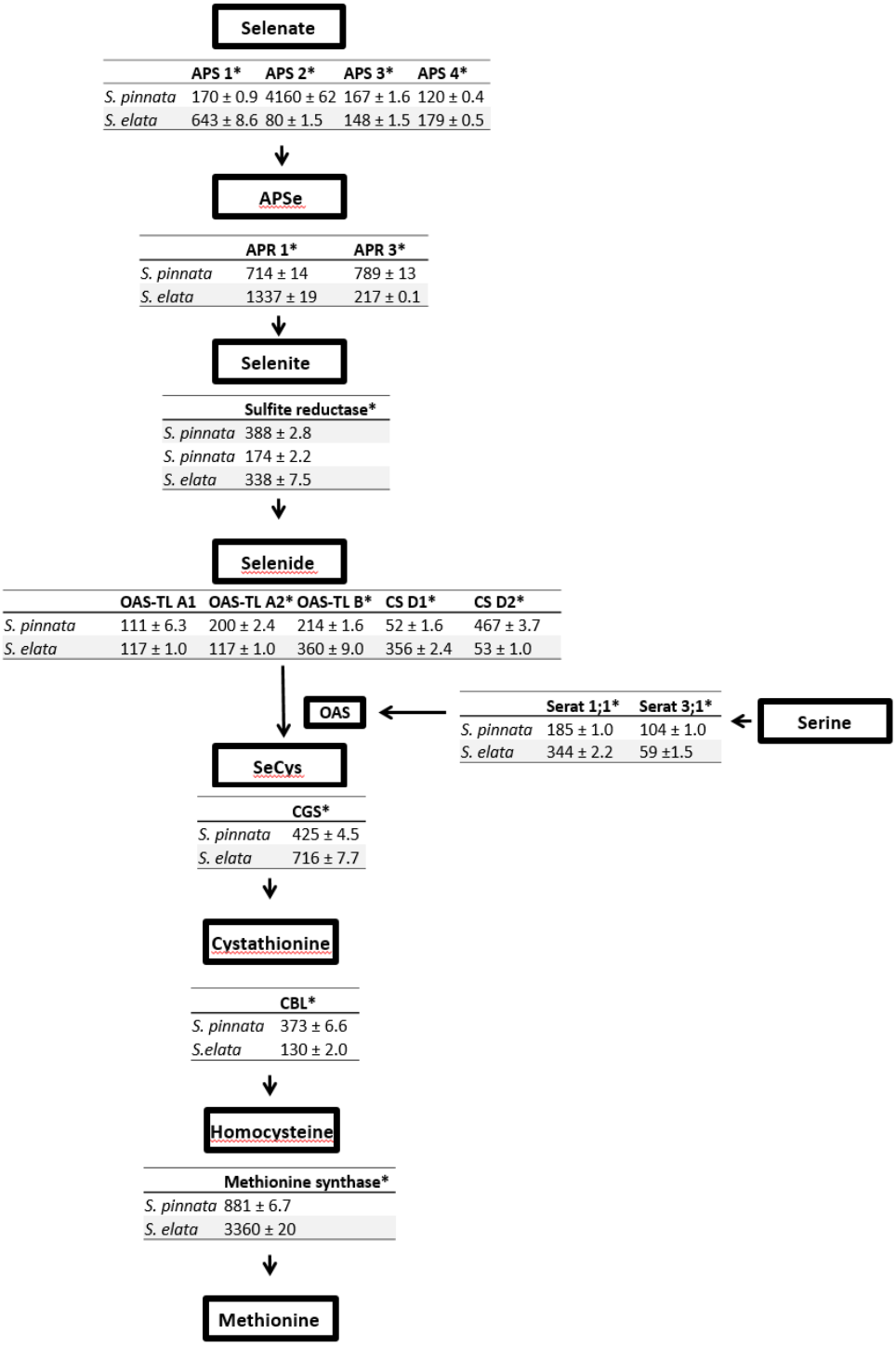

Figure 4.3. Transcript levels of S/Se assimilation pathway genes in 0 µM Se treated roots of *S. pinnata* and *S. elata*. All gene family members are shown that fit the species comparison criteria (materials and methods). The values are mean RPKM and SEM (n = 3). Stars denote significant differences between species (t-test, $p<0.05$).

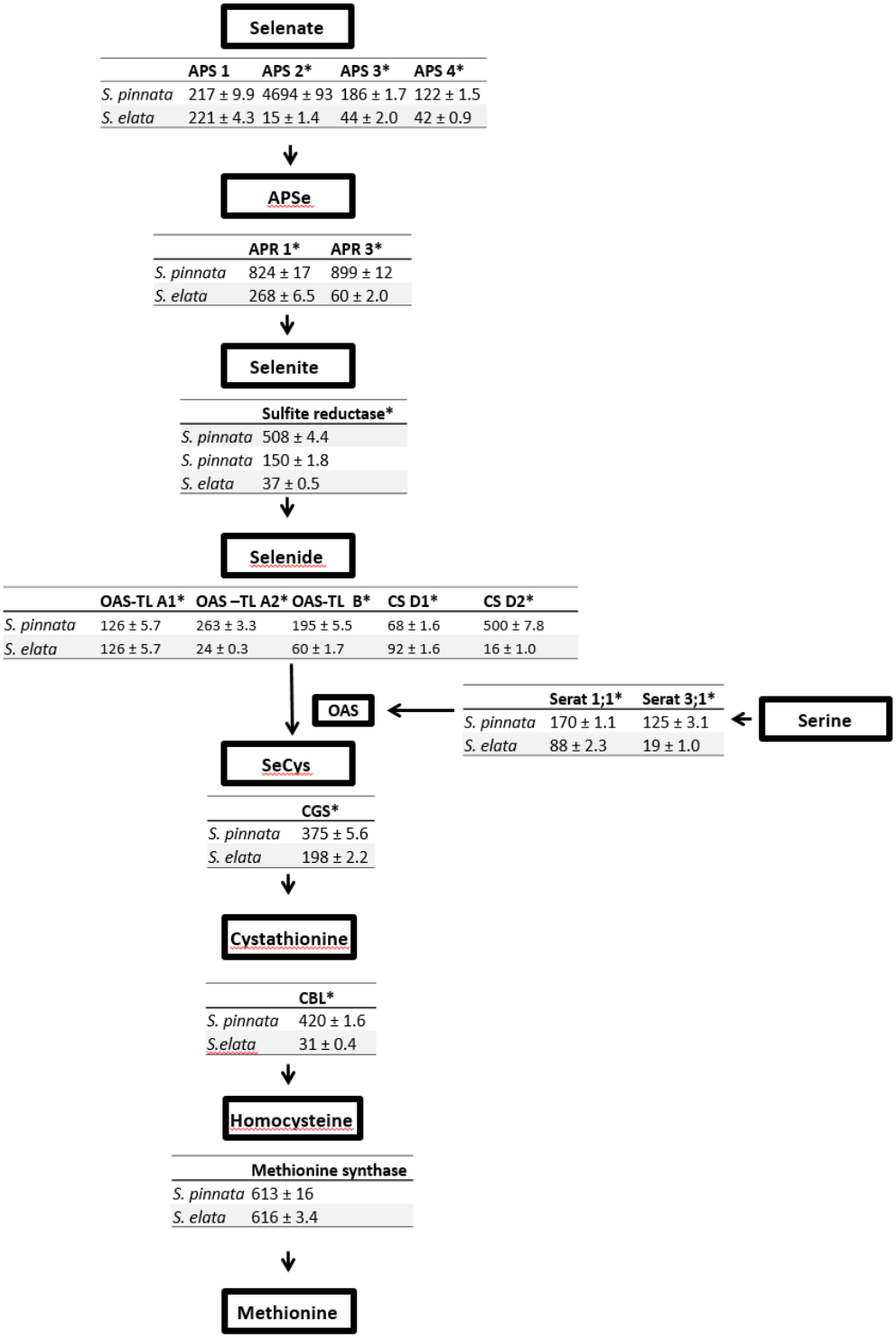

Figure 4.4. Transcript levels of S/Se assimilation pathway genes in 20 µM Se treated roots of *S. pinnata* and *S. elata*. All gene family members are shown that fit the species comparison criteria (materials and methods). The values are mean RPKM and SEM (n = 3). Stars denote significant differences between species (t-test, $p<0.05$).

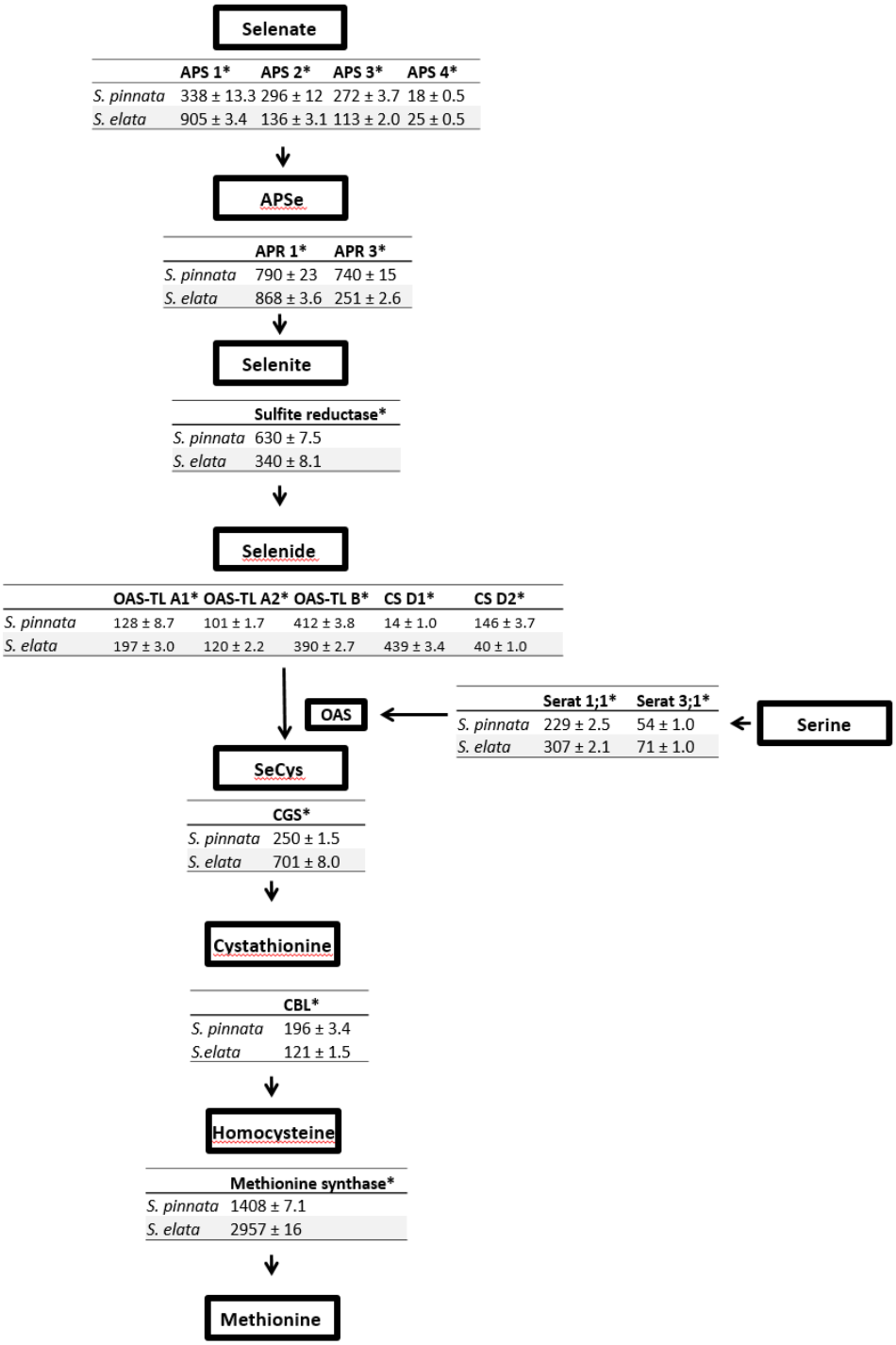

Figure 4.5. Transcript levels of S/Se assimilation pathway genes in 0 µM Se treated shoots of *S. pinnata* and *S. elata*. All gene family members are shown that fit the species comparison criteria (materials and methods). The values are mean RPKM and SEM (n = 3). Stars denote significant differences between species (t-test, $p<0.05$).

