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Sulfur-Responsive Elements in the 3′-Nontranscribed Intergenic Region Are Essential for the Induction of *SULFATE TRANSPORTER 2;1* Gene Expression in Arabidopsis Roots under Sulfur Deficiency

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Under sulfur deficiency (−S), plants induce expression of the sulfate transport systems in roots to increase uptake and root-to-shoot transport of sulfate. The low-affinity sulfate transporter SULTR2;1 is predominantly expressed in xylem parenchyma and pericycle cells in *Arabidopsis thaliana* roots under –S. The mechanisms underlying –S-inducible expression of *SULTR2;1* in roots have remained unclear, despite the possible significance of SULTR2;1 for acclimation to low-sulfur conditions. In this investigation, examination of deletions and base substitutions in the 3′-intergenic region of *SULTR2;1* revealed novel sulfur-responsive elements, SURE21A (5′-CAATGTATC-3′) and SURE21B (5′-CTAGTAC-3′), located downstream of the *SULTR2;1* 3′-untranslated region. SURE21A and SULTR21B effectively induced reporter gene expression from fusion constructs under –S in combination with minimal promoters or promoters not inducible by –S, suggesting their versatility in controlling transcription. T-DNA insertions near SURE21A and SULTR21B abolished −S-inducible expression of *SULTR2;1* in roots and reduced the uptake and root-to-shoot transport of sulfate. In addition, these mutations partially suppressed *SULTR2;1* expression in shoots, without changing its –S-responsive expression. These findings indicate that SULTR2;1 contributes to the increase in uptake and internal translocation of sulfate driven by gene expression induced under the control of sulfur-responsive elements in the 3′-nontranscribed intergenic region of *SULTR2;1*.

INTRODUCTION

Sulfur is an essential element required for plant growth. Sulfate is the major form of sulfur that plants can use for synthesizing sulfur-containing compounds, such as the amino acids cysteine and methionine, proteins, lipids, coenzymes, and various secondary metabolites (Leustek et al., 2000; Saito, 2004). The essentiality of these compounds clearly indicates the importance of sulfate uptake, distribution, and metabolism. Following uptake of sulfate from the soil, sulfate moves horizontally through the apoplast and symplast and is loaded into xylem to be transported to aerial parts of the plant. Sulfate transporters mediate the uptake and internal mobilization of sulfate.

The *Arabidopsis thaliana* genome encodes 12 sulfate transporters (SULTRs), classified into four groups (SULTR1, SULTR2, SULTR3, and SULTR4) based on the similarity in their protein sequences (Takahashi et al., 2012). Their biochemical properties, tissue localization, and functions in plants have been studied extensively (Davidian and Kopriva, 2010; Takahashi et al., 2011). The group 1 sulfate transporters consist of the high-affinity transporters SULTR1;1, SULTR1;2, and SULTR1;3. SULTR1;1 and SULTR1;2 are expressed in the epidermis and cortex of roots and facilitate the initial uptake of sulfate from the soil (Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). SULTR1;3 is localized in the phloem and mediates source-to-sink translocation of sulfate (Yoshimoto et al., 2003). The group 4 sulfate transporters SULTR4;1 and SULTR4;2 are localized to the tonoplast and are involved in remobilization of vacuolar sulfate pool (Kataoka et al., 2004a).

When the soil concentration of sulfate declines, plants increase the capacities of sulfate transport systems in roots. In Arabidopsis, −S induces the expression of several sulfate transporters, including *SULTR1;1*, *SULTR1;2*, *SULTR1;3*, *SULTR4;1*, and *SULTR4;2* (Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002; 2007; Kataoka et al., 2004a). These sulfate transporters are essential for the initial uptake and vascular translocation of sulfate and release of vacuolar sulfate to support efficient utilization of sulfate pools. Our recent studies have indicated several molecular mechanisms

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required for the –S-responsive gene expression of Arabidopsis *SULTRs* (Maruyama-Nakashita et al., 2005, 2006). A sulfur-responsive *cis*-acting element, comprising 16 bp of a DNA sequence named the sulfur-responsive element (SURE) occurs in the 5′-region of *SULTR1;1* and induces its gene expression in response to –S (Maruyama-Nakashita et al., 2005). An EIL-family transcription factor, SLIM1, has been identified as a transcriptional regulator controlling the main pathways of sulfate uptake and metabolism, including *SULTR1;1* and *SULTR1;2*, under –S conditions in Arabidopsis (Maruyama-Nakashita et al., 2006).

