Investigation of selenium tolerance mechanisms in
Arabidopsis thaliana

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To investigate selenium tolerance mechanisms in Arabidopsis thaliana, genetic and physiologic studies were performed in the three Arabidopsis accessions Landsberg erecta (Ler), Columbia (Col) and Wassilewskija (Ws). Accession Ler was significantly less tolerant to selenate than Ws and Col, whereas Ws was less tolerant to selenite than the others. Analysis of selenium tolerance in F1 and F2 plants obtained from crosses between these accessions suggest that multiple genes are involved in selenate tolerance and a single major gene controls selenite tolerance in these populations. Bulked segregant analysis in two F2 populations indicated that molecular marker ciw7 on chromosome 4 is linked to selenite tolerance, and three molecular markers on chromosomes 1, 3 and 5 (nga111, ciw4 and ciw8, respectively) are linked to selenate tolerance. The ecotypic variation in selenite tolerance appeared to be correlated with root levels of non-protein thiols. Also, the shoot tissue levels of selenocysteine (SeCys) and selenocystine were correlated with tolerance to both selenate and selenite. Judging from RT-PCR results, several sulfate transporters and S assimilatory enzymes appear to be upregulated by selenate and selenite at the transcriptional level. A potential SeCys methyltransferase was expressed at lower levels in selenite-sensitive Ws than in the other two, when grown on selenite. Together, these studies show that there is substantial intraspecific variation in tolerance to selenate and selenite in Arabidopsis, and provide insights into the genetic and biochemical mechanisms underlying the observed ecotypic differences.

Introduction

The element selenium (Se) is both essential for many organisms and toxic at higher levels (Birringer et al., 2002). Se is essential because the amino acid selenocysteine (SeCys) is present in the active site of certain essential enzymes (Stadtman, 1990). The toxicity of Se is

Abbreviations – APS, adenosine triphosphate sulfurylase; CGS, cystathionine γ-synthase; GSH, glutathione; HMT2, homocysteine methyltransferase; ICP-AES, inductively coupled plasma atomic emission spectrometry; LC-MS, liquid chromatography–mass spectrometry; NPT, non-protein thiol; OAS, O-acetyl-L-serine; PC, phytochelatin; RIL, recombinant inbred line; RT-PCR, reverse transcription polymerase chain reaction; SAT, serine acetyl transferase; SeCys, selenocysteine; SeCystine, selenocystine; SeMet, selenomethionine; SMT, selenocysteine methyltransferase; SSLP, simple sequence length polymorphism; TI, tolerance index.

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thought to be due to its chemical similarity to sulfur (S), leading to non-specific replacement of S by Se in proteins and other sulfur compounds (Anderson, 1993). Both the importance of Se to human health and Se contamination in the environment have become a focus of research in recent years. To clean up Se-contaminated water and soil, phytoremediation is emerging as an efficient and cost-effective method (Terry et al., 2000). Several plant species classified as Se hyperaccumulators have the ability to extract and accumulate large amounts of Se even from low external Se concentrations. Examples of hyperaccumulator plants are Astragalus bisulcatus (Byers, 1936) and Stanleya pinnata (Feist and Parker, 2001). Currently, the use of Se-hyperaccumulating plants is limited, due to their slow growth and undefined growth requirements. Although the level of Se tolerance and accumulation in non-hyperaccumulator species is much lower than those of Se hyperaccumulators, the development of transgenic crops with improved Se accumulation and tolerance traits has shown promising results (Pilon-Smits et al., 1999; Van Huyzen et al., 2003; LeDuc et al., 2004). To enhance the success of these transgenic approaches, better knowledge is needed of the Se transporters and enzymes that limit Se accumulation and tolerance in non-hyperaccumulator plants.

