New Phytologist

Research

Influence of sulfate supply on selenium uptake dynamics and expression of sulfate/selenate transporters in selenium hyperaccumulator and nonhyperaccumulator Brassicaceae

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Received: *5 June 2017*
Accepted: *8 September 2017*

New Phytologist (2017)
doi: 10.1111/nph.14838

Key words: *Brassica juncea*, competition, hyperaccumulation, selenate, *Stanleya elata*, *Stanleya pinnata*, substrate specificity, sulfate.

Summary

- *Stanleya pinnata* not only hyperaccumulates selenium (Se) to 0.5% of its dry weight, but also exhibits higher tissue Se-to-sulfur (S) ratios than other species and its surroundings.
- To investigate the mechanisms underlying this Se enrichment, we compared *S. pinnata* with the nonhyperaccumulators *S. elata* and *Brassica juncea* for selenate uptake in long- (9 d) and short-term (1 h) assays, using different concentrations of selenate and competitor sulfate. Different sulfate pre-treatments (0, 0.5, 5 mM, 3 d) were also tested for effects on selenate uptake and sulfate transporters' expression.
- Relative to nonhyperaccumulators, *S. pinnata* showed higher rates of root and shoot Se accumulation and less competitive inhibition by sulfate or by high-S pretreatment. The selenate uptake rate for *S. pinnata* (1 h) was three- to four-fold higher than for nonhyperaccumulators, and not significantly affected by 100-fold excess sulfate, which reduced selenate uptake by 100% in *S. elata* and 40% in *B. juncea*. Real-time reverse transcription PCR indicated constitutive upregulation in *S. pinnata* of sulfate transporters SULTR1;2 (root influx) and SULTR2;1 (translocation), but reduced SULTR1;1 expression (root influx).
- In *S. pinnata*, selenate uptake and translocation rates are constitutively elevated and relatively sulfate-independent. Underlying mechanisms likely include overexpression of SULTR1;2 and SULTR2;1, which may additionally have evolved enhanced specificity for selenate over sulfate.

Introduction

Selenium (Se) is an indispensable micronutrient for humans and animals, but although considered to be a beneficial element, its essentiality for plants has not yet been established (Sors *et al.*, 2005; Schiavon & Pilon-Smits, 2017a). Inadequate dietary Se intake by humans is responsible for a number of diseases, including thyroid disorders, reduced fertility and immune function, and may lead to increased risk of developing cancers and infections (Rayman, 2000, 2002). To date, between 0.5 and 1 billion people may suffer from Se deficiency worldwide and the number is likely growing (Combs, 2001; Jones *et al.*, 2017). Because crops represent the main source of dietary Se intake for most Se-deficient individuals, crop enrichment in this element might be envisioned as a tool to counteract the issue of Se deficiency (Wu *et al.*, 2015; Schiavon & Pilon-Smits, 2017b). Strategies commonly employed to biofortify plants with Se include Se fertilization, breeding crops with enhanced Se uptake traits and genetic manipulation to improve crop Se uptake capacity (Broadley *et al.*, 2006; Wu *et al.*, 2015; Schiavon & Pilon-Smits, 2017b). Problems can be posed not only by Se deficiency, but also by Se toxicity at elevated concentrations. Plants also can be used to phytoremediate Se in regions where Se concentration in soil is sufficiently high to pose a threat to wildlife, livestock and local populations (Schiavon & Pilon-Smits, 2017b). It is critical to unravel mechanisms of Se uptake by plants and the factors affecting it, in order to attain efficient Se enrichment in plants that can be used for either Se phytoremediation or human/animal nutrition purposes.

Selenium in soil occurs mainly as inorganic compounds, primarily in the form of selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}). Selenate is generally more abundant and available to plants than selenite in soils under oxidizing conditions and, owing to its chemical similarity to sulfate, it is absorbed by plants via sulfate permeases (Ellis & Salt, 2003; Sors *et al.*, 2005; Schiavon *et al.*, 2015). The role of root sulfate transporters in mediating selenate uptake by plants was first established in *Arabidopsis thaliana* mutants defective in the expression of a functional high-affinity sulfate transporter SULTR1;2 (Shibagaki *et al.*, 2002). Plants carrying this mutation were extremely resistant to selenate compared

to the wild-type. Consequently, SULTR1;2 has been recognized as the main portal for the influx of selenate into plant roots. The high-affinity sulfate transporter SULTR1;1 can also contribute to Se uptake, but its expression level is mainly detectable under S deficiency (Barberon *et al.*, 2008). Once absorbed, selenate can be loaded into the xylem, predominantly by the low-affinity sulfate transporter SULTR2;1, and transferred to the aerial parts of the plant, where it can enter the sulfur reductive assimilation pathway to be converted into seleno-amino acids. Additional sulfate transporters may be involved in the movement of selenate over plastid (SULTR3;1) and vacuolar (SULTR4;1 and SULTR4;2) membranes, or may assist in translocation (SULTR2;2, SULTR3;5) (Kataoka *et al.*, 2004; Cao *et al.*, 2013)

Depending on environmental conditions (Se and S, in particular), a single plant will express different sulfate transporters, which may differ in selectivity for sulfate vs selenate (White *et al.*, 2004). The inducible sulfate transport system in *Arabidopsis thaliana*, which is upregulated by low external sulfate concentration, has been reported to be more selective for sulfate over selenate than the constitutively expressed sulfate uptake system (White *et al.*, 2004).

Transport selectivity for selenate and sulfate also varies depending on the plant species (White *et al.*, 2004; White, 2016). Based on their capacity to accumulate Se in their natural environment, plants can be divided into three main groups: nonaccumulators, which contain less than 100 μg Se g^{-1} DW; secondary accumulators like *Brassica juncea* and *B. napus*, which thrive on soils with low-to-medium Se content and accumulate up to 1000 μg Se kg^{-1} DW; and hyperaccumulators, like some species of the genera *Stanleya* (Brassicaceae) and *Astragalus* (Fabaceae), which can accumulate over 1000 μg Se g^{-1} DW in all organs (0.1–1.5%) while growing on seleniferous soils containing 2–10 ppm Se (Terry *et al.*, 2000; Galeas *et al.*, 2007; Pilon-Smits & LeDuc, 2009). The high Se concentrations in hyperaccumulators are likely to serve ecological roles in protection from herbivores and pathogens, and perhaps in elemental allelopathy (El Mehdawi & Pilon-Smits, 2012). Differences in Se content between hyperaccumulators and nonhyperaccumulators might be due to differential regulation of sulfate transporters, as well as differences in kinetic properties and substrate specificities of these transporters (White *et al.*, 2004; Cabannes *et al.*, 2011; Schiavon *et al.*, 2015). Hyperaccumulators may have higher or more constitutive expression of one or more sulfate transporters involved in selenate uptake. In an earlier study, the transcript of *Sultr1;2* was found to be more abundant in the hyperaccumulator *Stanleya pinnata* compared to *B. juncea* (Schiavon *et al.*, 2015). There is also evidence that hyperaccumulators possess sulfate transporters that preferentially transport selenate over sulfate: Se hyperaccumulators are characterized by high Se/S ratios compared to nonhyperaccumulators and to their growth substrate (White *et al.*, 2007; Harris *et al.*, 2014; Schiavon *et al.*, 2015).

The goal of this study was to further elucidate how *S. pinnata* achieves its extraordinary Se concentrations, particularly its Se enrichment relative to S. Potential mechanisms explored here include differences in kinetic properties of sulfate/selenate transporters (i.e. higher specificity for selenate than sulfate) or in their regulation (lack of S-dependent repression, overall elevated expression level). The properties of the sulfate/selenate transport systems in *S. pinnata* were compared with the related nonhyperaccumulators *Stanleya elata* and *B. juncea*. Both species have been characterized earlier to accumulate less Se and be more Se-sensitive than *S. pinnata* (El Mehdawi & Pilon-Smits, 2012; Cappa *et al.*, 2014, 2015). The three species were compared with respect to their capacity to accumulate Se in the long term (9 d) and short term (1 h), in relation to the ambient sulfate concentration and plant S status. We also compared the transcript levels of *Sultr1;1*, *Sultr1;2* and *Sultr2;1* of these species under S-deplete, S-replete and excess-S conditions, and investigated the kinetics of selenate uptake in the presence or absence of the inhibitor sulfate.

