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Transcriptomic analysis of selenium accumulation in *Puccinellia distans* (Jacq.) Parl., a boron hyperaccumulator

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HIGHLIGHTS

- *Puccinellia distans* is a selenium accumulator.
- Accumulation results in many transcriptomic changes.
- Sulfur and selenocompound metabolism are up-regulated.
- Reactive oxygen species are produced and glutathione metabolism is altered.
- Jasmonic acid synthesis is induced.

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ABSTRACT

Selenium (Se) is present in a wide variety of natural and man-made materials on Earth. Plants are able to tolerate and (hyper)accumulate Se to different extents. In fact, some species can tolerate and accumulate multiple elements. *Puccinellia distans* (*P. distans*), weeping alkali grass, is known to hyperaccumulate extreme concentrations of boron and tolerate high levels of salinity, therefore, we investigated the Se accumulation and tolerance capacities of this species. In addition, *P. distans'* Se tolerance mechanism was studied using a transcriptomic approach. The results of this study indicated that, when grown in a hydroponic system containing 80 or 120 μM Se, *P. distans* shoots accumulated from 1500 to 2500-fold more Se than plants grown without the element. Thus, *P. distans* was discovered to be a novel Se accumulator plant. RNA sequencing results and biochemical analyses helped to shed light on the Se tolerance and accumulation mechanism of *P. distans*. Here, we suggest that upregulation of Se assimilation and stress response genes may be 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 hypothesize that Se accumulates in cells by sequestration of selenate in the vacuole.

1. Introduction

Selenium (Se) is a Group 6A metalloid element which has physical and chemical properties that are intermediate to metals and nonmetals (Fordyce, 2013). Se is similar to sulfur (S) in terms of its chemical properties. 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).

The soil concentration of Se varies significantly depending on local land conditions and is not distributed evenly across the Earth. Se toxicity for humans occurs in Se rich areas whereas Se deficiency occurs in Se poor soils (Yasin et al., 2015). Se toxicity is a common problem in many countries including parts of the USA, China, Canada and India (Schiavon and Pilon-Smits, 2017). Se deficiency also poses problems to organisms and is mostly seen in China, northwestern Europe, Australia, New Zealand, sub-Saharan Africa, southern Brazil and parts of the USA (Wu et al., 2015). Although Se is necessary for humans and other animals, it is not an essential element for plants (White, 2016). However, Se is beneficial for plant growth and is associated with increased antioxidant activity at low tissue levels (10 mg kg^{-1}) (Mora et al., 2015). Plants also use Se as an elemental defense against certain herbivores, fungal pathogens and nematodes (Hanson et al., 2003, 2004; Freeman et al., 2007, 2009; Quinn et al., 2010; Prins et al., 2019). Thus, Se provides

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ecological advantages to plants.

Se is easily taken up and metabolized by all plants because it is atomically similar to S. Selenate and selenite are the two major forms of Se that are available to plants (Sors et al., 2005). Uptake of selenate competes with that of sulfate because both enter the plant via sulfate transporters (SULTR) (Terry et al., 2000). There are five groups of SULTRs (SULTR 1–5), which have been studied widely in the model species *Arabidopsis thaliana.* The extent of Se accumulation varies among different species and plants are divided into three groups in terms of their Se accumulation capacity in the shoots. These groups are: Se non-accumulators, secondary accumulators and hyperaccumulators. According to one definition, 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 grown on seleniferous soils (Prins et al., 2011). Se hyperaccumulators take up Se at levels that are 100–1500 fold higher than non-accumulators, with accumulation of 1000–15,000 mg Se kg^{-1} in seleniferous soils (White, 2016).

Se hyperaccumulators can distinguish selenate and sulfate and prefer Se over S (Schiavon et al., 2015). In addition, sulfate uptake is restricted by selenate in these plants (Harris et al., 2014). Se hyperaccumulators have advanced sulfate/selenate uptake and assimilation due to the constitutive upregulation of genes involved in the antioxidant pathway and encoding the stress/defense hormones jasmonic acid, salicylic acid and ethylene (Schiavon and Pilon-Smits, 2017). Defense related genes are also constitutively upregulated in Se hyperaccumulator plants but their effects on hyperaccumulation capacity need further research (Tamaoki et al., 2008; Freeman et al., 2010). Furthermore, Se hyperaccumulators have detoxification mechanisms that allow them to eliminate damaged/misfolded proteins which are degraded by the proteasome and to minimize Se incorporation into proteins by assimilating or sequestering Se into different organs (Sors et al., 2005; Sabbagh and Van Hoewyk, 2012; Wang et al., 2018).

