Differential Regulation of the Expression of Two High-Affinity Sulfate Transporters, SULTR1.1 and SULTR1.2, in Arabidopsis[1][W][OA]

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The molecular mechanisms regulating the initial uptake of inorganic sulfate in plants are still largely unknown. The current model for the regulation of sulfate uptake and assimilation attributes positive and negative regulatory roles to *O*-acetyl-serine (*O*-acetyl-Ser) and glutathione, respectively. This model seems to suffer from exceptions and it has not yet been clearly validated whether intracellular *O*-acetyl-Ser and glutathione levels have impacts on regulation. The transcript level of the two high-affinity sulfate transporters *SULTR1.1* and *SULTR1.2* responsible for sulfate uptake from the soil solution was compared to the intracellular contents of *O*-acetyl-Ser, glutathione, and sulfate in roots of plants submitted to a wide diversity of experimental conditions. *SULTR1.1* and *SULTR1.2* were differentially expressed and neither of the genes was regulated in accordance with the current model. The *SULTR1.1* transcript level was mainly altered in response to the sulfur-related treatments. Split-root experiments show that the expression of *SULTR1.1* is locally regulated in response to sulfate starvation. In contrast, accumulation of *SULTR1.2* transcripts appeared to be mainly related to metabolic demand and is controlled by photoperiod. On the basis of the new molecular insights provided in this study, we suggest that the expression of the two transporters depends on different regulatory networks. We hypothesize that interplay between SULTR1.1 and SULTR1.2 transporters could be an important mechanism to regulate sulfate content in the roots.

Sulfur (S) is one of the most important macronutrients for plant growth and development. Higher plants acquire S predominantly in the form of anionic sulfate from the soil. In plastids, sulfate is reduced into sulfide, which then combines with *O*-acetyl-Ser to form Cys (Leustek et al., 2000). Subsequently, Cys is either converted into Met or directly incorporated into proteins or glutathione. S deficiency is a major abiotic stress that affects plant growth and crop productivity worldwide (McGrath et al., 1996). Plants mainly respond to S deficiency by regulating the expression of genes involved in the uptake and assimilation of sulfate and the remobilization of secondary metabolites (Maruyama-Nakashita et al., 2003, 2006). Numerous studies have shown that the regulation of sulfate transporters occurs predominantly at the mRNA level (Hawkesford et al., 1993; Smith et al., 1995, 1997; Hawkesford and Wray, 2000; Takahashi et al., 1997, 2000; Yoshimoto et al., 2002, Hopkins et al., 2005). Using different experimental conditions in various species, these studies led to the proposal of a general model describing the regulation of S uptake and assimilation (Hawkesford and Smith, 1997). In this model, positive and negative regulatory roles are attributed to *O*-acetyl-Ser and glutathione, respectively. The validity of this model can, however, be questioned in the light of a number of experimental outcomes as described below.

Glutathione is the major organic S-containing compound transported and stored in plants. It has an important role in regulating cellular S homeostasis and in controlling the redox cellular status (Noctor et al., 2002). Exogenous glutathione application causes a significant reduction in the expression of sulfate transporters and in the uptake of sulfate by roots (Herschbach and Rennenberg, 1994, Smith et al., 1997, Lappartient et al., 1999; Vidmar et al., 1999). In rapeseed (*Brassica*

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napus) seedlings, split-root experiments showed that glutathione acts as a long-distance signal from the shoot to the root: the negative regulation of sulfate uptake resulting from the application of glutathione in one part of the root system was also demonstrated in the untreated part of the root (Lappartient and Touraine, 1996). However, such a negative effect of glutathione on the root sulfate uptake capacity was not observed in poplar after an exposure of aerial organs to H_2S, although this treatment was shown to significantly increase leaf and root glutathione contents (Herschbach et al., 2000; Westerman et al., 2001). Also, the overexpression of the key enzyme for glutathione synthesis, γ-glutamyl-Cys synthase, which resulted in the overaccumulation of glutathione, did not induce any down-regulation of the expression of sulfate transporters (Herschbach et al., 2000; Hartmann et al., 2004). Finally, increasing the glutathione content by providing *O*-acetyl-Ser to barley (*Hordeum vulgare*) plants did not result in a reduction in either the transcript accumulation of sulfate transporters or the sulfate uptake (Smith et al., 1997). In this context, the role of glutathione in down-regulating transporters involved in sulfate uptake needs further investigation.

O-Acetyl-Ser, a precursor of Cys synthesis in higher plants, has been proposed to be a positive regulator of the expression of genes involved in uptake and assimilation of sulfate in plants. Indeed, *O*-acetyl-Ser treatments induce the elevation of sulfate uptake rate in *Lemna minor* (Neuenschwander et al., 1991), barley (Smith et al., 1997), and potato (*Solanum tuberosum*; Hopkins et al., 2005). *O*-Acetyl-Ser treatments also result in an increase in the expression of all the genes involved in the sulfate assimilation pathway in Arabidopsis (*Arabidopsis thaliana*; Koprivova et al., 2000). The hypothesis that *O*-acetyl-Ser is a strong positive regulator of the S-deficiency response in plants is strengthened by the fact that in bacteria, *O*-acetyl-Ser plays an important role in S homeostasis: it has been proposed to stimulate the formation of a transcription initiation complex comprising the homotetrameric CysB protein and an RNA polymerase, which is active on several promoters of the Cys regulon (Kredich, 1992). In plants, some studies however lead to different conclusions. *O*-Acetyl-Ser treatment of pumpkin leaf discs did not result in an increase in the sulfate uptake rate (Rennenberg, 1983). In potato, the increase in the mRNA relative abundance of the *StST1* sulfate transporter and the induction of sulfate uptake both precede the accumulation of *O*-acetyl-Ser (Hopkins et al., 2005). In addition, metabolite-to-gene network analysis showed that the level of expression of genes known to be induced by S deficiency is not correlated with *O*-acetyl-Ser accumulation (Hirai et al., 2005). Altogether, these observations suggest that *O*-acetyl-Ser cannot be the sole molecule sensed in response to S deficiency and that the main signal in the roots may be the depletion of intermediates of the sulfate assimilation pathway.

Concluding on the validity of the model proposed for the regulation of sulfate uptake transporters is not easy, mainly because the experiments performed to test the model used different plant species and different growth conditions. It is thus important (1) to extensively analyze to what extent the model is indeed valid and (2) to do the whole analysis in one species. In Arabidopsis, sulfate uptake at the root surface is suggested to be achieved by two high-affinity sulfate transporters (SULTR1.1 and SULTR1.2) that are coexpressed in root hairs and in root epidermal and cortical cells (Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002, 2007; El Kassis et al., 2007). *SULTR1.2* transcripts are more abundant than *SULTR1.1* ones (Fizames et al., 2004) and SULTR1.2 was shown to contribute to at least 80% of the sulfate uptake in standard growth conditions (Maruyama-Nakashita et al., 2003). Up to now, *SULTR1.1* and *SULTR1.2* were described to be tightly coregulated, although the magnitude of their up- or down-regulation is different in that *SULTR1.1* transcripts show greater changes in their abundance (Yoshimoto et al., 2002; Maruyama-Nakashita et al., 2003, 2004a, 2004b). Exogenous supplies of *O*-acetyl-Ser or glutathione were shown to regulate *SULTR1.1* and *SULTR1.2* expression in agreement with the proposed model (Maruyama-Nakashita et al., 2004b). However, such treatment data do not constitute evidence for a direct role of *O*-acetyl-Ser or glutathione in the regulation of *SULTR1.1* and *SULTR1.2*. In this study, we thus extensively tested whether *SULTR1.1* and *SULTR1.2* are indeed regulated in agreement with the proposed regulatory model. A standardized plant growth procedure was used to examine *SULTR1.1* and *SULTR1.2* gene expression in a wide variety of experimental conditions. Treatments known to have a pronounced effect on sulfate uptake, on S metabolite contents, or on the expression of sulfate transporters were used: S, nitrogen (N), or phosphorous (P) starvation, supplementation of the culture hydroponic solutions with either selenate, sugar, cadmium or sodium chloride, and photoperiod conditions according to the literature (Howden et al., 1995; Barroso et al., 1999; Lejay et al., 2003; Maruyama-Nakashita et al., 2004b; El kassis et al., 2007). By analyzing the root glutathione, *O*-acetyl-Ser and sulfate contents in conjunction with *SULTR1.1* and *SULTR1.2* transcript abundances, we show that the expression of these two transporters was not regulated in agreement with the current model. We propose that expression of *SULTR1.1* and *SULTR1.2* is regulated to a significant extent by distinct signaling pathways.

RESULTS

Design and Validity of the Experimental Setup

The main purpose of our experiments was to test the validity of the current model for the regulation of the expression of transporters involved in root sulfate uptake. This validity was assayed in the model plant Arabidopsis for the sulfate transporters *SULTR1.1* and *SULTR1.2*. A common standardized system was used

to assay a large set of different experimental conditions (see "Materials and Methods"). For every sample, *O*-acetyl-Ser, glutathione, and sulfate contents and transcript abundance of *SULTR1.1* and *SULTR1.2* were systematically determined (Table I).

SULTR1.1 and *SULTR1.2* mRNA accumulations were determined using quantitative PCR. Using this technique, the data must be normalized using control genes, expression of which remains invariant during the timescale of all the different experimental conditions assayed. Because no single reference gene has been previously described to have a stable expression level across all the treatments used in our study, we used four reference genes or gene pairs: the *ACTIN2-ACTIN8* pair, the 60S ribosomal protein L23a gene, *SCARECROW*, and a region common to *MAP3K-1* and *MAP3K-2*. These genes, already described to have invariant expression levels in different abiotic stress conditions, display different levels of relative expression (Charrier et al., 2002). Over the different treatments used in our experiments, the relative transcript levels of the four references commonly showed less than 2-fold variations (Supplemental Table S1). We then used the mean value of the expression levels of all the control genes or gene pairs to ensure maintaining of a stable and reliable reference point over 20 different conditions tested (Table I). In few cases, one of the treatments had a major impact on the mRNA accumulation of one of the reference genes. For example, the 1 mM glutathione treatment resulted in an approximately 5-times decrease in the accumulation of *ACTIN2-8* mRNA relative to that of the other reference genes. In such a situation, the mRNA accumulation value for this reference gene was not taken into account for standardization.

Our results presented in Table I are in agreement with previously documented expression patterns of *SULTR1.1* and *SULTR1.2* (Maruyama-Nakashita et al., 2003). *SULTR1.2* transcripts were indeed more abundant than *SULTR1.1* transcripts (approximately 10 times in our experiments; Table I). Sulfate starvation, application of *O*-acetyl-Ser, selenate or cadmium all induced an increase in *SULTR1.1* and/or *SULTR1.2* mRNA levels, while the application of glutathione resulted in a strong reduction in the *SULTR1.1* and/or *SULTR1.2* expression (Table I). Our experimental framework is thus validated.

Is Glutathione Involved in the Regulation of the Expression of *SULTR1.1* and *SULTR1.2*?

