THESIS

STUDIES ON SELENIUM HYPERACCUMULATOR STANLEYA PINNATA AND NONACCUMULATOR STANLEYA ELATA (BRASSICACEAE): FUNCTIONAL CHARACTERIZATION OF SELENATE TRANSPORTER SULTR1;2 IN YEAST AND DEVELOPMENT OF A MICROPROPAGATION PROTOCOL

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Zackary S Guignardi

Department of Biology

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Master's Committee:

Advisor: Elizabeth Pilon-Smits

Marinus Pilon
Tom Santangelo

ABSTRACT

STUDIES ON SELENIUM HYPERACCUMULATOR STANLEYA PINNATA AND NONACCUMULATOR STANLEYA ELATA (BRASSICACEAE): FUNCTIONAL CHARACTERIZATION OF SELENATE TRANSPORTER SULTR1;2 IN YEAST AND DEVELOPMENT OF A MICROPROPAGATION PROTOCOL

Stanleya pinnata is an herbaceous perennial species in the family Brassicaceae native to the western United States. This species is classified as a selenium (Se) hyperaccumulator, and can be found thriving on Se-rich soils. Selenium hyperaccumulators are plant species that have the capacity to accumulate Se over 1,000 mg kg^{-1} dry weight in their tissues, concentrations toxic to non-accumulator plant species as well as to herbivores and pathogens, which may explain why plants hyperaccumulate Se. Due to the chemical similarity of Se to sulfur (S), Se is believed to be transported and metabolized by the same proteins and enzymes, including sulfate transporters and the sulfate assimilation pathway. Selenate (SeO_4^{2-}), the predominant available form of Se in soil, is transported into the roots mainly via the high-affinity membrane transporter SULTR1;2. While most plants do not appear to discriminate between selenate and sulfate, and the two compounds compete for uptake, selenate uptake in Se hyperaccumulators is less inhibited by high sulfate concentrations. Since SULTR1;2 is the main portal of entry for selenate into the plant, it may be hypothesized that SULTR1;2 from the Se hyperaccumulator *S. pinnata* has intrinsic properties that allow this species to discriminate between sulfate and selenate and preferentially take up selenate. One of the objectives of this thesis project was to test this hypothesis, by means of functional characterization of SULTR1;2 from *S. pinnata* and from control species *Stanleya elata,* and *Arabidopsis thaliana* in the YSD1 yeast mutant which lacks its native sulfate transporters.

A secondary objective in this thesis project was to develop a micropropagation protocol for *Stanleya*. In order to effectively study Se hyperaccumulation in a laboratory setting, sufficient numbers of *S. pinnata* and *S. elata* plants need to be available. However, due to low rates of seed germination, vernalization requirements, self-incompatibility, and ineffectiveness of propagation by cuttings, conventional propagation methods via seed or vegetative cuttings severely limit the number of plants that can be cultivated at a time. In order to overcome these limits, a tissue culture micropropagation protocol for leaf explants of *S. pinnata* and *S. elata* was developed. This protocol will allow for the rapid reproduction of both *Stanleya* species, not only to be used in laboratory experiments, but also in industrial applications such as Se phytoremediation projects, as well as for horticultural and native landscaping purposes.

The first chapter of this thesis reviews plant Se uptake and metabolism, offering an overview of the current understanding of the Se assimilation pathway in plants, including mechanisms of accumulation and tolerance unique to Se hyperaccumulators. This chapter also outlines key proteins and enzymes in the Se assimilation pathway that are candidates for future experiments to determine the mechanisms of Se hyperaccumulation.

The second chapter describes the results from yeast studies, characterizing the selenate and sulfate transport capabilities of SULTR1;2 from hyperaccumulator *S. pinnata* and non-accumulators *S. elata,* and *A. thaliana,* and their selenate specificity, as judged from the effects of sulfate competition on selenate uptake. Interestingly, yeast transformed with SULTR1;2 from *S. pinnata* (SpSultr1;2) showed less inhibition of selenate uptake by high sulfate concentration, indicating that this species' selenate selectivity may be facilitated by the SULTR1;2 protein. While apparently more Se-specific, yeast transformed with *SpSultr1;2* overall took up less Se when compared to yeast expression SULTR1;2 from non-accumulators. It is feasible that a

mutation that changes the substrate specificity of SpSULTR1;2 also reduced its overall activity. In *S. pinnata, SpSultr1;2* transcript was found in earlier studies to be ~10-fold up-regulated when compared to *S. elata,* which may compensate for decreased activity. Identification of a selenate-specific transporter has applications for Se phytoremediation and biofortification. Constitutive overexpression of a hyperaccumulator selenate transporter in other plant species may increase their uptake of Se, even in the presence of high environmental S levels.

The third chapter of this thesis outlines the development of a fast and efficient tissue culture micropropagation protocol for *S. pinnata* and *S. elata.* Through the testing of multiple concentrations of hormones on *in vitro* callus formation, shoot induction and elongation, and root formation, followed by *ex vitro* acclimatization, both species of *Stanleya* were shown to be very amenable to micropropagation. Both exhibited rapid callus, shoot, and root induction under a wide range of 1-napthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), and indole-3-butyric acid (IBA) concentrations. Future experiments could explore the genetic transformation of *S. elata* plants with genes from *S. pinnata* to test their importance for Se accumulation and tolerance in this related non-accumulating species. This micropropagation protocol also opens up the possibility to cultivate the *Stanleya* species at a large scale for multiple applications including biofortification, phytoremediation, and native landscaping.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: BIOCHEMISTRY OF PLANT SE UPTAKE AND METABOLISM

1.1 Uptake and Translocation of Selenium

Plants take up Se primarily in two forms, either as selenate (SeO_4^{2-}) or selenite (SeO_3^{2}), but they have the capacity to take up organic Se compounds as well. However, plants are unable to take up elemental Se or metal selenide compounds (White & Broadley, 2009). Selenate is the most common form of Se taken up by plants and is the predominant bioavailable form in alkaline and well-oxidized soils, while selenite is the main identifiable bioavailable form in anaerobic soils and wetlands (Mikkelsen et al., 1989; White et al., 2007b; Fordyce, 2012). Due to its chemical similarities to sulfur (S), Se in the form of selenate is transported throughout the plant via the sulfate transport system. Sulfate transporters were first characterized in *Arabidopsis thaliana* selenate-resistant mutants (Shibagaki, 2002) and can be clustered into 4 main groups. Group 1 includes high affinity sulfate transporters, SULTR1;1 and SULTR1;2, which are the best-characterized and primarily found in the roots (Buchner, 2004). Group 2 transporters have a low affinity for sulfate, are found throughout the plant, and have a role in sulfate loading into the vascular systems, and thus in translocation. Two isoforms have been identified in *A. thaliana*, SULTR2:1 and SULTR2:2, both expressed in leaves and roots. AtSULTR2;1 localizes to the xylem parenchyma, as well as the phloem cells in leaves and pericycle cells in roots, while AtSULTR2;2 is found in the phloem cells in roots and the bundle sheath cells in leaves (Takahashi, 2000; Buchner, 2004). Group 3 sulfate transporters are only found in leaves, and do not show responsiveness to the sulfur status of the plant (Buchner, 2004). AtSULTR3;1 localizes to the chloroplasts, and loss of this transporter greatly reduced the sulfate uptake capacity of these organelles (Cao et al., 2013). Group 4 includes sulfate transporters localized in tonoplasts. In *A. thaliana*, AtSULTR4;1 and AtSULTR4;2 have been characterized as low affinity sulfate

transporters playing a role in sulfate vacuolar efflux, which may make sulfate more available for export via the vasculature; thus, AtSULTR4;1 and AtSULTR4;2 have been implicated to contribute to root-shoot translocation and the delivery of sulfate to developing seeds (Zuber et al., 2010).

Selenate enters the roots through the high affinity sulfate transporters SULTR1;1 and SULTR1;2, which are proton-sulfate symporters; for every molecule of selenate that enters the roots, 3 protons are also taken up (Lass and Ulrich-Eberius, 1984; Hawkesford et al., 1993) (Figure 1.1). The expression of SULTR1;1 and SULTR1;2 is controlled by the sulfur status of the plant. SULTR1;1 expression is lower and upregulated under S-deficient conditions, while SULTR1;2 is highly expressed under both S-sufficient and S-deficient conditions (White et al., 2007; El Kassis et al., 2007). Both SULTR1 transporters have the capacity to mediate selenate transport from the soil into the root cells, but there is unequal functional redundancy between these two transporters (Barberon, 2008). *Arabidopsis thaliana sultr1;2* mutants displayed a higher tolerance to selenate compared to *sultr1;1* mutants and wild-type plants, while *sultr1;1-sultr1;2* double mutants exhibited the greatest tolerance to selenate (Barberon, 2008). This suggests that SULTR1;2 is the main portal for selenate entry into the plant, compared to SULTR1;1. SULTR1;2 shares 70% amino acid homology with other high-affinity plant sulfate transporters, and is localized in the root hairs as well as the root epidermis and cortex (Takahashi, 2002). AtSULTR1;2 was found to complement the function of two yeast sulfate transporters located in the plasma membrane (Takahashi, 2002).

Recent research suggests that SULTR1 homologs found in Se hyperaccumulator species may have a preference for selenate transport over sulfate, which may explain the high Se/S ratio and Se hyperaccumulator status of these plants (White, 2015). SULTR1 sequences isolated from

several hyperaccumulator species in the genus *Astragalus* (Fabaceae) contain an alanine residue instead of the glycine found in SULTR1 isoforms of non-accumulating angiosperms, which may play a role in the preferential uptake of selenate over sulfate reported in these species (White, 2015; Cabannes et al., 2011).

While the high-affinity sulfate transporters are responsible for the transport of selenate into the plant, selenite is taken up through a separate pathway. It is believed that selenite uptake is mediated by root phosphate transporters. Studies in perennial ryegrass (*Lolium perenne* L. cv. Evening Shade) and strawberry clover (*Trifolium fragiferrum* L. cv. O'Conner) showed that selenite uptake was reduced by up to 50% in response to a 10-fold increase in phosphate treatment (Hopper & Parker, 1999). Another study has shown that the K_m of selenite influx increased in the presence of phosphate in wheat (*Triticum aestivum*) (Li et al., 2008). These results indicate the existence of competition for uptake between selenite and phosphate, suggesting the two molecules share a common transporter, as has been reported for yeast (Lazard et al., 2010).

Plants also have the capacity to take up organic forms of Se via amino acid permeases, which are plasma membrane-localized transporters mediating the uptake of amino acids in the cell (Figure 1.1). Two common forms of organic Se are selenocysteine (SeCys) and selenomethionine (SeMet). Normally, these products are formed from inorganic pools of Se through the S assimilation pathway, but there is evidence that plants can take up organic selenocompounds directly. Studies in durum wheat (*Triticum turgidum*) and spring canola (*Brassica napus*) showed that organic forms of Se, specifically selenomethionine and selenocystine, were taken up at rates over 20-fold higher than selenate or selenite (Zayed, et al., 1998; Kikkert & Berkelaar, 2013). A broad specificity amino acid permease isolated from *A.*

thaliana complemented proline uptake in yeast mutant strains, with the strongest competitors for proline uptake being cysteine and methionine (Frommer et al., 1993). It is conceivable that selenocysteine and selenomethionine are taken up by this amino acid transporter as well.

1.2 Conversion of Inorganic Selenium into Organic Forms: The First Steps of Selenium Assimilation

After uptake into the roots, selenate needs to be converted into a biologically active form for assimilation into the plant. This is carried out by the enzyme ATP sulfurylase, which couples selenate (or sulfate) to ATP, forming adenosine 5′-phosphoselenate (APSe) or adenosine 5′-phosphosulfate (APS) (Leustek, 1994; Pilon-Smits et al., 2009; Schiavon et al., 2015) (Figure 1.1). This step, which was found to be rate limiting in Se assimilation (Pilon-Smits et al., 2009) occurs in both the cytosol and plastids (White et al., 2007b; Pilon-Smits & LeDuc, 2009; Pilon-Smits, 2012).

First characterized during studies of S assimilation, ATP sulfurylase was found to be derepressed by a selenate concentration $1/10^{th}$ that of sulfate, indicating it is responsible for the assimilation of both molecules (Reuveny, 1977). There have been 4 isoforms of ATP sulfurylase identified in *A. thaliana* (APS1-4), all localizing to the plastids of cells (Anjum et al., 2015), but *A. thaliana* APS2 was found to have dual localization to both the plastids and cytosol (Bohrer et al., 2015).

ATP sulfurylase has been a target for genetic engineering of plants with higher Se uptake capacity, with the aim of developing plants for use in phytoremediation. Transgenic Indian mustard (*Brassica juncea)* overexpressing APS1 from *A. thaliana* showed increased selenate reduction, with roots and shoots containing mostly organic Se compounds compared to wild-type plants which mostly accumulated selenate (Pilon-Smits, 1999). Greenhouse experiments

conducted with *B. juncea* APS transgenics grown on naturally seleniferous soils demonstrated that these plants accumulated Se up to 3-fold higher than wild type plants (Van Huysen et al., 2004). Field experiments in California on Se-contaminated soil confirmed these findings, with APS transgenics accumulating 4-fold more Se than wild type plants (Bañuelos et al., 2005).

APSe is converted to selenite by the activity of APS reductase (APR) (Figure 1.1). This reaction happens exclusively in the plastids. APR is an essential enzyme and is reported to be another rate-limiting step in selenate assimilation (Setya et al., 1996; Suter et al., 2000; Sors et al., 2005). The reaction equilibrium of ATP sulfurylase favors the reverse direction, and so the products of this reaction need to be converted rapidly in order for assimilation to proceed (Sors et al., 2005; Saito, 2004). While native expression of APR in several *Astragalus* species was not found to correlate with Se hyperaccumulation, transgenic experiments have shown that overexpression of APR enhances selenate reduction into organic forms, thus suggesting a role for this enzyme in selenate assimilation (Sors et al., 2005). APR's role in the Se assimilation pathway is also supported by the fact that increased activity of this enzyme contributed to increased Se flux through the plant (Sors et al., 2005). *Apr2-1 Arabidopsis* mutants showed enhanced levels of selenate, but decreased levels of selenite, implicating APR2 in converting APSe into selenite (Grant et al., 2011). These mutants also had decreased selenate tolerance due to decreased levels of glutathione, which helps to prevent the formation of damaging superoxides in the cell (Grant et al., 2011).

The next step in the Se assimilation pathway is the reduction of selenite to selenide, for incorporation into organic molecules such as amino acids. The conversion of selenite into selenide may occur either enzymatically or non-enzymatically. Sulfite reductase (SiR) is responsible for the conversion of sulfite to sulfide during reductive sulfate assimilation

(Yarmolinsky et al., 2012), so it is not out of the question for the same enzyme to catalyze the reduction of selenite (Pilon-Smits, 2012; White, 2015) (Figure 1.1). There is a single copy of the gene coding for SiR in *A. thaliana* (Khan et al., 2010), and it has been found to localize to plastids (Armengaud et al., 1995; Bork et al., 1998). The conversion to selenide may also occur non-enzymatically through an interaction between selenite and reduced glutathione (GSH) (Anderson, 2001; Terry, 2000; Pilon-Smits, 2012). This conversion takes place in multiple steps, with selenite first converted to the organic molecule GSSeSG non-enzymatically, which is then converted to GSSeH and finally to selenide through the action of glutathione reductase (GR) using NADPH as a reductant (Hsieh & Ganther, 1975) (Figure 1.1). In support of a GR role in Se assimilation, yeast glutathione was shown to reduce selenite to selenide (Hsieh & Ganther, 1975). Thus, while the reduction of selenite may be non-enzymatic, the regeneration of reduced glutathione is mediated by the enzyme GR. It belongs to the oxidoreductase family of proteins, which require NADP+ or NAD+ to transfer electrons from one molecule to another (Price & Stevens, 1999). Glutathione reductase is responsible for converting glutathione from its oxidized state back to its reduced form, which is essential in numerous cellular processes such as combating oxidative stress, promoting enzyme stability, and the regulation of cell metabolism (Jocelyn, 1972; Williams, 1976). In plants, this enzyme is active in chloroplasts and cytosol (Foyer and Halliwell, 1976). The reduction of oxidized glutathione by GR in chloroplasts has been reported to be coupled to photosynthetic electron transport (Jablonski & Anderson, 1978; Schaedle and Bassham, 1977) and may suggest that the reduction of selenite to selenide occurs in the chloroplasts as part of a light-dependent reaction (Ng & Anderson, 1978).

Se toxicity in plants can be attributed to many factors, including oxidative stress, but the main cause is considered to be the misincorporation of selenoamino acids into proteins (Pilon-

Smits, 2012). Selenium can replace sulfur in the amino acids cysteine (Cys) and methionine (Met) to produce selenocysteine (SeCys) and selenomethionine (SeMet). The prevention of incorporating these selenoamino acids into proteins is a key feature of Se hyperaccumulator species, and is instrumental for their high Se tolerance (Brown & Shrift, 1982).

1.3 Formation and Processing of Seleno-Amino Acids: Mechanisms of Preventing Selenium Toxicity

The first step in the formation of selenoamino acids is carried out by the enzyme complex Cysteine synthase (CS), which catalyzes the formation of SeCys from O-acetylserine (OAS) and selenide (White, 2015; Pilon-Smits, 2012). This process occurs in the chloroplasts of cells, but also in the cytosol and mitochondria (Ng & Anderson, 1978; Wirtz et al., 2001) (Figure 1.1). During S assimilation, Cys is formed by the reaction between OAS and hydrogen sulfide (Giovanelli, 1990). Selenocysteine formation is identical to this reaction, with the substitution of hydrogen selenide as a reactant. Cysteine synthase is a complex formed by the association of two enzymes, serine acetyltransferase (SAT) and OAS thiol-lyase (OAS-TL) (Bogdanova & Hell, 1997). SeCys can be incorporated into proteins nonspecifically, which can lead to disruption of protein function and thus Se toxicity (Stadtman 1990; Neuhierl & Bock, 1996; Van Huysen et al., 2003). The prevention of non-specific incorporation of SeCys into proteins is crucial in preventing Se toxicity. The methylation of SeCys to form methyl-SeCys (MeSeCys) is a key mechanism used by hyperaccumulator species to reduce the amount of SeCys available for incorporation into proteins (Pilon-Smits et al., 2009). The enzyme SeCys methyltransferase (SMT) is responsible for this conversion (Neuhierl and Bock 1995). SMT is homologous to other enzymes with similar functions, such as YagD in *Escherichia coli,* a homocysteine methyltransferase (HMT) able to methylate both SeCys and homocysteine, and belongs to a class

of methyltransferases involved in the metabolism of S-methylmethionine (Neuhierl et al., 1999; Sors et al., 2005). SMT was also found to be highly homologous to HMTs isolated from *A. thaliana* and *Oryza sativa* (Sors et al., 2005), and is localized in the chloroplasts (Sors et al., 2009). SMT also shows a preference for the methylation of SeCys over Cys by at least 3 orders of magnitude (Neuhierl & Bock, 1996), further solidifying its role in conferring Se tolerance to plants (Neuhierl et al., 1999). SMT has been identified in multiple non-accumulator and Se hyperaccumulator species of *Astragalus* but only the isoform from the hyperaccumulators had the ability to produce MeSeCys, indicating its essential role in the ability to tolerate and accumulate high levels of Se (Sors et al., 2009). In fact, the main form of Se found in the hyperaccumulators *A. bisulcatus* and *Stanleya pinnata* is MeSeCys, due to the high activity of the SMT enzyme (Neuhierl et al., 1999; Birringer et al., 2002; Pickering et al., 2003; Sors et al., 2005; Freemen et al., 2006, 2010; Lindblom et al., 2013; Alford et al., 2014; White 2015), while selenate was the major Se compound found in related non-accumulator species (de Souza et al., 1998; Freeman et al., 2006; Pilon-Smits 2012). Although SMT is found to be highly expressed specifically in hyperaccumulators (Sors et al., 2009), some Se accumulator species, such as *Brassica oleracea (*Broccoli) also have an SMT enzyme, but it is expressed only in the presence of Se (Lyi et al., 2005; Pilon-Smits 2012). SMT has been used in transgenic studies to confer increased Se accumulation and tolerance in non-accumulating species. SMT isolated from *A. bisulcatus* induced the accumulation of MeSeCys and γ-glutamyl-MeSeCys in *A. thaliana*, and increased Se accumulation and volatilization in *B. juncea* (LeDuc et al., 2003; Ellis et al., 2004).

