Cooperative Ethylene and Jasmonic Acid Signaling Regulates Selenite Resistance in Arabidopsis[1[W][OA]

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Selenium (Se) is an essential element for many organisms, but excess Se is toxic. To better understand plant Se toxicity and resistance mechanisms, we compared the physiological and molecular responses of two Arabidopsis (Arabidopsis thaliana) accessions, Columbia (Col)-0 and Wassilewskija (Ws)-2, to selenite treatment. Measurement of root length Se tolerance index demonstrated a clear difference between selenite-resistant Col-0 and selenite-sensitive Ws-2. Macroarray analysis showed more pronounced selenite-induced increases in mRNA levels of ethylene- or jasmonic acid (JA)-biosynthesis and -inducible genes in Col-0 than in Ws-2. Indeed, Col-0 exhibited higher levels of ethylene and JA. The selenite-sensitive phenotype of Ws-2 was attenuated by treatment with ethylene precursor or methyl jasmonate (MeJA). Conversely, the selenite resistance of Col-0 was reduced in mutants impaired in ethylene or JA biosynthesis or signaling. Genes encoding sulfur (S) transporters and S assimilation enzymes were up-regulated by selenite in Col-0 but not Ws-2. Accordingly, Col-0 contained higher levels of total S and Se and of nonprotein thiols than Ws-2. Glutathione redox status was reduced by selenite in Ws-2 but not in Col-0. Furthermore, the generation of reactive oxygen species by selenite was higher in Col-0 than in Ws-2. Together, these results indicate that JA and ethylene play important roles in Se resistance in Arabidopsis. Reactive oxygen species may also have a signaling role, and the resistance mechanism appears to involve enhanced S uptake and reduction.

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laboratory-induced mutants or transgenic plants, another source of genetic variation can be found among naturally occurring populations of Arabidopsis (Koornneef et al., 2004). After comparison of Se resistance and genetic investigation of recombinant inbred lines between Arabidopsis (Arabidopsis thaliana) accessions Columbia (Col)-4 and Landsberg erecta-0, it was reported that the difference in selenate resistance between these accessions is controlled by multiple genes located on chromosomes 3 and 5 (Zhang et al., 2006b). In another study that compared selenate or selenite resistance and Se accumulation among 19 Arabidopsis accessions, no correlation was found between Se resistance and accumulation, either for selenite or selenite (SeO₃²⁻; Zhang et al., 2007). Although there are a few studies for natural variation of Se resistance in Arabidopsis ecotypes, the underlying mechanisms that cause the difference in Se resistance are largely unknown.

In this work, we investigated the natural difference in selenite resistance between Arabidopsis accessions Col-0 and Wassilewskija (Ws)-2. In a previous study, Zhang et al. (2006a) showed Ws was sensitive to selenite while Col was resistant. Here, the molecular mechanisms behind this physiological difference were further investigated. The new results indicate that jasmonic acid (JA) and ethylene play important roles in Se resistance in Arabidopsis. Reactive oxygen species (ROS) may also have a signaling role, and the resistance mechanism appears to involve enhanced S uptake and reduction.

RESULTS AND DISCUSSION

Ws-2 Is More Sensitive to Selenite Than Col-0

To quantify differences in selenite resistance between Col-0 and Ws-2, these accessions were grown on Murashige and Skoog medium containing different concentrations of sodium selenite for 7 d. The root growth in the absence of added Se was not different in both ecotypes (data not shown). With increasing selenite concentration in the medium, the selenite tolerance index decreased in both accessions, but the tolerance index in Ws-2 was significantly more affected than in Col-0 for the five highest selenite concentrations (Fig. 1). Thus, Ws-2 is more susceptible to selenite than Col-0. These results confirm the report by Zhang et al. (2006a) that an Arabidopsis Col accession was more resistant to selenite than Ws. The difference in tolerance index was most pronounced (2-fold) when plants were grown on medium containing 15 or 20 μM selenite, and 15 μM was chosen for all subsequent experiments.

Sulfur Transport and Assimilation Genes Are More Induced by Selenite in Col-0 Than Ws-2

Se is chemically similar to S and known to be taken up and assimilated by plants via the same transporters and enzymes (Terry et al., 2000). Therefore, it can be expected that high levels of Se treatment prevent S uptake and assimilation, resulting in S starvation. The magnitude of S starvation induced by Se may depend on S transport and assimilation activity. To compare Col-0 and Ws-2 in this respect, macroarray analysis was used to measure the selenite-related expression of a large set of genes encoding sulfate transporters, S assimilation proteins, iron (Fe)-S cluster-related proteins, homologs of selenoproteins and Se-binding proteins, and defense-related proteins. Of the 250 genes analyzed (Supplemental Table S1), 55 genes were found to be responsive only to selenite in Col-0 but not Ws-2, using a P value of less than 0.05 and a minimal fold-change of greater than two (Table I; Supplemental Fig. S1). Among these, three encode sulfate transporters, 24 are related to S assimilation, five are Fe-S cluster related, four encode homologs of selenoprotein or Se-binding proteins, and 12 are defense-related genes (Table I). Seven genes were up-regulated only by selenite in Ws-2 but not Col-0, and 27 genes were induced in both ecotypes (Supplemental Table S1; Supplemental Fig. S1). Thus, the general trend was that genes involved in S assimilation or defense were more up-regulated by selenite treatment in Col-0 than in Ws-2.

