# Characterization of a Selenate-Resistant Arabidopsis Mutant. Root Growth as a Potential Target for Selenate Toxicity[1][OA]

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Screening an Arabidopsis (*Arabidopsis thaliana*) T-DNA mutant library for selenate resistance enabled us to isolate a selenate-resistant mutant line (*sel1-11*). Molecular and genetic characterization showed that the mutant contained a lesion in the *SULTR1;2* gene that encodes a high affinity root sulfate transporter. We showed that *SULTR1;2* is the only gene among 13 mutated genes of the Arabidopsis sulfate transporter family whose mutation conferred selenate resistance to Arabidopsis. The selenate resistance phenotype of the *sel1-11* mutant was mirrored by an 8-fold increase of root growth in the presence of selenate as shown by the calculated lethal concentration values. The impairment of SULTR1;2 activity in *sel1-11* resulted in a reduced $^{35}S$-sulfate uptake capacity by both roots and calli and a reduced sulfate and selenate content in root, shoot, and calli. Comparing sulfate-to-selenate ratios instead of absolute sulfate and selenate contents in roots and shoots enabled us to gain better insight into the mechanism of selenate toxicity in Arabidopsis. Roots of the *sel1-11* mutant line showed a higher sulfate to selenate ratio than that of wild-type roots, while there were no significant differences in sulfate to selenate ratios in shoots of wild-type and mutant lines. These results indicated that the mechanism that confers the selenate resistance phenotype to the *sel1-11* line takes place rather in the roots. It might be in part the result of a lower selenate uptake and of a protective effect of sulfate against the toxic effects of selenate on root growth. These results revealed in plants a central and specific role of the transporter SULTR1;2 in selenate sensitivity; they further suggested that root growth and potentially the root tip activity might be a specific target of selenate toxicity in Arabidopsis.

In animals, both the beneficial and toxic effects of selenium are well documented (Ohlendorf et al., 1986; Läuchli, 1993; Spallholz, 1994; Arteel and Sies, 2001; El-Bayoumy, 2001; Tapiero et al., 2003; Schromburg et al., 2004). They are, however, poorly understood in plants and yeast (*Saccharomyces cerevisiae*; Läuchli, 1993; Terry et al., 2000). Although required in minute quantities (0.05–0.10 mg/kg) by animals to form the selenoenzymes, thioredoxin reductase and glutathione peroxidase, selenium quickly becomes toxic at higher concentrations (2–5 mg/kg dry food) causing mortality, developmental defects, and reproductive failure (Terry et al., 2000). Selenium has not yet been found to have any role in higher plants, although some resistant plants growing on seleniferous soils, including some species of Astragalus and Stanleya, are able to accumulate selenium to very high concentrations (Feist and Parker, 2001; Pickering et al., 2003). It has, however, been shown to be essential in *Chlamydomonas reinhardtii* (Novoselov et al., 2002). In most plants, protein synthesis is adversely affected, causing symptoms including chlorosis and stunting that mimic sulfate starvation, as well as withering and drying of leaves and premature death (Terry et al., 2000).

Sulfate ($SO_4^{2-}$) is the main source of sulfur taken up by roots from the soil solution. Because of the structural similarity of selenate ($SeO_4^{2-}$) to sulfate, selenium (as selenate) competes for access to membrane sulfate transporters and to enzymes of the sulfur assimilation pathway, leading to the reduction and assimilation of selenate to the selenium analogs of Cys and Met, seleno-Cys and seleno-Met, in plants (Leggett and Epstein, 1956; Läuchli, 1993; Terry et al., 2000; Sors

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et al., 2005b) and microorganisms (Tweedie and Segel, 1970; Breton and Surdin-Kerjan, 1977; Cherest et al., 1997). In Arabidopsis (*Arabidopsis thaliana*), sulfate uptake by roots and its subsequent transport throughout the plant is mediated by a family of sulfate transporters belonging to the SLC26 super family of anion transporters. This family is composed of 14 members that fall into five groups depending on sequence and functional homology (Buchner et al., 2004). The two sulfate transporters SULTR1;1 and SULTR1;2 belong to group 1, which is composed of genes encoding high affinity sulfate transporters that are expressed mainly in roots (Vidmar et al., 2000; Shibagaki et al., 2002). These two transporters are colocalized in the epidermis and cortex of roots and are probably responsible for the initial acquisition of sulfate from the soil solution (Shibagaki et al., 2002; Yoshimoto et al., 2002).

Plant responses to sulfur deficiency are relatively well documented in Arabidopsis and include the induction of *SULTR1;1* expression in roots, which contributes to the increased root sulfate uptake capacity (Takahashi et al., 2000; Yoshimoto et al., 2002). Expression studies of several other sulfate transporters, and of enzymes of the sulfur metabolic pathway, show that there is an increase in gene expression upon sulfate starvation and that this potentially contributes to greater sulfur use efficiency. Other genes encoding sulfate transporters that are involved in long-distance transport in plants, i.e. SULTR1;3, SULTR2;1, SULTR2;2, and SULTR3;5, have also been characterized in Arabidopsis (Takahashi et al., 2000; Yoshimoto et al., 2003; Kataoka et al., 2004a). Additionally, the transporters SULTR4;1 and SULTR4;2 also appear to be involved in sulfate accumulation by controlling transport across the tonoplast (Kataoka et al., 2004b). Once in leaves, sulfate is either accumulated in vacuoles or transferred to chloroplasts where Cys, Met, and several other important sulfur-containing compounds such as glutathione, are synthesized via the sulfate assimilation pathway (Leustek, 2002; Ravanel et al., 2004).

One of the main mechanisms of selenium toxicity in animals and plants is believed to be the nonspecific incorporation of selenium into proteins (Brown and Shrift, 1981; Stadtman, 1990; Terry et al., 2000). The substitution of Cys and Met by their selenoanalogs in proteins has been shown to impair protein synthesis, structure, function, and regulation; for example, selenocysteine has been shown to impede the formation of sulfur bridges (Gromer and Gross, 2002), while selenomethionine affects protein synthesis (Eustice et al., 1981). Furthermore, because Cys is an important component of the catalytic domain of many enzymes, its replacement by the much more reactive seleno-cysteine (Stadtman, 1996) may make such enzymes hypersensitive to the action of heavy metals, thereby leading to their inactivation (Bock et al., 1991).

One approach to elucidating mechanisms of selenium toxicity in plants is to use mutant lines. Screening of an ethyl methanesulfonate (EMS) mutant library of Arabidopsis led to the identification of allelic selenate-resistant mutants (*sel1*) that had lesions in the *SULTR1;2* gene encoding a root sulfate transporter (Shibagaki et al., 2002). These mutants displayed enhanced root growth in presence of selenate compared to wild type, but their selenate resistance phenotype has not been further characterized. A comprehensive study of the behavior under selenate treatment of *sel1* allelic mutants, isolated in our lab, was undertaken to fill this gap and led to a better understanding of selenate toxicity mechanisms in plants.

## RESULTS

### Isolation and Genetic Characterization of Selenate-Resistant Mutants

Selenate-resistant mutants of Arabidopsis were identified by germinating T2 seeds of a T-DNA mutagenized Wassilewskija (WS) ecotype population on a solid germinating medium (GM) containing 10 $\mu$M selenate and djenkolate as an organic sulfur source (Greenberg et al., 1964). Plants from the mutagenized population possessing roots with lengths $\geq$5 mm were selected for further analysis; under these conditions, root lengths of wild-type plants were no longer than 2 mm. From 30,000 T-DNA mutagenized T2 lines, 132 putative selenate-resistant mutants were isolated. After rescreening the progeny of the putative mutants, the selenate-resistant phenotype was confirmed for only four mutant lines: A25, B78, B181, and A251.