Materials and Methods

Plant material

Stanleya pinnata ((Pursh) Britton) seeds were obtained from Western Native Seed (Coaldale, CO, USA). *Stanleya elata* (M.E. Jones) seeds (accession #113) were collected from nonseleniferous soil in Nevada at 37°26.699′N 117°21.896′W, at an elevation of 1515 m above sea level. *Brassica juncea* (L.) Czern. was originally obtained from the US Department of Agriculture plant introduction station, as described previously (Pilon-Smits *et al.*, 1999).

Plant growth

Stanleya pinnata, *S. elata* and *B. juncea* seeds were surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10-min rinses in sterile water. Seeds were allowed to germinate on sterilized, wet filter paper in the dark at 23°C inside a plant growth cabinet. Once germinated, seedlings were transplanted into pots filled with Turface®/sand (2 : 1) and irrigated for 2 wk with 0.2× Hoagland nutrient solution (Hoagland & Arnon, 1938) containing 0.2 mM $MgSO_4$. Seedlings were placed inside a growth chamber at 24°C, under fluorescent lights, with a 16 h : 8 h, light : dark photoperiod. Then, plants of similar size were transferred to 10-l hydroponic-containers (density = 2.4 plant l^{-1}) and cultivated for 2 wk, while receiving 0.5× Hoagland nutrient solution (containing 0.5 mM $MgSO_4$). Subsequently, the plants were incubated with different Se and S concentrations, as described in further paragraphs.

Long-term selenate uptake experiment

In order to measure long-term selenate uptake, plants were transferred to 500-ml containers containing a continuously aerated 0.5× Hoagland nutrient solution either with 0.5 or 5 mM sulfate ($MgSO_4^{2-}$), plus one of the following sodium selenate (Na_2SeO_4) concentrations: 0 (control), 10, 20, 40 and 80 μM. For each selenate/sulfate combination tested, three containers were prepared with three plants each. The experiment lasted 9 d and the nutrient solution in the containers was replaced once, in the middle of the incubation period. At the end of this period, plants were harvested, carefully washed in distilled water,

separated into shoots and roots and dried at 50°C. Dry plant material was weighed and then used for elemental analysis.

Short-term selenate uptake experiments

Effect of sulfate pre-treatment and sulfate concentration on selenate uptake In order to test the effects on selenate uptake of plant sulfur (S) status and of competing sulfate in the incubation solution, plants were grown in hydroponics for 2 wk with 0.5× Hoagland nutrient solution, rinsed with deionized water and then transferred to 500-ml containers and cultivated for 3 d in 0.5× Hoagland solution with modified sulfate concentrations: 0, 0.5 (i.e. the S concentration of 0.5× Hoagland solution) and 5 mM. Then, for the short-term selenate uptake experiment the plants were incubated for 1 h in 2 mM MES buffer (pH 5.6) with 0, 0.5 or 5 mM sulfate and 50 μM selenate. The uptake experiment was conducted in 100-ml containers with one plant per container, and each treatment (selenate/sulfate combination) was replicated three times. We had previously determined 1 h to be an optimal duration for these studies, because the uptake was linear from 30 to 120 min and the resulting shoot selenium (Se) concentrations were well above the ICP-OES detection limit (results not shown). After 1 h uptake, the plants were transferred to an ice-cold desorption solution (2 mm $CaCl_2$, 2 mm MES, pH 5.6) for 2 min. Then the roots were blotted dry, shoots and roots were separated, dried at 50°C and used for the determination of Se and S concentrations. To assay the efficiency of Se desorption from the root cell apoplast, additional plants were harvested following 5 min in ice-cold uptake solution in the presence of different selenate/sulfate concentration combinations. The Se concentration measured in these plants was negligible (not shown).

Selenate uptake kinetics in the presence or absence of sulfate In order to investigate the effects of the competitor ion sulfate on selenate uptake rates, roots of plants grown in hydroponics for 2 wk with 0.5× Hoagland nutrient solution were first rinsed with deionized water and then plants were placed in a series of uptake solutions containing 0 (for the determination of the background concentration of Se in plants from the seed), 20, 40, 80, 120 or 200 μM selenate, with or without 5 mM sulfate, in 2 mM MES buffer (pH 5.6). The experiment was conducted in 100-ml containers with one plant per container, and each treatment (selenate/sulfate combination) was replicated three times. After the 60 min uptake period, the plants were desorbed for 2 min in ice-cold desorption solution as described in the previous section, and processed for elemental analysis.

Elemental analysis

Foliar and root tissues of *S. pinnata*, *S. elata* and *B. juncea* plants were dried for 48 h at 50°C and then digested in nitric acid as described by Zarcinas *et al.* (1987). Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used as described by Fassel (1978) to determine each digest's Se and S concentrations using appropriate quality controls and standards. The detection limit for this machine was 0.02 mg Se l^{-1} in the digest, corresponding with 1–2 mg Se kg^{-1} DW.

Gene expression via quantitative real-time PCR

For quantitative reverse transcription PCR (RT-qPCR) experiments, RNA was extracted from root material from *S. pinnata*, *S. elata* and *B. juncea* plants (three biological replicates) grown in hydroponics for 2 wk in 0.5× Hoagland solution containing 0.5 mM sulfate, and then treated for 3 d with 0.5× Hoagland solution modified to the following sulfate concentrations: 0, 0.5 mM or 5 mM. In addition, three plants supplied with 0.5 mM sulfate were exposed for 1 h to 50 μM sodium selenate. Root and leaf samples from three biological replicates were flash-frozen for gene expression analysis.

RNA extraction was performed using a phenol/chloroform protocol according to Sambrook & Russell (2001). After DNAse treatment, cDNA was prepared from 3 μg of RNA per sample, using 200 U of Superscript Reverse Transcriptase III (Life Technologies, Carlsbad, CA, USA) and oligodT as primer in 20-μl reaction volume. Mixtures were incubated at 37°C for 60 min, 70°C for 5 min and 4°C for 5 min to stop the reverse transcription reaction. Specific primer pairs of sequences were designed based on conserved sequences among Brassicaceae spp. (Table 1). Primers were tested for their activity at 58–67°C by conventional PCR. RT-qPCR analyses were performed using a thermal cycler (Roche 480) equipped with a 96-well plate system with the SYBR green PCR Master Mix reagent (Applied Biosystems, Foster City, CA, USA). Each qPCR reaction (10 μl final volume) contained 1 μl of diluted cDNA (1 : 10), 1 μl of primer couple (10 μM), and 5 μl of 2 × SYBR Green PCR Master Mix according to the manufacturer's instructions. The following thermal cycling profile was used for all PCRs: 95°C for 10 min, 50 cycles of 95°C for 15 s and 60°C for 1 min. The gene expression analysis for each biological replicate was evaluated in two technical replicates, which showed similar results (only one set of data is shown).