Genes specifically up-regulated in the selenite-resistant Col-0 accession but not in selenite-sensitive Ws-2 included three sulfate transporters (Sultr2;2, Sultr3;1, and Sultr3;5), three ATP sulfurylases (APS1, APS2, and APS4), three 5'-adenylylsulfate reductases (APR1, APR2, and APR3), a sulfite reductase (SIR), four Ser O-acetyltransferases (SAT1, SAT3, SAT52, and SAT106), three Cys synthases (CYSD1, CYSD2, and CYS1), all involved in sulfate to Cys assimilation, and two glutathione biosynthesis genes encoding γ-glutamyl-Cys synthetase (GSH1) and glutathione synthetase (GSH2; Table I). The specific induction by selenite of several of these genes in Col-0 plants but not Ws-2 was further

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**Figure 1.** Selenite tolerance index for Col-0 (black circles) and Ws-2 (white circles). Plants were grown on control medium or on medium with various concentrations of sodium selenite for 7 d, then measured for root length. Shown are the means ± SD (n = 20). Lowercase letters indicate significant differences between Col-0 and Ws-2 for a particular selenite concentration (P < 0.05).
Table I. Genes that were more induced in Col-0 than inWs-2 by15 μM selenite treatment

Macroarray analysis was carried out two times (experiments 1 and 2), and each macroarray membrane contained two duplicate spots per gene. Average andsd are calculated from fold-induction from the four replicate spots. Listed are genes where the fold-induction in Col-0 was >2, the fold-induction inWs-2 was <2, and the P value between the two accessions was <0.05.

| Gene | Annotation | MIPS Code | Col-0 (Fold-Induction) | We-2 (Fold-Induction) | P Value |
|------|------------|-----------|------------------------|-----------------------|---------|
|      |            |           | Experiment 1 | Experiment 2 | Average | Experiment 1 | Experiment 2 | Average |        |
|      |            |           | Spot 1 | Spot 2 |            | Spot 1 | Spot 2 |          | Spot 1 | Spot 2 |        |
|      |            |           |         |         |            |         |         |          |         |         |        |
| Fe-S cluster-related genes | | | | | | | | | | | |
| APO1 | Accumulation of PSI | At1g64810 | 2.86 | 1.84 | 3.15 | 1.81 | 2.42 | 0.69 | 0.79 | 1.05 | 0.89 | 1.38 | 1.03 | 0.25 | 0.011 |
| GRX5.1 | Chloroplastic glutaredoxin | At1g3805 | 2.53 | 1.96 | 3.02 | 1.90 | 2.35 | 0.53 | 1.06 | 0.91 | 1.03 | 1.09 | 1.02 | 0.08 | 0.007 |
| GRX | Chloroplastic glutaredoxin | At5g13810 | 2.66 | 2.34 | 2.52 | 1.95 | 2.37 | 0.31 | 1.63 | 1.18 | 1.79 | 1.64 | 1.56 | 0.27 | 0.004 |
| GRX | Chloroplastic glutaredoxin | At5g58510 | 2.54 | 1.63 | 4.06 | 1.43 | 2.41 | 1.20 | 0.53 | 1.05 | 0.58 | 1.33 | 0.87 | 0.38 | 0.038 |
| PSAD2 | PSII reaction center subunit II | At1g30110 | 2.69 | 1.73 | 2.67 | 2.11 | 2.30 | 0.47 | 1.25 | 1.10 | 1.16 | 1.30 | 1.20 | 0.09 | 0.008 |
| Selenoprotein | | | | | | | | | | | |
| SFP | Selenoprotein family protein | At1g5720 | 3.25 | 1.98 | 3.50 | 2.23 | 2.74 | 1.32 | 0.94 | 1.21 | 1.17 | 1.49 | 1.20 | 0.22 | 0.008 |
| HMT3 | Homo-Cys-S-methyltransferase 3 | At1g22740 | 2.32 | 2.12 | 1.88 | 2.15 | 2.12 | 0.38 | 0.96 | 1.20 | 0.86 | 1.66 | 1.17 | 0.36 | 0.018 |
| SEL7 | Selenoprotein related | At1g47100 | 1.99 | 2.51 | 2.13 | 2.11 | 2.22 | 0.22 | 1.28 | 1.23 | 1.58 | 1.72 | 1.45 | 0.24 | 0.002 |
| Se-binding protein | | | | | | | | | | | |
| Sulfur transporter | | | | | | | | | | | |
| Sultr2;2 | Sulfate transporter | At3g51895 | 2.06 | 1.78 | 2.64 | 1.96 | 2.11 | 0.37 | 0.60 | 0.74 | 0.58 | 1.03 | 0.74 | 0.17 | 0.001 |