Since Se is chemically similar to S, an essential plant macronutrient, Se tolerance may be governed by genes involved in S metabolism. Sulfate is transported over membranes by sulfate transporters (Leggett and Epstein, 1956), and the uptake and assimilation of selenate and sulfate are assumed to follow the same pathway (Ng and Anderson, 1979; Zayed and Terry, 1992; Anderson, 1993; Lauchli, 1993). In Arabidopsis, 14 putative sulfate transporter genes have been identified (Takahashi et al., 1999). Among the protein products of these, the sulfate transporters Sultr1;1 and Sultr1;2 are responsible for sulfate uptake from the soil into the plant root, and Sultr4;1 and Sultr4;2 are tonoplast proteins thought to control root-to-shoot translocation. Sulfate that accumulates in the vacuoles can be released by Sultr4;1 and Sultr4;2, and may subsequently enter the xylem stream (Kataoka et al., 2004).

As mentioned, selenate is thought to be incorporated through the sulfate reduction and assimilation pathway. Several in vitro studies have proposed that ATP sulfurylase (APS) is involved in the reduction of selenate as well as sulfate in plants (Shaw and Anderson, 1972; Dilworth and Bandurski, 1977; Burnell, 1981). The reduction of selenate to selenite was proposed to be rate-limiting for the selenate assimilation pathway on the basis of physiologic studies (de Souza et al., 1998). Indeed, overexpression studies showed that APS not only mediates selenate reduction in plants, but is also rate limiting for selenate uptake and assimilation (Pilon-Smits et al., 1999). Serine acetyltransferase (SAT) catalyzes the formation of O-acetyl-L-serine (OAS) from acety-CoA and L-serine. The OAS is subsequently coupled to sulfide to form cysteine. SAT is a major regulatory factor in the biosynthesis of cysteine in plants (Noji et al., 1998; Noji and Saito, 2002; Youssefian et al., 1993, 2001).

To prevent non-specific incorporation of SeCys into protein, plants can convert SeCys into volatile dimethylselenide, methylate it to methyl-SeCys, or break it down into elemental Se and alanine. Cystathionine-γ-synthase (CGS) is a rate-limiting enzyme for Se volatilization (Van Huyzen et al., 2003). It is thought to catalyze the conversion of SeCys to Se-cystathionine (Kim and Leustek, 1996), the first step in the conversion of SeCys to dimethylselenide. NiFS-like proteins can catalyze the formation of elemental S and alanine from cysteine or of elemental Se and alanine from SeCys. AtCpNiFS (Arabidopsis thaliana chloroplast NiFS-like protein) is the first characterized NiFS-like protein from higher plants (Pilon-Smits et al., 2002). Its overexpression in Arabidopsis was shown recently to lead to enhanced Se tolerance and accumulation (Van Hoewyk et al., 2005). Selenocysteine methyltransferase (SMT) is thought to be the main enzyme conferring Se tolerance to the Se hyperaccumulator Arabidopsis bisulcatus. SMT specifically methylates SeCys to produce the non-protein amino acid methylselenocysteine (Neuhierl and Böck, 1996). Arabidopsis and Indian mustard overexpressing SMT had increased Se tolerance (particularly to selenite) and accumulation, and volatilized higher levels of Se (Ellis et al., 2004, LeDuc et al., 2004). Recently, an SMT-like gene was discovered in Brassica oleracea (Lyi et al., 2005). The Arabidopsis thaliana HMT2 gene is its closest homolog.

A class of metabolites that may affect plant tolerance to Se and other stresses are the non-protein thiols (NPTs). These include cysteine, glutathione (GSH) and phytochelatins (PCs). All are redox active and play an important role in oxidative stress resistance in plants (Kunert and Foyer, 1993). Moreover, the thiol (–SH) group has metal-binding properties and is used by cells to detoxify excesses of various heavy metals (Zhou and Goldsbrough, 1994; Cockett, 2000). Levels of NPTs in the plant may therefore reflect the capability of plants to deal with environmental stress.

