ELUCIDATION OF SELENIUM TOLERANCE MECHANISMS IN *Puccinellia distans (Jacq.) Parl.* USING A TRANSCRIPTOMIC APPROACH

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ABSTRACT

ELUCIDATION OF SELENIUM TOLERANCE MECHANISMS IN *Puccinellia distans (Jacq.) Parl.* USING A TRANSCRIPTOMIC APPROACH

Selenium (Se) has a versatile chemistry. Therefore, it is present in a wide variety of materials on Earth. Although it is not an essential element for plants, Se provides several survival and ecological advantages. *Puccinellia distans* (*P.distans*) is weeping alkali grass which has a relatively fast growth rate and produces a large biomass. It can also hyperaccumulate extreme concentrations of boron and tolerate high levels of salinity. It is known that some plant species can tolerate high concentrations of soil salts and boron while hyperaccumulating Se in their leaves. Therefore, in this study, the Se accumulation and tolerance capacity of *P. distans* were investigated, and the plant's Se tolerance mechanism was elucidated using a transcriptomic approach. The results of this study indicated that *P. distans* accumulated more than 100 mg Se kg^{-1} in its shoots. Therefore, *P. distans* was discovered to be a novel Se accumulator plant. Biochemical analyses and RNA sequencing results helped us to decipher the Se tolerance and accumulation mechanism of *P. distans*. Accordingly, the tolerance mechanism was found to be associated with the upregulation of genes involved in sulfate, selenocompound and antioxidant pathways. Here, we suggest that upregulation of Se assimilation and stress responses genes may have been due to induction of jasmonic acid signaling. In addition, we propose that the cell wall may play an important role in restriction of Se movement to the cytoplasm. Also, we put forward that sequestration of selenate inside the vacuole may be a way that Se accumulates in cells.

ÖZET

Puccinellia distans (Jacq.) Parl.'DA SELENYUM TOLERANS MEKANİZMALARININ TRANSKRİPTOMİK YAKLAŞIM KULLANILARAK ANLAŞILMASI

Selenyum (Se), farklı durumlara adapte olabilen bir kimyaya sahiptir. Bu nedenle, dünya üzerinde birçok materyal içinde bulunabilir. Se bitkiler için gerekli bir element olmamasına karşın, bitkilere yaşamını sürdürme konusunda ve ekolojik anlamda birçok avantaj sağlar. *Puccinellia distans* (*P. distans*) nispeten hızlı büyüyen ve geniş biyokütle üreten bir çorak çimidir. Ayrıca, son derece yüksek konsantrasyonlarda boru biriktirebilir ve yüksek tuz konsantrasyonlarına dayanabilir. Günümüzde bazı bitki çeşitlerinin yüksek bor ve tuz konsantrasyonlarına dayanabilirken, selenyumu da yapraklarında hiperakümüle edebildiği bilinmektedir. Bu nedenle, bu çalışmada *P. distans* bitkisinin Se akümülasyon ve tolerans kapasitesi araştırılıp, tolerans mekanizması transkriptomik yaklaşım kullanılarak aydınlatılmaya çalışılmıştır. Çalışmanın sonuçları *P. distans*'ın >100 mg kg^{-1} 'dan fazla selenyumu yapraklarında biriktirdiğini göstermiştir. Bu nedenle, *P. distans*'ın yeni bir Se akümülatör bitki olduğu keşfedilmiştir. Biyokimyasal analizler ve RNA sekanslama sonuçları *P. distans* bitkisinin Se tolerans ve akümülasyon mekanizmasını açığa çıkarmak konusunda yardımcı olmuştur. Buna göre, tolerans mekanizmasının sülfat, selenyum bileşiği ve antioksidan yolaklarının upregulasyonuyla alakalı olduğu bulunmuştur. Burada, Se asimilasyon ve antioksidan yolaklarının ve stres cevap yolaklarının upregulasyonunun, jasmonik asit sinyalizasyonundan kaynaklanabileceğini, buna ek olarak hücre duvarının selenyumun sitoplazmaya geçişinin engellenmesinde önemli bir rolü olduğunu önerdik. Ayrıca, selenatın koful içeresinde tutulmasının da bir çeşit Se akümülasyon yolu olabileceğini ileri sürdük.

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CHAPTER 1

INTRODUCTION

1.1 Selenium

1.1.1 Properties of Selenium

Selenium (Se) was discovered by Jacob Berzelius in 1817 and named after the Greek goddess of the moon (Oldfield 1974). Se is used in a wide range of areas including agriculture, electronics, solar cells, glass industry, photocopying, cosmetics industry and medicine (Wadgaonkar et al. 2018). It is a Group 6A element with an atomic weight of 78.9 and is a metalloid. Therefore, it shows physical and chemical properties that are intermediate between metals and nonmetals (Fordyce 2013). Se is similar to sulfur (S) in terms of its chemical properties. They both have five valance states. These are 2-, 0, 2+, 4+ and 6+. Therefore, Se can exist as Se^{2-} (selenide), Se (elemental Se), $Se_2O_3^{2-}$ (thioselenate), SeO_3^{2-} (selenite) and SeO_4^{2-} (selenate). In aerobic and neutral to alkaline (oxidizing) conditions, Se speciation shifts towards selenate whereas selenide and elemental Se are the major species under anaerobic (reducing) conditions. Due to its versatile chemistry, Se is present in all natural materials on Earth; for example, rocks, soils, waters, air and biological tissues (Table 1). Although Se and S share some properties, they are differentiated from each other by significant characteristics. For example, the radius of Se (0.50 Å) is larger than S (0.37 Å) and the bond between two Se atoms is longer and weaker than the disulfide bond (Läuchli 1993).

Table 1. Selenium concentrations in natural materials
(Source: Fordyce 2013)

Material	**Total Se (mg kg^{-1})**
Earth's crust	0.05
Atmospheric dust (general)	0.05-10
Oil (general)	0.01-1.4
World freshwater (μg L^{-1})	0.02

(cont. on the next page)

Table 1. (cont.)

Material	Total Se (mg kg^{-1})
Polar ice water (general)	0.02
Sea water (general)	0.09
Animal tissue (general)	0.4-4
Air (ng m^{-3}) (general)	0.05-10
Soil (general)	0.4
Soil (seleniferous)	1-5000

1.1.2. Selenium in the Environment

Se in the Se cycle is derived from man-made sources (76,000- 88,000 tonnes $year^{-1}$) and natural systems including marine (38,250 tonnes $year^{-1}$), terrestrial (15,380 tonnes $year^{-1}$) and atmospheric (15,300 tonnes $year^{-1}$) sources. The major release of Se occurs through anthropogenic activities. Some of these activities are metal processing, burning of coal and fossil fuels, disposal of sewage sludge, and agricultural use of pesticides and phosphate fertilizers. A major pathway of Se loss from land in the Se cycle occurs through flux of Se from land into the oceans (14,000 tonnes $year^{-1}$). The average concentration of Se in seawater is relatively low (Figure 1), however, the residence time for Se ranges from 70 to 1,100 years. Therefore, the marine system is an important natural sink for Se. Rocks are the primary source of Se in the terrestrial system and Se enters the food chain via weathering of rocks, water-rock interactions and biological activities. In addition to this, volatilization of Se from soils, waters, biological organisms (plants, animals and microorganisms) and industrial activities leads to flux of Se to the atmosphere. Seventy six to 93% of total gaseous Se returns to the land surface in wet form (rain, snow, etc.) and more than 70% of that is in soluble form. This contributes 5,610 tonnes Se per year to Earth's surface (Fordyce 2013).

The soil concentration of Se changes significantly depending on local land conditions. Therefore, it is not distributed evenly across the Earth and ranges from 0 to 5000 μg Se g^{-1}. For example, it reaches up to 1200 μg Se g^{-1} in Se rich soils at a site near County Meath, Ireland (Wadgaonkar et al. 2018, Fordyce 2013). The normal range of Se in soil is 0.1-2 μg Se g^{-1}. Se deficient soils contain less than 0.05 μg Se g^{-1} and Se rich (or seleniferous) soils contain more than 5 μg Se g^{-1} (Wadgaonkar et al. 2018). The bioavailability of Se depends on several factors including pH, redox potential, the

chemical form and speciation of Se, soil properties (texture, mineral, etc.) and the presence of competitive ions (Wadgaonkar et al. 2018, Fordyce 2013)

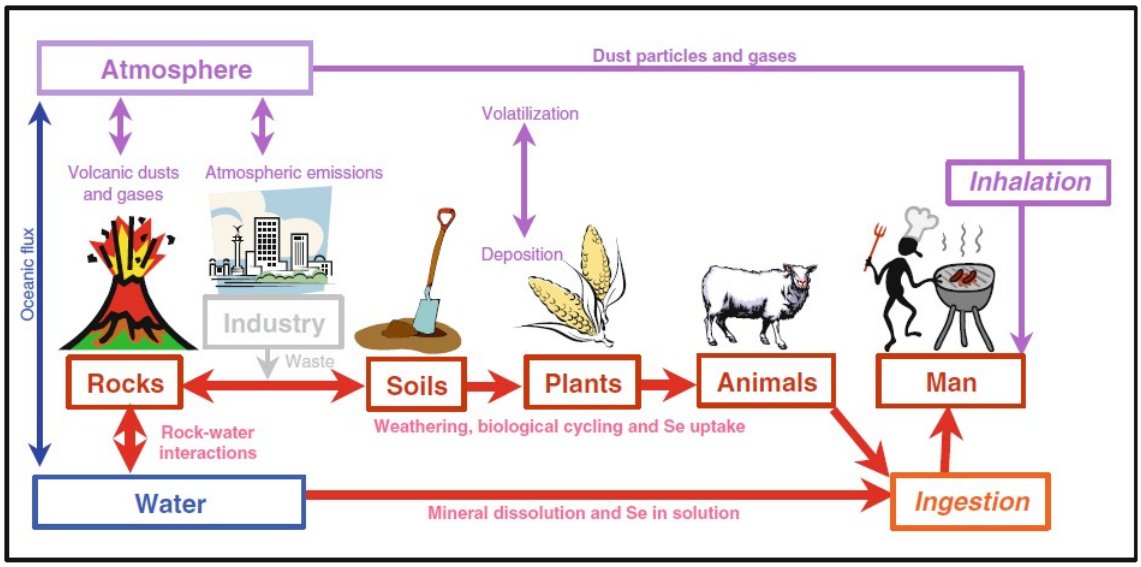

Figure 1. Schematic diagram of the Se cycle. Red arrows indicate the main pathways (Source: Fordyce 2013).

1.1.3. Se as a Nutrient: Toxicity and Deficiency

Se is an important trace element for humans and other animals because it is part of at least 25 different selenoproteins (e.g. glutathione peroxidase, thioredoxin reductase). These proteins generally contain a selenocysteine, the 21st amino acid, at their active site (Schiavon et al. 2015, Rayman 2000). Se enters the food chain through plants and algae (Fordyce 2013). The recommended daily uptake of Se is 55–70 µg Se day^{-1} (NIH, 2016). Because the minimum and maximum levels of Se as a nutrient are narrow, both deficiency and toxicity cause problems worldwide (Schiavon et al. 2015).

Se toxicity is prevalent in Se rich areas whereas Se deficiency occurs in Se poor soils (Yasin et al. 2015). Plants and animals can accumulate Se in greater concentrations than are present in their environment and bioaccumulation causes toxicity (Fordyce 2013). For example, Se toxicity is a common problem in many countries including parts of the USA, China, Canada and India (Schiavon and Pilon-Smits 2017). High doses of Se may increase the likelihood of having diabetes and hypercholesterolemia (Schiavon et al. 2015). In addition, it increases the risk of some cancers such as pancreatic and skin cancers (Fordyce 2013).

Like excess Se, Se deficiency also poses problems to organisms. It is mostly seen in China, northwestern Europe, Australia, New Zealand, sub-Saharan Africa, southern Brazil and parts of the USA (Wu et al. 2015). Se at low doses may lead to heart diseases (e.g. Keshan disease and Kashin-Beck disease), reduced fertility, irreversible brain injury and reduced immune function (Rayman 2000, 2012, Schiavon et al. 2015). It is also associated with decreased survival in HIV-infected patients (Rayman 2012).

Although Se is necessary for humans and animals, it is not an essential element for plants. However, Se is beneficial for plant growth and is associated with increased antioxidant activity at low tissue levels (10 mg kg^{-1}). Plants use Se as an elemental defense against certain herbivores such as the black tailed prairie dog (*Cynomys ludovicianus*), caterpillars (*Pieris rapae*), crickets (*Orthoptera*) and aphids (*Myzus persicae*) In addition, Se protects plants against some fungal pathogens (*Fusarium* and *Alternaria*). Thus, it provides ecological advantages to plants (Freeman et al. 2007, Freeman et al. 2009, Hanson et al. 2003, Hanson et al. 2004, Quinn et al. 2010, White 2016, Schiavon et al. 2017).

1.2 Metal/Metalloid Tolerance in Plants

Plant tolerance is the ability of a plant to survive in soil which is toxic to other plants of the same or different species. Other plants are used as a reference to determine the toxicity of the soil (Macnair et al. 1999). Toxicity symptoms are observed due to several reasons. Firstly, metals may bind to sulphydryl groups in proteins or interfere with the functional groups of important molecules and disrupt their function. Secondly, they can take the place of an essential element and cause deficiency of this element. Lastly, excess amount of the specific metal may lead to oxidative stress due to formation of free radicals and reactive oxygen species (ROS). ROS may lead to lipid peroxidation; ion leakage; and disruption of macromolecule, DNA and membrane integrity (Hall 2002, Rascio and Navari-Izzo 2011).

Metal tolerant plants have adaptation mechanisms at the cellular level that allow them to survive and grow on metalliferous soils. They develop avoidance and homeostatic mechanisms that keep toxic concentrations of metals away from their susceptible sites and prevent their accumulation. For example, the restriction in

movement of metals across the plasma membrane is one of them. A second mechanism is trapping metals in the apoplastic environment by binding them to the cell wall, root exudates or various ligands. A third method is the transport and accumulation of metals in vacuoles. A fourth mechanism is the repair and protection of plasma membrane. The final mechanism is the activity of mycorrhizae that are utilized by plants to tolerate metal (Hall 2002, Rascio and Navari-Izzo 2011).

In general, metal tolerant plants minimize the translocation of metals from roots to above ground tissues. Therefore, most of the metals are retained and detoxified in the roots. On the other hand, hyperaccumulator plants behave exactly opposite to tolerant plants and translocate and distribute metals to other plant tissues (Rascio and Navari-Izzo 2011).

1.3. Metal/Metalloid Hyperaccumulation in Plants

1.3.1. Hyperaccumulators

Hyperaccumulator plant species are of special importance in the plant kingdom because they pave the way for studies aimed at understanding adaptations to harsh conditions, the metal homeostasis mechanism and evolution of naturally selected complex traits (Hanikenne and Nouet 2011).

Hyperaccumulator is a term that is used to define plants which have the ability to take up large amounts of one or more metals/metalloids from the soil during normal growth and reproduction without suffering any phytotoxic effects. Hyperaccumulators share several common features that differentiate them from non-hyperaccumulator plants. Some of them are their enhanced ability to detoxify and translocate metal from their roots to shoots and store it in their above ground organs at concentrations 100 - 1000 fold higher than those found in non-hyperaccumulators (Peer et al. 2006, Rascio and Navari-Izzo 2011). Some examples of threshold values that are used for a definition of specific metal/metalloid hyperaccumulation are indicated in Table 2. There are at least 515 taxa from 60 plant families that have been defined as metal/metalloid hyperaccumulators. The Brassicaceae family constitutes 25% of hyperaccumulators, especially the genera *Thlaspi* and *Alyssum (R*ascio and Navari-Izzo 2011).

Table 2. Examples to the threshold values of some of the elements that are used to define a plant as a hyperaccumulator.

Element	Threshold values
Manganese and Zinc	>10 mg g^{-1}
Arsenic, Cobalt, Copper, Nickel and Selenium	>1 mg g^{-1}
Cadmium	>0.1 mg g^{-}

1.3.2. Selenium Tolerance and Hyperaccumulation

Se is easily taken up and metabolized by all plants because Se is atomically similar to S. Selenate and selenite are two of the available forms of Se that a plant takes up (Sors, Ellis, and Salt 2005). However, the extent of Se accumulation levels varies among different plant species. Plants are divided into three groups in terms of their Se accumulation capacity. These are: Se non-accumulators, secondary accumulators and hyperaccumulators. Se non-accumulators accumulate 10 - 100 mg Se kg^{-1} dry weight (DW) and Se secondary accumulators accumulate Se in the moderate range of 100 - 1000 mg Se kg^{-1} when growing on seleniferous soils. On the other hand, Se hyperaccumulators take up Se 100 - 1500 fold more than non-accumulators, typically 1000 - 15000 mg Se kg^{-1} from seleniferous soils (White 2016).

Se non-accumulators cannot differentiate selenate and sulfate. Therefore, the amount of selenate and sulfate taken up and incorporated into S compounds by non-accumulators is proportional to the amount that is present in the growth medium (Pilon-Smits, Winkel, and Lin 2017). Also, the Se/S ratio in tissues of non-accumulators is similar to the Se/S ratio of their growth medium. Se is accumulated mostly as inorganic Se in non-accumulators and secondary accumulators (Schiavon and Pilon-Smits 2017).

Hyperaccumulators of Se are found in at least 45 taxa from 14 genera and 6 families. Some genera of different families are *Astragalus* (Fabaceae); *Oonopsis*, *Xylorhiza*, *Symphyotrichum* (Asteraceae); *Castilleja angustifolia* var. *dubia*, *Coelospermum decipiens* (Rubiaceae), *Stanleya pinnata, S. bipinnata* (Brassicaceae); *Neptunia amplexicaulis* (Mimosaceae). The Scrophulariaceae, and Chenopodiaceae families also contain Se hyperaccumulator species (El Mehdawi et al. 2014, Reeves and Baker 1999, Rosenfeld 1964, Schiavon et al. 2017).

Se hyperaccumulators can distinguish selenate and sulfate and prefer Se over S. In addition, sulfate uptake is restricted by selenate in these plants (Harris, Schneberg, and Pilon-Smits 2014). Se hyperaccumulators also have other special features making them different from non-hyperaccumulators. For instance, they assimilate most of the Se into organic forms, distribute Se into various organs, and store it as methyl-selenocysteine in specific epidermal storage areas. Conversion of inorganic Se into organic forms contributes to increased Se tolerance because organic forms of Se cause less oxidative stress than inorganic Se (Van Hoewyk 2013). Moreover, Se hyperaccumulators have higher Se/S ratios in their tissues than their growth medium and other plants in the same environment (Prins et al. 2011, Schiavon et al. 2017). Although, both Se hyperaccumulators and non-accumulators accumulate Se in their reproductive tissues, Se hyperaccumulator flowers can contain relatively more Se in ovules and pollen compared to non-accumulators (Quinn et al. 2011). The sequestration pattern of Se in hyperaccumulators is thought to serve a protective function since the level of Se is high in important and sensitive organs including young leaves, pistil, anthers, seed and seedling, and tissues such as mesophyll (Schiavon et al. 2017).

Se hyperaccumulators have an advanced sulfate/selenate uptake and assimilation ability because genes involved in the antioxidant pathway and genes encoding stress/defense hormones (jasmonic acid, salicyclic acid and ethylene) are constitutively upregulated in these plants. Defense related genes are also constitutively upregulated in Se hyperaccumulator plants but their effects on plants' hyperaccumulation capacity still needs further research. Plant defensin protein (PDF) is an exception since its overexpression is known to lead to increased Se tolerance (Freeman et al. 2010, Schiavon et al. 2017, Tamaoki, Freeman, and Pilon-Smits 2008)

Furthermore, Se hyperaccumulators have advanced detoxification mechanisms that allow them to eliminate damaged/misfolded proteins to be degraded by the proteasome and minimize Se incorporation into proteins by assimilating or sequestering Se into different organs (Sabbagh and Van Hoewyk 2012, Sors, Ellis, and Salt 2005, Wang et al. 2018).

1.4. Molecular Mechanisms of Selenium Tolerance and Hyperaccumulation

1.4.1 Se Uptake and Transport

Although selenite enters plant cells through passive diffusion, selenate is taken up against its electrochemical potential gradient through active transport (Sors, Ellis, and Salt 2005, Terry et al. 2000). Uptake of selenate competes with that of sulfate because both enter the plant via sulfate transporters (SULTR) (Terry et al. 2000) (Figure 3). Sulfate transporters are proton/sulfate co-transporters ($3H^+/SO_4^{2-}$) (Hawkesford, Davidian, and Grignon 1993, Shibagaki et al. 2002). There are five groups of SULTRs (SULTR 1-5) (Table 2), which have been studied widely in the model species *Arabidopsis thaliana*. They have 12 transmembrane spanning domains. Both C- and N-termini reside in the cytoplasm. The C terminal site of SULTRs contain a Sulfate Transporter and Antisigma factor antagonist (STAS) domain which is proposed to interact with Se/S assimilation enzymes (such as cysteine synthase) for regulation of acquisition rate (Shibagaki and Grossman 2010, Vatansever et al. 2016).

Group 1 transporters constitute the high affinity SULTRs. They have a Michaelis constant (Km), which shows the affinity of the transporter for the specific substrate; the higher the Km value, the lower the affinity and vice versa (Boyle 2005). Hyperaccumulators constitutively overexpress these transporters encoding genes as compared to non-hyperaccumulators (Buchner, Takahashi, and Hawkesford 2004, Schiavon et al. 2017). Among these SULTR1;2 and SULTR1;1 are primarily expressed in the root (root tip and root epidermis). They facilitate the influx of sulfate (and thus, selenate) into the root symplast (Buchner, Takahashi, and Hawkesford 2004). Both selenate and sulfate are mainly taken up by SULTR 1;2 transporter. It is expressed under both sulfate-rich and sulfate-deficient conditions. In addition, the expression level of this gene is relatively independent from the external sulfate concentration and suggested to be regulated by metabolic demand and photoperiod. However, SULTR 1;1 expression depends on sulfate supply since its expression is induced under sulfate starvation (Buchner, Takahashi, and Hawkesford 2004, Rouached et al. 2008, Schiavon et al. 2015, Yoshimoto et al. 2002). In addition, it is thought to have higher specificity for sulfate than selenate (Takahashi et al. 2000). Another high affinity transporter is

SULTR1;3 which is localized in the phloem (mainly in transport phloem) of cotyledons, hypocotyls, and roots in *A. thaliana.* Specifically, it is expressed in sieve element-companion cell complexes in mature organs and companion cells in roots but not in young developing leaves. SULTR1;3 is suggested to function in source to sink transport of sulfate, specifically it recovers the sulfate that is leaked out from the sieve tube during long distance transport within the transport phloem (Yoshimoto et al. 2003).