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investigated by determining their expression profiles using semiquantitative reverse transcription (RT)-PCR. The mRNA levels of SIR, APR1, APR3, SAT52, GSH1, and GSH2 again increased only with selenite treatment in Col-0 but not in Ws-2 (Fig. 2A). Induction of APR2 and SAT1 with selenite in Ws-2 was lower than in Col-0 (Fig. 2A). Therefore, the results from the macroarray and semiquantitative RT-PCR approaches both indicate that the expression of several key genes involved in S uptake and assimilation are more enhanced upon selenite treatment in Col-0 than in Ws-2. In this context, it is interesting to note that both APS1 and a SAT gene (SAT1) are located in the chromosome 3 quantitative trait loci region that was shown earlier to be associated with selenate tolerance in Col (Zhang et al., 2006b). Coordinated induction of S uptake and assimilation genes, including Sultr2;1, APR2, APR3, and GSH1, has also been observed in S-starved plants (for review, see Hirai and Saito, 2004), suggesting that part of the selenite responses observed in this experiment could be induced by S deficiency. However, the selenite-induced genes encoding homologs of selenoproteins (At1g05720, At3g22740, and At3g47300) and a Se-binding protein (At5g40415) were not induced by S deficiency. Thus, the induction of some genes by selenite treatment may occur via S starvation, while others may be induced in a different, selenite-specific response.

**Levels of Selenite-Induced Expression in Ethylene- and JA-Modulated Genes Are Higher in Col-0 Than in Ws-2**

Earlier studies have shown that S deficiency induces the expression of 12-oxophytodienoate reductase, involved in JA biosynthesis (Hirai et al., 2003; Nikiforova et al., 2003). JA is known to be a plant hormone whose production is induced by various environmental stresses (Pieterse and van Loon, 1999). In addition to JA, salicylic acid (SA) and ethylene are also known as stress-inducible phytohormones (Nürnberger and Scheel, 2001). To study the involvement of JA, SA, and ethylene in selenite resistance, the selenite-related expression of genes either involved in the biosynthesis of these phytohormones or known to be responsive to these hormones was analyzed in the two accessions using macroarrays. The selenite-induced expression of all SA-related genes tested, i.e. the biosynthetically involved isochorismate synthase 1 (ICS1) and Phe ammonia-lyase 2 (PAL2), and the SA-responsive pathogenesis-related protein 1 (PR1), PR2, PR5, enhanced disease susceptibility 1 (EDS1), and EDS5 were all similar in Col-0 and Ws-2 (Supplemental Table S1). In contrast, the induction levels of genes known to be involved in ethylene or JA biosynthesis, i.e. 1-aminoacyclopropane-1-carboxylate synthase 1 (ACS1), 5-adenosyl-1-Met synthase 1 (SAM1), lipoxygenase 2 (LOX2), and allene oxide synthase (AOS), were remarkably higher in Col-0 than in Ws-2 (Table I). Moreover, induction of ethylene response factor 1 (ERF1), PR4, plant defensin 1.2 (PDF1.2), vegetative storage protein 1 (VSP1), proteinase inhibitor 2 (PIN2), and JA-responsive gene (JR), which are responsive to ethylene and/or JA, was also significantly more pronounced in Col-0 (Table I). To confirm the selenite-induced gene expression observed from macroarray experiments with an independent experimental approach and biological replicate, semiquantitative RT-PCR was performed for selected genes, which were indeed significantly more induced in Col-0 than in Ws-2 by the selenite treatment. Consistent with the macroarray data, the expression of genes ICS1 and PR1 was increased in both accessions, and the expression of genes ACS6, PR4, AOS, PIN2, and PDF1.2 was more induced by selenite in Col-0 than in Ws-2 (Fig. 2B). The expression of PR4 is known to be induced by ethylene (Lawton et al., 1994) and widely used as a good marker for ethylene signaling. Induction of the PIN2 gene is JA dependent (Farmer et al., 1992), and concomitant triggering of the ethylene and JA pathways is required for PDF1.2 induction (Penninckx et al., 1998). ACS is the rate-limiting enzyme and governs the major regulatory step in stress-induced ethylene production (Yang and Hoffman, 1984; Bleecker and Kende, 2000), and AOS is also a key enzyme in JA synthesis (Stenzel et al., 2004). Together, these results show that ethylene and JA biosynthesis and/or signaling are induced by selenite in Col-0 but much less in Ws-2.