As already mentioned, glutathione has been proposed to be a negative regulator of sulfate uptake and of the gene expression of transporters involved in

Table I. *Metabolite contents and accumulation of SULTR1.1 and SULTR1.2 mRNA*

Treatment[a]	Metabolites			mRNA Accumulation			
	SO_4^{2-}	GSH	*O*-Acetyl-Ser	*SULTR1.2*		*SULTR1.1*	
	μmol/g FW	*nmol/g FW*	*nmol/g FW*	$-\Delta Ct$[b]	$2^{-\Delta\Delta Ct}$	$-\Delta Ct$[b]	$2^{-\Delta\Delta Ct}$
Control 24 h	19.2 ± 1.7	120 ± 3	0.95 ± 0.32	0.85 ± 0.14	0.92 ± 0.01[c]	−1.59 ± 0.16	1.75 ± 0.05[c]
Control 72 h	19.3 ± 0.3[d]	110 ± 8	0.69 ± 0.11	0.97 ± 0.12	1.00 ± 0.01	−2.40 ± 0.24	1.00 ± 0.04
−S 24 h	17.2 ± 2	101 ± 7	1.38 ± 0.16	1.98 ± 0.13	2.02 ± 0.02	−0.78 ± 0.16	3.09 ± 0.08
−S 72 h	8.7 ± 0.8	85 ± 4	2.63 ± 0.17	1.63 ± 0.31	1.58 ± 0.04	−0.29 ± 0.49	4.32 ± 0.35
−N 24 h	20.2 ± 0.9	127 ± 7	1.57 ± 0.40	−0.33 ± 0.12	0.41 ± 0.00	−1.48 ± 0.28	1.89 ± 0.09
−P 24 h	19.1 ± 0.9	117 ± 8	1.45 ± 0.73	1.65 ± 0.20	1.61 ± 0.03	−1.41 ± 0.30	2.00 ± 0.10
0.5 mM Selenate	17.1 ± 0.7	96 ± 12	0.82 ± 0.18	1.69 ± 0.37	1.65 ± 0.05	−2.32 ± 0.36	1.06 ± 0.06
0.2 mM *O*-Acetyl-Ser	12.5 ± 1.8	239 ± 19	101.89 ± 7.08	3.03 ± 0.42	4.19 ± 0.15	−1.51 ± 0.24	1.86 ± 0.08
0.1 mM GSH	12.9 ± 0.6	141 ± 7	1.10 ± 0.18	1.27 ± 0.26	1.24 ± 0.03	−5.18 ± 0.21	0.15 ± 0.01
0.5 mM GSH	9.5 ± 0.2	164 ± 10	1.49 ± 0.16	−1.04 ± 0.71	0.25 ± 0.02	−7.57 ± 0.69	0.03 ± 0.00
1 mM GSH	8.8 ± 0.8	334 ± 16	2.53 ± 0.87	0.67 ± 0.41	0.81 ± 0.03	−8.20 ± 0.61	0.02 ± 0.00
20 μM Cadmium	23.1 ± 1	104 ± 6	6.73 ± 1.95	2.58 ± 0.07	3.06 ± 0.02	−0.70 ± 0.10	3.25 ± 0.06
In light (0 h[e])	20.0 ± 1	97 ± 10	0.54 ± 0.19	1.16 ± 0.21	1.14 ± 0.02	−2.26 ± 0.30	1.10 ± 0.06
In light (2 h)	20.9 ± 0.4	110 ± 5	1.69 ± 1.20	1.48 ± 0.14	1.43 ± 0.02	−2.31 ± 0.54	1.06 ± 0.10
In light (5 h)	19.3 ± 0.4	110 ± 6	1.31 ± 0.35	1.31 ± 0.15	1.27 ± 0.02	−1.81 ± 0.18	1.51 ± 0.04
In light (8 h[e])	20.1 ± 0.8	120 ± 9	2.13 ± 0.90	1.60 ± 0.22	1.55 ± 0.03	−1.74 ± 0.33	1.59 ± 0.09
In dark (5 h)	19.4 ± 1.7	105 ± 12	1.54 ± 0.81	1.72 ± 0.29	1.68 ± 0.04	−1.88 ± 0.41	1.43 ± 0.10
In dark (10 h)	17.8 ± 2	112 ± 9	1.30 ± 0.57	0.95 ± 0.18	0.99 ± 0.02	−2.31 ± 0.41	1.07 ± 0.07
0.1 M NaCl	13.0 ± 0.6	205 ± 8	1.20 ± 0.50	3.04 ± 0.30	4.21 ± 0.11	−2.85 ± 0.51	0.73 ± 0.06
0.2 M Mannitol	16.5 ± 0.8	129 ± 7	0.94 ± 0.29	1.83 ± 0.18	1.82 ± 0.03	−3.16 ± 0.26	0.59 ± 0.03
30 mM Suc	17.7 ± 0.8	63 ± 3	1.86 ± 0.79	3.12 ± 0.16	4.45 ± 0.06	−1.49 ± 0.35	1.88 ± 0.11
30 mM Mannitol	15.4 ± 1.1	86 ± 7	1.22 ± 0.42	1.78 ± 0.14	1.75 ± 0.02	−3.30 ± 0.38	0.54 ± 0.03

[a]The treatments are described in "Materials and Methods." [b]The $-\Delta$Ct value expresses in a logarithmic scale the mRNA abundance of the gene of interest relative to the average mRNA abundance of the housekeeping genes evaluated for the same treatment. [c]The $2^{-\Delta\Delta Ct}$ value expresses the mRNA abundance for the gene of interest in a given condition (treatment) relative to the average mRNA abundance for the same gene measured in the control treatment. A $2^{-\Delta\Delta Ct}$ value of 4 means that the mRNA is four times more abundant in plants submitted to the considered treatment than in control plants. [d]Average ± SD ($n \geq 4$). [e]0 h and 8 h in light designate the time points just before switching the lights on and off, respectively.

sulfate uptake. To test this hypothesis, the root intracellular glutathione content was measured in plants submitted to the different treatments and related to the *SULTR1.1* and *SULTR1.2* mRNA accumulations. The treatments corresponding to the supplementation of the culture medium with glutathione had the greatest impact on the root glutathione content, raising it 2- to 3-fold when compared to the control (Table I). They also resulted in reduced expression of the sulfate transporters: reduction of *SULTR1.2* mRNA accumulation was only significantly noticeable in plants treated with 0.5 mM glutathione, but *SULTR1.1* mRNA accumulation was significantly reduced by a factor varying from 6 to 50 in plants treated with 0.1 to 1.0 mM glutathione, respectively (Table I). These results supported the hypothesis that glutathione is a negative regulator of expression of *SULTR1.1* and *SULTR1.2* genes. However, when the glutathione treatments were not considered, no correlation could be found between the intracellular content of the reduced form of glutathione (GSH) and the mRNA accumulations of either *SULTR1.1* ($R^2 < 0.01$, $P = 0.685$) or *SULTR1.2* ($R^2 = 0.1$, $P = 0.171$; Fig. 1). This lack of correlation is reliable since it was established from a wide range of root glutathione contents varying from 63 ± 3 nmol.g^{-1} fresh weight (FW) to 239 ± 19 nmol.g^{-1} FW in response to the Suc and the *O*-acetyl-Ser treatments, respectively. Our data thus do not support any role of the root intracellular glutathione on the expression of either *SULTR1.1* or *SULTR1.2*.

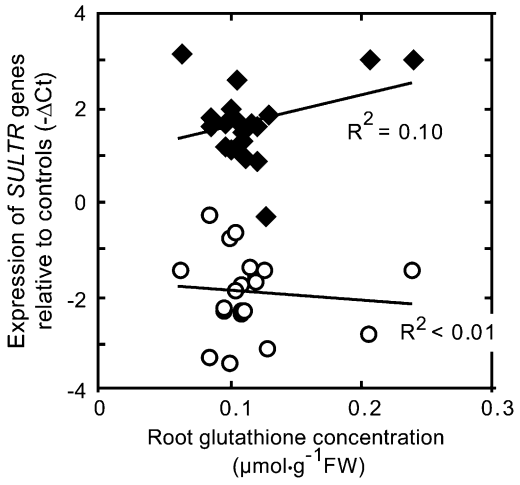

Figure 1. Relationship between glutathione content and accumulation of *SULTR1.1* and *SULTR1.2* mRNA in Arabidopsis roots. The mRNA accumulation of *SULTR1.1* (white circles) and *SULTR1.2* (black diamonds) is plotted against the root glutathione content. Accumulation of *SULTR1.1* and *SULTR1.2* mRNA is expressed relative to that of the reference genes as −ΔCt values. Each data point was obtained from the analysis of roots collected from a pool of six plants. For both *SULTR1.1* and *SULTR1.2*, each symbol represents one of the different treatments used in this work and is the average of four to six biological repeats. The three glutathione treatments were not considered in this analysis. The two black lines represent linear regression lines corresponding to the least square adjustment of all the data obtained for either the *SULTR1.1* or the *SULTR1.2* gene; the corresponding Pearson's correlation coefficients (R^2) are reported.

Is *O*-Acetyl-Ser Involved in the Regulation of the Expression of *SULTR.1.1* and *SULTR1.2*?

O-Acetyl-Ser has been proposed to be a positive regulator of sulfate uptake and of the genetic expression of transporters involved in sulfate uptake (Hawkesford and Smith, 1997). To test this hypothesis, the root intracellular *O*-acetyl-Ser content was measured in plants submitted to different treatments (Table I). Compared to the control treatment, the *O*-acetyl-Ser treatment resulted in an increase of over 100-fold in the root *O*-acetyl-Ser content. Interestingly, cadmium treatment also induced a significant overaccumulation of *O*-acetyl-Ser in roots, which corresponded to 8 to 10 times the *O*-acetyl-Ser content found in the control condition. A noteworthy increase of Ser acetyltransferase (SAT) gene expression from Arabidopsis has also been observed upon cadmium stress (Howarth et al., 2003). Apart from the *O*-acetyl-Ser and cadmium treatments, the *O*-acetyl-Ser content was evenly distributed within a 1 to 5 range, varying from 0.5 ± 0.2 nmol g^{-1} FW measured in control condition at dawn to 2.6 ± 0.2 nmol g^{-1} FW measured after 72 h of sulfate starvation. *O*-acetyl-Ser root contents were also high in response to the addition of glutathione or Suc and at the end of the light period (Table I).

The relationship between root *O*-acetyl-Ser contents and the mRNA accumulation of *SULTR1.1* and *SULTR1.2* was not as expected. The *O*-acetyl-Ser and cadmium treatments, which had the greatest impacts on the intracellular *O*-acetyl-Ser content, indeed induced approximately 2- to approximately 4-fold increases in mRNA accumulations of the two sulfate transporters (Table I). However, when the data obtained from plants submitted to the *O*-acetyl-Ser treatment were not taken into account, no correlation could be found between the intracellular *O*-acetyl-Ser content and the mRNA accumulations of either *SULTR1.1* ($R^2 = 0.02$, $P = 0.519$) or *SULTR1.2* ($R^2 = 0.06$, $P = 0.27$; Fig. 2). This conclusion, which is valid whether considering or not the cadmium treatment, is warranted by the fact that it has been obtained from the analysis of a wide range of *O*-acetyl-Ser contents. Our data are thus not in agreement with the hypothesis that intracellular *O*-acetyl-Ser plays a role in the gene expression of either *SULTR1.1* or *SULTR1.2*.