Genetic analysis indicated that the selenate-resistant phenotypes of the mutants A25, B78, and B181 are caused by single recessive mutations (Table I). Selenate sensitivity in the $F_2$ progeny of the three mutant lines segregated in a 3:1 ratio (sensitive:resistant), indicating that the mutant phenotypes are caused by a recessive mutation at a single locus. None of the four mutant lines showed resistance to kanamycin (data not shown), a character normally conferred by the T-DNA insertion (Bechtold and Pelletier, 1998), revealing that the mutations were not properly tagged by a T-DNA. Genetic complementation tests indicated that the four selenate-resistant mutants identified fell into the same complementation group (Table I). In all the crosses performed, all $F_2$ progeny tested were resistant to selenate. The results indicated that these mutants contained lesions at the same genetic locus.

### Mapping and Identification of the Mutations in the *SULTR1;2* Gene

To identify the gene responsible for the mutant phenotype, the lesions in A25 and B78 were genetically mapped. These two mutants were crossed with a wild-type plant from the Landsberg erecta ecotype. The $F_1$ plants were allowed to self fertilize and the resulting $F_2$ plants used to genetically map the mutation. $F_2$ seeds were germinated on solid GM medium containing 10 $\mu$M selenate and scored for selenate resistance. Total DNA was isolated from $F_2$ plants individually, and the segregation of molecular genetic

**Table I.** *Segregation of the selenate-resistant phenotype in $F_2$ Arabidopsis plants*

Wild-type WS and mutants plants (A25, B78, B181, A251, and *sel1-8*) are, respectively, not able or able to fully grow in vitro on a synthetic agarose medium containing 10 $\mu$M selenate in the presence of 100 $\mu$M djenkolate as sole sulfur source. *sel1-8* line is mutated in the gene *SULTR1;2* encoding a sulfate transporter. $F_2$ generation plants of each cross were counted after 10 d, according to the plant root length.

| Crosses | Generation | No. of Phenotypes | Plants Tested | | $\chi^{2a}$ |
|---|---|---|---|---|---|
| | | | Sen[b] | Res[b] | |
| A25 × WS | $F_2$ | 2,090 | 1,587 | 504 | 0.04[c] |
| B78 × WS | $F_2$ | 108 | 74 | 34 | 2.25[c] |
| B181 × WS | $F_2$ | 1,834 | 1,415 | 419 | 0.24[c] |
| A25 × B78 | $F_2$ | 192 | 0 | 192 | |
| A25 × B181 | $F_2$ | 177 | 0 | 177 | |
| A251 × B78 | $F_2$ | 204 | 0 | 204 | |
| A25 × *sel1-8* | $F_2$ | 643 | 0 | 643 | |
| B78 × *sel1-8* | $F_2$ | 519 | 0 | 519 | |
| A251 × *sel1-8* | $F_2$ | 223 | 0 | 223 | |

[a]$\chi^2$ values were calculated based on the expected ratio of 3:1 segregation. [b]Sen, Sensitive and Res, resistant to 10 $\mu$M selenate, are plants in which root length is shorter than 2 mm or longer than 10 mm, respectively. [c]$P < 0.05$.

markers was analyzed. The mutation was mapped to the lower arm of chromosome 1 near the marker alcohol dehydrogenase (ADH; Fig. 1A) by examining 51 and 76 $F_2$ progeny from A25 and B78, respectively. Nine allelic selenate-resistant mutants (*sel1-1* to *9*) were previously isolated by screening for plant growth on medium containing 10 $\mu$M selenate (*sel1-1* to *7*) or 20 $\mu$M selenate (*sel1-8* and *sel1-9*; Shibagaki et al., 2002). All of these mutant lines bear mutations in the *SULTR1;2* gene located at the lower arm of chromosome 1 near the ADH marker. To determine whether the mutants that we isolated were allelic with *sel1*, genetic complementation tests were performed by crossing the three mutant lines A25, B78, and A251 with *sel1-8*. All $F_2$ progeny from these crosses were selenate resistant (Table I), indicating that the mutants were all alleles of *sel1*. Subsequently, we have renamed the A25, B78, B181, and A251 mutant lines as *sel1-11*, *sel1-12*, *sel1-13*, and *sel1-14*, respectively.

We examined the nature of the mutations in the *SULTR1;2* gene in the four isolated mutant lines. PCR analysis indicated that *sel1-12* and *sel1-14* bear an important deletion in the lower arm of chromosome 1, covering 65 kb and concerning 16 different genes, including *SULTR1;2* (Fig. 1B). Sequencing of the *SULTR1;2* gene of *sel1-11* and *sel1-13* indicated the presence of the same microdeletion of seven bases at the start of exon number 2 (Fig. 1C). Bioinformatics analysis indicated that this microdeletion generates a frame shift, the creation of a premature stop codon and subsequently the synthesis of a truncated protein of 139 amino acids instead of the usual 653 amino acids of the SULTR1;2 native protein. Subsequently, we chose the *sel1-11* mutant line for further characterization. SULTR1;2 was previously characterized as a high affinity sulfate transporter (Yoshimoto et al., 2002; Shibagaki and Grossman, 2004). We confirmed the transport function for SULTR1;2 by complementing the yeast sulfate transport defective 1 strain (YSD1) yeast mutant defective in its sulfate transport capacity (Smith et al., 1995). The calculated kinetic parameters values, $K_m$ for sulfate (approximately 7.4 $\mu$M) and $V_{max}$ (approximately 0.6 nmol $h^{-2}$ [$10^8$ cellules]$^{-1}$), were consistent with previous reports (Shibagaki et al., 2002; Yoshimoto et al., 2002).

### *sel1* Plants Are the Only Mutants to Show Root Growth in the Presence of Selenate

We determined the root growth capacity of different Arabidopsis lines by comparing the Columbia (Col-0) wild type to 12 Col-0-derived homozygote T-DNA insertion mutant lines, each one bearing a mutation in one of the following sulfate transporter genes: *SULTR1;1*, *SULTR1;3*, *SULTR2;1*, *SULTR2;2*, *SULTR3;1*, *SULTR3;2*, *SULTR3;3*, *SULTR3;4*, *SULTR3;5*, *SULTR4;2*, *SULTR5;1*, and *SULTR5;2*. An additional Col-0 EMS-derived mutant *sel1-8* defective in the SULTR1;2 transporter was also studied. This mutant is allelic to the WS-derived T-DNA insertion mutant *sel1-11* (Table I). When germinated in vitro on sulfate-free agarose solid growth media containing djenkolate as the sole sulfur source, the roots of all lines grew normally (Fig. 2A). In the presence of 3 $\mu$M of selenate, root growth of the wild-type seedlings stopped following cotyledon emergence as did all the mutants defective in a sulfate transporter, except *sel1-8*, whose roots continued to grow, reaching approximately 1 cm in length 10 d after germination (Fig. 2A).