### Table 1. Continued from previous page.

| Gene | Annotation | MIPS Code | Col-0 (Fold-Induction) | Ws-2 (Fold-Induction) |
|------|------------|-----------|-----------------------|-----------------------|
|      |            |           | Experiment 1 | Experiment 2 | Spot 1 | Spot 2 | Spot 1 | Spot 2 | Average | SD | P Value |
|      |            |           | Col-0 | Col-0 | Average | SD | Col-0 | Col-0 | Average | SD | Col-0 |}\n
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#### Table 1. Continued from previous page.

| Gene | Annotation | MIPS Code | Col-0 (Fold-Induction) | Ws-2 (Fold-Induction) |
|------|------------|-----------|-----------------------|-----------------------|
|      |            |           | Experiment 1 | Experiment 2 | Spot 1 | Spot 2 | Spot 1 | Spot 2 | Average | SD | P Value |
|      |            |           | Col-0 | Col-0 | Average | SD | Col-0 | Col-0 | Average | SD | Col-0 |
Ethylene and JA Are Important Factors in the Difference in Selenite Resistance between Col-0 and Ws-2

The observation that the expression of known ethylene- and JA-inducible genes was more extensively enhanced by selenite in Col-0 than in Ws-2 may suggest that Col-0 generates higher levels of these phytohormones and/or is more sensitive to these hormones than Ws-2 under selenite treatment. To test these hypotheses, first the levels of these plant hormones were measured in Col-0 and Ws-2 plants treated with or without selenite. Free SA accumulation was similar in both accessions and increased 4-fold with selenite treatment in both (Fig. 3A). Interestingly, the shoot concentration of methyl jasmonate (MeJA) was approximately 2-fold higher in Col-0 than in Ws-2 (Fig. 3C) and also increased more with selenite treatment in Col-0 (7-fold) than in Ws-2 (2-fold). Ethylene generation from seedlings grown without selenite was not different in both accessions but increased more with selenite treatment in Col-0 (3-fold) than in Ws-2 (1.4-fold; Fig. 3E). Thus, the observed higher level of production of ethylene and JA in selenite-treated Col-0 in comparison with Ws-2 is in agreement with the observed higher level of induction of genes that contribute to ethylene (ACS6 and SAM1) and JA biosynthesis (AOS and LOX2; Table I; Fig. 2B). Also, the observed increase in SA levels upon selenite treatment, to a similar extent in Col-0 and Ws-2, corresponds well with the observed ICS1 and PAL2 expression levels.

The above results indicate that increased ethylene and JA signaling, caused by production of ethylene and JA by selenite treatment, correlates with enhanced selenite resistance in Col-0. Alternatively, Col-0 may be more sensitive to ethylene and/or JA than Ws-2. To compare phytohormone sensitivity between the two accessions, we examined the SA-, ethylene-, and JA-induced inhibition of root growth in seedlings of Col-0.
and Ws-2. Note that rather than ethylene gas, its precursor, 1-aminocyclopropane-1-carboxylate (ACC), was supplied. As shown in Supplemental Figure S2, the degree of inhibition was not statistically different between Col-0 and Ws-2 (approximately 20% and 45% inhibition at 1 and 10 µM SA; approximately 20% and 32% inhibition at 1 and 0.1 µM MeJA; approximately 15% and 42% inhibition at 0.1 and 1 µM ACC, respectively). This suggests that the enhanced ethylene and JA signaling in Col-0 was not due to higher ethylene or JA sensitivity.

To assess whether the selenite resistance of the sensitive accession Ws-2 is limited by its lower ethylene or JA concentration, we next tested whether selenite sensitivity could be mitigated via external supply with MeJA or ACC. Indeed, the selenite tolerance index of Ws-2 increased with increasing MeJA content in the medium (Fig. 3D). As a result, no significant difference in selenite tolerance index was observed any more between Col-0 and Ws-2 when grown on 0.5 or 1 µM MeJA (Fig. 3D). Treatment with ACC resulted in a similar trend. Accession Ws-2 showed lower resistance to selenite than Col-0 without ACC, but its selenite tolerance index increased with increasing ACC content in the media, and it became the same as Col-0 when the plants were grown at or above 0.5 µM ACC (Fig. 3F). In contrast, growing the plants on a series of different concentrations of SA had no positive effect on the selenite tolerance index of Ws-2 (Fig. 3B); Col-0 was even significantly inhibited by external SA (Fig. 3B).

