the plant journal

SEB Society for Experimental Biology

The Plant Journal (2014) **77,** 185–197 doi: 10.1111/tpj.12376

Aberrant gene expression in the Arabidopsis *SULTR1;2* mutants suggests a possible regulatory role for this sulfate transporter in response to sulfur nutrient status

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Received 21 June 2013; revised 17 October 2013; accepted 4 November 2013; published online 7 November 2013.

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SUMMARY

Sulfur is required for the biosynthesis of cysteine, methionine and numerous other metabolites, and thus is critical for cellular metabolism and various growth and developmental processes. Plants are able to sense their physiological state with respect to sulfur availability, but the sensor remains to be identified. Here we report the isolation and characterization of two novel allelic mutants of *Arabidopsis thaliana*, *sel1-15* and *sel1-16*, which show increased expression of a sulfur deficiency-activated gene β-glucosidase 28 (*BGLU28*). The mutants, which represent two different missense alleles of *SULTR1;2*, which encodes a high-affinity sulfate transporter, are defective in sulfate transport and as a result have a lower cellular sulfate level. However, when treated with a very high dose of sulfate, *sel1-15* and *sel1-16* accumulated similar amounts of internal sulfate and its metabolite glutathione (GSH) to wild-type, but showed higher expression of *BGLU28* and other sulfur deficiency-activated genes than wild-type. Reduced sensitivity to inhibition of gene expression was also observed in the *sel1* mutants when fed with the sulfate metabolites Cys and GSH. In addition, a *SULTR1;2* knockout allele also exhibits reduced inhibition in response to sulfate, Cys and GSH, consistent with the phenotype of *sel1-15* and *sel1-16*. Taken together, the genetic evidence suggests that, in addition to its known function as a high-affinity sulfate transporter, SULTR1;2 may have a regulatory role in response to sulfur nutrient status. The possibility that SULTR1;2 may function as a sensor of sulfur status or a component of a sulfur sensory mechanism is discussed.

Keywords: sulfate transporter, sulfur sensor, SULTR1;2, Arabidopsis, transceptor.

INTRODUCTION

Sulfur (S) is an essential mineral nutrient for all organisms. For plants, sulfate (SO_4^{2-}), the primary source for S assimilation, is taken up from the rhizosphere by roots and transported to shoots. Sulfate is reduced to sulfide, and is assimilated into Cys and Met (Leustek *et al.*, 2000; Takahashi *et al.*, 2011). In addition to their role in protein synthesis, these amino acids are used for production of a wide variety of S-containing compounds, including vitamins, cofactors and secondary compounds, some of which, like glucosinolates and glutathione (GSH), play critical roles in response to biotic and abiotic stresses (Leustek *et al.*, 2000; Rausch and Wachter, 2005; Takahashi *et al.*, 2011).

Plants sense and adapt to the level of S in their environment (Leustek *et al.*, 2000; Grossman and Takahashi, 2001; Takahashi *et al.*, 2011). When S is limiting, the expression of high-affinity sulfate transporters, assimilation enzymes and many other genes is induced, as is activation of some assimilation enzymes, notably regulation of the cysteine synthase complex (Leustek *et al.*, 2000; Saito, 2004; Hawkesford and De Kok, 2006; Davidian and Kopriva, 2010; Takahashi *et al.*, 2011). Conversely, feeding reduced sulfur compounds such as Cys or GSH to plants represses these processes. Recent systems studies have shown that S deprivation causes large-scale transcriptomic changes

involving about 1500 genes in Arabidopsis (Hirai *et al.*, 2003, 2004; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003). Among the major changes occurring after S starvation are repression of glucosinolate biosynthesis gene expression and induction of glucosinolate degradation gene expression. One of the most strongly and rapidly up-regulated S-response genes that has particular relevance to this study is At2g44460 (Maruyama-Nakashita *et al.*, 2003, 2006; Nikiforova *et al.*, 2003; Dan *et al.*, 2007; Amtmann and Armengaud, 2009).

At2g44460 was initially predicted to encode a putative thioglucosidase, and was recently annotated as *BGLU28*, encoding β-glucosidase 28. Although its physiological and biochemical function has not been demonstrated, BGLU28 has been hypothesized to function in the release of thiol groups by breaking down various gluconsinolates in response to sulfur deficiency (Maruyama-Nakashita *et al.*, 2003, 2006; Hirai *et al.*, 2005; Dan *et al.*, 2007). Two other genes, *LSU1* (*RESPONSE TO LOW SULFUR 1*; At3g49580) and *SDI1* (*SULPHUR DEFICIENCY-INDUCED 1*; At5g48850), are also known to be highly up-regulated by S deficiency (Maruyama-Nakashita *et al.*, 2006). The biochemical functions for these two genes remain unknown. Antisense suppression of *LSU1* in tobacco was shown to alter S metabolism and S-responsive gene expression, linking its biochemical function to plant responses to S deprivation (Lewandowska *et al.*, 2010). *SDI1* is annotated as a protein similar to male sterility family protein MS5, and its expression has been used as a biosensor of plant S nutritional status (Howarth *et al.*, 2009).

Sulfate transporters function in sulfate uptake and movement within the plant body. Initially characterized from legume (*Stylosanthes hamata*, Smith *et al.*, 1995), barley (*Hordeum vulgare*, Smith *et al.*, 1997) and Arabidopsis (Takahashi *et al.*, 1997), sulfate transporters are encoded by a gene family in various plant species (Leustek *et al.*, 2000; Grossman and Takahashi, 2001; Buchner *et al.*, 2004; Maruyama-Nakashita *et al.*, 2004; Rouached *et al.*, 2009; Davidian and Kopriva, 2010; Takahashi *et al.*, 2011). In Arabidopsis, there are 12 members organized into four groups, SULTR1 to SULTR4, each with specialized functions (Buchner *et al.*, 2004; Maruyama-Nakashita *et al.*, 2004; Rouached *et al.*, 2009; Davidian and Kopriva, 2010; Takahashi *et al.*, 2011). All of these transporters are predicted to contain up to 12 transmembrane (TM) helices and a conserved C-terminal STAS (sulfate transporter and anti-sigma factor antagonist) domain. Among these, the SULTR1 group has been extensively characterized. *SULTR1;3* is expressed in phloem and is involved in S redistribution from source to sink organs (Yoshimoto *et al.*, 2003). SULTR1;1 and SULTR1;2 are high-affinity transporters expressed in root cortical cells, and function in uptake of sulfate from the rhizosphere (Takahashi *et al.*, 2000; Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002; Maruyama-Nakashita *et al.*, 2004; Barberon *et al.*, 2008; Rouached *et al.*, 2008). However, these genes are not entirely redundant, with SULTR1;2 playing the primary role in sulfate uptake from the rhizospere (Yoshimoto *et al.*, 2002; Barberon *et al.*, 2008; Rouached *et al.*, 2008). Mutants of *SULTR1;2* were first isolated by screening Arabidopsis for selenate resistance (Shibagaki *et al.*, 2002; El–Kassis *et al.*, 2007), and thus were designated *sel1* mutants. Resistance is caused by the inability to take up selenate, a toxic sulfate analog. Structure-functional studies of SULTR1;2 revealed that amino acid residues identified by missense *sel1* alleles are critical for sulfate transport, including *sel1-1* (S96F), *sel1-3* (G509E) and *sel1-8* (I511T). Whereas S96 is located near the N-terminus, G509 and I511 are in the linker region between the last TM helix and the STAS domain. Three recent studies (Shibagaki and Grossman, 2004, 2006; Rouached *et al.*, 2005) revealed that the STAS domain and the linker connecting the domain to the rest of the protein are important for SULTR1;2 function and/or protein degradation. One response to S limitation involves the Cys regulatory complex, which is composed of Ser acetyltransferase and *O*-acetylserine (thiol) lyase (OASTL, the enzyme producing L-Cys) (Kumaran *et al.*, 2009; Yi *et al.*, 2010; Wirtz *et al.*, 2012). SULTR1;2 was shown to interact with OASTL, and the interaction results in control of both sulfate transport and OASTL activity, linking Cys synthesis with S availability (Shibagaki and Grossman, 2010). However, the sensor of S status that leads to changes in gene expression remains to be identified (Davidian and Kopriva, 2010; Takahashi *et al.*, 2011).