While the production of MeSeCys is critical to Se tolerance in plants, further processing of this molecule into volatile compounds serves as another mechanism by which plants tolerate high levels of Se. The volatile compound dimethyldiselenide (DMDSe) is formed by oxidation

and methylation of MeSeCys (Meija et al., 2002; Sors et al., 2005). First, MeSeCys is converted to methylselenocysteineselenideoxide (MeSeCysSeO), whose sulfur analog methylcysteinesulfoxide (MeCysSO) is responsible for many *Brassica* varieties' characteristic flavors (Chin & Lindsay, 1994). This compound is then converted to another key intermediate methaneselenol (CH_3SeH) via the action of the enzyme Cys sulfoxide lyase (Chin & Lindsay, 1994; Griffiths et al., 2002; Ellis & Salt, 2003). DMDSe production occurs in the leaves, and has been detected in the Se hyperaccumulator *Astragalus racemosus* (Evans et al., 1967). Volatile Se compounds have been hypothesized to aid in defense against herbivory. This is supported not only by the fact that the production of these volatiles occurs in the leaves, but that it also occurs primarily after tissue injury (Ellis & Salt, 2003).

The formation of SeMet occurs through the enzymatic conversion of SeCys. There are multiple steps involved in the synthesis of SeMet, which include potential targets for transgenic phytoremediation efforts. First, SeCys is converted to Se-cystathionine by the enzyme cystathionine-γ-synthase (CGS) (Pilon-Smits, 2012). CGS catalyzes the formation of Se-cystathionine via the condensation of O-phosphohomoserine (OPH) and SeCys (Van Huysen et al., 2013; Sors et al., 2005). CGS was shown to be a rate-limiting enzyme in the conversion of SeCys to volatile DMSe (Van Huysen et al., 2003). Transgenic *B. juncea* overexpressing CGS had 2-3 fold higher Se volatilization rates and concurrent 20-40% lower shoot and 50-70% lower root Se levels compared to wild type plants, highlighting the value of this approach for applications in Se phytoremediation (Van Huysen et al., 2003, 2004). Se-cystathionine is converted to Se-homocysteine via a reaction between Se-cystathionine and water, mediated by the enzyme cystathionine beta-lyase. This enzyme is shared in both the Se and S assimilation pathways, evidenced by the fact that cystathionine beta-lyase isolated from both Se

hyperaccumulator and non-accumulator plant species had the capacity to cleave both Se-cystathionine and cystathionine into Se-homocysteine and homocysteine, respectively (Sors et al., 2005; McCluskey et al., 1986). Finally, the conversion of Se-homocysteine to SeMet is catalyzed by the enzyme Met synthase. Met synthase has been isolated from plants from various angiosperm taxa, including *A. thaliana, Catharanthus roseus,* and *Coleus blumei* (Eichel et al., 1995; Petersen et al., 1995; Ravanel et al., 1998). Using methyl-tetrahydrofolate as a carbon donor, Met synthase catalyzes the conversion of Se-homocysteine to SeMet (Cossins & Chen, 1997).

Like SeCys, SeMet is subject to further processing steps that reduce its incorporation into proteins. The volatile Se compound DMSe is synthesized via the S volatilization pathway starting from SeMet (Tagmount et al., 2002). Enzymes involved in the S volatilization pathway and formation of dimethyl sulfide (DMS) have also been discovered to be involved in the production of DMSe (Terry & Zayed, 1994; Tagmount et al., 2002). The production of DMSe in plants is important not only as a defense against herbivores, but it also diverts large pools of potentially toxic SeMet to the significantly less toxic DMSe. DMSe was found to be almost 600 times less toxic than inorganic Se compounds (McConnell & Portman, 1952; Wilber, 1980). DMSe is the main volatile Se compound isolated from non-accumulator plant species, while DMDSe is primarily produced in hyperaccumulators (Pilon-Smits & LeDuc, 2009). The first step in the synthesis of DMSe is the methylation of SeMet to form Se-methyl Se-Met (SeMM) by the enzyme S-adenosyl-L-Met:Met-S-methyltransferase (MMT) (Tagmount et al., 2002). SeMM can be converted to DMSe by one of two pathways. SeMM may first be converted to the intermediate molecule 3-dimethylselenoniopropionate (DMSeP) (Kocsis et al., 1998). The sulfur analog DMSP is a biologically important molecule, playing important roles in osmoprotection of

plants and bacteria (Mason & Blunden, 1989; Paquet et al., 1994; Kocsis et al., 1998). The synthesis of DMSP has been detected in members of the family Poaceae, such as *Spartina alterniflora* (Kocsis et al., 1998), as well as members of the Asteraceae including *Melanthera biflora* (syn. = *Wollastonia biflora*) (Hanson et al., 1994; James et al., 1995) and *Ratibida pinnata* (Paquet et al., 1995). The synthesis of DMSe may also proceed directly from SeMM via the enzyme methylmethionine hydrolase (Mudd et al., 1990; Meija et al., 2002; Ellis & Salt, 2003).

Aside from volatilization, plants have another mechanism to help prevent Se toxicity. Selenocysteine lyase (SL) is an enzyme that breaks down SeCys into elemental Se and alanine, reducing the amount of free SeCys available for misincorporation into proteins (Van Hoewyk et al., 2005). Selenocysteine lyases are analogous to NifS-like Cys desulfurase proteins characterized in Arabidopsis (Ye et al., 2005), whose main role is to generate free S from Cys for the formation of FeS clusters (Pilon-Smits et al., 2002). There are two isoforms of SeCys lyase found in plants, with different subcellular localization patterns; one isoform localizes to the cytosol (Kushnir et al., 2001), and the other to mitochondria and plastids (Pilon-Smits et al., 2002). Overexpression of a chloroplast-localizing NifS protein from Arabidopsis (AtCpNifS) was found to increase Se tolerance by 1.9-fold and increased Se accumulation by 2.2-fold (Van Hoewyk, 2005). Similarly, expression of a mouse SL caused a 2-fold reduction in Se incorporation into proteins and a 1.5-fold increase in shoot Se concentration in Arabidopsis (Pilon, 2003), as well as a 2-fold increase in Se accumulation in Indian mustard in both lab (Garifullina et al., 2003) and field (Bañuelos et al., 2007) studies. Selenocysteine lyases not only help to reduce Se toxicity in plants, but also appear to be promising enzymes to exploit for phytoremediation purposes.

The mechanisms by which plants accumulate, assimilate, and tolerate Se mirror aspects of the S assimilation pathway, but the roles these two elements play in the plant are very different. By better understanding the pathways of Se assimilation, new approaches to developing plants for phytoremediation and biofortification can be exploited, and mechanisms that hyperaccumulator species exploit in their uptake and assimilation of Se can be further elucidated.

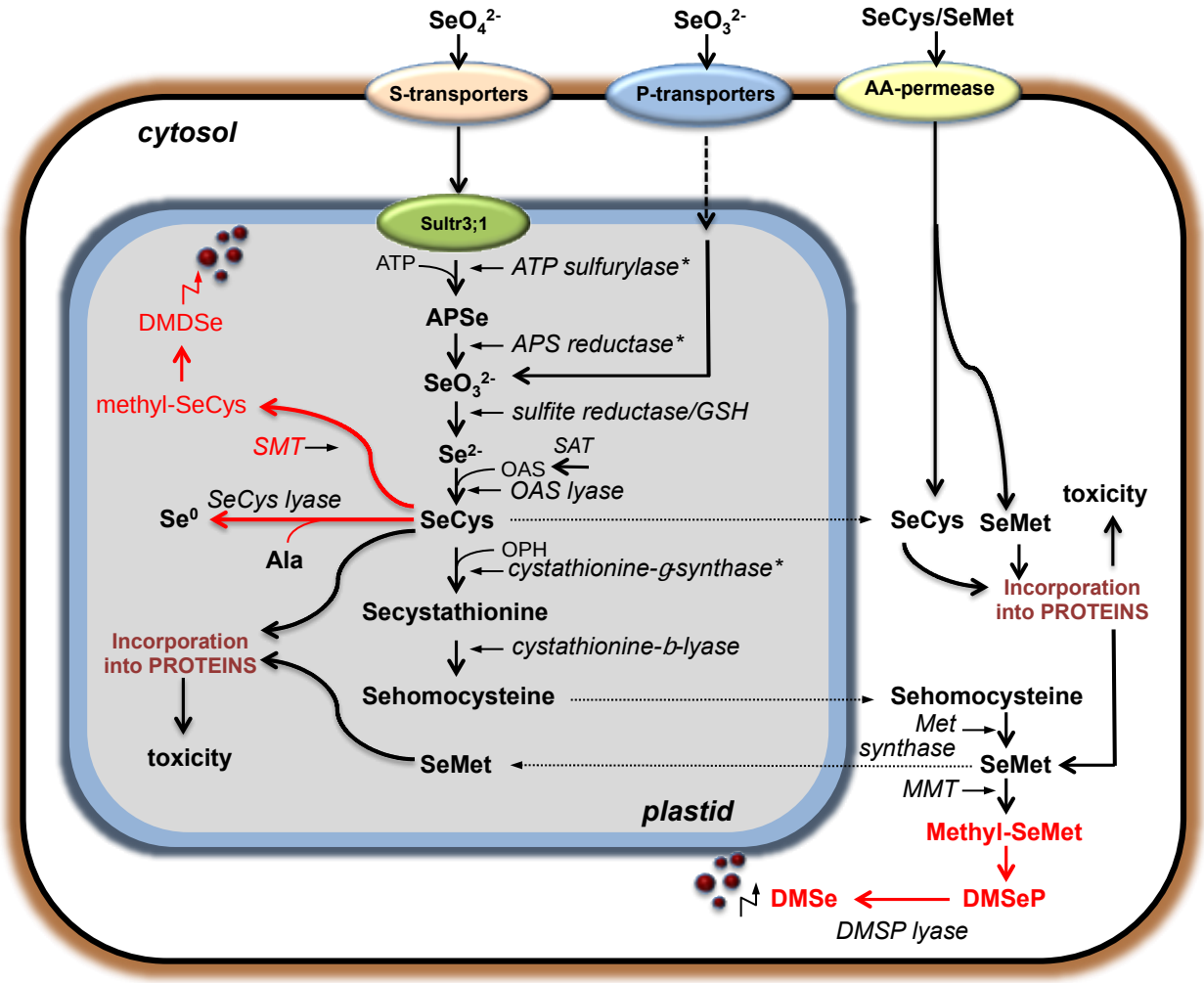

Figure 1.1) Schematic model of Se assimilation and metabolism in plant mesophyll cells. Red text and arrows indicate Se hyperaccumulator processes. Asterisks indicate enzymes overexpressed via genetic engineering. Sultr: sulfate/selenate cotransporters; APSe: adenosine phosphoselenate; GSH: glutathione; SAT: serine acetyltransferase; OAS: O-acetylserine; (Se)Cys: (seleno)cysteine; OPH: O-phosphohomoserine; (Se)Met: (seleno)methionine; MMT: methylmethionine methyltransferase; DMSeP: dimethylselenoproprionate; DM(D)Se: dimethyl(di)selenide (volatile); SMT: selenocysteine methyltransferase. Adapted from Schiavon & Pilon-Smits, 2016.

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CHAPTER 2: CHARACTERIZATION OF SELENATE TRANSPORT OF ROOT MEMBRANE TRANSPORTER SULTR1;2 FROM SELENIUM HYPERACCUMULATOR STANLEYA PINNATA AND NONACCUMULATORS STANLEYA ELATA AND ARABIDOPSIS THALIANA

2.1 Introduction

Selenium (Se) is a trace element naturally occurring in soils at concentrations between 0.1 and 5 parts per million (ppm) (https://mrdata.usgs.gov/geochem/doc/averages/se/usa.html). While Se has been found to be an essential microelement for animals and bacteria (Romero et al, 2005), it has not yet been found to be essential for plants (Schiavon et al, 2017). However, due to its chemical similarity to sulfur (S) plants still have the capacity to take up Se via the S assimilation pathway. Selenium can be found in soils in various forms, including the inorganic forms selenate ($Se0_4^{2-}$) and selenite (SeO_3^{2-}), and organic forms including methyl-selenocysteine and methyl-selenomethionine (Pyrzynska, 1996; Bauer, 1997). Selenate is the predominant form found in oxidizing conditions, and is the primary form taken up by plants (Bauer, 1997). Some plants have the capacity to accumulate and tolerate high levels of Se that would be fatal to other plant species. These species occur across different orders and families (Cappa et al, 2014, and are known as Se hyperaccumulators.

Selenium hyperaccumulators are defined as having concentrations of Se of over 1,000 mg kg^{-1} dry weight (El Mehdawi et al, 2012), with some species accumulating up to 10,000 mg kg^{-1} (Quinn et al, 2010). These elevated levels of Se can have adverse effects on overall plant health (Brown & Shrift, 1982), but hyperaccumulators have multiple mechanisms to circumvent and mitigate Se toxicity. They generally accumulate the methylated form of the seleno-amino acid selenocysteine (methylselenocysteine). Methylation of selenocysteine prevents its substitution

for cysteine in proteins, which likely disrupts proper protein folding due to inhibition of disulfide bridge formation (LeDuc et al, 2004). Hyperaccumulators also show different patterns of Se localization when compared to nonaccumulators, with hyperaccumulators preferentially sequestering Se in the outer margins of the leaves in epidermal cells, segregated from sensitive metabolic processes (Cappa et al, 2015). Another key difference observed in Se hyperaccumulators is elevated Se: S ratios when compared to nonaccumulators (White et al, 2007) and compared to their growth substrate, which may suggest that hyperaccumulators have the ability to preferentially accumulate Se over S analogues.

Due to its chemical similarity to S, Se is believed to be transported into plants via S transporters, and metabolized through many of the enzymes found in the S assimilation pathway (White, 2016). Both sulfate (SO_4^{2-}) and selenate are first transported from the soil into the roots via the high-affinity sulfate transporters SULTR1;1 and SULTR1;2, with SULTR1;2 being constitutively expressed over a wide range of sulfate concentrations in *Arabidopsis* and SULTR1;1 expressed only under S deficiency (Yoshimoto et al, 2002). Based on experiments exploring changes in root length in *Arabidopsis* knockouts grown on varying concentrations of selenate, SULTR1;2 has also been found to be the main portal of entry for selenate into the plant (El Kassis et al, 2007). In experiments exploring the effects of sulfate concentrations on selenate uptake, it has been shown that selenate concentrations in non-accumulator plants are significantly reduced when high sulfate levels are present in the environment (White et al, 2007). However, this level of reduction is variable across plant species, with Se hyperaccumulator species exhibiting less reduction of selenate uptake in the presence of high sulfate compared to nonaccumulator species (White et al, 2004; White et al, 2007). This ability to preferentially

transport higher levels of selenate even in the presence of high sulfate may be due to properties inherent to the SULTR1;2 homolog found in hyperaccumulators (Cabannes et al, 2011).

SULTR1;2 is a membrane protein belonging to the sulfate permease (SulP) family, found across many kingdoms of life including plants, animals, bacteria, fungi, and archaea (Alper & Sharma, 2013). SULTR1;2 is a sulfate/ proton cotransporter, transporting 3 protons across the plasma membrane for one sulfate ion (Shibagaki, 2002). This protein is characterized by having 12 membrane spanning domains, and a C-terminal Sulfate Transporter and Anti-Sigma factor antagonist (STAS) domain (Aravind & Koonin, 2000), which is responsible for sulfate transport (Rouached et al, 2005) and interactions with other sulfate assimilation enzymes such as cysteine synthase (Shibagaki & Grossman, 2010). While enzyme kinetics measurements for sulfate transport have been performed on SULTR1;2 isolated from the model plant *Arabidopsis thaliana* (Yoshimoto et al, 2002), and selenate resistance has been found in *sultr1;2 Arabidopsis* knockouts (Shibagaki, 2002), this protein has not been characterized in the Se hyperaccumulator *Stanleya pinnata*. Based on RNA-seq data (Pilon-Smits lab, unpublished), it appears that *Sultr1;2* from *S. pinnata* is constitutively expressed under S-deficient and S-sufficient conditions, and that its expression is upregulated 10-fold compared to the non-hyperaccumulator *Stanleya elata.*

Previous studies on the transport activity of SULTR1;2 have utilized radiolabeled sulfate in order to effectively determine the kinetics of sulfate transport for this protein (Yoshimoto et al, 2002). However, no commercial source of radiolabeled selenate is available. In order to effectively measure the selenate transport activity of SULTR1;2, inductively coupled plasma mass spectrometry (ICP-MS) can be utilized. ICP-MS can potentially detect concentrations of elements to 100 parts per trillion (ppt) (Shrivastava & Gupta, 2011), and in conjunction with

relatively low background levels of selenium in the environment, serves as a viable platform to test for the selenate transport activity of SULTR1;2.

The goal of this study is to determine if SULTR1;2 from the Se hyperaccumulator *S. pinnata* has altered selenate transport capabilities in the presence of high and low sulfate concentrations when compared to the non-hyperaccumulator species *S. elata* and *A. thaliana.* To explore this hypothesis, the *Saccharomyces cerevisiae* yeast sulfate uptake mutant YSD1 can be used (Yoshimoto et al, 2002). YSD1 is a yeast strain first isolated because of its resistance to selenate, and was later characterized by having mutations in the sulfate transporter gene *Sul1* (Smith et al, 1995). The SUL1 protein is one of two sulfate transporters found in *S. cerevisiae,* and is a high affinity sulfate transporter (K_m = 7.5 ± 0.6 μM SO_4^{2-}) with 12 membrane domains, similar to *Sultr1;2* in plants (Smith et al., 1995). This yeast strain and the yeast inducible expression vector pYES2 have been previously used to characterize sulfate uptake of plant transporters (Yoshimoto et al, 2002). However, the selenate transport activity of plant sulfate transporters has not been previously explored using this system. By using YSD1 to study the selenate transport activity of SULTR1;2, we can determine if the properties of SULTR1;2 from *S. pinnata* enable the yeast cell to discriminate between selenate and sulfate in high and low sulfate growth conditions.

2.2 Materials and Methods

2.2.1 Yeast Strains and Growth Conditions

The strains used for these studies are listed in Table 1. Media components were purchased from Difco (Detroit, MI), Sigma-Aldrich (St. Louis, MO), and Thermofisher (Waltham, MA). Yeast media types are listed in Table 3. Media for plates was supplemented

with 2% agar. All yeast liquid cultures and plates were incubated in an incubator set to 30°C. The OD_{600nm} of all cultures was measured using a Beckman DU 530 Spectrophotometer. Selenate added to media was from a 0.1 M stock solution prepared by dissolving 9.45 g sodium selenate (Acros Organics) in 500 ml of distilled H_2O.