Knowledge of the genes that have impacts on Se tolerance can provide insights into Se tolerance mechanisms. A common approach to identify candidate genes that control a specific trait is to utilize the genetic resources available for the model plant Arabidopsis.
were sterilized, vernalized at 4°C for 3 days, and sown on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 10 g L⁻¹ sucrose and 4 g L⁻¹ agar gel (Sigma), supplied with either selenite (20 μM), selenate (50 μM) or no Se. These concentrations were chosen because they each result in an approximately 50% reduction in fresh weight. The plates were placed vertically in a growth chamber (24°C, 12 h light/12 h dark photoperiod). After 10 days, the seedlings were harvested and seedling root length was measured as a parameter for Se tolerance (Murphy and Taiz, 1995). To correct for any differences in growth between the accessions under control conditions, the Se tolerance index (TI) was calculated as root length in the presence of Se divided by root length on control medium.

**Selenium and sulfur concentrations in plant tissues**

One hundred plants of each accession were grown from surface-sterilized seeds on MS agar medium containing selenite (20 μM), selenate (50 μM) or no Se. The plates were incubated horizontally in a growth chamber (24°C, 12 h light/12 h dark) for 3 weeks. Root and shoot material were harvested separately, rinsed with distilled water and dried at 70°C overnight. Three to five replicates consisting of several seedlings each (20 mg dry weight) were acid digested and analyzed for Se and S by inductively coupled plasma atomic emission spectrometry (ICP-AES) as described by Pilon-Smits et al. (1999).

**F₁ and F₂ Se tolerance segregation analysis**

Ten seeds of each of the three accessions were vernalized at 4°C for 3 days, and grown on soil at 25°C (14 h light/10 h dark). Ler (selenate sensitive) was used as female and crossed with Col (selenate tolerant). Ws (selenite sensitive) was used as female and crossed with Col (selenite tolerant). Both parental accessions used for the cross were allowed to produce extra seeds for comparison with the F₁. All crosses were successful, as confirmed by PCR using simple sequence length polymorphism (SSLP) molecular marker nga151 (Bell and Ecker, 1994). The F₁ hybrids were allowed to self-fertilize to generate a segregating population. F₂ seeds from a single F₁ plant were scored for selenate or selenite resistance by growing on half-strength MS medium with selenite (20 μM) or selenate (50 μM) together with the F₁ plants and the parental accessions. The plates were placed vertically in a growth chamber (24°C, 12 h light/12 h dark). After 10 days, the root lengths on media with and without Se were measured and the Se tolerance index (TI) was calculated as root length in the presence of Se divided by root length on control medium.

**Bulked segregant analysis**

Two F₂ populations from crosses Ler × Col (283 F₂ plants) and Ws × Col (468 F₂ plants) were used for bulked segregant analysis. DNA was isolated following the ‘shorty’ protocol described by the University of Wisconsin Arabidopsis knockout facility (http://...
The three accessions Ler, Col and Ws were grown for 3 weeks on half-strength MS medium containing 10 g L\(^{-1}\) sucrose and 4 g L\(^{-1}\) agar gel (Sigma) supplied with selenite (15 μM) or selenate (30 μM). Shoots and roots were harvested and stored at \(-80^\circ\)C. Subsequently, non-protein organic selenocompounds were analyzed by liquid chromatography–mass spectrometry (LS-MS) using a Hewlett Packard Agilent 1100 Series HPLC equipped with XCALIBUR software. The selenocompounds and non-protein thiols were separated at 15°C using a Phenomenex Hypersil 5 u C18 (ODS) column (250 x 2.00 mm, 5 μm) at a flow rate of 0.36 ml/min, using the two eluents (A) water + 0.1% formic acid, and (B) acetonitrile + 0.1% formic acid. The following program was used: 0–2 min, 100% A; 2–10 min, gradient 0–40% B; 10–12 min, 40–0% B; 12–17 min, 100% A. For each accession and treatment, three replicates of 100 mg of shoot material were ground in liquid nitrogen, and extracted overnight at 4°C in 50 mM HCl [2 : 1 (v/w) or 2 mL/g fresh weight] with 78% recovery of total extractable Se. Four selenocompounds were used as standards: selenocystine (SeCystine), methylSeCys, \(\gamma\)-glutamyl-SeCys, and selenomethionine (SeMet); they were purchased from PharmaSe (Lubbock, TX). The four different non-protein selenocompounds were measured at different mass ranges, taking into account the characteristic Se isotopic signatures. The following mass ranges were used: m/z = 329.00–339.00 for SeCystine \([M+H]^+ m/z = 336.9]\); m/z = 192.00–200.00 for SeMet \([M+H]^+ m/z = 197.9]\); m/z = 166.50–167.50 for methylSeCys \([M+H]^+ m/z = 183.9\) and \([M+H-\text{NH}_3]^+ m/z = 167.0]\); and m/z = 308.00–316.00 for \(\gamma\)-glutamyl-SeCys \([M+H]^+ m/z = 312.9\). In addition, SeCys could be detected at m/z = 164.00–174.00 \([M+H]^+ m/z = 168.9\). Note that an SeCys standard is not commercially available, due to the rate at which it is oxidized to SeCystine. In order to create SeCys, we reduced SeCystine with sodium borohydride. SeCystine (100 mM) was incubated for 24 h at 1:1 molar ratio with sodium borohydride at room temperature. Mass spectrometry was also used to confirm that the peaks integrated matched the specific Se isotopic signatures of all standards.