The importance of SA, ethylene, and JA for acquisition of selenite resistance in Arabidopsis was also investigated using mutants with defects in phytohormone biosynthesis or signaling. Mutants incapable of SA production (sid2) or signaling (npr1; Cao et al., 1997) showed no statistical difference in selenite tolerance index in comparison to their wild type, Col-0 (Fig. 4A). In contrast, selenite resistance in mutants defective in ethylene production (acs6) or signaling (ein3 and ein2) was less than that in wild-type Col-0 (Fig. 4B). Furthermore, an ethylene-overproducing mutant in the Col-0 background, eto1, showed higher resistance than wild-type Col-0 (Fig. 4B). Selenite resistance in a mutant deficient for JA signaling, jar1, was significantly lower than in Col-0 (Fig. 4C). Conversely, JA-hypersensitive mutant iop1 (Penninckx et al., 2003) and the constitutive JA-producing mutant cas1 (Kubigsteltig and Weiler, 2003) were more tolerant to selenite than their wild type, C24 (Fig. 4C). Taken together, these results indicate that the selenite susceptibility of Ws-2 is highly dependent on ethylene and JA biosynthesis, while there is no evidence of SA involvement.

In view of the finding that both ethylene and JA appear to play important roles in selenite resistance in Arabidopsis, it is interesting to note that in earlier studies JA was implicated to be involved in the regulation of S metabolism because S starvation induced genes involved in JA synthesis, as well as JA-responsive genes (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). The above-mentioned studies showed no indication that ethylene plays a role in S deficiency responses. However, a recent comprehensive gene expression analysis showed that transcripts regulating ethylene synthesis (ACS6) and signaling (ERF) were up-regulated by selenate treatment, and plants overexpressing ERF1 exhibited an increase in selenate resistance (Van Hoewyk et al., 2008). These results indicate that S resistance achieved through ethylene signaling is not mediated by S star-
It is known that reduced organic S metabolites compose a large fraction of the S pool (Nikiforova et al., 2006). To determine whether levels of reduced organic S metabolites are affected by selenite treatment, the levels of nonprotein thiols were measured in shoot tissue. Under control conditions, both accessions contained the same nonprotein thiol level (Fig. 5C). When grown with 15 μM selenite, the nonprotein thiol levels were reduced in both accessions compared to control conditions (Fig. 5C). However, the magnitude of this reduction in nonprotein thiol level was significantly higher in Ws-2 than in Col-0. As a result, selenite-treated Col-0 plants contained approximately 65% higher nonprotein thiol levels than Ws-2 plants on the same medium.

A large fraction of the nonprotein thiol pool consists of glutathione, which is involved in regulating the redox state of the cell as well as the storage and transport of reduced S in plants (Nikiforova et al., 2006). While the level of reduced glutathione (GSH) was not affected by selenite treatment in Col-0, it was nearly 2-fold lower in selenite-treated Ws-2 compared to control conditions and to selenite-treated Col-0 plants (Fig. 5D, white bars). The level of oxidized glutathione (GSSG) doubled with selenite treatment in both accessions (Fig. 5D, hatched bars); no significant difference in GSSG content was found between Col-0 and Ws-2 grown either without or with selenite. Therefore, it appears that S uptake is inhibited by selenite and that Col-0 is better able to maintain S status in the presence of selenite than Ws-2. Col-0 appears to also be better able to maintain a sufficiently large pool of reduced S compounds, especially glutathione, when treated with selenite, which may in part be responsible for its resistance. Incidentally, a reduction in GSH content was also observed in S-starved Arabidopsis (Hirai et al., 2003), indicating that selenite treatment induced S starvation in Ws-2 plants.

**Optimal Levels of ROS Are Necessary for Selenite Resistance**

From the experiments described above, it appears that ethylene and JA play important roles in selenite resistance in Arabidopsis. Ethylene and JA are known to be required for plant defense responses such as pathogen attack, drought stress, and resistance to air pollutants (Dong, 1998; Zhu, 2002; Overmyer et al., 2003). During defense responses to these environmental stresses, generation of ROS often precedes ethylene and JA production (Dong, 1998; Overmyer et al., 2003). Optimal levels of ROS act as signal molecules that activate defense responses (Overmyer et al., 2003). To obtain further insight into the biochemical mechanisms underlying the observed difference in selenite resistance between Col-0 and Ws-2, we compared ROS generation in these accessions.