Using a reporter gene-based, forward genetic approach, three mutants that alter the S-deficiency response have been isolated – *osh1* (Ohkama-Ohtsu *et al.*, 2004), *slim1* (Maruyama-Nakashita *et al.*, 2006) and *big* (Kasajima *et al.*, 2007; 7) – but none have been linked to S sensing. In this study, we screened for Arabidopsis mutants that show up-regulated *BGLU28* promoter activity (Dan *et al.*, 2007), and characterized two allelic recessive mutants (*sel1-15* and *sel1-16*) of *SULTR1;2*. Gene expression and metabolite analyses suggest that, in addition to its function as a high-affinity sulfate transporter, SULTR1;2 may also play a role in a regulatory or S-sensing/signaling mechanism.

RESULTS

Isolation of two novel *sel1* alleles that reduce Arabidopsis sensitivity to external sulfate

In an effort to identify sensing or signaling components involved in the S-deficiency response pathway, a genetic screen was developed using the At2g44460/*BLGU28* promoter:GUS transgenic plants constructed and characterized previously (Dan *et al.*, 2007). Several putative mutants were isolated that showed stronger GUS expression than wild-type (WT), and we report the characterization of two

monogenic recessive mutants in this paper. These mutants failed to complement each other, and, for reasons discussed below, were designated *sel1-15* and *sel1-16*, respectively. The *BGLU28* promoter activity in *sel1-15* and *sel1-16* is shown in Figure 1(a). On S-deficient medium (designated −S), they exhibit a similar GUS expression pattern; however, under S-sufficient conditions (designated +S), whereas the WT showed barely detectable GUS activity, the mutants retained comparatively high GUS expression, particularly in roots.

To test the possibility that *sel1-15* and *sel1-16* have a reduced inhibition response to external sulfate, real-time PCR was used to measure *BGLU28* expression in whole seedlings treated with a range of sulfate doses. After treatment for 48 h with −S medium, the two *sel1* alleles showed similar *BGLU28* expression to WT (Figure 1b). The

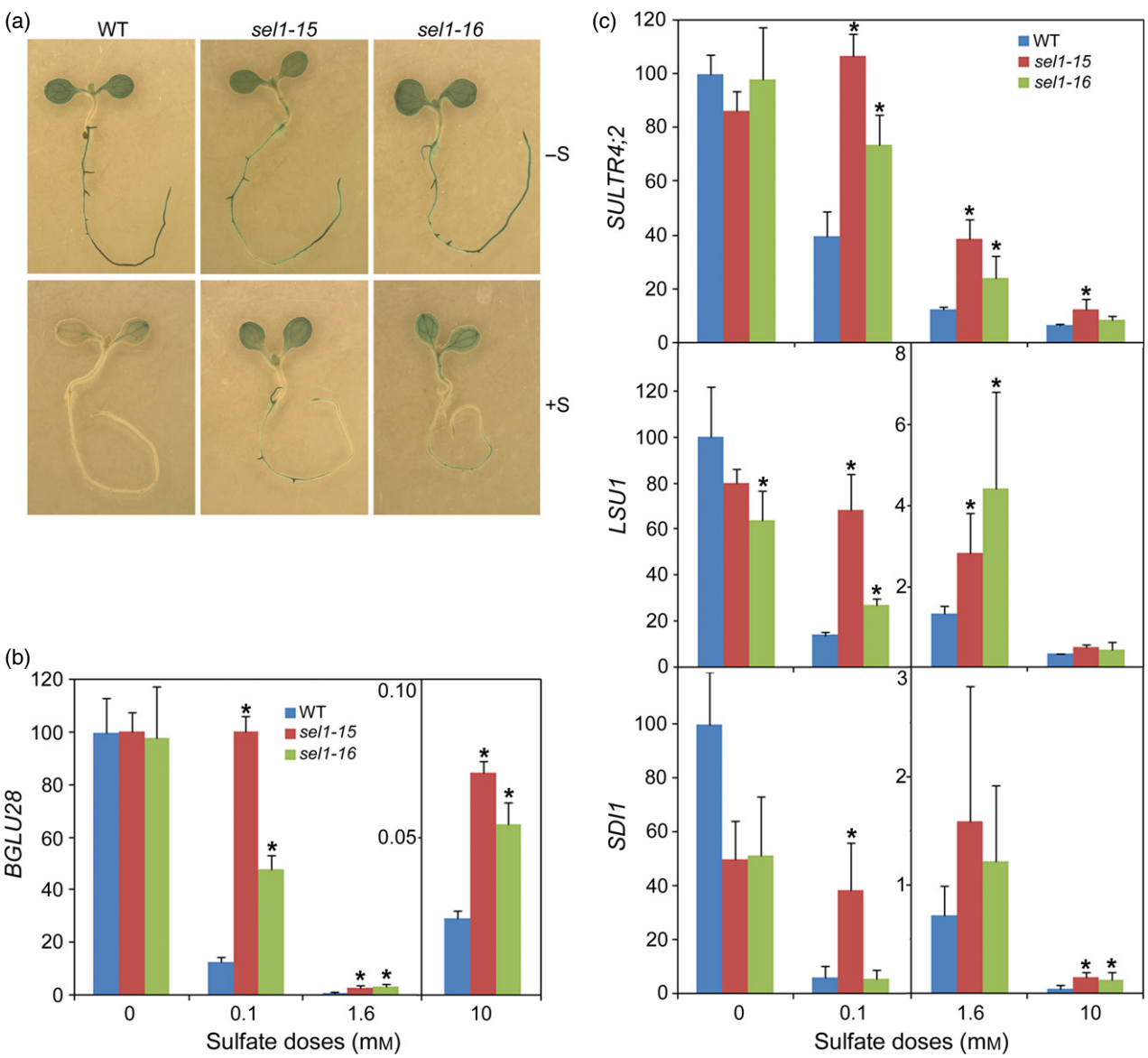

Figure 1. Isolation of two allelic mutants with altered response to external sulfate.
(a) Enhanced *BGLU28* promoter activity in *sel1-15* and *sel1-16*. Seedlings grown vertically on normal half-strength MS medium with 1% w/v sucrose for 4 days were then transferred to −S liquid medium (0. 0001 mM sulfate) or +S liquid medium (1.6 mM sulfate) for 2 days. WT, wild-type.
(b, c) Real-time PCR analysis of transcript levels for *BGLU28* (b) and three other S-responsive genes (c) in whole seedlings under various exogenous sulfate doses. Four-day-old vertically grown seedlings as described for (a) were treated in liquid medium with various doses of sulfate for 2 days before RNA extraction from whole seedlings. Relative mRNA levels were determined by normalizing their PCR threshold cycle numbers to that of the *ACT2* reference gene, and then presenting the data relative to WT treated on −S medium, which was set as a value of 100. Values are means and SD of three biological replicates. Asterisks indicate statistically significant differences ($P < 0.05$; ANOVA) between *sel1* and WT under the same sulfate dose.

BGLU28 expression level decreased with increasing sulfate concentration. However, while the WT expression level decreased significantly at 0.1 and 1.6 mM, the *BGLU28* expression level in *sel1-15* and *sel1-16* was 3–7 fold greater than in WT at these sulfate concentrations. The quantitative (reverse transcriptase [RT]-PCR) result shows that endogenous *BGLU28* mRNA expression under 0 and 1.6 mM sulfate is consistent with the *BGLU28* promoter activity as monitored by GUS expression (Figure 1a). At 10 mM sulfate, all genotypes had very little *BGLU28* mRNA, but expression in *sel1-15* and *sel1-16*, respectively, was still three- and twofold greater than in WT. Overall, this result indicates that these two mutants may be defective in S sensing/signaling and/or sulfate acquisition or metabolism, which leads to reduced control of *BGLU28* expression by sulfate.