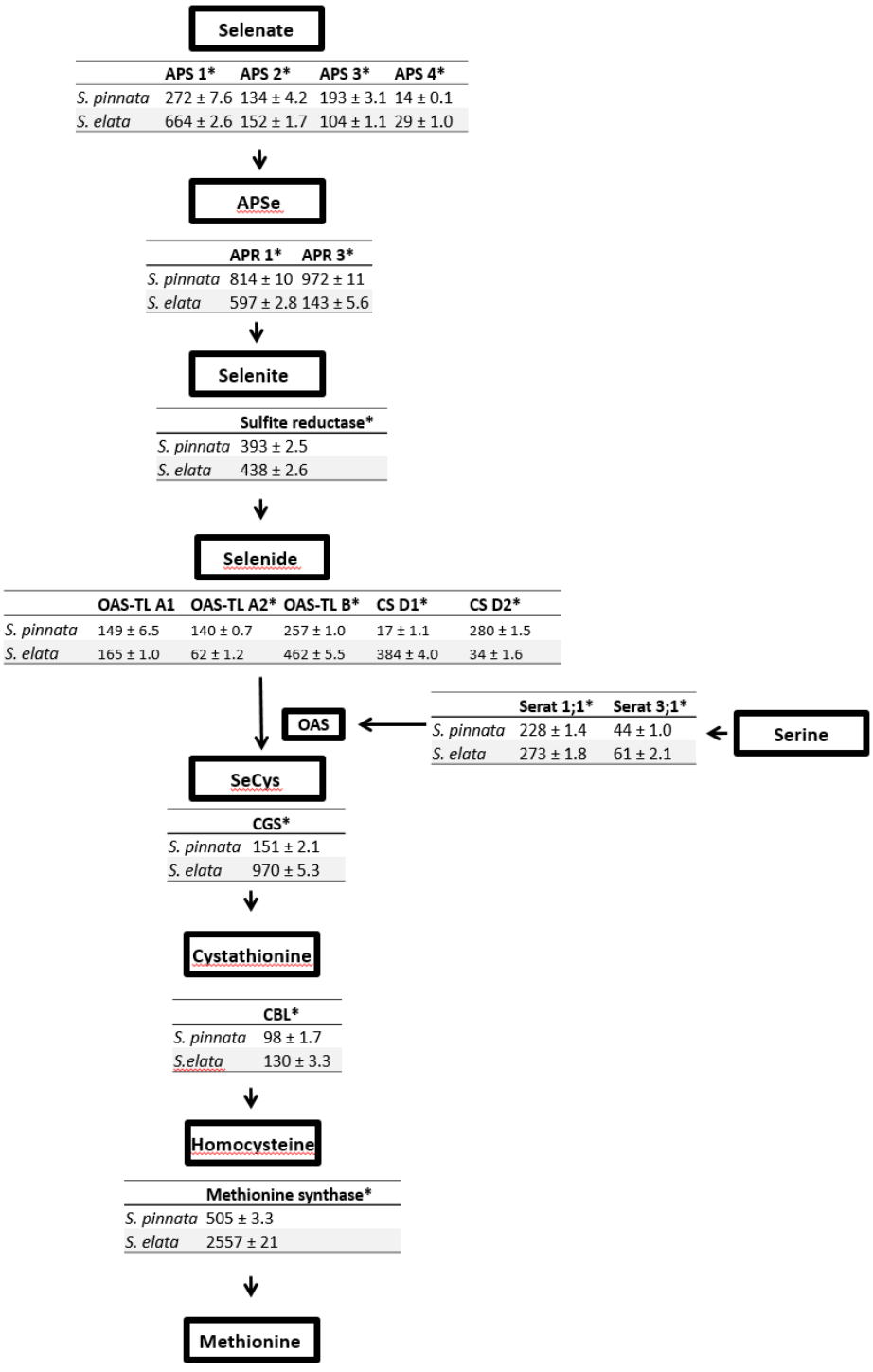

Figure 4.6. Transcript levels of S/Se assimilation pathway genes in 20 µM Se treated shoots of *S. pinnata* and *S. elata*. All gene family members are shown that fit the species comparison criteria (materials and methods). The values are mean RPKM and SEM (n = 3). Stars denote significant differences between species (t-test, $p<0.05$).

to ATP before reduction to sulfite/selenite; the product of the reaction is adenosine-5-phosphosulfate/phosphoselenate, respectively. We found four isoforms of *APS*. Interestingly, *APS2* was the isoform with the highest transcript level in the hyperaccumulator *S. pinnata*, but the isoform with the lowest abundance in the non-hyperaccumulator, *S. elata*. In the root, *S. pinnata* had a 52-fold higher *APS2* transcript level compared to *S. elata* when plants were not treated with Se (Fig. 4.3) and 300-fold higher *APS2* transcript level when the plants were grown with Se (Fig. 4.4). The difference was greater in the presence of Se because *S. elata* roots showed down-regulation of *APS* (all isoforms) in the presence of Se, whereas *S. pinnata* roots did not. It is interesting to note that there was a unique transcript of *APS2* (we had a poor alignment with *Arabidopsis* and *Brassica*) in *S. pinnata* that accounted for 1/3 of the total *APS2* transcript abundance (this transcript was not included in the Fig. 4.3 or 4.4 numbers) it was later confirmed via PCR on genomic DNA that this was not an assembly artifact (Suppl. Fig. 4.3). The expression level of *APS2* in roots of Se-treated *S. pinnata* was almost 4,700 RPKM (Fig. 4.4), which is extraordinarily high considering that the leaf transcript level of the most abundant protein, Rubisco SSU, was around 10,000 RPKM (not shown). In both the roots and shoots, *APS1* was the most transcribed *APS* isoform in *S. elata*. There was significantly more *APS1* in the shoots of *S. elata* as compared to *S. pinnata.*

The next enzyme in the S/Se assimilation pathway is APS reductase (*APR*), which is responsible for the reduction of APS to sulfite/selenite. We found three isoforms of APS reductase (*APR1-3*) in *S. pinnata*, but only *APR1* and *APR3* in *S. elata*. In the roots and shoots of *S. pinnata, APR1 and APR3* were significantly up-regulated with Se treatment for the majority of contigs. We found a different trend for *S. elata,* where both *APR1* and *APR3* were significantly down-regulated for the majority of contigs in the presence of Se, in both roots and

shoots. The greatest change was in the roots of *S. elata* with a 5-fold downregulation of *APR1* in the presence of Se. The largest difference between the two species was found for *APR3* in roots of plants treated with Se, where there was a 14-fold higher expression in *S. pinnata* than *S. elata* (Fig. 4.4).

Sulfite reductase is the next enzyme in the pathway. It reduces sulfite/selenite to sulfide/selenide, respectively, for incorporation into cysteine. We found much greater transcript abundance in the roots of *S. pinnata* compared to *S. elata* (Fig. 4.3, 4.4). However, it should be noted that there appeared to be two root transcripts for sulfite reductase in *S. pinnata*, with opposite Se regulation patterns. Thus, it is possible there are two isoforms of sulfite reductase in *S. pinnata* roots. This was not found in the shoots of *S. pinnata*, nor in either organ in *S. elata*.

The next step in the assimilation of S/Se is the incorporation of sulfide/selenide into cysteine. This involves the action of two enzymes. Serine is first activated by serine acetyltransferase (*Serat*) to O-acetylserine (OAS), which is combined to sulfide/selenide by O-acetylserine (thiol) lyase (*OAS-TL, CS*) to form cysteine. One of the few genes that was not significantly different in expression between the species was an isoform *OAS-TL (OAS-TLA)* in the non-Se treated roots. Interestingly, the two plant species appear to be utilizing different isoforms of *CS.* The transcript level for *CSD2* was 3 – 30 fold higher in *S. pinnata* depending on the treatment and organ, while in *S. elata* the transcript level was 1.3-31 fold higher for *CSD1* as compared to *S. pinnata*, depending on the treatment and organ.

Cysteine to methionine – Three enzymatic steps convert cysteine to methionine. In the roots of *S. elata* there were 4-fold higher methionine synthase (*MS*) transcript levels in the absence of Se, relative to *S. pinnata* (Fig. 4.3) and no significant differences between the two species in the Se treated roots (Fig. 4.4). In the shoots of Se-treated plants, *S. elata* had

significantly higher transcript levels than *S. pinnata* for all three enzymes involved in the conversion of cysteine to methionine (Fig. 4.6).

Discussion

The main question addressed in this study is: how is the Se hyperaccumulator *S. pinnata* different from the non-hyperaccumulator *S. elata* in terms of Se and S accumulation and selenate-dependent transcript abundance of genes involved in selenate transport and assimilation? Cappa et al. (2014) demonstrated that within the *Stanleya* clade, *S. elata* is the most different from *S. pinnata* with respect to Se physiology. *Stanleya elata* had the lowest Se accumulation in the field and was the only species that did not translocate S and Se differentially, as does the hyperaccumulator *S. pinnata*. It was found here that the transcript levels of many sulfate transporter homologs as well as many sulfate assimilation genes were higher in *S. pinnata* than *S. elata*. The biggest differences were found for the roots, especially in the presence of Se. However, it should be noted that overall expression levels for all transcripts were lower in Se treated roots in *S. elata*. Based on these findings, we hypothesize that Se assimilation occurs to a large extent in the root of the hyperaccumulator. Plastids are present in all plant cells, and there is no reason that Se assimilation cannot occur in the root, as long as there is sufficient reducing power and ATP.

A homolog of *APS2* showed the highest transcript abundance of any gene in the Se/S assimilation pathway in *S. pinnata*—two orders of magnitude more abundant than in *S. elata*. ATP sulfurylase was found to be a rate limiting enzyme for Se assimilation in *Brassica juncea* (Pilon-Smits et al., 1999), and thus increased transcript abundance may overcome a potential bottleneck for Se assimilation in *S. pinnata*. There are multiple isoforms of *APS*, both plastidic and cytosolic (Takahashi et al., 2011). The existence of a cytosolic *APS* isoform was confirmed

in *Arabidopsis* via cell fractionation and immunoblot assays (Rotte and Leustek, 2000). Hatzfeld et al. (2000) hypothesized that based on amino acid composition, specifically location of methionine residues, that the most likely candidate for cytosolic localization is *APS2*. The finding that *APS2* was the most abundant form in *S. pinnata* whereas *APS1* was most abundant in *S. elata* indicates that Se assimilation not only is occurring more in the roots in *S. pinnata* and in the shoots in *S. elata*, but may also be differentially compartmentalized within the cells in hyperaccumulators and nonaccumulators.