Relationship between Expression of the *SULTR1.1* and *SULTR1.2* Transporters and Sulfate Accumulation in Roots

Internal SO_4^{2-} has been proposed as a signal responsible for the repression of sulfate uptake (Smith, 1975, 1980; Jensén and König, 1982; Datko and Mudd, 1984; Lass and Ullrich-Eberius, 1984). Particularly, sulfate transporter expression has been considered to correlate negatively to the sulfate content. The rela-

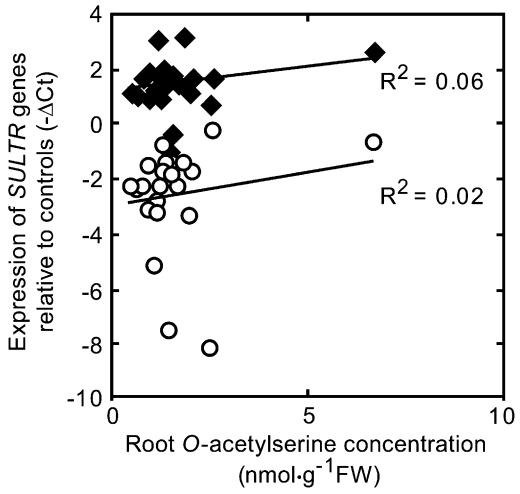

Figure 2. Relationship between *O*-acetyl-Ser content and accumulation of *SULTR1.1* and *SULTR1.2* mRNA in Arabidopsis roots. The mRNA accumulation of *SULTR1.1* (white circles) and *SULTR1.2* (black diamonds) is plotted against the root *O*-acetyl-Ser content. Accumulation of *SULTR1.1* and *SULTR1.2* mRNA is expressed relative to that of the reference genes as $-\Delta$Ct values. Each data point was obtained from the analysis of roots collected from a pool of six plants. For both *SULTR1.1* and *SULTR1.2*, each symbol represents one of the different treatments used in this work and is the average of four to six biological repeats. The *O*-acetyl-Ser treatment was not considered in this analysis. The two black lines represent linear regression lines corresponding to the least square adjustment of all the data obtained for either the *SULTR1.1* or the *SULTR1.2* gene; the corresponding Pearson's correlation coefficients (R^2) are reported.

tionship between sulfate transporter expression and sulfate content was then examined. As expected, 3 d after sulfate was withdrawn from the culture medium, the sulfate content in roots decreased to approximately 45% of the control value (8.7 $\pm$ 0.8 μmol g^{-1} FW compared to 19.5 $\pm$ 0.3 μmol g^{-1} FW; Table I). This decrease was negatively correlated to the increases in mRNA accumulations of both *SULTR1.1* (4.5-fold) and *SULTR1.2* (1.6-fold). The mechanism underlying this negative correlation is well known (Vidmar et al., 2000): when sulfate is withdrawn from the culture solution, the intracellular sulfate pool cannot be renewed; to overcome this sulfate shortage, the plant overexpresses transporters involved in sulfate uptake from the soil solution. When sulfate is present in a nonlimiting concentration in the culture medium, which is the case for all the treatments that we used apart from the sulfate deprivation, the situation may be completely different. Thus, for our further analysis of the relationship linking sulfate content and *SULTR1.1* and *SULTR1.2* mRNA accumulations in roots, we did not take into account the treatments in which sulfate was withdrawn from the culture medium.

Whatever the culture condition, the root sulfate content remained mostly stable (Table I). It was only markedly altered—actually lowered—in response to the *O*-acetyl-Ser, the glutathione, and the NaCl treatments. The root sulfate content thus displayed a 2.5 range of variation from 8.8 $\pm$ 0.8 μmol g^{-1} FW (glutathione treatment) to 23 $\pm$ 1 μmol g^{-1} FW (cadmium treatment). A positive correlation explaining 66% of the observed variability linked the *SULTR1.1* mRNA accumulation level and the root sulfate content (R^2 = 0.66, $P < 0.001$; Fig. 3A). Our analysis of this correlation excludes that an increase in the intracellular sulfate content could be the cause of an increase in the expression of *SULTR1.1*, since such a mechanism would lead to an indefinite increase in sulfate uptake and accumulation. We instead consider that the increase in sulfate accumulation was the result of the increase in *SULTR1.1* expression. In contrast, no

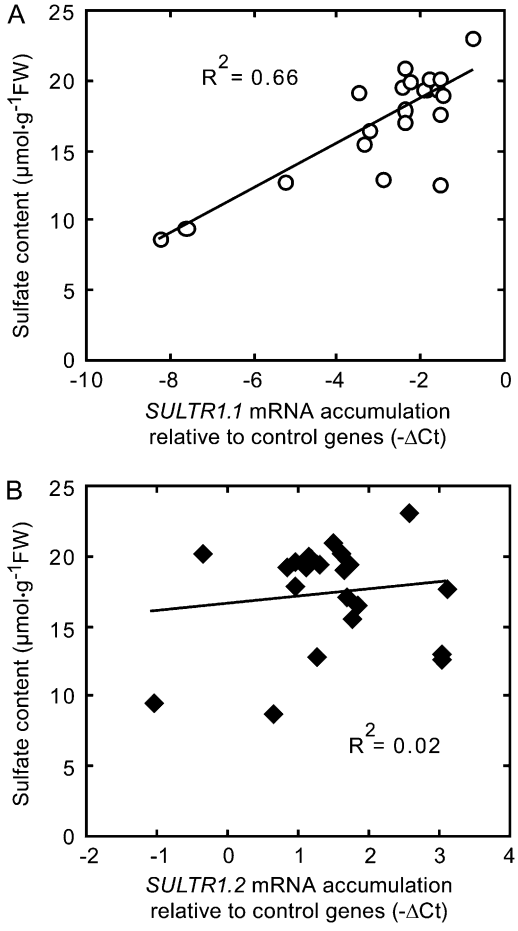

Figure 3. Relationship between sulfate content and accumulation of *SULTR1.1* and *SULTR1.2* mRNA in Arabidopsis roots. The root sulfate content is plotted against the accumulation of *SULTR1.1* (A) and *SULTR1.2* (B) mRNA inferred from quantitative PCR analysis. Accumulation of *SULTR1.1* and *SULTR1.2* mRNA is expressed relative to that of the reference genes as $-\Delta$Ct values. Each symbol represents one of the different treatments used in this work and is the average of four to six biological repeats. The sulfate-free treatments were not considered in this analysis. The two black lines represent linear regression lines corresponding to the least square adjustment of all the data obtained for either the *SULTR1.1* or the *SULTR1.2* gene; the corresponding Pearson's correlation coefficients (R^2) are reported.

correlation was observed between the mRNA accumulation of *SULTR1.2* and the root sulfate content (R^2 = 0.02, P = 0.55; Fig. 3B). Thus, the variation in the root sulfate content appeared to be dependent on the level of *SULTR1.1* mRNA accumulation but not on the level of *SULTR1.2* mRNA accumulation. This is surprising since *SULTR1.2* mRNAs were approximately 10 times more abundant than *SULTR1.1* mRNAs (Fizames et al., 2004) and also since SULTR1.2 is known as the major root transporter involved in sulfate uptake from the soil solution (Maruyama-Nakashita et al., 2004b).

Expression of *SULTR1.1* Is Locally Regulated in Response to the S Status

The existence of a shoot-to-root signal regulating the sulfate uptake activity has been suggested earlier in rapeseed seedlings (Lappartient and Touraine, 1996). To examine whether the expression of sulfate uptake transporters *SULTR1.1* and *SULTR1.2* is systemically regulated upon sulfate-deficient conditions or not, split-root experiments were carried out. In these experiments the root system was divided into two parts, and each part was individually submitted to either sulfate deficient (−S) or sufficient (+S) conditions. Split-roots submitted to +S/−S condition were used as the control conditions of the split-roots submitted to +S/+S and −S/−S treatments (Fig. 4). Three days after the −S treatment, sulfate, *O*-acetyl-Ser, and glutathione contents and mRNA accumulation for *SULTR1.1* and *SULTR1.2* have been measured in both +S and −S sides of all split-roots. Variations in *O*-acetyl-Ser and sulfate root contents in response to sulfate deficiency in split-roots were in agreement with results obtained in whole-root experiments. *O*-acetyl-Ser levels showed an apparent increase (approximately 30%) in all −S root sides compared to +S root sides (Fig. 4B). Sulfate content in −S side of the split-roots decreased to approximately 53% of that of the +S side (8.8 ± 1.8 μmol g^{-1} FW compared to 16.5 ± 1.2 μmol g^{-1} FW; Fig. 4A). Sulfate contents in the +S or −S sides were similar to that of +S/+S or −S/−S split-roots, respectively. This result indicates that sulfate content in S-deficient parts of the roots is not compensated by the S-sufficient parts. As expected, glutathione in −S/−S (83.5 ± 6.1 nmol g^{-1} FW) decreased compared to +S/+S (100.4 ± 7.0 nmol g^{-1} FW; Fig. 4A). However, unlike sulfate contents, no difference was observed for glutathione content in the −S side (109.1 ± 6.3 nmol g^{-1} FW) of the split-roots compared to +S/+S control roots (Fig. 4A). These results suggest that glutathione content in roots is not only dependent on local root sulfate content but also determined by the availability of glutathione in other tissues of the plant.

As in the whole-root experiments (Table I), relative accumulation of *SULTR1.2* mRNA in the −S side (1.52 ± 0.2) of split-roots was slightly induced upon sulfate starvation (compared to +S side, 1.14 ± 0.1; Fig. 4D). Interestingly, however, relative accumulation of

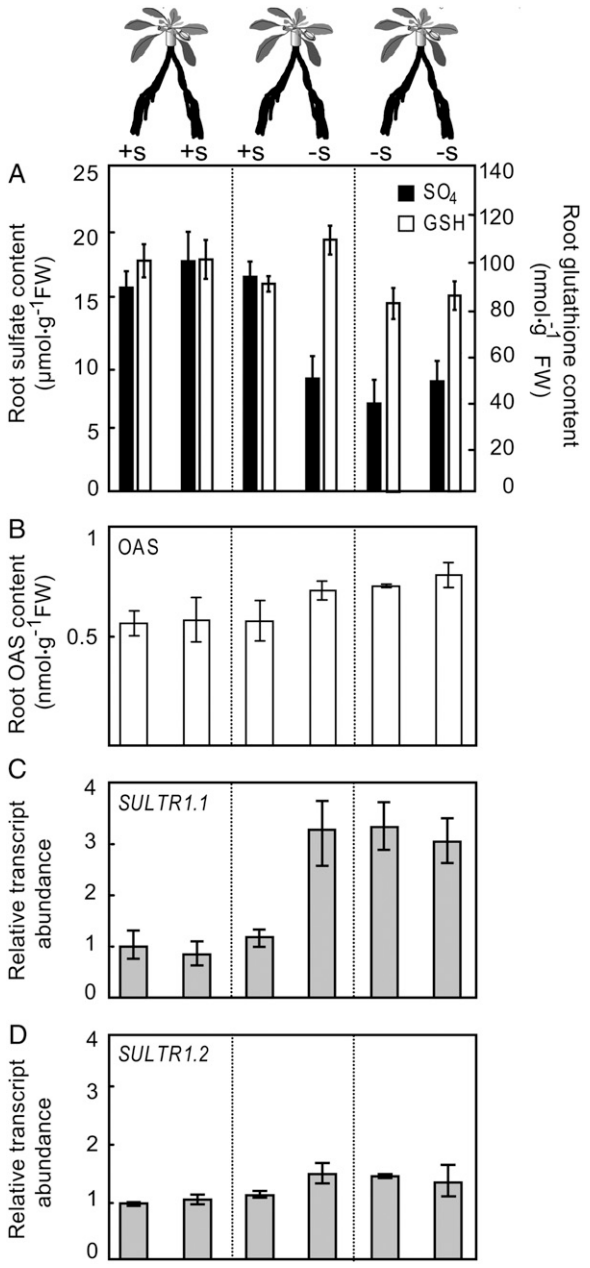

Figure 4. Effect of localized sulfate starvation on accumulation of sulfate, glutathione, *O*-acetyl-Ser, and *SULTR1.1* and *SULTR1.2* mRNA. Plants were grown hydroponically for 5 weeks, and then roots were split in two parts and maintained in complete nutrient solution for an additional week, before each part of split-roots was treated with either sulfate deficient (−S) or sufficient (+S) media. Each side of split-roots was separately harvested 3 d after the treatment, and accumulation of sulfate (black bars), glutathione (white bars; A), and *O*-acetyl-Ser (hatched bars; B) was determined. Abundance of *SULTR1.1* (C) and *SULTR1.2* (D) mRNA was normalized against their respective expression in +S/+S control conditions. Individual measurements were obtained from the analysis of roots collected from a pool of five plants. Error bars correspond to SD; biological repeats (n =6).