To assess whether the *sel1-15* and *sel1-16* mutations affect a general S response, we analyzed the expression of other known S-responsive genes in *sel1-15* and *sel1-16*. We first chose *SULTR4;2*, which is known to be up-regulated by sulfur deficiency (Kataoka *et al.*, 2004; Maruyama-Nakashita *et al.*, 2006; Dan *et al.*, 2007) and encodes a vacuolar transporter that functions to redistribute internal sulfate (Kataoka *et al.*, 2004). Expression of the *SULTR4;2* gene decreases with increasing sulfate doses, although the magnitude of decrease is smaller than *BGLU28* (Figure 1c), as previously reported (Maruyama-Nakashita *et al.*, 2006). However, whereas WT showed a 60% reduction of *SULTR4;2* mRNA at 0.1 mM sulfate, no reduction in expression was observed in *sel1-15*, and there was only a 26% reduction in *sel1-16*. At 1.6 mM sulfate, expression was 2-3 fold greater in *sel1-15* and *sel1-16* than in WT, and at 10 mM sulfate, expression in WT was reduced to 6.7% compared with −S medium, but expression in *sel1-15* was twofold greater than in WT. The mis-regulation of *SULTR4;2* expression in *sel1-15* and *sel1-16* further indicates that the mutants are less responsive to repression by external sulfate.

We then examined two other known sulfur deficiency-responsive genes, *LSU1* and *SDI1*. In a co-expression network constructed using the Arabidopsis gene–gene interaction dataset (Ma *et al.*, 2007) as previously described (Xin *et al.*, 2009), *LSU1* and *SDI1* exhibited functional interactions with *SULTR4;2* (Figure S1), consistent with their important physiological function in the S-starvation response. Under our experimental conditions, expression of these two genes also exhibited a dose–response pattern in WT (Figure 1c). Overall, these two genes had a higher expression level in the two *sel1* alleles than in WT at most sulfate doses (Figure 1c). Similar to *BGLU28* and *SULTR4;2*, the sensitivity of expression of *LSU1* in both mutants to increasing sulfate concentrations was reduced at 0.1 and 1.6 mM compared to WT. At 10 mM sulfate, the difference between the two mutants and WT was not statistically significant. For *SDI1*, the expression level in *sel1-15* and *sel1-16* at 1.6 mM was not statistically different from that in WT, but at 10 mM it was three to four times that in WT. In total, these results show that the *sel1-15* and *sel1-16* mutants exhibit a general reduction in response to external sulfate conditions as measured using several functionally demonstrated S-responsive genes.

Molecular cloning revealed that the two *sel1* mutants represent two novel missense alleles of *SULTR1;2*, and therefore they were designated *sel1-15* and *sel1-16*, based on the previously established *sel1* nomenclature (Shibagaki *et al.*, 2002; El–Kassis *et al.*, 2007). Functional complementation of *sel1-16* using the CaMV 35S promoter-driven *SULTR1;2* coding sequence (Figure 2a) showed that the mutation in *SULTR1;2* is responsible for the enhanced *BGLU28* expression phenotype in this mutant. *SULTR1;2* encodes a 653 amino acid, high-affinity sulfate transporter. Both *sel1* mutations alter the amino acid sequences close to the N-terminus: D108N for *sel1-15* and G208D for *sel1-16*. Comparison of these two mutation sites with the corresponding positions in the 11 other Arabidopsis SULTR proteins (Figure 2b) revealed that D108 is conserved only in the SULTR1 group transporters, while G208 is conserved in all of the SULTR proteins. SULTR1;2 has been described as a 12 TM helix-containing protein based entirely on transmembrane prediction algorithms rather than direct experimental evidence. Using various algorithms available on the internet for prediction of membrane protein topology, a consensus model was produced for SULTR1;2 (Figure 2c). The N- and C-termini are both predicted to be cytoplasmic, and the first two TM helices at the extreme N-terminus are predicted by some but not all modeling programs, while TM3-12 are high-probability membrane-spanning domains. A similar topology was proposed previously for the barley high-affinity sulfate transporter by comparison with various sulfate transporters from yeast and animals (Smith *et al.*, 1995), and this topology was experimentally confirmed for the closely related cyanobacterial homolog BicA (Shelden *et al.*, 2010). Based on this model, D108N is localized in TM1 or in the cytoplasm if TM1 does not exist, and G208D is localized in TM5.

Alteration of sulfate transport, metabolite accumulation and metabolism in *sel1-15* and *sel1-16*

As the *sel1* alleles characterized here carry mutations in the sulfate transporter SULTR1;2, one may argue that the reduced response in gene expression to increasing sulfate concentrations may be caused by a lower internal sulfate content in *sel1-15* and *sel1-16* rather than by the reduced sensitivity to sulfur nutrient status. Therefore, to distinguish these two possibilities, sulfate transport, S-metabolite accumulation and metabolism in these mutants was investigated. First, a functional complementation test was performed using a *Saccharomyces cerevisiae* sulfate transport mutant. Yeast strain CP154-7A, carrying deletions of

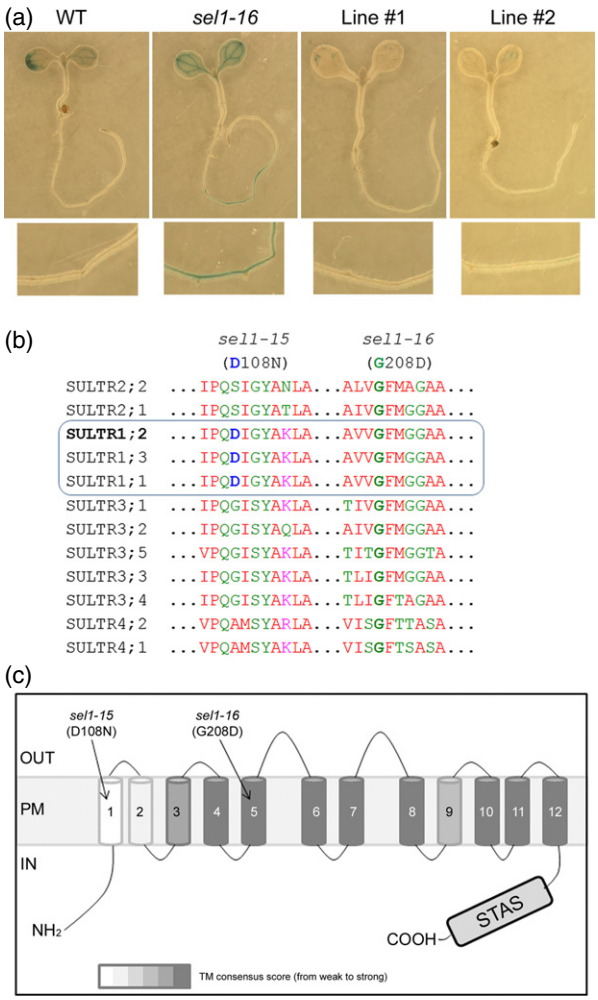

Figure 2. *sel1-15* and *sel1-16* have different mutations in the *SULTR1;2*-encoded high-affinity sulfate transporter.
(a) Complementation of *BGLU28* promoter:GUS activity in *sel1-16* by transgenic expression of CaMV 35S promoter-driven *SULTR1;2–GFP*. Whole seedlings from wild-type (WT), *sel1-16*, and two independent transformants of *sel1-16* with the *SULTR1;2-GFP* construct (lines #1 and #2) were treated with 1.6 mM sulfate for 2 days. Close-up views of the GUS staining in roots from similar regions for each genotype are shown in the lower panel.
(b) Schematic representation of the missense mutation sites in the SULTR1;2 protein. The region of SULTR1;2 with the *sel1-15* and *sel1-16* mutation sites is aligned with that of 11 other Arabidopsis SULTR proteins. The alignment was constructed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Three SULTR1 group transporters are circled. The four colors represent different amino acid properties: blue (acidic amino acids), magenta (basic amino acids), red (small and hydrophobic amino acids) and green (hydroxyl + sulfhydryl + amine amino acids and glycine).
(c) Diagram of the predicted SULTR1;2 topology. Increased strength of the prediction score for TM helices is indicated by increased depth of shading. TM1 and TM2, which have the lowest score, are shown in white. The conserved STAS domain is located at the C-terminus. PM, plasma membrane.

two sulfate transporter genes *sul1* and *sul2*, is defective in sulfate transport and is unable to grow at low sulfate concentration, but grows in the presence of Met as an S source (Cherest *et al.*, 1997). The coding sequences for WT and each of the newly isolated mutant alleles of *SULTR1;2* were cloned in a galactose-inducible expression plasmid. Quantitative analysis of yeast growth in liquid culture showed that WT *SULTR1;2* complemented growth of CP154-7A in the absence of Met (Figure S2a), as reported previously (Shibagaki *et al.*, 2002; Shibagaki and Grossman, 2004). However, yeast transformants expressing *sel1-15*, *sel1-16* and *sel1-8* did not grow on medium with 50 μM sulfate as the sole sulfur source, but grew well if the medium was supplemented with Met (Figure S2a). Therefore, this result shows that, similar to *sel1-8* (Shibagaki *et al.*, 2002), *sel1-15* and *sel1-16* failed to complement CP154-7A, indicating that *sel1-15* and *sel1-16* are defective in sulfate uptake.