2.2.2 E. coli and Yeast Transformation

DH5-α competent *E. coli* cells were used for all bacterial transformations (Taylor et al, 1993). Vectors were transformed into these cells via $CaCl_2$/ heat shock transformation. Ligated vector was added to 200 µL of cells in a 1.5 ml Eppendorf microcentrifuge tube on ice for 45 minutes. Then, the cells were heat shocked at 42°C on a VWR digital heatblock for 45 seconds, and then placed on ice for 5 minutes. 1 ml of liquid LB media was added to the cells, and they were incubated at 37°C for 1 hour. The cells were then plated on LB agar media supplemented with 100 ug / ml ampicillin. YSD1 yeast cells were used for all yeast transformations, and all transformations were carried out via the LiAc-mediated transformation procedure outlined in the Clontech Yeast Protocols Handbook (Clontech, 2008).

2.2.3 Sultr1;2 Amplification, Cloning, Plasmids, and Plasmid Purification

Primers used for amplification of Sultr1;2 cDNA from these three species are listed in Table 4. Previously generated cDNA from root tissue of *S. pinnata* (Western Native Seed, Coaldale, CO), *S. elata* (El Mehdawi et al, 2012), and *A. thaliana* (Genbank AB042322) was used for isolation of the Sultr1;2 open reading frame. PCR reactions were prepared and executed with the Novagen KOD HotStart DNA Polymerase kit per the manufacturer's instructions. PCR was performed in a Eppendorf Mastercycler gradient thermocycler with these cycling conditions; Initial denaturation for 2 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 20

seconds, annealing at 55°C for 10 seconds, and extension 70°C for 40 seconds, with a final extension at 70°C for 10 minutes. All restriction digestions were performed with Thermofisher FastDigest restriction enzymes incubated at 37°C on a Thermolyne Dri-Bath heating block for 15 minutes. To purify restriction fragments and PCR products, samples were loaded onto a 1% agarose TBE gel and run at 90V for 40 minutes. DNA bands were illuminated with a Fotodyne FOTO/ UV 26 illuminator, excised with a scalpel, and moved to a 1.5 ml Eppendrof microcentrifuge tube. The DNA was purified from the gel piece using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany). All ligations were performed with T4 Ligase (Thermofisher) at room temperature for 30 minutes.

After each ligation, plasmids were initially transformed into *E. coli* for screening on positive transformants and plasmid amplification. Plasmids were purified using the Qiagen Plasmid Miniprep kit, and sequence verified via Sanger sequencing through GeneWiz (http://www.genewiz.com) using the pYES2_F2, pYES2_R2, SpinSultr1;2_5FW_EcoRI, SpinelaSultr1;2_3Rev_EcoRI, AtSultr1;2_5Fw_EcoRI, AtSultr1;2_3Rev_EcoRI, SelaSultr1;2_5FW_EcoRI, Spin_Sultr1;2_QuarterFw, Spin_Sultr1;2_QuarterRev, SpinelaSultr1;2_centerFw, SpinelaSultr1;2_centralRev, SpinSultr1;2_ThreequarterFw, SpinSultr1;2_ThreequarterRev, AtSultr1;2_centF, AtSultr1;2_centR primers (Table 4). After verification of the plasmid sequences, plasmids were then cloned into YSD1 yeast cells for expression studies.

Plasmids used in this study are listed in Table 2. Sultr1;2 from *S. pinnata* was amplified using the 5'-SpSultr1;2_EcoRI and 3'-SpSultr1;2_EcoRI primer set, and *Sultr1;2* from *A. thaliana* was amplified using the 5'-AtSultr1;2_EcoRI 3'-AtSultr1;2_EcoRI primer set. After gel purification of the PCR products and the pYES2 plasmid digested with EcoRI, the 2.1-kb EcoRI

fragments containing the Sultr1;2 sequence from *S. pinnata* or *A. thaliana* were digested using the EcoRI restriction enzyme and cloned into pYES2 (digested with EcoRI and dephosphorylated) to produce pEPY1 and pEPY2. To generate variants of these plasmids with a Myc/6x His sequence for immunoblotting, the 339bp AvrII-SphI from pET28-At was ligated to pEPY2 that was digested with AvrII and SphI to create pEPY21. pEPY1 was used as the starting template for a PCR with the 5'-SpSultr1;2_EcoRI and 3'-SpSultr1;2_nostop_PacI primer set to generate a copy of Sultr1;2 from *S. pinnata* without the stop codon. This amplicon and pEPY21 were digested with the EcoRI and PacI restriction enzymes, and the 2.1kb PCR fragment and 5.9kb pEPY21 fragment were ligated together to generate pEPY11. pEPY31 was generated by first amplifying Sultr1;2 from *S. elata* cDNA using the 5'-SeSultr1;2_EcoRI/ 3'-SeSultr1;2_nostop_PacI primer set. The amplicon was purified via gel extraction, and was digested with the EcoRI and PacI restriction enzymes along with the pEPY21 vector. The DNA fragments from these reactions were separated via gel electrophoresis, purified via gel extraction, and the 2.1kb PCR fragment and the 5.9kb pEPY21 fragment were ligated to create pEPY31.

2.2.4 Sulfate Uptake Complementation

For the initial assessment of sulfate uptake complementation, single colonies of yeast strains SpSultr1;2, AtSultr1;2, and YSD1pY were streaked on SD-S (0.1 mM SO_4^{2-}) plates supplemented with a final concentration of 2% glucose or 2% galactose and grown for 5 days at 30°C. Growth of the cells in the presence of glucose (no expression of plant transporters) and galactose (induced expression of plant transporters) was assessed after 3 and 5 days. Complementation of sulfate uptake with SpSultr1;2, AtSultr1;2, and YSD1pY was repeated three times.

After the results of the first sulfate complementation experiment, single colonies of strains SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY were streaked on SD-S (0.1 mM SO_4^{2-}) plates supplemented with a final concentration of 2% galactose and grown for 5 days at 30°C. Growth of the cells was assessed after 3 and 5 days. Complementation of sulfate uptake with SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY was repeated twice.

2.2.5 Selenate Tolerance Assay

The YSD1 yeast strain, lacking sulfate transport activity, and yeast strain 22574d, which contains a functional copy of the Sul1 transporter (Jauniaux & Grenson, 1990) were used to assess the selenate tolerance of yeast. Cultures of yeast strains YSD1 and 22574d were grown overnight in either SD-U or SD-S media (0.1 mM SO_4^{2-}). 10 ul of serial dilutions of the overnight cultures were plated on SD-S media (0.1 mM SO_4^{2-}) supplemented with 0 µM, 50 µM, 100 µM, 500 µM, 1,000 µM, 2,500 µM, 5,000 µM, or 10,000 µM selenate. The cells were initially normalized to an OD_{600nm} of 1.0 before 10-fold serial dilutions were prepared to a final concentration of 1.0×10^{-5}. The cells were incubated at 30°C and growth of the cells was assessed after 3 and 5 days. Assessment of selenate tolerance in YSD1 and 22574d was conducted once.

Serial dilutions of strains SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY were incubated on SD-S (0.1 mM SO_4^{2-}) plates supplemented with either 0 µM, 10 µM, 25 µM, 50 µM, or 100 µM selenate. The cells were initially normalized to an OD_{600nm} of 1.0, before being diluted 10-fold to a final concentration of 1.0×10^{-5}. The cells were incubated at 30°C and growth of the cells was assessed after 3 and 5 days. Assessment of selenate tolerance in SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY was conducted twice.

2.2.6 Yeast Growth Curve Assay

To determine the effect of selenate on the growth of yeast cells in liquid culture, strains SpSultr1;2, AtSultr1;2, and YSD1pY were grown in the presence or absence of selenate and their growth curve was plotted over time. For the first experiment, 250 ml of SD-S (0.1 mM SO_4^{2-}) with or without 50 µM selenate was inoculated with SpSultr1;2, AtSultr1;2, or YSD1pY cells to a starting average OD_{600nm} of 0.03. The cultures were grown at 30°C in a shaking incubator set to 180 rpm, and the OD_{600nm} was measured at 15, 24, 39, 47, and 65 hours. For the second experiment cultures of SpSultr1;2, AtSultr1;2, or YSD1pY cells were inoculated and grown under the same conditions as the previous experiment, and the OD_{600nm} was measured at 22, 27, 46, 52, and 72 hours. These experiments were used to determine at what OD_{600nm} logarithmic growth began for these yeast strains.

2.2.7 Quantification of Selenate Uptake

SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY cells were inoculated in 175ml of SD-S (0.1 mM SO_4^{2-}) or SD-S (1.0 mM SO_4^{2-}) media and grown to log-phase overnight in 500 ml Erlenmeyer flasks at 30°C in a shaking incubator set to 180 rpm. After measuring the OD_{600nm}, the larger cultures were divided into three 250 ml baffled Nalgene Erlenmeyer flasks so that each flask contained 50 ml. Selenate was added to these flasks to a final concentration of 0 µM, 10 µM, 25 µM, 50 µM, or 100 µM, and the cells were incubated at 30°C in a shaking incubator set to 180 rpm for an additional hour. After one hour of incubation, the cultures were transferred to 50 ml conical tubes, and centrifuged in an Allegra 21R centrifuge at room temperate at 2,500 rpm for 5 minutes. The supernatant was removed and the cells were washed and re-suspended twice in 30ml of ice-cold 25 mM sodium phosphate (Thermofisher) monobasic buffer set to pH 7.5. The cells were centrifugated at 2,500 rpm for 5 min between each wash, and the supernatant

was removed. The pellets were transferred to 1.5 ml Eppendorf microcentrifuge tubes by re-suspending in 1ml ice-cold 25 mM sodium phosphate monobasic buffer, centrifugated in an Eppendorf 5415D centrifuge at 2,500 rpm for 5 minutes, and the supernatant was removed. The cell pellets were dried overnight in a Fisher Scientific Isotemp Incubator set to 55°C. The dry weight of the cell pellets was measured before acid digestion and ICP-MS elemental analysis. Three biological replicates for each yeast strain were analyzed for total Se content. To determine the statistical significance of selenate uptake data for the yeast strains tested, a 2-tailed student's T-test was performed. Values that were significantly different ($p < 0.05$), were indicated by different letters and asterisks in figures and tables.

2.2.8 ICP-MS Analysis

Elemental analysis was performed following a protocol previously developed for plant material (Prins et al, 2011) modified for yeast cells. Dried yeast cells (5-25 mg) were transferred to 50 ml glass test tubes and digested in 500 µl of 70% trace metal grade nitric acid (Thermofisher) at 60°C for 2 hours, followed by 130°C for 6 hours. The digested samples were diluted to 15 mL with distilled H_2O and analyzed for total Se and S via inductively coupled plasma mass spectrometry (HP Agilent 4500 ICP-MS) according to the manufacturer's instructions, using appropriate controls and standards. The detection limit of this machine was approximately 0.1 µg L^{-1} (ppb) in the digest. All values shown in the figures were well above this detection limit.

2.2.9 Protein Extraction, Quantification, and Immunodetection

The yeast protein extraction protocol was slightly modified from the protocol published by Zhang et al, 2011. SpSultr1;2t, SeSultr1;2t, AtSultr1;2t and YSD1pY yeast strains were

grown to an OD_{600nm} of 0.8 – 1.0 in 20 ml SD-U media supplemented with 2% galactose to induce expression of Sultr1;2. The cultures were centrifugated at 2,500 rpm for 5 minutes, and the supernatant was removed. The cell pellets were re-suspended in 5 ml ice-cold 2.0 M lithium acetate (ICN Biomedicals) for 5 minutes on ice before centrifugation and decanting of the supernatant. The cell pellets were re-suspended in 5 ml ice-cold 0.4 M sodium hydroxide (Thermofisher) for 5 minutes on ice, centrifuged, and the supernatant was removed. The cell pellets were then re-suspended in 100 µl extraction buffer consisting of 50 mM Tris-HCl pH 8.0, 2% sodium dodecyl sulfate (SDS) (Acros Organics), and 1 tablet of cOmplete protease inhibitor cocktail (Roche) (50 mg/ ml antipain, 40 mg/ ml bestatin, 20 mg/ ml chymostatin, 10 mg/ml E64, 10 mg/ml phosphoramidon, 50 mg/ ml pefabloc SC, and 2 mg/ ml aprotinin) in a total volume of 50 ml. The samples were then boiled in a water bath for 5 minutes, centrifugated, and the supernatant consisting of total yeast protein was moved to a clean 1.5 ml Eppendorf microcentrifuge tube. The concentration of total protein was quantified using the Pierce™ BCA Protein Assay Kit (Thermofisher) with a 5 µl aliquot of the protein extract. The remaining 95 ul of protein extract was diluted 2-fold with 95 ul of 2x Laemmli solubilization buffer consisting of 125 mM Tris-HCl pH 6.8, 50 mM dithiothreitol (DTT) (Sigma-Aldrich), 20% (w/ v) glycerol (Sigma-Aldrich), 4% (w/ v) SDS, and bromophenol blue (Bio-Rad). A protein extract of *E. coli* expressing Myc/6xHis tagged ATP sulfurylase 2 (APS2) from *S. pinnata* was used as a positive control for immunodetection.

20 ug of total protein was separated via SDS-PAGE (10%) (Bio-Rad) set at a constant current of 20 mA during the stacking phase, followed by 40 mA during the running phase for a total duration of 90 minutes. The proteins on the SDS gel were transferred to a 0.2µM nitrocellulose membrane (Bio-Rad) for 2 hours in a TE 22 Mini Tank Transfer Unit (GE) filled

with blotting buffer (see Appendix) at a constant voltage of 50 V. After blotting, the nitrocellulose membrane was incubated with an Anti-His mouse primary antibody (Sigma-Aldrich), then with a secondary antibody coupled to alkaline phosphatase (Sigma-Aldrich), followed by incubation of the membrane in detection buffer consisting of 1 NBT/BCIP tablet (Roche) dissolved in 10 ml distilled H_2O for 10 minutes for immunodetection.

A dot-blost assay was also performed with the protein extracts to determine the detection limit and quality of detection for a horseradish peroxidase monoclonal secondary antibody (Thermofisher). 10 ug of total protein was pipetted directly onto a nitrocellulose membrane and incubated in an Anti-His mouse primary antibody (Thermofisher), followed by incubation with the horseradish peroxidase secondary antibody. After incubation the nitrocellulose membrane was imaged via a chemiluminescent protocol using a Bio-Rad gel imager.

2.3 Results

2.3.1 Sultr1;2 Amplification, Cloning and Polypeptide Alignment

Initially, the full-length cDNAs of *Sultr1;2* from *S. pinnata* and *A. thaliana* were amplified using the SpinSultr1;2_5FW_EcoRI / SpinelaSultr1;2_3Rev_EcoRI and AtSultr1;2_5Fw_EcoRI / AtSultr1;2_3Rev_EcoRI primer sets (Table 4), respectively. The PCR products were restriction-digested and cloned into *E.coli* – yeast shuttle vector pYES2. After sequence analysis, the plasmids were transformed into yeast. YSD1 transformed with these constructs did not show complementation of sulfate uptake on SD-S (0.1 mM SO_4^{2-})(results not shown). Comparison of these sequences to published sequences of *A. thaliana Sultr1;2* in pYES2 (Yoshimoto et al, 2002) pointed to the presence of truncated 5' and 3' untranslated regions (UTR) in the new constructs as a possible reason for the lack of complementation, if they inhibit

expression. After determining that these constructs were non-functional in YSD1, new constructs were designed, using primers that began at the start and stop codons. Primers 5'-SpSultr1;2_EcoRI_noUTR / 3'-SpSultr1;2_EcoRI_noUTR and 5'-AtSultr1;2_EcoRI_noUTR / 3'-AtSultr1;2_EcoRI_noUTR (Table 4) were used to amplify the *Sultr1;2* genes from the previously cloned *S. pinnata* and *A. thaliana, Sultr1;2* sequences, removing the flanking UTR sequences. These new constructs, pEPY1 and pEPY2, served as templates for further construction of the tagged constructs pEPY11, pEPY21, and pEPY31. An illustration of the pEPY11, pEPY21, and pEPY31 constructs is presented in Figure 2.1. Comparison of the polypeptide sequences of SpSULTR1;2, SeSULTR1;2, and AtSULTR1;2 indicates that SpSULTR1;2 and SeSULTR1;2 share 96.85% homology, SpSULTR1;2 and AtSULTR1;2 share 92.78% homology, and AtSULTR1;2 and SeSULTR1;2 share 93.12% homology. SpSULTR1;2 contains 7 unique amino acid residues when compared to SeSULTR1;2 and AtSULTR1;2, with 1 unique residue in the third membrane spanning domain, and 3 unique residues in the C-terminal STAS domain (Figure 2.2).