Group 2 transporters are composed of low affinity SULTRs. There are two group 2 transporters in *A. thaliana*: SULTR2;1 and SULTR2;2. They are expressed both in root and shoot of the plant and mainly localized in vascular tissue. Therefore, they mediate the transfer of sulfate/selenate within the vascular cylinder and between organs. SULTR2;2 helps transport via phloem and causes an increase in the sulfate/selenate concentration in the root. In the shoot, it is responsible for uptake from the vessels for further sulfate/selenate assimilation (Baraniecka and Kopriva 2014). SULTR2;1 is the main pathway for the translocation of sulfate/selenate from roots to other organs. Its expression in the xylem parenchyma is involved in the reabsorption of sulfate/selenate for regulation of sulfate/selenate transport to shoots during deficiency, and further xylem transport (Buchner, Takahashi, and Hawkesford 2004). In addition, the activity of SULTR2;1 is regulated and reaches its maximum capacity through heterodimerization with SULTR3;5 to translocate sulfate/selenate to xylem parenchyma (Kataoka, Hayashi, et al. 2004).

Group 3 is the largest group of SULTR family proteins including SULTR3;1, 3;2, 3;3, 3;4 and 3;5. They are low affinity transporters (Talukdar and Talukdar 2015) and expressed in shoots (Vatansever et al. 2016, Yoshimoto et al. 2002). Among them, SULTR3;1 is localized in the chloroplast and involved in sulfate/selenate transport into the chloroplast. SULTR3;2, SULTR3;3, SULTR3;4 but not SULTR3;5 are also suggested to be chloroplast sulfate transporters. However, the functions of these group members are still unknown and remain to be elucidated (Cao et al. 2013).

Group 4 transporters contains two members: SULTR4;1 and SULTR4;2. They are localized in both roots and shoots, and they are responsible for export of selenate/sulfate from vacuoles (Kataoka, Watanabe-Takahashi, et al. 2004).

Group 5 is the last group of SULTR transporters. It has two members: SULTR5;1 and SULTR5;2. They are localized in the tonoplast. This group shares a low level of sequence similarity with Groups 1 to 4 and is significantly diverged from them (Buchner, Takahashi, and Hawkesford 2004, Hawkesford 2003). Indeed, Group 5

SULTR transporters have different functions. They are involved in molybdate transport and accumulation. In addition, their role in sulfate transport has not yet been confirmed and so they are not accepted as sulfur transporters (Baxter et al. 2008, Tomatsu et al. 2007, Takahashi et al. 2011, Gigolashvili and Kopriva 2014). SULTR5;1 and SULTR5;2 are also named MOT2 and MOT1, respectively.

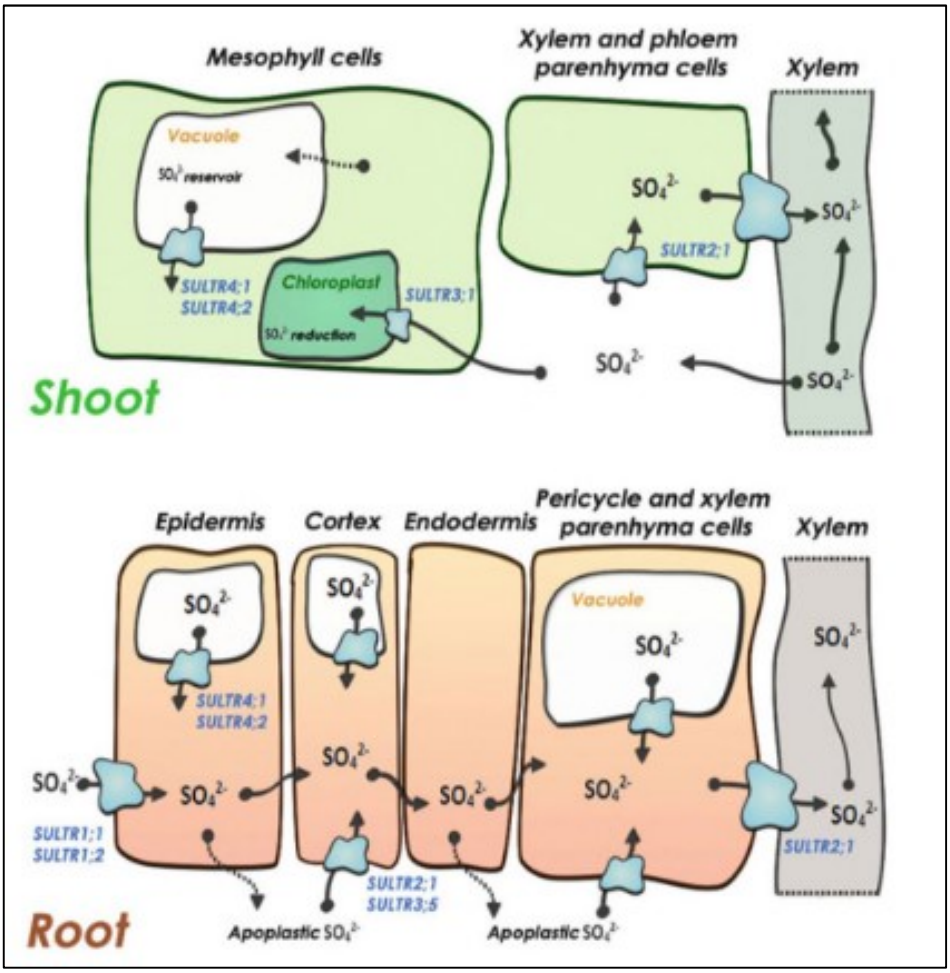

Figure 2. Sulfate transport system in *A. thaliana.* Blue shapes show the SULTRs and dashed arrows are transport pathways that remain to be elucidated (Source: Baraniecka and Kopriva, 2014)

1.4.2 Se Reduction, Assimilation and Volatilization

After selenate is taken up into roots, it can be moved to plastids for reductive assimilation (Figure 3). The first and rate limiting step is the activation of selenate by ATP sulfurylase (APS) (Pilon-Smits et al. 1999). In this process, ATP is coupled to

selenate to form adenosine 5'-phosphoselenate (APSe) which is subsequently reduced to selenite by APS reductase (APR) in another rate limiting step in the chloroplast.

Selenite is further reduced to selenide either non-enzymatically or enzymatically (Setya, Murillo, and Leustek 1996).. The non-enzymatic reduction is accomplished by glutathione (GSH) while the enzymatic pathway requires the action of sulfite reductase (Ng and Anderson 1978, Terry et al. 2000). After that, selenocysteine (SeCys) is produced from selenide by the action of two enzymes: serine acetyltransferase (SAT) and O-acetyl thiol-lyase (OAS-TL) which form a complex named cysteine synthase. Selenide is assimilated with O-acetylserine (OAS) by cysteine synthase and SeCys is formed (Bogdanova and Hell 1997, Ng and Anderson 1978). The SeCys can be utilized to synthesize glutathione, which is an important molecule to decrease oxidative stress in plants by gamma-glutamylcysteine synthetase and gluthathione synthetase in a two-step reaction (Noctor et al. 1998). In addition, SeCys can be converted into selenocystathionine (Secysth) by cystathionine gamma synthase (CGS) or methylated by SeCys methyltransferase (SMT) to form MeSeCys. Se hyperaccumulators can accumulate and tolerate higher concentrations of Se because they sequester Se into non-protein amino acids such as Se-methylselenocysteine (MeSeCys), γ-glutamyl-Se-methylselenocysteine (GGMeSeCys) and selenocystathionine. For example, *S. pinnata* accumulates MeSeCys, the Se hyperaccumulator *Neptunia amplexicaulis* stores Secysth and *Astragalus bisulcatus* accumulates GGMeSeCys in its seeds (Freeman et al. 2006, Pilon-Smits, Winkel, and Lin 2017, Peterson and Butler 1962). In this way, integration of Se into proteins is limited or prevented (Sors, Ellis, and Salt 2005, Çakır, Turgut-Kara, and Arı 2012).

SeCys can also be metabolized to selenomethionine (SeMet) through the Met biosynthesis pathway. SeMet is the precursor for the production of volatile Se compounds (Figure 4). For example, dimethylselenide (DMSe) is one of these volatile Se compounds and the main one in non-accumulators. The initial step in the production of DMSe is further methylation of SeMet to form Se-methylselenomethionine (MeSeMet) by S-adenosyl-L-methionine: L-methionine S-methyltransferase (MMT).

Table 3. Summary of sulfate transporter family proteins

	Members	Localization	Function	
Group 1	SULTR1;1	Root tip and root epidermis	The influx of sulfate/ selenate into the root symplast	Under sulfate starvation
	SULTR1;2			Both sulfate-rich and sulfate-starvation conditions
	SULTR1;3	Phloem of cotyledons, hypocotyls, and roots	Source to sink distribution	
Group 2	SULTR2;1	Shoot	Transfer of sulfate/selenate within the vascular cylinder and between organs.	Xylem parenchyma and phloem
		Root		Xylem parenchyma and pericycle cells
	SULTR2;2	Shoot		Xylem vessels
		Root		Phloem

(cont. on the next page)

Table 3. (cont.)

	Members	Localization	Function
Group 3	SULTR3;1 SULTR3;2 SULTR3;3 SULTR3;4	Shoot (SULTR3;1 is localized in chloroplast)	Influx of sulfate/selenate into chloroplast
	SULTR3;5	Root	Root to shoot transport of sulfate/selenate
Group 4	SULTR4;1 SULTR4;2	Root and Shoot	Efflux of sulfate/selenate from the vacuole
Group 5	SULTR5;1 (a.k.a MOT2) SULTR5;2 (a.k.a MOT1)	Tonoplast	Molybdate transport and accumulation

After that, MeSeMet is converted into DMSe by S-methylmethionine hydrolase (Lewis, Johnson, and Broyer 1974, Sors, Ellis, and Salt 2005). Dimethyldiselenide (DMDSe), another volatile Se compound, is produced from the oxidation and subsequent methylation of MeSeCys (Meija et al. 2002, Sors, Ellis, and Salt 2005).

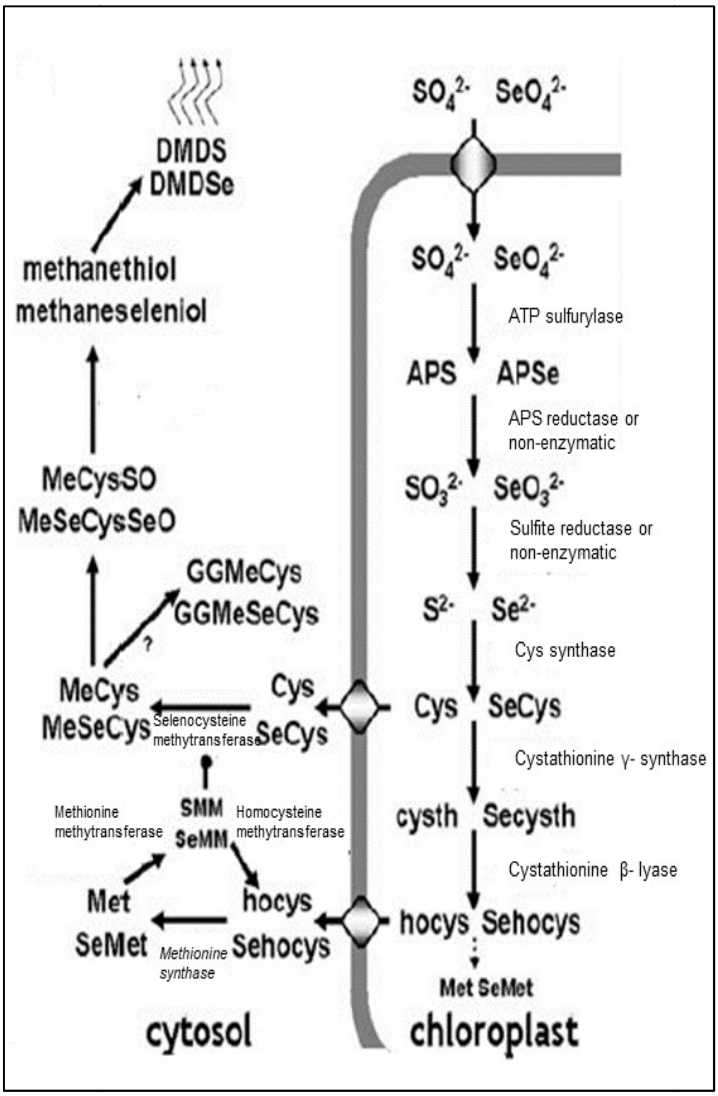

Figure 3. Model for selenate/sulfate reduction, assimilation and volatilization pathway (Source: Sors et al., 2005). Cys, cysteine; cysth, cystathionine; hocys, homocysteine; Met, methionine; GGMeSeCys, gamma-glutamyl-methylselenocysteine; MeSeCysSeO, methylselenocysteine

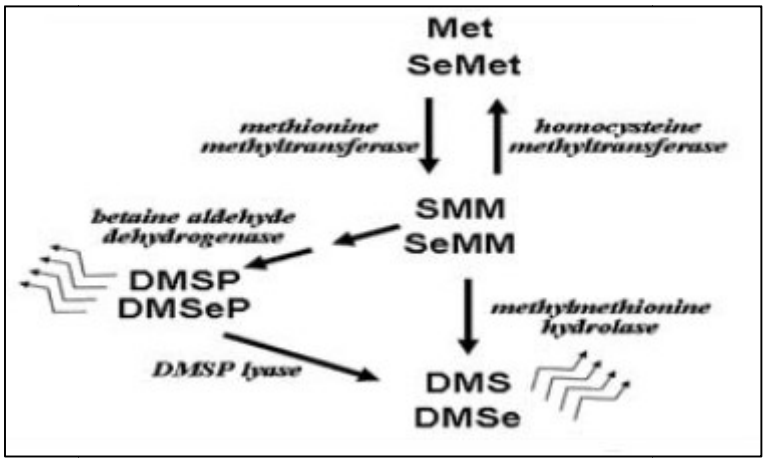

Figure 4. The production of volatile Se/S compounds from selenomethionine (SeMet) (Source: Sors et al., 2005). S, sulfur; Se, selenium; DMSeP (dimethylselenoniopropionate); SeMM (Se-methylmethionine)

The volatilization process is used by both non-accumulator and hyperaccumulator species to avoid the harmful effects of Se. Non-accumulator plants either lack the enzyme selenocysteine methyltransferase (SMT) or have the SMT enzyme without selenocysteine methyltransferase activity. Therefore, they cannot convert SeCys to MeSeCys. Instead, they reduce the toxicity by converting SeMet into DMSe (Wang et al. 2018, Sors, Martin, and Salt 2009). On the other hand, hyperaccumulator plants metabolize MeSeCys to produce mainly volatile DMDSe (Sors, Ellis, and Salt 2005, Wang et al. 2018).

1.5 Phytoremediation

Soil pollution due to metals and metalloids is different from air or water pollution because metals/metalloids remain in the soil for much longer periods of time than in air or water (Lasat, 2002). Therefore, removal and recovery of metals/metalloids from contaminated soil is important to reduce toxicity and protect ecosystems. A green solution to decontaminate polluted soil/environment is phytoremediation (Sarma 2011).

Phytoremediation is a method which utilizes naturally occurring or genetically engineered plants and associated microbes to recover the area from contamination (Sarma 2011). The use of plants for this purpose has several advantages. Firstly, it is cost effective, efficient and applicable in situ. Secondly, the biological properties and

physical structure of the soil are maintained because plants can clean contaminants without disturbing the composition and fertility of the soil. Indeed, they may increase the quality of the soil by producing organic compounds as a byproduct of natural processes. Moreover, plants protect soil from erosion and metal leaching. Its final advantage is that contaminants with a market value can be extracted by plants and harvested for further purposes (Ali, Khan, and Sajad 2013, Yang et al. 2005).

Selection of the appropriate plant is an important step to increase the effectiveness of a phytoremediation program. Grasses are thought to be the best candidate for phytoremediation because they have high growth rate, high biomass and fibrous root system. Fibrous rooting systems are preferred because they stabilize soil and cover larger soil surface which enhances root and soil contact. In addition, grasses can adapt to extreme conditions (Ali, Khan, and Sajad 2013, Sarma 2011).

1.6 Puccinellia distans

Puccinellia distans (P. distans) belongs to the Poaceae family. It is hexaploid, 2n=42 (Bowden 1961, Edgar 1996). *P. distans* is perennial, monocotyledonous and C3 facultative weeping alkali grass (Tarasoff, Mallory-Smith, and Ball 2007, Hajiboland, Dashtebani, and Aliasgharzad 2015). It is native to Eurasia and distributed throughout Europe, Africa, Asia, Australasia and USA (Barkworth, Capels, and Long 1993, Clayton et al. 2006). *P. distans* is a facultative halophytic plant and can tolerate high salinity levels (Akhzari et al. 2012, Hajiboland, Dashtebani, and Aliasgharzad 2015). Therefore, it can be found by the side of salted roads, marshes, meadows and ruderal areas (Bernhardt and Koch 2003, Bar, Doğanlar, and Frary 2015, Tarasoff, Mallory-Smith, and Ball 2007).

P. distans collected from a boron (B) mining area of Eskişehir, Turkey was identified to be extremely B-tolerant (Bar, Doğanlar, and Frary 2015, Babaoğlu et al. 2004, Stiles et al. 2010). It can tolerate extremely high levels of boron (B) (> 1250 mg B L^{-1}) and has the ability to accumulate approximately 6000 mg B kg^{-1} in its shoots under hydroponic conditions (Bar, Doğanlar, and Frary 2015, Padmanabhan, Babaoglu, and Terry 2012, Stiles et al. 2010). Padmanabhan et al. (2012) suggested that molecular mechanisms related to stress and defense are important for *P. distans* to tolerate boron toxicity. Bar et al. (2015) investigated the difference in protein profile of *P. distans*

control and 500 mg B L^{-1} applied stress groups. They pointed out three candidate proteins that might play roles in boron hyperaccumulation. These proteins were involved in malate synthesis pathway, iron storage and oxidative stress. Recently, Öztürk et al. (2018) elucidated the boron hyperaccumulation and tolerance mechanism using a transcriptomic approach. The results showed that 3312 transcripts of *P. distans* were differentially expressed (67% of the transcripts were up-regulated and 32 % were down-regulated) when it was exposed to 500 mg B L^{-1}. Transcriptomic changes specific to the boron tolerance and hyperaccumulation included genes associated with stress, the cell wall, transporters and plant hormones. The most significant transcriptional differences were observed in the malate pathway, cell wall components, a putative boron transporter and two putative aquaporins.

P. distans produces a large biomass, has a relatively fast growth rate and can tolerate harsh conditions. In addition, *P. distans* is widely distributed throughout the world (Padmanabhan, Babaoglu, and Terry 2012, Stiles et al. 2010). The potential of a US ecotype of *P. distans* for phytorestoration of boron contaminated soil was evaluated by Stiles et al. (2011). They concluded that *P. distans* can be used as initial vegetation to return B contaminated land to its natural state. Moreover, the work by Ghasemi et al. (2017) suggested that *P. distans* could be a suitable plant species for phytoextraction of lead (Pb). In addition, *Puccinellia frigida* which is a close relative of *P. distans* is found to tolerate higher concentrations of boron and proposed to have boron phytoremediation potential (Ramila et al. 2016). Overall, these studies point out that Puccinellia species could be useful candidate for phytoremediation of metals/metalloids.

1.7 Aim of the Study

Plants can show tolerance to several elements and their tolerance relies on similar mechanisms. It is known that some plant species can tolerate high concentrations of soil salts and boron while hyperaccumulating Se in their leaves. Because *P. distans* can withstand high levels of boron and salinity, it was proposed that it can be tolerant to Se. In this study, we would like to understand the Se tolerance capacity of *P. distans* and elucidate its Se tolerance mechanism using a transcriptomic approach. For this purpose, *P. distans* plants were grown hydroponically under high Se conditions. Se accumulation in treated and control plants was quantified by ICP-AES.

Some biochemical analyses were also carried out including the measurement of glutathione reductase, guaiacol peroxidase, and chlorophyll a and b content to determine their responses to Se stress. RNA sequencing was used to detect genes that are differentially expressed under Se stress conditions and the results were validated by qRT-PCR. These genes were annotated to determine their possible roles in accumulation and/or tolerance. Understanding the Se tolerance mechanism of *P. distans* is necessary since it allows us to exploit the plant itself or its genes for phytoremediation and biofortification. For example, the ability of *P. distans* to absorb and sequester Se can be harnessed to clean up excess Se from seleniferous and polluted soils and wastewater. In addition, the Se concentration of food crops can be increased through transfer of genes playing roles in Se hyperaccumulation. Farm animals could then be fed with forage with enhanced Se content. As a result of these processes, the products and the meat of the animals will be rich in Se. In this way, *P. distans* can directly or indirectly provide sufficient dietary Se to individuals in areas where Se deficiency is prevalent.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials and Selenium Treatment

P. distans seeds were obtained from a boron mining site, Kirka-Eskisehir, Turkey (39° 17′ 23.7156" and 30° 31′ 33.4812" (Babaoğlu et al. 2004). They were germinated in perlite for 7 weeks in a growth chamber. Then, they were transferred to full strength Hoagland solution and grown for one week. After that plants were grouped as stress and control and grown for an additional week in full strength Hoagland solution with and without 80 μM Na_2SeO_4, respectively. Selenium treatment was stopped, and plants were rinsed with sterile distilled water. The shoot tissues were frozen in liquid nitrogen and stored at -80°C.

2.2 Methods

2.2.1 RNA Isolation

Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Ontario, Canada) was used for RNA extraction from shoot tissue of control and stress groups. The obtained total RNA was treated with DNase I. Their quality and quantity were measured by a NanodropND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). They were stored at -80^{o}C.

2.2.2 RNA Sequencing and Analysis

Extracted RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with Agilent

TapeStation (Agilent Technologies, Palo Alto, CA, USA). RNA library preparation, sequencing reaction, and bioinformatics analysis were conducted at GENEWIZ, LLC (South Plainfield, NJ, USA).

RNA sequencing library preparation used the NEB Next Ultra RNA Library Prep Kit for Illumina by following the manufacturer's recommendations (NEB, Ipswich, MA, USA). The sequencing libraries were clustered on one lane of a flowcell. After clustering, the flowcell was loaded onto the Illumina HiSeq instrument according to manufacturer's instructions. The samples were sequenced using a 2x150 Paired End (PE) configuration. Image analysis and base calling were conducted by HiSeq Control Software (HCS).

Raw sequence data (.bcl files) generated from Illumina HiSeq were converted into .fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. After NGS analysis of RNA samples, all sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality at the end, using CLC Genomics Server version 9.0.1. After trimming, sequence reads shorter than 50 nucleotides were discarded. De novo transcriptome assembly was conducted with data from all 5 samples using the CLC Genomics Server (Figure 5). A reference genome was created using the assembled transcriptome described above. Using the CLC Genomics server, trimmed sequence reads were mapped to the reference genome for each sequenced sample. Gene read counts were measured and RPKM values were calculated in accordance with the mapping process. Unsupervised hierarchical clustering and principal component analysis were performed to examine sample separation. Comparison of gene expression between various treated samples and the control was conducted using the Wald test. Genes with a False-Discovery-Rate (FDR) < 0.05 and fold-change > 2 were selected as differentially expressed genes.