Puccinellia distans (P. distans) belongs to the Poaceae family and is hexaploid, 2n = 42 (Bowden, 1961; Edgar, 1996). *P. distans,* weeping alkali grass, is a perennial, monocotyledonous C3 plant which has a relatively fast growth rate and produces a large biomass (Tarasoff et al., 2007; Hajiboland et al., 2015). It is native to Eurasia and distributed throughout Europe, Africa, Asia, Australasia and the USA (Barkworth et al., 1993; Clayton et al., 2006). *P. distans* can withstand high levels of boron (0.5–1250 mg L^{-1}) and salinity (200 mM NaCl) and hyperaccumulate extreme concentrations of boron (Stiles et al., 2010; Akhzari et al., 2012; Dashtebani et al., 2014; Hajiboland et al., 2015; Ozturk et al., 2018). Plants that have tolerance to multiple elements and stresses may use similar mechanisms to achieve this tolerance. Because it is known that some plant species (for example, two hybrid poplar clones) can tolerate high concentrations of soil salts and boron while hyperaccumulating B and Se in their tissues, we hypothesized that *P. distans* may be tolerant to Se (Zhu and Bañuelos, 2017). To test this hypothesis, first, the Se tolerance and accumulation capacity of *P. distans* was elucidated. Then, the mechanisms responsible for Se tolerance and accumulation were investigated using RNA-Seq. Genes that were differentially expressed under Se stress conditions were detected, annotated and KEGG pathway analyses performed to determine their possible roles in accumulation and/or tolerance. Finally, gene expression and biochemical analyses were done to confirm some of the transcriptomic results and to assess the level of plant stress. As a result of this work, *P. distans* was discovered to be a novel Se accumulator. Discovery of new plants that can accumulate multiple metal/metalloids is of great importance because they help us to understand metal homeostasis and detoxification mechanisms in plants. Moreover, because *P. distans* can produce a large biomass, has a relatively fast growth rate and can tolerate and accumulate multiple metals, it could be a useful candidate for phytoremediation of metals/metalloids.

2. Materials and methods

2.1. Plant materials and selenium treatment

P. distans seeds were obtained from a boron mining site, Kirka-Eskişehir, Turkey (39° 17′ 23.7156″ and 30° 31′ 33.4812″) (Babaoğlu et al., 2004). Seeds were germinated in perlite and grown for 7 weeks in a growth chamber. Seedlings were transferred to plastic tanks (dimensions: 23 × 15 × 22 cm) containing 5 L full strength, aerated Hoagland solution (Hoagland and Arnon, 1938). They were grown for one week in the growth chamber at 25 °C with a 16 h photoperiod. Plants were then assigned into stress and control groups (3 seedlings per Se level in each container) and grown for an additional week in full strength Hoagland solution with and without Na_2SeO_4 at various levels: 0, 20, 40, 80 and 120 μM, respectively. To compensate for the loss of solution due to evaporation, 500 ml Hoagland solution with and without Na_2SeO_4 was added every three days. Plants were then rinsed with sterile distilled water. The shoot tissues were frozen in liquid nitrogen and stored at −80 °C for antioxidant analyses with tissue for the 80 μM treatment also used for RNA isolation. The same procedure was repeated three times to obtain biological replicates.

2.2. Determination of Se accumulation of P. distans

Frozen ground shoot tissue was lyophilized for 4 days at −20 °C. Elemental Se concentrations of the samples were determined using inductively-coupled plasma atomic emission spectroscopy (ICP-AES) at Muğla Sıtkı Koçman University (Muğla, Turkey). Measurements were done with three biological and three technical replicates.

2.3. RNA isolation, sequencing and analysis

A Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Ontario, Canada) was used for RNA extraction from shoot tissue of the control and stress groups which were grown as described above. Total RNA was treated with DNase I. RNA quality and quantity were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA samples were stored at −80 °C.

Extracted RNA samples were quantified using a 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 for the control and Se treated samples were conducted at GENEWIZ, LLC (South Plainfield, NJ, USA). RNA sequencing library preparation used the NEB Next Ultra RNA Library Prep Kit for Illumina 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 2 × 150 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 the samples using the CLC Genomics Server. A reference genome was created using a transcriptome assembled from RNA sequences isolated from shoots of *P. distans* plants treated with selenium, arsenic, sulfur, nickel as well as control plants. Using the CLC Genomics server, trimmed sequence reads were mapped to the reference genome for each sequenced sample. Gene read counts were measured and reads per kb per million (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 Se-treated and control samples was conducted using the Wald test. Genes with a False-Discovery-Rate (FDR) < 0.05 and fold-change > 2 were selected as differentially expressed.