Table 2.1) Strains used for this study

Strain	Genotype	Source/ reference
YSD1	(*MATα, his3, leu2, ura3, sul1*)	Smith et al, 1995, Donated from Takahashi Lab
22574d	(*matα ura3-1 gap1-1 put4-1 uga4-1*)	Jauniaux & Grenson, 1990, Donated from Bush Lab

SpSultr1;2	Same as YSD1, but with Sultr1;2 from *S. pinnata*	This study
AtSultr1;2	Same as YSD1, but with Sultr1;2 from *A. thaliana*	This study
SpSultr1;2t	Same as SpSultr1;2, but with a Myc/6xHis tag added at the C-terminus.	This study
SeSultr1;2t	Same as YSD1, but with Sultr1;2 from *S. elata* with a Myc/6xHis tag added at the C-terminus.	This study
AtSultr1;2t	Same as AtSultr1;2, but with a Myc/6xHis tag added at the C-terminus.	This study

Table 2.2) Plasmids used for this study

Plasmid	Description	Source/ reference
pYES2	Yeast expression vector	Thermofisher
pET28-At	Plasmid containing 339bp DNA sequence consisting of the 3' end of Sultr1;2 from *A. thaliana* with the stop codon removed and a Myc/6x His sequence at the C-terminus.	Genscript
pEPY1	pYES2 with Sultr1;2 from *S. pinnata* inserted at the EcoRI restriction site.	This study

pEPY2	pYES2 with Sultr1;2 from *A. thaliana* inserted at the EcoRI restriction site.	This study
pEPY11	pYES2T with Sultr1;2 from *S. pinnata* inserted at the EcoRI / PacI restriction sites.	This study
pEPY21	pYES2T with Sultr1;2 from *A. thaliana* inserted at the EcoRI / PacI restriction sites.	This study
pEPY31	pYES2T with Sultr1;2 from *S. elata* inserted at the EcoRI / PacI restriction sites.	This study

Table 2.3) Yeast media used for this study

Media	Components	Source/ reference
YPD	10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 100 ml 20% w/v glucose	Pilon et al, 1997
SD-U	1.92 g L^{-1} Yeast Synthetic Drop-out Medium Supplements (-ura), 5.0 g L^{-1} ammonium sulfate (Sigma-Aldrich), 1.7 g L^{-1} Yeast Nitrogen Base without amino	Guthrie & Fink, 1991

	acids and ammonium sulfate (Difco)	
SD-S (0.1 mM SO_4^{2-})	Appendix 2	Yoshimoto et al, 2002
SD-S (1.0 mM SO_4^{2-})	Appendix 2	Yoshimoto et al, 2002

Table 2.4) Primers used for this study

Primer Names	Primer Sequence	Source/ reference
5'-SpSultr1;2_EcoRI_noUTR	5'-GAGCGAATTCATGCCCGCGAGAGCTCATCCTATG-3'	This study
3'-SpSultr1;2_EcoRI_noUTR	5'-GAGCGAATTCTCAGACCTCGTCGGAGAGTTTTG-3'	This study
3'-SpSultr1;2_nostop_PacI	5'-GAGCTTAATTAAGACCTCGTCGGAGAGTTTTGG-3'	This study
5'-SeSultr1;2_EcoRI_noUTR	5'-GAGCGAAATCATGCCCGAGAGAGCTCATCCTATG-3'	This study
3'-SeSultr1;2_nostop_PacI	5'-GAGCTTAATTAAGACCTCGTCGGAGAGTTTTGG-3'	This study
5'-AtSultr1;2_EcoRI_noUTR	5'-GAGCGAATTCATGTCGTCAAGAGCTCACCC-3'	Yoshimoto et al, 2002
3'-AtSultr1;2_EcoRI_noUTR	5'-GCGCGAATTCTCAGACCTCGTTGGAGAG-3'	Yoshimoto et al, 2002
pYES2_F2	5'-AACCCCGGATCGGACTACTA-3'	This study
pYES2_R2	5'-CTTTTCGGTTAGAGCGGATG-3'	This study
SpinSultr1;2_5FW_EcoRI	5'-TGCAGAATTCACATTTAAGTCACCTACAAACCCA-3'	This study
SpinelaSultr1;2_3Rev_EcoRI	5'-TGCAGAATTCATTTCAGACCTCGTCGGAGAG-3'	This study
AtSultr1;2_5Fw_EcoRI	5'-GAGCGAATTCATGTCGTCAAGAGCTCACCC-3'	This study

AtSultr1;2_3Rev_EcoRI	5’-GCGCGAATTCTCAGACCTCGTTGGAGAG-3’	This study
SelaSultr1;2_5FW_EcoRI	5’-TGCAGAATTCACATTTAAGTCACCTACAAATCCA-3’	This study
Spin_Sultr1;2_QuarterFw	5’-CGGTTTATATTCGAGTTTTGTTCC-3’	This study
Spin_Sultr1;2_QuarterRev	5’-GGAACAAAACTCGAATATAAACC-3’	This study
SpinelaSultr1;2_centerFw	5’-CCTTAACAGAAGCTGTAGCGAT-3’	This study
SpinelaSultr1;2_centralRev	5’-GAAGAGCAATGTCAAGAGAACG-3’	This study
SpinSultr1;2_ThreequarterFw	5’-CCTGAAGCCACTATGGTTCCAG-3’	This study
SpinSultr1;2_ThreequarterRev	5’-CCCTGGAACCATAGTGGCTTC-3’	This study
AtSultr1;2_centF	5’-GACCTTCCTTCTCACGTCTAAGA-3’	This study
AtSultr1;2_centR	5’-CCCTTAGCAAGGTTATCACCAG-3’	This study

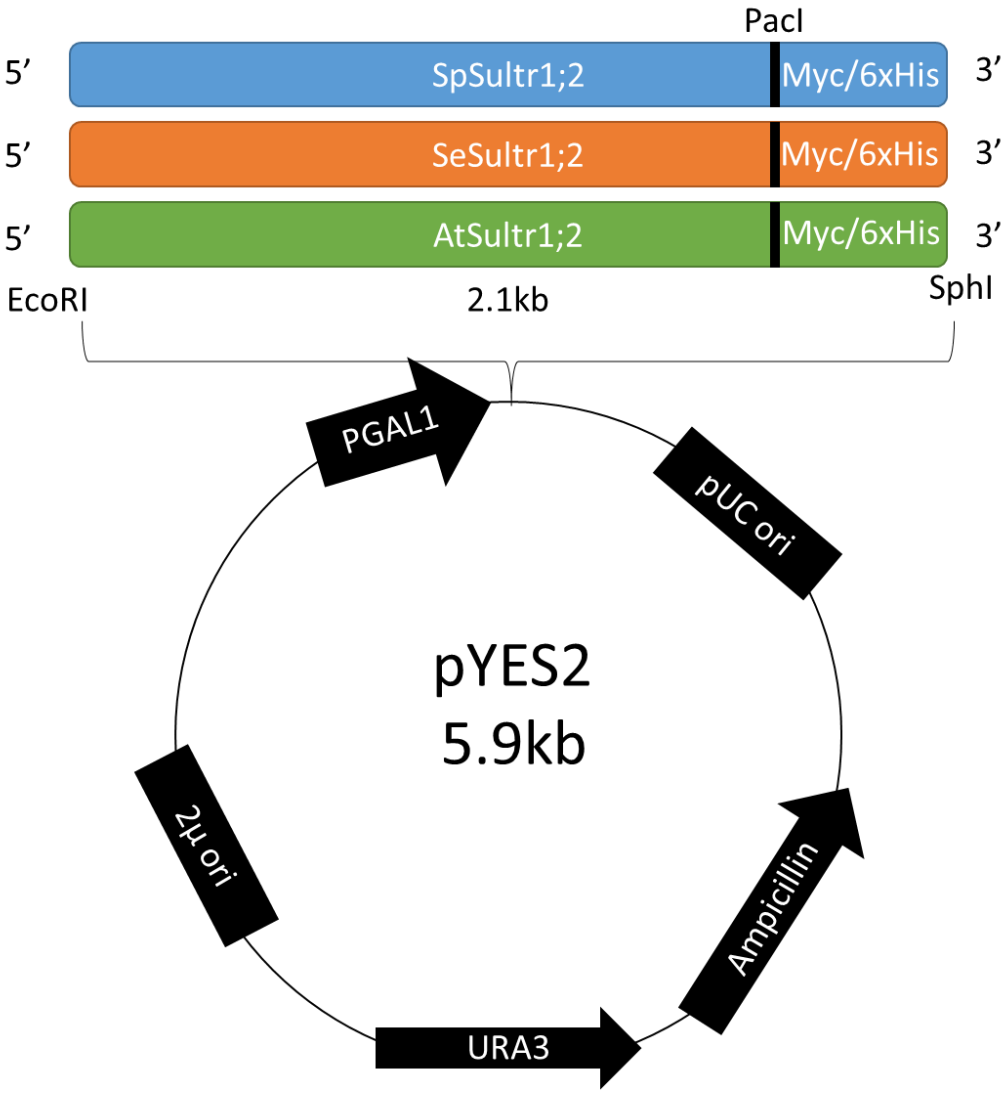

Figure 2.1) Outline of tagged Sultr1;2 constructs in yeast expression vector pYES2. The tagged open reading frame of Sultr1;2 was inserted into the EcoRI and SphI restriction sites behind the galactose inducible promoter PGAL1. The Myc/6xHis tag is separated from the rest of the Sultr1;2 gene body by a PacI restriction site. The URA3 gene codes for uracil production for selection after yeast transformation. The Ampicillin gene codes for ampicillin resistance in *E. coli.* The pUC ori is the origin of replication for *E. coli*, and 2µ ori is the origin of replication for yeast. Adapted from the pYES2 illustration developed by Thermofisher.

Figure 2.2) Amino acid alignment of the pEPY11, pEPY21, and pEPY31 yeast expression vectors. Membrane spanning domains are highlighted in red, C-terminal STAS domains are highlighted in green. Amino acid differences in *S. pinnata* are highlighted in yellow. Conserved arginine residues reported earlier to be important for sulfate binding are highlighted in blue.

2.3.2 Sulfate Uptake Complementation

SpSultr1;2, and AtSultr1;2 yeast strains showed complementation of the no-growth phenotype of YSD1 and of the negative control cells harboring the pYES2 vector on SD-S (0.1 mM SO_4^{2-}) plates (Figure 2.3a). Growth in the Sultr1;2 yeast lines was observed after 3 and 5 days. On SD-S (0.1 mM SO_4^{2-}) plates supplemented with 2% glucose, no growth was observed for all of the yeast lines after 3 and 5 days (Figure 2.3b), indicating that the ability to grow on SD-S depended on the expression of the *Sultr1;2* plant genes. Similar to the untagged versions of the genes, SpSultr1;2t, SeSultr1;2t, and AtSultr1;2t yeast strains also showed complementation of the no-growth phenotype after 3 and 5 days on SD-S (0.1 mM SO_4^{2-}) plates supplemented with 2% galactose (Figure 2.4). Together these results indicate that the proteins are all expressed and have sulfate transport capacity; also, the presence of a 3' protein purification tag does not appear to inhibit sulfate transport capacity.

2.3.3 Selenate Tolerance Assay

The 22574d yeast cells (containing a functional Sul1 gene) showed a reduction in growth when exposed to selenate concentrations upwards of 50 - 100 µM (Figure 2.5a). The reduction in growth was apparent starting at the 1 x 10^{-2} serial dilution. The YSD1 yeast cells did not grow on this medium, due to the lack of a functional SUL1 sulfate transporter (Figure 2.5a). Growth of YSD1 strains SpSultr1;2t, SeSultr1;2t, or AtSultr1;2t was reduced at selenate concentrations upwards of 50 -100 µM, as evidenced between the 1 x 10^{-1} and 1 x 10^{-2} serial dilution (Figure 2.5b). Based on these results, 50 µM selenate was chosen for future selenate uptake experiments as it is the concentration at which growth starts to be impeded in the wildtype yeast.

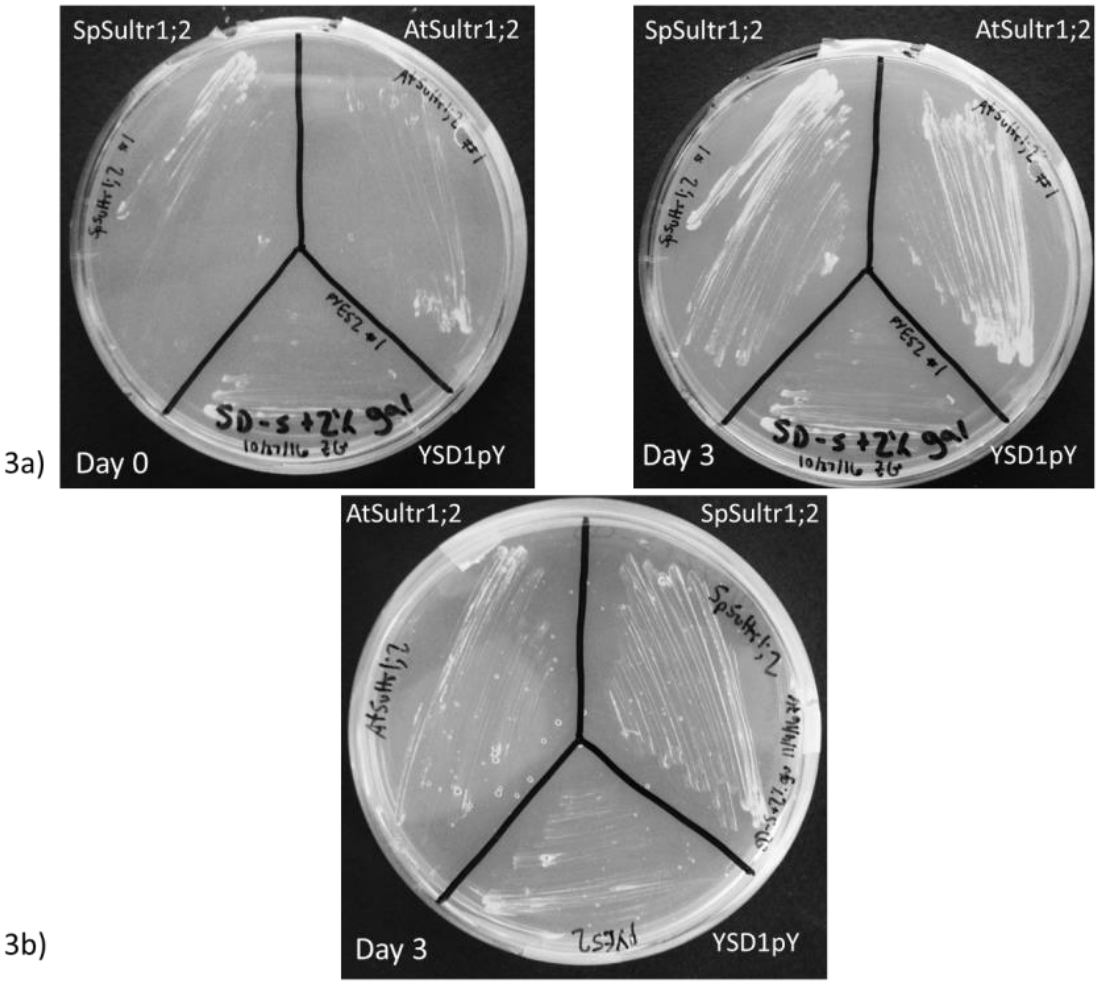

Figure 2.3) Functional complementation of sulfate uptake in SpSultr1;2, AtSultr1;2, and YSD1pY yeast cells. a) Complementation of sulfate uptake of SpSultr1;2 (top left sector) and AtSultr1;2 (top right sector) yeast cells on SD-S (0.1 mM SO_4^{2-}) media supplemented with 2% galactose to induce *Sultr1;2* gene expression. Growth was documented after 3 days of incubation at 30°C. No growth was observed for the YSD1pY cells after 3 days (bottom sector). b) Lack of sulfate uptake complementation of SpSultr1;2 (top right sector), AtSultr1;2 (top left sector), and YSD1pY (bottom sector) yeast cells on SD-S (0.1 mM SO_4^{2-}) media supplemented with 2% glucose. The plate was imaged after 3 days of incubation at 30°C.

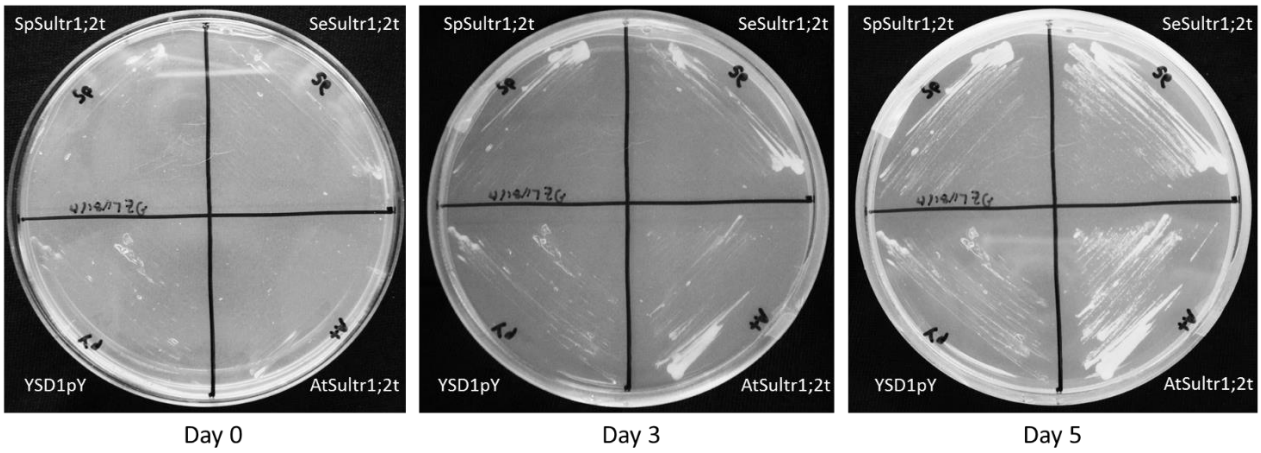

Figure 2.4) Functional complementation of sulfate uptake in SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY yeast cells. Complementation of sulfate uptake of SpSultr1;2t (top left sector), SeSultr1;2t (top right sector), and AtSultr1;2t (bottom right sector) yeast cells on SD-S (0.1 mM SO_4^{2-}) media supplemented with 2% galactose to induce *Sultr1;2* gene expression. Growth was documented after 3 and 5 days of incubation at 30°C. No growth was observed for the YSD1pY cells after 3 and 5 days (bottom left sector).

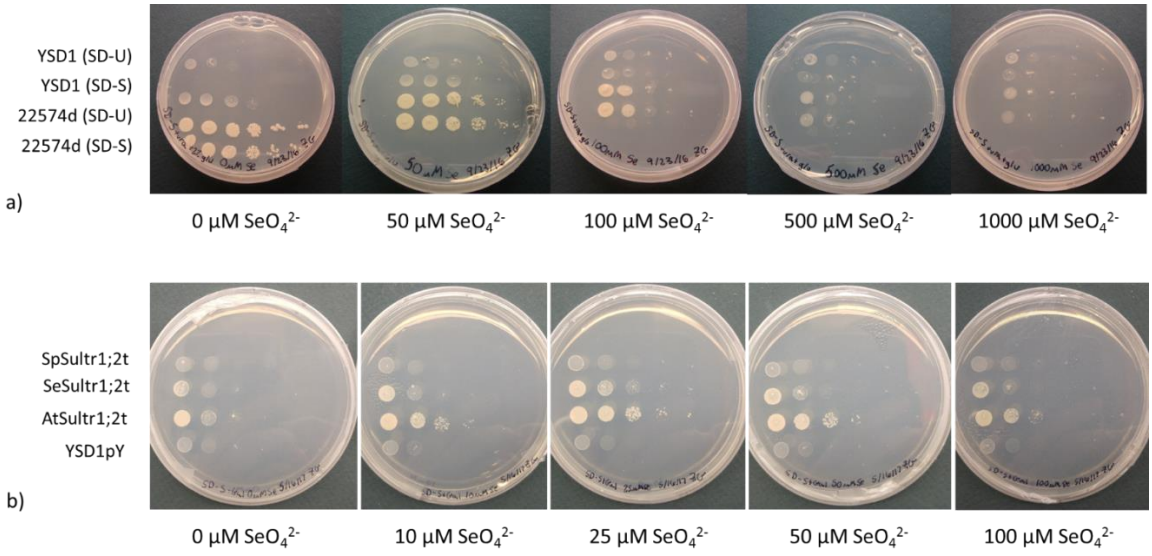

Figure 2.5) Selenate tolerance of yeast strains on SD-S (0.1 mM SO_4^{2-}) plates supplemented with varying concentrations of selenate. a) Sulfate transport deficient strain YSD1 (top 2 rows) and sulfate transport sufficient strain 22574d (bottom two rows) were gown either in sulfate sufficient (SD-U) or sulfate deficient (SD-S) media before being plated on SD-S (0.1 mM SO_4^{2-}) media with selenate concentrations ranging from 0 µM to 1000 µM. Serial dilutions of yeast cells range from a concentration of OD_{600nm} 1.0 (farthest left) to 1.0 x 10^{-5} (farthest right). b) Yeast strains SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY were grown on SD-S (0.1 mM SO_4^{2-}) media with selenate concentrations ranging from 0 µM to 100 µM. Serial dilutions of yeast cells range from a concentration of OD_{600nm} 1.0 to 1.0 x 10^{-5}.