SULTR1.1 mRNA was strongly increased in the −S side (3.24 ± 0.57), while it remained unchanged in the +S side (1.17 ± 0.15) relative to +S/+S control roots (1.0 ± 0.31). The induction in the −S side was nearly to the same extent as when both sides were starved for sulfate in −S/−S control roots (Fig. 4C). The confined induction of *SULTR1.1* expression in the −S side is consistent with a local but not a systemic regulation in response to localized S starvation.

From this data it is obvious that the regulation of the expression of sulfate uptake transporters in Arabidopsis is dependent on local root sulfate content and is not mediated by GSH, as it has been suggested earlier (Lappartient and Touraine, 1996). These results are in agreement with a previous report in which an obvious role for the shoot-to-root signaling to regulate sulfate uptake and expression of the sulfate transporters in *Brassica oleracea* was not found (Buchner et al., 2004).

SULTR1.1 and *SULTR1.2* Depend on Different Regulation Networks

The models currently for the genetic expression of sulfate uptake transporters seldom take into account that there are different genes encoding these transporters. In this study, analyses distinguishing the two high-affinity sulfate transporters *SULTR1.1* and *SULTR1.2* in Arabidopsis were performed. They showed that these transporters display similar alterations of the abundances of their transcripts in response to various treatments (Yoshimoto et al., 2002; Maruyama-Nakashita et al., 2003, 2004a, 2004b). *SULTR1.1* and *SULTR1.2* were thus considered as functionally redundant. When considering all our different experimental conditions, we observed a positive correlation explaining only approximately 26% of the observed variation between the mRNA abundances of *SULTR1.1* and *SULTR1.2* ($R^2 = 0.26$, $P = 0.001$; Fig. 5). This positive correlation was due to data points corresponding to the three glutathione treatments only. When these treatments were not considered, no correlation could be obtained between the mRNA abundances of *SULTR1.1* and *SULTR1.2* ($R^2 = 0.01$, $P = 0.651$; Fig. 5). The analysis of the results from the salt (100 mM NaCl) or the osmotic (200 mM mannitol) treatments gives a very good example of the lack of coregulation of the expressions of the two genes (Fig. 6): *SULTR1.2* transcripts were markedly overaccumulated in both conditions, while *SULTR1.1* transcripts were rather underaccumulated in the same conditions. The validity of this conclusion was confirmed by analyzing hundreds of DNA chips through the Genevestigator "Gene correlator" tool (http://www.genevestigator.ethz.ch; Zimmermann et al., 2004); the Pearson's correlation coefficient (R^2) determined from the analysis of the mRNA accumulations of *SULTR1.1* and *SULTR1.2* was also only 0.23 (Supplemental Fig. S1). Altogether, these results suggest that the expressions of *SULTR1.1* and *SULTR1.2* are depending to a large extent on different regulation networks.

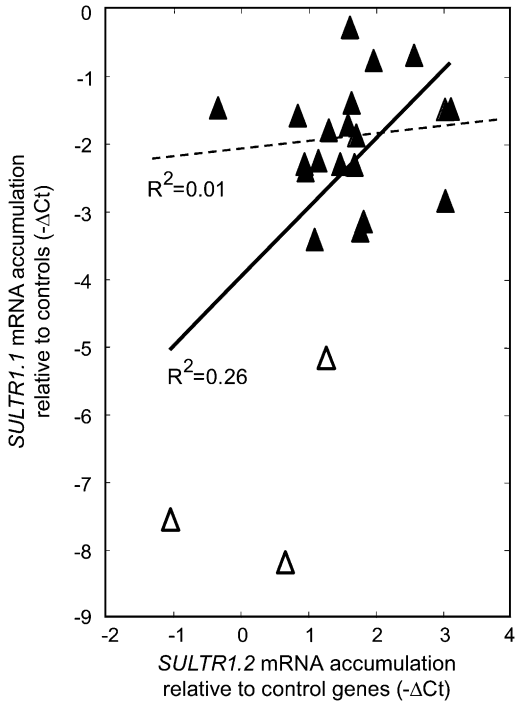

Figure 5. Comparison of *SULTR1.1* and *SULTR1.2* mRNA accumulation. Accumulation of *SULTR1.1* and *SULTR1.2* mRNA is expressed relative to that of the reference genes as −ΔCt values. Each data point was obtained from the analysis of roots collected from a pool of six plants. Each symbol represents one of the different treatments used in this work and is the average of four to six biological repeats. The white symbols correspond to the glutathione treatments. The black line represents the linear regression line corresponding to the least square adjustment of all the data, whereas the dotted line represents the linear regression line calculated without considering the glutathione treatments; the corresponding Pearson's correlation coefficients (R^2) are reported.

The contrasted mRNA accumulation profiles of *SULTR1.1* and *SULTR1.2* in response to the different culture treatments are presented in Figure 6. The order of magnitude of the between-treatment variation in mRNA accumulation was twice as high for *SULTR1.1* as for *SULTR1.2*. Moreover, the transcript abundance of *SULTR1.1* appeared less stable than that of *SULTR1.2*. Indeed, the three control treatments showed a marked variation between each other with respect to the mRNA accumulation of *SULTR1.1* but not to that of *SULTR1.2*. Transcript accumulations of *SULTR1.1* and *SULTR1.2* were depending on different factors. The *SULTR1.1* transcript level was mainly altered in response to the S demand: a marked increase in this level was only observed in response to S deficiency and supply of cadmium, while a significant decrease in this level was only noticed in response to the supply of glutathione. In contrast, the accumulation of *SULTR1.2* transcripts appeared to be mainly related to a broader carbon (C), N, and S demand: the abundance of *SULTR1.2* mRNAs was indeed significantly increased by the supply of Suc, *O*-acetyl-Ser,

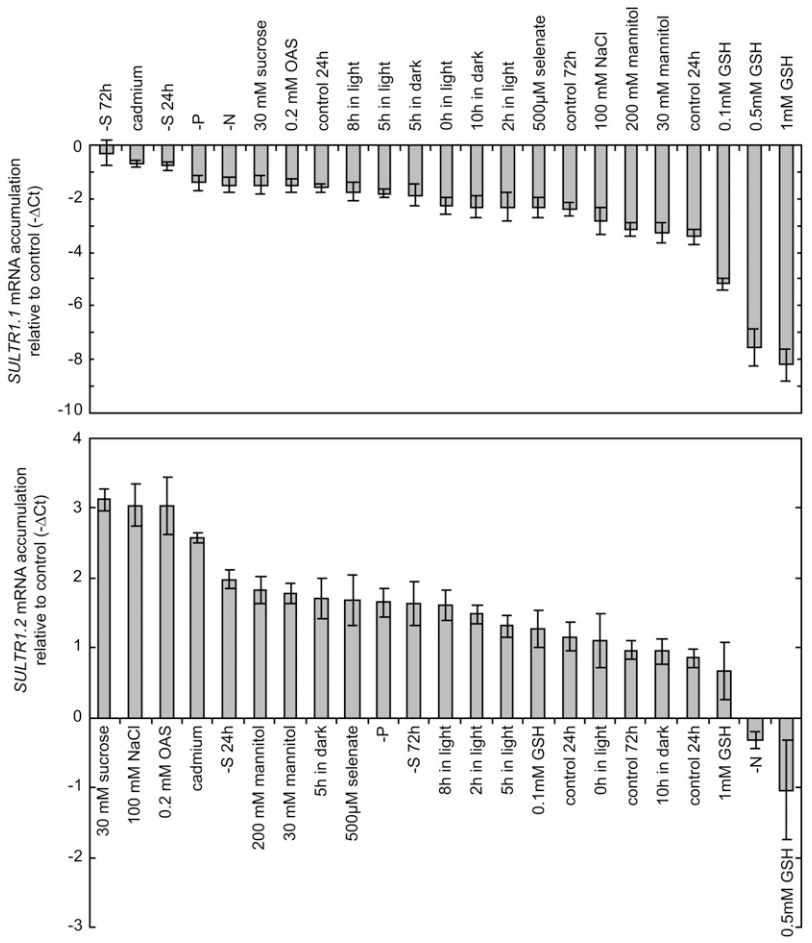

Figure 6. Effect of individual treatments on the accumulation of *SULTR1.1* and *SULTR1.2* mRNA. Accumulation of *SULTR1.1* and *SULTR1.2* mRNA is expressed relative to that of the reference genes as $-\Delta$Ct values. The treatments to which the plants were submitted are detailed in "Materials and Methods". Every data point was obtained from the analysis of roots collected from a pool of six plants. Error bars correspond to SD; biological repeats ($4 \leq n \leq 6$).

and markedly reduced by N deficiency and the supply of glutathione. A relationship between the *SULTR1.2* mRNA abundance and a photosynthesis-driven C, N, and S demand was also noticeable when analyzing the diurnal cycle (Fig. 7). *SULTR1.2* mRNA accumulation, which was the lowest at the end of the night, regularly increased during the daylight period to reach a maximum in the first part of the night. Such regulation was not observed for *SULTR1.1* (Table I). It should be noticed that this latter result appears in contradiction with a previous report showing a diurnal change in *SULTR1.1* mRNA (Lejay et al., 2003) similar to the one we observed for *SULTR1.2*. The discrepancy can be interpreted as follows. In the earlier study, mRNA abundance was determined by northern analysis using a *SULTR1.1* probe that showed a high similarity with the *SULTR1.2* sequence. Since *SULTR1.2* mRNAs are approximately 10 times more abundant than *SULTR1.1* ones, it is likely that the authors of this previous study actually analyzed the relative abundance of *SULTR1.2* mRNAs. Altogether, these results suggest that the level of mRNA transcripts of *SULTR1.2* depends on the general metabolic activity of the plant, while the level of mRNA transcripts of *SULTR1.1* is more related to the sole S status of the plants. This functional conclusion can be related to the structural observation that SULTR1.1 and not SULTR1.2 possesses in its promoter a regulatory element involved in the response to sulfate starvation (Maruyama-Nakashita et al., 2005).