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 searched against the *A. thaliana* and green plant proteomes separately. These proteomes were generated from the NCBI database and UniProt Knowledgebase, respectively. Protein matches obtained from the 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 used to determine the roles of the up and down-regulated transcripts.

2.4. 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 and transport and four transcripts from the top 50 up and down-regulated transcripts were selected for validation by qPCR. Transcript specific primers were designed using Primer3 software (Untergasser et al., 2012) (Table S1). Three biological and three technical replicates were performed for both control and stress samples. Actin gene expression was used as a reference and for normalization. Relative changes in expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Antioxidant analyses

2.5.1. Determination of glutathione reductase activity

Shoot tissue (1 g) of control and Se-treated plants was homogenized in 50 mM potassium phosphate buffer (pH 7). Homogenates were centrifuged at 15,000 g for 15 min. 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 et al., 1996). Absorbance values of samples were measured at 340 nm with a Thermo Scientific™ Multiskan™ GO Microplate spectrophotometer using three technical replicates. Enzyme activity was calculated using the formulae defined in the glutathione reductase assay kit (Cayman Chemical, Ann Arbor, MI USA).

$$\Delta A_{340} \Big/ min = \frac{|A_{340}\ (Time\ 2)\ -\ A_{340}\ (Time\ 1)|}{Time\ 2\ (min) - Time\ 1\ (min)} \tag{1}$$

$$Enzyme\ activity = \frac{\Delta A_{340}/min\ \times\ Final\ volume\ of\ assay\ (ml) \times\ Sample\ dilution}{NADPH\ extinction\ coefficient\ \left(\mu M^{-1}\right) \times\ Volume\ of\ plant\ extract\ (ml)} \tag{2}$$

2.5.2. Determination of guaiacol peroxidase activity

Shoot tissue (400 mg) of control and Se-treated plants was homogenized in 0.1 M cold sodium phosphate buffer (pH 7). The homogenates were centrifuged at 14,000 rpm for 25 min. Then the supernatant was mixed with 0.1 mM sodium phosphate buffer containing 5 mM H_2O_2 and 15 mM guaiacol (Birecka et al., 1973). Absorbance values of samples were measured at 470 nm using three technical replicates. The enzyme activity was calculated using the same formulae defined for glutathione reductase.

2.5.3. Chlorophyll measurement

Shoot tissue (1 mg) from control and Se-treated plants was ground to a fine powder by shaking for 1 min at 30 Hz using a TissueLyser II system (Qiagen Inc, USA). To extract chlorophyll, 1.0 ml of methanol was added to the ground sample and the sample was shaken for 2 min at 30 Hz. Samples were centrifuged for 2 min at 14,000 rpm (Warren, 2008). Absorbance of the supernatant was measured at 652 nm and 665 nm with a 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:

$$Chl\ a\ (\mu g\ /\ ml)\ =\ -\ 8.0962\ A652, 1\ cm\ +\ 16.5169\ A665, 1\ cm \tag{3}$$

$$Chl\ b\ (\mu g\ /\ ml)\ =\ 27.4405\ A652, 1\ cm\ -12.1688\ A665, 1\ cm \tag{4}$$

3. Result and discussion

3.1. Determination of Se accumulation by P. distans

After one week in Hoagland solution with up to 120 μM Se, no serious toxicity symptoms were observed in *P. distans* plants which had very slight signs of chlorosis but grew like the untreated control plants. The application of increasing selenate concentration resulted in significant ($p < 0.05$) increases in shoot Se concentration (Fig. 1). At 80 μM selenate, the average concentration of Se in the shoot was 142.5 mg kg^{-1} dry weight (DW), almost 1500-fold higher than that found in the control group (0.1 mg kg^{-1} DW). With the 120 μM selenate treatment, the average Se concentration difference between the control and treated group (254 mg kg^{-1} DW) reached 2500-fold.