For NPT analysis, three replicates of 100 mg of roots and shoots were ground in liquid nitrogen, and extracted and analyzed as described by Zhu et al (1999), using Ellman’s reagent. Ellman’s reagent detects free –SH groups. The extraction method used does not extract proteins, but it does extract small peptides and non-protein amino acids, including SeCys.

**Analysis of expression levels of S-related genes by RT-PCR**

The three accessions Ler, Col and Ws were grown for 3 weeks on half-strength MS medium containing 10 g L\(^{-1}\) sucrose and 4 g L\(^{-1}\) agar gel (Sigma) supplemented with selenite (20 μM) or selenate (50 μM), or without Se. Roots and shoots were harvested separately and stored at \(-80^\circ\)C for RNA isolation. Total RNA from shoots and roots was isolated by the TRIzol method (Invitrogen, Carlsbad, CA). DNase-treated total RNA was reverse-transcribed using the the First Strand cDNA synthesis kit (Fermentas International Inc., Burlington, Ontario, Canada). The products of this reaction were used as a template for PCR to amplify S-related genes using the Expand High Fidelity PCR system (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer’s instructions. Nine S-related genes were tested in this study. These included the genes for four sulfate transporters: Sultr 1;1, Sultr 1;2, Sultr 4;1 and Sultr 4;2. The others encode the enzymes AtCpNiF5 (SeCys lyase), CGS, APS1, SAT and the SMT homologue HMT2 (homocysteine methyltransferase). Ubiquitin was included as a control. Information about these genes and primers is included in Table 1.
Statistical analyses

Statistical analyses (ANOVA, Student’s t-test, chi-square, Shapiro–Wilk W-test for normal distribution) were performed using the software package JMP-IN from the SAS institute (Cary, NC).

Gel image analysis was performed using the IMAGEJ software program (National Institute of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) to quantify RT-PCR products.

Results

Significant differences in selenate and selenite TIs were observed between the three Arabidopsis thaliana accessions. TI was calculated as root length in the presence of Se divided by root length on control medium. When the three accessions were grown on selenate, the TIs of Col and Ws were two to three times higher than that of Ler. When they were grown on selenite, the TIs of Col and Ler were two to three times higher than that of Ws (Fig. 1A). Therefore, Col appears to be tolerant to both selenate and selenite, Ler is tolerant to selenite but sensitive to selenate, and Ws is tolerant to selenate but not selenite. Se accumulation in the shoots of the three accessions were different on selenate but the same on selenite (Fig. 1B). The selenate-sensitive accession Ler accumulated significantly higher levels of Se in shoots compared to Col and Ws when grown on selenate.