DISCUSSION

The molecular mechanisms underlying the regulation of sulfate uptake by roots remain largely unknown. The current model attributes positive and negative regulatory roles to *O*-acetyl-Ser and glutathione, respectively (Hawkesford and Smith, 1997). However, the model is questioned in the light of a number of experimental outcomes. Data are still lacking to demonstrate that *O*-acetyl-Ser and glutathione are indeed molecular signals regulating either sulfate uptake or the gene expression of the transporters responsible for it. Several studies showed that regulation of sulfate transporters occurs predominantly at the mRNA level (Hawkesford et al., 1993; Smith et al., 1995, 1997; Hawkesford and Wray, 2000; Takahashi et al., 2000; Yoshimoto et al., 2002, Hopkins et al., 2005). Our objective was to test the current model by analyzing the mRNA accumulation of the two Arabidopsis high-affinity sulfate transporters that are involved

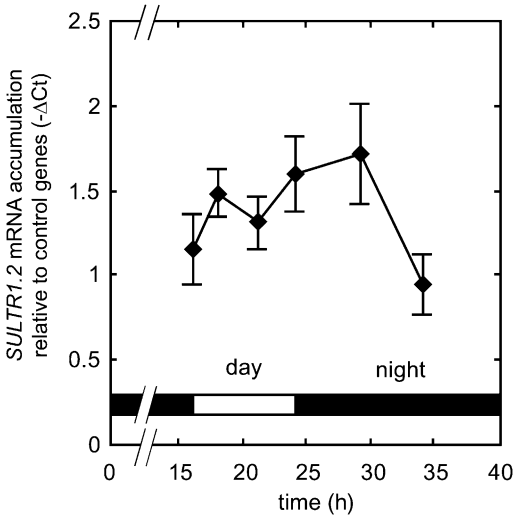

Figure 7. Accumulation of *SULTR1.2* mRNA during day and night. Roots of 6-week-old plants grown under an 8-h-day/16-h-night cycle from germination were collected just before switching to day, and 2, 5, and 8 h thereafter. Additionally samples were collected 5 and 10 h after switching back to night. Accumulation of *SULTR1.2* mRNA is expressed relative to that of the reference genes as $-\Delta$Ct values. Every data point was obtained from the analysis of roots collected from a pool of six plants. Error bars correspond to SD; biological repeats ($n = 6$).

in sulfate uptake from the soil solution, SULTR1.1 and SULTR1.2. This study used two strategies that have not been previously utilized to investigate *SULTR* gene expression. First, a common standardized system was used to assay a large set of different experimental conditions, including conditions that have not been tested before for their effect on the expression of sulfate transporters. Second, the transcript levels of *SULTR1.1* and *SULTR1.2* were systematically compared to each other but also to the root sulfate, glutathione, and *O*-acetyl-Ser contents.

Role of Glutathione in the Regulation of *SULTR1.1* and *SULTR1.2*

Numerous studies have shown that glutathione application to plants results in a repression of the expression of sulfate transporters with no exception (Brunold and Schmidt, 1978; Hawkesford and Smith, 1997; Smith et al., 1997; Lappartient et al., 1999; Vidmar et al., 1999, 2000; Vauclare et al., 2002; Maruyama-Nakashita et al., 2004b). Our data are in full agreement: exogenous applications of glutathione indeed resulted in a reduction in the transcript accumulation of *SULTR1.1* and *SULTR1.2*. However, given the many aspects of cellular metabolism that the glutathione is engaged in (Noctor et al., 2002), such feeding data do not constitute evidence for a direct role in the regulation of *SULTR1.1* and *SULTR1.2*. Indeed, the analysis of our whole data set indicates that there is no correlation between the root glutathione contents and the mRNA levels of either *SULTR1.1* or *SULTR1.2* (Fig. 1). Such a lack of relationship between increased levels of glutathione and decreased accumulation of sulfate transporter transcripts in roots has already been documented in barley (Smith et al., 1997), and in maize (*Zea mays*; Nocito et al., 2006). Our results give a broader experimental basis to this observation.

The down-regulation of the expression of sulfate transporters in response to glutathione has actually only been demonstrated following exogenous applications of glutathione. One explanation could be that plants seem to be able to distinguish between the glutathione pool synthesized in the cell and glutathione taken up by the roots from the nutrient solution, and to respond differently in the two situations (Hartmann et al., 2004). Another explanation could be that our glutathione measurements gave access to the average of different cell pool contents that would mask a great heterogeneity in the distribution of glutathione within the root or even within the cells. Intercompartmental variations in glutathione content and redox status were indeed suggested to be crucial in signaling (Noctor et al., 2002). These two explanations might merge: an exogenous application of glutathione perceived at the root epidermis may down-regulate *SULTR1.1* and *SULTR1.2* since these transporters are expressed in the outer cell layers of the roots, whereas increases in endogenous glutathione contents in inner cell layers resulting from different treatments would not. To our knowledge, it is still impossible to analyze the subcellular distribution of glutathione, and thus to analyze more deeply the effect of glutathione on the expression of *SULTR1.1* and *SULTR1.2*. The above-mentioned explanations still remain insufficient to interpret the whole set of data. Indeed, the N shortage and the 200 mM mannitol treatments both resulted in a similar glutathione accumulation but in a greater than 4-fold variation in *SULTR1.2* mRNA accumulation between each other (Table I). Similar discrepancies can be found through the pairwise analysis of many different treatments. It seems unlikely that glutathione would be systematically distributed in different cells or subcellular compartments in response to the different treatments.

Glutathione and sulfate are both translocated within the sieve sap (Rennenberg et al., 1979), although glutathione is the major form of long-distance transport of reduced S in plants (Rennenberg et al., 1979; Herschbach et al., 2000). Interestingly, split-root experiments revealed that glutathione content in the $-$S side of the split-roots, unlike sulfate content, remains high, and is comparable to the glutathione content of +S/+S control roots (Fig. 4A). Two possible explanations may account for the observed high levels of glutathione in $-$S side of the split-roots. First, the sulfate translocated from S-sufficient parts of the plant and the metabolized sulfate in the $-$S side of roots could contribute to maintain the high levels of glutathione in the $-$S side of the root. However, because there is no compensation of sulfate content in S-deficient parts of the root by the S-sufficient parts (Fig. 4A), such

an explanation cannot be deemed entirely convincing. An alternative explanation would be that the glutathione supply from the S-sufficient parts of the plant could maintain high glutathione levels in the −S side of the roots. Our results suggest that the glutathione content in roots is not only dependent on local root sulfate contents but also determined by the availability of glutathione in other tissues of the plant. Furthermore, glutathione has been postulated as a systemic regulator; however, using split-root systems no evidence was found for a systemic regulation of the expression of *SULTR1.1* and *SULTR1.2* in response to sulfate starvation (Fig. 4). These results are in full agreement with a previous report in which the significance of nonprotein thiols as signal in the shoot/root regulation of sulfate uptake and expression of the sulfate transporters in *B. oleracea* appears to be limited (Buchner et al., 2004). Taken together, our results strongly argue against a relay model in which glutathione would be a significant intermediary in the repression of the expression of either *SULTR1.1* or *SULTR1.2*, even if it may be a component of this regulation.

Role of *O*-Acetyl-Ser in the Regulation of *SULTR1.1* and *SULTR1.2*

O-Acetyl-Ser has been proposed to be a positive regulator of the expression of sulfate transporters (Hawkesford and Smith, 1997). This proposal was mainly supported by the strong parallelism found between the increase in *O*-acetyl-Ser content and the transcript abundance of sulfate transporters in both *O*-acetyl-Ser feeding and S-depletion experiments (Smith et al., 1997; Kim et al., 1999). Considering similar experimental conditions, our results are in agreement with these earlier observations (Table I). However, the analysis of our whole set of experimental conditions clearly showed that there is no correlation between the root *O*-acetyl-Ser content and the mRNA levels of either *SULTR1.1* or *SULTR1.2* (Fig. 2). This result suggests that *O*-acetyl-Ser does not play a major role in the regulation of these two genes. This conclusion is corroborated by the recent finding that *O*-acetyl-Ser accumulation in S-starved potato plants was preceded by the increase in both the *StST1* sulfate transporter mRNA level and the induction of sulfate uptake (Hopkins et al., 2005). As already detailed in the case of glutathione, it is not obvious to understand why exogenous application of *O*-acetyl-Ser results in an increase of the transcript level of either *SULTR1.1* or *SULTR1.2* while changes in endogenous *O*-acetyl-Ser contents are not correlated to changes in the transcript level of these two genes. Differences in subcellular distributions of *O*-acetyl-Ser may be part of the explanation. There is, however, a specific feature of *O*-acetyl-Ser that may help to interpret our results. *O*-acetyl-Ser is synthesized from Ser and acetyl-coenzyme A by the Cys synthase protein complex. This complex is also involved in the synthesis of Cys from *O*-acetyl-Ser and sulfide. Importantly, dissociation and stabilization of the Cys synthase complex is achieved by *O*-acetyl-Ser and to a lesser extent Cys on one side, and sulfide on the other side, respectively (Wirtz and Hell, 2004). When the complex is formed, which is mainly dependent on the availability of sulfide, *O*-acetyl-Ser is metabolized to Cys. However, when sulfide is lacking or when there would be an excess of *O*-acetyl-Ser compared to sulfide, part of *O*-acetyl-Ser would not be metabolized. The current hypothesis is that in that case, free *O*-acetyl-Ser would play a role in up-regulating the expression of sulfate transporters to ultimately increase the sulfide pool. In that context, it would not be the root *O*-acetyl-Ser content alone that would be the critical variable regulating the expression of sulfate uptake transporters, but the resultant relative contents of *O*-acetyl-Ser, sulfide, and maybe Cys.

Relationship between Expression of *SULTR1.1* and *SULTR1.2* and Sulfate Content in Roots

Our work aimed at characterizing the regulation of *SULTR1.1* and *SULTR1.2* at the mRNA accumulation level and not at analyzing the role of these transporters in sulfate uptake, and we concluded that the root sulfate content did not play any role in the regulation of either *SULTR1.1* or *SULTR1.2* gene expression when sulfate supply from the medium is not limited. However, our analysis surprisingly revealed that the root sulfate content showed a strong positive correlation with mRNA accumulation of the less expressed of the sulfate transporters *SULTR1.1* and not with mRNA accumulation of the more expressed *SULTR1.2*. The contradiction might be only apparent. First, considering that the regulation of *SULTR1.2* might be dependent on the metabolic demand, it should be reasonable to consider that the sulfate taken up following the increase in *SULTR1.2* expression is rapidly reduced and metabolized and thus not accumulated. The fact that *SULTR1.1* transcripts are approximately 10 times less abundant than those of *SULTR1.2* should not be readily interpreted to mean *SULTR1.1* has a secondary role. *SULTR1.1* transcript and protein accumulation increases under sulfate starvation (Yoshimoto et al., 2007). The increase in SULTR1.1 protein levels, following the increase in its transcript levels, could partially provide an explanation for its contribution in accumulation of sulfate in roots. It is also possible that a synergistic functional interaction between SULTR1.1 and SULTR1.2 could explain why an increase in *SULTR1.1* expression had a major impact on root sulfate accumulation. Consistent with this idea, two other sulfate transporters, SULTR2.1 and SULTR3.5, have been shown to synergistically interplay to increase the sulfate transport capacity in yeast (Kataoka et al., 2004). In plants, hetero-oligomerization of different subunits appears to be a typical feature to alter transport properties as exemplified in the case of Suc transporters (Reinders et al., 2002), ammonium transporters (Ludewig et al., 2003), and channels (e.g.