### *SULTR1;2* Mutation Results in an 8-Fold Increase in Selenate Resistance

We quantified the selenate resistance of WS and the *sel1-11* mutant by establishing the lethal concentration ($LC_{50}$) curves of root length as a function of selenate concentration in the sulfate-free agarose growth medium. The calculated $LC_{50}$ value represents the concentration of selenate in the growth medium that

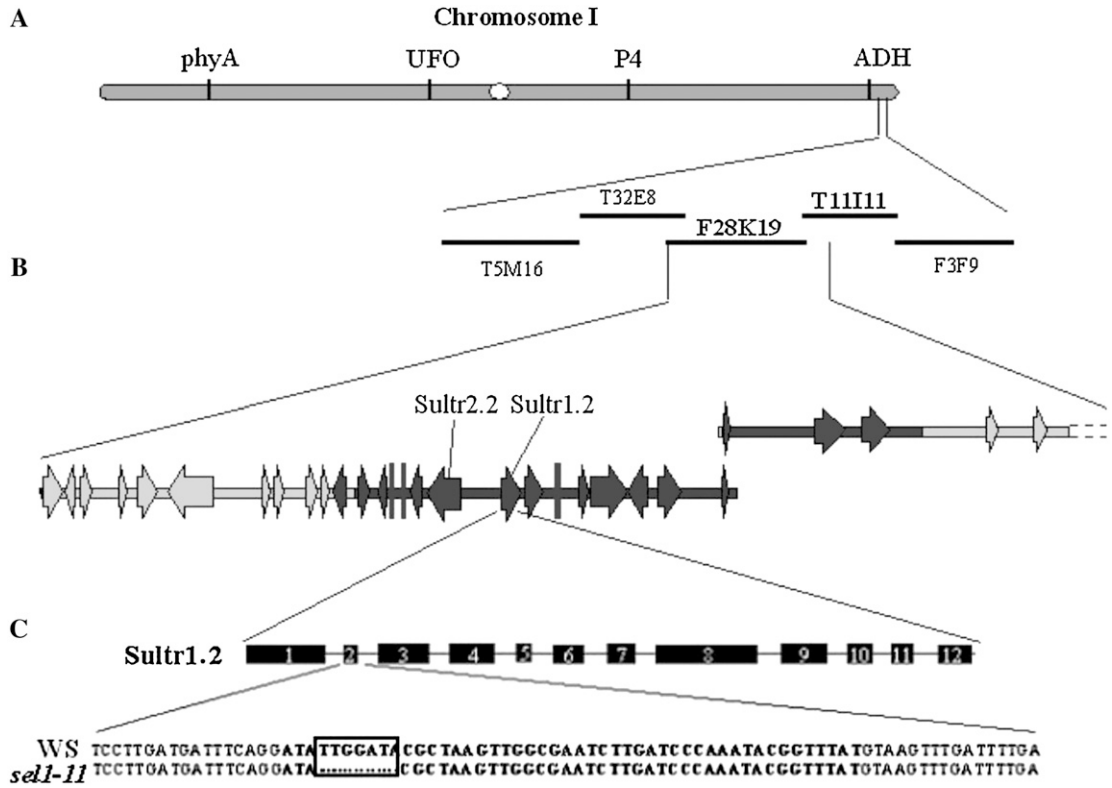


**Figure 1.** Localization of deletions in *SULTR1;2* gene in *sel1-11* and *sel1-12* mutants. A, Position of the ADH marker on the lower arm of the chromosome 1. B, Structure of the 65-kb deletion in the *sel1-12* and *sel1-14* mutant lines shared by the bacterial artificial chromosomes F28K19 and T11I11. Black arrows represent deleted genes. C, Structure of the *SULTR1;2* gene with the localization of the seven bases deletion in the gene of the *sel1-11* and *sel1-13* mutant lines at the beginning of exon number 2.

causes 50% reduction in root length compared to the control. Seeds were germinated on a solid GM medium containing djenkolate as sole sulfur source and amended with selenate in concentrations ranging from 0 to 50 $\mu$M. Seedlings were allowed to grow for 1 week before their root length was measured (Fig. 2B). The calculated $LC_{50}$ for the wild-type seedlings was 1.5 $\mu$M, while that for *sel1-11* seedlings was 12.7 $\mu$M, which represents an 8-fold increase in selenate resistance of the mutant plants compared to the wild type. Furthermore, the selenate toxicity threshold, which represents the minimal selenate concentration that affects root growth, was less than 0.5 $\mu$M for the wild-type seedlings, while *sel1-11* seedlings can withstand up to 2 $\mu$M of selenate in the external medium without any adverse effect on root growth (Fig. 2B). In the range of higher external concentrations of selenate (20–50 $\mu$M), root length of wild-type seedlings was below 10% of that of control plants roots. Although root length of *sel1-11* seedlings was markedly reduced, it was never lower than 25% of untreated control plants roots.

### *SULTR1;1* and *SULTR1;2* Gene Expression in WS and *sel1-11* Roots

Expression of the *SULTR1;1* and *SULTR1;2* genes was determined by reverse transcription (RT)-PCR in the roots of wild type and *sel1-11* mutants in response to sulfate and selenate availability in the culture medium. For this purpose, WS and *sel1-11* seedlings were allowed to grow for 8 d on a GM medium containing 1 mM sulfate and then transferred for 2 d to the same medium containing: 1 mM sulfate (+S), no sulfate (−S), or 1 mM sulfate and 0.25 mM selenate (+Se). Plants were then harvested and RT-PCR performed on total RNA extracted from roots (Fig. 3).

The *SULTR1;2* gene was strongly expressed in both WS and *sel1-11* roots independent of sulfate and selenate availability in the medium. The small deletion in the *SULTR1;2* gene of *sel1-11* did not seem to affect the transcription of the gene or the level of its expression in *sel1-11* roots. The *SULTR1;1* gene expression in WS roots is repressed when sufficient sulfate is supplied to the plants but increased under sulfate deficiency conditions and by selenate presence in the medium. In *sel1-11* roots, *SULTR1;1* expression was induced even under sulfate sufficiency conditions, suggesting a functional redundancy in the absence of SULTR1;2 sulfate transport activity.

### Sulfate Uptake Capacity Is Impaired in *sel1-11* Roots

Because expression of the *SULTR1;2* gene was localized primarily in the root epidermis and cortex