All quantifications were normalized to the *actin1* gene used as housekeeping gene and amplified simultaneously under the same

Table 1 Sequences of primers used in quantitative real time RT-PCR experiments

Gene name	Forward primer 5′–3′	Reverse primer 5′–3′
Bj/Sp/SeSultr1;1	TGTTCATCACACCGCTCTTC	TGCTGCGTCAATGTCAATAAG
BjSultr1;2	ATGGCTGGATGTCAAACTGC	TCAGAGGAATCACTGCGTTG
Sp/SeSultr1;2	TAGTGATTGCTGCGAGGATG	CGTCGTTCTCTTGACATTGC
BjSultr2;1	TTGGGCTACAAGAAACTCGTC	CTGAAAATCCCGAAAGAAGC
Sp/SeSultr2;1	CATCGCCGTCTCACACCC	ATCGTTGCCGTTGTTGCTTT
Bj/Sp/SeActin1	AGCATGAAGATCAAGGTGGTG	CTGACTCATCGTACTCTCCCT

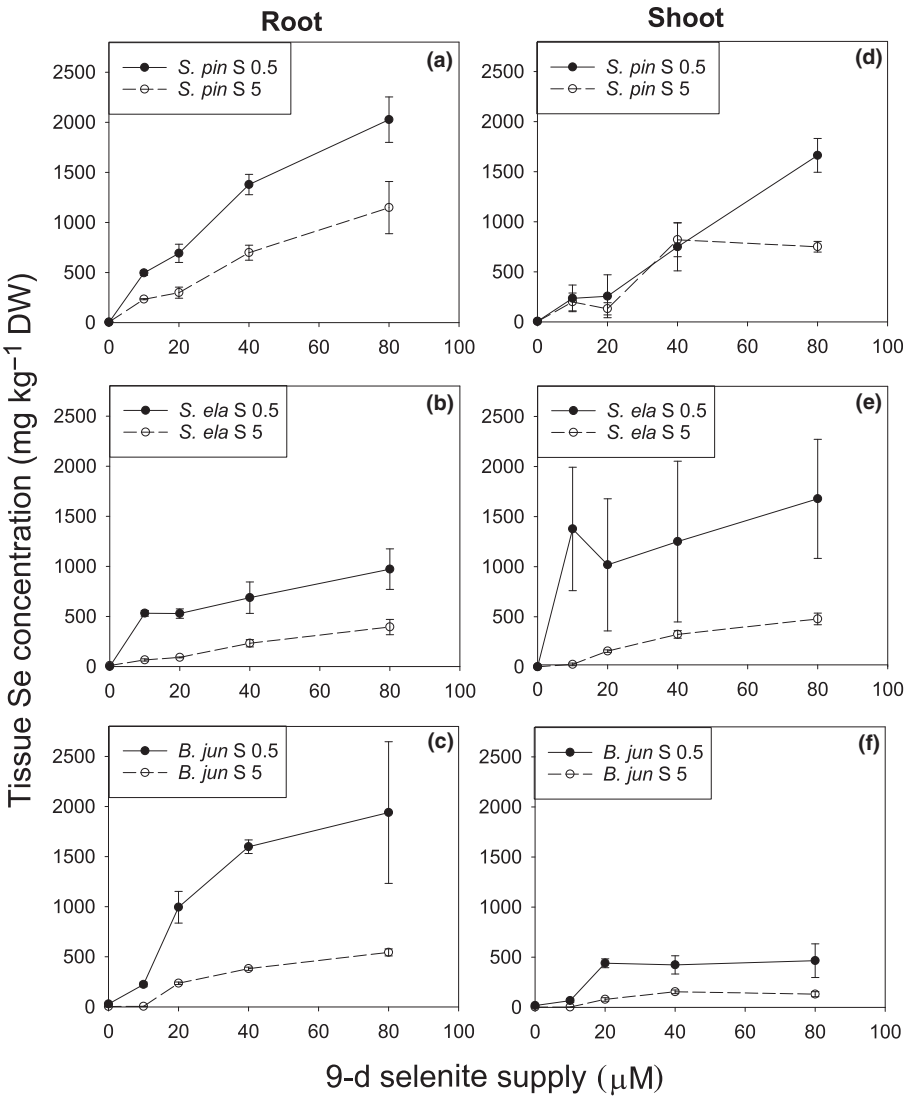

Fig. 1 Root and shoot selenium (Se) accumulation of 4-wk-old *Stanleya pinnata* (a, d; *S. pin*), *S. elata* (b, e; *S. ela*) and *Brassica juncea* (c, f; *B. jun*) plants grown in hydroponics with 0.5× Hoagland nutrient solution, supplemented during the last 9 d with different concentrations of Na_2SeO_4 (0–80 μM) and either 5 mM sulfate or no additional sulfur (0.5 mM). Values shown are the mean ± SEM ($n = 3$). The experiment was replicated twice and only data from one representative experiment are shown.

conditions. The obtained CT values were analysed with the Q-gene software by averaging three independently calculated normalized expression values for each sample. Expression values are given as the mean of the normalized expression values of the triplicates, calculated according to Eqn 2 of the Q-GENE software (Muller *et al.*, 2002).

Statistical analysis

The software JMP-IN (v.3.2.6; SAS Institute, Cary, NC, USA) was used for statistical data analysis, using Student's *t*-test or analysis of variance (ANOVA) followed by the post hoc Tukey–Kramer test. All datasets were tested for normal distribution and equal variance.

Determination of V_{max} and K_m were performed by SIGMAPLOT software (v.13), using the Michaelis–Menten equation. For the kinetics calculations, first any background Se originating from the seeds (as determined from the control treatment without added selenate) was subtracted from the root and shoot concentration data. For calculation of selenate uptake rate per g root DW over the 1 h experiment, the total amount of Se accumulated in the plant was divided by root DW, via the equation $([Se]_{root} \times DW_{root} + [Se]_{shoot} \times DW_{shoot})/DW$.

Results

Long-term Se uptake/accumulation experiment: influence of ambient sulfate concentration

The Se hyperaccumulator *S. pinnata* and the nonhyperaccumulators *S. elata* and *B. juncea* were treated with a range of selenate concentrations in the presence of either of two sulfate concentrations, 0.5 (normal S concentration in RT-qPCR Hoagland solution) and 5 mM (10-fold excess), in order to evaluate the effect of the presence of the competitor ion sulfate on Se uptake and accumulation by the three plant species in the long term (9 d). When exposed to selenate in the presence of low S, *S. pinnata* and *B. juncea* accumulated Se to similar concentrations in their roots, whereas *S. elata* contained two-fold lower Se concentration (Fig. 1a–c). Exposure to 10-fold higher S concentration (5 mM)

resulted in a decrease in Se accumulation in the roots of all three plant species, but the inhibitory effect was more pronounced for *B. juncea* and *S. elata*. At 80 μM Se, the sulfate-mediated reduction in root Se accumulation was 45% for *S. pinnata*, whereas the other two species showed 60–70% reduction. As a result, the root Se concentration under high S conditions in *S. pinnata* was two- to four-fold higher than those of the nonhyperaccumulators.

With respect to the shoot, *B. juncea* possessed a very low capacity to accumulate Se (Fig. 1f) compared to *S. pinnata* (Fig. 1d) and *S. elata* (Fig. 1e), regardless of the ambient S concentration. Shoot Se accumulation in *B. juncea* was 5- and 10-fold lower than *S. pinnata* under 0.5 mM S and 5 mM S, respectively. In *S. elata* and *S. pinnata*, Se shoot accumulation was not significantly different when plants were grown with 0.5 mM S (Fig. 1d, e). High-S treatment caused a pronounced reduction (70%) of Se concentration in *S. elata* and *B. juncea*, but in *S. pinnata* the Se concentration in the shoot was the same for plants supplied with 0.5 mM S or 5 mM S for all Se treatments except the 80 μM Se application (45% reduction). As a result, the shoot Se concentration under high-S conditions in *S. pinnata* was two- to four-fold higher than those of the nonhyperaccumulators.