K^+ channels; Obrdlik et al., 2004). From these considerations, we hypothesize, that interplay between SULTR1.1 and SULTR1.2 transporters could be an important mechanism to regulate sulfate content in the roots.

Regulation of the Expression of *SULTR1.1* and *SULTR1.2* Involves Partially Independent Signaling Pathways

SULTR1.1 and SULTR1.2 are the two high-affinity sulfate transporters that are expressed in the same set of cells at the root-soil interface and contribute to sulfate uptake from the soil solution (Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002; El Kassis et al., 2007). They have up to now been considered as equivalent but not equal (Maruyama-Nakashita et al., 2003). The concept of equivalence was established on the basis that these two transporters showed similar biochemical characteristics and similar regulations of expression (Yoshimoto et al., 2002; Maruyama-Nakashita et al., 2003, 2004a, 2004b). But these two transporters were not considered as equal, since SULTR1.1 plays a minor role in comparison to SULTR1.2; *SULTR1.1* mRNAs are approximately 10 times less abundant than *SULTR1.2* ones and SULTR1.1 contributes to less than 30% of the sulfate uptake (Shibagaki et al., 2002; Yoshimoto et al., 2002; El Kassis et al., 2007). Our results are not in agreement with the concept of equivalence, at least as far as regulation of expression is considered. *SULTR1.1* and *SULTR1.2* were shown to be coregulated only to a limited extent ($r^2 \sim 0.26$) and this weak correlation between their levels of expression was only attributable to the glutathione treatments (Fig. 5). Data extracted from the available sets of Arabidopsis microarray data revealed a similar weak correlation ($r^2 \sim 0.25$) between *SULTR1.1* and *SULTR1.2* mRNA accumulations (Supplemental Fig. S1; Zimmerman et al., 2004). Thus, regulation of the expression of *SULTR1.1* and *SULTR1.2* involves to a significant extent independent signaling pathways.

Our analysis suggests a specialization in the regulation of these two transporters. The abundance of *SULTR1.1* mRNA transcripts was mainly altered in response to S-related treatments, whereas the level of *SULTR1.2* mRNA transcripts was mainly altered by changes in the C, N, and S demands (Fig. 8). Interestingly, we found that *SULTR1.1* expression is locally regulated in response to sulfate starvation treatment, whereas such a regulation is not observed for *SULTR1.2* as demonstrated by results obtained in split-root experiments (Fig. 4, C and D). The identification of an S specificity of the *SULTR1.1* regulation is consistent with the presence of a S-responsive cis-regulatory element (SURE element) in the *SULTR1.1* promoter but not in the *SULTR1.2* promoter (Maruyama-Nakashita et al., 2005). In Arabidopsis, this element has been identified in promoters of different S-responsive genes and was shown to be necessary and sufficient for mediating a negative regulation by sulfate, Cys, or glutathione in the *SULTR1.1* promoter (Maruyama-Nakashita et al., 2005). The differential regulation of *SULTR1.1* and *SULTR1.2* is further strengthened by the recent identification of a trans-regulatory element, the EIL3 transcription factor, which strongly alters the transcription of *SULTR1.2* but has a limited effect on *SULTR1.1* (Maruyama-Nakashita et al., 2006). These results support our analysis that *SULTR1.1* and *SULTR1.2* are subjected to some extent to different regulatory networks.

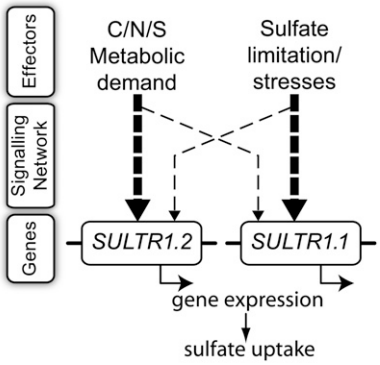

Figure 8. Schematic representation for the differential regulation of *SULTR1.1* and *SULTR1.2*. In the model, the expression of *SULTR1.2* is predominantly controlled by metabolic demand (S/N/C), while limitation in sulfate availability in conjunction with stress conditions results in the activation of *SULTR1.1* expression. In this scheme, the thick and thin hashed arrows indicate the major and minor signaling networks, respectively.

Our proposal that *SULTR1.2* is transcriptionally regulated by the metabolic demand is mainly supported by our data showing that the expression of *SULTR1.2* is diurnally regulated and is also dependant on the S, N, and C availabilities (Table I; Fig. 7). In this context, sulfate uptake would appear to be coordinated with sulfate assimilation since regulation of the expression of adenosine 5-phosphosulfate reductase, the key enzyme of sulfate assimilation pathway, has also been shown to be dependent from the S, N, and C demands (Kopriva et al., 2002). Sulfate uptake from the soil solution was shown to be essentially ensured by the *SULTR1.2* sulfate transporter (Maruyama-Nakashita et al., 2003). It would actually not be surprising that the transporter that plays a major role in sulfate uptake is regulated depending on the metabolic demand. All together, these arguments suggest that the metabolic demand plays a major role in the regulation of *SULTR1.2* expression.

Similar to the case of *SULTR1.2*, other transporters involved in the uptake of essential macronutrients such as N (e.g. NRT2.1), are also transcriptionally regulated depending on the S, N, and C demands (Lejay et al., 2003). This is reminiscent of data showing that sulfate assimilation is well coordinated with nitrate and C assimilation (Kopriva and Rennenberg, 2004). Interestingly, recent data have confirmed close connections between C, N, and S utilization and

cytokinin in plants, pointing out to novel metabolic and transport processes that might be under the influence of cytokinin (Ohkama et al., 2002; Maruyama-Nakashita et al., 2004c; Brenner et al., 2005; Sakakibara et al., 2006). Cytokinins have been reported to signal the plant N, S, sugar, or P status (Sakakibara et al., 1999; Martín et al., 2000; Gessler et al., 2004; Franco-Zorrilla et al., 2005). Also, synthesis of cytokinins depends on the availability of different macronutrients: cytokinin contents are increased by nitrate supply (Takei et al., 2001; Collier et al., 2003), and decreased by N or P starvation (Salama and Wareing, 1979; Horgan and Wareing, 1980). Finally, applications of exogenous cytokinin were shown to repress the expression of transporters involved in sulfate, nitrate, or phosphate uptake and to reduce the net nitrate uptake (Martín et al., 2000; Maruyama-Nakashita et al., 2004b; Brenner et al., 2005; Dluzniewska et al., 2006). It has been well demonstrated that cytokinins significantly down-regulate *SULTR1.1* and *SULTR1.2* with major impact on the latter; however, it should be noted that this negative effect is independent of the S limitation response (Maruyama-Nakashita et al., 2004c). Taken together, cytokinins, as common integrative mediators in signaling of plant nutritional status, could take part in regulation of *SULTR1.2* expression in response to metabolic demand.

In conclusion, this work is the first extensive comparison of the effect of a wide set of culture conditions on accumulation of *SULTR1.1* and *SULTR1.2* transcripts. The expression of *SULTR1.1* and *SULTR1.2* was shown not to be regulated consistently to the current model. Our results also show for the first time that the regulation of these two high-affinity sulfate transporters depend to a significant extent on different signaling pathways, and raise the possibility of an interplay between SULTR1.1 and SULTR1.2 to regulate the sulfate content in the roots. These observations indicate that the regulation of the root sulfate uptake is more complex than previously thought and pave the way for further work reconsidering the nature of signal(s) involved in the regulation of the expression of *SULTR1.1* and *SULTR1.2*. Determining additional level of regulation acting on the sulfate transporters will be required to fully appreciate the regulation mechanisms for sulfate transport in plants.

MATERIALS AND METHODS

Plant Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) plants used in all experiments were obtained from the Nottingham Arabidopsis Stock Centre (Columbia [Col-8] ecotype; reference N60000). Plants were grown hydroponically under nonsterile conditions. Seeds were germinated directly on top of pierced Eppendorf tubes filled with sand and placed on rafts floating in a nonsterile hydroponic solution, as described previously by Lejay et al. (1999). Plants were germinated and grown for 1 week in tap water then in the following nutrient solution: 0.5 mM KNO_3; 1 mM $MgSO_4$; 1 mM KH_2PO_4; 0.25 mM $Ca(NO_3)_2$; l00 μM NaFeEDTA; 30 μM H_3BO_3; l0 μM $MnCl_2$; l μM $CuCl_2$; 1 μM $ZnCl_2$; 0.1 μM $(NH_4)_6Mo_7O_{24}$; and 50 μM KCl. The nutrient solution was renewed every 4 d and on the day before the beginning of every treatment. Plants were grown in a growth chamber under the following environmental conditions: light/dark cycle of 8 h/16 h, light intensity of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperature of 24/20°C, and RH of 75%. The treatments were applied to 6-week-old plants. For the control condition, plants were kept in culture medium with the above-mentioned composition. For the sulfate- (−S), phosphate- (−P), or N-free (−N) treatments, plants were transferred into nutrient solutions of similar composition as described above, except that 1 mM $MgSO_4$, 1 mM KH_2PO_4, or 0.5 mM KNO_3 and 0.25 mM $Ca(NO_3)_2$ were replaced by 1 mM $MgCl_2$, 1 mM KCl, or 0.5 mM KCl and 0.25 mM $CaCl_2$, respectively. Plants were grown in media lacking S for either 24 or 72 h, and in media lacking N or P for 24 h only. Other treatments consisted of growing the plants in the above-mentioned nutrient solution supplemented with 0.1, 0.5 or 1 mM glutathione, 0.2 mM *O*-acetyl-Ser, 20 μM cadmium ($CdCl_2$), 0.5 mM selenate (Na_2SeO_2), 100 mM NaCl, or 200 mM mannitol for 24 h. A Suc treatment was performed by adding 30 mM Suc to the nutrient solution immediately after light was switched off; the plants were then grown for 4 h in this solution, as previously described in Lejay et al. (2003). A treatment with 30 mM mannitol was used as an osmotic control for the Suc treatment. At the end of the treatments, roots were cut, immediately frozen in liquid N, and stored at −80°C.

The split-root experiments were essentially performed as previously described by Gansel et al. (2001). Briefly, plants were grown hydroponically on complete media as described above. After 2 weeks, seedlings were removed so that only one seedling per tube was maintained. The root system of 5-week-old plants was gently separated into two approximately equal parts, and each part was transferred to separate containers supplied with 1 mM $MgSO_4$ solution and allowed to adapt for 3 d to split-root conditions. After washing the roots with double deionized water, the localized supply treatments were initiated by supplying each part of the root with either a sulfate-free (−S) or complete nutrient solution (+S). The side of the root system containing the main root was treated with +S or −S in an equal number of replicates. After 3 d of treatment, root metabolite (glutathione, *O*-acetyl-Ser, and sulfate) contents were expressed on an FW basis.