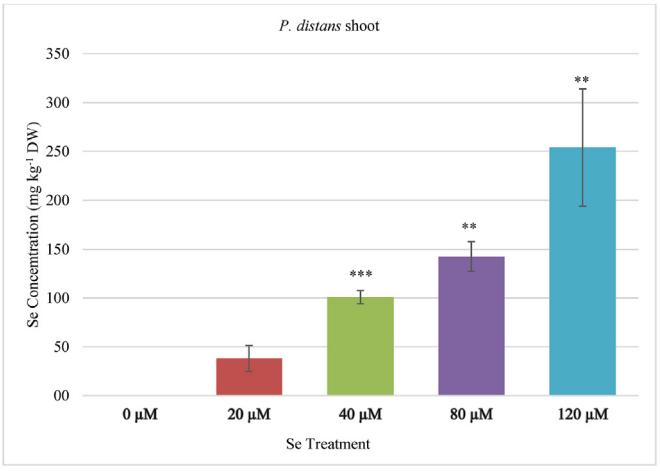

Fig. 1. Selenium concentration in shoots of control (0 µM) and treated plants (20, 40, 80 and 120 µM). Plants were treated with Se for 7 d. Values in the graph represent means (±SD) of three biological and three technical replicates. Asterisks (**) and (***) indicate $0.001 < p \leq 0.01$ and $p \leq 0.001$, respectively, as determined by Student's t-test.

These results indicated that *P. distans* could both tolerate and accumulate very high levels of Se. Tolerance was indicated by the ability of the plants to survive and continue to grow under high Se concentration. Accumulator plants are classified based on the amount of the element they accumulate in their tissues or by their capacity to accumulate the element compared to non-accumulators. 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* was found to be a novel Se accumulator. Indeed, *P. distans* may have an even greater capacity to accumulate Se than determined in this study 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. In addition, a time course of Se accumulation at different levels of exposure should be performed.

3.2. Transcriptome analysis of P. distans using RNA-Seq

3.2.1. De novo transcriptome assembly of reference genome

The reference genome was created using five cDNA libraries generated from mRNA from untreated control plant shoots as well as samples treated with selenium, arsenic, sulfur and nickel. The libraries were sequenced using Illumina HiSeq which resulted in 454×10^6 raw reads. After adapter trimming, sequence reads shorter than 50 nucleotides were discarded resulting in ~99.5% of the original reads. De novo transcriptome assembly led to association of 78,520 transcripts with the reference genome. 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.

3.2.2. Gene expression analysis

Sequence fragments from the control and Se treated samples 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. 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%). Gene expression data were visualized with a heatmap (Fig. S1). The top 100 up- and downregulated transcripts are listed in Table S2.

3.2.3. Gene ontology analysis for RNA-Seq data

Blast2Go analysis annotated 1492 transcripts to associated gene ontology (GO) terms. 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 (Fig. 2).

3.2.3.1. Biological process. The roles of the differentially expressed transcripts in the 22 biological process terms were examined by expanding the processes to descendant terms. The descendant

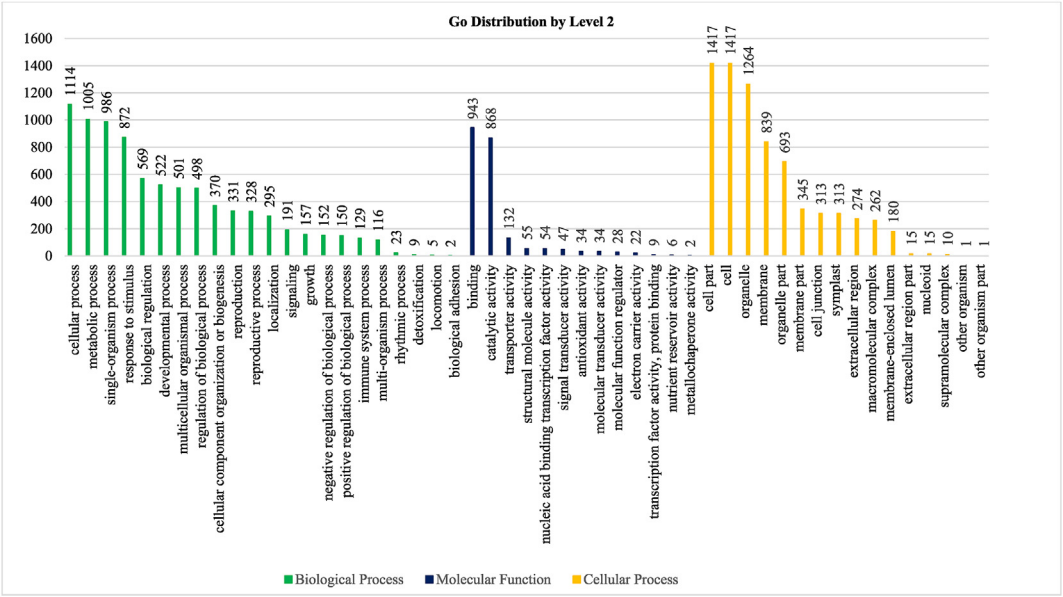

Fig. 2. GO annotation level distribution statistics of *P. distans* transcripts.