It is interesting that while most of the genes in the S/Se assimilation pathway were significantly upregulated in the hyperaccumulator in the presence of Se, there was already high transcript abundance in the absence of Se. It should noted however, the seeds of *S. pinnata* contain Se, and this in part may explain the high transcript abundance of many of the above mentioned enzymes in the absence of Se treatment. This indicates that the whole pathway has high constitutive expression in *S. pinnata* and may explain the Se hyperaccumulation capacity of *S. pinnata*. *Stanleya elata* had much lower expression levels of many of the S/Se assimilation genes in the root and resembled a more typical non-hyperaccumulator plant, in that the majority of the Se assimilation transcripts were found to be more abundant in the shoot. In addition to *APS2*, sulfate/selenate transporter 1;2 stood out in its degree of overexpression in the hyperaccumulator (Fig. 4.2). *Sultr1;2* has been implicated in several studies to be involved in Se uptake in *Arabidopsis*. Thus, it is likely that the high constitutive transcript abundance of *sultr1;2* in *S. pinnata* is one of its key mechanisms of Se hyperaccumulation. Other sulfate/selenate transporters were also upregulated in *S. pinnata*, and these may further contribute in hyperaccumulation, and by transporting selenate out of the root vacuole so that it stays available for assimilation (*Sultr4;1, 4;2*), or by mediating uptake into the root pericycle (*Sultr2;1, Sultr2;2*

and *Sultr3;5*) for translocation from root to shoot and from leaves to reproductive organs (Fig. 4.2). The enhanced expression levels of these genes may explain the high shoot/root and fruit/leaf Se ratios found in *S. pinnata* relative to non-hyperaccumulator relatives (Cappa et al., 2014).

It is thought that the main Se tolerance mechanism in *Astragalus* is the methylation of SeCys via the enzyme *SMT* (Sors et al., 2005). We did not find *SMT* homologs in our *Stanleya* transcriptome data, although previous studies identified methyl-SeCys in *S. pinnata*, in addition to Se-cystathionine (Freeman et al., 2006). The transcriptome data did not provide evidence that *S. pinnata* was different from *S. elata* in relation to the accumulation of Se-cystathionine in *S. pinnata*; root *CGS* transcript levels were higher in *S. pinnata* than *S. elata* when supplied with Se, but so were root *CBL* levels, and in the shoots *CGS* transcript levels were actually lower in *S. pinnata*.

There are many follow-up studies to be conducted in light of these data. The Se assimilation capacity of roots of *S. pinnata* could be experimentally tested. A transgenic *Brassica juncea* line for *APS1* was found to have increased Se tolerance, associated with higher levels of selenate assimilation to organic Se in root and shoot (Pilon-Smits et al, 1999). However, when tested whether the roots were capable of selenate assimilation without the shoot present, neither the transgenic nor wild type individuals were capable of assimilating Se in the roots. A similar test could be done with *S. pinnata*. If indeed *S. pinnata* is capable of assimilating Se in the root, this likely contributes to the ability of *S. pinnata* to tolerate and accumulate such high concentrations of Se. Other Se hyperaccumulator plants may also be tested for their ability to assimilate Se in their roots. It is interesting to note, in this respect, an analysis of guttation (= xylem) fluid from the hyperaccumulator *Astragaulus bisulcatus* showed the presence of organic

Se in the forms of methyl-SeCys and γ-glutamyl-SeCys (unpublished results), which suggests this species, too, assimilates Se in its root. Selenium hyperaccumulation evolved convergently in different genera, yet it appears that many of the mechanisms are similar in the different clades. Another intriguing finding is the unique transcript for *APS2* in *S. pinnata*. This gene could be cloned and functionally tested to see if this allele may contribute to the increased tolerance and accumulation of Se in *S. pinnata*.

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Supplementary Material: Chapter 1

Supplemental Table 1.1. Occurrence of hyperaccumulators across angiosperms.

Order	Family	Species	Element(s)	Reference
Apiales	Araliaceae	*Chengiopanax sciadopylloides*	Mn	Mizuno et al 2008
	Iridaceae	*Cipura aff. xanthomelas*	Ni	Reeves et al 2007
		Gladiolus gregarius	Co	Malaisse et al 1994
		Iris ensata	Cr	Usman et al 2012
Asterales	Argophyllaceae	*Argophyllum grunowii*	Ni	Reeves and Baker 2000
		Argophyllum laxum	Ni	Reeves and Baker 2000
	Asteraceae	*Achillea tenuifollia*	Mo	Boojar and Tavakkoli 2011
		Ansiopappus chinensis	Cu	Brooks et al 1986
		Ansiopappus davyi	Co	Brooks et al 1980
		Berkheya coddii	Ni	Robinson et al 1997
		Berkheya nivea	Ni	Smith et al 2001
		Centaurea (13 taxa)	Ni	Reeves and Adigüzel 2008
		Centaurea virgata	Mn	Lorestani et al 2012
		Chromolaena nr meyeri	Ni	Reeves et al 2007
		Chromolaena sp. nov.	Ni	Reeves et al 2007
		Dicoma niccolifera	Ni	Brooks and Yang 1984
		Gochnatia crassifolia	Ni	Reeves et al 1999
		Gochnatia recurva	Ni	Reeves et al 1999
		Gutenbergia pubescens	Co and Cu	Brooks et al 1986
		Gynura pseudochina	Cd and Zn	Panitlertumpai et al 2013
		Haplopappus condensata	Se	Reeves and Baker 2000
		Haplopappus fremontii	Se	Reeves and Baker 2000
		Koanophyllon grandiceps	Ni	Reeves et al 1999
		Koanophyllon prinodes	Ni	Reeves et al 1999
		Leucanthemopsis alpina	Ni	Reeves and Baker 2000
		Machaeranthera (4 taxa)	Se	Reeves and Baker 2000
		Millotia myosotidifolia	Cu	Reeves and Baker 2000

		Oonopsis (4 taxa)	Se	Beath et al 1940
		Pentacalia (11 taxa)	Ni	Reeves et al 1999
		Picris divaricata	Cd and Zn	Broadhurst et al 2013
		Porophyllum aff. angustissimum	Ni	Reeves et al 2007
		Scariola orientalis	Cu and Pb	Lorestani et al 2012
		Senecio (8 taxa)	Ni	Reeves et al 1999
		Senecio sp.	Pb	Bech et al 2012
		Shafera platyphylla	Ni	Reeves et al 1999
		Solidago hispida	Ni	Reeves and Baker 2000
		Stevia nr parvifolia	Ni	Reeves et al 2007
		Tagetes patula	Cd	Wei et al 2012
		Vernonia petersii	Cu	Brooks et al 1986
		Xylorhiza (8 taxa)	Se	Beath et al 1940
	Campanulaceae	*Campanula scheuchzeri*	Ni	Reeves and Baker 2000
Brassicales	Brassicaceae	*Alyssum* (48 taxa)	Ni	Reeves and Baker 2000
		Arabidopsis hallerii	Cd and Zn	Bert et al 2003
		Arabis gemmifera	Cd and Zn	Kubota and Takenaka 2003
		Arabis paniculata	Cd and Zn	Zeng et al 2011
		Biscutella laevigata	Tl	Poscic et al 2012
		Bornmuellera (6 taxa)	Ni	Reeves et al 2009
		Cardamine resedifolia	Ni	Reeves and Baker 2000
		Cardaminopsis halleri	Zn	Macnair and Smirnoff 1999
		Cardaria draba	Cu	Lorestani et al 2012
		Cochlearia (3 species)	Ni	Krämer 2010
		Hesperis persica	As	Krämer 2010
		Isatis capadocica	As	Krämer 2010
		Noccaea caerulescens	Cd, Ni and Zn	Krämer 2010
		Noccaea praecox	Cd and Zn	Krämer 2010
		Noccaea (6 taxa)	Zn	Reeves and Baker 2000, Krämer 2010

		Noccaea (21 taxa)	Ni	Brooks and Reeves 1983, Krämer 2010
		Peltaria emarginata	Ni	Reeves et al 1980
		Pseudosempervivum sempervivum	Ni	Reeves et al 2009
		Rorippa globosa	Cd	Sun et al 2010
		Stanleya bipinnata	Se	Beath et al 1940
		Stanleya pinnata	Se	Beath et al 1940
		Streptanthus polygaloides	Ni	Reeves et al 1981
	Resedaceae	*Reseda alba*	Cu	Lorestani et al 2012
Buxales	Buxaceae	*Buxus* (17 taxa)	Ni	Reeves et al 1996
Caryophyllales	Amaranthaceae	*Celosia trigyna*	Cu	Reeves and Baker 2000
		Pandiaka metallorum	Cu	Brooks et al 1980
		Arthrocnemum macrostachyum	Cd	Redondo-Gomez et al 2010
		Beta vularis	Cd	Chen et al 2013
		Pfaffia glomerata	Cd	Gomes et al 2013
		Pfaffia sarcophylla	Ni	Reeves et al 2007
	Caryophyllaceae	*Minuartia patula*	Pb	Wenzel and Jokwer 1999
		Minuartia (5 taxa)	Ni	Reeves and Baker 2000
		Polycarpaea synandra	Pb and Zn	Reeves and Baker 2000
		Silene cobalticola	Cu	Brooks et al 1980
	Chenopodiaceae	*Atriplex confertiflora*	Se	Reeves and Baker 2000
		Salsola kali	Cr	de la Rosa et al 2004
	Phytolaccaceae	*Phytolacca acinosa*	Mn	Xue et al 2005
		Phytolacca americana	Mn and Cd	Yang 2013
	Plumbaginaceae	*Armeria maritima*	Pb	Reeves and Baker 2000
	Polygonaceae	*Polygonum hydropiper*	Mn	Yang 2013
		Polygonum pubescens	Mn	Deng et al 2010
		Rumex acetosa	Pb and Zn	Reeves and Baker 2000
Celastrales	Celastraceae	*Maytenus* (4 taxa)	Mn	Reeves and Baker 2000
		Stackhousia tryonii	Ni	Bhatia et al 2002
Commelinales	Commelinaceae	*Commelina communis*	Cu	Wang et al 2011

		Commelina zigzag	Cu	Brooks et al 1980
		Cyanotis longifolia	Co	Brooks et al 1980
Ericales	Balsaminaceae	*Impatiens walleriana*	Cd	Wei et al 2013
	Sapotaceae	*Pouteria oxyedra*	Ni	Reeves and Baker 2000
		Sebertia acumunata	Ni	Jaffré 1976
Fabales	Fabaceae	*Acacia cana*	Se	Reeves and Baker 2000
		Anthyllis sp.	Ni	Reeves and Baker 2000
		Astragalus (24 taxa)	Se	Beath et al 1940
		Camptosema aff. ellipticum	Ni	Reeves et al 2007
		Crotalaria cobalticola	Co	Brooks et al 1980
		Melilotus alba	Pb	Fernandez et al 2012
		Neptunia amplexicaulis	Se	Reeves and Baker 2000
		Pearsonia metallifera	Ni	Brooks and Yang 1984
		Prosopis laevigata	Cr and Cd	Buendia-Gonzalez et al 2010
		Vigna dolomitica	Cu	Brooks et al 1980
		Sesbania drummondii	Pb	Sahi et al 2002
		Trifolium pallescens	Ni	Reeves and Baker 2000
Gentianales	Apocynaceae	*Alyxia rubricaulis*	Mn	Reeves and Baker 2000
	Rubiaceae	*Ariadne shaferi*	Ni	Reeves et al 1999
		Mitracarpus sp.	Ni	Reeves and Baker 2000
		Morinda reticulata	Se	Reeves and Baker 2000
		Phyllomelia coronata	Ni	Reeves and Baker 2000
		Psychotria (5 taxa)	Ni	Reeves et al 1999
		Richardia grandifora	Ni	Reeves et al 2007
		Rondeletia sp. (2)	Ni	Reeves and Baker 2000
Lamiales	Acanthaceae	*Blepharis acuminata*	Ni	Brooks and Yang 1984
		Justicia lanstyakii	Ni	Reeves et al 2007
		Lophostachys villosa	Ni	Reeves et al 2007
		Phidiasia lindavii	Ni	Reeves et al 1999
		Ruellia geminiflora	Ni	Reeves et al 2007
	Lamiaceae	*Aeollanthus homblei*	Cu	Reeves and Baker 2000
		Aeollanthus saxatilis	Co	Brooks et al 1980