Real-Time Quantitative Reverse-Transcription PCR

Total RNA was extracted from frozen root tissues using the Plant RNeasy extraction kit (QIAGEN). Any residual genomic DNA was eliminated using an RNAse-free DNAse I (QIAGEN). Total RNA was quantified with a UV spectrophotometer. Two micrograms of total RNA were reverse transcribed using the SuperscriptIII RT kit (Life Technologies/Gibco-BRL). Complementary DNA (cDNA) was kept at −20°C until analyzed. Real-time reverse-transcription PCR was performed with an ABI Prism 7700 sequence detection system, using the SYBR green PCR master mix kit (Applied Biosystems). Reactions were performed in MicroAmp 96-well plates (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). PCR reactions were performed in a final volume of 25 μL containing 300 nM each of the forward and reverse primers, 12.5 μL of the SYBR green master mix, and 5 μL of a 1:50 cDNA dilution. All PCR reactions were performed in triplicate. Samples were submitted to 50°C for 2 min, then to 95°C for 10 min, and finally to 40 cycles of 95°C for 15 s followed by 60°C for 1 min, except for *SULTR1.1* and *SULTR1.2* for which the annealing/extension temperature was adjusted to 65°C. Data were analyzed using the Sequence Detection System software version 1.7 (Applied Biosystems). The specificity of the amplified PCR products was systematically confirmed by a melting curve analysis using the ABI PRISM Dissociation Analysis software (Applied Biosystems). Performing real-time PCR on RNA samples that were not subjected to cDNA synthesis checked the absence of genomic DNA contamination. We also checked that reactions performed without template did not result in any PCR product.

In addition to the two genes of interest, *SULTR1.1* (At4g08620) and *SULTR1.2* (At1g78000), six genes were considered for the standardization of real-time PCR data. These genes were mainly chosen based on previous analyses (Charrier et al., 2002). The corresponding primers were designed from a region common to *ACTIN2* (At5g09810) and *ACTIN8* (At1g49240; 5′-ggtaacattgtgctcagtggtgg-3′ and 5′-aacgaccttatcttcatgctgc-3′); from the 60S ribosomal protein L23a gene (At2g39460; 5′-ctgacaagaagaagattaaggatgctg-3′ and 5′-atccaaagcatcatagtctggtgtaa-3′); from *SCARECROW* (At3g54220; 5′-aagcgactctactgttgggaatg-3′ and 5′-aaactaagaacgaggcgtccaag-3′); and from a region common to *MAP3K-1* (At3g13530) and *MAP3K-2* (At3g07980; 5′-gagatggacaacgttcaggagg-3′ and 5′-ccccaagggatattatcgttttg-3′). Specific primers for the *SULTR* genes were previously described (Maruyama-Nakashita et al., 2004a, 2004b): 5′-gccatcacaatcgctctccaa-3′ and 5′-ttgccaattccacccatgc-3′ for *SULTR1.1*

and 5′-ggatccagagatggctacatga-3′ and 5′-tcgatgtccgtaacaggtgac-3′ for *SULTR1.2*. Design of the primers took into account the amplicon length, the optimal PCR annealing temperature, the lack of primer dimerization, and the PCR efficiency. For each primer pair, the PCR efficiency E was determined after the analysis of serial 1:10 dilutions of a plasmidic solution by using the equation $E = [(10^{-1/s}) - 1]\cdot 100$, where "s" is the slope of the linear regression of the threshold cycle (C_T) values per the $\log_{10}$ values of the starting DNA copy numbers. The above-mentioned primer pairs all resulted in measured PCR efficiencies of 100% ± 3%.

Quantification of the relative transcripts levels was performed using the comparative C_T method (Livak and Schmittgen, 2001). For every data point, the threshold cycle (C_T) value was the average of the C_T values obtained from the triplicate PCR analysis. For every treatment, four to six biological replicates were performed, leading to four to six corresponding samples. For every sample, the relative gene expression of each of the *SULTR* genes $\Delta C_{T,SULTR}$ was expressed following normalization against the average of the C_T values obtained for the genes used for standardization: $\Delta C_{T,SULTR} = C_{T,SULTR} -$ (average $C_{T,standards}$). When a treatment of interest (TOI) was compared to a reference treatment (RT), for instance for pairwise comparisons in chart bars, the relative expression of a SULTR gene was expressed as a $\Delta\Delta$Ct value calculated as follows: $\Delta\Delta Ct = \Delta C_{T,TOI} - \Delta C_{T,RT}$. The fold change in relative gene expression was determined as $2^{-\Delta\Delta CT}$.

Statistical Analysis

For statistical analysis of quantitative data, the Analysis Toolpak add-in program for Excel 2004 for Macintosh (Microsoft) was used to calculate the square of Pearson product-moment correlation coefficient (R^2) and Student's t tests to establish if the correlation coefficient is significantly different from zero. For all the t-test analyses the difference is considered statistically significant with a probability of $P < 0.05$.

Glutathione Measurements

The glutathione extraction protocol was adapted from Creissen et al. (1999) and glutathione was analyzed using monobromobimane (Newton et al., 1981). Briefly, weighed root material (approximately 20 mg) was extracted with 3 volumes of 0.1 M HCl during 15 min at 4°C. The insoluble residues were removed by centrifuging at 20,000*g* for 15 min at 4°C. Extracts were neutralized by adding an equal volume of 0.1 M NaOH. Aliquots of 50 μL were subjected to derivation by adding 35 μL of water, 10 μL of 1 M Tris-HCl, pH 8, and 5 μL of 10 mM monobromobimane (Interchim). Samples were incubated for 15 min at 37°C before the addition of 45 μL of pure acetic acid. Separation of monobromobimane-derivatized thiols was achieved by reversed-phase HPLC (Nucleodur C18 column, 250 × 4.6 mm; Macherey-Nagel). The compounds were eluted at a flow rate of 0.8 mL/min using the following two-phase acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid: acetonitrile concentration was raised from 0% to 10% (v/v) in 15 min, then from 10% to 99.9% in 2 min. Fluorescence of glutathione derivatives was measured with a ProStar Fluorescence detector (Varian; excitation 385 nm; emission 484 nm). Identification and quantification of glutathione were performed by comparison of retention times and peak areas with samples containing known amounts of pure synthetic glutathione (G4251; Sigma-Aldrich) and analyzed using the same procedure.

Sulfate Measurements

Weighed fresh roots were grounded into powder in liquid N and extracted in water by incubation for 30 min at 70°C. The extract was centrifuged at 20,000*g* for 30 min, and the supernatant filtered through a 0.45-μm filter unit. Ion concentrations were determined by high-pressure ionic chromatography (ICS-2500 apparatus; Dionex) using the AS11 anion exchanging column (Dionex) and a NaOH gradient. NaOH concentration was raised from 3.5 to 5 mM in 3 min, then linearly from 5 to 35.4 mM in 8 min. Identification and quantification of sulfate were performed by comparison of the retention times and peak areas—integrated using the Chromeleon software (Dionex)—with the standards.

O-Acetyl-Ser Measurements

Metabolites were extracted with 3 volumes of 0.1 M HCl using 0.02 g FW of root material that was grinded in liquid N to a fine powder. Cell debris were sedimented by centrifuging at 16,800*g* for 15 min at 4°C. The resulting supernatants were used for metabolite analysis after a short-term storage at −80°C. *O*-Acetyl-Ser was quantified using the AccQ-Tag fluorescence dye (Waters) after separation by reversed-phase HPLC on a Nova-Pak C18 3.9 × 150 mm column (pore size 4 μm) according to Wirtz et al. (2006). Chromatograms were recorded with a Jasco 920 fluorescent detector at 250-nm excitation wavelength and 395-nm emission wavelength and processed with the Millenium[32] software.

Supplemental Data

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

Supplemental Figure S1. Relationship between *SULTR1.1* and *SULTR1.2* expression signal values obtained through Genevestigator.

Supplemental Table S1. Accumulation of *SCARECROW*, *ACTIN2/8*, *MAP3K-1/2*, and 60S ribosomal protein L23A gene mRNA.

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LITERATURE CITED

Barroso C, Romero LC, Cejudo FJ, Vega JM, Gotor C (1999) Salt-specific regulation of the cytosolic *O*-acetylserine(thiol)lyase gene from Arabidopsis thaliana is dependent on abscisic acid. Plant Mol Biol **40:** 729–736

Brenner WG, Romanov GA, Kollmer I, Burkle L, Schmulling T (2005) Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J **44:** 314–333

Brunold C, Schmidt A (1978) Regulation of sulfate assimilation in plants: 7. Cysteine inactivation of adenosine 50-phosphosulfate sulfotransferase in *Lemna minor* L. Plant Physiol **61:** 342–347

Buchner P, Stuiver CE, Westerman S, Wirtz M, Hell R, Hawkesford MJ, De Kok LJ (2004) Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H2S and pedospheric sulfate nutrition. Plant Physiol **136:** 3396–3408

Charrier B, Champion A, Henry Y, Kreis M (2002) Expression profiling of the whole Arabidopsis shaggy-like kinase multigene family by real-time reverse transcriptase-polymerase chain reaction. Plant Physiol **130:** 577–590

Collier MD, Fotelli MN, Nahm M, Kopriva S, Rennenberg H, Hanke DE, Gessler A (2003) Regulation of nitrogen uptake by Fagus sylvatica on a whole plant level: interactions between cytokinins and soluble N compounds. Plant Cell Environ **26:** 1549–1560

Creissen G, Firmin J, Fryer M, Kular B, Leyland N, Reynolds H, Pastori G, Wellburn F, Baker N, Wellburn A, et al (1999) Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. Plant Cell **11:** 1277–1292

Datko AH, Mudd SH (1984) Sulfate uptake and its regulation in *Lemna paucicostata* Hegelm. 6746. Plant Physiol **75:** 466–473

Dluzniewska P, Gessler A, Kopriva S, Strnad M, Novak O, Dietrich H, Rennenberg H (2006) Exogenous supply of glutamine and active cytokinin to the roots reduces NO^{3-} uptake rates in poplar. Plant Cell Environ **29:** 1284–1297

El Kassis E, Cathala N, Rouached H, Fourcroy P, Berthomieu P, Terry N, Davidian JC (2007) Characterization of a selenate-resistant Arabidopsis mutant. Root growth as a potential target for selenate toxicity. Plant Physiol **143:** 1231–1241

Fizames C, Munos S, Cazettes C, Nacry P, Boucherez J, Gaymard F, Piquemal D, Delorme V, Commes T, Doumas P, et al (2004) The Arabidopsis root transcriptome by serial analysis of gene expression. Gene identification using the genome sequence. Plant Physiol **134:** 67–80

Franco-Zorrilla JM, Martin AC, Leyva A, Paz-Ares J (2005) Interaction between phosphate-starvation, sugar, and cytokinin signaling in Arabidopsis and the roles of cytokinin receptors CRE1/AHK4 and AHK3. Plant Physiol **138:** 847–857

Gansel X, Munos S, Tillard P, Gojon A (2001) Differential regulation of the NO_3^- and NH_4^+ transporter genes *AtNrt2.1* and *AtAmt1.1* in Arabidopsis: relation with long-distance and local controls by N status of the plant. Plant J **26:** 143–155

Gessler A, Kopriva S, Rennenberg H (2004) Regulation of nitrate uptake at the whole-tree level: interaction between nitrogen compounds, cytokinins and carbon metabolism. Tree Physiol **24:** 1313–1321

Hartmann T, Honicke P, Wirtz M, Hell R, Rennenberg H, Kopriva S (2004) Regulation of sulphate assimilation by glutathione in poplars (Populus tremula x P. alba) of wild type and overexpressing gamma-glutamylcysteine synthetase in the cytosol. J Exp Bot **55:** 837–845

Hawkesford MJ, Davidian JC, Grignon C (1993) Sulphate/proton cotransport in plasma-membrane vesicles isolated from roots of Brassica napus L.: increased transport in membranes isolated from sulfur-starved plants. Planta **190:** 297–307

Hawkesford MJ, Smith FW (1997) Molecular biology of higher plant sulphate transporters. *In* WJ Cram, LJ De Kok, C Brunold, H Rennenberg, eds, Sulfur Metabolism in Higher Plants: Molecular, Ecophysiological and Nutritional Aspects. Backhuys Publishers, Leiden, The Netherlands, pp 13–25.