Next, the sulfate uptake capacity of Arabidopsis WT and *sel1* mutant plants was measured under conditions that selectively measure high-affinity transport (20 μM [^{35}S]Na_2SO_4) and both high- and low-affinity transport (500 μM [^{35}S]Na_2SO_4). In agreement with the yeast complementation experiment (Figure S2a), *sel1-15* and *sel1-16* plants are defective in sulfate uptake whether measured under high- or low-affinity conditions, and also whether the plants were pre-grown on +S or −S medium (*SULTR1;2* expression is known to be induced by sulfur deficiency) (Figure S2b). Furthermore, the analysis showed that *sel1-15* and *sel1-16* are as defective in sulfate uptake as a T-DNA null mutant of *SULTR1;2*, *sel1-10* (Maruyama-Nakashita *et al.*, 2003). Therefore, the data indicate that D108 and G208 are critical for sulfate transport activity. The fact that sulfate uptake capacity was induced in WT and all of the *sel1* alleles is probably a result of induced expression of other sulfate transporters.

The sulfate content in whole seedlings of the mutants was measured to determine the ability of *sel1-15* and *sel1-16* to maintain an internal sulfate pool. The growth and treatment conditions were identical to those for the gene expression experiments. Prior to initiating the treatment, there was no statistically significant difference between *sel1-16* and WT, but sulfate was significantly lower in *sel1-15* compared to WT (Figure S2c). At 0 mM sulfate, the difference in internal sulfate content between the two *sel1* mutants and WT was small but statistically significant. Although there was no difference in internal sulfate content in the *sel1* mutants and WT at 0.1 mM sulfate, the two mutants accumulated 50% less sulfate than WT at 1.6 mM sulfate. Therefore, the transport-defective *sel1-15* and *sel1-16* alleles show the greatest decrease in internal sulfate content when treated with 1.6 mM sulfate. Interestingly, at 10 mM, *sel1-15* and *sel1-16* had similar internal sulfate levels as WT, probably as a result of low-affinity sulfate transporters that function when external sulfate concentration is high. GSH content was also measured in the roots of the mutants (Figure S2d), and the results revealed that *sel1-15* and *sel1-16* are able to synthesize GSH from sulfate that is taken up. At 0 mM sulfate, *sel1-15* but not *sel1-16* had

slightly lower GSH than WT. At both 0.1 and1.6 mM sulfate, *sel1-15* and *sel1-16* had significantly lower GSH than WT, but these two mutants retained similar GSH content as WT when treated with 10 mM sulfate.

sel1-15 and *sel1-16* show significantly reduced *BGLU28* repression in response to external sulfate in the root system

As indicated in Figure 1(a), *BGLU28* promoter activity shows greater differential regulation by sulfate in roots compared with the shoot, and therefore further experiments focused on roots. Plants were treated for 2 days with −S medium and medium containing 10 mM sulfate, a very high sulfate dose that does not lead to any difference in either internal cellular sulfate or GSH among the genotypes (Figure S2c,d). The relative gene expression level is shown in Table S1. From these data, the fold inhibition was calculated and is given in Figure 3. We found that the root system in WT is indeed much more sensitive to down-regulation of *BGLU28* expression by the very high dose of sulfate (approximately 106 000-fold difference between −S and +S medium; Figure 3) compared with whole seedlings (approximately 4000 fold difference between −S and +S medium; Figure 1b). Interestingly, the inhibition of *BGLU28* in *sel1-15* and *sel1-16* roots by 10 mM sulfate was only approximately 2000- and 4500-fold, respectively, compared to the −S control. This result reflects a 20-50-fold reduction of the response to 10 mM sulfate compared to WT. Three other genes, *SULTR4;2*, *LSU1* and *SDI1*, also exhibited 2-10-fold reduction in the response to 10 mM sulfate in *sel1-1*5 and *sel1-16* compared to WT (Figure 3).

Taken together, the results show that *sel1-15* and *sel1-16* are defective in sulfate transport. However, the mutants accumulated similar level of internal sulfate in whole seedlings and the sulfate assimilation end-product GSH in roots compared with WT when treated with 10 mM sulfate. This suggests that the much higher expression of the S-responsive genes in roots of the *sel1-15* and *sel1-16* plants treated with a high concentration of sulfate is unlikely to be caused by an intracellular sulfur deficiency. At 10 mM external sulfate, other sulfate transporters are able to maintain the internal sulfate level. The gene expression defect therefore suggests that *sel1-15* and *sel1-16* show a reduced response to a high concentration of external sulfate.

sel1-15 and *sel1-16* show a reduced response to externally applied Cys and GSH

To further explore the hypothesized S-response defect, gene expression was analyzed in the roots of *sel1-15* and *sel1-16* plants treated with Cys or GSH. These compounds are the end-products of S assimilation and are known to repress expression of S-response genes. GUS activity was reduced to an undetectable level in *BGLU28* promoter:GUS reporter plants treated with 1 mM Cys, but *sel1-15* and *sel1-16* retained considerable GUS activity (Figure 4a). To exclude the possibility that the higher *BGLU28* promoter activity in *sel1-15* and *sel1-16* was the result of compromised Cys uptake and metabolism caused by the *SULTR1;2* mutations, Cys uptake was measured in the two mutants. The results showed that *sel1-15* and *sel1-16* were completely unaffected with regard to Cys uptake either before or after treatment with 1 mM Cys (Figure 4b). *sel1-15* and *sel1-16* were further analyzed to determine whether Cys metabolism into GSH is affected in the mutant roots. As expected, except at 0 mM Cys where *sel1-15* roots have a slightly lower GSH level than WT, there was no significant difference between *sel1-15* and WT or between *sel1-16* and

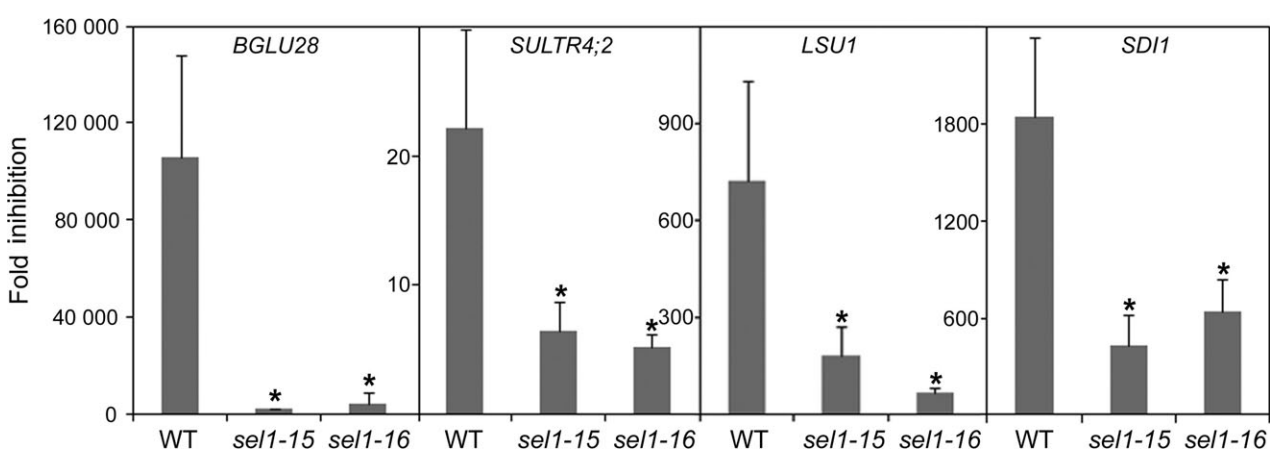

Figure 3. Reduction of the sensitivity to 10 mM sulfate in the roots of *sel1-15* and *sel1-16* alleles.
Plants were treated as described in Figure 1(b, c), and gene expression was measured using real-time reverse transcriptase (RT)-PCR analysis of RNA extracted from roots of the treated seedlings, using *ACT2* as an internal control. Fold inhibition for each genotype was expressed as the ratio of gene expression for the 0 mM control versus the 10 mM sulfate treatment. Values are means and SD of three independent replicates. Asterisks indicate statistically significant differences ($P < 0.05$; Student's *t* test) between wild-type (WT) and *sel1* alleles.