		Aeollanthus subacaulis	Co and Cu	Brooks et al 1986, 1980
		Becium grandiflorum	Cu	Brooks et al 1986
		Haumaniastrum homblei	Cu	Brooks et al 1980
		Haumaniastrum katagense	Cu	Brooks et al 1980
		Haumaniastrum robertii	Co and Cu	Brooks et al 1980
		Haumaniastrum rosulatum	Cu	Brooks et al 1986
	Linderniaceae	*Crepidorhopalon perennis*	Co and Cu	Brooks et al 1986
		Crepidorhopalon tenuis	Co and Cu	Brooks et al 1986
		Linderia damblonii	Co	Brooks et al 1980
		Linderia perennis	Co and Cu	Brooks et al 1980
	Oleaceae	*Chionanthus domingensis*	Ni	Reeves and Baker 2000
	Orobanchaceae	*Alectra sessiliflora*	Co	Brooks et al 1986
		Buchnera henriquesii	Co and Cu	Brooks et al 1980
		Castilleja chromosa	Se	Reeves and Baker 2000
		Esterhazya sp.	Ni	Reeves et al 2007
		Melasma welwitschii	Co	Brooks et al 1980
		Sopubia (4 taxa)	Co	Brooks et al 1980, 1987
		Striga hermontheca	Cu	Brooks et al 1986
	Plantaginaceae	*Linaria alpina*	Ni	Reeves and Baker 2000
	Verbenaceae	*Lippia* (5 taxa)	Ni	Reeves et al 2007
Magnoliales	Myristicaceae	*Myristica laurifolia*	Ni	Reeves and Baker 2000
Malpighiales	Clusiaceae	*Garcinia* (4 taxa)	Ni	Reeves et al 1999
		Garcinia amplexicaulis	Mn	Reeves and Baker 2000
	Dichapetalaceae	*Dichapetalum gelniodes*	Ni	Reeves and Baker 2000
	Euphorbiaceae	*Baloghia sp.*	Ni	Reeves and Baker 2000
		Bonania (3 taxa)	Ni	Reeves et al 1996
		Cleidion viellardii	Ni	Reeves and Baker 2000
		Cnidoscolus sp. nov.	Ni	Reeves et al 2007
		Croton campestris	Ni	Reeves et al 2007
		Euphorbia macroclada	Fe	Lorestani et al 2012
		Euphorbia (3 taxa)	Ni	Reeves et al 1996, Reeves et al 2007
		Gymnanthes recurva	Ni	Reeves et al 1996

		Leucocroton (28 taxa)	Ni	Reeves et al 1996
		Monadenium cupricola	Cu	Brooks et al 1986
		Sapium erythrospermum	Ni	Reeves et al 1996
	Ochnaceae	*Brackenridgea palustris*	Ni	Reeves and Baker 2000
		Ouratea nitida	Ni	Reeves et al 1999
		Ouratea striata	Ni	Reeves et al 1999
	Passifloraceae	*Piriqueta sidifolia*	Ni	Reeves et al 2007
		Piriqueta sp.	Ni	Reeves et al 2007
		Turnera (5 taxa)	Ni	Reeves et al 2007
	Phyllanthaceae	*Phyllanthus* (39 taxa)	Ni	Reeves et al 1996
		Phyllanthus williamioides	Cu	Reeves et al 1999
		Savia (3 taxa)	Ni	Reeves et al 1996
	Salicaceae	*Casearia melistaurum*	Ni	Jaffre et al 1979
		Homalium (7 taxa)	Ni	Jaffre et al 1979
		Lasiochlamys peltata	Ni	Jaffre et al 1979
		Xylosma (10 taxa)	Ni	Jaffre et al 1979
	Violaceae	*Agatea deplanchei*	Ni	Reeves and Baker 2000
		Hybanthus (3 taxa)	Ni	Reeves and Baker 2000
		Rinorea bengalensis	Ni	Reeves and Baker 2000
		Rinorea javanica	Ni	Brooks et al 1977
		Viola baoshanensis	Cd	Wu et al 2010
		Viola calaminaria	Zn	Reeves and Baker 2000
Malvales	Cistaceae	*Cistus incanus*	Pb	Reeves and Baker 2000
	Dipterocarpaceae	*Shorea tenuiramulosa*	Ni	Reeves and Baker 2000
	Malvaceae	*Hibiscus rhodanthus*	Co	Brooks et al 1986
		Sida linifolia	Ni	Reeves et al 2007
		Tetralix (5 taxa)	Ni	Reeves et al 1999
		Trichospermum kjellbergii	Ni	Reeves and Baker 2000
		Triumfetta dekindtiana	Cu	Brooks et al 1986
		Triumfetta digitata	Cu	Brooks et al 1980
		Triumfetta welwitschii	Co	Brooks et al 1986
Myrtales	Myrtaceae	*Eugenia clusioides*	Mn	Reeves and Baker 2000

		Gossia bidwilli	Mn	Fernando et al 2013
		Gossia fragrantissima	Mn	Fernando et al 2013
		Mosiera (4 taxa)	Ni	Reeves et al 1999
		Psidium araneosum	Ni	Reeves et al 1999
		Psidium havanense	Ni	Reeves et al 1999
	Lythraceae	*Cuphea aff. erectifolia*	Ni	Reeves et al 2007
Oxalidales	Cunoniaceae	*Geissois* (7 taxa)	Ni	Jaffré et al 1979
		Agrophyllum (2 taxa)	Ni	Jaffré et al 1979
Pandanales	Velloziacae	*Vellozia* sp.	Ni	Reeves and Baker 2000
		Xerophyta retinervis	Co	Brooks et al 1986
Proteales	Proteaceae	*Beaupreposis paniculata*	Mn	Reeves and Baker 2000
		Macadamia angustifolia	Mn	Reeves and Baker 2000
		Macadamia neurophylla	Mn	Reeves and Baker 2000
Poales	Cyperaceae	*Ascolepis metallorum*	Cu	Brooks et al 1980
		Bulbostylis cupricola	Cu	Brooks et al 1986
		Bulbostylis mucronata	Co and Cu	Brooks et al 1980
		Bulbostylis pseudoperennis	Co and Cu	Brooks et al 1986
	Juncaceae	*Luzula lutea*	Ni	Reeves and Baker 2000
	Poaceae	*Agrostis stolonifera*	Pb	Reeves and Baker 2000
		Agrostis tenuis	Pb	Reeves and Baker 2000
		Arrhenatherum elatius	Pb	Reeves and Baker 2000
		Eragrostis boehmii	Cu	Brooks et al 1980
		Eragrostis racemosa	Cu	Reeves and Baker 2000
		Festuca ovina	Pb	Reeves and Baker 2000
		Leersia hexandra	Cr	Zhang et al 2007
		Rendlia altera	Cu	Reeves and Baker 2000
		Spartina argentinensis	Cr	Redondo-Gomez et al 2011
		Sporobolus congoensis	Cu	Malaisse et al 1994
		Trisetum distichophyllum	Ni	Reeves and Baker 2000
Ranunculales	Papaveraceae	*Corydalis davidii*	Zn	Lin et al 2012
	Ranunculaceae	*Ranunculus glacialis*	Ni	Reeves and Baker 2000
Rosales	Rosaceae	*Potentilla griffithii*	Cd and Zn	Qiu et al 2011

Sapindales	Anacardiaceae	*Rhus wildii*	Ni	Brooks and Yang 1984
	Meliaceae	*Walsura monophylla*	Ni	Reeves and Baker 2000
Saxifragales	Crassulaceae	*Crassula alba*	Co	Reeves and Baker 2000
		Crassula helmsii	Cu	Küpper et al 2009
		Crassula vaginata	Co	Brooks et al 1980
		Sedum alfredii	Cd and Zn	Yang et al 2004
	Saxifragaceae	*Saxifraga* (3 taxa)	Ni	Reeves and Baker 2000
Solanales	Convolvulaceae	*Ipomoea alpina*	Cu	Brooks et al 1980
		Ipomoea aff. echioides	Ni	Reeves et al 2007
		Merremia xanthophylla	Ni	Brooks and Yang 1984
	Solanaceae	*Solanum nigrum*	Cd	Wei et al 2013
Unplaced	Boraginaceae	*Heliotropium salicoides*	Ni	Reeves et al 2007
	Oncothecaceae	*Oncotheca balansae*	Ni	Reeves and Baker 2000

Supplemental Table 1.1 References

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Supplementary Material: Chapter 2

Appendix 2.1

Taxon, Voucher, Collection locale, Herbarium.

Stanleya albescens M.E.Jones, JJ & PJ Cappa 38, Colorado, CS. ***Stanleya albescens*** M.E.Jones, JJ & PJ Cappa 42, Colorado, CS. ***Stanleya albescens*** M.E.Jones, JJ & PJ Cappa 43, Colorado, CS. ***Stanleya elata*** M.E.Jones, JJ & PJ Cappa 54, Nevada, CS. ***Stanleya elata*** M.E.Jones, JJ & PJ Cappa 67, Nevada, CS. ***Stanleya elata*** M.E.Jones, JJ & PJ Cappa 69, Nevada, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***integrifolia*** (E.James) Rollins, JJ & PJ Cappa 28, Utah, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***integrifolia*** (E.James) Rollins, JJ & PJ Cappa 31, Utah, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***integrifolia*** (E.James) Rollins, JJ & PJ Cappa 35, Utah, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***inyoensis*** (Munz & Roos) Reveal, JJ & PJ Cappa 63, Nevada, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 4, Wyoming, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 6, Wyoming, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 47, Colorado, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 52, Utah, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 90, Oregon, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 132, Colorado, CS. ***Stanleya pinnata*** (Pursh) Britton var. ***pinnata***, JJ & PJ Cappa 140, Wyoming, CS. ***Stanleya tomentosa*** Parry, JJ & PJ Cappa 9, Wyoming, CS. ***Stanleya tomentosa*** Parry, JJ & PJ Cappa 12, Wyoming, CS. ***Stanleya tomentosa*** Parry, JJ & PJ Cappa 15, Wyoming, CS. ***Stanleya viridiflora*** Nuttall, JJ & PJ Cappa 1, Wyoming, CS. ***Stanleya viridiflora*** Nuttall, JJ & PJ Cappa 19, Idaho, CS. ***Stanleya viridiflora*** Nuttall, JJ & PJ Cappa 25, Utah, CS. ***Thelypodium laciniatum*** Endl., JJ & PJ Cappa 117, California, CS.

Supplemental Table 2.1. Tissue Se and S concentrations and soil Se concentrations (mg kg^{-1} DW) in *Stanleya* and *Thelypodium* field-collected samples. Values are shown for individual plants and collection locales. The first number indicates the population for a given taxon, and the second number is the collection number. For taxon averages, see Table 2.1. BD indicates below detection limit, ND indicates not determined and * indicates pooled samples from multiple plants.