Selenium tolerance of F1 and F2 offspring

Crosses were made between selenite-sensitive Ws and selenite-tolerant Col, as well as between selenate-sensitive Ler and selenate-tolerant Col. The F1 was confirmed using PCR with SSLP marker nga151. The two F1 populations and their parent lines were then tested for Se tolerance to investigate the mode of inheritance. From the cross Ws · Col, the mean root length of the F1 on selenite was the same as that of the sensitive parent Ws (Fig. 2A). The segregation of selenite tolerance in the F2 from Ws · Col was non-normal (n = 468, P < 0.05, Shapiro–Wilk W-test) and corresponded with a 3 : 1 ratio of sensitive to tolerant individuals (P < 0.05, chi-square test, Fig. 2B). The F1 from cross Ler × Col

| Table 1. Oligonucleotide sequence of primers used in RT-PCR reactions. |
|-------------------------|-------------------------|-------------------------|-------------------------|
| Primer name             | Forward primer (5’-3’)   | Reverse primer (5’-3’)  | AGI locus               |
| Ubiq (ubiquitin extension protein 2) | CCAAGATCCAGGACAAGAAAGGA | TGGAGACGAGCTATAACACTTG | At2g36170.1             |
| Sultr1;1 (sulfate transporter)    | GCCATCAAATGCTTCCAA   | TGCCAAATCCACCCATTG    | At4g08620.1             |
| Sultr1;2 (sulfate transporter)    | GGCATCACAGAGGCTTACATG | TCAGATGCTGCATAGGTTGAC | At1g78000.2             |
| Sultr4;1 (sulfate transporter)    | TGCACACCTTCTAAGAGTCCTTC | AATTGTTGGAAGGCGCTTTCCG | At5g13550.1             |
| Sultr4;2 (sulfate transporter)    | GATGACACCTTCTGTATTCACCC | TATTGTTGGAAGGCGCTTTCCG | At1g32520.1             |
| SMT/HMT2 (homocysteine S-methyltransferase 2) | TCCCAACAGTAAAGGCTTACGGC | GGGGACGTACGTTTACCCAACC | At3g63250.1             |
| CGS (cystathionine γ-synthase) | CTCGAAAGCGTCAGAGAGGTT | GACCAACACCTCATAACCACTGCA | At3g01210.1             |
| APS1 (ATP-sulfurylase 1) | CTGGCTAGTACGTTAAAGAGT | ACCTGGAGCCGTCGATACC | At3g22890.1             |
| AtCpNifS (cysteine desulfurase) | TGCCTTCTTCTCTCCTATGTTGAGGCC | TGCGCGATCCACTTTGGAAAGTTGAA | At1g08490.1             |
| SAT (serine O-acetyltransferase) | ATGCCGAACCGTGGTTAATCAAGAAGC | GAAGCGCTTTGAAGGCGCTTTCCG | At1g55920.1             |

Fig. 1. Se tolerance and accumulation in the three accessions. (A) Tolerance index (root length + Se/–Se × 100%) of three accessions for selenate and selenite. (B) Shoot Se concentration (mg kg⁻¹ dry weight) in the three accessions grown on selenate or selenite.
showed an intermediate phenotype between both parents, and the F2 selenate tolerance showed a non-normal distribution (n = 238, P < 0.05, Shapiro–Wilk W-test; Fig. 2A,B). These results suggest that a single major gene may control selenite tolerance whereas a few genes are involved in selenate tolerance in these populations.

**Bulked segregant analysis**

For both selenate and selenite, DNA was isolated from a group of tolerant and sensitive F2 plants and pooled into two bulked samples each. The pooled DNA samples were then used for bulked segregant analysis, using primer pairs distributed over the five *Arabidopsis* chromosomes. Polymorphisms that corresponded with selenate tolerance were detected for molecular markers nga111, ciw4 and ciw8 (results not shown). Sixteen individual selenate-tolerant F2 plants were subsequently tested with these markers. For marker nga111, 15 selenate-tolerant F2 plants had either one band derived from tolerant parent Col or two bands, indicating heterozygosity; one plant did not produce any band, possibly due to poor-quality DNA (Fig. 3A); this plant was excluded for the other two markers. For markers ciw8 and ciw4, 14 of the 15 plants showed either the Col-specific band or two bands, and only one F2 plant showed only the Ler-specific band. Thus, molecular marker nga111 on chromosome 1, marker ciw4 on chromosome 3 and marker ciw8 on chromosome 5 showed a clear bias towards the Col-specific band in the selenate-tolerant plants. This suggests that the selenate tolerance trait is controlled by loci linked to these three markers in this population from Ler·Col. In the selenite-related bulked segregant analysis, the molecular marker ciw7 showed linkage with selenite tolerance. Twelve of 14 selenite-tolerant F2 plants had the Col-specific band and all appear to be homozygous (Fig. 3B). This indicates that a selenite tolerance gene is located on the long arm of chromosome 4 in this population from Ws·Col, and the tolerance allele appears to be recessive.