Annotation, gene ontology mapping of differentially expressed genes and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of the selected transcripts were conducted using Blast2GO (version 4.1.9). Transcripts were first blasted against *A. thaliana* and green plant proteomes separately. These proteomes were generated from the NCBI database and UniProt Knowledgebase, respectively. Hits obtained by the blast search were then mapped to the associated GO terms and transcripts were functionally annotated. Patternscan and HMMPfam searches were conducted on the InterPro database to further increase the quality of functional annotation. KEGG pathway analysis of the selected transcripts which were successfully

annotated was done to discover the role of the up and down-regulated transcripts in enzymatic pathways.

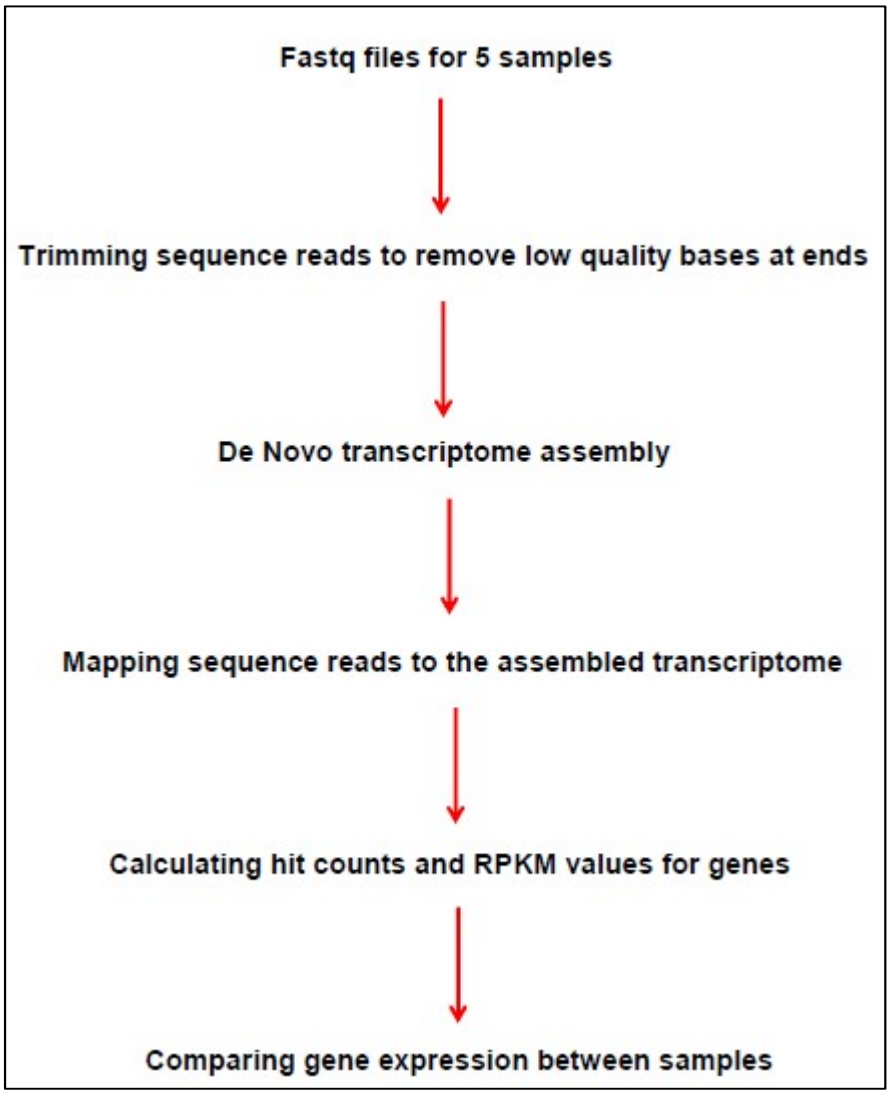

Figure 5. Workflow of RNA-Seq data analysis

2.2.3 Quantitative Real-Time PCR

RT-qPCR was used to confirm the results obtained from RNA-Seq analysis. cDNAs were synthesized from 1 µg extracted total RNA of control and stress groups using GoTaq® 2-Step RT-qPCR kit (PROMEGA). qPCR reactions were carried out in the LightCycler® 480 Instrument II (Roche, Basel, Switzerland). Eight transcripts involved in antioxidant and redox metabolism, sulfur assimilation, transport and four transcripts from the top 50 up and downregulated transcripts were selected for

validation by qPCR. The transcript specific primers were designed using Primer3 software (Untergasser et al. 2012) (Table 4). Three biological and three technical repeats were carried out for both control and stress samples. The expression of actin was used as a reference gene and for normalization. Relative changes in expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 4. RT-qPCR primer sequences of transcripts

Transcript Name	**Forward Primer (5'–3', top)** **Reverse Primer (5'–3', bottom)**
Transcript_112	TTAGACATTGAGCCTGGAAC
	TTTACAGCTCCGAGACAAAA
Transcript_2159	CATTCCCATCTTGTGCTTTC
	TGTGCAGAGTTCATCATCAA
Transcript_2796	ATGACACAAGTTCTGACCTC
	CTTCCGAAGATGACAAATGC
Transcript_3369	CGTACAAAGAACCACTACCT
	GTCAAGTCCATCCAGATCTC
Transcript_6651	GCTGCCAAGTAAATTTCGTT
	TCAACCAAACACACCCTAAT
Transcript_6816	TTATGCCTGGGGAGTTTTAC
	TGGTCGACATGTAACTTGTT
Transcript_25145	CTCAAGGAAGAACACCGATC
	TTCCAATGGCTAGATCACTC
Transcript_27690	GTGGACTGTGCTATGGATAG
	GCTCGCCACTATATGATGAA
Transcript_29272	GACTGAATGCAACTGGAATG
	ATTGGGTATTGGTGTGAGAC
Transcript_39773	GGGAATACTACAGGAAGCAG
	GGATATCTTCATGGCGAACT
Transcript_41354	AAGGCAAAGGAAAGTGAAGA
	TGTCGTATATTATTGCCCGG
Transcript_49788	CAGAGGGTGTTAACAGATCC
	AGTTGTTCTTTCTTCGGGTT
Actin	CCATTGGTGCTGAGCGTTT
	GCTTCCATCCCAATGAAGGA

2.2.4 Determination of Leaf Selenium Content

P. distans (four controls and four Se treated) were grown under the same conditions as the samples used for RNA-Seq. After selenium treatment, the shoots were separated from roots. They were ground with liquid nitrogen and lyophilized for 4 days at -20^{o}C. Elemental Se concentrations of shoot samples were determined using inductively-coupled plasma atomic emission spectroscopy (ICP-AES) in Muğla Sıtkı Koçman University. Measurements were done using three biological and three technical replicates.

2.2.5 Antioxidant Analyses

2.2.5.1 Determination of Glutathione Reductase

Shoot tissue (1 g) was homogenized in 50 mM potassium phosphate buffer (pH=7). Homogenates were centrifuged at 15,000 g for 15 min and supernatant was transferred to a new tube. The supernatant was mixed with a solution containing 50 mM HEPES (pH=8), 0.5 mM EDTA, 20 mM glutathione oxidized and 2.5 mM NADPH (Sgherri, Pinzincp, and Navari-Izzo 1996). After that the absorbance values of samples were measured at 340 nm by Thermo Scientific™ Multiskan™ GO Microplate spectrophotometer using three technical replicates. The enzyme activity was calculated using the formulae defined in the glutathione reductase assay kit.

$$\Delta A_{340}/\text{min} = \frac{|A_{340}\ (\text{Time 2}) - A_{340}\ (\text{Time 1})|}{\text{Time 2 (min)-Time 1 (min)}} \quad (2.1)$$

$$\text{Enzyme activity} = \frac{\Delta A_{340}/\text{min} \times \text{Final volume of assay (ml)} \times \text{Sample dilution}}{\text{NADPH extinction coefficient } (\mu M^{-1}) \times \text{Volume of plant extract (ml)}} \quad (2.2)$$

2.2.5.2 Determination of Guaiacol Peroxidase Activity

Shoot tissue (400 mg) was homogenized in 0.1 M cold sodium phosphate buffer (pH=7). The homogenates were centrifuged at 14,000 rpm for 25 min and the supernatant was transferred to a new tube. Then 0.1 mM sodium phosphate buffer containing 5 mM H_2O_2 and 15 mM guaiacol were mixed with the supernatant (Birecka,

Briber, and Catalfamo 1973). After that the absorbance values of samples were measured at 470 nm by Thermo Scientific™ Multiskan™ GO Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) using three technical replicates. The enzyme activity was calculated using the same formulae defined in section 2.2.5.1.

2.2.6 Chlorophyll Measurement

Shoot tissue (1 mg) was ground to a fine powder by shaking for 1 minute at 30 Hz using TissueLyser II system (Qiagen Inc, USA). To extract chlorophyll, 1.00 ml of methanol was added to the ground sample and the sample was shaken for 2 minutes at 30 Hz. Samples were centrifuged for 2 minutes at 14,000 rpm. (Warren 2008). Supernatant was transferred to a new tube and its absorbance was measured at 652 nm and 665 nm by Thermo Scientific™ Multiskan™ GO Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) using three technical replicates. The chlorophyll a (Chl a) and chlorophyll b (Chl b) concentrations were calculated using the formulae of Ritchie (2006).

$$\text{Chl a } (\mu g/ml) = -8.0962\ A_{652,1\ cm} + 16.5169\ A_{665,1\ cm} \quad (2.3)$$

$$\text{Chl b } (\mu g/ml) = 27.4405\ A_{652,1\ cm} - 12.1688\ A_{665,1\ cm} \quad (2.4)$$

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Tissue Selenium Concentration Determination of *P. distans*

Se concentration in shoot tissue of *P. distans* was significantly increased by the application of increasing selenate concentration ($p < 0.05$) (Figure 6). The average concentration of Se in shoot was 142.5 mg kg^{-1} when plant was treated with 80 µM selenate. This was almost 1500-fold higher than that found in the control group (0.1 mg kg^{-1}). With the application of 120 µM selenate, the average Se concentration difference between control and treated group (254 mg kg^{-1}) reached up to 2500-fold.

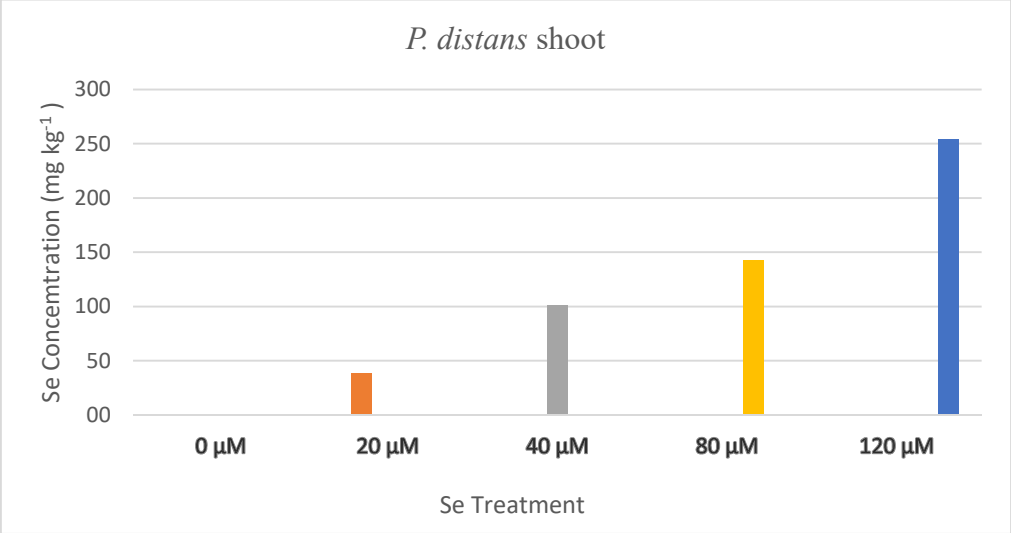

Figure 6. Selenium concentration in shoots of control (0 µM) and treated plants (20, 40, 80 and 120 µM). Values in the graph represent mean of three biological and three technical replicates.

These results indicated that *P. distans* could tolerate and accumulate more than 100 mg Se kg^{-1} dry weight (DW) in its shoots when it was treated with 80 µM and 120 µM. Tolerance was indicated by the ability of the plants to survive under high Se concentration and to continue to grow. Plants that are able to accumulate tissue Se concentrations >100 mg Se kg^{-1} DW are defined as Se accumulators (White 2016).

Therefore, *P. distans* is a novel Se accumulator plant. In the previous study, *S. pinnata* (an extreme Se hyperaccumulator) was grown on agar medium supplemented with 0, 20, 80 or 160 μM of sodium selenate. Then, selenium concentration in the shoots and growth inhibition was detected to understand the plant's tolerance to Se. It was found that *S. pinnata* accumulated 2000 mg Se kg^{-1} DW and did not display any significant growth reduction, thus indicating hypertolerance to Se (Cappa et al. 2015).

3.2 Transcriptome Analysis of *P. distans* using RNA-Seq

3.2.1 De Novo Transcriptome Assembly Analysis

Four cDNA libraries generated from mRNA samples treated with selenium, arsenic, sulfur, nickel and a control shoot sample were used for the investigation of stress response of *P. distans* at the transcriptomic level. Generated libraries were then sequenced using Illumina HiSeq. Illumina HiSeq sequencing resulted in 454 x 10^6 raw reads (Table 5). CLC Genomics server was used to trim the reads from adapter sequences to prevent adapter disruption of the assembly. After trimming, sequence reads shorter than 50 nucleotides were discarded resulting in ~99.5 % of the original reads. De novo transcriptome assembly was conducted with data from all 5 samples. As a result, 78,520 transcripts were assembled (Table 7).

Table 5. Summary statistics of RNA-Seq of *P. distans*

	Count	**Average Length**	**Total bases (bp)**
Reads	454 x 10^6	131.7	59.9 x 10^{10}
Matched	361 x 10^6	129.6	46.7 x 10^{10}
Not Matched	94 x 10^6	140.0	13 x 10^{10}
Contigs	78.5 x 10^3	796.0	62 x 10^6
Reads in pairs	274 x 10^6	112.7	
Broken paired reads	86 x 10^6	139.4	

Maximum and minimum transcript lengths were 404 and 14,460 nt, respectively. The average length was 796 nt. The N50 value was 773 which means that, among the

contigs that represent at least half of the assembled nucleotides, the smallest contig length was 773 nt (Table 6).

Table 6. Summary statistics of transcript measurements

Parameter	Length
N75	621
N50	773
N25	1,084
Minimum	404
Maximum	14,460
Average	796
Count	78,520
Total	62.5×10^7

3.2.2 Gene Expression Analysis

After the assembly of the reference genome using the generated transcriptome, sequence fragments were mapped to the reference genome using CLC Genomics server. Over 64% of the fragments (29.3×10^7) were successfully aligned from 45.4×10^7 total fragments. For the detection of differentially expressed transcripts, mapped fragments were used to calculate reads per kb per million (RPKM) values for each transcript. Comparison of RPKM values of transcripts belonging to control and stress induced samples allowed detection of up and down regulated transcripts.

A total of 3525 transcripts, 4.5% of the total, were differentially expressed in the control versus Se treated plants. Almost half of these differentially expressed transcripts were annotated to genes. The percent of upregulated genes (57.7%) was slightly higher than downregulated genes (42.3%) (Table 7). Gene expression data were visualized with a heatmap (Figure 7). The top 100 up- and downregulated transcripts are listed in Table 8.

Table 7. Summary statistics of the comparison of gene expression, annotation and gene ontology (GO) mapping

	Number	Percentage
Total transcripts	78520	
Differentially expressed transcripts	3525	4.5
Annotated (Gene)	1822	51.7
Annotated (GO Term)	1492	81.9
Upregulated genes	861	57.7
Downregulated genes	631	42.3

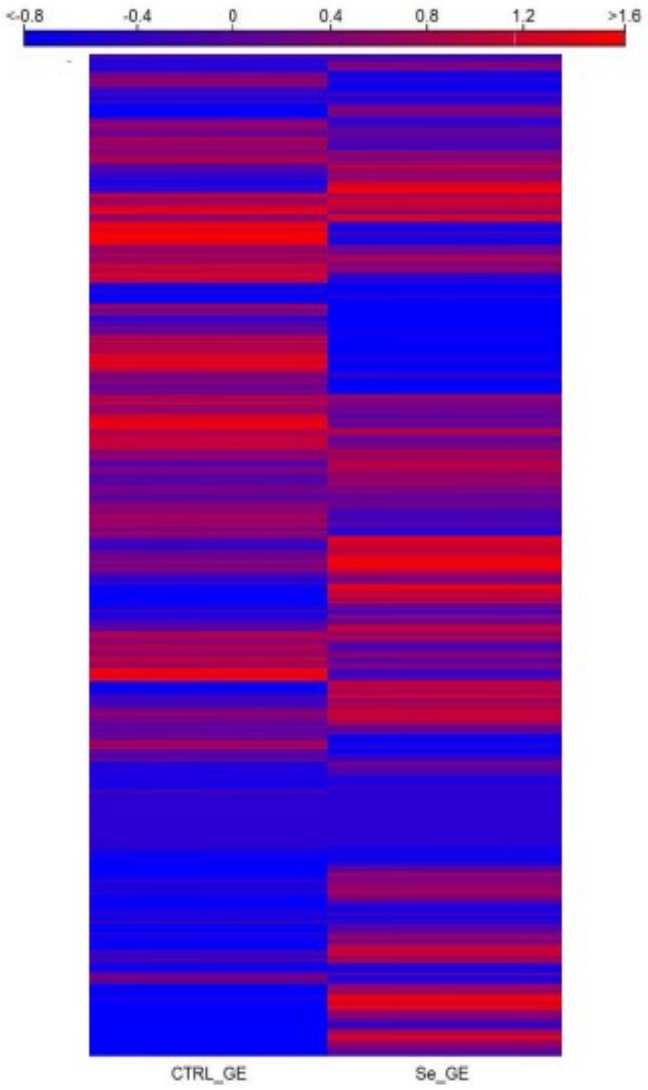

Figure 7. Heat map of log transformed RPKM values of 3525 differentially expressed transcripts from RNA-Seq of control (left) and selenium (right) treated samples. RPKM ranged from - 0.8 (blue) to 1.6 (red).

Table 8. The top 100 differentially expressed transcript in response to 80 µM Se treatment in *P. distans* shoots. Transcripts with a False-Discovery-Rate (FDR) < 0.05 and fold-change > 2 were selected as differentially expressed transcripts. Increased expression is indicated with positive (+) and decreased expression is indicated with negative (-) values.

Increased Expression		
Sequence Name	**Sequence Description**	**Fold Change**
Transcript_504	iaa-leucine resistant 2	407.6
Transcript_5752	A0A0J7YP30_BETVUUncharacterized protein (Fragment)	212.3
Transcript_3369	HSP20-like chaperones superfamily	87.4
Transcript_75950	Cytochrome c oxidase, subunit III	74.9
Transcript_548	heat shock protein 101	71.3
Transcript_10894	HXXXD-type acyl-transferase family	57.9
Transcript_46264	splicing factor Prp18 family protein	53.7
Transcript_6910	N-terminal nucleophile aminohydrolases (Ntn hydrolases) superfamily	52.3
Transcript_2532	senescence regulator (Protein of unknown function, DUF584)	49.9
Transcript_42592	A0A1D5SEC9_WHEATUncharacterized protein	48.1
Transcript_55125	diacylglycerol kinase 5	43.1
Transcript_12213	Peroxidase superfamily protein	36.8
Transcript_22794	C2H2-type zinc finger family	34.0
Transcript_2193	A0A1E5WHH8_9POAL kinase chloroplastic	32.5
Transcript_50309	NP_568842.1phosphofructokinase 7	31.8
Transcript_22653	cytochrome P450, family 94 subfamily B, polypeptide 1	31.8
Transcript_22653	A0A1E5V391_9POALCytochrome P450	31.8
Transcript_8359	BCL-2-associated athanogene 6	31.6
Transcript_330	NAD(P)-linked oxidoreductase superfamily protein	31.3
Transcript_29272	Heat shock protein 70 (Hsp 70) family protein	31.1
Transcript_40561	DHHC-type zinc finger family	30.4
Transcript_6921	UDP-Glycosyltransferase superfamily	27.8
Transcript_2771	NAD(P)-linked oxidoreductase superfamily	27.7
Transcript_33185	F2DPI4_HORVV OS=Hordeum vulgare vulgare PE=2 SV=1	27.6
Transcript_57642	cytokinin oxidase/ dehydrogenase 1	26.9
Transcript_28306	WWE Protein-protein interaction domain family	26.3
Transcript_58547	cytochrome P450, family 79, subfamily B polypeptide 3	24.8
Transcript_54747	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	24.8
Transcript_8872	heat shock protein 70 (Hsp 70) family	24.5
Transcript_26394	FKBP-type peptidyl-prolyl cis-trans isomerase family	24.3

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Table 8. (cont.)

Sequence Name	Sequence Description	Fold Change
Transcript_5062	DNA-directed RNA polymerase subunit beta, putative (DUF630 and DUF632)	24.1
Transcript_25501	MAC/Perforin domain-containing protein	23.7
Transcript_21098	RNI-like superfamily protein	23.5
Transcript_39654	aluminum induced protein with YGL and LRDR motifs	23.3
Transcript_9717	HSP20-like chaperones superfamily protein	22.6
Transcript_194	Chaperone protein htpG family protein	22.3
Transcript_32558	Transducin/WD40 repeat-like superfamily protein	22.0
Transcript_8290	F2DVX4_HORVV OS=Hordeum vulgare vulgare PE=2 SV=1	21.8
Transcript_12155	ubiquitin extension protein 1	21.3
Transcript_16723	PDI-like 1-4	21.3
Transcript_12155	A0A0V0GFL3_SOLCH ovule (Fragment)	21.3
Transcript_17394	Ribosomal protein L34e superfamily protein	21.3
Transcript_13911	Plant VAMP (vesicle-associated membrane protein) family protein	21.3
Transcript_12866	Integrase-type DNA-binding superfamily protein	21.3
Transcript_43776	transferring glycosyl group transferase (DUF604)	21.3
Transcript_17028	Chaperone DNAJ-domain superfamily protein	20.7
Transcript_13028	ADP-ribosylation factor-like A1A	19.8
Transcript_40146	Insulinase (Peptidase family M16) family protein	19.8
Transcript_5986	Heat shock protein 70 (Hsp 70) family protein	19.8
	Decreased expression	
Transcript_1645	A0A0V0GHI9_SOLCH ovule (Fragment)	-6522.6
Transcript_69038	NADH dehydrogenase subunit 4 (mitochondrion)	-172.4
Transcript_77662	glyceraldehyde 3-phosphate dehydrogenase A subunit 2	-146.7
Transcript_196	E1CJ62_9ROSIDehydration responsive (Fragment)	-120.2
Transcript_42210	A0A0F7BID3_9CHLOATP synthase F0 subunit 8	-112.1
Transcript_5999	NADH dehydrogenase subunit 7 (mitochondrion)	-94.1
Transcript_41242	apocytochrome B (mitochondrion)	-86.6
Transcript_57092	NADH dehydrogenase subunit 1 (mitochondrion)	-72.5
Transcript_34746	ribosomal protein S12 (chloroplast)	-54.7
Transcript_11996	ribosomal protein L2 family	-48.2
Transcript_8347	NP_176702.2transmembrane protein, putative (DUF2358)	-41.5
Transcript_51188	Ribosomal protein L16p/ L10e family protein	-41.4
Transcript_21927	NP_568447.1 30S ribosomal protein	-38.2
Transcript_77909	Ribosomal protein L6 family protein	-37.1
Transcript_75360	DEA(D/H)-box RNA helicase family protein	-37.1

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Table 8. (cont.)