The analysis of S concentration in tissues revealed that *S. pinnata* plants grown without Se were the highest in S, with values three-fold greater compared to those measured in *B. juncea* (Fig. 2). *Stanleya elata* plants displayed intermediate values of S accumulation, in both roots and shoots. In general, a decrease in S concentration in *S. pinnata* and *S. elata* plants in response to increasing Se concentration was evident, although it was more pronounced for the hyperaccumulator, especially in the shoot of plants grown with the lower S concentration of 0.5 mM. An opposite trend was observed for *B. juncea*, as in this case plants tended to accumulate more S in the presence of Se. In the shoot particularly, values of S concentration were two-fold higher in plants grown with Se compared to untreated plants.

Short-term experiment I: effects of S pre-treatment and Se/S ratio in incubation solution

Effects on selenate uptake Plants of *S. pinnata*, *S. elata* and *B. juncea* were pretreated for 3 d with different sulfate concentrations (0, 0.5 mM and 5 mM) and then incubated for 1 h with 50 μM selenate in the absence or presence of competitor

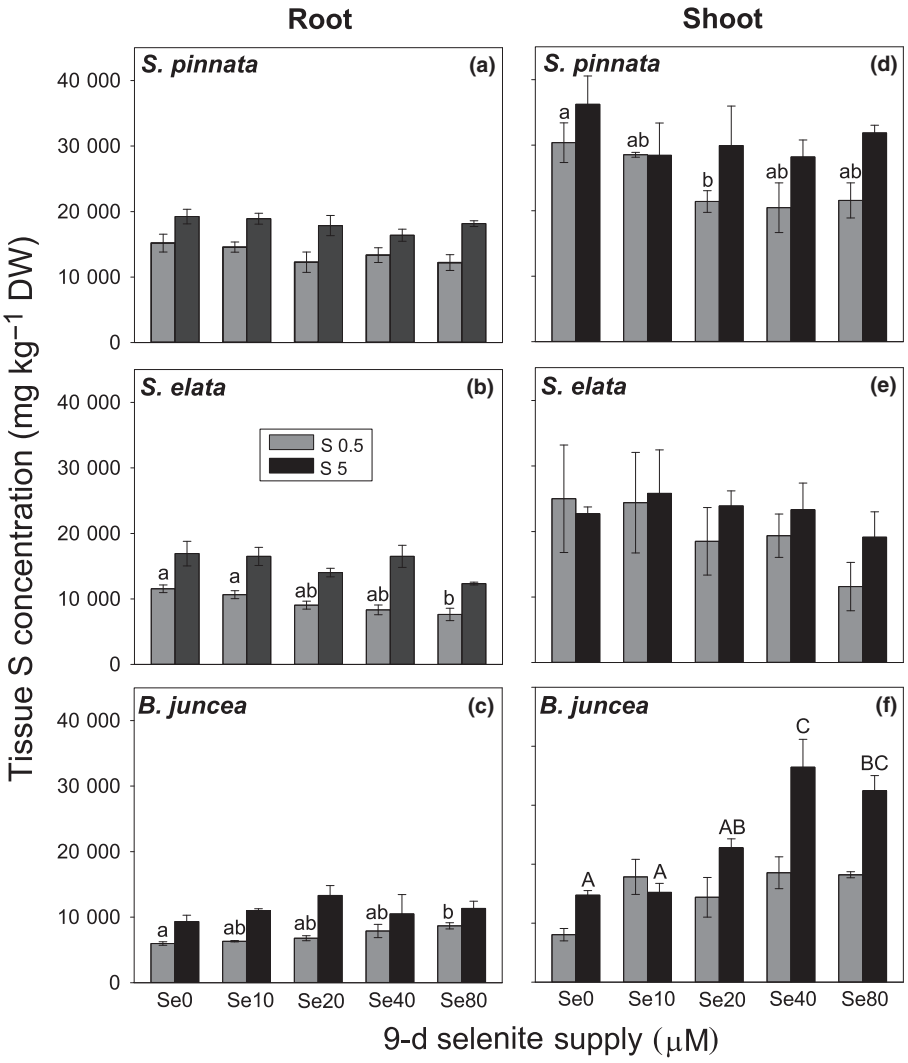

Fig. 2 Root and shoot sulfur (S) accumulation of 4-wk-old *Stanleya pinnata* (a, d), *S. elata* (b, e) and *Brassica juncea* (c, f) plants grown for 9 d in hydroponics with 0.5× Hoagland nutrient solution, supplemented during the last 9 d with different concentrations of Na_2SeO_4 (0–80 μM) and either 5 mM sulfate or no additional S (0.5 mM). Values shown are the mean ± SEM ($n = 3$). Different letters above bars denote statistically different means (ANOVA, $P < 0.05$).

sulfate (0, 0.5 mM and 5 mM). The hyperaccumulator *S. pinnata* differed from the other two species in that its selenate uptake was less affected by competing sulfate in the incubation solution. In the shoot, *S. pinnata* Se concentrations were not even significantly reduced by the highest competing sulfate concentration (Fig. 3; Supporting Information Fig. S1). In general, the reduction in Se accumulation by competing sulfate was more prominent for plants that had been pre-treated for 3 d without S (Fig. 3). Plants pretreated without S also generally showed the highest Se uptake, whereas the plants given the highest S pretreatment took up the least selenate (Fig. 3). However, *S. pinnata* selenate uptake was less affected by the 3 d S pretreatment compared to the other species, and shoot *S. pinnata* Se concentrations did not differ at all with sulfate pretreatment (Figs 3, S1). In the other two species, there was a dramatic negative effect of sulfate in the 3 d pretreatment on root and shoot Se accumulation, already apparent at 0.5 mM sulfate (Fig. 3b–f). The tissue concentrations of S were not significantly different among the 3 d S pretreatments (Fig. S2).

As a result of Se accumulation in *S. pinnata* being less negatively affected by sulfate in the uptake solution or in the pretreatment solution, the tissue Se concentrations in *S. pinnata* were higher compared to the other two species when the plants were pretreated with standard 0.5× Hoagland (0.5 mM sulfate) solution as well as after pretreatment with excess S; *S. pinnata* also accumulated more Se when any competing sulfate was present during the 1 h selenate uptake period. In the absence of competing sulfate, selenate uptake was comparable for the two *Stanleya* species (NS), whereas *B. juncea* showed lower Se accumulation than the other two species under all conditions, particularly in the shoot (Fig. 3).

Gene expression analysis of sulfate transporters In order to gain better insight into the molecular mechanisms underlying the observed differences between Se hyperaccumulator *S. pinnata*