Table 1
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

Table 2
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, seq-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

terms were sorted based on the percentage of transcripts in each category (Table 1). A total of 17.7% of all transcripts were related to the 13 abiotic stress response terms that fell in the top 20. Seven of the remaining terms were related to developmental process (2.2%) and biotic stress response (2.2%). Some examples of response to abiotic stimulus were salt stress (2.4%), cadmium (Cd) ions (2.1%) and water deprivation (1.1%). Altered expression of transcripts related to salt stress and water deprivation might have resulted from growth in 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, creates a reducing environment and reactive oxygen species (ROS) production (Tedeschini et al., 2015). Therefore, it is not surprising that 2.7% of the transcripts were related to oxidative stress and oxidation-reduction reactions. The differential abundance of transcripts related to Cd may be due to the known links between Se and Cd transport and tolerance. It has been reported that Se affects the expression of genes encoding transporters with roles in Cd uptake and translocation to the upper part of the plant (Cui et al., 2018; Ismael et al., 2019). In addition, Se protects plants from Cd toxicity by decreasing the amount of ROS and lipid peroxidation while increasing the activities of various antioxidant enzymes (Khan et al., 2015; Ahmad et al., 2016). Therefore, it is not surprising that Se triggered changes in the expression of transcripts involved in the plant's response to cadmium stress.

3.2.3.2. Molecular function. GO annotation level distribution statistics grouped differentially expressed transcripts into 13 subcategories. The majority of the transcripts fell into the binding and catalytic activity subcategories (Fig. 2). Expansion of subcategories into descendant terms showed that the binding processes included protein, ATP, metal, mRNA and DNA binding. Specifically, 7.5% of all transcripts appeared to be part of zinc, copper, iron and general metal binding (Table 2). In addition, Se treatment led to altered gene expression in 7.3% of all transcripts which were directly related to transcription and translation processes. The relevant terms included mRNA binding, DNA binding, transcription factor activity, structural constituent of ribosome, sequence specific DNA binding and RNA binding.

3.2.3.3. Cellular component. Sixteen subcategories of cellular components were further expanded to descendant terms and the top 20 are provided (Table 3). According to this analysis, 3919 transcripts were associated with at least one cellular component. Since selenate is taken up through the plasma membrane and either stored in vacuoles or moves to plastids for further reductive assimilation (Ramos et al., 2011), 1433 transcripts (26.5%) were related with plasma membrane, chloroplast and vacuole associated GO-terms.

3.2.4. KEGG pathway analysis

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 S3). In some cases, more than one transcript was 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 (Fig. S2). Hydrolases (34.9%), transferases (26.9%) and oxidoreductases (22.7%) were the dominant groups. The most represented main KEGG pathway was metabolism with 1313 transcripts (95.9%).

Table 3
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

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.

3.2.4.1. Sulfur and selenocompound metabolism. Eight differentially expressed transcripts (seven upregulated and one downregulated) were found to encode eight enzymes in sulfur metabolism based on the KEGG pathway (Fig. S3). The change in the expression of two enzymes (adenylylsulfate reductase and phosphoadenosine phosphosulfate reductase) could not be determined because they were encoded by the same transcript.

The first and rate limiting step for selenate assimilation is the activation of selenate to adenosine 5′- phosphoselenate (APSe) by ATP sulfurylase (or ATP:sulfate adenylyl transferase; APS) (Pilon-Smits et al., 1999; Sors et al., 2005; Ramos et al., 2011). *APS1* was expressed at 4.3-fold higher levels under selenium stress in this study. A previous study showed that when the *APS1* gene was 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). Transgenic plants were more tolerant to Se and performed better. 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) and its expression was 4.5-fold higher in the stress as compared to the control group.

According to our findings, the expression of both sulfite reductase and serine *O*-acetyltransferase was upregulated while the expression of cysteine synthase was downregulated. Upregulation of sulfite reductase causes production of excess sulfide which prevents dissociation of *O*-acetylserine (thiol)-lyase from serine *O*-acetlytransferase. Association of these two enzymes reduces the activity of *O*-acetylserine (thiol)-lyase (Feldman-Salit et al., 2009). We found that the expression of the transcript encoding *O*-acetylserine (thiol)-lyase was downregulated by Se stress. This should result in a decrease in the conversion of selenide/sulfide to selenocysteine/cysteine. These results indicated that *P. distans* may decrease the production of selenocysteine to keep Se away from proteins.