Sequence Name	Sequence Description	Fold Change
Transcript_26964	Ribonuclease H-like superfamily protein	-34.8
Transcript_25145	NP_197943.1BURP domain-containing protein	-31.7
Transcript_21789	ribosomal protein L23 (chloroplast)	-31.4
Transcript_41032	P-loop containing nucleoside triphosphate hydrolases superfamily protein	-30.4
Transcript_70837	thymidylate synthase 2	-28.1
Transcript_77657	Peptidase S24/ S26A/ S26B/ S26C family protein	-28.1
Transcript_18958	Ion protease 4	-28.1
Transcript_16491	protein kinase family protein	-27.0
Transcript_61963	Zinc finger, C3HC4 type (RING finger) family protein	-27.0
Transcript_16597	Fatty acid hydroxylase superfamily protein	-24.8
Transcript_35323	myosin-binding protein (Protein of unknown function, DUF593)	-24.8
Transcript_46485	ATP binding cassette protein 1	-24.8
Transcript_71329	Protein kinase superfamily protein	-24.8
Transcript_5304	RIBOSOMAL RNA PROCESSING 5	-24.8
Transcript_6991	from the Czech 'roh' meaning 'corner'	-24.3
Transcript_41382	UDP-Glycosyltransferase superfamily protein	-23.6
Transcript_66092	Trypsin family protein with PDZ domain-containing protein	-23.6
Transcript_41382	A0A1D5ZL17_WHEATGlycosyltransferase	-23.6
Transcript_19467	J3LQ64_ORYBRATP synthase subunit beta	-23.4
Transcript_6764	Ca(2+)-dependent nuclease family protein	-22.5
Transcript_36326	histidine phosphotransfer protein 6	-22.5
Transcript_44155	YELLOW STRIPE like 3	-22.5
Transcript_5533	Protein kinase superfamily protein	-22.5
Transcript_11028	A0A1E5VRX3_9POALPentatricopeptide repeat-containing	-22.5
Transcript_35902	F6IAY1_HORVVNAC transcription factor	-21.4
Transcript_46219	NP_199512.2RNA helicase	-21.4
Transcript_44981	nuclear RNA polymerase D2B	-21.4
Transcript_11203	phosphate transporter 2	-21.4
Transcript_7631	Replication protein A, subunit RPA32	-21.4
Transcript_46283	glucose-induced degradation-like protein	-21.4
Transcript_25932	A0A1E5V7N4_9POAL helicase MAGATAMA 3	-21.4
Transcript_44950	Glycosyl hydrolase family protein	-20.7
Transcript_112	MATE efflux family protein	-20.3
Transcript_40150	heat shock protein 101	-20.3

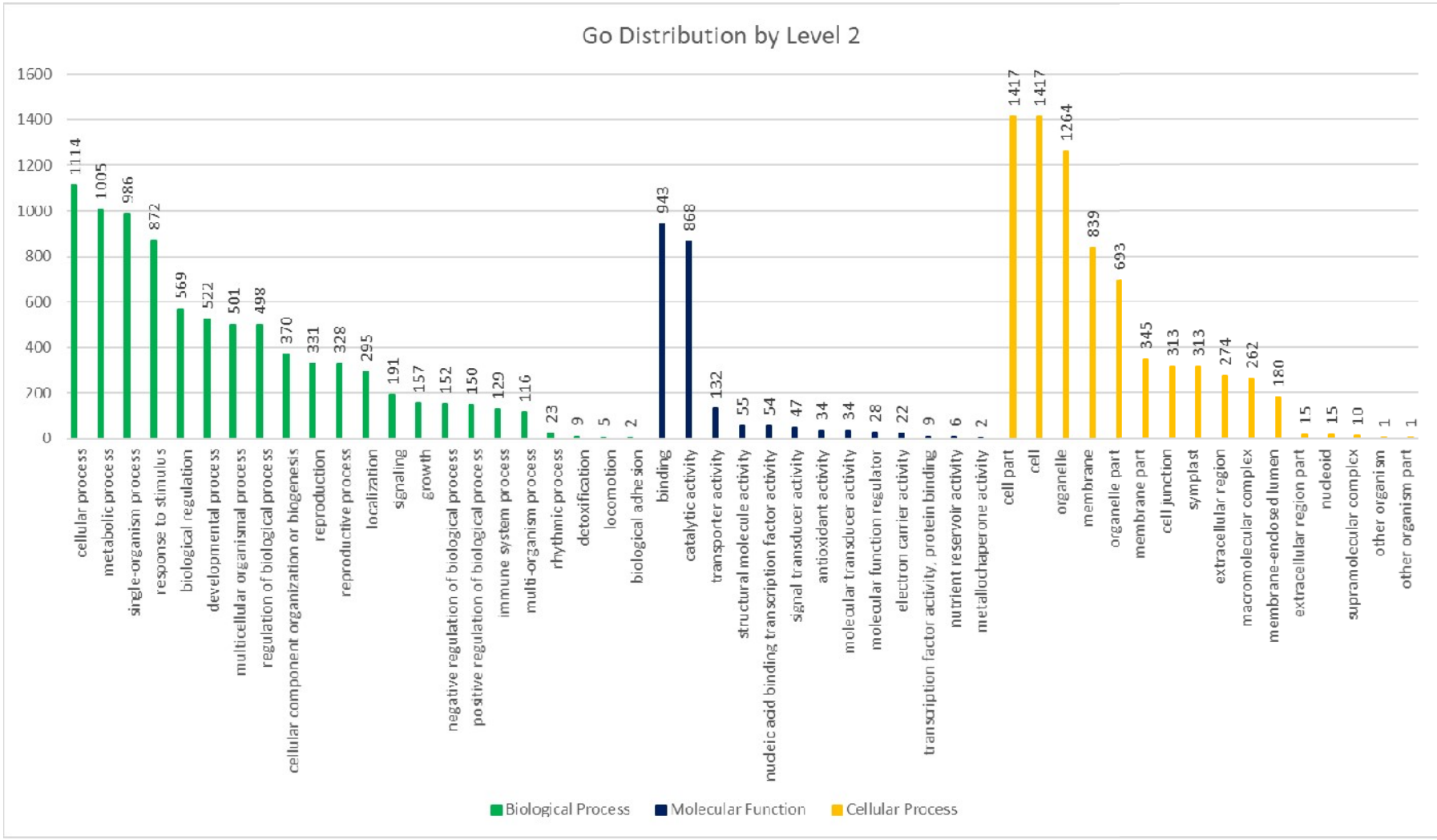

Figure 8. GO annotation level distribution statistics of *P. distans* transcript

3. 3 Gene Ontology Analysis for RNA-Seq data

Blast2Go analysis annotated 1492 transcripts to associated gene ontology (GO) terms (Table 7). Based on the analysis, 1251 (83.8%), 1211 (81.2%) and 1431 (95.9%) transcripts were annotated to three main categories: biological process, molecular function and cellular component, respectively. There were 22 biological process, 13 molecular function and 16 cellular process subcategories (Figure 8)

3.3.1 Biological Process

Twenty-two main terms under biological process were expanded to descendant terms. The descendant terms were sorted based on the percentage of transcripts (Table 9). A total of 17.7% of the top 20 were related to abiotic stress response, 2.2% to developmental process and 2.2% to biotic stress response. Selenium has been suggested to protect plants from abiotic stresses including salinity, water, cold, heat, high light and metal (Feng, Wei, and Tu 2013). Similar to other studies we found that out of 20, thirteen transcripts were associated with abiotic stress responses which were given on table 8. Also, these transcripts comprised 15.1% of the total descendant terms. Some examples of response to abiotic stimulus are salt stress (2.4%) and water deprivation (1.1%) which are similar to each other in terms of osmotic disturbance. This might have resulted from the presence of a high concentration of selenium because metals are known to cause osmotic disturbances and changes in water transport (Rucinska-Sobkowiak 2016). Water deprivation in turn increases reducing power and reactive oxygen species (ROS) production (Tedeschini et al. 2015). Therefore, 2.7% of the transcripts were related to oxidative stress and oxidation-reduction reactions. In addition, the antioxidant capacity of Se is suggested to affect pollen development under water stress (Tedeschini et al. 2015). A similar relationship was observed in the RNA-Seq results because the transcripts related to pollen development (0.6%) and pollen tube growth (0.6%) were altered under Se stress conditions. In addition, a high concentration of Se application resulted in changes similar to those caused by biotic stress due to changes in the transcription of genes with roles in the S assimilation pathway and methionine metabolism. Methionine and selenomethionine are S/Se amino acids and the

precursors of aliphatic glucosinolates. Glucosinolates are involve in plant-pathogen/herbivore interactions. Therefore, 2.2% of the transcripts were associated with defense response to bacteria and fungi.

Table 9. Top 20 descendant terms of biological function

Biological Process GO Term	**Percentage of transcripts (%)**
response to salt stress	2.4
response to cadmium ion	2.1
oxidation-reduction process	1.8
response to cold	1.5
response to cytokinin	1.4
defense response to bacterium	1.2
response to abscisic acid	1.2
response to water deprivation	1.1
response to wounding	1.1
embryo development ending in seed dormancy	1.0
defense response to fungus	1.0
response to oxidative stress	0.9
response to karrikin	0.8
response to heat	0.8
regulation of transcription, DNA-templated	0.8
response to hydrogen peroxide	0.6
response to light stimulus	0.6
response to high light intensity	0.6
pollen development	0.6
pollen tube growth	0.6

3.3.2 Molecular Function

There were 13 subcategories of molecular function according to GO annotation level distribution statistics. These subcategories were further divided into descendant terms. The top 20 descendant terms came from two main molecular function categories: binding (31.1%) and catalytic activity (4.2%) (Table 10). The transcripts functions in binding were related to protein, ATP, metal, RNA, DNA and protein binding. A total of 7.3% of the top 20 descendant terms were directly related to transcription and translation processes. These terms included mRNA binding, DNA binding, transcription factor activity, sequence-specific DNA binding, structural constituent of ribosome,

sequence specific DNA binding and RNA binding. In addition, Se treatment resulted in changes in the expression level of transcripts involved in metal binding. This constituted 7.4% of the total and included the terms: zinc ion, metal ion, copper and iron binding.

Table 10. Top 20 descendant terms of molecular function

Molecular Function GO Term	Percentage of transcripts (%)
Protein binding	8.4
ATP binding	5.1
Zinc ion binding	3.1
Metal ion binding	2.1
mRNA binding	1.9
Heme binding	1.4
Copper ion binding	1.4
Protein homodimerization activity	1.3
DNA binding	1.3
Transcription factor activity, sequence-specific DNA binding	1.3
Protein serine/threonine kinase activity	1.1
Structural constituent of ribosome	1.1
Sequence-specific DNA binding	1.1
Identical protein binding	1.0
Iron ion binding	0.9
Calmodulin binding	0.8
Unfolded protein binding	0.8
Kinase activity	0.8
RNA binding	0.7

3.3.3 Cellular Components

Sixteen subcategories of cellular components were further divided into descendant terms and the top 20 are listed (Table 11). According to this analysis, 3919 transcripts were associated with at least one cellular component. In addition, 1433 of the total transcripts (26.5%) were observed to be related with plasma membrane, chloroplast and vacuole associated GO-terms. This was an expected result since selenate/sulfate is taken up through the plasma membrane and transported across the chloroplast envelope for its reduction in the chloroplast. Also, excess selenate/sulfate could be stored in the vacuole as part of the metal tolerance mechanism.

Table 11. Top 20 descendant terms of cellular component

Cellular component GO Term	Percentage of transcripts (%)
plasma membrane	8.1
cytosol	7.0
nucleus	6.5
plasmodesma	5.7
chloroplast	4.8
mitochondrion	4.6
chloroplast stroma	3.5
Golgi apparatus	3.4
integral component of membrane	3.4
vacuolar membrane	3.4
chloroplast envelope	3.1
apoplast	2.8
cell wall	2.4
nucleolus	2.3
endoplasmic reticulum	2.2
extracellular region	2.0
vacuole	1.8
chloroplast thylakoid membrane	1.7
membrane	1.7
plant-type cell wall	1.6

3.4 KEGG Pathway Analysis for RNA-Seq data

A total of 1357 differentially expressed genes had matches in the KEGG database. The genes were associated with 715 enzymes and 121 KEGG pathways (Table 12). In some cases, more than one transcript were connected to the same enzyme. This might have been due to the presence of sequences encoding different portions of a single transcript or isoforms of the same protein (Hyun et al. 2012, Wang et al. 2013). The enzymes were further categorized into six groups based on their Enzyme Commission (EC) numbers (Figure 9). Hydrolases (34.9%), transferases (26.9%) and oxidoreductases (22.7%) were the dominant groups. The most represented main KEGG pathway was metabolic pathway with 1313 transcripts (95.9%). The metabolic pathways included 11 different subcategories. Of these categories, 274 (20.9%) transcripts were involved in carbon metabolism, 160 (12.2%) in metabolism of cofactors and vitamins, 145 (11%) in nucleotide metabolism, 142 (10.8%) in amino acid

metabolism, 128 (9.7%) in xenobiotics biodegradation and metabolism, 127 (9.7%) in lipid metabolism and 91 (6.9%) in energy metabolism subcategories. The remaining 246 (18.8%) transcripts were associated with other metabolism subcategories.

Table 12. Pathway assignment of differentially expressed genes based on the KEGG pathway database

Pathway ID	Pathways	#Sequences	#Enzymes
01130	Biosynthesis of antibiotics	108	80
00010	Glycolysis / Gluconeogenesis	47	22
00500	Starch and sucrose metabolism	19	17
00520	Amino sugar and nucleotide sugar metabolism	17	13
00620	Pyruvate metabolism	17	11
00051	Fructose and mannose metabolism	15	10
00630	Glyoxylate and dicarboxylate metabolism	13	10
00052	Galactose metabolism	13	10
00650	Butanoate metabolism	12	10
00562	Inositol phosphate metabolism	10	9
00030	Pentose phosphate pathway	18	8
00020	Citrate cycle (TCA cycle)	10	8
00053	Ascorbate and aldarate metabolism	13	7
00040	Pentose and glucuronate interconversions	13	6
00640	Propanoate metabolism	9	5
00521	Streptomycin biosynthesis	6	5
00960	Tropane,piperidine and pyridine alkaloid biosynthesis	5	5
00941	Flavonoid biosynthesis	7	4
00950	Isoquinoline alkaloid biosynthesis	5	4
00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	4	4
00401	Novobiocin biosynthesis	3	4
00944	Flavone and flavonol biosynthesis	7	2
00524	Neomycin, kanamycin and gentamicin biosynthesis	1	2
00525	Acarbose and validamycin biosynthesis	2	1
00402	Benzoxazinoid biosynthesis	1	1
00232	Caffeine metabolism	1	1
00261	Monobactam biosynthesis	1	1
00405	Phenazine biosynthesis	1	1
00901	Indole alkaloid biosynthesis	1	1
00660	C5-Branched dibasic acid metabolism	1	1
00966	Glucosinolate biosynthesis	1	1
00332	Carbapenem biosynthesis	1	1
00710	Carbon fixation in photosynthetic organisms	23	13
00920	Sulfur metabolism	11	10
00680	Methane metabolism	18	9

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Table 12 (cont.)

Pathway ID	Pathways	#Sequences	#Enzymes
00910	Nitrogen metabolism	8	9
00720	Carbon fixation pathways in prokaryotes	13	7
00190	Oxidative phosphorylation	17	6
00195	Photosynthesis	1	1
00561	Glycerolipid metabolism	23	13
00071	Fatty acid degradation	18	12
00564	Glycerophospholipid metabolism	11	10
00592	Alpha-Linolenic acid metabolism	19	9
00062	Fatty acid elongation	8	7
01040	Biosynthesis of unsaturated fatty acids	8	6
00100	Steroid biosynthesis	4	6
00590	Arachidonic acid metabolism	8	4
00600	Sphingolipid metabolism	5	4
00565	Ether lipid metabolism	3	3
00073	Cutin, suberine and wax biosynthesis	5	2
00591	Linoleic acid metabolism	4	2
00061	Fatty acid biosynthesis	4	2
00072	Synthesis and degradation of ketone bodies	2	2
00120	Primary bile acid biosynthesis	3	1
00140	Steroid hormone biosynthesis	2	1
00230	Purine metabolism	128	17
00240	Pyrimidine metabolism	17	10
00270	Cysteine and methionine metabolism	16	19
00400	Phenylalanine, tyrosine and tryptophan biosynthesis	11	16
00360	Phenylalanine metabolism	18	12
00250	Alanine, aspartate and glutamate metabolism	11	10
00350	Tyrosine metabolism	14	9
00260	Glycine, serine and threonine metabolism	14	9
00380	Tryptophan metabolism	12	9
00280	Valine, leucine and isoleucine degradation	10	8
00330	Arginine and proline metabolism	9	7
00220	Arginine biosynthesis	8	6
00310	Lysine degradation	9	5
00290	Valine, leucine and isoleucine biosynthesis	3	3
00340	Histidine metabolism	4	2
00300	Lysine biosynthesis	3	1
00480	Glutathione metabolism	16	11
00460	Cyanoamino acid metabolism	7	5
00450	Selenocompound metabolism	9	4
00410	Beta-Alanine metabolism	8	3
00430	Taurine and hypotaurine metabolism	2	1

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Table 12 (cont.)

Pathway ID	Pathways	#Sequences	#Enzymes
00471	D-Glutamine and D-glutamate metabolism	2	1
00440	Phosphonate and phosphinate metabolism	1	1
00511	Other glycan degradation	3	3
00603	Glycosphingolipid biosynthesis -globo and isoglobo series	2	1
00604	Glycosphingolipid biosynthesis- ganglio series	1	1
00510	N-Glycan biosynthesis	1	1
00531	Glycosaminoglycan degradation	1	1
00740	Riboflavin metabolism	11	6
00860	Porphyrin and chlorophyll metabolism	7	6
00830	Retinol metabolism	12	4
00130	Ubiquinone and other terpenoid-quinone biosynthesis	7	4
00770	Pantothenate and CoA biosynthesis	4	4
00730	Thiamine metabolism	108	3
00760	Nicotinate and nicotinamide metabolism	4	3
00750	Vitamin B6 metabolism	4	3
00790	Folate biosynthesis	3	2
00900	Terpenoid backbone biosynthesis	8	12
00281	Geraniol degradation	6	4
00906	Carotenoid biosynthesis	5	4
00908	Zeatin biosynthesis	6	3
00902	Monoterpenoid biosynthesis	2	3
00903	Limonene and pinene degradation	6	2
00981	Insect hormone biosynthesis	3	1
00523	Polyketide sugar unit biosynthesis	2	1
01055	Biosynthesis of vancomycin group antibiotics	2	1
00904	Diterpenoid biosynthesis	1	1
00940	Phenylpropanoid biosynthesis	44	15
00983	Drug metabolism-other enzymes	24	7
00362	Benzoate degradation	7	6
00627	Aminobenzoate degradation	36	4
00930	Caprolactam degradation	12	4
00982	Drug metabolism- cytochrome P450	12	3
00980	Metabolism of xenobiotics by cytochrome P450	12	3
00625	Chloroalkane and chloroalkene degradation	9	3
00623	Toluene degradation	5	2
00643	Styrene degradation	2	2
00626	Naphthalene degradation	5	1
00622	Xylene degradation	2	1
00984	Steroid degradation	1	1
00642	Ethylbenzene degradation	1	1
00970	Aminoacyl-tRNA biosynthesis	3	3

(cont. on the next page)

Table 12 (cont.)

Pathway ID	Pathways	#Sequences	#Enzymes
04070	Phosphatidylinositol signaling system	9	8
04150	mTOR signaling pathway	2	1
04660	T cell receptor signaling pathway	16	2
04658	Th1 and Th2 cell differentiation	14	1

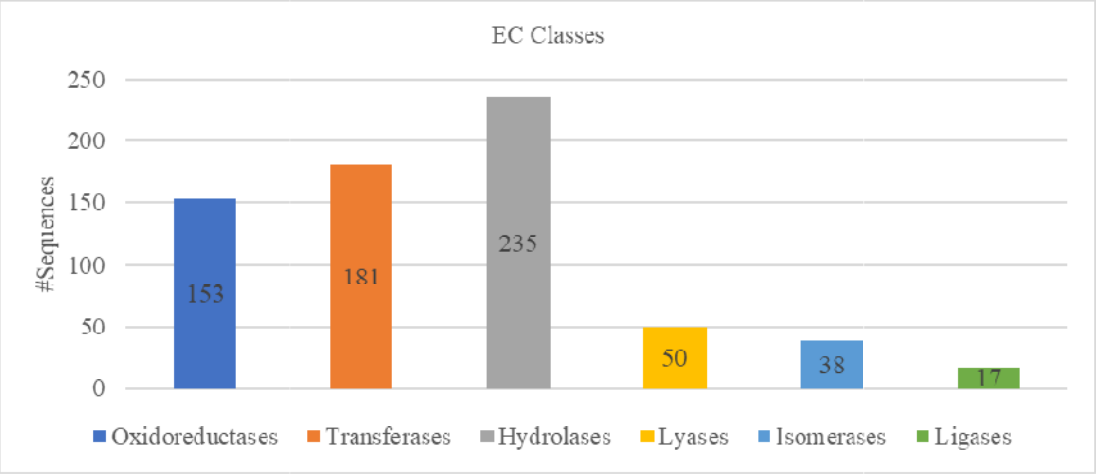

Figure 9. Enzyme classification based on KEGG pathway analysis

3.5 Sulfate Metabolism

Eight differentially expressed transcripts were found to encode eight enzymes in sulfur metabolism based on the KEGG pathway (Figure 10). Seven transcripts were upregulated and one transcript was downregulated. In addition, adenylylsulfate reductase (aprA, EC 1.8.99.2) and phosphoadenosine phosphosulfate reductase (cysH, EC 1.8.4.8) were encoded by the same transcript. Therefore, their expression levels could not be assigned and are, therefore marked with yellow in the figure.

After Se is transported into shoots, it is either stored in vacuoles or moves to plastids for further reductive assimilation (Ramos et al. 2011). The first and rate limiting step is the activation of selenate to adenosine 5'- phosphoselenate (APSe) by ATP sulfurylase (or ATP:sulfate adenylyl transferase; APS)(Pilon-Smits et al. 1999, Ramos et al. 2011, Sors, Ellis, and Salt 2005). A previous study showed that when the *Aps1* gene is overexpressed in *Brassica juncea*, plants accumulated 1.5 to 2-fold more Se in their shoots and reduced selenate at increased rates (Pilon-Smits et al. 1999). Also,

plants were tolerant to Se and performed better. *APS1* was expressed at 4.3-fold higher levels under selenium stress in this study as expected. The next and second rate limiting step in selenate assimilation is the formation of selenite. In this step, APS reductase (APR) reduces activated selenate into selenite. The expression of *APR1* in *P. distans* was 2.3-fold higher in selenate treated plants than in the control group. The third step is carried out by sulfite reductase (SIR) which reduces selenite to selenide. *SIR* had a 4.5-fold increased expression under Se-stress in our study.