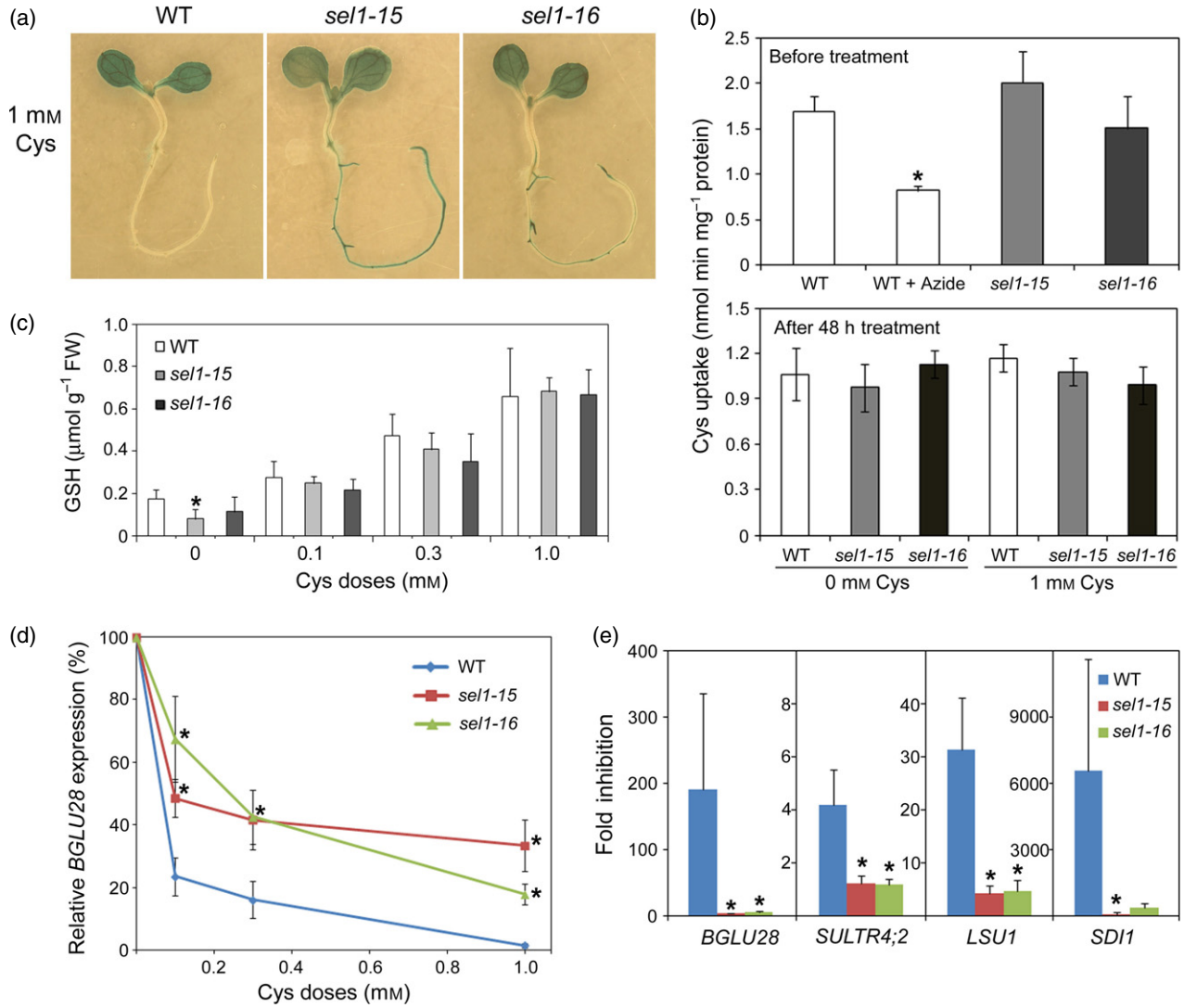

Figure 4. *sel1-15* and *sel1-16* reduced the sensitivity to externally applied Cys.
(a) *BGLU28* promoter:GUS activities in *sel1-15* and *sel1-16* in response to 1 mM Cys. Four-day-old seedlings as described in Figure 1(c) were treated with −S liquid medium supplemented with 1 mM Cys for 2 days before GUS staining.
(b) Cys uptake in roots. [^{35}S]-L-Cys uptake was measured in 4-day-old seedlings. Seedlings were either grown on +S medium (top), or treated with 1 mM Cys for 48 h before performing the uptake experiment (bottom). Sodium azide (0.1 mM) was added to WT samples during the uptake experiment (labeled WT + Azide). Azide-dependent inhibition of uptake is an indication that L-Cys transport is an energy-dependent process. Values are means and SD of four replicates, each replicate consisting of five plants.
(c) GSH content in the roots of Cys-treated *sel1-15* and *sel1-16*. Four-day-old seedlings as described in Figure 1(b) were treated in −S liquid medium supplemented with various doses of L-Cys for 2 days before GSH measurement. Values are means and SD of five or six replicates, each replicate consisting of a pool of 4–5 roots.
(d) Dose–response curve for *BGLU28* gene expression change in the roots of *sel1-15* and *sel1-16*. Plants were treated exactly as in (c), and gene expression was assessed as described in Figure 1(b) except that each genotype at 0 mM Cys was set to 100, and gene expression under all other Cys doses was normalized to the 0 mM Cys control for each genotype. Values are means ± SEM of three independent replicates.
(e) Fold inhibition for *BGLU28*, *SULLTR4;2*, *LSU1* and *SDI1* genes in roots in response to 1 mM Cys. Fold inhibition for gene expression was expressed as in Figure 3. Values are means and SEM of three independent replicates.
Asterisks indicate statistically significant differences ($P < 0.05$; ANOVA) between *sel1* and WT (or between WT + Azide and WT in Figure 5b) under the same nutrient treatment conditions.

WT under all conditions tested (Figure 4c). The result also indicates that Cys is readily utilized by plants, and most likely is taken up via a SULTR1;2-independent mechanism.

Therefore, *BGLU28* expression was examined after treatment with Cys. Relative gene expression levels are presented in Table S2. The dose–response pattern between *sel1* and WT, calculated as the change in gene expression, is shown in Figure 4(d). The results confirm that endogenous *BGLU28* expression in WT is significantly decreased with increasing Cys doses, but *sel1-15*

and *sel1-16* showed a reduced capacity for Cys-dependent reduction of gene expression (Figure 4d). Except for the marginally significant difference between *sel1-15* and WT at 0.3 mM Cys (*P* value 0.0505), the differences between the two *sel1* mutants and WT under the other conditions are significant. For example, at 1 mM Cys, while WT exhibited 190-fold inhibition compared to the 0 mM Cys control, *sel1-15* and *sel1-16* showed only 3.6- and 6-fold inhibition, respectively, (Figure 4e). Three other genes, *SULTR4;2*, *LSU1* and *SDI1*, also exhibited reduced inhibition (Figure 4e and Table S2).