In contrast to the group 1 and 4 sulfate transporters, the physiological functions of group 2 and 3 sulfate transporters are not well understood. SULTR2;1, a group 2 sulfate transporter, exhibits a low-affinity sulfate transport activity in yeast and is expressed in the xylem and phloem parenchyma cells of leaves and xylem parenchyma and pericycle cells of roots in Arabidopsis (Takahashi et al., 1997, 2000). Based on its tissue-specific localization, SULTR2;1 has been suggested to mediate the uptake of sulfate from the apoplast within the vascular bundle. Furthermore, it has been considered to function as a component of a sulfate transport system that possibly involves a functional interplay with SULTR3;5 mediating the root-to-shoot transport of sulfate in Arabidopsis (Kataoka et al., 2004b). Simultaneous expression of *SULTR2;1* and *SULTR3;5* in yeast enhances sulfate uptake capacity compared with the expression of either *SULTR2;1* or *SULTR3;5* (Kataoka et al., 2004b). The synergistic contribution of SULTR2;1 and SULTR3;5 to root-to-shoot transport of sulfate is suggested based on the overlap of tissue-specific gene expression in the xylem parenchyma and pericycle cells of Arabidopsis roots (Takahashi et al., 1997, 2000; Kataoka et al., 2004b). Because the coexpression of *SULTR2;1* with *SULTR3;5* increased sulfate uptake activity in yeast, the inducible expression of *SULTR2;1* in roots has been suggested to act as a key factor in increasing root-to-shoot transport of sulfate under –S conditions (Kataoka et al., 2004b).

Expression of *SULTR2;1* shows complicated responses to –S conditions. The transcript level of *SULTR2;1* is highly upregulated in response to −S in roots but is decreased in shoots (Takahashi et al., 2000). The repression of *SULTR2;1* in shoots involves microRNA-395 (miR395), which is induced by −S in a SLIM1-dependent manner in phloem (Kawashima et al., 2009) and targets *SULTR2;1* mRNA (Jones-Rhoades and Bartel, 2004; Allen et al., 2005; Kawashima et al., 2009). However, this post-transcriptional regulatory mechanism contrasts with the situation in roots, where the *SULTR2;1* mRNA level increases significantly under –S conditions; nevertheless, miR395 accumulates to high levels in roots, as in shoots (Kawashima et al., 2009). This disagreement between miR395 and *SULTR2;1* accumulations has been suggested to be due to their cell-type-specific expression in root vascular tissues; i.e., the expression of miR395 is restricted in the phloem companion cells, which leaves the target *SULTR2;1* mRNA to remain intact and accumulate in xylem parenchyma and pericycle cells (Kawashima et al., 2009). Thus, an alternative regulatory mechanism independent of SLIM1 and miR395 must underlie the –S-responsive induction of *SULTR2;1* expression in roots, particularly in cell types where an induced expression of sulfate transporters (i.e., SULTR2;1) can increase the flux of sulfate loaded to the xylem stream and transferred from roots to aerial organs. This study reveals the presence of *cis*-acting regulatory elements in the 3′-nontranscribed intergenic region for the –S-responsive transcriptional regulation of *SULTR2;1* in Arabidopsis. Molecular dissection of regulatory elements and disruption of their function in T-DNA knockout lines demonstrate the significance of this mechanism for the –S-responsive induction of *SULTR2;1* in roots.