According to our findings the expression of both sulfite reductase and serine O-acetyltransferase were upregulated, the expression of cysteine synthase was downregulated. Upregulation of sulfite reductase causes production of excess sulfide. The presence of excess sulfide prevents dissociation of O-acetylserine (thiol)-lyase with serine O-acetlytransferase. Association of serine O-acetlytransferase with O-acetylserine (thiol)-lyase reduces the activity of O-acetylserine (thiol)-lyase (Feldman-Salit et al. 2009). Therefore, there was a decrease in the conversion of selenide/sulfide to selenocysteine/cysteine. These results indicated that *P. distans* may have tried to decrease the production of selenocysteine to keep Se away from proteins.

3.6 Cell Wall Metabolism

The cell wall is an important part of metal stress tolerance and it is required for stress sensing and signal transduction. Lignification of cell wall is one part of this tolerance system. Production of lignin decreases the flexibility of the cell wall. Therefore, the cell wall become stronger and more rigid. As mentioned before, metal stress causes water deprivation due to osmotic disturbance. Lignification helps cells to retain water inside the cytoplasm even under low water potential because it makes the cell wall less permeable to water. In addition, lignin is composed of various functional groups.

Thus, it can bind keep multiple metal ions ($Cu^{2+,}$ Cd^{2+}, Pb^{2+}, etc.) and decreases transport of metals into the vascular bundles (Liu, Luo, and Zheng 2018, Luisa et al. 2004). Moreover, it was shown that As (V) and Cu treatment led to upregulation of the genes mentioned below. Upregulation of lignin biosynthesis enzymes were found to protect plants against adverse effect of metals (Chandran et al. 2008, Ali et al. 2006).

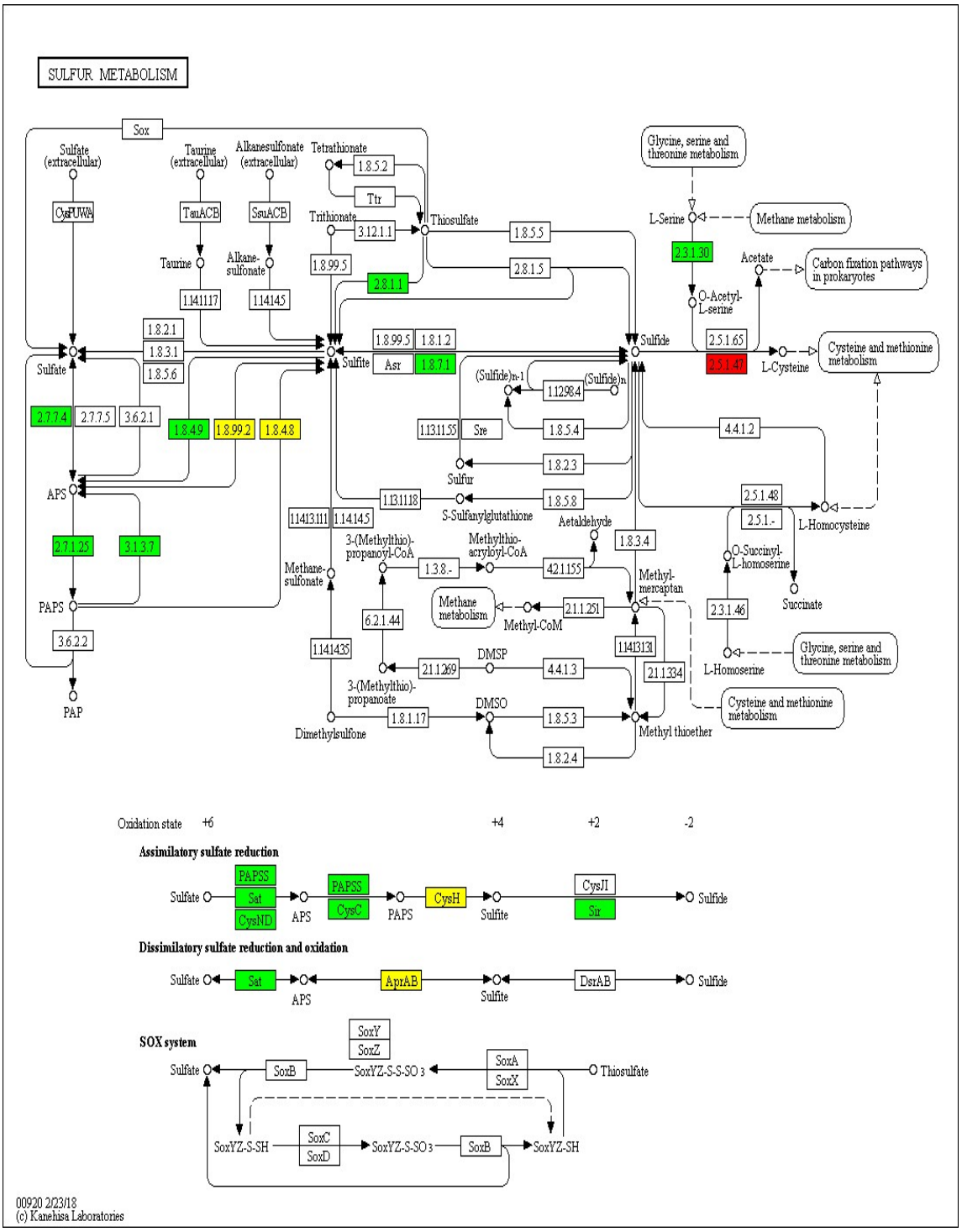

Figure 10. Expression pattern of transcripts involved in sulfur metabolism (KEGG pathway: map00920). The green and red color correspond to up- and downregulated transcripts, respectively. The yellow color represents the transcripts whose expression level could not be determined. Adenylyl-sulfate reductase (EC 1.8.4.9), sulfite reductase (EC 1.8.7.1), serine O-acetyltransferase (EC 2.3.1.30), 3'-phosphoadenosine 5'-phosphosulfate synthase (EC 2.7.7.4 and EC 2.7.1.25), thiosulfate/3-mercaptopyruvate sulfurtransferase (EC 2.8.1.1), 3'(2'), 5'-bisphosphate nucleotidase (EC 3.1.3.7), O-acetylserine (thiol)-lyase (EC 2.5.1.47), adenylylsulfate

reductase (EC 1.8.99.2) and phosphoadenosine phosphosulfate reductase (EC 1.8.4.8)

RNA Seq analysis in this study showed that Se had a positive effect on lignin production because in addition to guaiacol peroxidase, the expression of transcripts encoding laccase, cinnamoyl-CoA reductase (CCR), phenylalanine ammonia-lyase (PAL), hydroxycinnamyl alchocol dehydrogenase (CAD) and 4 coumarate CoA-ligase (4CL) were upregulated (Figure 11 and Table 13).

The results suggested that *P. distans* may have tolerated Se and protected itself from the adverse of Se by trapping it in the cell wall and restricting its movement across the cell membrane. To understand whether Se binds to cell wall or not, the selenium content of a whole cell and protoplast can be compared. Reduction in the selenium content in protoplasts compared to whole cell indicates that some Se is trapped in the cell wall before it enters inside the cell. Also, cellular fractionation and electrothermal atomic absorption spectrometry can be used to find relative contribution of Se in the cell wall. The next step should be to find the candidate molecules that Se can interact with. In the previous studies with yeasts, it was suggested that polysaccharides (especially mannan) could be a good candidate molecule (Kieliszek et al. 2015).

3.7 RT-qPCR Results of Transcript Analysis

A total of 50 transcripts were chosen for the next step in this study. Among them, 20 were eliminated because either we could not obtain any amplification from PCR or the size of the amplicon was not the same as it should have been. RT-qPCR were carried out with the remaining primers. However, some primers were found to bind nonspecifically to random sequences. The remaining 12 transcripts were used for verification of RNA-Seq analysis. The up- and downregulated transcripts are shown in Figure 12.

Transcript_112 was found to be downregulated by 20% according to RT-qPCR. This result is consistent with the RNA-Seq results (-20.3-fold). Transcript_112 encoded MATE-efflux transporter which is responsible for sequestration of flavonoids from cytoplasm to vacuoles (Eckardt 2001). Plant decreased the expression of this transporter because it needed flavonoids in the cytoplasm to protect itself from oxidative stress.

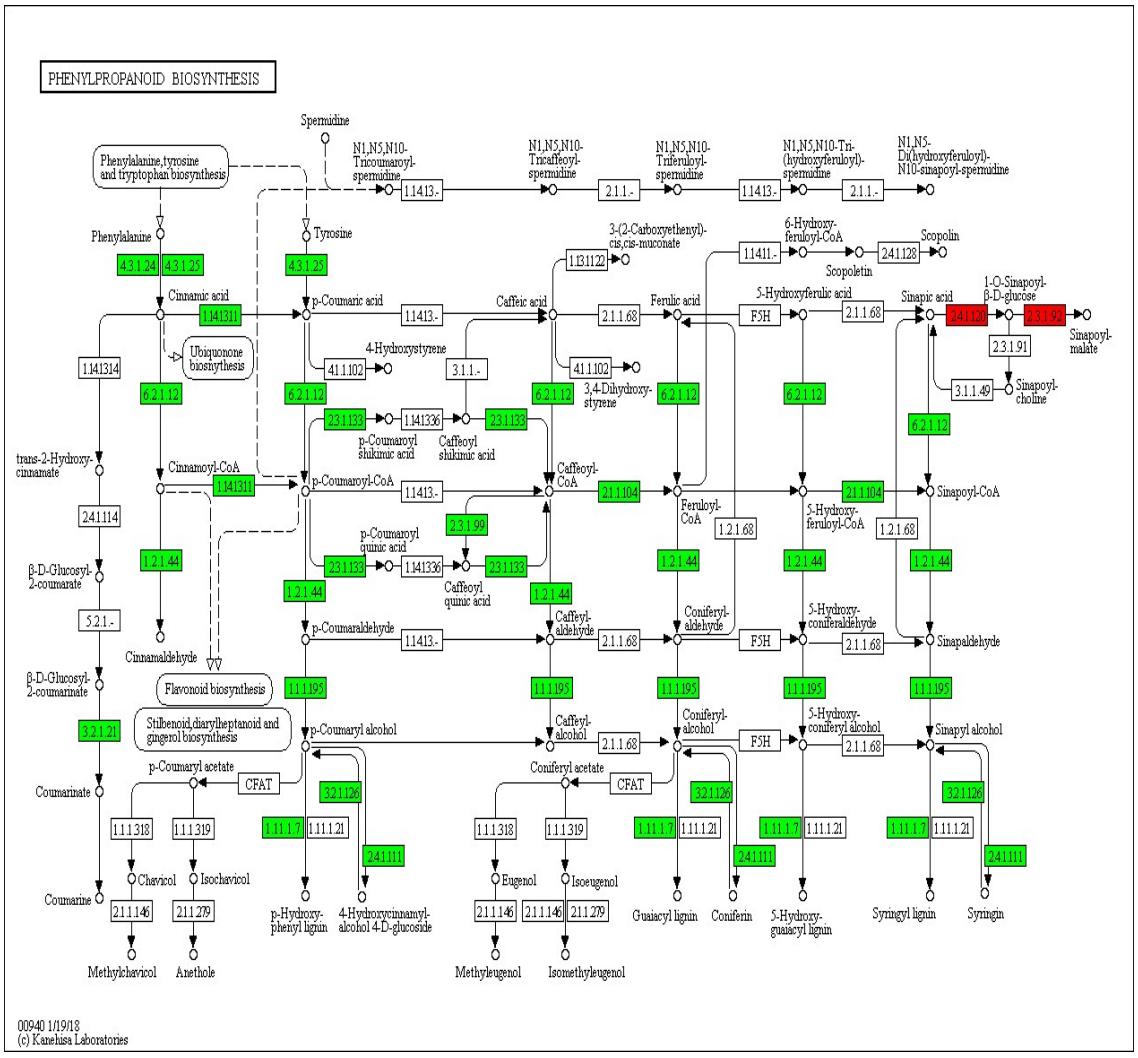

Figure 11. Expression pattern of transcripts involved in phenylpropanoid biosynthesis (KEGG pathway: map00940). The green and red color correspond to up- and downregulated transcripts, respectively. Cinnamyl-alcohol dehydrogenase (EC: 1.1.1.195), cinnamoyl-CoA reductase (EC: 1.2.1.44), guaiacol peroxidase (EC: 1.11.1.7), trans-cinnamate 4-monooxygenase (EC: 1.14.13.11), caffeoyl-CoA O-methyltransferase (EC: 2.1.1.104), shikimate O-hydroxycinnamoyltransferase (EC: 2.3.1.133), coniferyl-alcohol glucosyltransferase (EC: 2.4.1.111), sinapate 1-glucosyltransferase (EC: 2.4.1.120), beta-glucosidase (EC: 3.2.1.21), coniferin beta-glucosidase (EC: 3.2.1.126), phenylalanine ammonia-lyase (EC: 4.3.1.24), phenylalanine/tyrosine ammonia-lyase (EC: 4.3.1.25), 4-coumarate--CoA ligase (EC: 6.2.1.12).

Table 13. Differentially expressed transcripts and corresponding enzyme names in phenylpropanoid biosynthesis in response to 80 μM Se treatment in *P. distans* shoots. Increased expression is indicated with positive (+) and decreased expression is indicated with negative (-) values.

Transcript Name	Fold Change	Enzyme Names	Enzyme Code
Transcript_17899	4.4	Guaiacol peroxidase	1.11.1.7
Transcript_5701	2.3	Guaiacol peroxidase	1.11.1.7
Transcript_21544	-5.6	Guaiacol peroxidase	1.11.1.7
Transcript_517	3.6	Guaiacol peroxidase	1.11.1.7
Transcript_12213	36.8	Guaiacol peroxidase	1.11.1.7
Transcript_14010	2.8	Guaiacol peroxidase	1.11.1.7
Transcript_33513	4.9	Cinnamyl-alcohol dehydrogenase	1.1.1.195
Transcript_26093	8.7	Cinnamyl-alcohol dehydrogenase	1.1.1.195
Transcript_11687	4.4	Cinnamyl-alcohol dehydrogenase	1.1.1.195
Transcript_1326	3.6	Cinnamyl-alcohol dehydrogenase	1.1.1.195
Transcript_74757	-8.5	Serine carboxypeptidase-like 8	2.3.1.92
Transcript_31560	5.5	Quinate O-hydroxycinnamoyltransferase;	2.3.1.99
Transcript_19349	18.8	Quinate O-hydroxycinnamoyltransferase;	2.3.1.99
Transcript_6806	10.3	Cinnamoyl-CoA reductase	1.2.1.44
Transcript_26093	8.7	Cinnamoyl-CoA reductase	1.2.1.44
Transcript_33895	-3.2	Cinnamoyl-CoA reductase	1.2.1.44
Transcript_8587	2.7	Trans-cinnamate 4-monooxygenase	1.14.13.11
Transcript_12622	10.8	Coniferyl-alcohol glucosyltransferase	2.4.1.111
Transcript_6921	27.8	Coniferyl-alcohol glucosyltransferase	2.4.1.111
Transcript_6418	3.7	Coniferyl-alcohol glucosyltransferase	2.4.1.111
Transcript_31560	5.5	Shikimate O-hydroxycinnamoyltransferase	2.3.1.133
Transcript_19349	18.8	Shikimate O-hydroxycinnamoyltransferase	2.3.1.133
Transcript_41382	-23.6	Sinapate 1-glucosyltransferase	2.4.1.120
Transcript_7083	6.3	4-coumarate--CoA ligase	6.2.1.12
Transcript_10541	5.8	4-coumarate--CoA ligase	6.2.1.12
Transcript_11819	5.6	Caffeoyl-CoA O-methyltransferase	2.1.1.104
Transcript_12179	2.6	Phenylalanine ammonia-lyase	4.3.1.24
Transcript_3895	2.5	Phenylalanine ammonia-lyase	4.3.1.24
Transcript_3794	2.0	Phenylalanine ammonia-lyase	4.3.1.24
Transcript_4628	2.3	Phenylalanine ammonia-lyase	4.3.1.24
Transcript_9564	-4.2	Phenylalanine ammonia-lyase	4.3.1.24
Transcript_12179	2.6	Phenylalanine ammonia-lyase	4.3.1.25
Transcript_3895	2.5	Phenylalanine ammonia-lyase	4.3.1.25
Transcript_3794	2.0	Phenylalanine ammonia-lyase	4.3.1.25
Transcript_4628	2.3	Phenylalanine ammonia-lyase	4.3.1.25

(cont. on the next page)

Table 13 (cont.)

Transcript Name	Fold Change	Enzyme Names	Enzyme Code
Transcript_9564	-4.2	Phenylalanine ammonia-lyase	4.3.1.25
Transcript_42464	5.4	Coniferin beta-glucosidase	3.2.1.126
Transcript_18491	8.3	Beta-glucosidase	3.2.1.21
Transcript_42464	5.4	Beta-glucosidase	3.2.1.21
Transcript_22869	-2.8	Beta-glucosidase	3.2.1.21

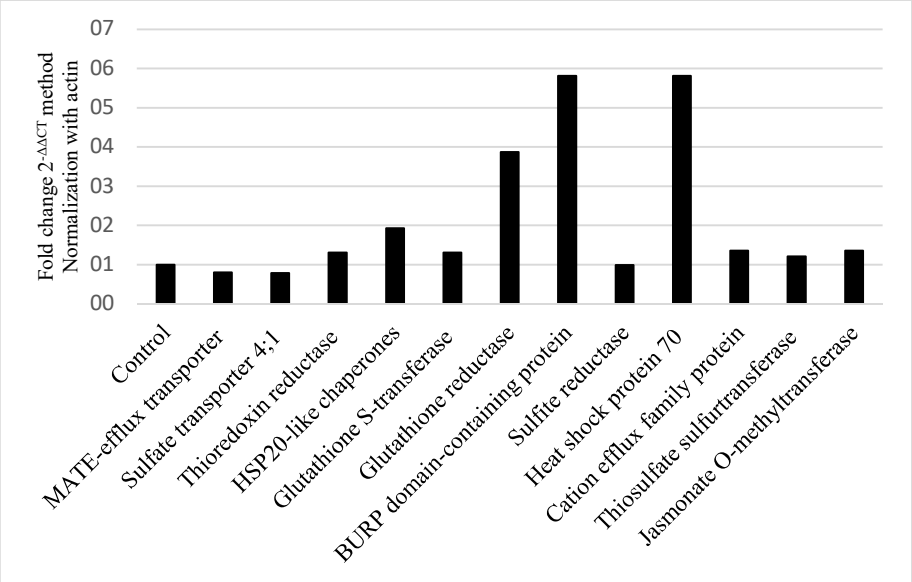

Figure 12. RT-qPCR results of 12 selected transcripts.

Transcript_2159 encodes the sulfate transporter 4;1 (SULTR4;1) which is responsible for export of sulfate/selenate from vacuoles. qRT-PCR result showed that the expression of transcript_2159 was decreased 30% in Se-treated plants compared to control.

The expression of two transcripts involved in glutathione metabolism were increased. Glutathione S-transferase (GST, EC 2.5.1.18) encoded by transcript_6651 increased 30% compared to control group. Similarly, increased expression (3.2-fold) of transcript_6651 was also obtained with RNA-Seq analysis. GST has a role in the transfer of selanate ion to glutathione to make R-S-glutathione. The chelation step of selenate with glutathione is necessary because it helps selenate to be transported into the vacuole through ABC transporter (a.k.a glutathione S-conjugate-transporting ATPase 4) (Zhou et al. 2018). Here, the expression of three transcripts encoding ABCC.4 were

found to be upregulated according to RNA-Seq analysis (Transcript 3063, 2.3-fold; transcript 53629, 3.2-fold and transcript 33226, 6.15-fold).

Downregulation of SULTR4;1 and upregulation of GST and ABCC.4 expression indicated that the plant tended to sequester selenate inside the vacuoles to accumulate Se (Figure 13). It may also have been a protective mechanism of *P. distans* against the adverse effects of Se.

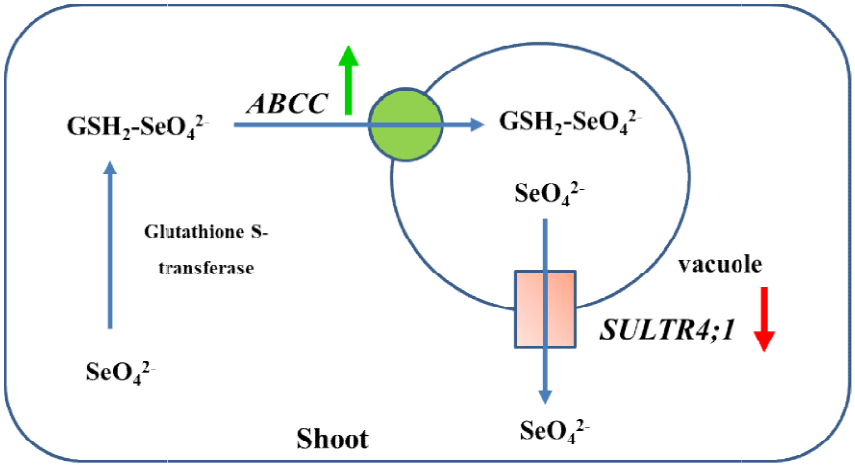

Figure 13. Mechanism for the selenium sequestration in vacuole. The green arrow indicates the upregulation and red arrow indicates the downregulation of gene expression.

Hsp70 and Hsp20 are molecular chaperones located in the cytosol. Hsp70 blocks accumulation of proteins and directs them to fold in the correct way. Hsp20 keeps proteins in a competent state for folding. Hsp20 also helps them to disaggregate through the Hsp70/90 chaperone system. Therefore, Hsp20 and Hps70 work in cooperation to protect plants from the effect of stress (Park and Seo 2015, Al-Whaibi 2011). The expression of heat shock protein Hsp70 and Hsp20 were upregulated 31.1 and 87.4-fold, respectively based on the RNA-Seq analysis. Based on RT-PCR, we found that the expression of Hsp70 (transcript_29272) and Hsp20 (transcript_3369) were increased 5.8 and 1.9-fold, respectively. Thus, RT-qPCR results showed a similar trend with the RNA-Seq results. In addition to these, 25 transcripts were found to encode heat shock proteins and their expression was upregulated according to RNA-Seq analysis. These findings suggested that *P. distans* was able to cope with the adverse effect of Se on protein folding and disaggregation through activating the molecular chaperone systems.

Se stress-induced ROS stimulate the same signaling pathway that is triggered in response to pathogens. These cascades of events in the signaling pathway lead to jasmonate (JA) production (Tamaoki, Freeman, and Pilon-Smits 2008). JA is important for Se tolerance because JA signaling has been reported to positively regulate sulfur and antioxidant metabolism and increase Se resistance. After jasmonate is synthesized, it is converted to methyl-jasmonate (MeJA) by S-adenosyl-L methionine-dependent methyltransferase (a.k.a jasmonate O-methyltransferase) (Seo et al. 2001). MeJA is a volatile compound and shown to be constitutively present in high amount in the Se hyperaccumulator *S. pinnata* (Pilon-Smits, Winkel, and Lin 2017). It can diffuse through the membrane and act as an inter- and intracellular regulator. Therefore, it is a good signal transducer candidate to control jasmonate responsive plant responses. In this study, an increase in the expression of transcript_49788 (13.9-fold) encoding S-adenosyl-L methionine-dependent methyltransferase was observed and verified with RT-qPCR.