The mutants were also found to be less responsive to GSH treatment. The gene expression level was significantly reduced after treatment with 1 mM GSH (Table S2), ranging from 15-fold inhibition for *SULTR4;2* to 208-fold inhibition for *BGLU28* in WT (Figure 5). By comparison, *sel1-15* and *sel1-16* only exhibited 30- and 22-fold inhibition, respectively, for *BGLU28*. All other genes were similarly affected in *sel1-15* and *sel1-16*, except for *SDI1* in *sel1-16* (Figure 5). These results clearly demonstrate that *sel1-15* and *sel1-16* show a reduced response to external Cys and GSH treatments which led to similar amounts of internal GSH in roots of WT and the two mutants.

A *SULTR1;2* knockout mutant shows reduced response to sulfate, Cys and GSH

To examine whether the gene expression defect is a specific property of the *sel1-15* and *sel1-16* missense mutations, the S response in gene expression was examined in a *SULTR1;2* null mutant. RT-PCR analysis of a *SULTR1;2* transposon line (stock number CS116961, with a transposon inserted into the 7th exon), designated *sel1-18*, showed that no intact *SULTR1;2* mRNA was expressed (Figure S3a). Furthermore, sulfate transport in *sel1-18* was reduced to the same degree as in *sel1-15* and *sel1-16* (Figure S3b), and was similar to that in *sel1-10*, a previously studied null allele (Maruyama-Nakashita *et al.*, 2003). Therefore, s*el1-18* represents a second null allele of *SULTR1;2*. A *BGLU28* promoter:luciferase (LUC) reporter construct was introgressed into the same locus in *sel1-18* and its WT Col parent. Using the growth and treatment conditions described above, it was found that *sel1-18* shows stronger LUC activity at 1.6 mM sulfate than WT (Figure S3c). The coding sequence of *SULTR1;2* but not *sel1-15* is able to complement the higher LUC activity in *sel1-18* at 1.6 mM sulfate (Figure S3c), confirming that the increased *BGLU28* promoter activity on +S medium is caused by the loss of *SULTR1;2* function. To determine whether the new null mutant shows a decreased response to sulfate and organic S sources, as *sel1-15* and *sel1-16* do, GSH content and gene expression in roots were measured in *sel1-18* after treatments with high concentrations of sulfate, Cys and GSH. At 10 mM sulfate, WT and *sel1-18* had similar contents of GSH in roots (0.63 ± 0.16 and 0.74 ± 0.18 μmol per g fresh weight, respectively). In the presence of 1 mM Cys or 1 mM GSH, both mutants had similar amounts of internal GSH as WT in roots (Figure 6a). LUC activity is given in Table S3, and the corresponding fold inhibition calculation compared with sulfur deficiency is shown in Figure 6(b). The results show that LUC in WT was inhibited 1356-, 756- and 188-fold by 10 mM sulfate, 1 mM Cys and 1 mM GSH, respectively, but was reduced only 29-, 14- and 6-fold, respectively, in *sel1-18*. This difference represents a 31–54-fold reduction in the response of *sel1-18* to sulfate and the organic S metabolites compared to WT. Therefore, these findings for *sel1-18*

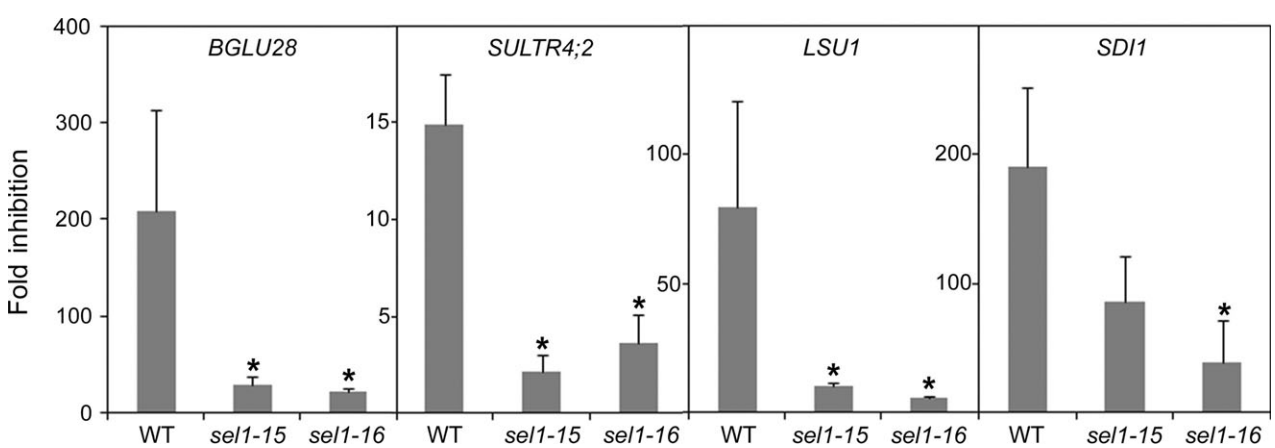

Figure 5. *sel1-15* and *sel1-16* show reduced sensitivity to externally applied GSH.
Four-day-old seedlings as described in Figure 1(b) were treated in −S liquid medium supplemented with 0 or 1 0mM GSH for 2 days, and gene expression was measured using real-time reverse transcriptase (RT)-PCR analysis of RNA extracted from the roots, using *ACT2* as an internal control. Fold inhibition for each genotype was expressed as the ratio of gene expression for the 0 mM control versus the 1 mM GSH treatment. Values are means and SD of three independent replicates. Asterisks indicate statistically significant differences ($P < 0.05$; Student's *t* test) between wild-type (WT) and *sel1* mutants.

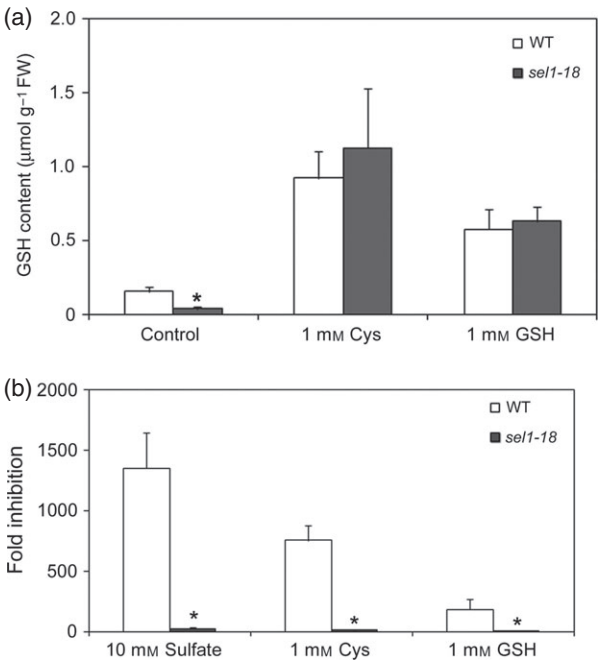

Figure 6. A *SULTR1;2* knockout mutant shows reduced sensitivity to sulfate, Cys and GSH.

(a) GSH content in *sel1-18* roots after treatment of whole seedling for 2 days with −S liquid medium (control), or −S medium supplemented with 1 mM Cys or 1 mM GSH. Values are means and SD of three replicates each with 4–5 roots.

(b) Fold inhibition for the *BGLU28* promoter:LUC activity in the roots of *sel1-18* in response to 10 mM sulfate, 1 mM Cys or 1 mM GSH for 2 days, compared to the −S control and using *ACT2* as an internal control. LUC activity is expressed as luminescence units per cm of roots. Values are means and SEM of 9–11 plants.

The treatments are indicated on the *x* axis. White bars, WT; gray bars, *sel1-18*. Asterisks indicate statistically significant differences ($P < 0.05$; Student's *t* test) between wild-type (WT) and *sel1-18*.

are in agreement with the results showing a reduced response of *sel1-15* and *sel1-16* to both inorganic and organic forms of S.

DISCUSSION

SULTR1;2 has been described as functioning as a high-affinity sulfate transporter (Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002; Maruyama-Nakashita *et al.*, 2003; El–Kassis *et al.*, 2007). The two novel alleles of *SULTR1;2* reported here, *sel1-15* (D108N) and *sel1-16* (G208D), are defective in sulfate uptake, similar to the null allele *sel1-10* (Maruyama-Nakashita *et al.*, 2003). Consequently, these two mutants accumulate less sulfate than WT and less of one assimilation end-product, GSH, when plants are grown at an intermediate sulfate level. The sulfate transport defect is more pronounced when uptake is measured under high-affinity conditions, and this activity is inducible by S starvation. These results demonstrate a transport function defect in the *SULTR1;2* mutants, consistent with previously published analyses of SULTR1;2 mutants (Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002; Maruyama-Nakashita *et al.*, 2003; El–Kassis *et al.*, 2007).