RESULTS

The 3′-Downstream Intergenic Region of *SULTR2;1* Controls –S-Inducible Expression in Roots

To identify the *cis*-acting molecular mechanisms controlling –S-inducible expression of *SULTR2;1* in Arabidopsis roots, regulatory functions of 5′-upstream and 3′-downstream intergenic regions flanking *SULTR2;1* (Figure 1A) were investigated by expressing their green fluorescent protein (GFP) fusion genes in transgenic Arabidopsis. Fusion genes containing the fragments, a 2535-bp 5′-upstream region of *SULTR2;1* ($P_{SULTR2;1}$), *GFP* coding region, and either the 1077-bp 3′-downstream region of *SULTR2;1* ($T_{SULTR2;1}$) or the nopaline synthase gene terminator (T_{NOS}) were constructed for generating transgenic plants (Figures 1A and 1B). The fusion gene constructs, $P_{SULTR2;1}$:*GFP*:$T_{SULTR2;1}$ (2;1PGT) and $P_{SULTR2;1}$:*GFP*:T_{NOS} (2;1PGN), were introduced into Arabidopsis plants (ecotype Columbia-0 [Col-0]) by *Agrobacterium tumefaciens* infection. T2 progenies of these transgenic Arabidopsis plants were grown on culture media containing 1500 μM sulfate (+S) or 15 μM sulfate (–S) for 10 d, and the GFP accumulation in plants was visualized (Figure 1B). The fluorescent images indicated significant accumulation of GFP in the root tissues of 2;1PGT transgenic lines under –S conditions (Figure 1B). In contrast, GFP expression was slightly reduced in the root tissues of 2;1PGN transgenic lines under –S (Figure 1B). These results suggested that –S-inducible transcript accumulation of *SULTR2;1* was controlled in the roots by the 3′-downstream region rather than the 5′-upstream region of *SULTR2;1*.

Previous studies based on in situ hybridization and reporter analysis using a 2990-bp 5′-upstream region of *SULTR2;1* and a uidA gene indicated predominant localization of *SULTR2;1* gene expression in the vascular tissues in both leaves and roots (Takahashi et al., 1997, 2000). The tissue localizations of GFP expression in 2;1PGT plants under +S in this study were basically identical to these previously reported results. GFP accumulated in the vascular tissues of roots, especially in basal region of primary roots and lateral root initiation zones under +S (Figure 1C, a to d), and these GFP signals significantly increased under –S (Figure 1C, e to h). In addition, weaker GFP fluorescence was detected in both cortex and epidermis in the roots of 2;1PGT plants grown under –S (Figure 1C, e to h).

Identification of Sulfur-Responsive Elements in the 3′-Downstream Intergenic Region of *SULTR2;1*

To determine the sulfur-responsive element in the 3′-downstream region of *SULTR2;1*, a series of 3′-truncated fragments were fused to the luciferase (*Luc*) gene, and these fusion genes were