**Measurement of non-protein organic selenocompounds and non-protein thiols**

Using LC-MS, shoot samples of the three accessions treated with selenate or selenite were analyzed for the
non-protein organic selenocompounds SeCys, SeCystine (the oxidized form of SeCys), methylSeCys, γ-glutamyl-SeCys, and SeMet. Of these compounds, only SeCys and SeCystine were detected. Interestingly, when treated with selenite, the selenite-tolerant accessions Ler and Col had significantly higher levels of SeCys and SeCystine in their tissue compared with selenite-sensitive accession Ws, which did not have detectable amounts of both compounds (Fig. 4B). Similarly, when treated with selenate, the tolerant accessions Ws and Col had higher levels of SeCystine compared with selenate-sensitive accession Ler; SeCys was undetectable (Fig. 4A). Thus, the presence and levels of SeCys and SeCystine were correlated with tolerance to both selenate and selenite, and may in part explain the ecotypic variation in Se tolerance in these three accessions.

When NPT levels were compared in the three accessions, the selenite-sensitive accession Ws had significantly lower amounts of NPTs in roots than the selenite-tolerant accessions Ler and Col (Fig. 5B). No differences in NPT concentration were found among the three accessions when plants were grown on selenate or under control conditions (Fig. 5). Therefore, one or more NPTs may contribute to selenite tolerance in these accessions. This result is in agreement with the finding that SeCys levels were correlated with Se tolerance, as SeCys is also an NPT (more accurately, a selenol).

Expression levels of S-related genes according to RT-PCR

Transcript levels of S-related genes were compared between the accessions using RT-PCR. The intensity of the bands was quantified with software program IMAGE J and normalized relative to ubiquitin, which is considered to be constitutively expressed. Still, ubiquitin did appear to be affected to some extent by selenate and selenite, especially in roots (Fig. 6). Despite this complication, and although RT-PCR is only a semiquantitative method of comparing transcript levels, some interesting differences in transcript levels were observed between different accessions and treatments.

The sulfate transporters Sultr 4;1 and Sultr 4;2 showed a similar pattern of gene expression in response to selenate and selenite (Fig. 6). Both Sultr4 transporters were generally more expressed in shoot than in root and were upregulated by Se. Sultr1;2 was also upregulated by selenate and selenite in shoots of all three accessions. Under control conditions, Sultr1;2 expression was greater in roots than in shoots, but in the presence of Se it was similar in both organs. Very low expression levels were observed for Sultr1;1 under most conditions, but it was clearly upregulated by selenate in roots of Ler and in both shoots and roots of Ws.

We also examined the expression levels of the S assimilatory enzymes APS1, CGS, SAT and HMT2, as well as the plastidic SeCys lyase AtCpNiFS (Fig. 6). No variation in expression levels were found for APS1 and AtCpNiFS in any of the three accessions. AtCpNiFS was expressed more in shoots than in roots; for APS1, there were no differences. Interestingly, the SMT homolog HMT2 was downregulated by selenite in the roots of the selenite-sensitive accession Ws only. Although CGS was not amplified very consistently, there was a trend for it to be upregulated by selenite in roots. There also appeared to be upregulation of CGS by selenite and...
selenite in shoots, but only in accession Ws. SAT was generally upregulated by selenate and selenite in shoots, as well as by selenite in roots.