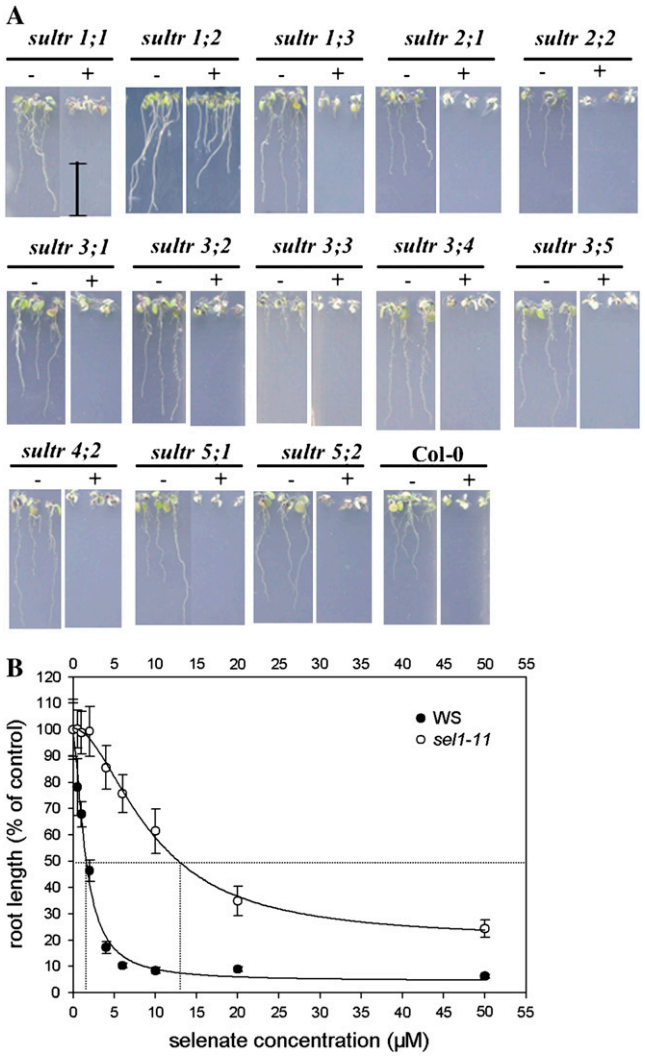


**Figure 2.** Effects of selenate on root growth of wild-type and derived mutant lines. A, Ten-day-old seedlings were grown in vitro on a solid sulfate-free agarose nutrient medium containing djenkolate as sole sulfur source either in absence (−) or in presence (+) of 3 $\mu$M selenate. Seeds grown were from wild-type Col-0, T-DNA insertion Col-0-derived mutant lines defective in sulfate transporters (*sultr1;1*, *sultr2;1*, *sultr2;2*, *sultr3;1*, *sultr3;2*, *sultr3;3*, *sultr3;4*, *sultr3;5*, *sultr4;2*, *sultr5;1*, and *sultr5;2*), and Col-0-derived EMS-mutagenized mutant *sel1-8* defective in *SULTR1;2* (instead of the mutant *sel1-11* derived from the WS ecotype). The black bar represents 10 mm. B, Root length as a function of a selenate concentration in the medium. Root length of 1-week-old wild-type and *sel1-11* seedlings are expressed as a percentage of that of the selenate nontreated plants. Dashed lines represent selenate concentration corresponding to a 50% reduction of root length ($LC_{50}$). Seeds were germinated on a GM medium containing djenkolate as sole sulfur source and various selenate concentrations ranging from 0 to 50 $\mu$M. Seedlings were allowed to grow for 1 week before roots were scanned and their length measured. Each value represents the mean of 25 measurements. Error bars represent confidence intervals ($P = 0.05$).

(Shibagaki et al., 2002), we checked whether sulfate uptake capacity of roots of *sel1-11* plants was impaired. Wild-type and *sel1-11* plants were grown hydroponically for 4 weeks on a modified Hoagland medium containing 1 mM sulfate as the sole sulfur source. Plants were then transferred to an identical medium containing either no sulfate (−S), 1 mM sulfate (+S), or 1 mM sulfate and 0.5 mM selenate (+Se). Sulfate $^{35}S$-labeled root uptake capacities for each set of treated plants were determined at 0, 24, 48, and 72 h following their transfer (Fig. 4). Sulfate uptake of roots of wild-type and *sel1-11* plants transferred from a sulfate-sufficient medium to an identical medium (+S) did not show significant variation, indicating that the plant transfer by itself did not impair root sulfate uptake capacity. Sulfate uptake capacity of the roots of (+S) s*el1-11* plants was 35% lower than that of wild-type plants. Sulfate uptake capacity by roots of WS and *sel1-11* plants were both induced in response to sulfate starvation. However, under these (−S) conditions, sulfate uptake by roots of *sel1-11* plants represented only 40% of that of the wild type (Fig. 4). A pretreatment in the presence of selenate markedly reduced root sulfate uptake capacity of both wild-type and *sel1-11* plants. Interestingly, under these conditions, there was no significant difference between wild-type and mutant plants in terms of root sulfate uptake capacity.

### Sulfate and Selenate Accumulation in Roots and Leaves of *sel1-11* Are Reduced

Because sulfate uptake by roots of *sel1-11* plants was impaired, we investigated sulfate and selenate accumulation in roots and leaves of *sel1-11* to determine if they were also reduced. Wild-type and *sel1-11* seedlings were allowed to grow for 8 d on a solid GM medium containing 1 mM sulfate as sole sulfur source. Seedlings were then transferred for 2 d to an identical medium containing either 1 mM sulfate (+S), no sulfate (−S), or 1 mM sulfate and 0.25 mM selenate (+Se). Roots and leaves were harvested separately, washed, and their sulfate and selenium contents measured. Selenate could not easily be measured by ionic chromatography due to cosegregation with fumarate, which was also significantly present in leaves. Instead, we routinely measured selenium contents, because we demonstrated, using x-ray absorption spectroscopy (Pickering et al., 2003), that in our wild-type and mutant lines, selenium was mainly found as selenate in both roots and shoots (data not shown). When grown on +S medium, sulfate content of roots and leaves of *sel1-11* seedlings was markedly reduced compared to wild-type plants (Fig. 5A). This result is in agreement with a reduced sulfate uptake capacity by roots of the mutant line *sel1-11* (Fig. 4). When transferred for 2 d on sulfate-depleted medium, sulfate contents of leaves and roots of both wild-type and *sel1-11* seedlings were decreased. However, when plants were transferred to a selenate-containing medium, sulfate contents were lowered in roots and increased in leaves of both WS and *sel1-11* seedlings. It is noteworthy that regardless of the treatments, no significant differences were detected between the two lines WS and *sel1-11* in their respective roots and leaves, chloride, nitrate, and phosphate contents (data

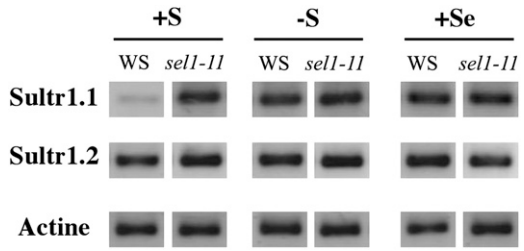


**Figure 3.** Expression of *SULTR1;1* and *SULTR1;2* in roots of wild-type WS and mutant *sel1-11 lines*. Expression analysis of the genes is done by RT-PCR (30 cycles) using total RNA extracted from roots of 10-d-old seedlings cultivated in vitro. Seedlings were allowed to grow for 8 d on a synthetic medium containing 1 mM sulfate and then transferred on the same medium containing: 1 mM sulfate (+S), no sulfate (−S), 1 mM sulfate, and 0.25 mM selenate (+Se). Actin expression is used to verify equal loading on the gel.

not shown). As expected, selenium was detected only in selenate-treated plants (Fig. 5B). Similar to sulfate, selenium contents of both roots and leaves of *sel1-11* seedlings were highly diminished compared to the wild type. Interestingly, the sulfate to selenium ratio in roots of *sel1-11* seedlings was significantly higher than that calculated for roots of WS (Fig. 5C), while sulfate to selenium ratios in leaves of WS and *sel1-11* seedlings were not significantly different.

### Sulfate Uptake Capacity and Selenium Accumulation in Wild-Type and *sel1-11* Callus Culture

To study the consequences of *SULTR1;2* mutation at the cellular level, we used calli produced from WS and *sel1-11* seeds by hormone treatment. Selenium accumulation in calli was determined over time after incubation in the presence of 10 $\mu$M selenate and djenkolate as sole sulfur source (Fig. 6A). Selenium accumulation was significantly reduced (75% decrease) in *sel1-11* callus compared to wild type after 96 h incubation in presence of selenate.

Sulfate uptake capacity measurements of calli of wild type and *sel1-11* lines were performed under the same experimental conditions as for selenium accumulation (Fig. 6B). In the presence of djenkolate as sole sulfur source, sulfate uptake capacity in *sel1-11* callus showed a 45% decrease compared to wild type. Addition of 10 $\mu$M selenate resulted in a decrease in the sulfate capacity of wild-type calli to reach a level similar of that of *sel1-11*. These results are consistent with those obtained for whole plants (Figs. 4 and 5).