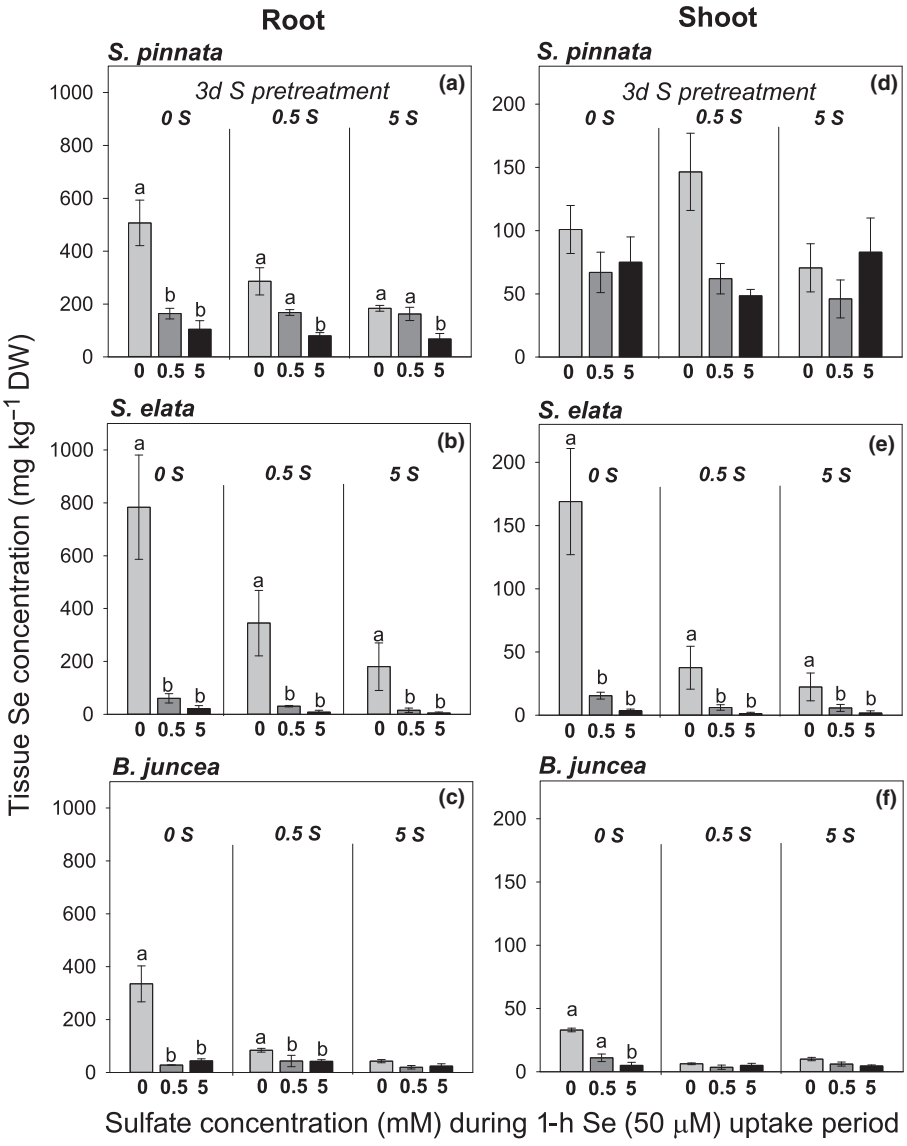

Fig. 3 Root and shoot selenium (Se) accumulation of 4-wk-old *Stanleya pinnata* (a, d), *S. elata* (b, e) and *Brassica juncea* (c, f) plants pre-treated for 3 d with 0.5× Hoagland nutrient solution modified to contain 0, 0.5 or 5 mM sulfate, and then incubated for 1 h with 50 μM Na_2SeO_4 in combination with 0, 0.5 or 5 mM sulfate. Values shown are the mean ± SEM ($n = 3$). Different letters above bars denote statistically different means, comparing the three sulfate concentrations during the 1 h incubation (ANOVA, $P < 0.05$).

and the nonhyperaccumulators *S. elata* and *B. juncea,* with respect to selenate accumulation under various S conditions, a transcript expression analysis was performed for several relevant root sulfate transporters (*Sultr1;1, Sultr1;2* and *Sultr2;1*), using plants pretreated for 3 d with the three sulfate concentrations (0, 0.5, 5 mM) as described above.

The transcript abundance of the high-affinity sulfate transporter *Sultr1;1* showed a strong dependence on S pretreatment concentration (Fig. 4a). The transcript levels indicate strong upregulation after 3 d of S depletion in all three plant species. However, its expression level was significantly higher (*c.* 30-fold) in the nonhyperaccumulators than in *S. pinnata*. In all three plant species, *Sultr1;1* was considerably downregulated under S-sufficient conditions (0.5 or 5 mM S), but it was upregulated by Se, especially in *S. elata*.

The transcript levels of the high-affinity sulfate transporter *Sultr1;2*, which is considered the main portal for the entry of selenate into root cells, were overall similar between *S. pinnata* and the Se nonhyperaccumulators under S starvation (Fig. 4b). However, in S-replete or S-excess conditions, *Sultr1;2* transcript abundance was up to seven-fold (0.5 S) higher in *S. pinnata* compared to22–42-fold (5 mM S) higher in *S. elata* and *B. juncea*. Interestingly, in *S. pinnata* the expression of *Sultr1;2* was not affected by S pretreatment, whereas *Sultr1;2* was downregulated by 0.5 and 5 mM S in *S. elata* and *B. juncea*. Furthermore, 1 h exposure to selenate appeared to upregulate *Sultr1;2* in the nonhyperaccumulators, but not in *S. pinnata*.

A striking difference in expression was found for the low-affinity sulfate transporter *Sultr2;1* – the main transporter for root-to-shoot sulfate transport (Kawashima *et al.*, 2011; Maruyama-Nakashita *et al.*, 2015), which was much more highly expressed in *S. pinnata* than in the nonhyperaccumulators under all conditions (Fig. 4c). Specifically, *Sultr2;1* transcript levels in *S. pinnata* were 35–50-fold higher compared to *S. elata*, and 300–1500-fold higher than in *B. juncea*. The *Sultr2;1* transcript levels were indicative of upregulation under S-depleted conditions in all three plant species.

Short-term experiment II: selenate uptake kinetics

An additional short-term selenate uptake experiment was performed using *S. pinnata*, *S. elata* and *B. juncea* plants pretreated with standard 0.5× Hoagland solution, to determine the kinetics of selenate uptake in the presence or absence of the competitive inhibitor sulfate. Incubation for 1 h in a range of selenate concentrations resulted in higher overall root and shoot Se accumulation in *S. pinnata* (Fig. 5a,d) compared to *S. elata* (Fig. 5b,e) and *B. juncea* (Fig. 5c,f), regardless of sulfate concentration. Specifically, in the presence of 0.5 mM sulfate, Se accumulation in *S. pinnata* was three-fold more pronounced than in *S. elata*, and four-fold greater than in *B. juncea*. When *S. pinnata* and *S. elata* plants were incubated in selenate in the competing presence of 5 mM sulfate, a reduction in root Se accumulation was evident in the roots for all species, whereas shoot Se accumulation was affected only in *S. elata*. Strikingly, in *S. elata* plants the uptake of selenate was so strongly inhibited by 5 mM sulfate that no Se could be detected in roots and shoots. *Brassica juncea* accumulated very low concentrations of Se in root and shoot in this short-duration experiment as compared to the other plant species. In the shoots of this species in particular, values of Se accumulation were up to 10-fold lower than in *S. pinnata* incubated with Se and without S.