Hawkesford MJ, Wray JL (2000) Molecular genetics of sulphate assimilation. Adv Bot Res **33:** 159–223

Herschbach C, Rennenberg H (1994) Influence of glutathione (GSH) on sulfate uptake and transport in tobacco plants. J Exp Bot **45:** 1069–1076

Herschbach C, van Der Zalm E, Schneider A, Jouanin L, De Kok LJ, Rennenberg H (2000) Regulation of sulfur nutrition in wild-type and transgenic poplar over-expressing gamma-glutamylcysteine synthetase in the cytosol as affected by atmospheric H2S. Plant Physiol **124:** 461–473

Hirai MY, Klein M, Fujikawa Y, Yano M, Goodenowe DB, Yamazaki Y, Kanaya S, Nakamura Y, Kitayama M, Suzuki H, et al (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J Biol Chem **280:** 25590–25595

Hopkins L, Parmar S, Blaszczyk A, Hesse H, Hoefgen R, Hawkesford MJ (2005) *O*-acetylserine and the regulation of expression of genes encoding components for sulfate uptake and assimilation in potato. Plant Physiol **138:** 433–440

Horgan JM, Wareing PF (1980) Cytokinins and the growth responses of seedlings of Betula pendula Roth. and Acer pseudoplatanus L. to nitrogen and phosphorus deficiency. J Exp Bot **31:** 525–532

Howarth JR, Domínguez-Solís JR, Gutiérrez-Alcalá G, Wray JL, Romero LC, Gotor C (2003) The serine acetyltransferase gene family in Arabidopsis thaliana and the regulation of its expression by cadmium. Plant Mol Biol **51:** 589–598

Howden R, Goldsbrough PB, Andersen CR, Cobbett CS (1995) Cadmium-sensitive, cad1 mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol **107:** 1059–1066

Jensén P, König T (1982) Development of regulation mechanisms for SO42− influx in spring wheat roots. Physiol Plant **55:** 459–464

Kataoka T, Hayashi N, Yamaya T, Takahashi H (2004) Root-to-shoot transport of sulfate in Arabidopsis. Evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. Plant Physiol **136:** 4198–4204

Kim H, Hirai MY, Hayashi H, Chino M, Naito S, Fujiwara T (1999) Role of O-acetyl-l-serine in the coordinated regulation of the expression of a soybean seed storage-protein gene by sulfur and nitrogen nutrition. Planta **209:** 282–289

Kopriva S, Rennenberg H (2004) Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Bot **55:** 1831–1842

Koprivova A, Suter M, den Camp RO, Brunold C, Kopriva S (2000) Regulation of sulfate assimilation by nitrogen in Arabidopsis. Plant Physiol **122:** 737–746

Kopriva S, Suter M, von Ballmoos P, Hesse H, Krahenbuhl U, Rennenberg H, Brunold C (2002) Interaction of sulfate assimilation with carbon and nitrogen metabolism in *Lemna minor.* Plant Physiol **130:** 1406–1413

Kredich NM (1992) The molecular basis for positive regulation of cys promoters in Salmonella typhimurium and Escherichia coli. Mol Microbiol **6:** 2747–2753

Lappartient AG, Touraine B (1996) Demand-driven control of root ATP sulfurylase activity and SO42− uptake in intact canola (the role of phloem-translocated glutathione). Plant Physiol **111:** 147–157

Lappartient AG, Vidmar JJ, Leustek T, Glass AD, Touraine B (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. Plant J **18:** 89–95

Lass B, Ullrich-Eberius CI (1984) Evidence for proton: sulfate cotransport and its kinetics in Lemna gibba G1. Planta **161:** 53–60

Lejay L, Gansel X, Cerezo M, Tillard P, Muller C, Krapp A, von Wiren N, Daniel-Vedele F, Gojon A (2003) Regulation of root ion transporters by photosynthesis: functional importance and relation with hexokinase. Plant Cell **15:** 2218–2232

Lejay L, Tillard P, Lepetit M, Olive F, Filleur S, Daniel-Vedele F, Gojon A (1999) Molecular and functional regulation of two NO3− uptake systems by N- and C-status of Arabidopsis plants. Plant J **18:** 509–519

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol **51:** 141–165

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25:** 402–408

Ludewig U, Wilken S, Wu B, Jost W, Obrdlik P, El Bakkoury M, Marini AM, Andre B, Hamacher T, Boles E, et al (2003) Homo- and hetero-oligomerization of ammonium transporter-1 NH4 uniporters. J Biol Chem **278:** 45603–45610

Martín AC, del Pozo JC, Iglesias J, Rubio V, Solano R, de La Pena A, Leyva A, Paz-Ares J (2000) Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis. Plant J **24:** 559–567

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. Plant Physiol **132:** 597–605

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) *Arabidopsis* SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell **18:** 3235–3251

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J **42:** 305–314

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Yamaya T, Takahashi H (2004a) Induction of SULTR1;1 sulfate transporter in Arabidopsis roots involves protein phosphorylation/dephosphorylation circuit for transcriptional regulation. Plant Cell Physiol **45:** 340–345

Maruyama-Nakashita A, Nakamura Y, Yamaya T, Takahashi H (2004b) Regulation of high-affinity sulphate transporters in plants: towards systematic analysis of sulfur signalling and regulation. J Exp Bot **55:** 1843–1849

Maruyama-Nakashita A, Nakamura Y, Yamaya T, Takahashi H (2004c) A novel regulatory pathway of sulfate uptake in Arabidopsis roots: implication of CRE1/WOL/AHK4-mediated cytokinin-dependent regulation. Plant J **38:** 779–789

McGrath SP, Zhao FJ, Withers PJA (1996) Development of sulfur deficiency in crops and its treatment. Proceedings of the Fertiliser Society, No. 379. The Fertiliser Society, Peterborough,UK

Neuenschwander U, Suter M, Brunold C (1991) Regulation of sulfate assimilation by light and *O*-acetyl-L-serine in *Lemna minor* L. Plant Physiol **97:** 253–258

Newton GL, Dorian R, Fahey RC (1981) Analysis of biological thiols: derivatization with monobromobimane and separation by reverse-phase high-performance liquid chromatography. Anal Biochem **114:** 383–387

Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian JC, Sacchi GA (2006) Heavy metal stress and sulfate uptake in maize roots. Plant Physiol **141:** 1138–1148

Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J Exp Bot **53:** 1283–1304

Obrdlik P, El-Bakkoury M, Hamacher T, Cappellaro C, Vilarino C, Fleischer C, Ellerbrok H, Kamuzinzi R, Ledent V, Blaudez D, et al (2004) K+ channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions. Proc Natl Acad Sci USA **101:** 12242–12247

Ohkama N, Takei K, Sakakibara H, Hayashi H, Yoneyama T, Fujiwara T (2002) Regulation of sulfur-responsive gene expression by exogenously applied cytokinins in *Arabidopsis thaliana.* Plant Cell Physiol **43:** 1493–1501

Reinders A, Schulze W, Kuhn C, Barker L, Schulz A, Ward JM, Frommer WB (2002) Protein-protein interactions between sucrose transporters of different affinities colocalized in the same enucleate sieve element. Plant Cell **14:** 1567–1577

Rennenberg H (1983) Role of *O*-acetylserine in hydrogen sulfide emission from pumpkin leaves in response to sulfate. Plant Physiol **73:** 560–565

Rennenberg H, Schmitz K, Bergmann L (1979) Long distance transport of sulfur in *Nicotiana tabacum.* Planta **147:** 57–62

Sakakibara H, Hayakawa A, Deji A, Gawronski SW, Sugiyama T (1999) His-Asp phosphotransfer possibly involved in the nitrogen signal transduction mediated by cytokinin in maize: molecular cloning of cDNAs for two-component regulatory factors and demonstration of phosphotransfer activity in vitro. Plant Mol Biol **41:** 563–573

Sakakibara H, Takei K, Hirose N (2006) Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends Plant Sci **11:** 440–448

Salama AD, Wareing PF (1979) Effects of mineral nutrition on endogenous cytokinins in plants of sunflower (Helianthus annus L.). J Exp Bot **30:** 971–981

Shibagaki N, Rose A, McDermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of Arabidopsis thaliana identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J **29:** 475–486

Smith FW, Ealing PM, Hawkesford MJ, Clarkson DT (1995) Plant members of a family of sulfate transporters reveal functional subtypes. Proc Natl Acad Sci USA **92:** 9373–9377

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vanden Berg PJ, Belcher AR, Warrilow AG (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. Plant J **12:** 875–884

Smith IK (1975) Sulfate transport in cultured tobacco cells. Plant Physiol **55:** 303–307

Smith IK (1980) Regulation of sulfate assimilation in tobacco cells: effect of nitrogen and sulfur nutrition on sulfate permease and *O*-acetylserine sulfhydrylase. Plant Physiol **66:** 877–883

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in Arabidopsis thaliana. Plant J **23:** 171–182

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, Engler JA, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in Arabidopsis thaliana. Proc Natl Acad Sci USA **94:** 11102–11107

Takei K, Sakakibara H, Sugiyama T (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. J Biol Chem **276:** 26405–26410

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krahenbuhl U, den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in Arabidopsis thaliana: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulfurylase to negative control by thiols. Plant J **31:** 729–740

Vidmar JJ, Schjoerring JK, Touraine B, Glass AD (1999) Regulation of the hvst1 gene encoding a high-affinity sulfate transporter from Hordeum vulgare. Plant Mol Biol **40:** 883–892

Vidmar JJ, Tagmount A, Cathala N, Touraine B, Davidian JCE (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from Arabidopsis thaliana. FEBS Lett **475:** 65–69

Westerman S, Stulen I, Suter M, Brunold C, De Kok LJ (2001) Atmospheric H2S as sulfur source for Brassica oleracea: consequences for the activity of the enzymes of the assimilatory sulphate reduction pathway. Plant Physiol Biochem **39:** 425–432

Wirtz M, Droux M, Hell R (2006) *O*-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in Arabidopsis thaliana. J Exp Bot **55:** 1785–1798

Wirtz M, Hell R (2004) Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties. J Plant Physiol **163:** 273–286

Yoshimoto N, Inoue E, Watanabe-Takahashi A, Saito K, Takahashi H (2007) Posttranscriptional regulation of high-affinity sulfate transporters in Arabidopsis by sulfur nutrition. Plant Physiol **145:** 378–388

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. Plant J **29:** 465–473

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol **136:** 2621–2632