The elevated expression of JA biosynthesis genes encoding eight different enzymes were also found in our transcriptome analysis (Table 14 and Figure 13). The key enzymes of JA biosynthesis (acyl-CoA oxidase and linoleate 13S-lipoxygenase) were two of the upregulated biosynthesis genes. Similarly, previous studies showed that the genes encoding these enzymes were constitutively upregulated in *S. pinnata* compared to non-accumulator relatives (Pilon-Smits, Winkel, and Lin 2017, Wang et al. 2018, Tamaoki, Freeman, and Pilon-Smits 2008).

Transcript_41354 encoding enzyme thiosulfate sulfurtransferase (EC: 2.8.1.1) was upregulated by 8.9-fold. This enzyme catalyzes the conversion reaction of thiosulfate to sulfite. Sulfite is an important component of the sulfur assimilation metabolism. Therefore, the expression of this transcript was checked with RT-qPCR. An increase (20%) in expression was confirmed.

In this study, it was observed that the expression of transcript_2796 encoding thioredoxin reductase was upregulated in Se treated plants compared to control based on both the RNA-Seq (10.56-fold) and RT-PCR (30%) results. Thioredoxin reductase converts selenite to hydrogen selenide (Figure 14). Afterwards, hydrogen selenide is used as a reactant to produce selenocysteine. The production of selenocysteine is identical to that of cysteine. Therefore, the same enzymes including O-acetylserine (thiol)-lyase are used in the reaction (Pilon-Smits, Winkel, and Lin 2017).

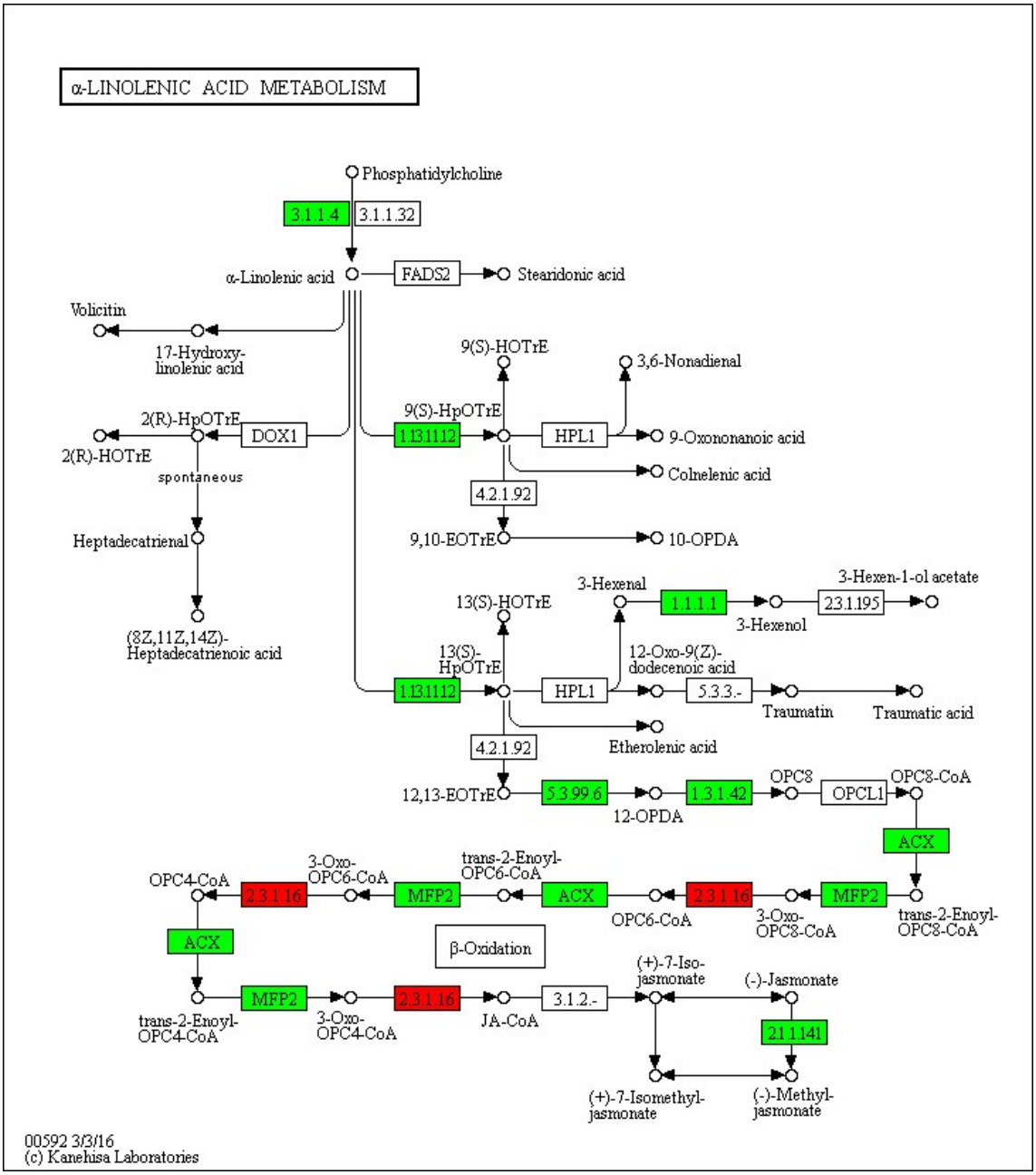

Figure 14. Expression pattern of transcripts involved in alpha-linolenic acid metabolism (KEGG pathway: map00592). The green and red color correspond to up- and downregulated transcripts, respectively. Alcohol dehydrogenase (EC 1.1.1.1), 12-oxophytodienoate reductase (EC 1.3.1.42), acyl-CoA oxidase (ACX, EC 1.3.3.6), linoleate 13S-lipoxygenase (EC 1.13.11.12), S-adenosyl-L methionine-dependent methyltransferase (EC 2.1.1.141), acetyl-CoA C-acyltransferase (EC 2.3.1.16), phospholipase A2 (EC 3.1.1.4), enoyl-CoA hydratase (MFP2, EC 4.2.1.17), allene-oxide cyclase (EC 5.3.99.6).

Table 14. Differentially expressed transcripts and corresponding enzyme names in alpha-linolenic acid metabolism pathway in response to 80 µM Se treatment in *P. distans* shoots. Increased expression is indicated with positive (+) and decreased expression is indicated with negative (-) values.

Transcript Name	Fold Change	Enzyme Names	Enzyme Code
Transcript_37606	5.4	Alcohol dehydrogenase	1.1.1.1
Transcript_17887	4.2	Alcohol dehydrogenase	1.1.1.1
Transcript_43832	2.7	Alcohol dehydrogenase	1.1.1.1
Transcript_26093	8.7	Alcohol dehydrogenase	1.1.1.1
Transcript_10632	6.7	Alcohol dehydrogenase	1.1.1.1
Transcript_66496	9.0	12-oxophytodienoate reductase	1.3.1.42
Transcript_5211	2.4	12-oxophytodienoate reductase	1.3.1.42
Transcript_47188	-10.7	12-oxophytodienoate reductase	1.3.1.42
Transcript_9947	3.9	Acyl-CoA oxidase	1.3.3.6
Transcript_9217	3.8	Linoleate 13S-lipoxygenase	1.13.11.12
Transcript_3677	9.7	Linoleate 13S-lipoxygenase	1.13.11.12
Transcript_24068	4.5	Linoleate 13S-lipoxygenase	1.13.11.12
Transcript_49788	13.9	Jasmonate O-methyltransferase	2.1.1.141
Transcript_32756	-3.7	Acetyl-CoA C-acyltransferase	2.3.1.16
Transcript_3584	6.3	Phospholipase A2	3.1.1.4
Transcript_24219	4.4	Enoyl-CoA hydratase	4.2.1.17
Transcript_6292	2.6	Enoyl-CoA hydratase	4.2.1.17
Transcript_10663	5.8	Enoyl-CoA hydratase	4.2.1.17
Transcript_3602	2.5	Allene-oxide cyclase	5.3.99.6

As can be seen in Figure 10, the expression of the transcript encoding O-acetylserine (thiol)-lyase was downregulated. In other words, cells tried to decrease the production of both cysteine and selenocysteine. Although cysteine is an essential amino acid for proteins, selenocysteine causes toxicity if it is nonspecifically incorporated into proteins. According to the RNA-Seq results and selenocompound metabolism pathway, here, *P. distans* used two additional mechanism to prevent nonspecific incorporation of selenium into proteins. First, it reduced the attachment of selenomethionine to selenomethionyl-tRNA (Met) by decreasing the expression of methionyl-tRNA synthetase enzyme. Second, it decreased the expression of cystathionine gamma-lyase to keep selenocysteine in a methylated form and so to decrease the amount of available selenocysteine for incorporation into proteins.

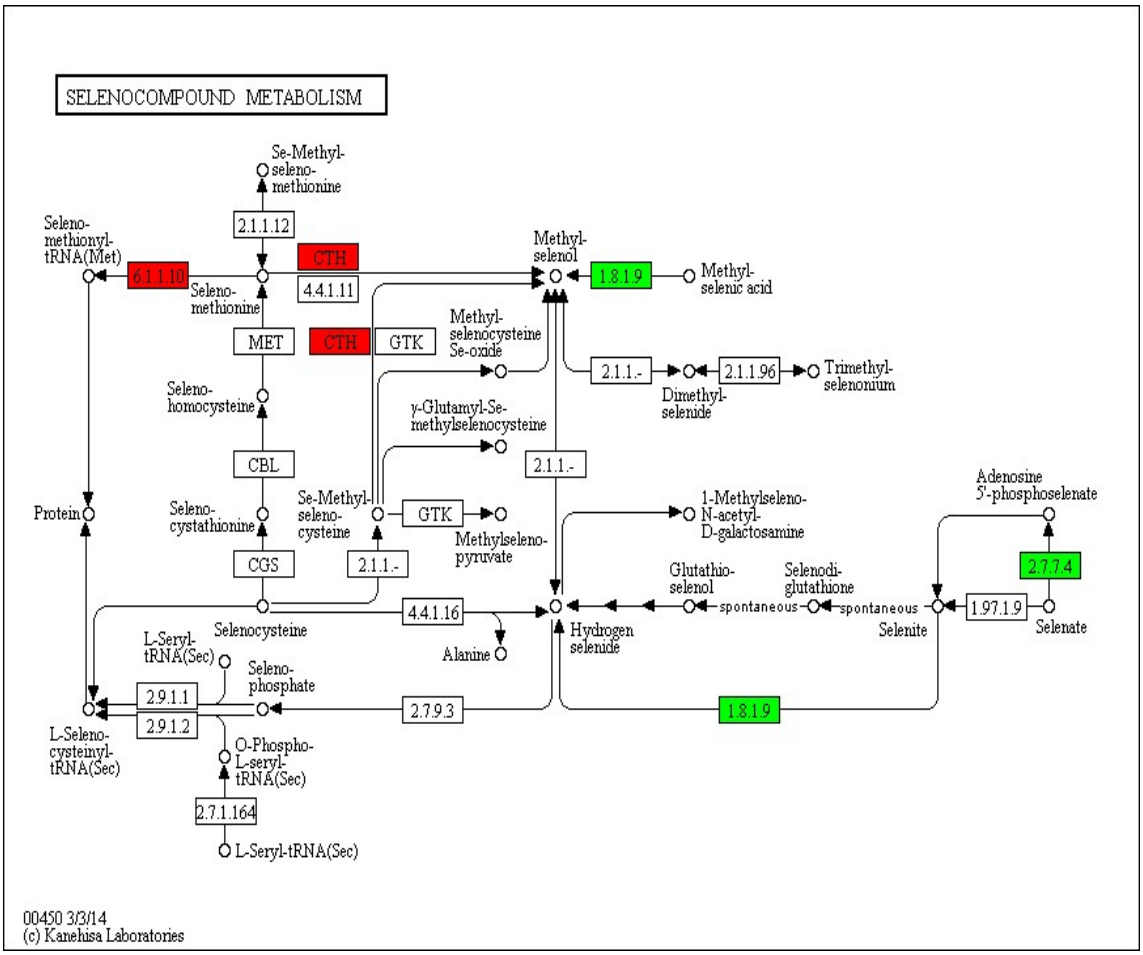

Figure 15. Expression pattern of transcripts involved in selenocompound metabolism (KEGG pathway: map00450). The green and red color correspond to up- and downregulated transcripts, respectively. The yellow color represents the transcripts for which expression level could not be determined. Thioredoxin reductase (NADPH) (EC 1.8.1.9), 3'-phosphoadenosine 5'-phosphosulfate synthase (EC 2.7.7.4), cystathionine gamma-lyase (CTH; EC 4.4.1.1), methionyl-tRNA synthetase (EC 6.1.1.10).

Two RT-qPCR results did not show similar trends with the RNA-Seq results. The expression of transcript_25145 and transcript_39773 were downregulated by 31.7 and 10.7-fold, respectively, according to RNA-Seq. However, RT-qPCR indicated that the expression of transcript_25154 and transcript_39773 were upregulated 5.8 and 1.4-fold. This lack of correlation between RNA-Seq and RT-qPCR may be due to the use of different parameters in calculation and the normalization processes of each technique.

3.8 Physiological Characterization of Selenium-Stressed *P. distans*

3.8.1 Chlorophyll Content

The porphyrin and chlorophyll metabolism pathway (Figure 16) is composed of several parts: 5-aminolevulinic acid (ALA) formation, proto IX formation, heme formation, chlorophyll formation and chlorophyll breakdown. In this study, we analyzed chlorophyll content of Se treated *P. distans* plants since chlorosis is one of the important effects of Se toxicity. According to RNA-Seq analysis, there were no differentially expressed transcripts involved in chlorophyll degradation. However, three transcripts (transcript_1562, transcript_9089, transcript_9144) playing roles in chlorophyll synthesis were found to be downregulated (Table 15). This indicated that chlorosis was attributable to a decrease in chlorophyll biosynthesis.

Table 15. Differentially expressed transcripts and corresponding enzyme names in porphyrin and chlorophyll metabolism pathway in response to 80 µM Se treatment in *P. distans* shoots. Increased expression is indicated with positive (+) and decreased expression is indicated with negative (-) values.

Transcript Name	Fold Change	Enzyme Names	Enzyme Code
Transcript_1562	-2.1	Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase	1.14.13.81
Transcript_9144	-2.6	Chlorophyllide a oxygenase	1.14.13.122
Transcript_3500	-3.1	Glutamyl-tRNA reductase	1.2.1.70
Transcript_11804	-3.4	Glucuronosyltransferase	2.4.1.17
Transcript_4222	3.6	Glucuronosyltransferase	2.4.1.17
Transcript_9089	-3.2	Magnesium chelatase subunit H	6.6.1.1
Transcript_57445	-3.7	Glutamyl-tRNA synthetase	6.1.1.17

The chlorophyll a, b and total chlorophyll contents of control and stress groups were determined to confirm RNA-Seq results. The chlorophyll a, b and the total chlorophyll contents amount of the plants were decreased (Figure 15) was verified. This also confirmed that plants were under Se stress.

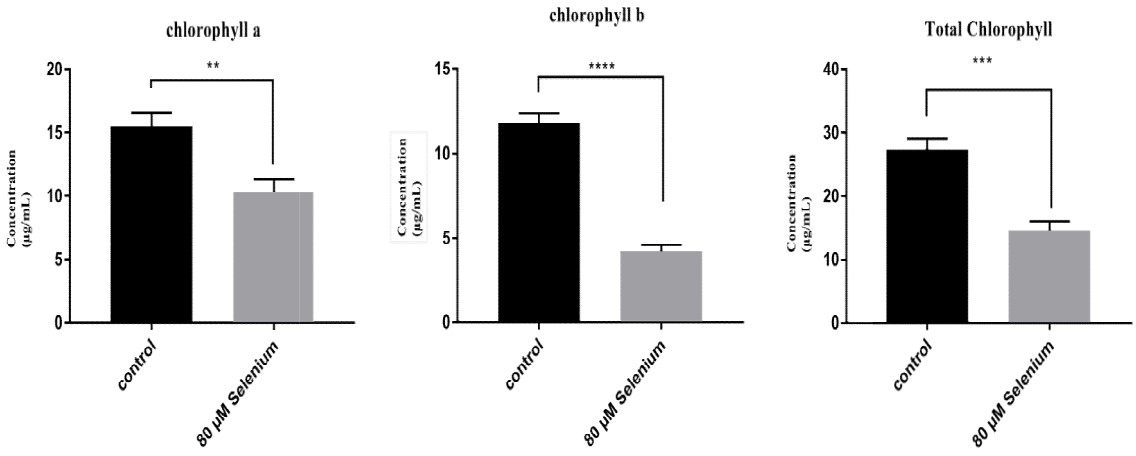

Figure 16. Measurement of chlorophyll a (A), chlorophyll b (B) and total chlorophyll (C) content of control and 80 µM selenium treated *P. distans* plants. The data represent the mean ± SD ($p<0.0001$) values of three replicates (paired student t-test). Asterisks (*) indicate significant differences.

In general, a decrease in chlorophyll could be explained by the S-deficiency response induced by selenate (Schiavon et al. 2017). Low levels of S can cause an alteration in carbon and nitrogen metabolism in plants. One consequence of inadequate carbon and nitrogen levels is the decrease in chlorophyll content (Lunde et al. 2008). More specifically, the observed decrease in chlorophyll a amount was caused by the reduced expression of two transcripts: transcript_9089 and transcript_1562 (Table 15). These encode magnesium chelatase subunit H and magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase enzymes, respectively. These enzymes carry out the consecutive steps of chlorophyll a synthesis from protoporphyrin IX. Synthesis of chlorophyll a is an important step because it is the precursor of chlorophyll b. The enzyme responsible for this reaction is chlorophyllide *a* oxygenase. In this study, the transcript encoding chlorophyllide a oxygenase was found to be downregulated by 2.6 fold. This could explain the observed decrease in chlorophyll b in *P. distans*.

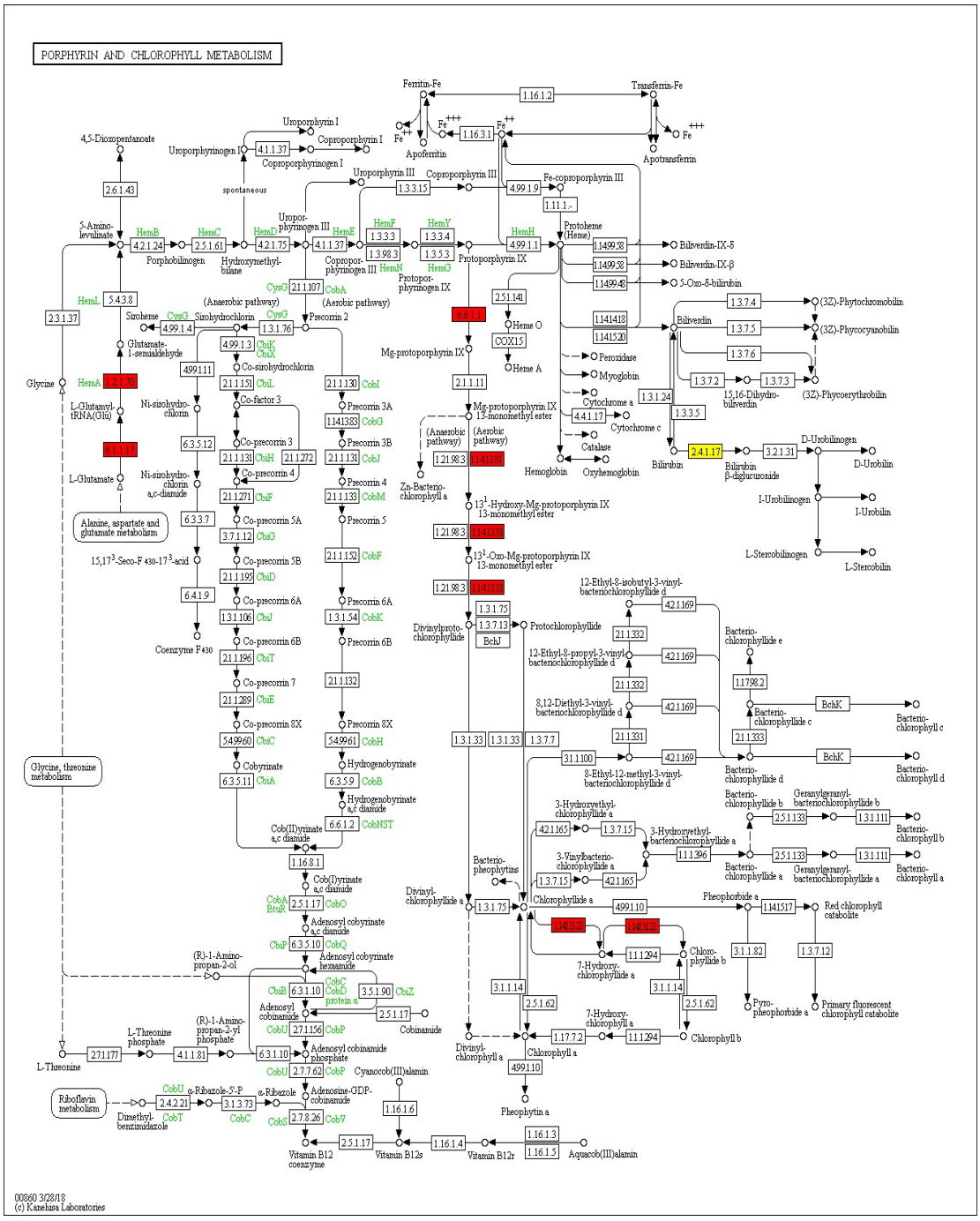

Figure 17. Expression pattern of transcripts involved in porphyrin and chlorophyll metabolism (KEGG pathway: map00860). The red color corresponds to downregulated transcripts. The yellow color represents the transcripts for which expression level could not be determined. Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase (EC 1.14.13.82), chlorophyllide a oxygenase (EC 1.14.13.122), glutamyl-tRNA reductase (EC 1.2.1.70), glucuronosyltransferase (EC 2.4.1.17), glutamyl-tRNA synthetase (EC 6.1.1.17), magnesium chelatase (EC 6.6.1.1).

3.8.2 Antioxidant and Redox Capacity

ROS is the main component of oxidative stress signaling. These molecules are continuously produced in plant cells as by-products of metabolic reactions and in excess under metal stress. ROS are generated in certain locations in the cell: plasma membrane, mitochondria, nucleus, chloroplast and cell wall (Gill and Tuteja 2010). ROS may affect important macromolecules including lipids, proteins and nucleic acids negatively. However, plants have defense mechanisms (both enzymatic and non-enzymatic) to protect themselves against oxidative agents (Gajewska and Sklodowska 2007). Selenium has a positive effect on the amount and the activity of the antioxidant enzymes and metabolites. Therefore, changes in the expression and the activity of antioxidant system in *P. distans* are discussed here.