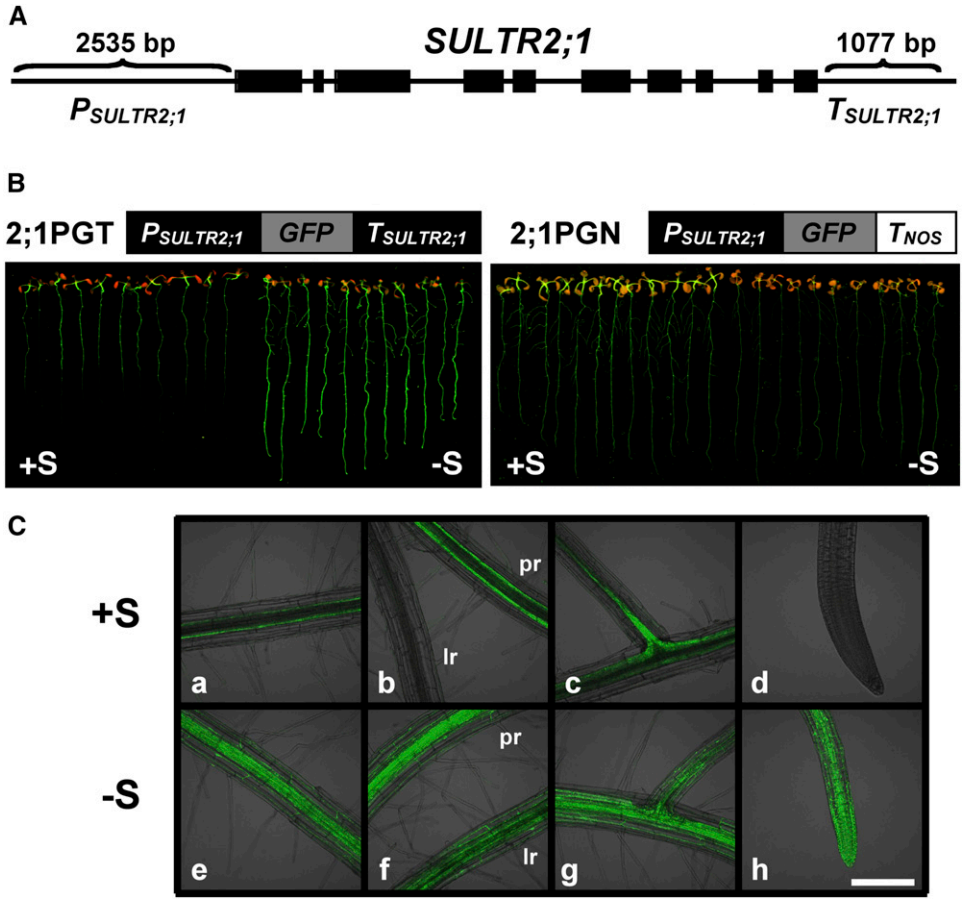

Figure 1. The –S-Inducible Expression of *SULTR2;1* in Roots Is Controlled by the 3′-Downstream Region.

(A) Genomic structure of *SULTR2;1*. Thick black bars represent exons. $P_{SULTR2;1}$ (2535-bp upstream sequence from the translational initiation codon of *SULTR2;1*) and $T_{SULTR2;1}$ (1077-bp downstream sequence from the translational termination codon of *SULTR2;1*) indicate the intergenic regions used for making *GFP* fusion constructs.

(B) GFP accumulation in $P_{SULTR2;1}$:*GFP*:$T_{SULTR2;1}$ (2;1PGT) and $P_{SULTR2;1}$:*GFP*:T_{NOS} (2;1PGN) plants grown under +S and –S conditions. Fusion gene constructs used for transformation of Arabidopsis are shown above the fluorescence images. T_{NOS} indicates the terminator sequence of nopaline synthase. 2;1PGT and 2;1PGN plants were grown for 10 d with 1500 μM (+S) or 15 μM of sulfate (–S). Fluorescence of GFP was visualized under an image analyzer as described in Methods.

(C) Tissue distribution of GFP fluorescence in 2;1PGT plant roots grown under +S or –S conditions. Fluorescence images in basal region of roots (a and e), primary roots and lateral roots (b and f), lateral root initiation zone (c and g), and root tip (d and h) of 2;1PGT plants grown under +S (a to d) and –S (e to h) conditions were visualized using laser confocal microscopy as described in Methods. pr, primary root; lr, lateral root. Bar = 200 μm.

introduced into Arabidopsis for monitoring Luc reporter activities in the transgenic plants (Figure 2). Twelve independent fragments of the 3′-region of *SULTR2;1* truncated at positions +1077, +663, +459, +449, +431, +411, +391, +372, +360, +352, +332, and +267 from the end of the *SULTR2;1* coding region were prepared and fused to the *Luc* gene placed downstream of $P_{SULTR2;1}$. These *Luc* fusion constructs were designated 2;1PLT1077, 2;1PLT663, 2;1PLT459, 2;1PLT449, 2;1PLT431, 2;1PLT411, 2;1PLT391, 2;1PLT372, 2;1PLT360, 2;1PLT352, 2;1PLT332, and 2;1PLT267, respectively. Luc activities in roots were determined using T2 progeny of the transgenic Arabidopsis plants grown under +S and –S conditions (Figure 2).