Latitude	Longitude	Taxa	Leaf [Se]	Leaf [S]	Fruit [Se]	Fruit [S]	Soil [Se]
38°37'42"N	107°59'6"W	*S. albescens* 1-38	9	16092	27	3232	BD
		S. albescens 1-39	43	21960	34	6769	0.9
		S. albescens 1-40	BD	16049	9	1772	0.6
		S. albescens 1-41	BD	10149	79	3211	BD
38°21'41"N	107°47'23"W	*S. albescens* 2-42	16	12454	14	2522	170.3
38°29'55"N	107°44'16"W	*S. albescens* 3-43	BD	18581	188	2910	5.3
41°19'49"N	110°25'18"W	*S. bipinnata* 1-73	16	14545	ND	ND	1.1
		S. bipinnata 1-74	11	16085	ND	ND	0.7
		S. bipinnata 1-75	BD	15181	106	8802	0.3
		S. bipinnata 1-76	5	14984	ND	ND	0.6
		S. bipinnata 1-84	42	42602	380	13497	4.9
		S. bipinnata 1-85	17	50520	30	17440	3.4
		S. bipinnata 1-86	37	50287	99	13132	5.2
		S. bipinnata 1-87	35	44373	227	12817	5.4
41°6'58"N	108°48'18"W	*S. bipinnata* 2-77	BD	6306	BD	7693	0.1
		S. bipinnata 2-78	BD	7085	ND	ND	BD
		S. bipinnata 2-79	BD	10118	8	8368	0.1
		S. bipinnata 2-81	9	27078	2	11535	0.1
		S. bipinnata 2-82	21	29642	9	12895	BD
		S. bipinnata 2-83	28	18260	8	8832	0.1
36°16'44"N	115°27'30"W	*S. elata* 1-54	BD	3045	ND	ND	0.1
		S. elata 1-55	BD	3430	BD	10948	0.1

		S. elata 1-56	BD	1373	BD	8124	BD
36°16'35"N	115°29'53"W	*S. elata* 2-58	BD	4915	BD	7655	BD
		S. elata 2-59	BD	2974	ND	ND	BD
36°16'36"N	115°30'12"W	*S. elata* 3-60	5	2757	BD	3978	11.2
		S. elata 3-61	11	2127	BD	2435	7.6
		S. elata 3-62	BD	2455	BD	5976	0.6
37°32'13"N	117°11"39"W	*S. elata* 4-67	BD	8443	BD	8977	0.2
		S. elata 4-68	BD	11785	BD	7194	0.2
38°11'36"N	117°59"15"W	*S. elata* 5-69	BD	12771	BD	7818	0.3
		S. elata 5-70	BD	17766	ND	ND	0.3
		S. elata 5-71	BD	10508	BD	6600	0.4
		S. elata 5-72	BD	10106	BD	8246	0.3
39°1'58"N	118°55'19"W	*S. elata* 6-91	ND	ND	1	14782	4.5
		S. elata 6-92	14	20911	BD	8770	2.7
38°11'36"N	117°59"15"W	*S. elata* 7-97	ND	ND	8	11822	3.1
		S. elata 7-98	12	42398	2	21450	5.3
		S. elata 7-99	10	45132	5	15537	2.4
		S. elata 7-100	3	14054	BD	7668	0.3
37°33'17"N	117°11'52"W	*S. elata* 8-104	ND	ND	15	10985	5.3
		S. elata 8-105	6	5194	3	9995	0.8
		S. elata 8-106	8	23628	5	10635	2.7
		S. elata 8-107	6	14145	2	12937	0.5
		S. elata 8-109	ND	ND	1	10919	3.3
37°26'41"N	117°21'53"W	*S. elata* 9-110	11	21773	8	7860	4.5
		S. elata 9-111	ND	ND	3	9365	6.1

		S. elata 9-112	ND	ND	6	22416	5.6
		S. elata 9-113	BD	11764	22	10361	2.8
40°58'13"N	109°42'56"W	*S. pinnata* var. *integrifolia* 1-28	19	17062	153	7503	BD
		S. pinnata var. *integrifolia* 1-29	BD	20927	71	4402	BD
		S. pinnata var. *integrifolia* 1-30	BD	18423	16	8320	BD
40°56'0"N	109°43'0"W	*S. pinnata* var. *integrifolia* 2-31	BD	30559	23	7242	BD
		S. pinnata var. *integrifolia* 2-32	BD	25714	BD	7508	BD
		S. pinnata var. *integrifolia* 2-33	23	15858	103	5774	37.6
		S. pinnata var. *integrifolia* 2-34	BD	11375	30	4623	4.8
40°55'31"N	109°42'53"W	*S. pinnata* var. *integrifolia* 3-35	10	16093	59	4849	BD
		S. pinnata var. *integrifolia* 3-36	32	15858	61	7537	BD
		S. pinnata var. *integrifolia* 3-37	52	21054	35	5796	BD
37°2'42"N	116°46'12"W	*S. pinnata* var. *inyoensis* 1-63	BD	10280	111	5213	BD
		S. pinnata var. *inyoensis* 1-64	BD	13570	18	5155	BD
		S. pinnata var. *inyoensis* 1-65	BD	3336	3	4194	BD
		S. pinnata var. *inyoensis* 1-66	BD	9224	10	5158	BD
38°29'49"N	118°10'18"W	*S. pinnata* var. *inyoensis* 2-93	13	20530	11	12081	1.7
		S. pinnata var. *inyoensis* 2-94	14	19742	75	13002	0.4
		S. pinnata var. *inyoensis* 2-95	BD	12826	ND	ND	3.6
		S. pinnata var. *inyoensis* 2-96	7	23959	9	6038	1.1
38°4'11"N	118°0'526"W	*S. pinnata* var. *inyoensis* 3-101	25	26821	2	6219	16.3
		S. pinnata var. *inyoensis* 3-102	6	15592	BD	8077	4.8
		S. pinnata var. *inyoensis* 3-103	15	16164	1	10431	5.0
43°47'41"N	108°28'1"W	*S. pinnata* var. *pinnata* 1-4	55	11694	1614	4817	3.7
		S. pinnata var. *pinnata* 1-5	55	12996	2122	8259	5.4

44°4'7"N	109°3'45"W	*S. pinnata* var. *pinnata* 2-6	66	28878	212	14221	5.2
		S. pinnata var. *pinnata* 2-7	99	24382	526	13277	1.0
		S. pinnata var. *pinnata* 2-8	BD	10943	197	10562	BD
39°6'57"N	108°32'59"W	*S. pinnata* var. *pinnata* 3-44	15	15913	702	7499	BD
		S. pinnata var. *pinnata* 3-45	6	20784	751	5624	BD
		S. pinnata var. *pinnata* 3-46	BD	23806	162	6358	BD
39°7'18"N	108°43'55"W	*S. pinnata* var. *pinnata* 4-47	BD	15029	13	6196	3.6
		S. pinnata var. *pinnata* 4-48	BD	16434	132	7710	4.3
		S. pinnata var. *pinnata* 4-49	29	8983	625	5299	4.0
		S. pinnata var. *pinnata* 4-50	15	11215	276	5985	BD
38°56'30"N	109°32'11"W	*S. pinnata* var. *pinnata* 5-51	138	18849	469	5518	BD
		S. pinnata var. *pinnata* 5-52	5	15005	183	5922	0.3
		S. pinnata var. *pinnata* 5-53	10	12241	314	6157	BD
42°48'20"N	117°43'51"W	*S. pinnata* var. *pinnata* 6-88	66	48652	562	12263	6.1
		S. pinnata var. *pinnata* 6-89	12	42140	169	10306	5.5
		S. pinnata var. *pinnata* 6-90	14	24363	120	9075	6.0
38°22’7”N	104°56’53”W	*S. pinnata* var. *pinnata* 7-130	266	23049	492	3492	13.9
		S. pinnata var. *pinnata* 7-131	5	9029	500	5123	BD
		S. pinnata var. *pinnata* 7-132	346	8996	2139	4493	14.6
		S. pinnata var. *pinnata* 7-133	30	24995	119	3910	5.6
		S. pinnata var. *pinnata* 7-134	123	18075	1264	4241	2.7
		S. pinnata var. *pinnata* 7-135	23	26907	261	4625	10.7
		S. pinnata var. *pinnata* 7-136	1304	20599	856	7962	9.8
38°15’14”N	104°58’50”W	*S. pinnata* var. *pinnata* 8-137	89	17150	635	5567	4.7
		S. pinnata var. *pinnata* 8-138	31	35188	2548	8332	12.6
		S. pinnata var. *pinnata* 8-139	120	30858	991	6989	12.9
		S. pinnata var. *pinnata* 8-140	190	19990	623	5941	13.5

41°14’21”N	105°51’6”W	*S. pinnata* var. *pinnata* 9-141	1341	13478	3214	4782	5.9
		S. pinnata var. *pinnata* 9-142	1369	6732	3293	5313	12.7
		S. pinnata var. *pinnata* 9-143	763	20072	605	5508	9.3
		S. pinnata var. *pinnata* 9-144	3807	6509	5622	3264	3.6
40°32’42”N	105°7’52”W	*S. pinnata* var. *pinnata* 10-146	812	10377	2565	5856	BD
		S. pinnata var. *pinnata* 10-147	1026	12587	2215	4370	BD
		S. pinnata var. *pinnata* 10-148	1273	9198	5497	3375	BD
29°31’N	103°36’W	*S. pinnata* var. *texana* 1-128	BD	9124	ND	ND	ND
		S. pinnata var. *texana* 1-129	19	12338	ND	ND	ND
		S. pinnata var. *texana* 1-130	17	20432	ND	ND	ND
44°3'20"N	109°2'10"W	*S. tomentosa* 1-9	24	14393	43	9369	0.8
		S. tomentosa 1-10	BD	18891	47	10701	BD
		S. tomentosa 1-11	61	13071	35	10152	BD
44°47'9"N	107°58'29"W	*S. tomentosa* 2-12	143	17138	489	7974	BD
		S. tomentosa 2-13	18	9758	683	6573	0.1
		S. tomentosa 2-14	47	10341	34	10139	0.2
44°47'19"N	107°58'12"W	*S. tomentosa* 3-15	BD	10103	BD	10602	2.1
		S. tomentosa 3-16	15	30011	BD	12676	1.7
		S. tomentosa 3-17	15	10024	19	12487	BD
42°18'22"N	106°25'59"W	*S. viridiflora* 1-1	76	8672	BD	5158	33.3
		S. viridiflora 1-2	51	7379	331	4817	8.4
		S. viridiflora 1-3	BD	7125	BD	4360	3.2
44°14'1"N	112°57'15"W	*S. viridiflora* 2-18	18	9990	12	5243	BD
		S. viridiflora 2-19	BD	4606	14	3449	BD
		S. viridiflora 2-20	31	10721	8	5848	BD
		S. viridiflora 2-21	10	8559	80	5279	BD

41°0'491"N	110°10'54"W	*S. viridiflora* 3-25	BD	7891	22	4620	BD
		S. viridiflora 3-26	BD	5490	6	2600	BD
		S. viridiflora 3-27	53	14538	52	6291	BD
41°19'50"N	110°25'21"W	*S. viridiflora* 4-123	12	24094	25	9816	6.0
		S. viridiflora 4-124	6	12955	48	7527	1.5
		S. viridiflora 4-125	11	18898	84	8594	1.9
37°4'20"N	118°15'22"W	*T. laciniatum* 1-114	4	27389	12	14546	3.5*
		T. laciniatum 1-115	BD	22313*	11	12333	3.5*
		T. laciniatum 1-116	BD	22313*	BD	26076	3.5*
37°24'24"N	118°32'32"W	*T. laciniatum* 2-117	BD	24236*	10	14652	7.4
		T. laciniatum 2-118	ND	ND	BD	13033	6.9
		T. laciniatum 2-119	BD	24236*	13	10529	10.5
37°39'37"N	118°49'43"W	*T. laciniatum* 3-120	ND	ND	BD	16749	ND
		T. laciniatum 3-121	ND	ND	8	12951	ND

Supplemental Table 2.2. Comparison of Se and S tissue accumulation for each taxon as a measure of interdependence between the two elements. Soil and Se vs. S columns list p-values from correlation analyses ($p < 0.05$ denotes significant correlation and is shown in bold). The rho columns indicate the nature of the correlation (positive or negative). Total Se levels in leaves and fruit were tested against total Se concentration in the soil (soil column) and tissue Se and S levels were tested against each other (Se vs. S column). All datasets have a non-normal distribution and unequal variances. Therefore, Spearman's Rho was used to test for correlations. Values given are for all individuals tested within a taxon.