Before ROS detection, we measured reduced (AsA) and oxidized (DHA) ascorbic acid content in the plants because AsA is the major antioxidant molecule in...
plants (Smirnoff et al., 2001). The AsA content was similar in the two accessions and not affected by selenite treatment (Fig. 6A). The level of DHA was also similar in both accessions under control conditions but increased significantly in Col-0 when plants were treated with selenite, in contrast to Ws-2 (Fig. 6A). The 
vtc1 mutant (Conklin et al., 1996) that is impaired in AsA production was also used in this experiment. As expected, the 
vtc1 mutant contained significantly lower levels of AsA compared to the wild type (Col-0), which were not affected by selenite treatment; the DHA level was also reduced under control conditions but increased with selenite treatment, similar to the Col-0 wild type (Fig. 6A). The AsA redox state was lower (i.e. more oxidized) in the presence of selenite in all plant types (Fig. 6B). Under control conditions, the AsA redox state was similar in Col-0 and Ws-2, but due to the higher DHA level in selenite-treated Col-0, the AsA redox state was significantly lower in Col-0 than in Ws-2 on selenite medium (Fig. 6B). The AsA redox state in the 
vtc1 mutant was significantly lower than in Col-0 and Ws-2, both with and without selenite.

To assay cellular hydrogen peroxide and superoxide accumulation, we performed in situ ROS detection as shown in Figure 6, C to E. The top row shows the accumulation of hydrogen peroxide in Col-0 (C), Ws-2 (D), and 
vtc1 (E). Hydrogen peroxide is visualized in situ as a reddish-brown precipitate, as 3,3′-diaminobenzidine (DAB) polymerizes on contact with hydrogen peroxide in a reaction requiring peroxidase (Torres et al., 2002). Brown precipitates were observed in selenite-treated Col-0, Ws-2, and 
vtc1 leaves, but almost no stain was detected in untreated plants. The density of brown precipitates in selenite-treated Col-0 appears higher than that in Ws-2 (Fig. 6, A and B). The leaves of the 
vtc1 mutant were stained more than Col-0 and Ws-2, likely due to its lack of AsA and low ROS-scavenging ability (Fig. 6B). The bottom row shows the accumulation of superoxide in Col-0 (C), Ws-2 (D), and 
vtc1 (E), monitored in situ via the precipitation of purple formazan from the reaction of nitro blue tetrazolium (NBT) with superoxide. Similar to the DAB staining, selenite-treated plants showed more superoxide than control plants, and Col-0 accumulated more superoxide than Ws-2; as expected, a high level of superoxide was also detected in the selenite-treated 
vtc1 mutant. Thus, selenite treatment resulted in the

![Figure 6](Plant Physiol. Vol. 146, 2008)
taken in selenite-induced SA production, we tested the selenite resistance of two double mutants: a vtc1/ein2 mutant that lacks AsA biosynthesis and ethylene signaling, and a vtc1/sid2 mutant lacking AsA and SA biosynthesis. The selenite resistance in the vtc1/ein2 mutant was not different from the vtc1 mutant, but the selenite resistance in the vtc1/sid2 mutant was restored to the level of Col-0 (Fig. 4D). The finding that the vtc1 mutant, which produces more SA when treated with selenite, is more selenite sensitive but if SA production is knocked out in vtc1/sid2 selenite then resistance is restored, suggests that SA inhibits the acquisition of selenite resistance. Indeed, we show that treatment of SA in Col-0 inhibited selenite resistance (see Fig. 3B). The next intriguing question is how the SA inhibits selenite resistance in plants. Our results indicate SA may inhibit JA and/or ethylene signaling because we observed that selenite induction of PR4 (a marker for ethylene signaling) and PIN2 (a marker for JA signaling) in Col-0 was completely repressed in the vtc1 mutant (Fig. 6F). Inhibition of JA and/or ethylene signaling by SA was also observed earlier in plants suffering biotic and abiotic stresses (Pena-Cortes et al., 1993; Doares et al., 1995; Berrocal-Lobo et al., 2002). Therefore, it appears that in the selenite-treated vtc1 mutant, an increase in SA production inhibited JA and ethylene signaling, leading to impaired Se resistance. Indeed, the vtc1 mutant showed no selenite-related induction of SIR and SAT1, whose expression is regulated by JA and/or ethylene (see Figs. 2A and 6F).

Taken together, our results suggest that an excess level of ROS production such as in the vtc1 mutant leads to a high level of SA, which inhibits JA and ethylene signaling, thereby impeding S assimilation and selenite resistance. However, a low ROS response such as in Ws-2 appears to be associated with a low Se resistance as well. Thus, an optimal level of ROS may be needed to acquire selenite resistance. Recently, ROS induction upon selenite treatment was also observed in a cell suspension of coffee (Gomes et al., 2007). The cellular mechanisms regulating ROS production in response to Se in plants are not clear at this point and will require further study. In this context, it is interesting to note that Zhang et al. (2006a) showed that a molecular marker on chromosome 4 (ciw7) appeared to be linked to selenite resistance in Col. Although no ethylene- or JA-related gene is found around the marker, a potential defense-related gene, LSD1-like 2 (LOL2; At4g21610), is located close to the marker. LOL2 encodes a member of a small family of LSD1 proteins that contain three highly related zinc fingers, and may function as either a transcriptional regulator or a scaffold protein (Dietrich et al., 1997). Eppel et al. (2003) showed LOL and LSD1 may function as antagonistic transcriptional regulators or scaffolds that control attenuation of cell death through regulation of ROS and/or SA production level. In future studies, it will be interesting to test the involvement of LOL genes in selenite tolerance in Arabidopsis.