The RNA-Seq results and selenocompound metabolism pathway (Fig. S4) suggest that *P. distans* used two additional mechanisms to prevent nonspecific incorporation of Se into proteins. First, it reduced the attachment of selenomethionine to selenomethionyl-tRNA (Met) by decreasing the expression of methionyl-tRNA synthetase by 2.4-fold. Second, it decreased the expression of cystathionine gamma-lyase (2.3-fold) to keep selenocysteine in a methylated form thereby decreasing the amount of available selenocysteine for incorporation into proteins.

3.2.4.2. Cell wall metabolism. Lignification of the cell wall is an important part of stress sensing and metal stress tolerance (Moura et al., 2010). 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 cinnamoyl-CoA reductase (CCR), phenylalanine ammonia-lyase (PAL), cinnamyl alcohol dehydrogenase (CAD) and 4 coumarate CoA-ligase (4CL) were upregulated (Fig. S5 and Table S4).

The results suggested that increased lignification may have helped *P. distans* to tolerate Se better by trapping the element in the cell wall and restricting its movement across the cell membrane. To understand whether Se binds to the cell wall or not, the Se content of a whole cell and protoplast can be compared. Reduction in the Se content in protoplasts compared to whole cell would indicate that some Se is trapped in the cell wall before it enters the cell. The next step should be to find the molecules with which Se interacts. In previous studies with yeast, it was suggested that polysaccharides (especially mannan) could be a good candidate for this interaction (Kieliszek et al., 2015). Additionally, lignification may have improved the durability of the cell wall by increasing its thickness and changing its mechanical properties (Cui et al., 2018).

3.2.5. Verification of differential expression

A total of 12 transcripts were chosen for verification of RNA-Seq analysis. The up- and downregulated transcripts are shown in Fig. 3. Transcript_112 was found to be downregulated by 20% according to RT-qPCR which was consistent with the RNA-Seq results (−20.3-fold). Transcript_112 encodes a MATE-efflux transporter which is responsible for sequestration of flavonoids from the cytoplasm into the vacuole (Eckardt, 2001). Perhaps the expression of this transporter was reduced under Se stress in order to maintain flavonoids in the cytoplasm as protection from oxidative stress. The expression of transcript_2159 encoding the sulfate transporter 4; 1 (SULTR4; 1, export of sulfate/selenate from vacuoles) was decreased 30% in Se-treated plants compared to control based on qRT-PCR result.

The expression of glutathione S-transferase (GST, EC 2.5.1.18) encoded by transcript_6651 increased 30% compared to the control group. Similarly, the transcript level of GST was 3.2-fold higher in the stress group relative to control in RNA-Seq analysis. GST has a role in the transfer of the selenate ion to glutathione to make glutathione-S conjugate. The chelation of selenate with glutathione is necessary because it helps the transport of selenate into the vacuole via the ABC transporter (a.k.a glutathione S-conjugate-transporting ATPase 4) (Zhou et al., 2018). In this work, the expression of three transcripts encoding ABCC.4 were upregulated according to RNA-Seq analysis. Transcript 3063 was upregulated 2.3-fold, transcript 53629 was upregulated 3.2-fold and transcript 33226 was upregulated 6.2-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 (Fig. 4). This response may also be a protective mechanism of the plant against the adverse effects of Se.

Heat shock proteins (Hsp) 70 and 20 are molecular chaperones

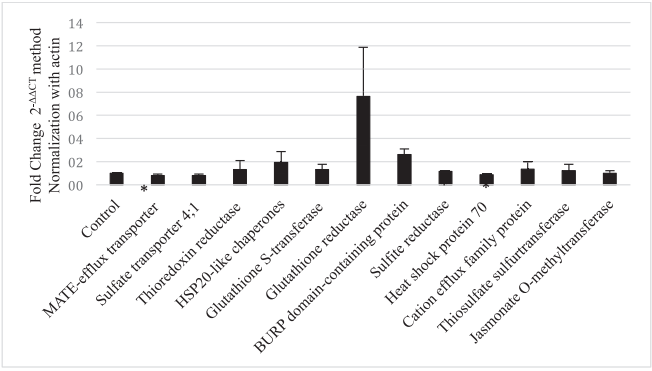

Fig. 3. RT-qPCR results of 12 selected transcripts amplified in P. distans shoots treated with 80 μM Se. The data represent the mean ± SD ($p > 0.05$) values of three replicates (paired Student's t-test). Asterisks (*) indicate significant differences at $p \leq 0.05$ with respect to control.