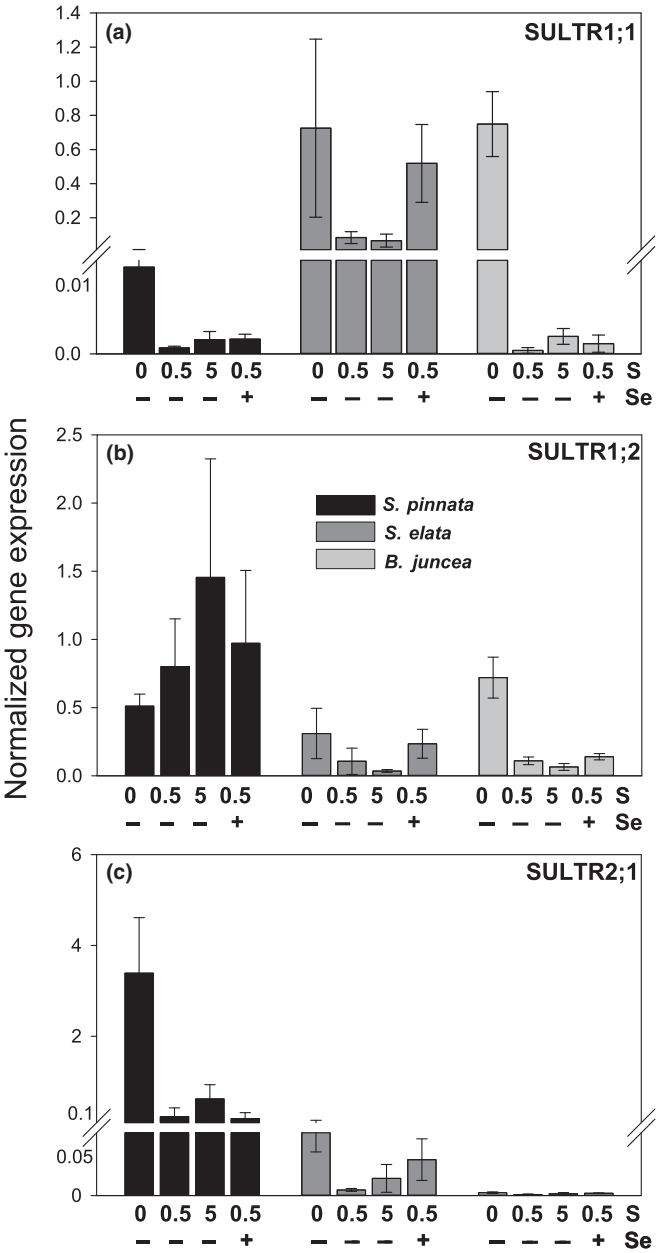

Fig. 4 Expression profiling by quantitative real time PCR of sulfate transporters *Sultr1;1* (a), *Sultr1;2* (b) and *Sultr2;1* (c), in roots of 4-wk-old *Stanleya pinnata*, *S. elata* and *Brassica juncea* plants grown for for 3 d with 0.5× Hoagland nutrient solution containing 0, 0.5 or 5 mM sulfate. Using a second set of plants supplied with 0.5 mM sulfate, the gene expression of sulfate transporters was also tested after 1 h incubation with 50 μM Na_2SeO_4. Data shown are the mean ± SEM of three biological replicates, and represent one of two technical replicates. Letters above bars indicate significant differences between the means, comparing treatments (ANOVA, $P < 0.05$).

In order to estimate root selenate uptake rate over the 1 h experiment, the total plant Se content (accumulated Se in shoot + root) was calculated and divided by root FW. Figure 6 shows Michaelis–Menten curve fits for the resultant uptake data for *S. pinnata*, *S. elata* and *B. juncea*.

Both in the absence and presence of competing sulfate, *S. pinnata* exhibited higher selenate uptake rates than *S. elata* and *B. juncea* (Fig. 6; Table 2). The fitted V_{max} values for *S. pinnata* were not significantly affected by sulfate in the incubation solution. However, the Se nonhyperaccumulators showed a substantial reduction in selenate uptake rates in the presence of the competitor sulfate. In *S. elata* in particular, no uptake of Se could be measured in 5 mM S, as mentioned.

The fitted K_m values for selenate uptake when incubated with Se for 1 h in the absence of S, revealed no appreciable differences between the three plant species (Table 2). However, when the substrate specificity for selenate was calculated from V_{max}/K_m, *S. pinnata* displayed a ~three-fold higher specificity for selenate as compared to *S. elata* and *B. juncea* (Table 2).

Discussion

The goal of this study was to determine whether selenium (Se) hyperaccumulation and Se enrichment in *Stanleya pinnata* is mediated by higher selenate-to-sulfate specificity of sulfate transporters in addition to the constitutive high expression of sulfate/selenate transporters. In this study, *S. pinnata* accumulated more Se than the two nonhyperaccumulator species, not only in the long term (9 d), as has been reported before, but it also showed three-fold higher short-term (1 h) selenate uptake rate on an equal root weight basis, when measured in the absence of competing sulfate. In the presence of sulfate, *S. pinnata* distinguished itself even more from the nonhyperaccumulator species: it was able to maintain high rates of selenate uptake and translocation under 100-fold higher competing sulfate concentration; moreover, its selenate uptake capacity was hardly diminished after 3 d of high-sulfur (S) pretreatment. Together, these findings are suggestive of enhanced selenate-over-sulfate transport specificity in the hyperaccumulator, and less repression of sulfate/selenate transporters by high S concentrations.

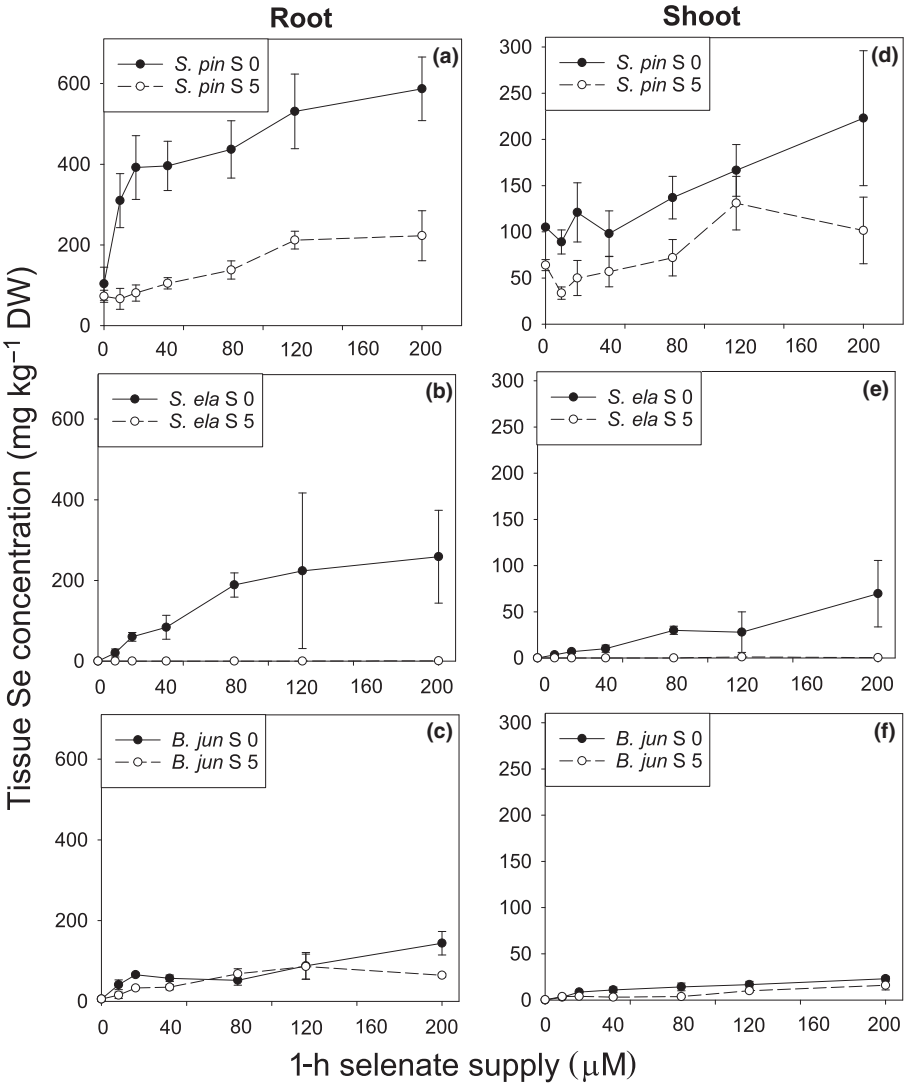

Fig. 5 Root and shoot selenium (Se) accumulation of 4-wk-old *Stanleya pinnata* (a, d; *S. pin*), *S. elata* (b, e; *S. ela*) and *Brassica juncea* (c, f; *B. jun*) plants incubated for 1 h with Na_2SeO_4 concentrations ranging from 0 to 200 μM in the presence of either 0 or 5 mM sulfate. Values shown are the mean ± SEM ($n = 3$).