Taxa	**Tissue**	**Soil**	**Rho**	**Se vs. S**	**Rho**
S. albescens	Leaves	0.511	0.339	0.439	0.395
S. bipinnata	Leaves	**0.030**	0.579	<**0.001**	0.816
S. elata	Leaves	<**0.001**	0.749	**0.011**	0.510
S. pinnata var. *integrifolia*	Leaves	0.899	0.046	0.437	0.278
S. pinnata var. *inyoensis*	Leaves	**0.009**	0.742	<**0.001**	0.858
S. pinnata var. *pinnata*	Leaves	0.090	0.283	0.141	0.247
S. tomentosa	Leaves	0.445	-0.293	0.814	0.092
S. viridiflora	Leaves	0.239	0.351	0.068	0.498
T. laciniatum	Leaves	0.510	-0.395	0.148	0.745
S. albescens	Fruit	0.957	-0.029	0.356	0.486
S. bipinnata	Fruit	**0.028**	0.689	0.104	0.552
S. elata	Fruit	0.240	0.239	0.559	0.120
S. pinnata var. *integrifolia*	Fruit	0.549	0.216	0.331	0.345
S. pinnata var. *inyoensis*	Fruit	**0.026**	-0.694	0.865	0.067
S. pinnata var. *pinnata*	Fruit	0.818	0.039	**0.007**	0.441
S. tomentosa	Fruit	0.132	-0.542	**0.017**	0.762
S. viridiflora	Fruit	0.915	0.033	0.083	0.498
T. laciniatum	Fruit	0.260	0.453	0.076	0.659

Supplementary Material: Chapter 3

Appendix 3.1

List of taxa sampled with taxonomic authorities, accession information, herbarium voucher information (if applicable, for a complete list of *Stanleya* and *Thelypodium* voucher information see Cappa *et al*., 2014) and TAIR and GenBank accession numbers (CHS, ITS, LD, and SAT, respectively, NA not sequenced) for new sequences generated for this study.

Arabidopsis thaliana (L.) Heynh. – NM_121396, U43224, 4010725328, CP002688

Brassica rapa L.– EF408923, EM:DU833313, KBrH028K09, AC189438

Stanleya albescens M.E.Jones – *J.J. and P.J. Cappa 38* (CS); KJ953717, KJ953750, NA, KJ953787

Stanleya albescens M.E.Jones – *J.J. and P.J. Cappa 42* (CS); KJ953718, KJ953751, KJ953817, KJ953788

Stanleya albescens M.E.Jones – *J.J. and P.J. Cappa WNS*; KJ953719, KJ953752, KJ953818, NA

Stanleya bipinnata Greene – *R.C. and Kathryn Rollins 79155* (MO); KJ953723, KJ953756, KJ953822, NA

Stanleya bipinnata Greene – *R.C. Rollins 57265* (MO); KJ953722, KJ953755, KJ953821, NA

Stanleya bipinnata Greene – *J.J. and P.J. Cappa 82*; KJ953720, KJ953753, KJ953819, KJ953789

Stanleya bipinnata Greene – *J.J. and P.J. Cappa 87*; KJ953721, KJ953754, KJ953820, KJ953790

Stanleya confertiflora (B.L. Robinson) Howell – *N. Taylor* (OSC); KJ953727, KJ953760, KJ953824, KJ953793

Stanleya confertiflora (B.L. Robinson) Howell – *Yates 1013* (OSC); KJ953724, KJ953757, KJ953823, KJ953791

Stanleya confertiflora (B.L. Robinson) Howell – *Holmgren 13154* (OSC); KJ953725, KJ953758, NA, KJ953792

Stanleya confertiflora (B.L. Robinson) Howell – *Hitchcock and Muhlick 21123* (RM); KJ953726, KJ953759, NA, NA

Stanleya elata M.E.Jones – *J.J. and P.J. Cappa 92*; NA, KJ953761, NA, KJ953794

Stanleya elata M.E.Jones – *J.J. and P.J. Cappa 99*; KJ953728, KJ953762, KJ953825, KJ953795

Stanleya elata M.E.Jones – *J.J. and P.J. Cappa 113*; KJ953729, KJ953763, KJ953826, KJ953796

Stanleya pinnata (Pursh) Britton var. ***integrifolia*** (E.James) Rollins – *J.J. and P.J. Cappa 30*; KJ953730, KJ953764, KJ953827, NA

Stanleya pinnata (Pursh) Britton var. ***integrifolia*** (E.James) Rollins – *J.J. and P.J. Cappa 34*; KJ953731, KJ953765, KJ953828, KJ953797

Stanleya pinnata (Pursh) Britton var. ***integrifolia*** (E.James) Rollins – *J.J. and P.J. Cappa 36*; NA, KJ953766, KJ953829, KJ953798

Stanleya pinnata (Pursh) Britton var. ***inyoensis*** (Munz & Roos) Reveal – *J.J. and P.J. Cappa 66*; KJ953732, KJ953767, KJ953830, NA

Stanleya pinnata (Pursh) Britton var. ***inyoensis*** (Munz & Roos) Reveal – *J.J. and P.J. Cappa 95*; KJ953733, KJ953768, KJ953831, KJ953799

Stanleya pinnata (Pursh) Britton var. ***inyoensis*** (Munz & Roos) Reveal – *J.J. and P.J. Cappa 102*; KJ953734, KJ953769, KJ953832, KJ953800

Stanleya pinnata (Pursh) Britton var. ***pinnata*** – *J.J. and P.J. Cappa 6* (CS); KJ953735, KJ953770, KJ953833, KJ953801

Stanleya pinnata (Pursh) Britton var. ***pinnata*** – *J.J. and P.J. Cappa 47* (CS); KJ953736, KJ953771, KJ953834, KJ953802

Stanleya pinnata (Pursh) Britton var. ***pinnata*** – *J.J. and P.J. Cappa 90* (CS); KJ953737, KJ953772, KJ953835, KJ953803

Stanleya pinnata (Pursh) Britton var. ***pinnata*** – *J.J. and P.J. Cappa 143*; NA, KJ953773, NA, KJ953804

Stanleya pinnata (Pursh) Britton var. ***pinnata*** – *J.J. and P.J. Cappa PR*; KJ953738, KJ953774, KJ953836, KJ953805

Stanleya pinnata (Pursh) Britton var. ***texana*** B.L. Turner – *J.J. and P.J. Cappa 128*; NA, KJ953775, KJ953837, KJ953806

Stanleya pinnata (Pursh) Britton var. ***texana*** B.L. Turner – *A.M Powell 3604* (MO); KJ953739, KJ953776, KJ953838, KJ953807

Stanleya pinnata (Pursh) Britton var. ***texana*** B.L. Turner – *A. Nelson and R.A. Nelson* 5027 (MO); KJ953740, KJ953777, NA, KJ953808

Stanleya tomentosa Parry – *J.J. and P.J. Cappa 10*; KJ953741, KJ953778, KJ953839, KJ953809

Stanleya tomentosa Parry – *J.J. and P.J. Cappa 13*; KJ953742, KJ953779, KJ953840, KJ953810

Stanleya tomentosa Parry – *J.J. and P.J. Cappa 16*; KJ953743, KJ953780, KJ953841, KJ953811

Stanleya viridiflora Nutt. – *J.J. and P.J. Cappa 1* (CS); KJ953744, KJ953781, KJ953842, KJ953812

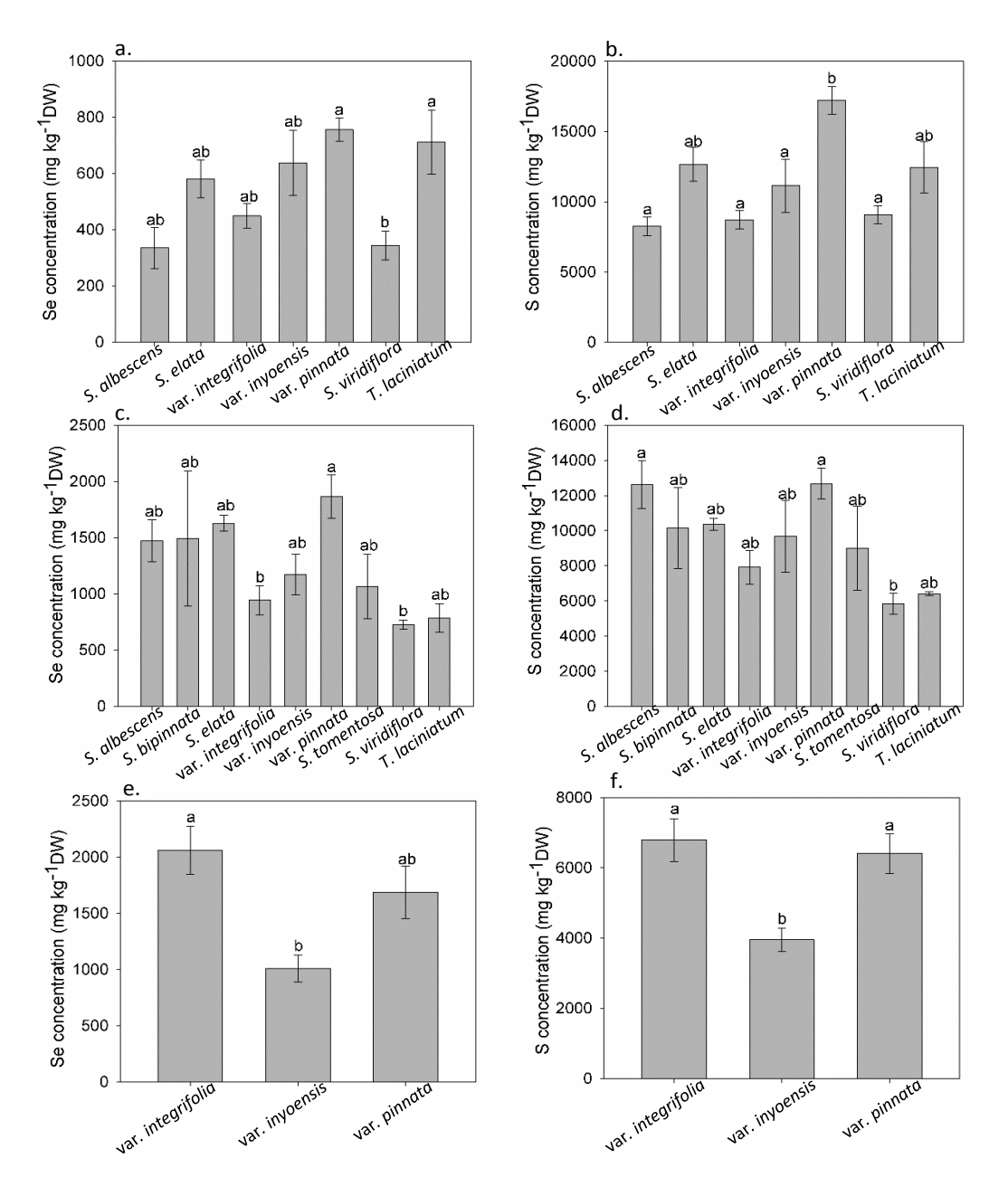
Stanleya viridiflora Nutt. – *J.J. and P.J. Cappa 25* (CS); KJ953745, KJ953782, KJ953843, KJ953813