Of particular note, *BGLU28* is the most strongly up-regulated gene in the *sel1-10* null mutant of *SULTR1;2* (Maruyama-Nakashita *et al.*, 2003), and using a *BGLU28* promoter-based mutant screen in the present study, we have identified the *sel1-15* and *sel1-16* alleles of *SULTR1;2*. This indicates that SULTR1;2 is at least one of the major regulators of *BGLU28* expression in response to sulfur nutrient status. Importantly, two types of physiological studies suggest that S deficiency caused by the transport defect in the *SULTR1;2* mutants cannot fully explain the increased *BGLU28* expression in *sel1* alleles. First, a sulfate dose–response experiment showed that whole seedlings of the *sel1-15* and *sel1-16* mutants have higher gene expression levels than WT at most of the sulfate doses. The elevated expression in the mutants is similar to several previous studies showing that loss-of-function mutants of *SULTR1;2* show elevated expression of many S-response genes (Maruyama-Nakashita *et al.*, 2003; El–Kassis *et al.*, 2007; Lee *et al.*, 2012), but these reports attributed the gene expression phenotype to the lower cellular content of sulfate and S-assimilation end-products resulting from the transport defect. Their interpretation was based on data from plants grown on media containing intermediate or low levels of sulfate. However, in the present study, we also included a high sulfate dose (10 mM), and found that, under this condition, the mutants and WT have similar contents of internal sulfate and GSH. Both sulfate and GSH are central metabolites of S assimilation from which all other S compounds are synthesized, and their internal concentrations are known to correlate with external S supply. Therefore, analysis of these two compounds suggests that, under 10 mM sulfate, the mutants have similar internal sulfur nutrient status. It is likely that, at 10 mM sulfate, low-affinity sulfate transporters bypass SULTR1;2 and so overcome the transport defect. Indeed, our experiments indicate that sulfate transport is not entirely abolished in *sel1-15* and *sel1-16* or even the null allele *sel1-18*. Further, Arabidopsis plants express both low-affinity sulfate transporters (such as SULTR2;1 and SULTR2;2) and high-affinity sulfate transporters (such as SULTR1;2 and SULTR1;1) in roots. Therefore, higher gene expression in *sel1-15* and *sel1-16* strongly indicates that SULTR1;2 alters gene expression irrespective of external or internal sulfate concentrations when a super-optimal dose is applied externally. Furthermore, we examined gene expression in the root system, as SULTR1;2 is primarily expressed in root cortical cells and is the major high-affinity sulfate transporter in the root system (expression in shoots is only detected in trichomes) (Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002, 2007; El–Kassis *et al.*, 2007; Barberon *et al.*, 2008). As expected, we observed a much greater difference

of gene expression in roots between mutants and WT compared with the difference in expression in whole seedlings when plants are treated with 10 mM sulfate. Moreover, a similarly reduced response to 10 mM sulfate was also observed in *sel1-18*, a T-DNA insertion mutant with complete loss of *SULTR1;2* mRNA expression.

Second, the S-assimilation end-product feeding experiments showed that a higher level of gene expression is maintained in the *sel1* mutants than WT even when plants have sufficient Cys and GSH. We have found that Cys uptake is independent of the SULTR1;2 transporter, and consequently, accumulation of internal GSH is not affected by the *SULTR1;2* mutations; however, gene expression inhibition by Cys is reduced in the mutants. This reduction is much stronger than in the 10 mM sulfate treatment. Furthermore, the reduced response of gene expression to Cys and GSH is not restricted to the *sel1-15* and *sel1-16* missense mutations. The *SULTR1;2* null mutant *sel1-18* also exhibits a considerable reduced response to Cys and GSH. Therefore, the results from the S-assimilation end-product feeding experiments not only show that mis-regulation of gene expression in the *sel1* mutants is not entirely under the control of the internal level of S metabolites, but also that the reduced response in the mutants to a high concentration of sulfate is not due to a defect in transporting sulfate or using sulfate as a nutrient. A similar experiment in which the nitrate assimilation products ammonium and glutamine were added to make up the same level of total nitrogen in the medium has been performed to illustrate the role of nitrate transporter NRT1.1 in nitrate sensing (Munos *et al.*, 2004; Remans *et al.*, 2006). If SULTR1;2 has a comparable sensing function as NRT1.1, it is less likely that the *sel1* mutants are directly involved in sensing the status of both sulfate and its assimilation products. Instead, it is more likely that the plant S-sensing or signaling machinery is responsive to feedback inhibition, and that the *sel1* mutations alter the end-product feedback regulation.

It should be noted that a mutant termed *sac1* has been isolated from *Chlamydomonas reinhardtii* showing that SAC1 is required for many responses that accompany acclimation of cells to S limitation; this mutant shows aberrant global responses to S deprivation (Davies *et al.*, 1994, 1996). Because of the pleiotropic effects of this mutant, SAC1 was proposed to function as a sensor of S status or as a component of the signal transduction chain just downstream of the sensory apparatus (Davies *et al.*, 1996). Although so far there is no direct experimental evidence for this hypothesis (Pollock *et al.*, 2005), it raises the intriguing possibility that the sulfate transporter may act as a sensor or a component of sensing/signaling network. As SULTR1;2 is not localized to the nucleus, it is less likely that SULTR1;2 may directly affect transcription of *BGLU28* and other S-responsive genes. Thus, there are at least two possibilities to explain the regulatory function of SULTR1;2. One is that SULTR1;2 does not sense sulfate or sulfur nutrient status but may regulate a signaling pathway or network that leads to the regulation of gene expression. Alternatively, SULTR1;2 may function as an S sensor or a component of sensing complex, which controls a signaling pathway or network to regulate gene expression.

Although our present evidence cannot distinguish these two possibilities, the second hypothesis that SULTR1;2 may act as a component of an S-sensing complex or even a sensor is very attractive and deserves further study. A possible mode of action is that SULTR1;2 is able to sense S status through its interaction with OASTL, a component of the Cys synthase complex when partnered with the serine acetyltransferase, the enzyme that synthesizes *O*-acetylserine (Kumaran *et al.*, 2009; Yi *et al.*, 2010; Wirtz *et al.*, 2012). A recent study has demonstrated that the STAS domain of SULTR1;2 forms a binding site for the cytosolic isoform of OASTL, and the interaction caused reciprocal activation/inactivation of the partners when analyzed in a yeast system (Shibagaki and Grossman, 2010). This finding was interpreted as indicating the existence of a direct connection between sulfate transport and sulfate assimilation. Interestingly, a loss-of-function mutant of this *OASTL* gene exhibits an abnormal response of gene expression (Alvarez *et al.*, 2010). Although the physiological relevance of such a functional interaction in plants remains to be demonstrated, these findings raise an intriguing possibility that the SULTR1;2 sensor may either directly control the assimilation pathway or integrate the sensing/signaling pathway with the assimilation pathway to control the gene expression response through OASTL. However, our data do not appear to support a direct role for SULTR1;2 in regulating OASTL. This is because, in the presence of a high sulfate (10 mM), there is no difference in internal sulfate or GSH content, indicating that elimination of SULTR1;2 does not markedly alter sulfate assimilation under this condition. Nevertheless, under normal physiological conditions, it is possible that SULTR1:2 and OASTL form a sensor complex. This possibility is intriguing, as we have observed that the *sel1-15*, *sel1-16* and *sel1-18* alleles are still responsive (albeit to a significantly reduced extent) to increasing S doses. Indeed, under 10 mM sulfate, compared to WT, the mutants exhibit only a minimally reduced gene expression inhibition response for *BGLU28*. Under 1 mM GSH or Cys, the mutants reduced the *BGLU28* expression inhibition response more dramatically. We also note that the degree of expression inhibition in *sel1* varied among genes and in response to various S treatments; for example, the *sel1* mutants showed a more dramatically reduced *SULTR4;2* expression inhibition response, particularly in the presence of Cys. It is very likely that other proteins besides SULTR1;2 are also involved in S sensing or

signaling. Perhaps other sulfate transporters have partially redundant or overlapping functions with SULTR1;2 in the hypothetical sensor complex involving OASTL; alternatively, other proteins unrelated to sulfate transporters may also act as sensors of sulfur nutrient status.