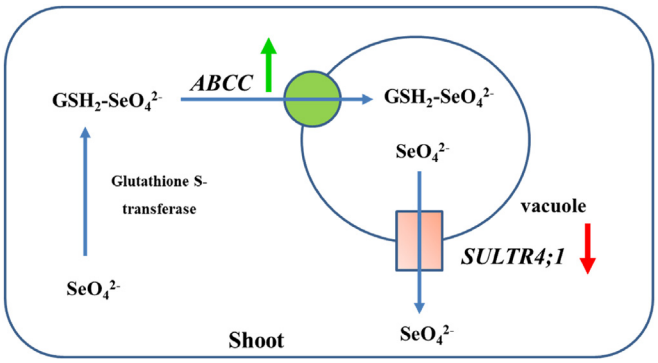

Fig. 4. Mechanism for selenium sequestration in the vacuole. The green arrow indicates upregulation and the red arrow indicates downregulation of gene expression as observed in transcriptomic analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in the cytoplasm and they work in cooperation to maintain protein homeostasis (Al-Whaibi, 2011; Park and Seo, 2015). The expression of heat shock proteins Hsp70 and Hsp20 were upregulated 31.1 and 87.4-fold, respectively according to the RNA-Seq analysis. Although the RT-qPCR result of Hsp70 (transcript_29272) did not agree with the RNA-Seq results, the expression analysis indicated that expression of Hsp20 (transcript_3369) increased 1.9-fold.

Jasmonate (JA) is synthesized from alpha-linolenic acid and is important for Se tolerance because JA signaling has been reported to positively regulate sulfur and antioxidant metabolism and increase resistance to Se stress. 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 at a high level in the Se hyperaccumulator *S. pinnata* (Pilon-Smits et al., 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 but it could not be verified with RT-qPCR. The transcriptome analysis also showed elevated expression of JA biosynthesis genes encoding eight different enzymes (Table S5 and Fig. S6) including the key enzymes acyl-CoA oxidase and linoleate 13S-lipoxygenase. Similarly, previous studies showed that the genes encoding these enzymes were constitutively upregulated in *S. pinnata* compared to non-accumulator relatives (Tamaoki et al., 2008; Pilon-Smits et al., 2017; Wang et al., 2018).

The expression of many transcripts involved in sulfur metabolism were altered due to Se treatment. For example, expression of transcript_41354 encoding thiosulfate sulfurtransferase which catalyzes the conversion of thiosulfate to sulfite was upregulated by 8.9-fold based on RNA-Seq with a 20% increase in expression confirmed by RT-qPCR. Thioredoxin reductase converts selenite to hydrogen selenide (Fig. S4). The expression of transcript_2796 encoding thioredoxin reductase was expressed more in Se treated plants compared to control based on both the RNA-Seq (10.6-fold) and RT-qPCR (30%) results.

RT-qPCR results of transcripts encoding BURP domain-containing protein and cation efflux family protein did not show similar trends with the RNA-Seq results. 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.

3.3. Biochemical parameters

The amounts of chlorophylls and antioxidant compounds in Se-treated and control plants were compared to assess the level of stress experienced by the plants and to see if the results agreed with expectations based on the transcriptome analysis.

3.3.1. Chlorophyll content

Chlorophyll content of Se treated *P. distans* plants was analyzed to check their stress level since Se accumulation led to slight chlorosis in shoots. Se treated plants showed significant ($p < 0.0001$) decreases in chlorophyll *a* (33%), chlorophyll *b* (64.3%) and total chlorophyll (46.5%) contents as compared to control plants. The reduced chlorophyll levels were more likely due to a decrease in chlorophyll biosynthesis rather than an increase in chlorophyll breakdown because the expression of enzymes (magnesium chelatase subunit H and magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase) carrying out the consecutive steps of chlorophyll *a* synthesis from protoporphyrin IX were downregulated according to RNA-Seq analysis (Table S6). Synthesis of chlorophyll *a* is an important step because it is the precursor of chlorophyll *b* (Fig. S7). The transcript encoding chlorophyllide a oxygenase which catalyzes this conversion was downregulated by 2.6-fold in our study. A decrease in chlorophyll content could also 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 a decrease in chlorophyll content (Lunde et al., 2008).

3.3.2. Antioxidant and redox capacity

Selenium has a positive effect on the amount and activity of antioxidant enzymes and metabolites. ROS are 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 (Gajewska and Sklodowska, 2007). To understand the effect of Se treatment on the antioxidant system, the activity of glutathione reductase (GR) and guaiacol peroxidase (POD) were measured.