Stanleya viridiflora Nutt. – *J.J. and P.J. Cappa 124*; KJ953746, KJ953783, KJ953844, KJ953814

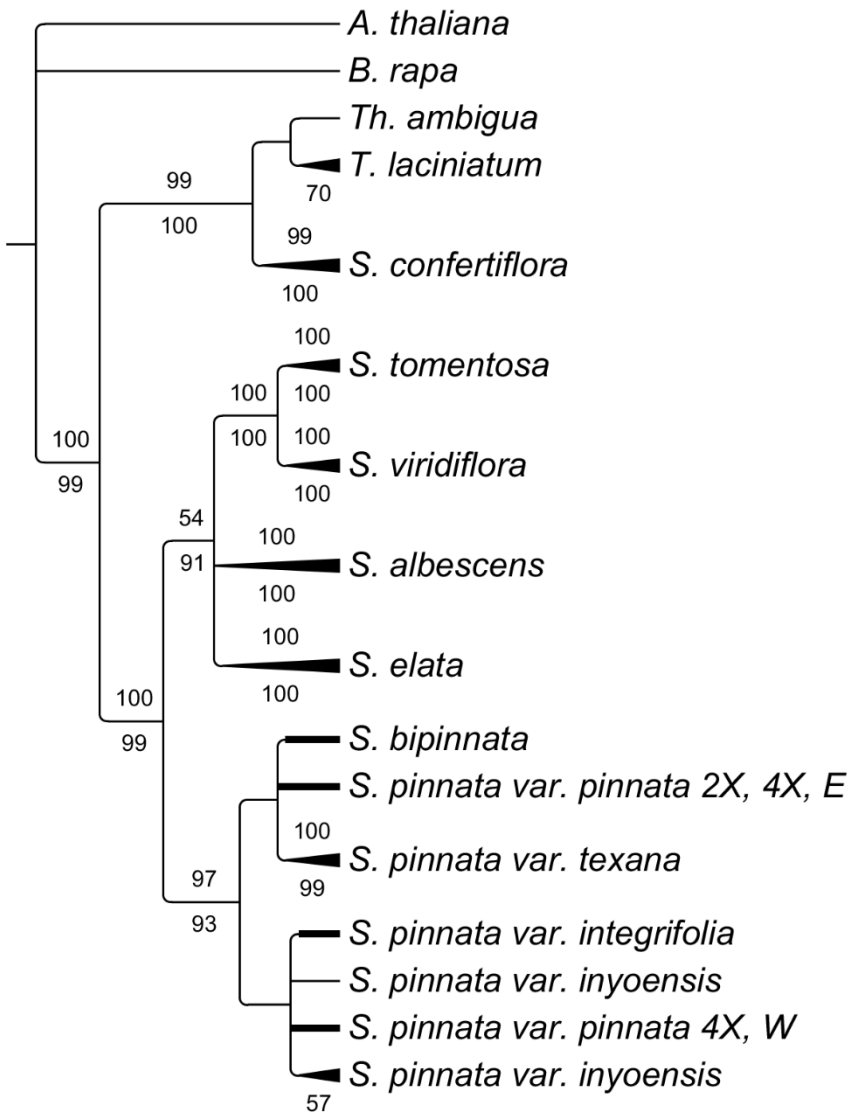
Thelypodium laciniatum Endl. – *Bourell 5992* (NY); KJ953716, KJ953749, NA, KJ953786

Thelypodium laciniatum Endl. – *J.J. and P.J. Cappa 116*; KJ953715, KJ953748, KJ953816, KJ953785

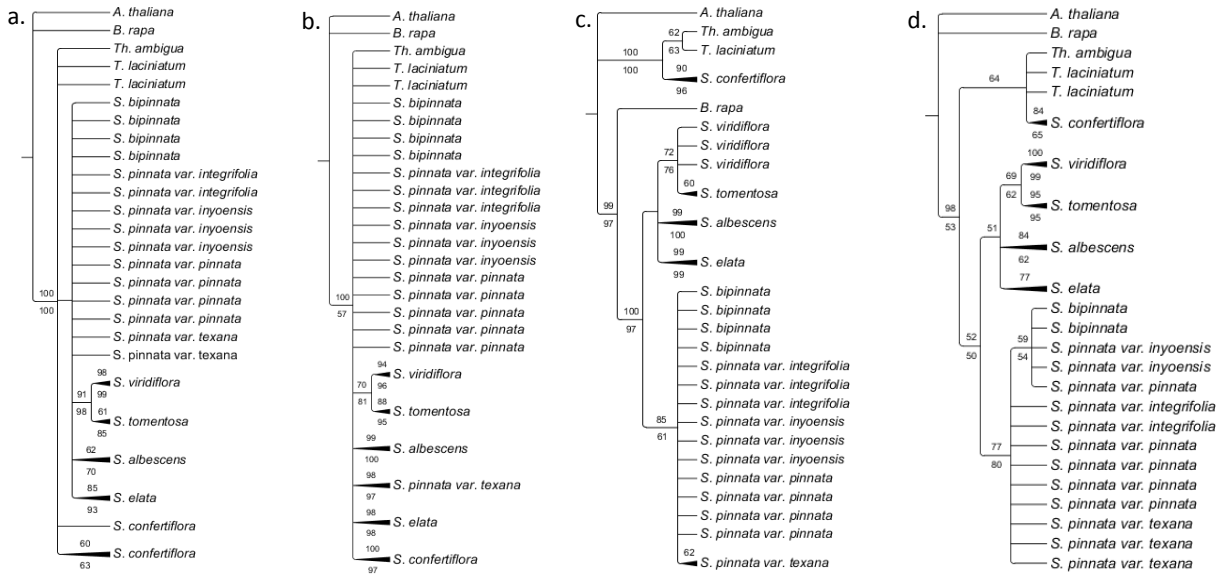
Thelypodiopsis ambigua (S.Watson) Al-Shehbaz – *Welsh and Atwood 26920* (NY); KJ953714, KJ953747, KJ953815, KJ953784



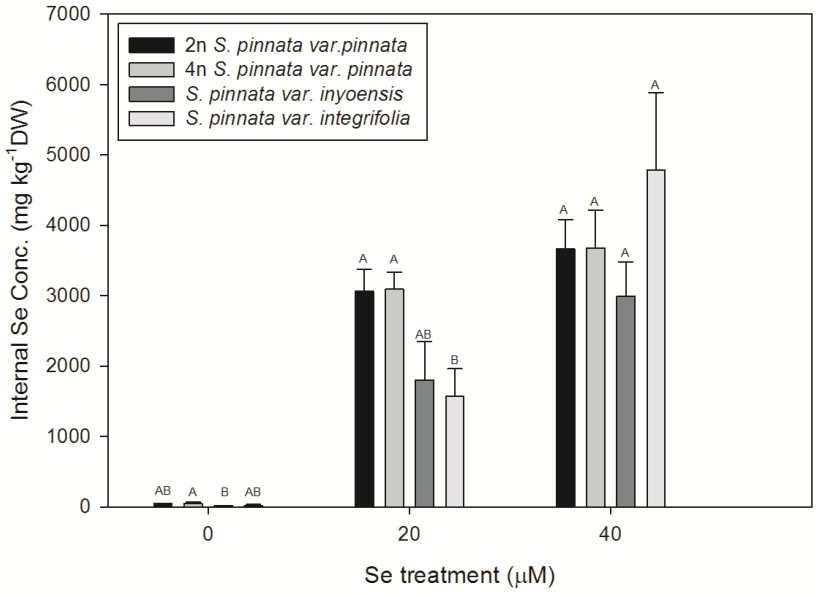
Supplemental figure 3.1. Selenium (left column) and sulfur (right column) concentrations in shoots of different *Stanleya* and *Thelypodium* species grown on agar medium supplemented with different concentrations of sodium selenate. a and b. 20µM, c and d. 80 µM, e and f. 160 µM. *Stanleya pinnata* taxa are indicated only by their varieties. Note: At the highest Se concentration, only the *S. pinnata* varieties survived. Shown are the mean and SEM of a minimum of six replicates. Letters above bars denote significant differences between the means, determined by ANOVA with a post hoc Tukey-Kramer analysis.



Supplemental figure 3.2. Strict consensus of the most parsimonious trees of molecular data only. Clade symbols represent 2-4 individuals per taxon. Values above and below the branches represent parsimony jackknife and likelihood bootstrap support values ≥ 50%, respectively. Values next to *S. pinnata* var. *pinnata* indicate diploid (2X) or tetraploid (4X) and collection site east slope (E) or west slope (W).



Supplemental figure 3.3. Strict consensus of most parsimonious trees for molecular data. Clade symbols represent 2-4 individuals per taxon. Values above and below the branches represent parsimony jackknife and likelihood bootstrap support values ≥ 50%, respectively. a. CHS, b. ITS, c. LD, d. SAT.

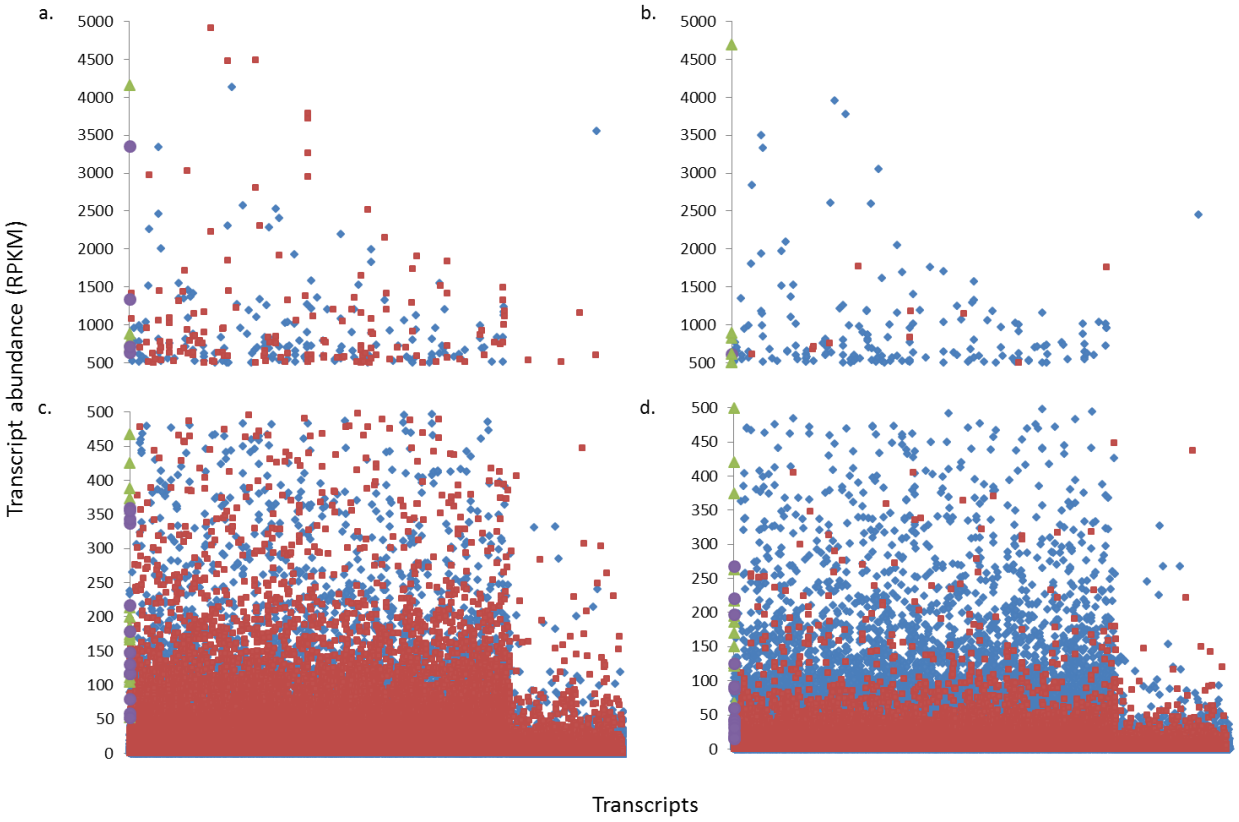


Supplemental Figure 3.4. Leaf Se accumulation in *Stanleya pinnata* varieties and ploidy variants. Plants were grown on Turface® for 3 months in a greenhouse. Selenium was supplied as sodium selenate in half strength Hoaglands solution. Shown are the mean and SEM of a minimum of five replicates. Letters above bars denote significant differences between means, determined by ANOVA with a post hoc Tukey-Kramer analysis.