2.3.4 Yeast Growth Curve Assay

Both SpSultr1;2 and AtSultr1;2 strains grew better in the SD-S (0.1 mM SO_4^{2-}) media when compared to YSD1pY (Fig. 2.6). In the absence of selenate, AtSultr1;2 yeast cells grew the best, compared to SpSultr1;2 and YSD1pY (Fig. 2.6, 2.7). Both SpSultr1;2 and AtSultr1;2 cells showed a significant reduction in growth in the presence of selenate, whereas YSD1pY showed no reduction in growth (Fig. 2.6, 2.7). The increased growth on SD-S, combined with the reduction in growth in the presence of selenate confirm that both SULTR1;2 homologs from *S. pinnata* and *A. thaliana* have the capability to transport both sulfate and selenate. These experiments also were used to estimate at which cell density logarithmic growth was occurring for future selenate uptake experiments. From the growth curves illustrated in Figures 2.6 and 2.7, it was determined that the logarithmic phase of growth began at an OD_{600nm} between 0.1 and 0.2

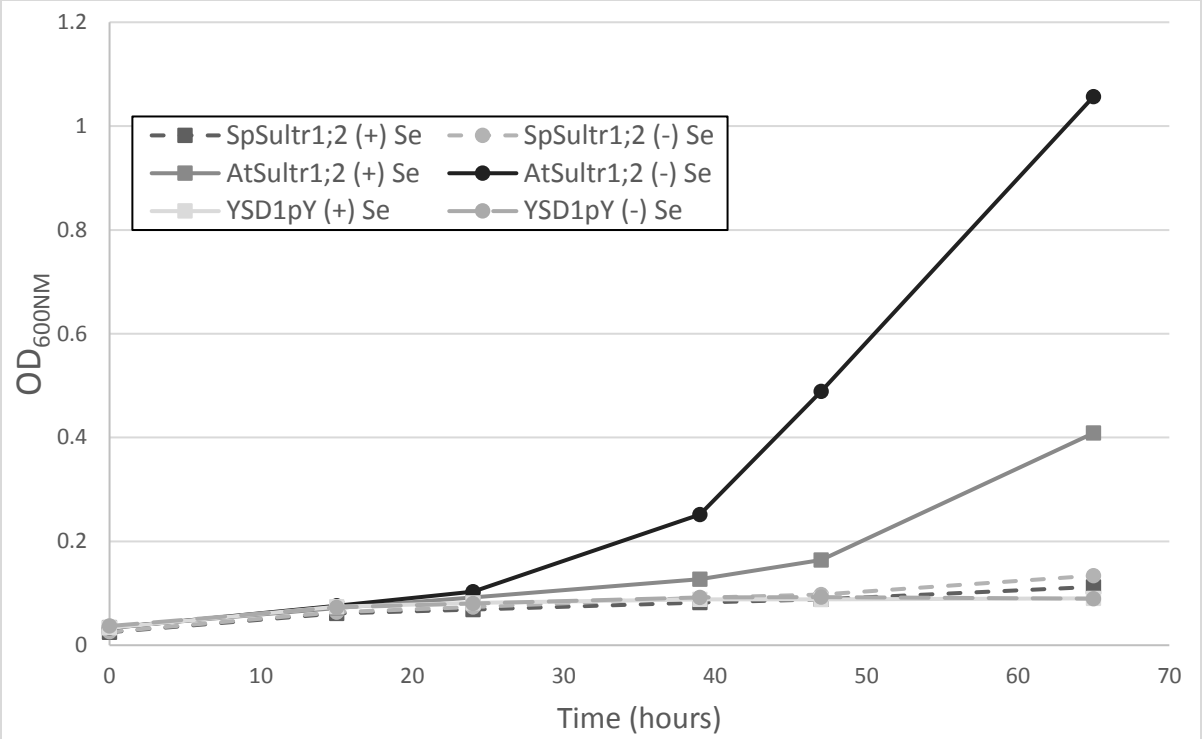

Figure 2.6) Growth curve of SpSultr1;2, AtSultr1;2, and YSD1pY yeast grown in the presence or absence of 50 µM selenate. The cultures were grown in a shaking incubator set to 30°C and 180 rpm for a total duration of 65 hours. Growth curves of cells grown in the presence of selenate are denoted with squares. Growth curves of cells grown in the absence of selenate are denoted with circles.

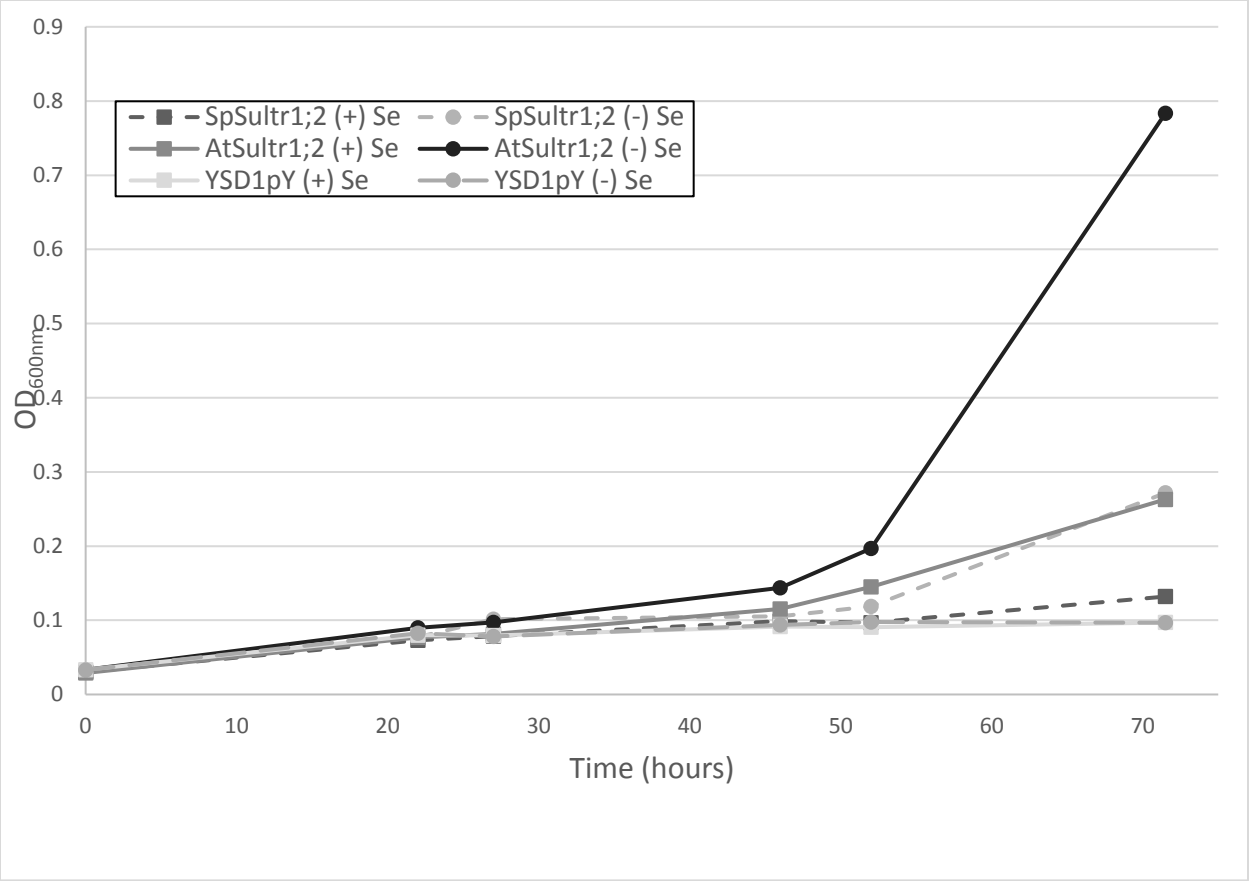

Figure 2.7) Growth curve of SpSultr1;2, AtSultr1;2, and YSD1pY yeast grown in the presence or absence of 50 µM selenate. The cultures were grown in a shaking incubator set to 30°C and 180 rpm for a total duration of 72 hours. Growth curves of cells grown in the presence of selenate are denoted with squares. Growth curves of cells grown in the absence of selenate are denoted with circles.

2.3.5 Sultr1;2 Immunodetection

The MycHis tag was added to the constructs so as to be able to compare their expression levels using immunoblotting. However, the SULTR1;2 protein from the YSD1 strains SpSultr1;2t, SeSultr1;2t, and AtSultr1;2t was not detectable by the alkaline phosphatase immunodetection protocol (Fig. 2.8). The positive control consisting of Myc/6x His tagged ATP sulfurylase (APS) 2 did produce a visible band on the nitrocellulose membrane after incubation of the membrane in AP buffer for 10 minutes. Thus, the relative expression of the plant proteins could not be determined using this method, perhaps because expression was low. For the dot-

blot, incubation with the horseradish peroxidase secondary antibody did result in positive detection of the SULTR1;2 proteins (Fig. 2.9). However, due to the varying concentrations of the protein extracts, different volumes of the protein extracts were loaded onto the membrane for each of the yeast lines. This resulted in diffusion of some of the protein extracts across the membrane surface, leading to fainter detection of SULTR1;2 in some of the yeast lines (Fig. 2.9). In subsequent selenate uptake experiments the strains were normalized on a yeast dry weight basis instead.

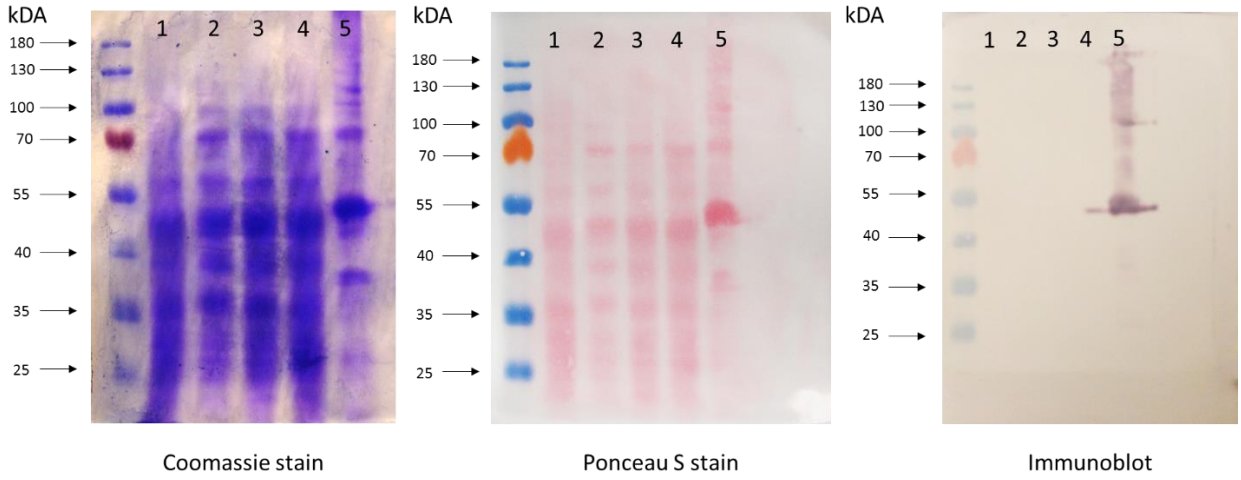

Figure 2.8) Coomassie Brilliant Blue stain of the protein gel (total protein), Ponceau S stain of the blotted proteins (total protein), and Immunodetection using anti-His antibody of protein extracts from yeast lines SpSultr1;2t (1), SeSultr1;2t (2), AtSultr1;2t (3), YSD1pY (4), and *E. coli* cells expressing Myc/6xHis tagged APS2 from *S. pinnata* (5). The expected Sultr1;2-MycHis protein size is predicted to be 78 kDa.

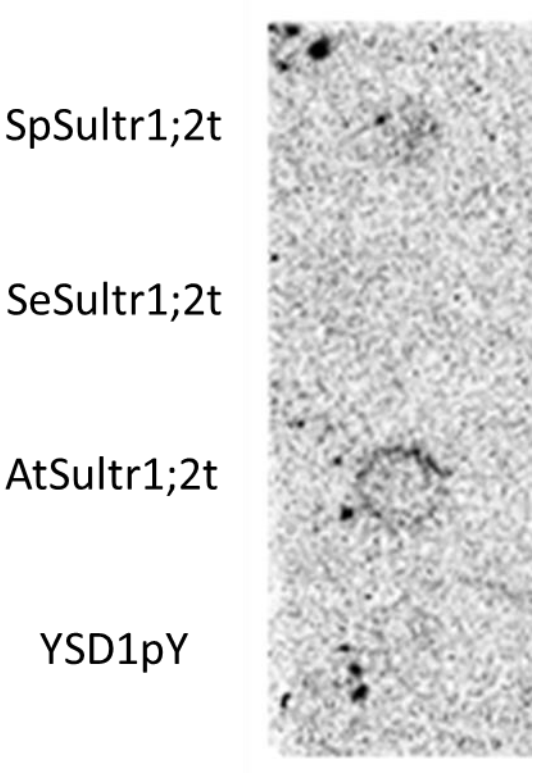

Figure 2.9) Immunodetection of tagged SULTR1;2 proteins from SpSultr1;2t, SeSultr1;2t, AtSultr1;2t. and YSD1pY yeast strains. 10 µg of total protein was loaded directly onto the nitrocellulose membrane, followed by incubation with an anti-His primary antibody and horseradish peroxidase secondary antibody. The membrane was detected via chemiluminescence for 5 minutes using a Bio-Rad gel imaging system.

2.3.6 Quantification of Selenate Uptake

After 1 hour of incubation in low (0.1 mM SO_4^{2-}) or high (1.0 mM SO_4^{2-}) sulfate conditions and 50 uM selenate, yeast strains SpSultr1;2t, SeSultr1;2t, and AtSultr1;2t all showed a significant ($p < 0.05$) increase in selenate content when compared to the negative control YSD1pY cells at 1.0 mM sulfate (Figure 2.10). SpSultr1;2t cells had significantly lower rates of uptake at both the 0.1 mM and 1.0 mM sulfate concentrations when compared to SeSultr1;2t or AtSultr1;2t cells (Figure 2.10). AtSultr1;2t, and the YSD1pY cells showed a significant ($p < 0.05$) reduction in selenate uptake between the 0.1 mM and 1.0 mM sulfate treatments, while

reduction in selenate uptake in the SeSultr1;2t strain was not significant (p = 0.06). However, this reduction in selenate uptake between high and low sulfate treatments was not observed in the SpSultr1;2t cells (Fig. 2.10).

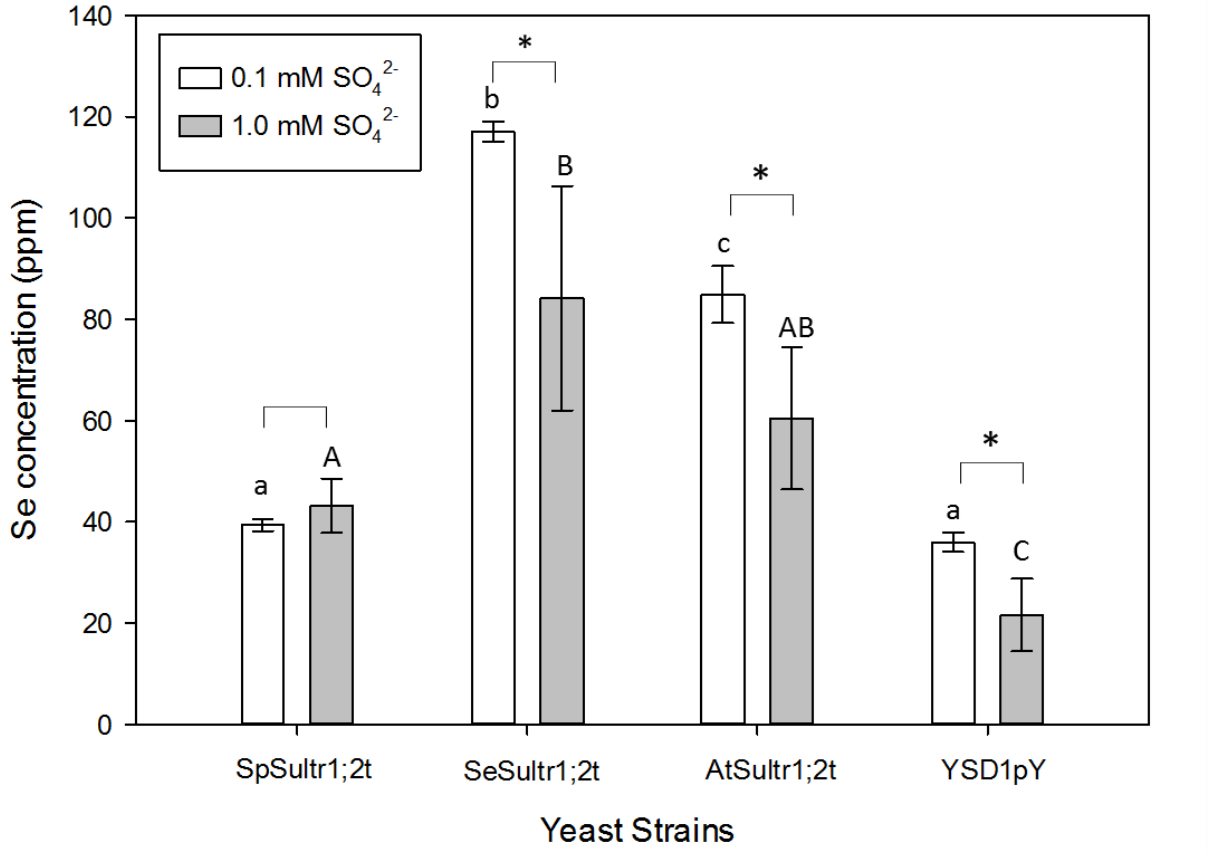

Figure 2.10) Selenate accumulation by yeast strains SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY (negative control). Yeast cells were grown to log-phase in either SD-S (0.1 mM SO_4^{2-}) or SD-S (1.0 mM SO_4^{2-}) media before incubation for 1 hour with 50 µM selenate at 30°C while shaking at 180 rpm. Total Se content of dried yeast pellets was analyzed via ICP-MS. Three biological replicates for each yeast strain were analyzed for total Se content. Significant differences ($p < 0.05$) between the 0.1 mM SO_4^{2-} and 1.0 mM SO_4^{2-} treatments within each strain are denoted with an asterisk. Significant differences ($p < 0.05$) for Se levels between SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY at the 0.1 mM SO_4^{2-} treatment are denoted with lowercase letters. Significant differences ($p < 0.05$) for Se levels between SpSultr1;2t, SeSultr1;2t, AtSultr1;2t, and YSD1pY at the 1.0 mM SO_4^{2-} treatment are denoted with uppercase letters.

Table 2.5) Statistical analysis of selenate uptake between yeast lines under 0.1 mM or 1.0 mM SO_4^{2-} conditions.

[SO_4^{2-}]	**SpSultr1;2t**	**SeSultr1;2t**	**AtSultr1;2t**	**YSD1pY**
0.1 mM SO_4^{2-}	**39.32a**	**116.99b**	**84.88c**	**35.97a**
1.0 mM SO_4^{2-}	**43.15A**	**84.12B**	**60.44AB**	**21.63C**

Table 2.6. Statistical anlysis of selenate uptake under 0.1 mM or 1.0 mM SO_4^{2-} conditions within yeast lines.