### Selenate Inhibitory Effect on Root Growth Is a Relatively Rapid Process

To test if root growth is a specific target for selenate toxicity, plants were grown normally on a solid GM medium in the absence of selenate for 8 d and then transferred to an identical medium plus or minus 10 $\mu$M selenate, in the presence of djenkolate as sole sulfur source. The petri dishes containing the transferred plants were then rotated 180° so the plants were upside down. As shown in Figure 7, root growth of wild-type seedlings stops after their transfer to a plate containing selenate. Root growth of *sel1-11* seedlings continued after transfer to a selenate-containing medium but at a lower rate compared to the nontreated plants (Fig. 7).

## DISCUSSION

Screening an Arabidopsis T-DNA mutant library for selenate resistance enabled us to isolate several resistant mutant lines, which were shown to be alleles of *sel1* selenate-resistant mutants deleted in the *SULTR1;2* gene (Shibagaki et al., 2002). Functional complementation of a yeast mutant strain lacking sulfate transport capacity confirmed that *SULTR1;2* encodes a high affinity sulfate transporter ($K_m$ approximately 7.4 $\mu$M). Furthermore, our results show that the deletion of the *SULTR1;2* gene leads to a significant reduction of root sulfate uptake capacity and an impaired response to sulfur deprivation (Fig. 4) and that sulfate accumulation by roots and leaves is markedly reduced in the *sel1-11* mutant compared to the wild type (Fig. 5A). This is in agreement with previously obtained results (Shibagaki et al., 2002; Maruyama-Nakashita et al., 2003). Thus, SULTR1;2, which is clearly localized in the outer cell layers of the root as well as in the root tip (Shibagaki et al., 2002; Yoshimoto et al., 2002), appears to play a major role in the acquisition of sulfate by roots of Arabidopsis, and its deletion leads to an inefficient synthesis of downstream sulfur containing

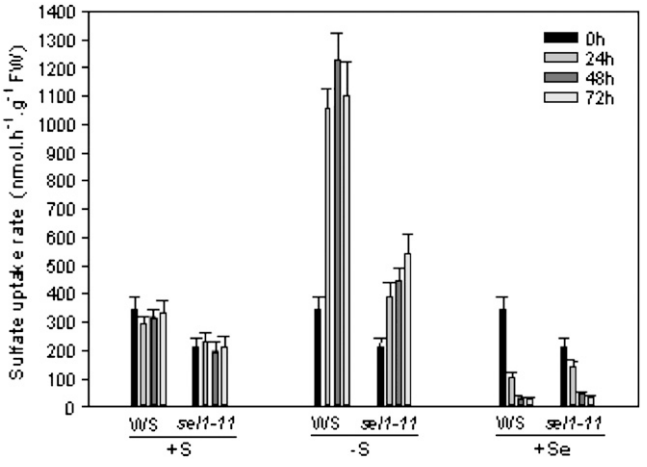


**Figure 4.** Effect of *SULTR1;2* mutation on sulfate uptake by roots. Four-week-old WS and *sel1-11* plants were grown hydroponically and pretreated for 0, 24, 48, or 72 h on sulfate sufficient (+S) or sulfate deficient (−S) medium or on a sulfate sufficient medium containing 0.5 mM selenate (+Se). Plants were then incubated for 10 min in a medium containing $^{35}S$-labeled 100 $\mu$M sulfate at pH 5, maintained at 25°C, before roots were harvested and $^{35}S$ radioactivity measured. Each value represents the mean of six to eight measurements. Error bars represent confidence intervals ($P = 0.05$).

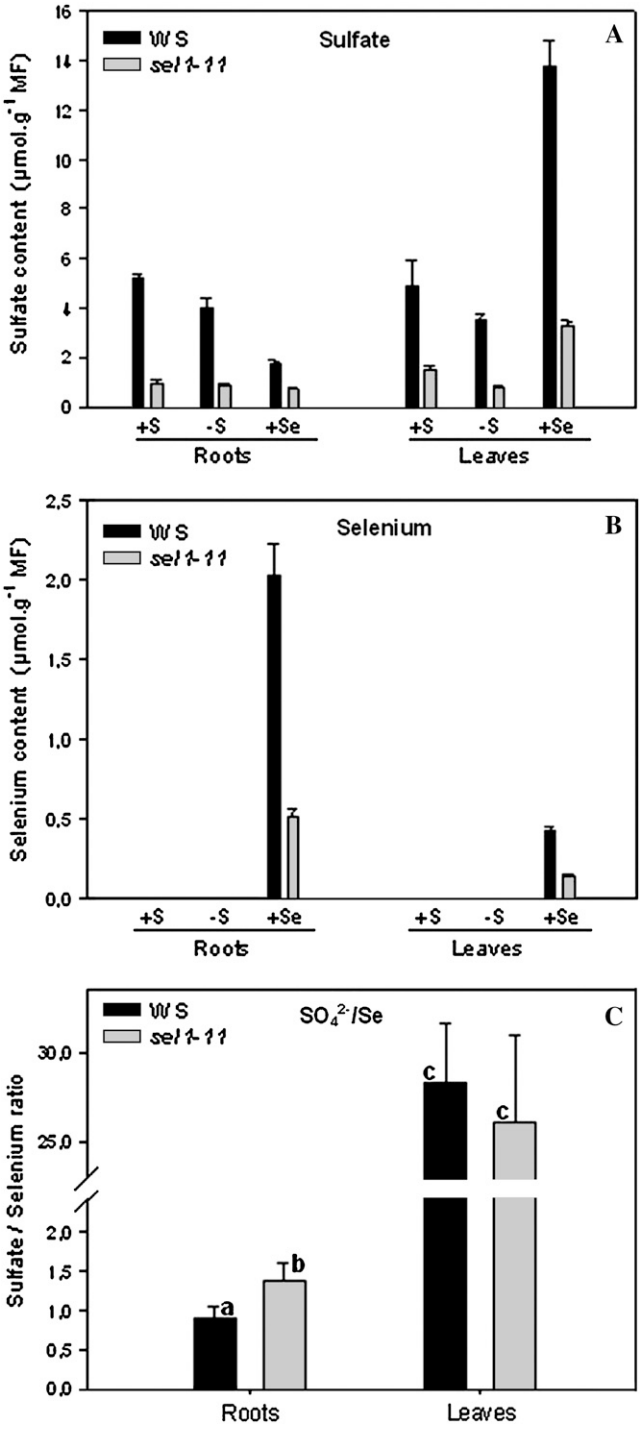


**Figure 5.** Sulfate and selenium contents of roots and leaves of wild-type WS and mutant *sel1-11* lines. WS and *sel1-11* seedlings were grown for 8 d on solid GM medium containing 1 mM sulfate as sole sulfur source then transferred for 2 d on an identical medium containing 1 mM sulfate (+S), or no sulfur (−S), or 1 mM sulfate and 0.25 mM selenate (+Se). Sulfate contents (A) were measured by high performance ion chromatography. Selenium contents (B) were measured by atomic absorption spectrometry coupled to a graphite furnace. C, Sulfate to selenium ratios in roots and leaves are calculated using data presented in A and B. Each value corresponds to the mean of five measurements. Error bars represent confidence intervals ($P = 0.05$). Groups of results with no significant statistical difference (Newman-Keuls test; $P < 0.05$) are designated by identical letters.

amino acids and other metabolites (Maruyama-Nakashita et al., 2003).