**Discussion**

In this study, it was found that the *Arabidopsis* accession Ler was significantly less tolerant to selenate than accessions Ws and Col, whereas Ws was less tolerant to selenite than the other two. Segregation of Se tolerance in offspring of accessions with contrasting tolerance phenotypes indicated that multiple genes may be involved in selenate tolerance, whereas a single major gene may control selenite tolerance. A molecular marker on chromosome 4 (ciw7) appeared to be linked to selenite tolerance, and three molecular markers on chromosome 1 (nga111), chromosome 3 (ciw4) and chromosome 5 (ciw8) were apparently linked to selenate tolerance. As for the potential biochemical mechanisms underlying Se tolerance, root NPT levels appeared to correlate with selenite tolerance. Also, shoot SeCys and SeCystine levels were correlated with selenate and selenite tolerance. Several sulfate transporters and S assimilatory enzymes appear to be upregulated by selenate and selenite according to RT-PCR. Of the genes examined, that encoding for one SeCys methyltransferase homolog (HMT2) was expressed at lower levels in selenite-sensitive Ws than in the other two accessions, when grown on selenite. Overall, these studies show that there is substantial intraspecific variation in tolerance to selenate and selenite in *Arabidopsis*, and provide some insights into the genetic and biochemical mechanisms underlying the observed ecotypic differences.

Bulked segregant analysis indicated that loci on chromosomes 1, 3 and 5 were involved in selenate tolerance. These findings are in agreement with those of a Quantitative Trait Loci (QTL) study (Zhang et al., 2006a) of selenate tolerance in a recombinant inbred line (RIL) population from a cross between Ler-0 and Col-4 (Lister and

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**Fig. 4.** Levels of the non-protein organic selenocompounds SeCys and SeCystine in the three accessions grown on selenate (A) or selenite (B). The data shown represent the mean and standard error of the mean (*n* = 3) of the peak area of these two compounds detected by LC-MS. ND, not detectable. Note: there was no detectable SeCys in any of the accessions when grown on selenate.
Many were clearly upregulated by selenate and/or the S/Se assimilation pathway? Among the genes tested by control Se tolerance, and are the encoded proteins part of the multigenic nature of selenate tolerance. Is in agreement with findings for other accessions (Dean, 1993). Both approaches revealed that selenate tolerance was governed by loci located on chromosomes 3 and 5 in populations from Ler × Col. The hypothesis that there is a single major gene responsible for selenite tolerance, but multiple genes involved in selenate tolerance, is in agreement with findings for other Arabidopsis accessions (Dijon G × Col-PRL, Dijon G × Estland) (Zhang et al., 2006b).

The finding that different numbers of genes and different loci appear to be involved in tolerance to selenate and selenite may reflect different uptake mechanisms for these two Se species, and different enzymes limiting their assimilation and detoxification in the plant. Three stages can be discerned in Se assimilation: uptake of Se from the environment, reduction of selenate to selenite, and assimilation of selenite into organic Se compounds. Selenate is taken up actively by sulfate transporters and enzymatically converted to selenite, whereas selenite is thought to be taken up passively (de Souza et al., 1998). These extra activities involved in selenate uptake and reduction may be additional points of control for selenate tolerance, explaining the multigenic nature of selenate tolerance.

The next intriguing question is: what are these genes that control Se tolerance, and are the encoded proteins part of the S/Se assimilation pathway? Among the genes tested by RT-PCR, many were clearly upregulated by selenate and/or selenite. There does not appear to be a clear correlation between the expression of any of the genes examined and Se tolerance or accumulation. However, an interesting observation is that on selenite medium, the SMT homolog HMT2 was expressed to a much lower level in roots of the selenite-sensitive accession Ws than in the other accessions. As selenite-treated plants are known to accumulate an organic form of Se (de Souza et al., 1998), the main selenite tolerance mechanism may involve the detoxification or sequestration of this organic selenocompound. If HMT2 expression does indeed affect Se tolerance, this effect may be direct or indirect. The lower HMT2 expression level in Ws could explain its sensitivity to selenite, if this enzyme was able to detoxify SeCys via methylation, preventing its non-specific incorporation into protein (LeDuc et al., 2004). However, no methyl-SeCys was detectable by LC-MS in any of the three accessions, so this compound does not appear to be very abundant. Moreover, HMT2 has been reported to be unable to use SeCys as a substrate in vitro (Ranocha et al., 2000). Alternatively, HMT2 may have an indirect effect on Se tolerance by affecting S metabolism.