Yeast Strain	**0.1 mM SO_4^-**	**1.0 mM SO_4^{2-}**	**$p < 0.05$**
SpSultr1;2t	**39.32**	**43.15**	
SeSultr1;2t	**116.99**	**84.12**	
AtSultr1;2t	**84.88**	**60.44**	*****
YSD1pY	**35.97**	**21.63**	*****

2.4 Discussion

The results presented in this study indicate that SULTR1;2 from *S. pinnata* may have enhanced selenate to sulfate substrate specificity when compared to SULTR1;2 from *S. elata* and *A. thaliana.* Based on the data from the selenate uptake experiment, the SpSultr1;2t yeast cells had no significant difference in selenate uptake in the presence of both high (1.0 mM SO_4^{2-}) or low (0.1 mM SO_4^{2-}) sulfate concentrations, and showed higher Se accumulation than the

negative control strain expressing the empty vector (YSD1pY). In contrast, selenate uptake in SeSultr1;2t and AtSultr1;2t strains as well as the YSD1pY control yeast strains all showed a significant reduction in selenate uptake between low and high sulfate conditions. This tentative conclusion is based on one uptake experiment with three biological replicates, and therefore still preliminary, yet encouraging. While SpSULTR1;2 may have enhanced selenate specificity, it is not an exclusive selenate transporter: it also transports sulfate, evidenced by its capacity to complement growth of YSD1 cells on SD-S media.

These results help support and explain previous findings of enhanced Se-specific uptake in Se hyperaccumulators, as evidenced by higher Se: S tissue ratios when compared to non-hyperaccumulators (Schiavon et al, 2017). The observation of a similar selenate-specificity in yeast transgenics transformed with *SpSultr1;2* suggests that Se specificity in *S. pinnata* is determined during uptake of selenate from the soil via this root membrane transporter, one of the first, key steps of the Se assimilation pathway in plants.

While SpSultr1;2t showed evidence of higher selenate specificity, it also appeared to have reduced overall transport activity, as indicated by the observed lower yeast Se accumulation of SpSultr1;2t compared to SeSultr1;2t and AtSultr1;2t. These results were also seen in the untagged SpSultr1;2 and AtSultr1;2 strains, where SpSultr1;2 grew less well on SD-S (0.1 mM SO_4^{2-}) media when compared to AtSultr1;2 (Fig. 2.5, 2.6). It should be noted that it is not certain whether all proteins were expressed at the same level; the data are currently normalized based on yeast biomass. However, assuming equal expression of these proteins in yeast, the hyperaccumulator SULTR1;2 appears less active as a transporter. The intuitively contradicting findings that the hyperaccumulator takes up more selenate, yet has a less active transporter may be explained by observations of gene expression *in planta.* Based on RNA-seq and RT-PCR data

generated in the Pilon-Smits lab (unpublished), *S. pinnata* has at least 10-fold higher expression of *Sultr1;2* compared to *S. elata.* The overexpression of *Sultr1;2* in the hyperaccumulator may have evolved as an adaptation to compensate for a decrease in overall transport activity that co-occurred with enhanced selenate uptake specificity. The possible presence of multiple copies of *Sultr1;2* in the *S. pinnata* genome, with one or more of these copies having higher selenate specificity, could also explain the results generated in this study. It is possible that there are multiple copies of *Sultr1;2* in *S. pinnata,* with only some conferring selenate specificity due to mutations in the amino acid sequence, allowing this species to accumulate high levels of Se without sacrificing sulfate uptake. Gene duplication events of transporters have been previously reported for some metal hyperaccumulators (Cappa et al, 2014).

If indeed SpSULTR1;2 has enhanced selenate specificity but lower overall transport capacity, as compared to SeSULTR1;2 and AtSULTR1;2, the differences in the amino acid sequence of SULTR1;2 from *S. pinnata* as compared to the other two species may explain these kinetic differences. Studies on the structure of the C-terminal STAS domain of *A. thaliana* Sultr1;2 have identified the Thr-587, Cys-645, and Cys-646 amino acid residues as essential for sulfate transport and protein-protein interactions (Rouached et al, 2005). However, no studies have explored the effects of modifications to the STAS domain on selenate transport activity. Amino acid differences in the STAS domain of Sultr1;2 from *S. pinnata,* specifically His-570 (proline in *S. elata* and *A. thaliana*) and Leu-577 (histidine in *S. elata* and *A. thaliana*) (Figure 2.2) may be responsible for altered selenate transport activity. Comparison of the selenate transport capacity of YSD1 expressing with *SULTR1;2* from *S. pinnata* with amino acid substitutions at these positions may help to identify the key amino acid residues responsible for discriminating between sulfate and selenate in the presence of high sulfate concentrations.

Follow up studies may also include expression of the SpSULTR1;2t in transgenic plants, and analysis of the effects on selenate to sulfate uptake specificity. If the capacity of *S. pinnata* for Se enrichment could be transferred to other plant species, this may hold great promise for future phytoremediation and biofortification applications. Previous studies have shown that expression of enzymes involved in Se assimilation in crop species can enhance the overall Se concentration found in the plants (LeDuc et al, 2004). Since SULTR1;2 is the main portal of entry for selenate into the plant (El Kassis et al, 2007), expression of SpSULTR1;2 in other species may also increase overall selenate concentrations.

While functional complementation of sulfate uptake was observed in the tagged and untagged *Sultr1;2* constructs (Fig. 2.3a,b; Fig. 2.4), it is not known if the C-terminal Myc/6xHis tag interferes with protein localization or sulfate transport. The tagged *Sultr1;2* constructs should be used to confirm equal expression of these proteins in YSD1, but future selenate uptake experiments to confirm these results should (also) be done with YSD1 transformed with the untagged Sultr1;2 constructs. This would ensure that selenate uptake is not affected by any interactions between the Myc/6x His protein tag and the STAS domain. However, it may be important to consider that while it has been shown that mutations and deletions in the STAS domain affect sulfate transport activity (Rouached et al, 2005) it has not been reported that additions to the STAS domain affect sulfate transport. Furthermore, SULTR1;2-GFP fusion proteins have been found to function and localize properly in *Arabidopsis* mutants (Yoshimoto et al, 2002).

In conclusion, this study of sulfate-dependent selenate transport activity of plant transporters in a yeast model system provides preliminary evidence for selenate specificity in *S. pinnata* SULTR1;2. This finding is of significance, since selenate specificity in a sulfate

transporter has not been reported until now. Furthermore, these findings help to further elucidate the mechanisms and evolution of Se hyperaccumulation, and may have applications in future Se phytoremediation or biofortification.

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CHAPTER 3: A TISSUE CULTURE MICROPROPAGATION AND REGENERATION PROTOCOL FOR SELENIUM HYPERACCUMULATOR STANLEYA PINNATA AND NON-ACCUMULATOR STANLEYA ELATA

3.1 Introduction

Stanleya pinnata is in the mustard family Brassicaceae, and is native to the United States from the western Plains states to California. Commonly known as Prince's Plume, it is typically found on selenium-rich soils, and has long been characterized as a selenium (Se) hyperaccumulator, with the ability to accumulate over 1,000 mg / kg dry weight of this normally toxic element (Mehdawi et al, 2011; Galeas et al, 2007). However, not all varieties of *S. pinnata* accumulate high levels of Se, and the hyperaccumulation phenotype is variable among *S. pinnata* accessions (Feist & Parker, 2001; Cappa et al., 2014). By potentially identifying ecotypes of this species with high Se accumulation capacity, tissue culture can allow for the large-scale production of Se hyperaccumulating plants to be used in experimental studies, as well as phytoremediation or biofortification projects. *Stanleya elata,* commonly known as Panamint Prince's Plume, is a closely related species to *S. pinnata* native to Nevada, Arizona and California in the United States (Cappa et al., 2015). Because *S. elata* does not accumulate high levels of Se, it has been frequently used as a contrasting species for *S. pinnata* to investigate mechanisms of Se hyperaccumulation (El Mehdawi et al, 2012; Cappa et al, 2014; Cappa et al, 2015). While commercial seed sources are available for *S. pinnata,* no seed source is available for *S. elata,* making seed collection laborious and difficult, as it is necessary to travel to remote desert locations to collect seeds from wild populations. Rates of germination for these two species have been found to be relatively low, requiring large quantities of seeds to be sown to grow enough plants for research applications. Obtaining more seeds from established plants is

also a challenge, due to a long vernalization period needed before flowering, self-incompatibility and low seed yields. Vegetative propagation is also difficult, especially in *S. pinnata,* due to this plant's propensity to form basal rosettes and short internode lengths. Because of difficulties surrounding the current cultivation of these two species, tissue culture micropropagation offers a potential avenue for the large scale production these plants to be used for research and horticultural applications.

While no protocol for the micropropagation and transformation of *Stanleya* species has yet been reported, there have been many publications on tissue culture in other Brassicaceae species, which served as a starting point for the development of this protocol. Many experiments have been previously conducted in the crop species Indian mustard (*Brassica juncea)* showing high frequency of callus induction (Glimelius, 1984) and shoot regeneration (Hachey et al, 1991; Pua et al, 1993; Guo et al, 2005). Outlined in this manuscript is a rapid and efficient protocol for callus induction, shoot regeneration, rooting, and acclimatization of two species in the genus *Stanleya,* the Se hyperaccumulator *S. pinnata,* and the closely related non-accumulator *S. elata.*

3.2 Materials and Methods

All media were formulated with 4.43 g L^{-1} Murashige and Skoog (MS) basal salts (Sigma-Aldrich, St. Louis, MO) (Murashige & Skoog, 1962) supplemented with 1 mL L^{-1} Gamborg's B5 vitamins (Sigma-Aldrich G1019), 30 g L^{-1} sucrose (Sigma-Aldrich S0389), and 6.8 g L^{-1} Phyto Agar (RPI A20300). This basal medium was diluted to ½ strength for seed germination and root induction. The MS medium was supplemented with varying concentrations of benzylaminopurine (BAP) (Sigma-Aldrich B3408), 1-Napthaleneaceticacid (NAA) (Sigma-Aldrich N0640), and Indole-3-butyric acid (IBA) (Sigma-Aldrich I5386) for callus, shoot, and root induction experiments. 50 mg / ml stocks of NAA, BAP, and IBA were prepared by first

dissolving 50 mg of the salt stocks in 1 -2 ml of 1 N NaOH before being brought up to a final volume of 50 ml with distilled H_2O in 100 ml Erlenmeyer flasks. The hormone stocks were then filter-sterilized using a 50 ml syringe (Thermofisher) and 0.22 µm nylon 25 mm diameter syringe filter (Thermofisher) into a 50 ml sterile conical tube (Falcon) Seeds and explants were cultured either on 100 x 15 mm petri plates (VWR 25384-088) or Magenta GA-7 vessels (Sigma-Aldrich V8505). All explants were grown in a Percival CU36L growth chamber with 4100K 17W T8 fluorescent bulbs (Sylvania 21770 – FO17/741/ECO) set at 25.1°C on a 16 hour light, 8 hour dark light cycle. All explants were cut using #10 Carbon steel blades (Glassvan 2001T-10) mounted to a #3 scalpel handle (Glassvan ISO 7740).

3.2.1 Seed Germination and Callus Induction

Seeds of *S. pinnata* were ordered from Western Native Seed while seeds of *S. elata* were collected from wild populations in remote locations between Las Vegas and Reno, Nevada along US Route 95. Approximately 100 seeds of *S. pinnata* and 200 seeds of *S. elata* were surface sterilized in 50 ml conical tubes by washing once with 25 ml 70% v/v ethanol for one minute, followed by one wash with 25 ml 10% v/v household bleach (Clorox) for 15 minutes, and 4 washes with 25 ml sterile distilled H_2O for 5 minutes each. Seeds were stratified in the fridge at 4°C in the dark for 7 days before being sown on ½ strength MS media with 15 g/L sucrose in Magenta boxes for 2 weeks. Germination occurred after 5 days for both species. The seedlings grew 3 sets of true leaves in the first 2 weeks, which were used to cut 1 cm^3 leaf squares. The leaf explants were cultivated on full-strength MS medium with 0.5 mg/L NAA and 0.5 mg/L BAP, 1.0 mg/L NAA and BAP, 1.5 mg/L NAA and BAP, or 2.0 mg/L NAA and BAP. Previous protocols published for Brassicaceae species report similar ranges of these plant growth regulators (Pua et al, 1993; Guo et al, 2005). The leaf explants were inserted perpendicular to the

media, with half of the cut edge exposed to the media and the other half exposed to the air. 15 explants were cultured on a single 100 x 15 mm petri plate with 4 plates used for each different hormone formulation, for a total of 40 explants per treatment or 160 explants total for each species. The number of leaf explants that produced visible callus was assessed every 2 days for a total duration of 15 days.

3.2.2 Shoot Initiation and Elongation

Callus explants produced from the callus induction experiment were excised from the initial leaf explant and cultured on full-strength MS media in 100 x 15 mm petri plates supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA, 1.0 mg/L BAP and 0.1 mg/L NAA, 1.5 mg/L BAP and 0.1 mg/L NAA, or 2.0 mg/L BAP and 0.1 mg/L NAA for a total of 50 explants per treatment, or 250 explants total for each species. The callus explants were assessed for average number of explants that formed shoots, as well as average number of shoots formed from each explant every 2 days for a total duration of 15 days. After 15 days, the explants were sub-cultured and moved to magenta boxes with the same media formulations to undergo elongation for a period of 15 days.

3.2.3 Root Induction

After 15 days on the different shoot induction media, the shoots that formed from the callus material were excised from the callus and cultured on full-strength MS media in magenta vessels with no hormones, or supplemented with 0.5 mg/ L, 1.0 mg/ L, or 2.0 mg/ L IBA, with 3 shoots per magenta box for a total of 27 shoots per treatment, and 108 shoots for each species. The shoot explants were assessed for average number of shoots that formed roots over a total period of 30 days.

3.2.4 Acclimatization

Explants that formed roots *in vitro* were moved to an *ex vitro* 5 L hydroponic system filled with distilled H_2O to begin the acclimatization (hardening) process. A total of 12 plants were in each hydroponic system, and the plants were covered with a plastic dome to maintain high humidity for a week before being opened gradually over a period of 3 weeks to reduce the humidity. The plastic dome remained over the plants for a total duration of 3 weeks before being removed. After 2 weeks, the distilled H_2O in the hydroponics bucket was switched to 1/10 strength Hoagland's solution (Hoagland & Arnon, 1938) for 1 week before being switched to 1/5 strength Hoagland's solution for the remainder of the experiment. Plants moved to the *ex vitro* hydroponic system were assessed for percent mortality over a total period of 30 days.

3.3 Results

3.3.1 Seed germination and callus Induction

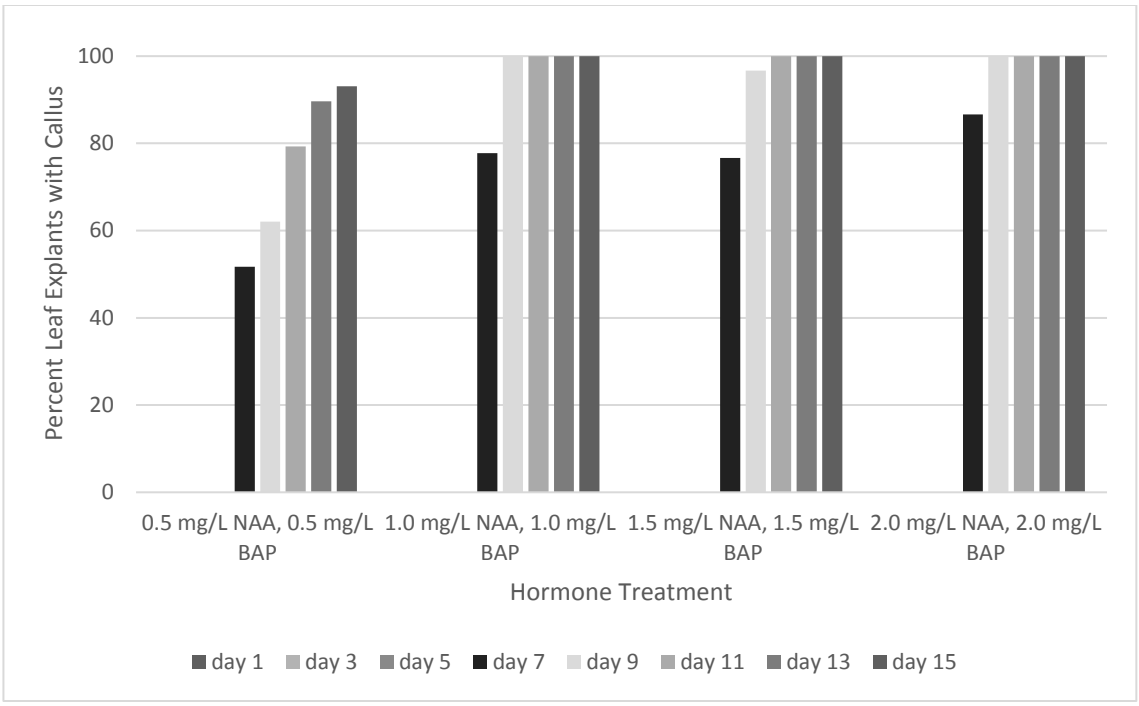
Approximately 11% of *S. pinnata* seeds and 64% of *S. elata* seeds had germinated after 10 days. All of the hormone treatments tested resulted in callus induction after 15 days, for both *S. pinnata* and *S. elata* (Fig. 3.1a,b). *S. elata* leaf explants exhibited faster initiation of callus induction, with callus being observed after 5 days on the various callus induction media formulations. No callus was observed on the *S. pinnata* leaf explants until 9 days after being cultured on the various callus induction media formulations. MS media supplemented with 1.0 mg/L BAP and NAA or 2.0 mg/L BAP and NAA resulted in 100% of the *S. pinnata* leaf cuttings producing callus after 15 days (Fig. 3.1a). However, the 2.0 mg/L BAP and NAA media formulation resulted in a slightly higher percentage of leaf explants forming callus in 7 days compared to the 1.0 mg/L BAP and NAA media formulation (Fig. 3.1a). For *S. elata*, the 0.5

mg/L BAP and NAA, 1.5 mg/L BAP and NAA, and 2.0 mg/L BAP and NAA media formulations resulted in 100% callus induction after 15 days (Fig. 3.1b). However, the 1.5 mg/L BAP and NAA media formulation resulted in the fastest induction of callus from leaf explants, with 92% of the leaf explants showing callus formation after 5 days (Fig. 3.1b).