GR recycles glutathione disulfide to glutathione, a non-enzymatic antioxidant (Bela et al., 2015). Se treatment led to a significant increase (almost 2-fold, $p < 0.001$) in GR activity in Se-treated plants compared to control (Fig. 5). This increase in GR activity confirmed the RNA-Seq results which indicated that the expression of transcript_8650 and transcript_6816 encoding GR were upregulated 2.5 and 5.5-fold, respectively. In addition, GR was expressed 7.6 times more in Se-treated plants compared to the control group according to RT-qPCR results.

POD is involved in ROS elimination and is a key enzyme in the biosynthesis of lignin. Guaiacol peroxidase takes part in the cross-linking of monolignols (coniferyl and sinopyl alcohol) to produce guaiacyl and syringyl lignin. Here, the activities of POD in Se treated plants and control groups were found to be almost the same (Fig. 5). However, Se treatment caused the upregulation of transcripts encoding POD (Table S7). Thus, the enzyme activity assay did not show a similar trend as the quantitative transcriptome data. This may have been due to post-transcriptional or translational regulation. The disparity between enzyme activity and mRNA level may also be linked to several other biological factors (ex. codon bias, protein half-lives, regulatory proteins and small RNAs) and some methodological constraints (Glanemann et al., 2003; Maier et al., 2009).

In previous work, the expression of additional antioxidant enzymes was shown to be upregulated in Se treated plants as compared to control (Freeman et al., 2010; Wang et al., 2018). Here,

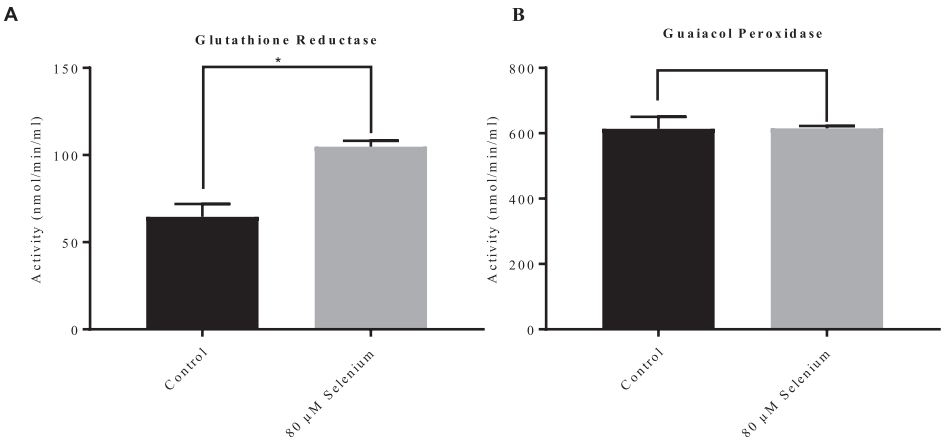

Fig. 5. A) Glutathione reductase and B) guaiacol peroxidase activity of control and 80 µM selenium treated *P. distans* plants. The data represent the mean ± SD ($p < 0.001$) values of three replicates (paired Student's t-test). Asterisks (*) indicate significant differences.

we found that in addition to GR and POD, transcription of catalase, glutathione peroxidase, superoxide dismutase, and glutathione S-transferase were upregulated (Table S7 and Fig. S8). Although most of the antioxidant enzymes were upregulated in Se-treated *P. distans*, the expression of ascorbate peroxidase was downregulated.

4. Conclusion

This study identified *P. distans*, weeping alkali grass, as a novel Se-accumulator and provides a preliminary model for this plant's Se tolerance and accumulation mechanism. According to this model, after Se is taken up by the root, it triggers the production of ROS. In turn, ROS production induces JA synthesis and JA signaling upregulates both antioxidant and defense response genes and Se/S assimilation genes to enhance the capacity of *P. distans* to cope with Se stress. However, the localization, distribution and chemical speciation of Se in the shoot are still unknown and must be investigated. The effect of different Se concentrations on the abundance and/or activity of sulfate transporters should also be studied. Overall, a better understanding of the selenium tolerance mechanism in *P. distans* could pave a path toward exploitation of this species for phytoremediation and biofortification.

Author contributions

ABK performed laboratory experiments; MDM performed bio-informatics analysis; SD and AF designed the study; ABK and AF drafted and revised the manuscript. All authors read and agreed upon the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125665.

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