Glutathione (GSH) is a non-enzymatic antioxidant with various fundamental functions such as detoxification and redox homeostasis. It is also linked to responses in oxidative stress signaling and acts as an antioxidant barrier. It reduces ROS and is oxidized to glutathione disulfide (GSSG). This mechanism protects sensitive parts of the cell against damaging effects of ROS (Bela et al. 2015). Therefore, GSH is an indispensable part of the plant's defense system in response to metal ions. At the end of each reaction, however, GSSG should be recycled to GSH. This reaction is carried out by glutathione reductase (GR).

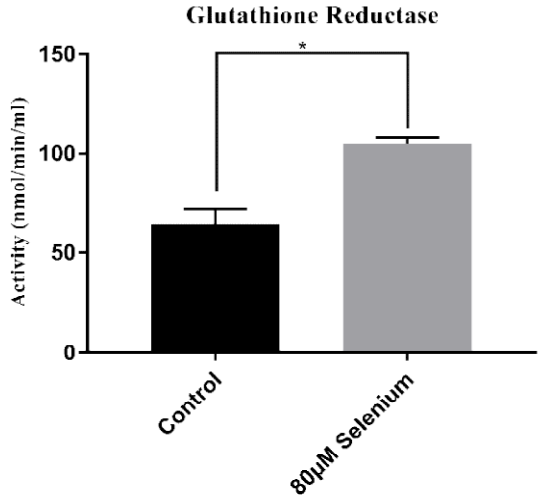

Figure 18. Measurement of GR activity of control and 80 µM selenium treated *P. distans* plants. The data represent the mean ± SD ($p<0.001$) values of three replicates (paired student t-test). Asterisks (*) indicate significant differences.

In this study, Se treatment led to upregulation of transcript_8650 and transcript_6816 expression encoding GR by 2.52 and 5.51-fold. This RNA-Seq analysis result was verified by both RT-qPCR and GR enzyme assay. According to RT-qPCR results, transcript_6816 was expressed 3.9 times more in Se-treated plants compared to control group. In addition, the activity of GR in Se treated plants were found to be almost 2 times more than in the control based on the enzyme activity assay (Figure 17). These results confirmed that Se treatment caused upregulation of GR expression.

Transcription of several antioxidant enzymes including glutathione reductase (EC 1.8.1.7), catalase (EC 1.11.1.6), glutathione peroxidase (EC:1.11.1.9), superoxide dismutase (EC 1.15.1.1), glutathione S-transferase (EC 2.5.1.18) were also upregulated (Table 16 and Figure 18). Similarly, in previous studies, the expressions of these antioxidant enzymes were upregulated in Se treated plants compared to control. In the same studies, they also observed that the expression of these enzymes was higher in Se hyperaccumulator plants compared to non-accumulators when plants were treated with selenate (Freeman et al. 2010, Wang et al. 2018). Although most of the antioxidant enzymes were upregulated in Se-treated *P. distans*, the expression of ascorbate peroxidase (EC:1.11.1.11) was downregulated. However, overall, increased expression and activity of antioxidant enzymes enhance the capacity of *P. distans* to cope with the Se stress through relief of oxidative stress.

Another antioxidant enzyme involved in ROS elimination is guaiacol peroxidase (POD, EC 1.11.1.7). It is also a key enzyme in the biosynthesis of lignin which is the structural component of the secondary cell wall. Guaiacol peroxidase takes part in the cross-linking of monolignols (coniferyl and sinopyl alcohol) to produce guaiacyl and syringyl lignin. Here, Se treatment caused the upregulation of transcripts encoding POD (Table 16). To verify RNA-Seq results, the enzyme activity of POD was measured. The activity of POD in Se treated plants and control groups were found to be almost the same (Figure 19).

The protein activity assay did not show a similar trend as the quantitative transcriptome data. This may have been due to the fact that the presence of more mRNA does not always mean that mRNAs will get translated into proteins since translation can be regulated at different levels. The disparity between enzyme activity and mRNA may have also linked to several other biological factors (ex. codon bias, protein half-lives

and, regulatory proteins and sRNAs) and some methodological constraints (Maier, Guell, and Serrano 2009, Glanemann et al. 2003).

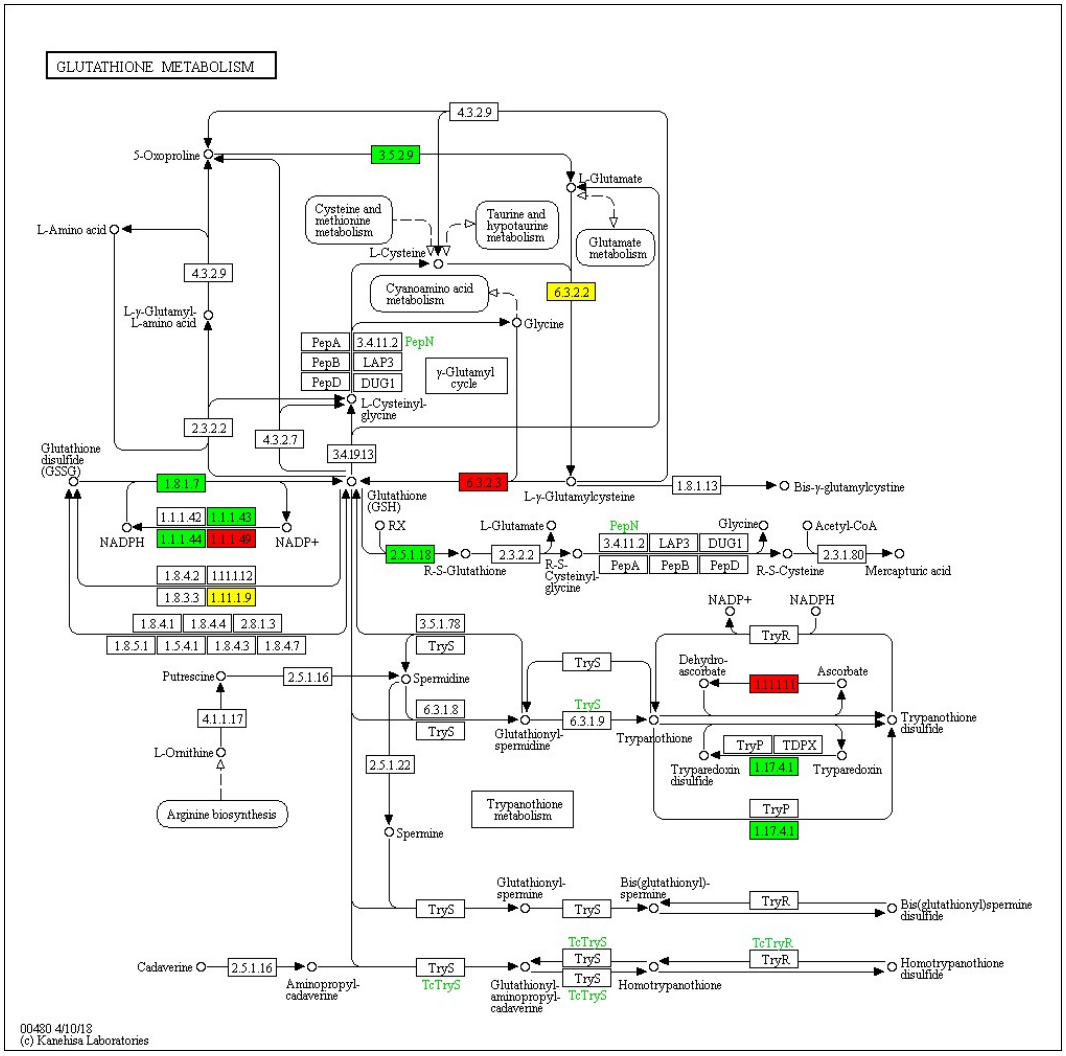

Figure 19. Expression pattern of transcripts involved in glutathione metabolism (KEGG pathway: map00480). The green and red color correspond to up- and downregulated transcripts, respectively. The yellow color represents the transcripts for which expression level could not be determined. Phosphogluconate 2-dehydrogenase (EC:1.1.1.43), 6-phosphogluconate dehydrogenase (EC:1.1.1.44), glucose-6-phosphate 1-dehydrogenase (EC:1.1.1.49), glutathione reductase (EC 1.8.1.7), glutathione peroxidase (EC:1.11.1.9), ascorbate peroxidase (EC:1.11.1.11), ribonucleoside-diphosphate reductase (EC:1.17.4.1), glutathione S-transferase (EC 2.5.1.18), 5-oxoprolinase (ATP-hydrolysing) (EC:3.5.2.9), glutamate-cysteine ligase catalytic subunit (EC:6.3.2.2), glutathione synthase (EC:6.3.2.3).

Table 16. Differentially expressed transcripts and corresponding enzyme names which are responsible for antioxidant scavenging in response to 80 µM Se treatment in *P. distans* shoots. Increased expression is indicated with positive (+) and decreased expression is indicated with negative (-) values.

Transcript Name	Fold Change	Enzyme Names	Enzyme Code
Transcript_8650	2.5	Glutathione reductase	1.8.1.7
Transcript_6816	5.5	Glutathione reductase	1.8.1.7
Transcript_15998	2.3	Catalase	1.11.1.6
Transcript_2547	2.6	Catalase	1.11.1.6
Transcript_17889	4.4	Guaiacol peroxidase	1.11.1.7
Transcript_5701	2.3	Guaiacol peroxidase	1.11.1.7
Transcript_517	3.6	Guaiacol peroxidase	1.11.1.7
Transcript_12213	36.8	Guaiacol peroxidase	1.11.1.7
Transcript_14010	2.8	Guaiacol peroxidase	1.11.1.7
Transcript_1570	2.6	Glutathione peroxidase	1.11.1.9
Transcript_1873	4.4	Glutathione peroxidase	1.11.1.9
Transcript_875	6.8	Glutathione peroxidase	1.11.1.9
Transcript_16187	-3.4	Ascorbate peroxidase	1.11.1.11
Transcript_51844	-2.0	Ascorbate peroxidase	1.11.1.11
Transcript_3601	2.6	Superoxide dismutase	1.15.1.1
Transcript_6651	3.2	Glutathione S-transferase	2.5.1.18
Transcript_9391	3.9	Glutathione S-transferase	2.5.1.18
Transcript_1873	4.4	Glutathione S-transferase	2.5.1.18

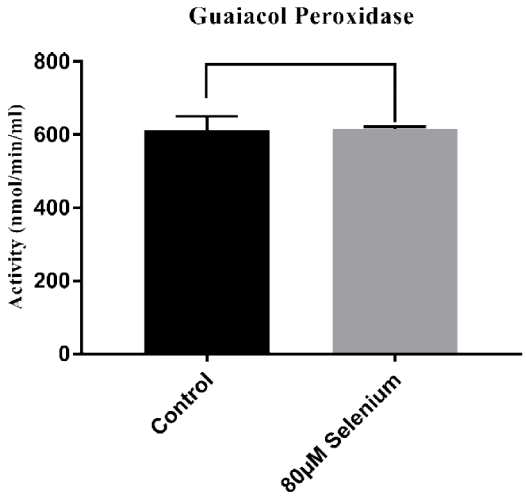

Figure 20. Measurement of POD activity of control and 80 µM selenium treated *P. distans* plants. The data represent the mean ± SD values of three replicates.

CHAPTER 4

CONCLUSION

P. distans is known to hyperaccumulate boron and tolerate high salt concentrations. Here, Se accumulation capacity of *P. distans* was determined. The results showed that it can accumulate more than 100 mg Se kg^{-1} dry weight (DW) in its shoots when it was treated with 80 μM. Also, the Se tolerance and accumulation mechanism of *P. distans* was investigated and a model was proposed for these. According this model, after Se was taken up by the plant, some Se may have been trapped in the cell wall and its movement restricted across the cell membrane. Meanwhile, Se may have triggered the production of ROS in the plant. Se stress induced ROS production may have led to JA production. In this study, the expression of genes in the sulfate/selenate and antioxidant metabolisms were found to be upregulated. Therefore, we suggested that the increased activities of these pathways may been due to the positive effect of JA signaling on the stress response genes (antioxidant and defence) and Se/S assimilation genes.

P. distans was suggested to use two pathways (sulfate and selenocompound) and three strategies to prevent nonspecific incorporation of selenocysteine and selenomethionine into proteins to protect itself against the adverse effect of Se. First, it decreased the production of selenocysteine downregulating the expression of O-acetylserine (thiol)-lyase. Second, it kept selenocysteine in a methylated form, decreasing the expression of cystathione gamma-lyase. Third, it reduced the attachment of selenomethionine to selenomethionyl-tRNA (Met) to keep selenomethione away from proteins.

P. distans was also shown to use the antioxidant system as a protection mechanism. Antioxidant enzymes protect plants against oxidative stress. Here, most of the antioxidant enzymes were upregulated. This suggested that *P. distans* was able to cope with Se stress through relief of oxidative stress. This study also showed that *P. distans* activated the molecular chaperone system by upregulating the expression of heat shock proteins to prevent aggregation of misfolded proteins due to nonspecific incorporation of Se into proteins and activity of ROS.

Finally, *P. distans* was found to increase the expression of glutathione S transferase and ABC transporter while downregulating the expression of SULTR4;1. These results suggested that *P. distans* tended to sequester selenate inside the vacuole to accumulate Se.

Overall, discovery of new metal/metalloid accumulator plants is of great importance because they help us to understand metal homeostasis and detoxification mechanisms in plants. In this study, *P. distans* was found to be a novel Se accumulator. It appears that *P. distans* may have an even greater capacity to accumulate Se because the 120 µM Se application did not lead to any major phenotypic changes in the plant. Thus, the species may actually be a hyperaccumulator, but this must be tested in future studies that use even higher concentrations of Se in order to determine the limit of *P. distans* tolerance and accumulation. Also, further studies should be performed to determine the localization, distribution and chemical speciation of Se in shoot. The effect of different Se concentration on the abundance and/or activity of sulfate transporters could also be an interesting research topic. In addition, understanding of the selenium tolerance mechanism better in *P. distans* could pave a path to exploit plants for phytoremediation and biofortification in the future.

REFERENCES

Akhzari, D., A. Sepehry, M. Pessarakli, and H. Barani. 2012. "Studying the effect of Salinity Stress on the Growth of Various Halophytic Plant Species (Agropyron elongatum, Kochia prostrata and Puccinellia distans)." *World Applied Science Journal* 16 (7):998-1003.

Al-Whaibi, Mohamed H. 2011. "Plant heat-shock proteins: A mini review." *Journal of King Saud University - Science* 23 (2):139-150. doi: 10.1016/j.jksus.2010.06.022.

Ali, H., E. Khan, and M. A. Sajad. 2013. "Phytoremediation of heavy metals--concepts and applications." *Chemosphere* 91 (7):869-81. doi:10.1016/j.chemosphere.2013.01.075.

Ali, M. B., N. Singh, A. M. Shohael, E. J. Hahn, and K. Y. Paek. 2006. "Phenolics metabolism and lignin synthesis in root suspension cultures of Panax ginseng in response to copper stress." *Plant Science* 171 (1):147-154. doi: 10.1016/j.plantsci.2006.03.005.

Babaoğlu, M., S. Gezgin, A. Topal, B. Sade, and H. Dural. 2004. "Gypsophila sphaerocephala Fenzl ex Tchihat.: A Boron Hyperaccumulator Plant Species That May Phytoremediate Soils with Toxic B Levels." *Turkish Journal of Botany* 28:273–278.

Bar, Cantuğ, Sami Doğanlar, and Anne Frary. 2015. "Genetic relationships among Eurasian Puccinellia distans genotypes." *Biochemical Systematics and Ecology* 62:20-24. doi: 10.1016/j.bse.2015.07.031.

Baraniecka, P., and S. Kopriva. 2014. "Macronutrient Use Efficiency – Sulfur in Arabidopsis thaliana." In *Nutrient Use Efficiency in Plants: Concepts and Approaches*, edited by Malcolm J. Hawkesford, Stanislav Kopriva and Luit J. De Kok, 51-91. Cham: Springer International Publishing.

Barkworth, M. E., K. M. Capels, and S. Long. 1993. "Flora of North America: North of Mexico. Magnoliophyta : Commelinidae (in Part) : Poaceae." In *Flora of North America: North of Mexico*, 473. Oxford University Press.

Baxter, I., B. Muthukumar, H. C. Park, P. Buchner, B. Lahner, J. Danku, K. Zhao, J. Lee, M. J. Hawkesford, M. L. Guerinot, and D. E. Salt. 2008. "Variation in molybdenum content across broadly distributed populations of Arabidopsis thaliana is controlled by a mitochondrial molybdenum transporter (MOT1)." *PLoS Genet* 4 (2):e1000004. doi: 10.1371/journal.pgen.1000004.

Bela, K., E. Horvath, A. Galle, L. Szabados, I. Tari, and J. Csiszar. 2015. "Plant glutathione peroxidases: emerging role of the antioxidant enzymes in plant development and stress responses." *J Plant Physiol* 176:192-201. doi: 10.1016/j.jplph.2014.12.014.

Bernhardt, Karl-Georg, and Marcus Koch. 2003. "Restoration of a salt marsh system: temporal change of plant species diversity and composition." *Basic and Applied Ecology* 4 (5):441-451. doi: 10.1078/1439-1791-00180.

Birecka, H., K. A. Briber, and J. L. Catalfamo. 1973. "Comparative studies on tobacco pith and sweet potato root isoperoxidases in relation to injury, indoleacetic Acid, and ethylene effects." *Plant Physiol* 52 (1):43-9.

Bogdanova, Natascha, and Rudiger Hell. 1997. "Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from Arabidopsis thaliana." *The Plant Journal* 11 (2):251-262. doi: 10.1046/j.1365-313X.1997.11020251.x.

Bowden, Wray M. 1961. "Chromosome Numbers and Taxonomic Notes on Northern Grasses: Iv. Tribe Festuceae: Poa, and Puccinellia." *Canadian Journal of Botany* 39 (1):123-138. doi: 10.1139/b61-010.

Boyle, J. 2005. *Lehninger principles of biochemistry* Edited by D. Nelson and M. Cox. 4 ed. Vol. 33, *Biochemistry and Molecular Biology Education*.

Buchner, P., H. Takahashi, and M. J. Hawkesford. 2004. "Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport." *J Exp Bot* 55 (404):1765-73. doi: 10.1093/jxb/erh206.

Cao, M. J., Z. Wang, M. Wirtz, R. Hell, D. J. Oliver, and C. B. Xiang. 2013. "SULTR3;1 is a chloroplast-localized sulfate transporter in Arabidopsis thaliana." *Plant J* 73 (4):607-16. doi: 10.1111/tpj.12059.

Cappa, J. J., C. Yetter, S. Fakra, P. J. Cappa, R. DeTar, C. Landes, E. A. Pilon-Smits, and M. P. Simmons. 2015. "Evolution of selenium hyperaccumulation in Stanleya (Brassicaceae) as inferred from phylogeny, physiology and X-ray microprobe analysis." *New Phytol* 205 (2):583-95. doi: 10.1111/nph.13071.

Chandran, D., N. Sharopova, S. Ivashuta, J. S. Gantt, K. A. Vandenbosch, and D. A. Samac. 2008. "Transcriptome profiling identified novel genes associated with aluminum toxicity, resistance and tolerance in Medicago truncatula." *Planta* 228 (1):151-66. doi: 10.1007/s00425-008-0726-0.

Clayton, W.D., M.S. Vorontsova, K.T. Harman, and H. Williamson. 2006. "GrassBase - The Online World Grass Flora."

Çakır, Ö. , N. Turgut-Kara, and Ş. Arı. 2012. "Advances in Selected Plant Physiology Aspects." In *Selenium Metabolism in Plants: Molecular Approaches*.

Eckardt, Nancy A. 2001. "Move It on Out with MATEs." *The Plant Cell* 13 (7):1477-1480. doi: 10.1105/tpc.13.7.1477.

Edgar, E. 1996. "{iPuccinellia} Parl. (Gramineae: Poeae) in New Zealand." *New Zealand Journal of Botany* 34 (1):17-32. doi: 10.1080/0028825x.1996.10412688.

El Mehdawi, A. F., R. J. Reynolds, C. N. Prins, S. D. Lindblom, J. J. Cappa, S. C. Fakra, and E. A. Pilon-Smits. 2014. "Analysis of selenium accumulation,

speciation and tolerance of potential selenium hyperaccumulator Symphyotrichum ericoides." *Physiol Plant* 152 (1):70-83. doi: 10.1111/ppl.12149.

Feldman-Salit, A., M. Wirtz, R. Hell, and R. C. Wade. 2009. "A mechanistic model of the cysteine synthase complex." *J Mol Biol* 386 (1):37-59. doi: 10.1016/j.jmb.2008.08.075.

Feng, R. W., C. Y. Wei, and S. X. Tu. 2013. "The roles of selenium in protecting plants against abiotic stresses." *Environmental and Experimental Botany* 87:58-68. doi: 10.1016/j.envexpbot.2012.09.002.

Fordyce, F. 2013. "Selenium deficiency and toxicity in the environment." In *Essentials of Medical Geology*, edited by O. Selinus, 373-415. Springer Netherlands.

Freeman, J. L., S. D. Lindblom, C. F. Quinn, S. Fakra, M. A. Marcus, and E. A. Pilon-Smits. 2007. "Selenium accumulation protects plants from herbivory by Orthoptera via toxicity and deterrence." *New Phytol* 175 (3):490-500. doi: 10.1111/j.1469-8137.2007.02119.x.

Freeman, J. L., C. F. Quinn, S. D. Lindblom, E. M. Klamper, and E. A. Pilon-Smits. 2009. "Selenium protects the hyperaccumulator Stanleya pinnata against black-tailed prairie dog herbivory in native seleniferous habitats." *Am J Bot* 96 (6):1075-85. doi: 10.3732/ajb.0800287.

Freeman, J. L., M. Tamaoki, C. Stushnoff, C. F. Quinn, J. J. Cappa, J. Devonshire, S. C. Fakra, M. A. Marcus, S. P. McGrath, D. Van Hoewyk, and E. A. Pilon-Smits. 2010. "Molecular mechanisms of selenium tolerance and hyperaccumulation in Stanleya pinnata." *Plant Physiol* 153 (4):1630-52. doi: 10.1104/pp.110.156570.

Freeman, J. L., L. H. Zhang, M. A. Marcus, S. Fakra, S. P. McGrath, and E. A. Pilon-Smits. 2006. "Spatial imaging, speciation, and quantification of selenium in the hyperaccumulator plants Astragalus bisulcatus and Stanleya pinnata." *Plant Physiol* 142 (1):124-34. doi: 10.1104/pp.106.081158.

Gajewska, E., and M. Sklodowska. 2007. "Effect of nickel on ROS content and antioxidative enzyme activities in wheat leaves." *Biometals* 20 (1):27-36. doi: 10.1007/s10534-006-9011-5.