3.3.2 Shoot Initiation / Elongation

Stanleya pinnata callus explants responded the best to the 1.5 mg/L BAP 0.1 mg/L NAA hormone treatment, with an average of 88% of calli forming shoots after 15 days (Fig. 3.2a). This hormone treatment also resulted in the highest number of shoots per callus explant, with an average of 3.3 shoots formed from each callus (Fig. 3.2b). *Stanleya elata* callus explants also responded best to the 1.5 mg/L BAP 0.1 mg/L NAA hormone treatment, with 92% of calli forming shoots after 15 days (Fig. 3.3a). However, the 0.5 mg/L BAP 0.1 mg/L NAA hormone treatment resulted in the most shoots per callus for *S. elata,* with an average of 3.7 shoots per callus formed compared to an average of 2.8 shoots per callus formed on the 1.5 mg/L BAP 0.1 mg/L NAA hormone treatment (Fig. 3.3b). Overall, *S. elata* callus explants responded better to the shoot induction media formulations, with more of the *S. elata* calli forming shoots, as well as more shoots per callus when compared to *S. pinnata* callus explants. After being moved to Magenta boxes containing the same hormone concentrations for shoot induction, shoots of both species elongated at similar rates over 15 days (Fig. 3.4a,b).

a)



b)

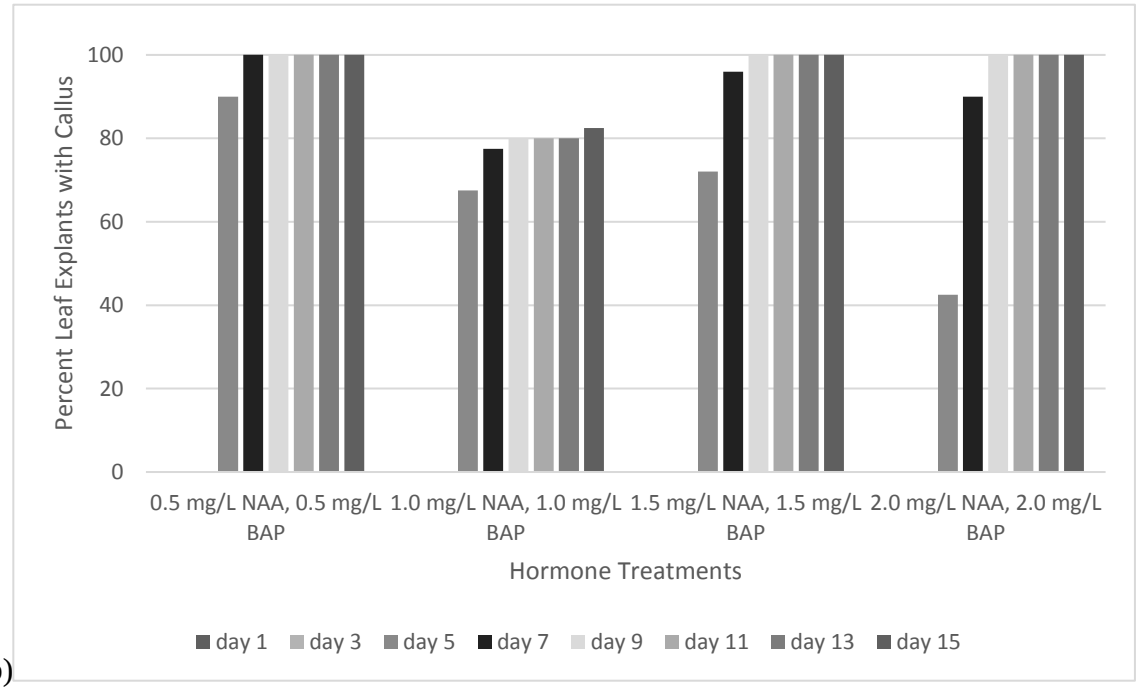
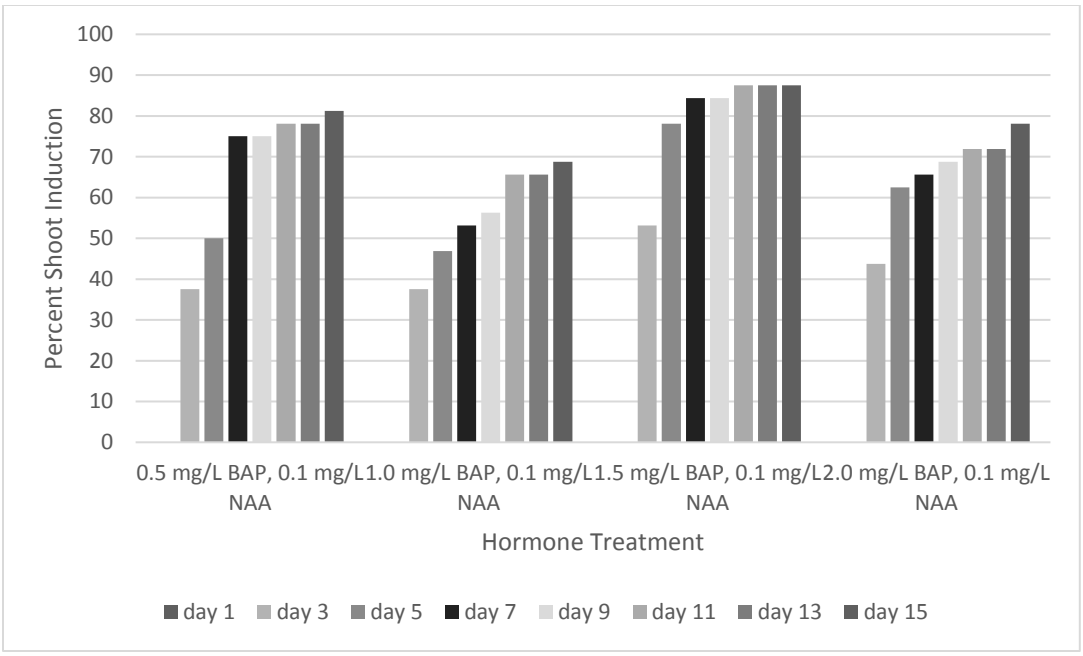


Figure 3.1) Callus induction from leaf explants of *S. pinnata* (a) and *S. elata* (b). 40 explants for each hormone treatment were grown on petri plates for 15 days. Assessment of callus formation was performed every 2 days (N = 160).

a)



b)

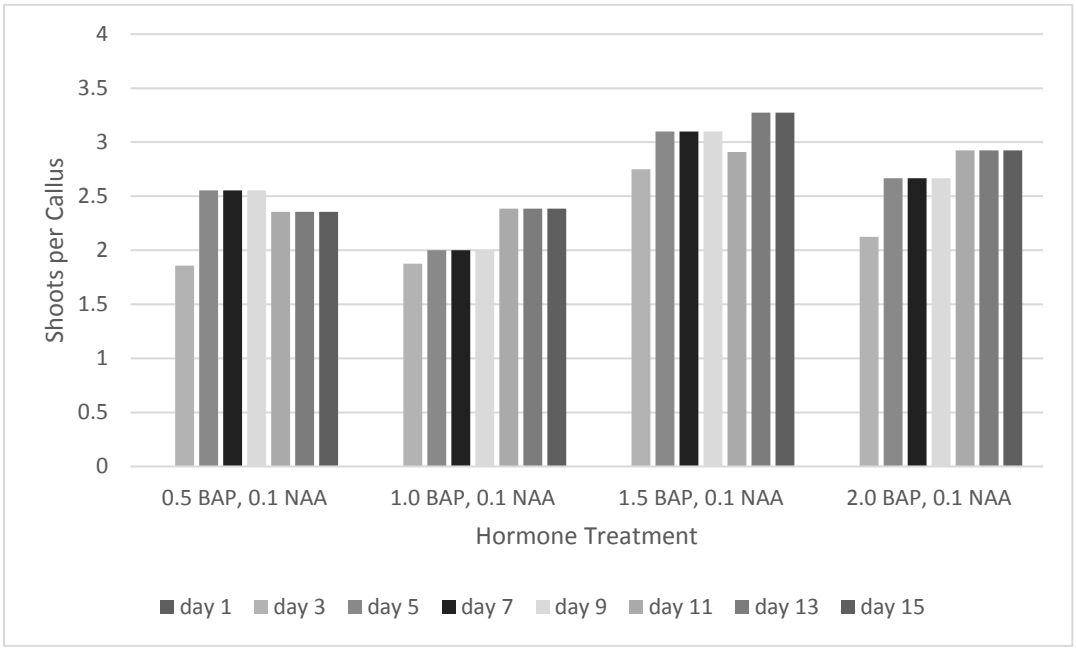
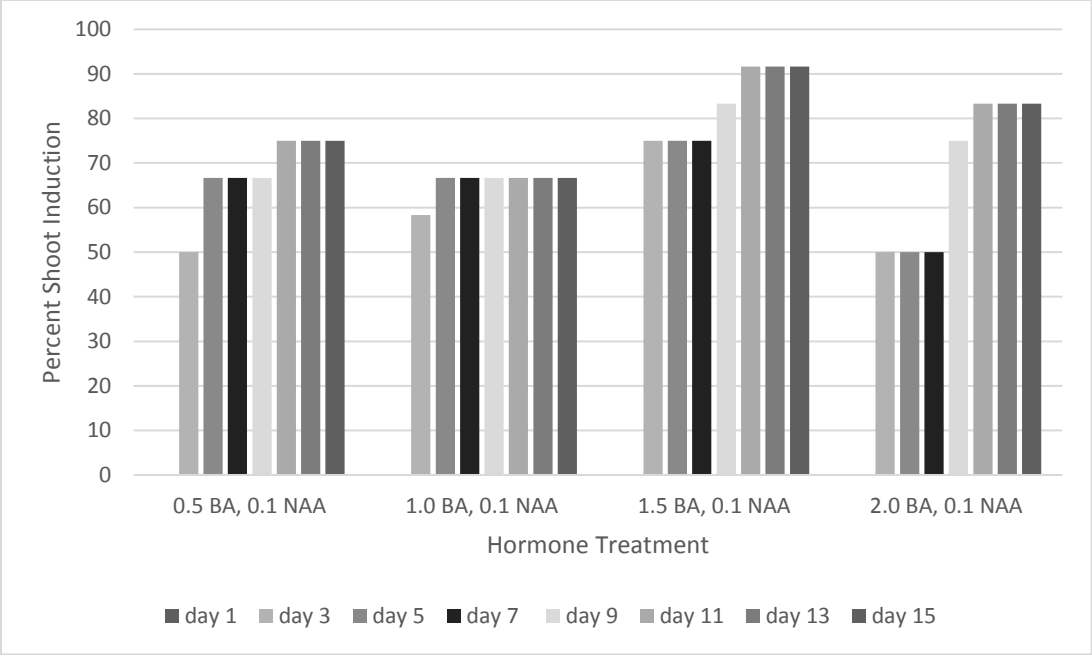


Figure 3.2) Rates of shoot induction (a) and average number of shoots per callus (b) from callus explants of *S. pinnata.* 50 explants for each hormone treatment were initially grown on petri plates for 15 days, then moved to Magenta boxes for another 15 days for elongation (N = 200).

a)



b)

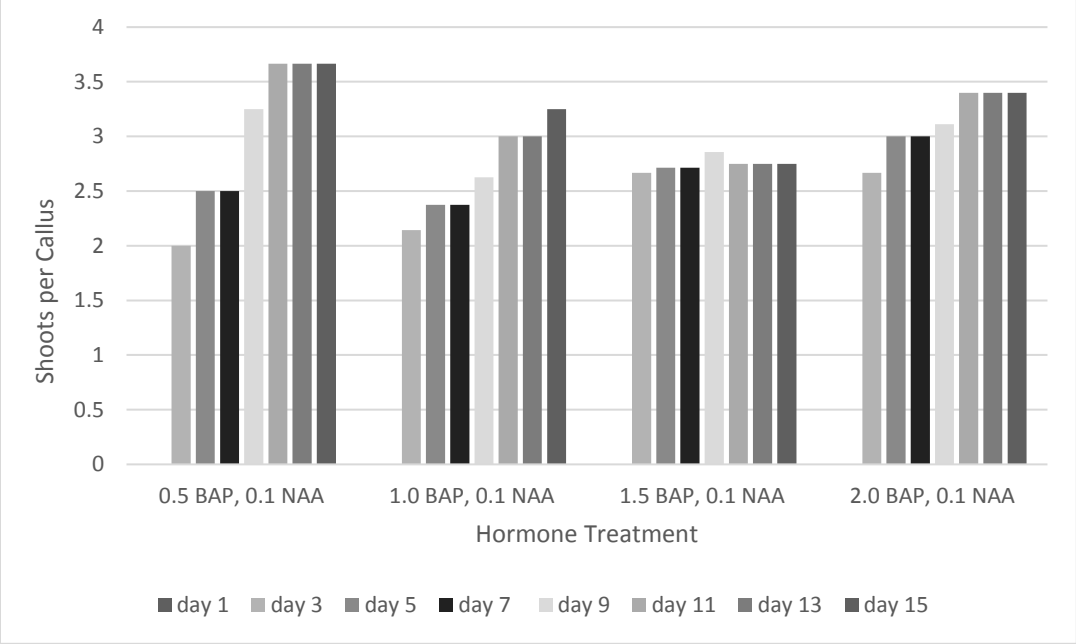
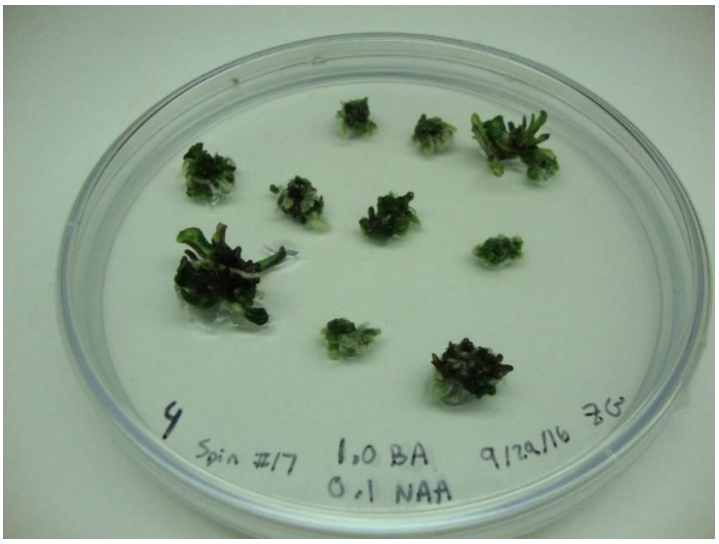


Figure 3.3) Rates of shoot induction (a) and average number of shoots per callus (b) from callus explants of *S. elata.* 50 explants for each hormone treatment were initially grown on petri plates for 15 days, then moved to Magenta boxes for another 15 days for elongation (N = 200).

a)



b)

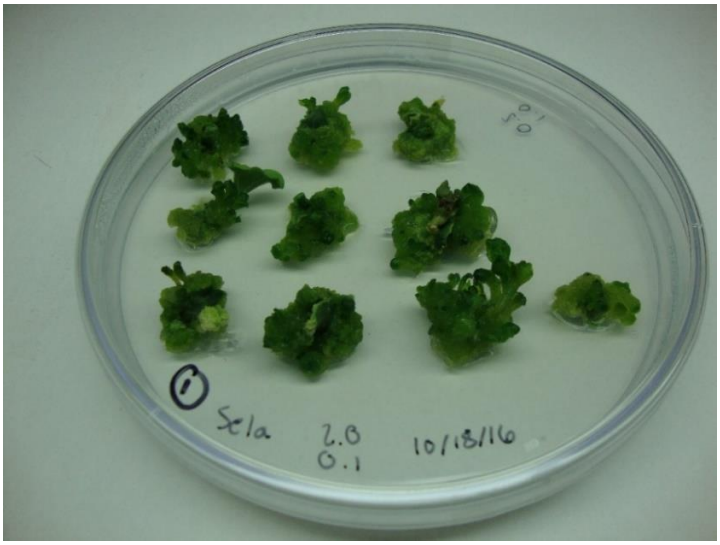


Figure 3.4) Shoot Induction of *S. pinnata* (a, left) and *S. elata* (b, left) callus explants after 15 days on MS media supplemented with 1.0 mg/ L BAP and 0.1 mg/ L NAA (*S. pinnata)* or 2.0 mg/ L BAP and 0.1 mg/ L NAA (*S. elata).* Elongation of shoots of *S. pinnata* (a, right) and *S. elata* (b, right) was done in Magenta boxes on MS media supplemented with 0.5 – 2.0 mg/L BAP and 0.1 mg/L NAA for 15 days.

3.3.3 Root Induction

Shoots of *S. pinnata* exhibited the highest percentage of root induction on full-strength MS supplemented with 0.5 mg/L IBA, with 53% of explants forming roots after a period of 30 days (Fig. 3.5). Shoots of *S. elata* showed the highest percentage of root induction on full-strength MS supplemented with 1.0 mg/L IBA, with 75% of the explants forming roots after 30 days (Fig. 3.5). Both *S. pinnata* and *S. elata* exhibited the lowest percentage of root induction on full-strength MS without hormones, at 17% and 13.3% root induction, respectively (Fig. 3.5). The number of *S. pinnata* shoots that formed roots across the various hormone treatments was lower compared to *S. elata* (Fig. 3.5). Based on visual observation, *S. pinnata* plants generally formed longer roots *in vitro* whereas *S. elata* roots were shorter with more root hairs (Fig. 3.6).

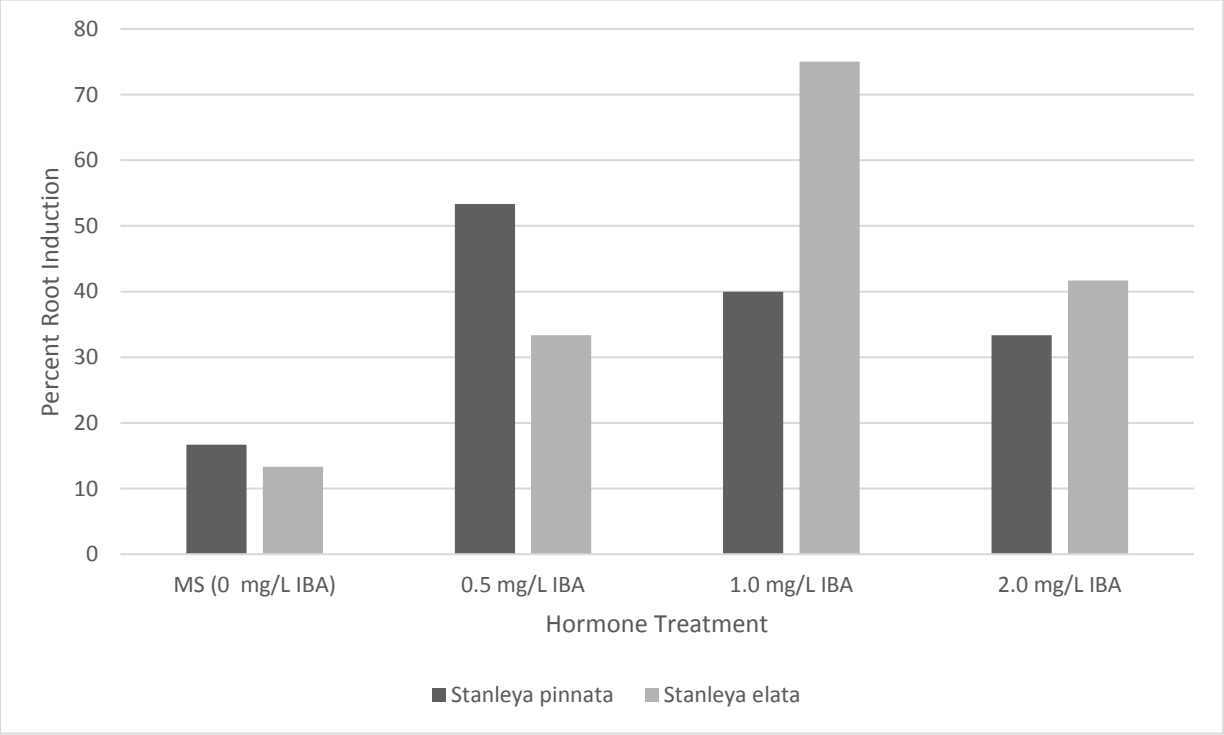


Figure 3.5) Root Induction of *S. pinnata* and *S. elata* shoots. 27 shoots per hormone treatment were grown in Magenta boxes for a period of 30 days (N = 216).

a)

b)

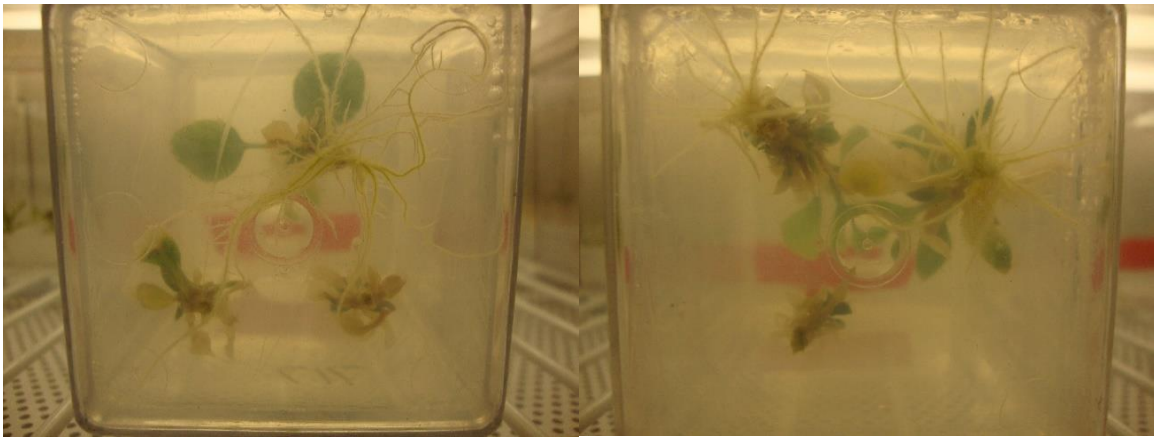


Figure 3.6) Root Induction of *S. pinnata* (a) and *S, elata* (b) shoots grown on MS media supplemented with 1.0 mg/ L IBA for 30 days.