Another possible mode of action that should be considered in the future is that SULTR1;2 may act as a putative transporting receptor. Studies in yeast and recently in animals and plants have revealed the existence of three types of nutrient sensors: classical receptors (such as the G protein-coupled receptor Gpr1), non-transporting receptors (such as Snf3 and Gcr1, which have non-functional transporter domains), and transporting receptors (such as Gap1, which contains functional transporter domains) (Thevelein and Voordeckers, 2009). The latter two transporter-related receptors, collectively termed 'transceptors', are thought to be evolutionary intermediates between nutrient transporters and classical receptors of chemical signals (Thevelein and Voordeckers, 2009). Various dual-function metabolite transporters/sensors have been identified in several organisms, such as the amino acid transporter/receptor Gap1 (Van Zeebroeck *et al.*, 2009) and the glucose transporter/sensor GLUT2 in non-plant systems (Leturque *et al.*, 2009). In Arabidopsis, the nitrate transporters NRT1.1 and NRT2.1 have been reported to play such a regulatory role, with NRT1.1 being the first demonstrated nitrate receptor/sensor (Munos *et al.*, 2004; Little *et al.*, 2005; Remans *et al.*, 2006; Ho *et al.*, 2009). It has been shown that a mutation of P492 (in the *chl1-9* allele) in the TM9–TM10 intracellular loop abolishes nitrate transport but retains the normal nitrate response, suggesting that the sensor function may be decoupled from the uptake function (Ho *et al.*, 2009). However, another possibility exists that transport and sensing functions are coupled. Recent work with the yeast amino acid transceptor Gap1 indicates that the same binding site functions in both transport and signaling, and that a conformational change after the ligand-binding step triggers activation of a signaling pathway (Thevelein and Voordeckers, 2009; Van Zeebroeck *et al.*, 2009). For SULTR1;2, several amino acid residues that are necessary for sulfate transport have been identified (Shibagaki *et al.*, 2002; Shibagaki and Grossman, 2006; El-Kassis *et al.*, 2007), but their involvement in sensing remains unknown. Our results suggest that residues D108 (*sel1-15*) and G208 (*sel1-16*), which are located in the predicted TM1 and TM5, respectively, are important for sulfate transport and are also required for the sensing function. This indicates that TM1 and TM5 may potentially be a site for future investigation regarding coupled transport and sensing functions in SULTR1;2. Unfortunately, the membrane topology of SULTR1;2 has not yet been experimentally characterized, so it is only possible to speculate on the position of this domain based on membrane topology predictions. Therefore, future research must focus on the membrane topology of SULTR1;2 in order to map the sensing domain and to understand how it functions. Understanding the sensing/signaling mechanism of SULTR1;2 will provide useful information regarding the evolution of inorganic ion receptors and sensing in plants.

EXPERIMENTAL PROCEDURES

Plant materials and nutrient treatments

Arabidopsis thaliana ecotype Columbia, a transgenic line GHF1, which contains the At2g44460/*BGLU28* promoter:GUS construct (Dan *et al.*, 2007), and the *sel1-15* and *sel1-16* mutants derived from this transgenic line were used in this study. Seedlings were grown vertically on agar-solidified Murashige and Skoog (MS) medium for 4 days, and then transferred to liquid medium containing various doses of sulfate, Cys or GSH. As described previously (Dan *et al.*, 2007), the −S medium contained 0.0001 mM sulfate from residual $CuSO_4$, and the +S medium was prepared by adding 1.6 mM sulfate to the −S medium.

Mutant isolation, genetic characterization and gene cloning

Two allelic, recessive mutants (designated *sel1-15* and *sel1-16*) that showed strong GUS staining in roots were obtained after screening the mutagenized GHF1 seedlings as described in detail in Methods S1. They were then back-crossed to GHF1, and the homozygous plants recovered were used in this study. For gene cloning, the *sel1-16* allele was used for molecular mapping. SSLP markers were used to map the *sel1-16* mutation to an 81 kb BAC clone F28K19, containing 25 putative genes including two *SULTR* genes, At1 g77990 (*SULTR2;2*) and At1 g78000 (*SULTR1;2*). Primers (Table S4) were designed to sequence the coding regions of these two genes in *sel1-16* and subsequently *sel1-15*.

Yeast complementation

Complementation was performed using the *sul1 sul2* double mutant of *Saccharomyces cerevisiae* CP154-7A (Cherest *et al.*, 1997), and its wild-type parental strain W303-1B was used as a control. Yeast expression constructs with coding sequences of wild-type *SULT1;2* and its mutant forms *sel1-15*, *sel1-16* and *sel1-8* (Maruyama-Nakashita *et al.*, 2003; Barberon *et al.*, 2008) were transformed into CP154-7A. Transformants for each construct were grown in minimal sulfate medium (0.05 mM) in the presence or absence of 0.4 mM Met and the presence of 2% galactose. Absorbance at 600 nm was monitored every 8 h in liquid culture for quantitative analysis.

Sulfate uptake, sulfate and GSH content measurement

For uptake, 6-day-old seedlings grown on +S medium were transferred to agarose-solidified +S or −S medium for sulfate uptake measurement. [^{35}S]Na_2SO_4 was used to determine the uptake, as described in Methods S1. For internal sulfate content measurement, growth conditions were exactly the same as for sulfate treatments, as described in detail in Methods S1. A Dionex (http://www.dionex.com/en-us/index.html) HPLC was used, as described in detail in Methods S1. For GSH content measurement, plants were treated exactly the same as in the corresponding treatments for gene expression. GSH content in roots was measured using the Promega (http://www.promega.com/) GSH-Glo kit according to the manufacturer's instructions.

Real-time RT-PCR analysis

RNA extraction, reverse transcription and real-time PCR reactions were performed as described previously (Xin *et al.*, 2009), and the gene-specific primers are listed in Table S4.

Luciferase (LUC) and β-glucuronidase (GUS) assays

BGLU28 promoter:LUC seedlings were scanned, and the root from each plant was then cut off and transferred to a 96-well white microtiter plate. LUC activity was measured in each cell by dispensing 100 μl luciferase assay buffer, and luminescence was read using a Synergy 2 microplate luminometer (Bio-Tek Instruments, http://www.biotek.com/), as described in detail in Methods S1. The GUS assay was performed as described in Methods S1.

ACKNOWLEDGEMENTS

We thank Dominique Thomas (Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique) for providing the yeast CP154-7A strain, Françoise Gosti (Biochimie et Physiologie Moléculaire des Plantes, National Institute for Agricultural Research) for *sel1-8* seeds, Hideki Takahashi (RIKEN Plant Science Center) for *sel1-10* seeds, and Tom Schultz (Department of Cell Biology, Scripps Research Institute) for the pZPXΩLuc+ vector. We are grateful to Dustin Carver, an undergraduate student at Rutgers University in the Biotechnology Major, for constructing pMDC123-LUC. Funding for this research was initially provided in part by US National Institutes of Health grant 3S06GM008225-20S1 and a Professional Staff Congress-City University of New York grant (to Z. -L. Z.), and a US Department of Agriculture Hatch grant (to T. L.), and is currently provided by collaborative US National Science Foundation grants (IOS-1121521 to T.L. and IOS-1121551 to Z. -L.Z.).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. A gene–gene interaction network involving *BGLU28* and *SULTR4;2*.

Figure S2. Sulfate transport and accumulation in *sel1-15* and *sel1-16* under various conditions.

Figure S3. Characterization of *sel1-18*.

Table S1. Gene expression levels in roots of the *sel1* mutants in response to 10 mM sulfate and 1 mM GSH.

Table S2. Gene expression levels in roots of the *sel1* mutants in response to Cys treatment.

Table S3. *BGLU28* promoter:LUC activity in roots of *sel1-18*.

Table S4. Primers used in the current study.

Methods S1. Extended experimental procedures.

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