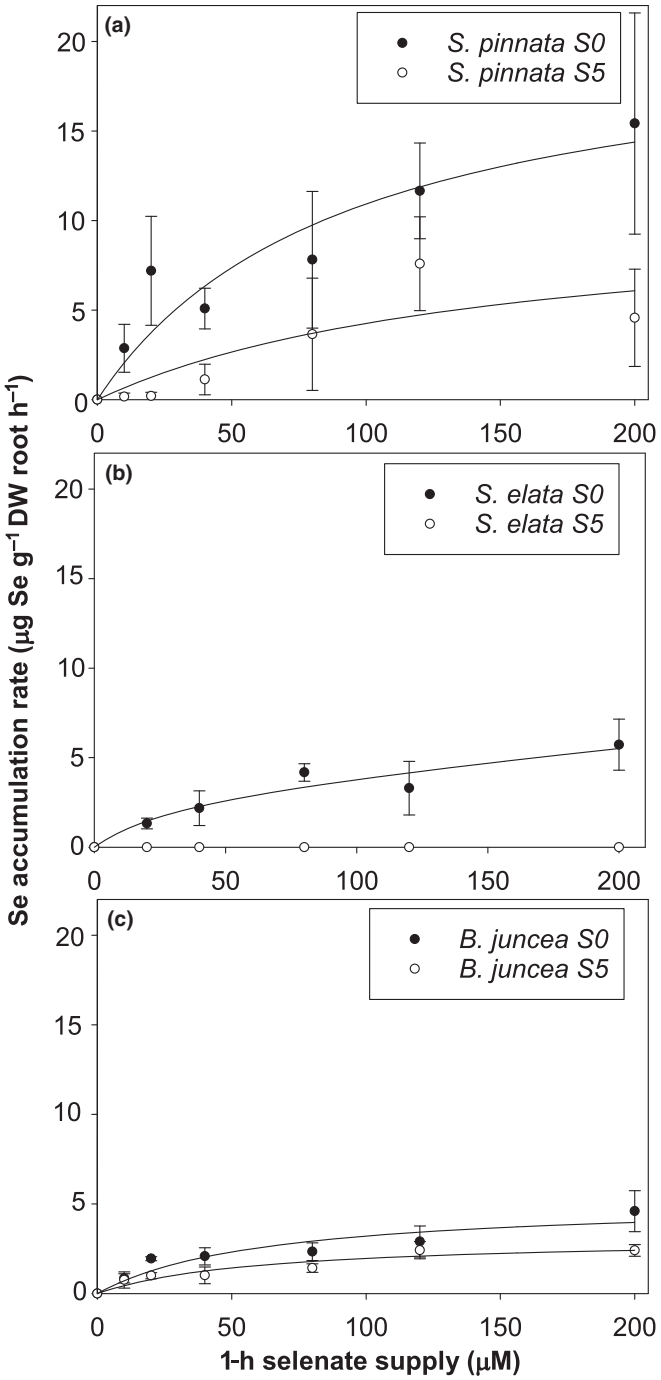

Fig. 6 Selenium (Se) accumulation rate in 4-wk-old *Stanleya pinnata* (a), *S. elata* (b) and *Brassica juncea* (c) plants incubated for 1 h with Na_2SeO_4 concentrations ranging from 0 to 200 μM in the presence of either 0 or 5 mM sulfate (calculated from data shown in Fig. 5, as described in the Materials and Methods section). Values shown are the mean ± SEM ($n = 3$).

These physiological results can be explained at least in part by the observed differential expression of three sulfate/selenate transporters, as depicted in Fig. 7. Compared to the other two species, *S. pinnata* had an order of magnitude higher transcript levels of *Sultr1;2* (high-affinity transporter mediating uptake into roots) and *Sultr2;1* (low-affinity transporter mediating xylem loading for transport to shoot), and the expression of *Sultr1;2* was not repressed under high-S pretreatment in the hyperaccumulator. Only when induced by 3 d of S starvation did the nonhyperaccumulators' cumulative *Sultr1* transcript levels match those of the hyperaccumulator. However, in that situation the hyperaccumulator still differed from the nonhyperaccumulators with respect to the relative contributions of *Sultr1;1* and *Sultr1;2*: *S. pinnata* had negligible transcript levels of *Sultr1;1* and very high *Sultr1;2* levels, whereas in *S. elata Sultr1;1* was clearly the most abundant transcript and in *Brassica juncea* both transporters were expressed at similar transcript levels.

The constitutive, high expression in *S. pinnata* of the two main transporters responsible for selenate uptake into the root and translocation to the shoot, and the absence of S-mediated regulation of *SpSultr1;2* are prominently different from nonhyperaccumulators (Buchner *et al.*, 2004; Rouached *et al.*, 2009; Takahashi *et al.*, 2011) and likely crucial for its capacity to hyperaccumulate Se. The mechanism underlying this deregulated overexpression awaits further genomic studies. In analogy with reported metal hyperaccumulation mechanisms, the overexpression may be due in part to one or more gene duplication events, which increase transport capacity and also allow for unconstrained evolution of one of the gene copies toward altered specificity (Hanikenne *et al.*, 2008; Lochlainn *et al.*, 2011; Craciun *et al.*, 2012). Both overexpression and deregulation may also be achieved via mutations in individual cis-regulatory sequences of the transporters (promoters, enhancers), or by changes in the coding sequences that influence regulatory protein–protein interactions, for example in the STAS domain of *Sultr1;2* (Rouached *et al.*, 2005; Shibagaki & Grossman, 2006; Takahashi *et al.*, 2011). It is also possible that the deregulation is a result of a change in a trans-regulatory component such as a transcription factor. It is interesting to note, however, that *Sultr2;1* is regulated by transcription factor SLIM via miRNA395, whereas *Sultr1;2* is not (Liang *et al.*, 2010; Kawashima *et al.*, 2011). Also, *Sultr1;1*, a gene normally upregulated under S starvation, appears to be downregulated in the hyperaccumulator, so it is not part of a concerted upregulation of *Sultr* genes due to perceived S starvation.

The typically observed high Se : S ratio in *S. pinnata* relative to other species and relative to its growth medium may be due in part to its exclusive use of SULTR1;2 for root uptake, if SULTR1;2 is relatively more selenate-specific and less sulfate-specific than SULTR1;1. There are mixed reports in this respect from studies with *Arabidopsis thaliana* (White *et al.*, 2004; El Kassis *et al.*, 2007; Barberon *et al.*, 2008). Alternatively, or additionally, the capacity of *S. pinnata* to enrich itself with Se over S may be due to an uniquely high selenate specificity of the hyperaccumulator's SULTR1;2 protein. More detailed functional analyses of isolated transporters are needed, but it is interesting to note that recent cloning and sequencing of *S. pinnata* and *S. elata Sultr1;2* cDNA showed seven amino acid differences, including three in the STAS domain and one in a membrane spanning domain (unpublished results). Also worth pointing out is that the 1 h selenate accumulation kinetics indicated a three-fold higher V_{max}/K_m ratio, indicative of higher selenate specificity, in the hyperaccumulator compared to

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Table 2 Kinetic parameters of selenate uptake in the selenium (Se)-hyperaccumulator *Stanleya pinnata* and nonhyperaccumulators *S. elata* and *Brassica juncea*

	S. pinnata		*S. elata*		*B. juncea*	
	0 S	5 mM S	0 S	5 mM S	0 S	5 mM S
V_{max}	21 ± 6.1	11 ± 8.5	8 ± 2.5	ND	5 ± 1.2	3 ± 0.6
K_m	93 ± 58	149 ± 22	110 ± 68	ND	62 ± 35	60 ± 30
V_{max}/K_m	0.23	0.08	0.07	ND	0.08	0.05

V_{max} is expressed as μg Se g^{-1} DW root h^{-1}, K_m is expressed as μM, specificity for selenate uptake is given by the ratio V_{max} to K_m. ND, not determined (no detectable Se).