3.3.4 Acclimatization / Hardening

Both *S. pinnata* and *S. elata* exhibited a high percentage of survivability during the acclimatization process, with *83% of S. pinnata* and 92% of *S. elata* plants acclimating to the *ex vitro* environment over a period of 30 days. In general, fully acclimatized *S. pinnata* and *S. elata* plants produced through the micropropagation protocol exhibited no visible differences when compared to plants grown from seed. However, *S. pinnata* plants produced from tissue culture

did seem to have longer internodes when compared to plants grown from seed in the same hydroponic system (Fig. 3.7).

a)

b)

Figure 3.7) Full acclimatization of *S. pinnata* (a) and *S. elata* (b) plants in the hydroponic system after 30 days.

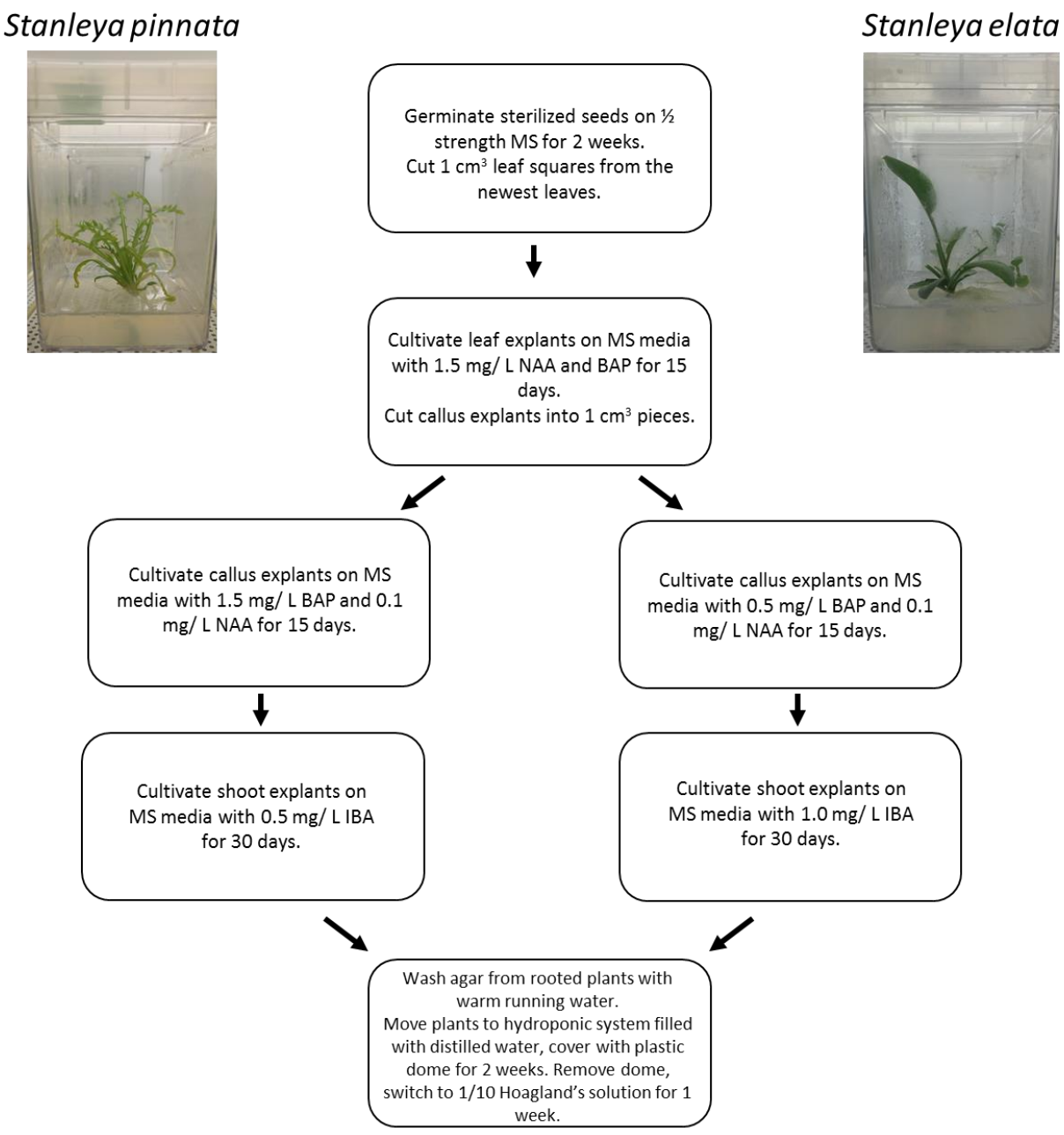


Figure 3.8) Flowchart outlining micropropagation protocol for *S. pinnata* and *S. elata*. Instructions for micropropagation of *S. pinnata* are on the left side, and instructions for micropropagation of *S. elata* are on the right. Steps in which the process is the same for both species are in the middle of the diagram.

3.4 Discussion

This study outlines an efficient and effective callus induction and plant regeneration protocol for the micropropagation of two Brassicaceae species, the Se hyperaccumulator *S. pinnata,* and related non-hyperaccumulator *S. elata.* In the case of *S. pinnata*, this protocol allows for the identification and clonal propagation of high Se-accumulating lines, which could pave the way for production of clones at a large scale to be used for industrial applications. Populations of *S. pinnata* grown via tissue culture could be planted in areas with high levels of Se as a method of phytoremediation to reduce Se levels. Alternatively, large quantities of Se-laden *S. pinnata* could be harvested and applied as green manure to agricultural fields to increase the Se content in crop species. *S. pinnata* synthesizes higher levels of Se-methylselenocysteine (SeMC) compared to nonaccumulator plant species (Freeman et al, 2010), which is a known anti-carcinogen and positively affect human health (Yang & Jia, 2014). Because of this, a fertilizer consisting of ground up *S. pinnata* could be applied to crop fields to enhance their nutritive qualities. Having a protocol for callus induction and regeneration also provides a first step toward developing a genetic transformation protocol for both species. Through such systems, novel genes from *S. pinnata* can be introduced and expressed in *S. elata* or could be knocked out in *S. pinnata* via CRISPR / Cas9 to further elucidate the mechanisms of Se hyperaccumulation. Both species of *Stanleya* also show extreme drought tolerance and the ability to thrive in poor soils, produce large number of flowers, and attract various pollinators, making them excellent candidates for use in native landscaping (Kratsch and Hunter, 2009). The use of micropropagation for these species may lead to the introduction of *Stanleya* to the horticultural industry.

Both *S. pinnata* and *S. elata* exhibited equal, high responses in terms of callus induction. High rates of callus induction in the presence of a range of BAP and NAA concentrations have been previously reported for other Brassicaceae species (Murata & Orton, 1987; Burbulis et al, 2009), and these rates are similar to the results from callus induction trials of *Stanleya* presented in this study. Previous studies have also shown that callus induction is affected more by different plant growth regulators, as opposed to a variation in the concentration of these plant growth regulators (Burbulis et al, 2009). The results seen for these species of *Stanleya*, which showed similar rates of callus induction across the ranges of BAP and NAA concentrations tested, is similar to results published for micropropagation of other Brassicaceae species (Ravanfar et al, 2017). *S. pinnata* had a lower percentage of shoot induction, as well as a lower number of shoots per callus when compared to *S. elata.* A strong genotype influence on shoot induction and general amenability to tissue culture has been reported for various Brassicaceae species, which may explain this variability in species of *Stanleya* (Glimelius, 1984; Murata & Orton, 1987; Akasaka-Kennedy et al, 2005).

Stanleya pinnata also displayed a significantly lower rate of root induction compared to *S. elata.* The morphology of the roots formed in culture was also different between the two species, with *S. pinnata* generally forming longer roots with little to no root hairs and *S. elata* generally forming shorter roots with a relatively high density of root hairs. Both species showed similar amenability to acclimating to the *ex vitro* environment, indicating that the physiology of the roots did not influence the overall health of the plant. Possible modifications to the rooting protocol that may increase the percentage of root induction include reducing the concentration of MS salts and sucrose by ½, and supplementing IBA with another auxin, such as NAA. Studies in other Brassicaceae species have shown efficient root induction with NAA, but usually in

conjunction with other auxins (Prevalek-Kozlina et al, 1997; Kaviani et al, 2011; Massoumi & Klerk, 2013). As a whole, the rate of root induction in these species of *Stanleya* using this protocol are comparable to the results from other studies in Brassicaceae species. In general, both *S. pinnata* and *S. elata* show high amenability to the tissue culture process, as has been reported for many species in the mustard family (Poulsen, 1996).

Both *S. pinnata* and *S. elata* are currently considered relatively obscure plants, and have only been used for research in an academic setting. However, through the utilization of tissue culture micropropagation, the regeneration protocol outlined above paves the way for the large-scale production of these species to be used in a variety of applications to benefit human health, and the environment.

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APPENDIX A

Location of Relevant Plasmids and Glycerol Stocks. List and diagram of location of glycerol stocks within the box labelled "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017" stored in the -80°C freezer, and list and diagram of location of relevant plasmids stored in the -20°C freezer in the box labelled "ZG pYES2 plasmids 05/30/2017".

SpSultr1;2 in *E. coli*: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 2-1

AtSultr1;2 in *E. coli*: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 3-1

SpSultr1;2t in yeast: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 4-6

SeSultr1;2t in yeast: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 5-6

AtSultr1;2t in yeast: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 6-6

YSD1pY in yeast: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 7-6

22574d yeast: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 2-4

YSD1 yeast: -80°C freezer, "ZG Sultr1;2 strain Glycerol Stocks 05/30/2017", 3-4

	1	2	3	4	5	6	7
1		SpSultr1;2 #8 no UTR *E. coli*	AtSultr1;2 #8 no UTR *E. coli*				
2							
3							
4		22574d *S. cerevisiae*	YSD1 *S. cerevisiae*				
5							

6				SpSultr1;2t #1 *S. cerevisiae*	SeSultr1;2t #1 *S. cerevisiae*	AtSultr1;2t #1 *S. cerevisiae*	YSD1pY (pYES2) *S. cerevisiae*

pEPY1 (SpSultr1;2 no tag): -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 2-2

pEPY2 (AtSultr1;2 no tag): -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 3-2

pEPY11 (SpSultr1;2 tag): -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 2-4

pEPY21 (AtSultr1;2 tag): -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 3-4

pEPY31 (SeSultr1;2 tag): -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 4-4

pYES2: -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 4-2

pET28-At (AtSultr1;2 3' end with tag): -20°C freezer, "ZG pYES2 plasmids 05/30/2017", 5-4

	1	2	3	4	5	6	7	8	9
1									
2		pEPY1	pEPY2	pYES2					
3									
4		pEPY11	pEPY21	pEPY31	pET28-At				
5									
6									
7									
8									
9									

APPENDIX B

SD-S Media Components, and instructions for preparation of 1 L of SD-S (0.1 mM SO_4^{2-}) yeast media.

100 X KNP stock		(g/100 mL)
ammonium chloride	NH_4Cl	20
potassium phosphate dibasic	K_2HPO_4	1.25
potassium phosphtate monobasic	KH_2PO_4	8.75

100 X Ca Na Mg stock		(g/100mL)
sodium chloride	NaCl	1
calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	1
magnesium chloride hexahydrate	$MgCl_2 \cdot 6H_2O$	4

1000 X micro element stock		(mg/100mL)
boric acid	H_3BO_3	50.0
copper chloride	$CuCl_2$	3.0
potassium iodide	KI	10.0
manganese chlororide tetrahydrate	$MnCl_2 \cdot 4H_2O$	35.0
ammonium molybdate tetrahydrate	$(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$	15.0
zinc chloride	$ZnCl_2$	20.0
EDTA ferric sodium salt	NaFe EDTA	50.0

1000 X vitamin stock	(mg/100ml)
biotin	2.0
calcium pantothenate	200
folic acid	0.20
myo-inositol	1000
nicotinic acid (niacin)	40
4-aminobenzoic acid	20
pyridoxine hydrochloride	40
riboflavin	20
thiamine hydrochloride	40

100 X $MgCl_2$ stock		(g/100mL)
magensium chloride hexahydrate	$MgCl_2 \cdot 6H_2O$	4.07

100 X $MgSO_4$ stock		(g/100mL)
magensium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	4.93

100 X Amino Acids		(mg/100ml)
isoleucine	Ile	300
valine	Val	1500
adenine hydrochloride	Ade	200
arginine	Arg	200
histidine	His	200
leucine	Leu	1000
lysine	Lys	300
methionine	Met	200
phenylalanine	Phe	500
threonine	Thr	2000
tryptophan	Trp	200
tyrosine	Tyr	300
uracil	Ura	200

20% (w/v) Galactose		(g/L)
galactose	Galactose	200

Autoclave all stock solutions except 20% (w/v) galactose, which should be filter-sterilized.

SD-S (0.1 mM SO_4^{2-}) [1L]:

- 10 ml 100x KNP
- 10 ml 100x Ca Na Mg
- 1 ml 1000x micro elements
- 1 ml 1000x vitamins
- 9.5 ml 100x $MgCl_2$
- 0.5 ml 100x $MgSO_4$

Bring volume up to 858 ml with distilled H_2O, and autoclave. After autoclaving, add:

- 100 ml 20% (w/v) filter-sterilized galactose
- 10 ml 100x Amino Acid stock solution

APPENDIX C

Compiled sequences of tagged and untagged open reading frames of Sultr1;2 from *S. pinnata, S. elata,* and *A. thaliana.* Raw Sanger sequencing reads and chromatograms are located in the Pilon-Smits lab folder on the CSU Biology Pangaea server.

>AtSULTR1;2
GAATTCATGTCGTCAAGAGCTCACCCTGTGGACGGAAGTCCGGCGACGGACGGTGGACATGTTCCGATGAAACCTTC
ACCCACTCGGCATAAAGTTGGAATCCCACCAAAGCAAAACATGTTCAAGGATTTCATGTACACATTCAAAGAAACTT
TCTTTCATGATGATCCTCTTAGGGATTTTAAGGATCAGCCTAAGTCTAAGCAGTTTATGCTCGGTCTCCAATCCGTC
TTCCCGGTCTTCGATTGGGGACGTAACTACACTTTCAAGAAGTTCCGAGGTGATCTCATCTCCGGTTTAACCATTGC
AAGTCTCTGCATTCCTCAGGATATTGGATACGCTAAGTTGGCGAATCTTGATCCCAAATACGGTTTATATTCGAGTT
TTGTTCCTCCATTGGTGTATGCTTGTATGGGAAGTTCTAGGGATATAGCAATAGGACCTGTCGCTGTGGTTTCGCTG
TTGCTAGGCACATTGCTTCGAGCTGAGATTGATCCAAACACAAGTCCAGATGAATATCTCCGCCTTGCCTTCACTGC
TACGTTTTTCGCCGGTATAACCGAAGCAGCCCTTGGATTCTTCAGATTAGGATTCTTGATCGATTTCCTTTCCCACG
CGGCTGTGGTTGGCTTCATGGGCGGCGCAGCCATCACTATCGCTCTTCAGCAGCTTAAAGGCTTCCTCGGGATCAAG
AAATTCACCAAGAAAACTGATATTATTTCTGTTCTTGAATCCGTTTTCAAAGCAGCTCATCACGGCTGGAATTGGCA
GACTATACTCATTGGTGCATCATTCTTGACCTTCCTTCTCACGTCTAAGATCATTGGGAAGAAGAGCAAGAAACTA
TTCTGGGTACCAGCTATTGCGCCATTGATATCAGTTATCGTTTCCACCTTCTTTGTCTACATAACCCGAGCCGACAA
ACAAGGAGTCCAAATCGTGAAACACCTTGACCAAGGAATCAACCCTTCCTCGTTCCATCTAATCTACTTCACTGGTG
ATAACCTTGCTAAGGGCATCCGCATCGGTGTAGTCGCTGGCATGGTCGCTTTAACAGAAGCTGTAGCGATTGGAAGA
ACCTTTGCTGCAATGAAAGACTACCAAATCGACGGTAACAAAGAGATGGTAGCATTAGGTATGATGAACGTAGTTGG
ATCGATGTCTTCTTGCTACGTAGCTACCGGATCTTTCTCAAGATCAGCTGTCAATTTCATGGCTGGATGTCAAACAG
CGGTTTCAAACATCATAATGTCAATTGTTGTTCTCTTGACATTGCTCTTCCTTACTCCTCTCTTCAAGTACACTCCA
AACGCCATCCTCGCAGCTATCATCATCAACGCTGTGATTCCTTTGATCGATATCCAAGCTGCTATTTTGATCTTCAA
GGTTGATAAGCTCGATTTCATCGCCTGTATTGGAGCATTCTTTGGCGTCATCTTTGTTTCTGTTGAGATCGGACTTC
TTATTGCCGTCTCGATCTCGTTTGCTAAGATCCTCTTGCAAGTAACAAGACCTAGAACTGCAGTTCTCGGAAATATT
CCAAGAACTTCGGTTTACAGAAATATTCAACAGTATCCTGAAGCCACTATGGTTCCAGGGGTTCTTACTATTCGTGT
TGACTCCGCCATTTACTTCTCCAACTCAAATTATGTTAGAGAAAGGATCCAGAGATGGCTACATGAGGAAGAAGAAA
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GGTATTCACGCATTAGAAGACTTATACAAGTCTCTCCAGAAAAGAGACATTCAGTTGATTCTGGCGAATCCTGGACC
GTTGGTGATAGGCAAGCTACACTTGTCGCACTTTGCCGACATGTTAGGACAAGACAATATCTATCTAACGGTGGCTG
ATGCCGTCGAGGCTTGCTGTCCAAAACTCTCCAACGAGGTCTGAGAATTC

>Sp_Sultr1;2
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