CONCLUSION

The results presented here indicate that the higher selenite resistance of accession Col-0 compared to Ws-2 is dependent on its higher level of selenite-induced JA and ethylene synthesis. Selenite-related ROS production was also higher in Col-0 than in Ws-2. This may indicate that JA and ethylene production require an optimal level of ROS production to lead to Se resistance in plants. The resistance mechanism may involve JA- and ethylene-enhanced S uptake and assimilation, as observed in Col-0. The higher levels of organic S compounds observed in Col-0 may enable it to more efficiently prevent Se analogs from replacing S in proteins and other S compounds. However, Se levels were also higher in Col-0 plants compared to Ws-2. It is intriguing to speculate that the Se-binding protein homolog that was induced by selenite in Col-0 but not in Ws-2 may play an additional role in alleviating Se toxicity. This would be in agreement with the study by Agalou et al. (2005), where overexpression of Se-binding protein resulted in enhanced Se resistance. Another possibility for increasing Se resistance in Col-0 is caused by its higher antioxidant levels, because higher Se causes the extra oxidative stress. JA might also be involved in this process because GSH and AsA biosynthetic pathways were enhanced after MeJA treatment (Sasaki-Sekimoto et al., 2005).
Se is an essential element for animals, including humans (Rayman, 2000). The recommended dietary allowance is 40 to 70 μg/d. However, human diets in several countries lack sufficient Se, which leads to enhance susceptibility to cancer, viral infections, and heart problems (Rayman, 2000). On the other hand, soils containing >0.5 mg Se kg⁻¹ are considered seleniferous, and forage produced on such soils often contains more than the maximum permissible level for animal consumption. Soils with elevated levels of Se are found in many countries, including Australia, China, India, and the United States (Dhillon and Dhillon, 2003). Se accumulation by plants may help alleviate both Se deficiency and toxicity. A better understanding of the mechanisms and rate-limiting factors controlling plant Se uptake and assimilation will be vital for the optimal use of plants to alleviate dietary Se deficiency or for cleanup of Se-polluted areas. Several transgenic plants with enhanced Se accumulation and resistance have already been developed (Pilon-Smits et al., 1999; Agalou et al., 2005; Bañuelos et al., 2005, 2007; Van Hoeyyk et al., 2005) and may be useful for Se phytoremediation (for review, see Pilson-Smits and Freeman, 2006). The new knowledge obtained here of the genes and processes that impact Se accumulation and resistance in Arabidopsis may lead to further development of plants with increased Se content.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Selenite Resistance Assays

Seeds of Arabidopsis (Arabidopsis thaliana) Col-0, WS-2, npr1, acs6, ein3, ein2, etiol, jar1, and vtc1 were obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, OH). The sid2 mutant and opr1 mutant were obtained from Christiane Nawrath (University of Fribourg) and Bart PHJ. Thromma (Wageningen University), respectively. C24 and ehl1 mutant were provided from Ines Kubisteltig, Ruhr-Universität Bochum. The double mutants vtc1/sid2 and etiol/ein2 were created by selecting F2 individuals from the cross between vtc1/sid2 or etiol/ein2. For selecting vtc1/sid2 double mutants, F2 plants that showed low levels of AsA were identified as described by Conklin et al. (2000). F1 lines lacking ozone-inducible SA accumulation were selected, then the point mutation of the ICS1 gene in the sid2 mutant was identified with DNA sequencing. For selecting etiol/ein2 double mutants, F2 plants lacking AsA biosynthesis were identified as described above. Ethylene-insensitive F1 lines were selected on plates containing 20 μM ACC for screening for the lack of the triple response (Guzman and Ecker, 1990). Seeds were surface sterilized with 15% bleach and germinated on agar plates containing 0.5× Murashige and Skoog medium with and without 15 μM sodium selenite. Total RNA was isolated from shoots as described above. Five micrograms of DNase-treated total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas International) following the manufacturer’s instructions. PCR reactions were carried out as described previously (Schiavon et al., 2007). A list of primers used in these experiments is presented in Supplemental Table S2.

Expression Analysis via Semiquantitative RT-PCR

Plants were grown for 7 d on 0.5× Murashige and Skoog medium with or without 15 μM sodium selenite. Total RNA was isolated from shoots as described above. Five micrograms of DNase-treated total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas International) following the manufacturer’s instructions. PCR reactions were carried out as described previously (Schiavon et al., 2007). A list of primers used in these experiments is presented in Supplemental Table S2.