In addition to sulfate transport, SULTR1;2 also appears to be involved in the transport of selenate, as suggested by the fact that the mutation of *SULTR1;2* in *sel1-11* led to a reduction in root and leaf selenium contents (Fig. 5). The role of SULTR1;2 as a major sulfate and selenate transporter is further confirmed by the fact that three different attempts to isolate selenate-resistant mutants through mutant library screening done by our laboratory and two other research teams (Rose, 1997; Shibagaki et al., 2002) all eventually led to the identification of *SULTR1;2*-affected mutants. It has to be acknowledged that the screening protocols used in these studies, i.e. root growth in presence of 10 $\mu$M selenate, may have favored the identification of the *sel1* mutants over others that might have shown different or more discrete phenotypes if treated with lower selenate concentrations. Nevertheless, we clearly demonstrated that in Arabidopsis, among the 13 homozygote lines, each of them bearing a mutation in a different sulfate transporter, the mutation of the gene encoding the sulfate transporter SULTR1;2 is the only one that conferred resistance to selenate when root growth was chosen as a selection criterion (Fig. 2A). It is possible that other classes of selenate-resistant mutants could still be found, as indicated by the quantitative trait loci (QTL) study that identified QTLs for selenate resistance that were independent of any sulfate transporter (Zhang et al., 2006a, 2006b). The fact that in these last studies no identified QTL actually included *sultr1;2* does not in any way cast doubt on our results; it merely confirms the fact that different selection criteria (or different definitions of selenate resistance) may lead to the identification of different classes of mutants and genes. With regard to selenate tolerance, potentially interesting genes encoding enzymes of the sulfur metabolic pathway (e.g. ATP sulfurylase or Ser *O*-acetyltransferase) have been identified in the QTL region on chromosome 3 of Arabidopsis (Zhang et al., 2006b). Overexpression of these genes has been shown to be useful in understanding their role in selenate metabolism in Arabidopsis (Sors et al., 2005a). A beneficial impact of ATP sulfurylase overexpression in response to selenate treatment has indeed been observed in Arabidopsis (Sors et al., 2005a) and Indian mustard (*Brassica juncea*; Pilon-Smits et al., 1999); however, such an effect has not been found in tobacco (*Nicotiana tabacum*; Hatzfeld et al., 1998).

*SULTR1;2* is not the only gene implicated in sulfate acquisition by roots from the soil solution. *SULTR1;1* gene, which also encodes a root-expressed high affinity sulfate transporter (Takahashi et al., 2000; Vidmar et al., 2000), has been shown to colocalize with SULTR1;2 in the outer cell layers of the root (Takahashi et al., 2000). *SULTR1;1* gene expression is induced in roots of *sel1-11* even under sulfate sufficiency conditions, whereas it is

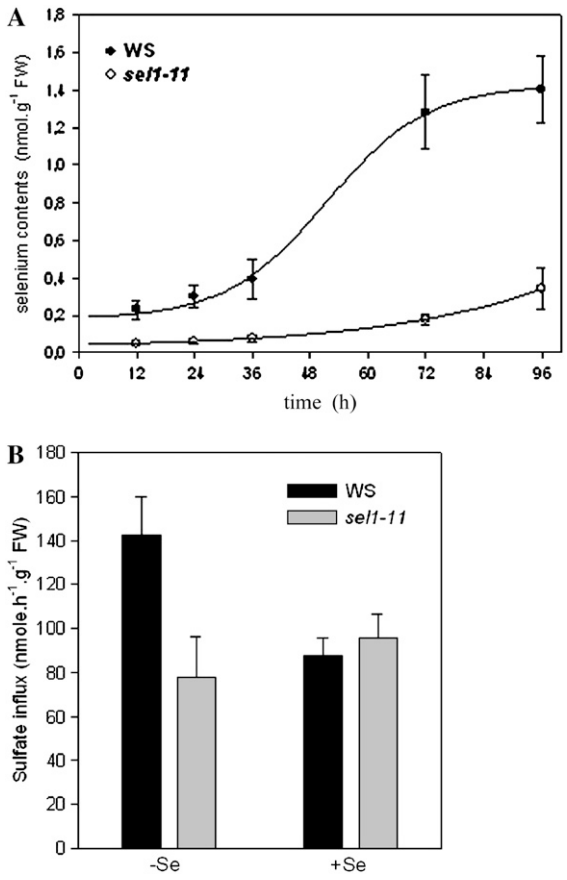


**Figure 6.** Selenate accumulation and sulfate uptake capacity of callus of wild-type WS and mutant *sel1-11* lines. A, Time-dependent selenium accumulation. One-month-old calli were transferred on a liquid modified Hoagland medium containing 100 $\mu$M djenkolate as sole sulfur source and selenate 10 $\mu$M. Selenium accumulated in calli was quantified by atomic absorption spectrophotometry coupled to a graphite furnace. B, Sulfate uptake capacity. Calli of each line were transferred on a liquid modified Hoagland medium containing 100 $\mu$M djenkolate as sole sulfur source in absence (−Se) or presence (+Se) of 10 $\mu$M selenate for 24 h, before measuring short-term sulfate influx during 10 min. Each value represents the mean of six independent measurements. Error bars represent confidence intervals ($P = 0.05$).

weakly expressed in WS roots (Fig. 3). This is probably to compensate for the absence of SULTR1;2 sulfate transport activity in *sel1-11* roots. *SULTR1;1* is strongly expressed along with *SULTR1;2* in roots of WS and *sel1-11* in response to sulfate deficiency and to selenate presence (Fig. 3). Our results show that the redundancy between the SULTR1;1 and SULTR1;2 functions is only partial. On the one hand, *SULTR1;1* induction in *sel1-11* roots leads to only a partial restoration of root sulfate uptake capacity (Fig. 4) and root and shoot sulfate contents (Fig. 5, A and B). On the other hand, characterization of a *SULTR1;1* T-DNA mutant showed that no selenate resistance phenotype was associated with *SULTR1;1* mutation (Fig. 2A).

*SULTR1;1* and *SULTR1;2* genes show similar expression patterns under sulfate deficiency (−S) and selenate (+Se) treatment (Fig. 3). Based on similar results obtained in wild-type Arabidopsis plants, it has been suggested that plant selenate treatment might mimic sulfate deficiency (Takahashi et al., 2000). Our results do not support this view. Sulfate starvation (−S) resulted in an increase in root sulfate uptake capacity (Fig. 4) and in a reduction of root and leaf sulfate contents in both wild-type and mutant plants (Fig. 5A), while upon selenate treatments (+Se), wild-type as well as *sel1-11* mutant plants exhibit a severe reduction in their root sulfate uptake capacity (Fig. 4). Similar results were also found in wild-type callus cultures (Fig. 6B). On the other hand, selenate treatment (+Se) resulted in a 2-fold increase in leaf sulfate contents and a more pronounced reduction in root sulfate content of both wild-type and mutant lines (Fig. 5A), which is consistent with results previously obtained in wild-type Arabidopsis lines, showing that selenate treatment tended to increase shoot sulfur (White et al., 2004) and shoot sulfate (Yoshimoto et al., 2002) contents. Together, these observations suggest that the increase of leaf sulfate content is more likely to be a result of sulfate relocation from roots to leaves rather than an increase in sulfate acquisition by roots. The effect of selenate on root sulfate uptake capacity cannot be attributed to a repression of the expression of sulfate transporters, because *SULTR1;1* and *SULTR1;2* are strongly expressed in response to selenate treatment (Fig. 3). It is more likely that selenate acts posttranscriptionally to inhibit the activity of sulfate transporters.