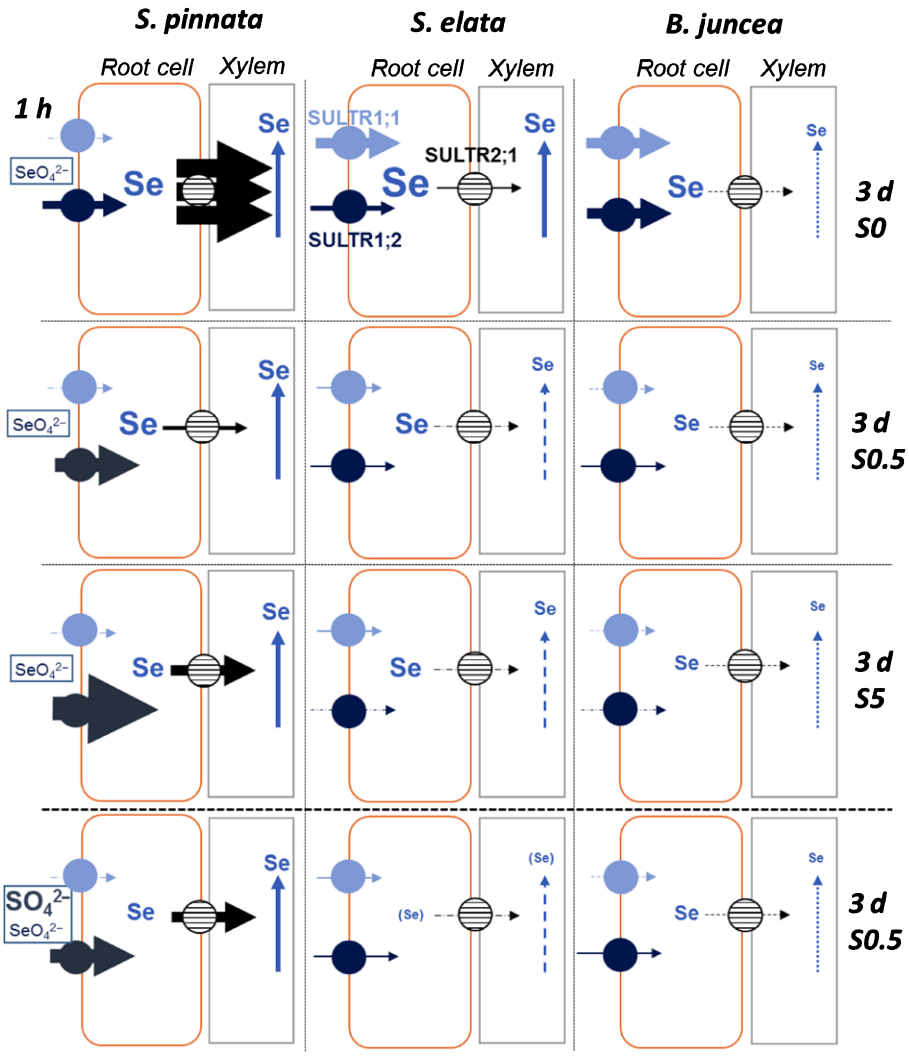

Fig. 7 Schematic overview of selenium (Se) uptake, translocation and accumulation in *Stanleya pinnata*, *S. elata* and *Brassica juncea* plants that were pre-treated for 3 d with different sulfur (S) concentrations and then incubated for 1 h with selenate without any competing sulfate (three top rows) or in the presence of excess sulfate (bottom row). Variation in root and shoot Se accumulation between species is indicated by different font size of 'Se' in root cell and xylem, respectively. Fluxes of selenate through *Sultr1;1*, *Sultr1;2* and *Sultr2;1* are indicated by arrows, the thickness of which is related to the gene expression of sulfate transporters in the three plant species (from Fig. 4).

S. elata and *B. juncea*. In the presence of competing sulfate, the apparent difference was even larger, but the ratio could not be determined for *S. elata* because selenate uptake was totally inhibited. Incidentally, the K_m values calculated here for selenate accumulation were intermediate between those reported for high-affinity sulfate transport systems (K_m 1.5–10 μM) and low-affinity plant sulfate transporters (400–1200 μM) (Takahashi *et al.*, 2000; Buchner *et al.*, 2004), which is probably because here the collective Se accumulated over 1 h in root and shoot was calculated, and thus the K_m values observed reflect the combined properties of various high- and low-affinity selenate/sulfate transporters into and within the plant.

The *S. pinnata* low-affinity SULTR2;1 may also contribute to selenate selectivity during the process of root-to-shoot translocation, considering its high expression levels and the observation that the differences between *S. pinnata* and the other species were

often most pronounced in the shoots. Incidentally, the shoot Se concentrations across the plant species did not always correspond with the *Sultr2;1* transcript levels, perhaps because other *Sultr* genes (*Sultr2;2, Sultr3;5*) also contribute to translocation (Takahashi *et al.*, 2000, 2011; Kataoka *et al.*, 2004; Gigolashvili & Kopriva, 2014), or because some Se may be assimilated in the root of the hyperaccumulator and translocated in organic form (Schiavon *et al.*, 2015). It is interesting to note that higher group 2 *Sultr* transcript levels were also reported in Se hyperaccumulating *Astragalus* species (*A. bisulcatus*, *A. racemosus*) as compared to nonhyperaccumulator species *A. drummondii and A. glycyphyllos* (Cabannes *et al.*, 2011). In the *Astragalus* study no individual group 2 genes were distinguished, so the effects of *Sultr2;1* and *Sultr2;2* cannot be determined.

The *S. pinnata* SULTR1;2 may have enhanced Se:S specificity, but still appears to have significant sulfate transport capacity as well, considering that transcript levels of *Sultr1;1* were negligible in *S. pinnata*, so SULTR1;2 likely is the sole root uptake pathway for sulfate, and *S. pinnata* had equal or higher S concentrations compared to nonhyperaccumulators. Interesting to note here is the declining trend of S accumulation in *S. pinnata* with increasing selenate supply, in contrast to the nonhyperaccumulators. This provides additional evidence that the sulfate transport system in the hyperaccumulator is more specific for selenate than sulfate compared to nonhyperaccumulators like *B. juncea*, for which actually an increase in S concentration was measured in the presence of selenate. This likely was due to the observed upregulation of *Sultr* genes.

Why would nature select for enhanced expression of sulfate/selenate transporters and for enhanced selenate specificity in a seleniferous habitat? Together, these mechanisms enable the hyperaccumulator to maintain high Se concentrations, even in high-S environments. Having enhanced Se accumulation likely offers *S. pinnata* physiological benefits, as it grows significantly better when supplied with Se (El Mehdawi & Pilon-Smits, 2012). Over the course of evolution, ever increasing Se accumulation was likely selected for because of incremental ecological advantages such as herbivory protection and allelopathic benefits (El Mehdawi & Pilon-Smits, 2012).

Future studies may focus on deeper investigation of the selenate-specificity mechanisms of *S. pinnata* SULTR1;2 and SULTR2;1. Better insight into mechanisms of sulfate/selenate specificity will be of broad interest for the field of enzymology, and may lead to applications in medicine. Also, if the ability to accumulate Se, even in the presence of high S concentrations, can be transferred from *S. pinnata* to other plant species via genetic engineering, this will have important applications in Se biofortification and phytoremediation, because these are often hampered by high S concentrations.

Acknowledgements

Funding was provided by National Science Foundation grant IOS-1456361 to E.A.H.P-S.

Author contributions

A.F.E.M. and M.S. planned and carried out the experiments and prepared the manuscript; Y.J., Z.S.G. and A.E. provided technical assistance; and M.P. and E.A.H.P-S. coordinated the project and helped prepare the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Heat map representation of the relative Se accumulation in roots and leaves of *S. pinnata*, *S. elata* and *B. juncea* plants as a function of the S pre-treatments and the Se : S ratios in the 1 h incubation solution.

Fig. S2 Root and shoot sulfur (S) accumulation of *S. pinnata*, *S. elata* and *B. juncea* pre-treated for 3 d with nutrient solution containing 0, 0.5 or 5 mM sulfate.

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