In Vitro Treatments and in Situ ROS Detection

The effect of exogenous SA, ACC, or MeJA on plant selenite resistance was analyzed by sowing sterilized Arabidopsis seeds on plates containing 0.5× Murashige and Skoog with a range of concentrations of SA, ACC, or MeJA with or without 15 μM sodium selenite. Seedlings were grown on vertical plates for 7 d. Seminal root length was measured from digital photographs of the seedlings with the ImageJ program (http://rsb.info.nih.gov/ij/). The Se tolerance index was calculated as described above.

Measurement of SA, MeJA, and JA

MeJA, salicylate, and jasmonate levels in shoot tissue were determined in plants grown as described above with or without 15 μM sodium selenite for 7 d. For measurement of SA and MeJA, the extracts were prepared as described by Wilpert et al. (1998). The extracts were analyzed by liquid chromatography-mass spectrometry (LC-MS) using a Hewlett-Packard Agilent 1100 series HPLC and a Finnigan LCQDuo thermosto MS system equipped with Xcalibur software. Through 30 mL injections, these extracts were separated at 40°C using a Phenomenex Hypersil 5-mm C18 (ODS) column (250 × 2 mm, 5 mm) at a flow rate of 0.32 mL/min using two eluents: A, water + 0.1% formic acid; and B, 100% methanol + 0.1% formic acid. The following gradient program was used during the 23-min run: 0 to 7 min, 50% A and 50% B; 7 to 9 min, 30% A and 70% B; 9 to 12 min, 100% B; 12 to 13 min, 50% A and 50% B, with a 10-min postrun, column wash 50% A and 50% B. Standard curves were established using chemicals purchased from Sigma Chemical; MeJA (catalog no. 392707) had a retention time of 2.5 min, SA (catalog no. A–6262) had a retention time of 4.45 min, and jasmonate (catalog no. D2500) had a retention time of 6.85 min. Through MS, the different metabolites were measured at their appropriate masses and retention times observed for each of the standards. The MS detector settings were 1 to 3.5 min in positive ion mode using parameters generated with the MeJA standard and the automated tune program, 3.5 to 5.5 min in negative ion mode using parameters generated with the SA standard and the automated tune program, and 5.5 to 13 min in negative ion mode using parameters generated with the JA standard and the automated tune program. Samples were kept at room temperature (25°C) in the autosampler. The previously published (Wilpert et al., 1998) and observed precursor and product ions for these standards were exactly the same. These masses and those from the dimers for MeJA and JA were used to quantify these compounds.
Measurement of Ethylene, Nonprotein Thiols, Ascorbic Acid, and Glutathione

For measurement of ethylene, 10 seedlings were enclosed in a 60-mL vial and incubated for 12 h with illumination in a growth chamber. A 25-mL gaseous phase of the vial was subjected to a Fisons 8000 gas chromatograph equipped with a flame ionization detector. A 2-m Altec Haysep N 80/100 column was used with isothermal oven temperature at 70°C and flame ionization detector at 200°C. The program was 2 min in length with the ethylene peak running from 1.180 to 1.633 min. Ethylene peak area was determined by the PeakSimple program (ver. 3.39, 6 channel; SRI Instruments). The amount of ethylene generated from the seedlings was estimated from the peak area compared to that of ethylene standard.

Measurement of nonprotein thiol levels was performed using Ellman’s reagent as described (Zhu et al., 1999). To measure total AsA, DHA, GSH, and GSSG, 100 mg of fresh plants was homogenized in 2 mL of cold 5% (w/v) metaphosphoric acid with sea sand. The ASA and DHA contents were quantified as described previously (Tamaoki et al., 2003a). GSH and GSSG contents were measured as described by Yoshida et al. (2006). All experiments were carried out in three replicates, each consisting of 20 to 30 pooled plants.

Quantification of Se and S Accumulation

Col-0 and Ws-2 plants were grown on 0.5× Murashige and Skoog agar medium with or without 15 μM selenite in a growth chamber for 3 weeks. Root and shoot materials were harvested separately, rinsed with distilled water, and dried at 37°C for a week. Three replicates consisting of 30 to 50 seedlings were acid-digested and analyzed for Se and S by inductively coupled plasma atomic emission spectrometry as described by Pilon-Smits et al. (1999).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Identified up-regulated selenite-responsive genes (P < 0.05, fold change >2) that are categorized with biological function.

Supplemental Figure S2. Sensitivity to SA, ACC, and MelA in seedlings of Col-0 and Ws-2.

Supplemental Table S1. Macroarray study showing induction of genes in Col-0 and Ws-2 by 15 μM selenite treatment.

Supplemental Table S2. Primers used in semiquantitative RT-PCR.

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