It is noteworthy that no difference is observed in root sulfate uptake capacity of wild-type and mutant plants under selenate treatment. A similar result is obtained in selenate-treated calli (Fig. 6B). This is rather surprising, because the commonly accepted hypothesis attributes the selenate resistance phenotype of the *sel1* mutants to a reduced root sulfate uptake capacity compared to wild type, leading to a reduced selenate entry in mutant

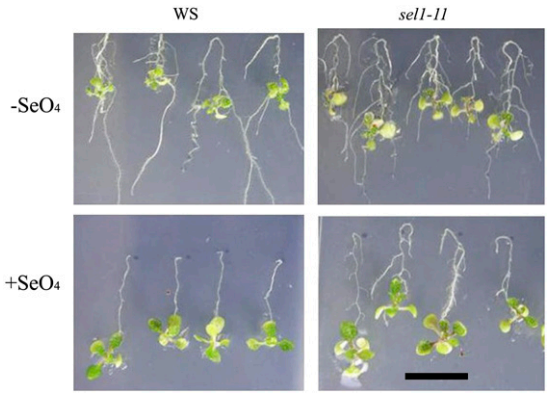


**Figure 7.** Effect of selenate on root growth of wild-type WS and mutant *sel1-11 lines*. Wild-type and mutant seedlings were allowed to grow for 8 d on an agarose GM medium in absence of selenate and in presence of 100 $\mu$M djenkolate as sole sulfur source. Seedlings were then transferred upside down to an identical agarose GM medium containing (bottom row $+SeO_4$) or not containing (top row $-SeO_4$) 10 $\mu$M selenate. Photographs were taken 4 d after the plant transfer. The black bar represents 5 mm.

plants. While a reduction in selenium accumulation is indeed observed in mutant plants compared to the wild type under selenate treatment (Fig. 5B), a comparable root sulfate uptake capacity between both lines is observed under the same conditions. This discrepancy suggests that a more complex determinant confers selenate resistance. The fact that in response to a 48-h selenate treatment, roots of both wild-type and mutant plants showed similar sulfate uptake capacity (Fig. 4) but divergent selenium contents (Fig. 5B) indicated that SULTR1;1 and SULTR1;2 could differ in their affinity for selenate. Because both transporters are active in the roots of wild-type plants and only SULTR1;1 is active in the roots of the mutant *sel1-11*, we can expect that the affinity of SULTR1;1 for selenate might be lower than that expected for SULTR1;2. If this is the case, then SULTR1;1 activity in *sel1-11* roots should be able to maintain a comparable root sulfate uptake capacity to that measured in selenate-treated wild-type plants while transporting less selenate. It has been suggested previously that in wild-type Arabidopsis plants, sulfate transporters might show contrasting selectivities toward sulfate and selenate based on the comparison of sulfate to selenate ratios in the culture medium and in plant tissues (White et al., 2004).

The *sel1-11* mutant shows enhanced root growth in the presence of toxic external concentrations of selenate (Fig. 2, A and B). The reduced sensitivity of *sel1-11* root growth to selenate toxicity is mirrored by an 8-fold increase in $LC_{50}$ value for selenate and a more than 4-fold increase in the selenate toxicity threshold. Moreover, *sel1-11* shows enhanced root growth compared to the wild type even in the presence of relatively high levels of selenate in the medium (up to 50 $\mu$M; Fig. 2B). The selenate-resistant phenotype of the *sel1-11* mutant could be attributed to a limitation of selenium accumulation in leaves and roots when plants are treated with selenate (Fig. 5B). However, selenate toxicity is generally attributed to the ability of selenate to compete with sulfate for its access to sulfate transporters and to the sulfur metabolic pathway. This would lead to the disruption of sulfur metabolism and to a reduced synthesis of important sulfur-containing compounds, such as Cys, Met, and glutathione, and to the disruption of many cellular processes. Therefore, we propose that selenate toxicity could be better evaluated if sulfate to selenate ratios in tissues are considered instead of absolute selenate contents. Wild-type and *sel1-11* selenate-treated seedlings showed a comparable sulfate to selenium ratio in leaves (Fig. 5C). However, *sel1-11* roots showed a statistically significant higher sulfate to selenate ratio, suggesting that the selenate resistance mechanism of the *sel1-11* mutant might be root specific and consist of the combined effect of a reduced selenate content and a protective role of sulfate against selenate toxicity in roots of the mutant line. The fact that callus cultures showed the same pattern in response to selenate presence in the medium as that of whole plants in terms of selenate accumulation (Fig. 6A) and sulfate uptake capacity (Fig. 6B) suggested that the selenate resistance mechanism in *sel1-11* takes place at the cellular level rather than the tissue or organ level.

The activity of the rapidly dividing cells in the root meristem is vital for root growth, and we propose that the root apical region might be the target of selenate toxicity in roots. This view is supported by the fact that *SULTR1;2* gene promoter fused to $\beta$-glucuronidase reporter gene was clearly shown to be localized in the root tip and the root cap (Shibagaki et al., 2002). The mutation of *SULTR1;2* could therefore restrict selenate transport into the particularly sensitive cells of the root tip, including the root cap cells, so that the toxic effects of selenium on root growth would be delayed. This hypothesis is supported by the results obtained when 8-d-old plants are transferred on a selenate-containing medium (Fig. 7). The fact that root growth of wild-type seedlings is halted relatively soon after transfer suggests a specific effect of selenate toxicity on root growth, supported by the root tip activity, rather than a general toxic effect on the plant as a whole.

The isolation and characterization of a selenate-resistant mutant, *sel1-11*, enabled the identification of the *SULTR1;2* gene as a major contributor in the acquisition of not only sulfate but also of selenate by Arabidopsis roots. We revealed the peculiarity of SULTR1;2 in Arabidopsis, demonstrating that it is, among 13 mutated sulfate transporters, the only one whose mutation conferred resistance to selenate. We therefore highlighted that SULTR1;2 function is not fully redundant, neither with its root-localized structural homolog SULTR1;1 nor with 12 other putative Arabidopsis sulfate transporters. The analysis of the selenate-resistant phenotype of the *sel1-11* mutant suggests that root growth, particularly activity at the root tip, might be a major target of selenate toxicity in Arabidopsis.

## MATERIALS AND METHODS

### Plant Material

Arabidopsis (*Arabidopsis thaliana*) L. Heynh ecotype WS was used in this study, and the population of T-DNA mutants (around 30,000 lines) were provided by the Station de Génétique et d'Amélioration des Plantes of the Institut National de la Recherche Agronomique, Versailles, France. Seeds of T-DNA insertion Col-0 mutant lines defective in sulfate transporters were provided by Max Planck Institut *sultrl1-3* line (121G06) and by the Arabidopsis Biological Resource Center (Ohio State University) *sultr1-1* (SALK-093256); *sultr2-1* (SALK-109907); *sultr2-2* (SALK-054730); *sultr3-1* (SALK-023190); *sultr3-2* (SALK-023980); *sultr3-3* (SALK-031340); *sultr3-4* (SALK-100362); *sultr3-5* (NASC-N112372); *sultr4-2* (SALK-103827); *sultr5-1* (SALK-015044); and *sultr5-2* (SALK-118311). EMS-mutagenized seeds of *sel1-8* (Col) line were kindly provided by Dr. N. Shibagaki (Carnegie Institution Washington, Palo Alto). The mutant *sultr4-1* was not found in seed stock center during the time span of this work.

### *Hydroponic Cultures*

Plants were grown for 4 weeks under a short-day cycle (8/16 h 24°C/20°C day/night) on a modified Hoagland liquid medium containing: 1 mM $KH_2PO_4$, 1 mM $MgSO_4$, 0.5 mM $KNO_3$, 0.25 mM $Ca(NO_3)_2$, 100 $\mu$M NaFeEDTA, 10 $\mu$M $MnCl_2$, 1 $\mu$M $CuCl_2$, 1 $\mu$M $ZnCl_2$, 30 $\mu$M $H_3BO_3$, 50 $\mu$M KCl, and 0.1 $\mu$M $(NH_4)_6MO_7O_{24}$ $4H_2O$. Sulfate salts were replaced by chloride salts for all sulfate depletion treatments.