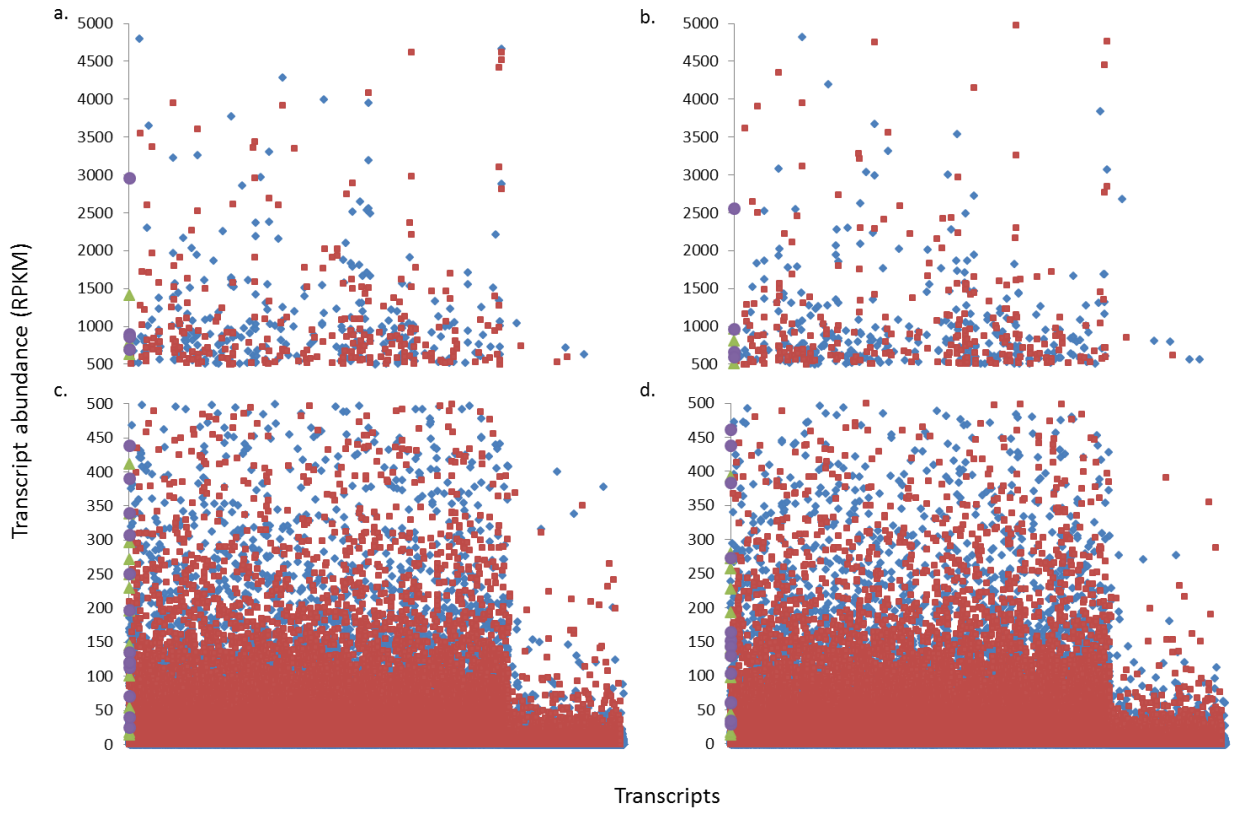
Supplemental Table 3.1. Morphological characters and chromosome numbers used in phylogenetic analysis shown in Figure 5.

Character	Character states
Plant duration	0 = annual, 1 = perennial, 2 = biennial
Basal leaf duration	0 = present when flowering, 1 = absent when flowering
Basal leaf margin	0 = margin entire, 1 = margin pinnatifid, 2 = lobed, 3 = dentate, 4 = laciniate, 5 = runcinate, 6 = bipinnatifid
Cauline leaf base shape	0 = bases acuminate, 1 = bases auriculate, 2 = hastate, 3 = cuneate
Cauline leaf margin	0 = margin entire, 1 = margin pinnatifid, 2 = lobed, 3 = dentate, 4 = laciniate, 5 = runcinate, 6 = bipinnatifid
Cauline leaf attachment	0 = sessile, 1 = petiolate, 2 = subsessile
Leaf pubescence	0 = glabrous, 1 = puberulent, 2 = tomentose, 3 = forked
Flower color	0 = yellow, 1 = white, 2 = purple
Petal claw pubescence	0 = present, 1 = absent
Gynophore presence	0 = present, 1 = absent
Filament base pubescence	0 = present, 1 = absent
Filament lengths	0 = equal, 1 = tetradynamous, 2 = subequal
Stamen position	0 = exserted, 1 = included
Silique shape	0 = subterete, 1 = terete, 2 = flattened
Chromosome number (n)	0 = 14, 1 = 28, 2 = 13, 3 = 11, 4 = 10, 5 = 5

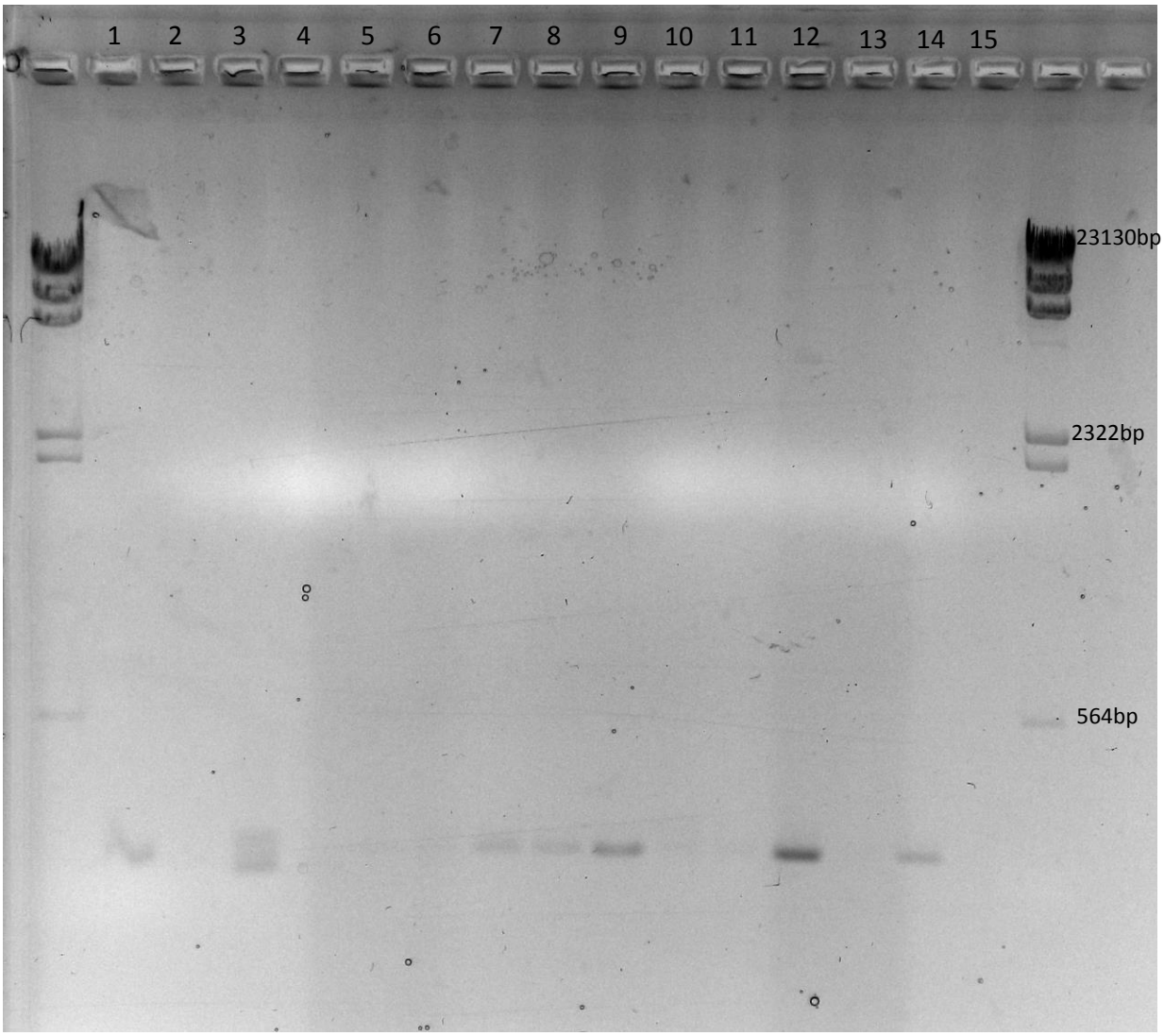
Supplementary Material: Chapter 4



Supplemental Figure 4.1. Transcript abundance in roots. Blue diamonds represent the RPKM of all transcripts in *S. pinnata* roots. Red squares represent the RPKM of all transcripts in *S. elata* roots. Green triangles represent sulfur assimilation genes in *S. pinnata* roots. Purple circles represent sulfur assimilation genes in *S. elata* roots. a. Total transcript abundance for genes with an expression level between 500-5,000 for roots treated without Se. b. Total transcript abundance for genes with an expression level between 500-5,000 for roots treated with 20 µM Na_2SeO_4. c. Total transcript abundance for genes with an expression level between 0-500 for roots treated without Se. d. Total transcript abundance for genes with an expression level between 0-500 for roots treated with 20 µM Na_2SeO_4.



Supplemental Figure 4.2. Transcript abundance in shoots. Blue diamonds represent the RPKM of all transcripts in *S. pinnata* roots. Red squares represent the RPKM of all transcripts in *S. elata* roots. Green triangles represent sulfur assimilation genes in *S. pinnata* roots. Purple circles represent sulfur assimilation genes in *S. elata* roots. a. Total transcript abundance for genes with an expression level between 500-5,000 for shoots treated without Se. b. Total transcript abundance for genes with an expression level between 500-5,000 for shoots treated with 20 µM Na_2SeO_4. c. Total transcript abundance for genes with an expression level between 0-500 for shoots treated without Se. d. Total transcript abundance for genes with an expression level between 0-500 for shoots treated with 20 µM Na_2SeO_4.



Supplemental Figure 4.3. 1% Agarose gel of APS2 genomic PCR reaction. The expected size PCR product is 243 bp. PCR primers sequences are APS2F1: 5' GCTTGCAAGAAATGATAAGCCT 3' and APS2R1: 5' GCGTACACTCATCGACTCCC 3'. Samples from left to right are: 1. *Thelypodium laciniatum*, 2. *S. confertiflora*, 3. *S. viridiflora*, 4. *S. tomentosa*, 5. *S. elata*, 6. *S. bipinnata*, 7. *S. pinnata* var. *texana*, 8. *S. pinnata* var. *integrifolia*, 9. *S. pinnata* var. *inyoensis*, 10-14. *S. pinnata* var. *pinnata* (multiple accessions) and 15. Negative control.