The selenate-sensitive Ler accession showed higher Se accumulation in shoots than the other two accessions, suggesting that the sensitivity may be due to a difference in selenate uptake and/or translocation. However, no clear difference was found for the four sulfate transporter genes tested. Still, since there are 14 sulfate transporters in Arabidopsis, others than the ones tested may be responsible for the observed accumulation difference. In addition, the tolerance mechanism may involve subtle sequence differences leading to differences in kinetic properties of the gene products, rather than differences in gene expression levels. Such differences are not revealed by RT-PCR, but may be by locus mapping using offspring of crosses between tolerant and sensitive accessions, followed by sequence comparison of the tolerant and sensitive accessions at the identified locus.

Two additional biochemical approaches were used to obtain insights into the mechanisms that may contribute to the variation in Se tolerance in these three accessions. Se affected NPT levels in these three accessions differently, which could in part explain their differences in tolerance. The lower root levels of NPTs in the selenite-sensitive Ws accession may offer them less protection from selenium-induced oxidative stress. It is also possible that certain thiols bind selenite directly, as they do arsenite (Pickering et al., 2000).

Another intriguing finding was that shoot SeCys and SeCystine levels were correlated with both selenate and selenite tolerance in these three accessions. A similar correlation was found in selected RILs from a Col-4 × Ler-0 cross that differ in selenate tolerance: the two selenate-tolerant RILs CS1927 and CS1989 with Se TIs of 0.60 and 0.75,
respectively, contained significant SeCystine levels (13.2 and 65.7 nmol g\(^{-1}\) fresh weight respectively) and no SeCys, whereas no detectable SeCystine or SeCys was present in the two selenate-sensitive RILs CS1948 and CS1949, with Tls of 0.17 and 0.20, respectively (unpublished results). If indeed SeCystine levels are correlated with tolerance, this may be because oxidation of SeCys to SeCystine prevents SeCys incorporation into protein. The correlation between Se tolerance and higher SeCys pools is more difficult to explain, but may reflect reduced incorporation into protein due to, for example, lower activity or specificity of the aminoacyl-tRNA transferase that non-specifically shuttles SeCys towards translation, replacing cysteine.

In general, more SeCys was observed in the selenite-treated plants than in those treated with selenate. Selenite is known to be easily assimilated into organic forms, whereas selenate is accumulated in unaltered form (de Souza et al., 1998). Perhaps the higher accumulation of selenate creates more oxidative stress, leading to more oxidation of SeCys to SeCystine. The quick conversion of selenite to organic Se may explain the higher overall levels of the organic selenocompounds SeCys and SeCystine. In a previous study, we found no significant correlation between Se tolerance and total Se concentration in 19 Arabidopsis accessions (Zhang et al., 2006b). However, Se tolerance may be correlated with tissue levels of specific selenocompounds, as was found here for SeCys and SeCystine. Continued dissection and comparison of levels of Se metabolites and Se-related gene expression should yield more detailed insights into the mechanisms that control Se tolerance.

In conclusion, this study offers some insights into the genetic basis and potential biochemical mechanisms underlying Se tolerance in these Arabidopsis accessions. The results from the bulked segregant analyses could be the starting point for the fine mapping of Se tolerance genes. Greater knowledge of the molecular mechanisms of Se tolerance will be helpful for manipulation of Se metabolism to meet the needs of human health, and to clean up Se contamination in the environment.

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**References**


Lister C, Dean C (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in Arabidopsis thaliana. Plant J 4: 745–750
Ng BH, Anderson JW (1979) Light-dependent incorporation of selenite and sulphite into selenocysteine and cysteine by isolated pea chloroplasts. Phytochemistry 18: 573–580
Youssefian S, Nakamura M, Sano H (1993) Tobacco plants transformed with the O-acetylserine (thiol) lyase gene of wheat are resistant to toxic levels of hydrogen sulphide gas. Plant J 4: 759–769