Ghasemi, F., M. Ebrahimi, and M. Pozesh Shirazi. 2017. "Lead phytoremediation capacity of Puccinellia distans (Jacq.) Parl. using EDTA and DTPA and associated potential leaching risk." *Global NEST Journal* 19 (3):359 - 366.

Gigolashvili, T., and S. Kopriva. 2014. "Transporters in plant sulfur metabolism." *Front Plant Sci* 5 (442):442. doi: 10.3389/fpls.2014.00442.

Gill, S. S., and N. Tuteja. 2010. "Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants." *Plant Physiol Biochem* 48 (12):909-30. doi: 10.1016/j.plaphy.2010.08.016.

Glanemann, C., A. Loos, N. Gorret, L. B. Willis, X. M. O'Brien, P. A. Lessard, and A. J. Sinskey. 2003. "Disparity between changes in mRNA abundance and enzyme

activity in Corynebacterium glutamicum: implications for DNA microarray analysis." *Appl Microbiol Biotechnol* 61 (1):61-8. doi: 10.1007/s00253-002-1191-5.

Hajiboland, R., F. Dashtebani, and N. Aliasgharzad. 2015. "Physiological responses of halophytic C4 grass Aeluropus littoralis to salinity and arbuscular mycorrhizal fungi colonization." *Photosynthetica* 53 (4):572-584. doi: 10.1007/s11099-015-0131-4.

Hall, J. L. 2002. "Cellular mechanisms for heavy metal detoxification and tolerance." *Journal of Experimental Botany* 53 (366):1-11. doi: 10.1093/jexbot/53.366.1.

Hanikenne, M., and C. Nouet. 2011. "Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics." *Curr Opin Plant Biol* 14 (3):252-9. doi: 10.1016/j.pbi.2011.04.003.

Hanson, B., G. F. Garifullina, S. D. Lindblom, A. Wangeline, A. Ackley, K. Kramer, A. P. Norton, C.B. Lawrence, and E.A. H. Pilon-Smits. 2003. "Selenium accumulation protects Brassica juncea from invertebrate herbivory and fungal infection." *New Phytologist* 159 (2):461-469. doi: 10.1046/j.1469-8137.2003.00786.x.

Hanson, B., S. D. Lindblom, M. L. Loeffler, and E. A. H. Pilon-Smits. 2004. "Selenium protects plants from phloem-feeding aphids due to both deterrence and toxicity." *New Phytologist* 162 (3):655-662. doi: 10.1111/j.1469-8137.2004.01067.x.

Harris, J., K. A. Schneberg, and E. A. Pilon-Smits. 2014. "Sulfur-selenium-molybdenum interactions distinguish selenium hyperaccumulator Stanleya pinnata from non-hyperaccumulator Brassica juncea (Brassicaceae)." *Planta* 239 (2):479-91. doi: 10.1007/s00425-013-1996-8.

Hawkesford, M. J. 2003. "Transporter gene families in plants: the sulphate transporter gene family - redundancy or specialization?" *Physiologia Plantarum* 117 (2):155-163. doi: 10.1034/j.1399-3054.2003.00034.x.

Hawkesford, MalcolmJ, Jean-Claude Davidian, and Claude Grignon. 1993. "Sulphate/proton cotransport in plasma-membrane vesicles isolated from roots of Brassica napus L.: increased transport in membranes isolated from sulphur-starved plants." *Planta* 190 (3):297. doi: 10.1007/bf00196957.

Hyun, T. K., Y. Rim, H. J. Jang, C. H. Kim, J. Park, R. Kumar, S. Lee, B. C. Kim, J. Bhak, B. Nguyen-Quoc, S. W. Kim, S. Y. Lee, and J. Y. Kim. 2012. "De novo transcriptome sequencing of Momordica cochinchinensis to identify genes involved in the carotenoid biosynthesis." *Plant Mol Biol* 79 (4-5):413-27. doi: 10.1007/s11103-012-9919-9.

Kataoka, T., N. Hayashi, T. Yamaya, and H. Takahashi. 2004. "Root-to-shoot transport of sulfate in Arabidopsis. Evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature." *Plant Physiol* 136 (4):4198-204. doi: 10.1104/pp.104.045625.

Kataoka, T., A. Watanabe-Takahashi, N. Hayashi, M. Ohnishi, T. Mimura, P. Buchner, M. J. Hawkesford, T. Yamaya, and H. Takahashi. 2004. "Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis." *Plant Cell* 16 (10):2693-704. doi: 10.1105/tpc.104.023960.

Kieliszek, M., S. Blazejak, I. Gientka, and A. Bzducha-Wrobel. 2015. "Accumulation and metabolism of selenium by yeast cells." *Appl Microbiol Biotechnol* 99 (13):5373-82. doi: 10.1007/s00253-015-6650-x.

Läuchli, A. 1993. "Selenium in Plants: Uptake, Functions, and Environmental Toxicity." *Botanica Acta* 106 (6):455-468. doi: 10.1111/j.1438-8677.1993.tb00774.x.

Lewis, B. G., C. M. Johnson, and T. C. Broyer. 1974. "Volatile selenium in higher plants the production of dimethyl selenide in cabbage leaves by enzymatic cleavage of Se-methyl selenomethionine selenonium salt." *Plant and Soil* 40 (1):107-118. doi: 10.1007/bf00011413.

Liu, Q., L. Luo, and L. Zheng. 2018. "Lignins: Biosynthesis and Biological Functions in Plants." *Int J Mol Sci* 19 (2). doi: 10.3390/ijms19020335.

Livak, K. J., and T. D. Schmittgen. 2001. "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." *Methods* 25 (4):402-8. doi: 10.1006/meth.2001.1262.

Luisa, Ederli, Reale Lara, Ferranti Francesco, and Pasqualini Stefania. 2004. "Responses induced by high concentration of cadmium in Phragmites australis roots." *Physiologia Plantarum* 121 (1):66-74. doi: doi:10.1111/j.0031-9317.2004.00295.x.

Lunde, C., A. Zygadlo, H. T. Simonsen, P. L. Nielsen, A. Blennow, and A. Haldrup. 2008. "Sulfur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways." *Physiol Plant* 134 (3):508-21. doi: 10.1111/j.1399-3054.2008.01159.x.

Macnair, M. R., V. Bert, S. B. Huitson, P. Saumitou-Laprade, and D. Petit. 1999. "Zinc tolerance and hyperaccumulation are genetically independent characters." *Proc Biol Sci* 266 (1434):2175-9. doi: 10.1098/rspb.1999.0905.

Maier, T., M. Guell, and L. Serrano. 2009. "Correlation of mRNA and protein in complex biological samples." *FEBS Lett* 583 (24):3966-73. doi: 10.1016/j.febslet.2009.10.036.

Meija, Juris, Maria Montes-Bayón, Danika L. Le Duc, Norman Terry, and Joseph A. Caruso. 2002. "Simultaneous Monitoring of Volatile Selenium and Sulfur Species from Se Accumulating Plants (Wild Type and Genetically Modified) by GC/MS and GC/ICPMS Using Solid-Phase Microextraction for Sample Introduction." *Analytical Chemistry* 74 (22):5837-5844. doi: 10.1021/ac020285t.

Ng, B. H., and J. W. Anderson. 1978. "Synthesis of Selenocysteine by Cysteine Synthases from Selenium Accumulator and Non-Accumulator Plants." *Phytochemistry* 17 (12):2069-2074. doi: 10.1016/S0031-9422(00)89282-1.

Noctor, G., A. C. M. Arisi, L. Jouanin, K. J. Kunert, H. Rennenberg, and C. H. Foyer. 1998. "Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants." *Journal of Experimental Botany* 49 (321):623-647. doi: 10.1093/jxb/49.321.623.

Oldfield, J. E. 1974. "The selenium story: Some reflections on the “Moon-Metal”." *New Zealand Veterinary Journal* 22 (6):85-94. doi: 10.1080/00480169.1974.34140.

Padmanabhan, P., M. Babaoglu, and N. Terry. 2012. "A comparative transcriptomic analysis of the extremely boron tolerant plant Puccinellia distans with the moderately boron tolerant Gypsophila arrostil." *Plant Cell Rep* 31 (8):1407-13. doi: 10.1007/s00299-012-1256-6.

Park, C. J., and Y. S. Seo. 2015. "Heat Shock Proteins: A Review of the Molecular Chaperones for Plant Immunity." *Plant Pathol J* 31 (4):323-33. doi: 10.5423/PPJ.RW.08.2015.0150.

Peer, W. A., I. R. Baxter, E. L. Richards, J. L. Freeman, and A. S. Murphy. 2006. "Phytoremediation and hyperaccumulator plants." In *Molecular Biology of Metal Homeostasis and Detoxification: From Microbes to Man*, edited by Markus J. Tamas and Enrico Martinoia, 299-340. Berlin, Heidelberg: Springer Berlin Heidelberg.

Peterson, PJ, and GW Butler. 1962. "The Uptake and Assimilation of Selenite by Higher Plants." *Australian Journal of Biological Sciences* 15 (1):126-146. doi: https://doi.org/10.1071/BI9620126.

Pilon-Smits, E. A., S. Hwang, C. Mel Lytle, Y. Zhu, J. C. Tai, R. C. Bravo, Y. Chen, T. Leustek, and N. Terry. 1999. "Overexpression of ATP sulfurylase in indian mustard leads to increased selenate uptake, reduction, and tolerance." *Plant Physiol* 119 (1):123-32. doi: 10.1104/pp.119.1.123.

Pilon-Smits, Elizabeth A. H., Lenny H. E. Winkel, and Zhi-Qing Lin. 2017. *Selenium in plants*. Vol. 11.

Prins, C. N., L. J. Hantzis, C. F. Quinn, and E. A. Pilon-Smits. 2011. "Effects of selenium accumulation on reproductive functions in Brassica juncea and Stanleya pinnata." *J Exp Bot* 62 (15):5633-40. doi: 10.1093/jxb/err247.

Quinn, C. F., J. L. Freeman, R. J. Reynolds, J. J. Cappa, S. C. Fakra, M. A. Marcus, S. D. Lindblom, E. K. Quinn, L. E. Bennett, and E. A. Pilon-Smits. 2010. "Selenium hyperaccumulation offers protection from cell disruptor herbivores." *BMC Ecol* 10 (1):19. doi: 10.1186/1472-6785-10-19.

Quinn, C. F., C. N. Prins, J. L. Freeman, A. M. Gross, L. J. Hantzis, R. J. Reynolds, Si Yang, P. A. Covey, G. S. Banuelos, I. J. Pickering, S. C. Fakra, M. A. Marcus, H. S. Arathi, and E. A. Pilon-Smits. 2011. "Selenium accumulation in flowers

and its effects on pollination." *New Phytol* 192 (3):727-37. doi: 10.1111/j.1469-8137.2011.03832.x.

Ramila, C. D. P., S. A. Contreras, C. Di Domenico, M. A. Molina-Montenegro, A. Vega, M. Handford, C. A. Bonilla, and G. E. Pizarro. 2016. "Boron stress response and accumulation potential of the extremely tolerant species Puccinellia frigida." *J Hazard Mater* 317:476-484. doi: 10.1016/j.jhazmat.2016.05.086.

Ramos, S. J., M. A. Rutzke, R. J. Hayes, V. Faquin, L. R. Guilherme, and L. Li. 2011. "Selenium accumulation in lettuce germplasm." *Planta* 233 (4):649-60. doi: 10.1007/s00425-010-1323-6.

Rascio, N., and F. Navari-Izzo. 2011. "Heavy metal hyperaccumulating plants: how and why do they do it? And what makes them so interesting?" *Plant Sci* 180 (2):169-81. doi: 10.1016/j.plantsci.2010.08.016.

Rayman, M. P. 2000. "The importance of selenium to human health." *Lancet* 356 (9225):233-41. doi: 10.1016/S0140-6736(00)02490-9.

Rayman, M. P. 2012. "Selenium and human health." *Lancet* 379 (9822):1256-68. doi: 10.1016/S0140-6736(11)61452-9.

Reeves, R., and A. Baker. 1999. "Metal Accumulating Plants." In *Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment*, edited by I. Raskin and B. D. Ensley, 193-229. John Wiley & Sons.

Ritchie, R. J. 2006. "Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents." *Photosynth Res* 89 (1):27-41. doi: 10.1007/s11120-006-9065-9.

Rosenfeld, I. 1964. "Selenium: geobotany, biochemistry, toxicity, and nutrition." In. New York: Academic Press.

Rouached, H., M. Wirtz, R. Alary, R. Hell, A. B. Arpat, J. C. Davidian, P. Fourcroy, and P. Berthomieu. 2008. "Differential regulation of the expression of two high-affinity sulfate transporters, SULTR1.1 and SULTR1.2, in Arabidopsis." *Plant Physiol* 147 (2):897-911. doi: 10.1104/pp.108.118612.

Rucinska-Sobkowiak, R. 2016. "Water relations in plants subjected to heavy metal stresses." *Acta Physiologiae Plantarum* 38 (11):257. doi: 10.1007/s11738-016-2277-5.

Sabbagh, M., and D. Van Hoewyk. 2012. "Malformed selenoproteins are removed by the ubiquitin--proteasome pathway in Stanleya pinnata." *Plant Cell Physiol* 53 (3):555-64. doi: 10.1093/pcp/pcs015.

Sarma, Hemen. 2011. "Metal Hyperaccumulation in Plants: A Review Focusing on Phytoremediation Technology." *Journal of Environmental Science and Technology* 4:118-138. doi: 10.3923/jest.2011.118.138

Schiavon, M., L. W. Lima, Y. Jiang, and M. J. Hawkesford. 2017. "Effects of Selenium on Plant Metabolism and Implications for Crops and Consumers." In *Selenium in plants*, edited by E. A. H. Pilon-Smits, L. H. E. Winkel and Z. Q. Lin, 257-270. Springer International Publishing.

Schiavon, M., and E. A. Pilon-Smits. 2017. "The fascinating facets of plant selenium accumulation - biochemistry, physiology, evolution and ecology." *New Phytol* 213 (4):1582-1596. doi: 10.1111/nph.14378.

Schiavon, M., M. Pilon, M. Malagoli, and E. A. Pilon-Smits. 2015. "Exploring the importance of sulfate transporters and ATP sulphurylases for selenium hyperaccumulation-a comparison of Stanleya pinnata and Brassica juncea (Brassicaceae)." *Front Plant Sci* 6 (2):2. doi: 10.3389/fpls.2015.00002.

Seo, H. S., J. T. Song, J. J. Cheong, Y. H. Lee, Y. W. Lee, I. Hwang, J. S. Lee, and Y. D. Choi. 2001. "Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses." *Proc Natl Acad Sci U S A* 98 (8):4788-93. doi: 10.1073/pnas.081557298.

Setya, A., M. Murillo, and T. Leustek. 1996. "Sulfate reduction in higher plants: molecular evidence for a novel 5'-adenylylsulfate reductase." *Proc Natl Acad Sci U S A* 93 (23):13383-8. doi: 10.1073/pnas.93.23.13383.

Sgherri, Cristina L. M., Calogero Pinzincp, and Flavia Navari-Izzo. 1996. "Sunflower seedlings subjected to increasing stress by water deficit: Changes in O2-production Aated to the composition of thylakoid membranes." *Physiologia Plantarum* 96 (3):446-452. doi: 10.1111/j.1399-3054.1996.tb00457.x.

Shibagaki, N., and A. R. Grossman. 2010. "Binding of cysteine synthase to the STAS domain of sulfate transporter and its regulatory consequences." *J Biol Chem* 285 (32):25094-102. doi: 10.1074/jbc.M110.126888.

Shibagaki, N., A. Rose, J. P. McDermott, T. Fujiwara, H. Hayashi, T. Yoneyama, and J. P. Davies. 2002. "Selenate-resistant mutants of Arabidopsis thaliana identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots." *Plant J* 29 (4):475-86. doi: 10.1046/j.0960-7412.2001.01232.x.

Sors, T. G., D. R. Ellis, and D. E. Salt. 2005. "Selenium uptake, translocation, assimilation and metabolic fate in plants." *Photosynth Res* 86 (3):373-89. doi: 10.1007/s11120-005-5222-9.

Sors, T. G., C. P. Martin, and D. E. Salt. 2009. "Characterization of selenocysteine methyltransferases from Astragalus species with contrasting selenium accumulation capacity." *Plant J* 59 (1):110-22. doi: 10.1111/j.1365-313X.2009.03855.x.

Stiles, A. R., D. Bautista, E. Atalay, M. Babaoglu, and N. Terry. 2010. "Mechanisms of boron tolerance and accumulation in plants: a physiological comparison of the extremely boron-tolerant plant species, Puccinellia distans, with the moderately boron-tolerant Gypsophila arrostil." *Environ Sci Technol* 44 (18):7089-95. doi: 10.1021/es1016334.

Takahashi, H., P. Buchner, N. Yoshimoto, M. J. Hawkesford, and S. H. Shiu. 2011. "Evolutionary relationships and functional diversity of plant sulfate transporters." *Front Plant Sci* 2 (119):119. doi: 10.3389/fpls.2011.00119.

Takahashi, H., A. Watanabe-Takahashi, F. W. Smith, M. Blake-Kalff, M. J. Hawkesford, and K. Saito. 2000. "The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in Arabidopsis thaliana." *Plant J* 23 (2):171-82. doi: 10.1046/j.1365-313x.2000.00768.x.

Talukdar, D., and T. Talukdar. 2015. "Thiolomics: Molecular Mechanisms of Thiol-Cascade in Plant Growth and Nutrition." In *PlantOmics: The Omics of Plant Science*, edited by Debmalya Barh, Muhammad Sarwar Khan and Eric Davies, 491-532. New Delhi: Springer India.

Tamaoki, M., J. L. Freeman, and E. A. Pilon-Smits. 2008. "Cooperative ethylene and jasmonic acid signaling regulates selenite resistance in Arabidopsis." *Plant Physiol* 146 (3):1219-30. doi: 10.1104/pp.107.110742.

Tarasoff, C. S., C. A. Mallory-Smith, and D. A. Ball. 2007. "Comparative plant responses of Puccinellia distans and Puccinellia nuttalliana to sodic versus normal soil types." *Journal of Arid Environments* 70 (3):403-417. doi: 10.1016/j.jaridenv.2007.01.008.

Tedeschini, Emma, Primo Proietti, Veronica Timorato, Roberto D'Amato, Luigi Nasini, Daniele Dei Buono, Daniela Businelli, and Giuseppe Frenguelli. 2015. "Selenium as stressor and antioxidant affects pollen performance in Olea europaea." *Flora - Morphology, Distribution, Functional Ecology of Plants* 215:16-22. doi: 10.1016/j.flora.2015.05.009.

Terry, N., A. M. Zayed, M. P. De Souza, and A. S. Tarun. 2000. "Selenium in Higher Plants." *Annu Rev Plant Physiol Plant Mol Biol* 51 (1):401-432. doi: 10.1146/annurev.arplant.51.1.401.

Tomatsu, H., J. Takano, H. Takahashi, A. Watanabe-Takahashi, N. Shibagaki, and T. Fujiwara. 2007. "An Arabidopsis thaliana high-affinity molybdate transporter required for efficient uptake of molybdate from soil." *Proc Natl Acad Sci U S A* 104 (47):18807-12. doi: 10.1073/pnas.0706373104.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B. C. Faircloth, M. Remm, and S. G. Rozen. 2012. "Primer3--new capabilities and interfaces." *Nucleic Acids Res* 40 (15):e115. doi: 10.1093/nar/gks596.

Van Hoewyk, D. 2013. "A tale of two toxicities: malformed selenoproteins and oxidative stress both contribute to selenium stress in plants." *Ann Bot* 112 (6):965-72. doi: 10.1093/aob/mct163.

Vatansever, R., I. Koc, I. I. Ozyigit, U. Sen, M. E. Uras, N. A. Anjum, E. Pereira, and E. Filiz. 2016. "Genome-wide identification and expression analysis of sulfate transporter (SULTR) genes in potato (Solanum tuberosum L.)." *Planta* 244 (6):1167-1183. doi: 10.1007/s00425-016-2575-6.

Wadgaonkar, Shrutika L., Yarlagadda V. Nancharaiah, Giovanni Esposito, and Piet N. L. Lens. 2018. "Environmental impact and bioremediation of seleniferous soils and sediments." *Critical Reviews in Biotechnology*:1-16. doi: 10.1080/07388551.2017.1420623.

Wang, J., J. J. Cappa, J. P. Harris, P. P. Edger, W. Zhou, J. C. Pires, M. Adair, S. A. Unruh, M. P. Simmons, M. Schiavon, and E. A. H. Pilon-Smits. 2018. "Transcriptome-wide comparison of selenium hyperaccumulator and nonaccumulator Stanleya species provides new insight into key processes mediating the hyperaccumulation syndrome." *Plant Biotechnol J* 0 (0). doi: 10.1111/pbi.12897.

Wang, Y., L. Xu, Y. Chen, H. Shen, Y. Gong, C. Limera, and L. Liu. 2013. "Transcriptome profiling of radish (Raphanus sativus L.) root and identification of genes involved in response to Lead (Pb) stress with next generation sequencing." *PLoS One* 8 (6):e66539. doi: 10.1371/journal.pone.0066539.

Warren, C. R. 2008. "Rapid measurement of chlorophylls with a microplate reader." *Journal of Plant Nutrition* 31 (7):1321-1332. doi: 10.1080/01904160802135092.

White, P. J. 2016. "Selenium accumulation by plants." *Ann Bot* 117 (2):217-35. doi: 10.1093/aob/mcv180.

Wu, Z., G. S. Banuelos, Z. Q. Lin, Y. Liu, L. Yuan, X. Yin, and M. Li. 2015. "Biofortification and phytoremediation of selenium in China." *Front Plant Sci* 6 (136):136. doi: 10.3389/fpls.2015.00136.

Yang, X., Y. Feng, Z. He, and P. J. Stoffella. 2005. "Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation." *J Trace Elem Med Biol* 18 (4):339-53. doi: 10.1016/j.jtemb.2005.02.007.

Yasin, M., A. F. El Mehdawi, C. E. Jahn, A. Anwar, M. F. S. Turner, M. Faisal, and E. A. H. Pilon-Smits. 2015. "Seleniferous soils as a source for production of selenium-enriched foods and potential of bacteria to enhance plant selenium uptake." *Plant and Soil* 386 (1-2):385-394. doi: 10.1007/s11104-014-2270-y.

Yoshimoto, N., E. Inoue, K. Saito, T. Yamaya, and H. Takahashi. 2003. "Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in Arabidopsis." *Plant Physiol* 131 (4):1511-7. doi: 10.1104/pp.014712.

Yoshimoto, Naoko, Hideki Takahashi, Frank W. Smith, Tomoyuki Yamaya, and Kazuki Saito. 2002. "Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots." *The Plant Journal* 29 (4):465-473. doi: 10.1046/j.0960-7412.2001.01231.x.

Zhou, Yifeng, Qiaoyu Tang, Meiru Wu, Di Mou, Hui Liu, Shouchuang Wang, Chi Zhang, Li Ding, and Jie Luo. 2018. "Comparative transcriptomics provides novel insights into the mechanisms of selenium tolerance in the hyperaccumulator plant Cardamine hupingshanensis." *Scientific Reports* 8 (1):2789. doi: 10.1038/s41598-018-21268-2.