### *In Vitro Cultures*

Surface-sterilized seeds were placed on a solidified soluble-sulfate-free agarose 0.8% (w/v) GM containing: 5 mM $KNO_3$, 5 mM $Ca(NO_3)_2$, 1 mM $NaH_2PO_4$, 0.1 mM FeNaEDTA, 1 mM $MgCl_2$, 50 $\mu$M $H_3BO_3$, 50 $\mu$M $MnCl_2$, 15 $\mu$M $ZnCl_2$, 3 $\mu$M $NaMoO_4$, 2.5 $\mu$M KI, 0.05 $\mu$M $CuCl_2$, 0.045 $\mu$M $CoCl_2$, and 10 mM Glc. Seedlings were grown vertically in such a way that roots were closely growing at the surface of the agarose, under a short-day cycle (14/10 h 25°C/22°C day/night). Sulfur was supplied either as potassium sulfate (generally 1 mM) or as an organic form, djenkolate (100 $\mu$M), as described. Selenate was added as sodium selenate.

### *Callus Culture*

Calli were produced from Arabidopsis seeds as starting material. Sterilized seeds were cultivated for 2 weeks on solid Murashige and Skoog medium (Sigma) containing Suc 2% (w/v), 10 mg/L *myo*-inositol, 100 $\mu$g/L nicotinic acid, 1 mg/L thiamine-HCl, 100 $\mu$g/L pyridoxin-HCl, 400 $\mu$g/L Gly, 0.23 $\mu$M kinetin, and 4.5 $\mu$M 2,4-dichlorophenoyacetic acid. Neoformed calli were then transferred for an additional month to the same medium but in the presence of 0.46 $\mu$M kinetin and 2.25 $\mu$M 2,4-dichlorophenoyacetic acid. Calli were then collected, transferred in a modified Hoagland liquid medium, and agitated for the duration of the appropriate treatment.

### Screening for Selenate-Resistant Mutants

Seeds of 30,000 T-DNA insertion mutant lines were surface-sterilized, sown, and grown in vitro for 2 weeks on an agarose GM containing 10 $\mu$M selenate and 100 $\mu$M djenkolate as the sole sulfur source. Seedlings, whose roots were at least 5 mm long, were selected and transferred for recovery to a nonselective Murashige and Skoog medium lacking selenate for 1 week; they were then transferred to peat and allowed to self fertilize. Seeds from the candidate lines were grown in vitro on agarose for an additional identical screening in the presence of 10 $\mu$M selenate.

### Sulfate and Selenate Analyses

Soluble anions were extracted by incubating 50 mg of fresh weight plant material in 2 mL of deionized water and heated at 70°C for 30 min. Sulfate, as well as major inorganic and organic anions, was analyzed by high performance ionic chromatography (LC20, Dionex) using an IonPaq AS11 column and a sodium hydroxide (1–22 mM) linear gradient. Plant samples for selenium speciation were immediately kept on dry ice after harvest, then ground in liquid nitrogen, and prepared as previously described for Se K-edge x-ray absorption spectroscopy (Pilon-Smits et al., 1999; Pickering et al., 2003). Selenate tissue contents were extracted in a 100-mM HCl solution heated for 30 min at 70°C and determined as selenium by atomic absorption spectrophotometry (Varian SpectAA 220) coupled to a graphite oven.

### Root Length Measurements

Plants were grown vertically in petri dishes on appropriate selective media. Roots were regularly scanned using an Epson Perfection 1240U scanner and their length measurements carried out using the OPTIMAS software (OPTIMAS Image Analysis system 6.1, Media Cybernetic). Data were analyzed using the SigmaPlot 7 analysis software (SPSS).

### RNA and DNA Extraction

Plants were harvested, rapidly weighed, then flash frozen in liquid nitrogen and stored at −80°C until extraction. RNA and DNA were extracted using the RNeasy Plant Mini kit (Qiagen) and the GeneElute Plant Genomic DNA kit (Sigma), respectively, following the manufacturer's instructions.

### RT-PCR Analysis

The cDNA first-strand synthesis was done using the Omniscript reverse transcriptase (Qiagen). DNA amplifications by PCR were done using Taq DNA Polymerase (Invitrogen). Thirty cycles were generally used during PCR unless otherwise indicated. Actin was used to normalize cDNA loading. The following primers were used to amplify the corresponding genes: *SULTR1;1*F (5′-CTACAGTATCCGGACGCTGCCCAAA-3′); *SULTR1;1*R (5′-CGATATTAAGTTTGTTGCTCAGCCACTTCC-3′); *SULTR1;2*F (5′-AGCAGCAAGCCTACCTAGGATTCA-3′); *SULTR1;2*R (5′-CTTGACCCCTTGGTGTGATAGAAGAATC-3′); *Actin*F (5′-GGTAACATTGTGCTCAGTGGTGG-3′); and *Actin*R (5′-AACGACCTTAATCTTCATGCTGC-3′).

### *SULTR1;2* Sequencing

The genomic sequence of the *SULTR1;2* gene was amplified by PCR using a Pfu high fidelity DNA polymerase (Promega) and the following primers: forward primer *SULTR1;2*F2 (5′-CCTGGTTCGATACCATTACTCCATCCAC-3′); and reverse primer *SULTR1;2*R2 (5′-CTTGACCCCTTGGTGTGATAGAAGAATC-3′). This fragment was cloned in the pCR-Script Amp SK(+) plasmid (Stratagene) at the Srf1 restriction site. The sequencing was done by Genome Express.

### Yeast Expression Studies

The yeast (*Saccharomyces cerevisiae*) strain used is YSD1 (Smith et al., 1995) transformed with the plasmid pYES2 (Invitrogen) harboring the SULTR1;2 cDNA (Rouached et al., 2005). Yeast transformation were performed using the S.c. EasyComp Transformation kit (Invitrogen) following the manufacturer's instructions, and yeast culture, drop tests, and short-term sulfate influx measurements were performed as previously described (Cherest et al., 1997; Howarth et al., 2003).

### Sulfate Uptake Measurements

Sulfate influx measurements were performed using whole plants grown hydroponically for 4 weeks. Roots of whole plants were placed in a 100-$\mu$M $K_2SO_4$ solution at pH 5.0 (adjusted with MES buffer), aerated, and thermoregulated at 25°C for 10 min and then transferred in an identical solution and pulsed for an additional 10 min in the presence of 0.03 $\mu$Ci/mL (1.12 kBq/mL) of the radiotracer $^{35}S$-$SO_4$ (Amersham Biosciences). The roots were then washed for 1 min in an ice-cold $K_2SO_4$ 5 mM solution, harvested, blotted with paper towel, and their soluble contents extracted in a 100-mM HCl solution heated for 30 min at 70°C. The radioactivity of an aliquot of the acid extract was determined after addition of a scintillation liquid (Ultima gold, Packard), using a $\beta$ scintillation counter (Packard Tri-Carb 2101 TR).

Sulfate influx measurements were performed using 4-week-old calli using similar conditions as described for root measurements, except that the calli were handled using a vacuum filtration device.

### Statistical Analysis

ANOVA was used for statistical analysis of the data. Mean separation procedures were carried out using the multiple range tests with Fisher's LSD procedure ($P < 0.05$).

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