Plants expressing 2;1PLT1077, 2;1PLT663, and 2;1PLT459 showed significantly higher levels of Luc activities under –S compared with those on +S conditions. The Luc activity was 2.4- to 4.3-fold higher in –S than in +S (Figure 2A). However, the Luc activities were lower under –S relative to +S in other transgenic plants, 2;1PLT449, 2;1PLT431, 2;1PLT411, 2;1PLT391, 2;1PLT372, 2;1PLT360, 2;1PLT352, 2;1PLT332, and 2;1PLT267 (Figure 2A). These transgenic lines showing reduction in –S responses were further classified into two groups based on their –S/+S ratios of Luc activities. In one group, from 2;1PLT449 to 2;1PLT372, the –S/+S ratios of Luc activities were relatively high (from 0.12 to 0.48), whereas in the second group, from 2;1PLT360 to 2;1PLT267, the –S/+S ratios of Luc activity were low (from 0.009 to 0.022). These results indicated the necessity of the 12-bp region between +372 and +360 and the 10-bp region between +459 and +449 for either inducing Luc

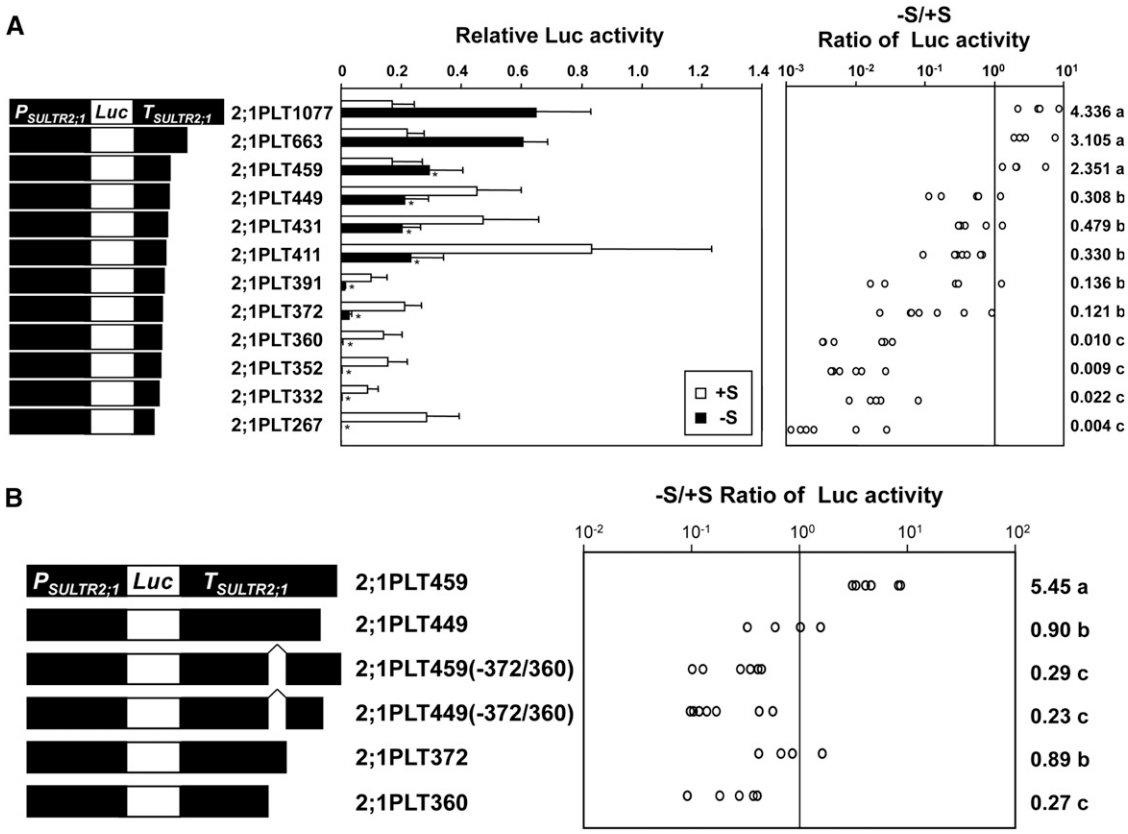

Figure 2. Deletion Analysis of *SULTR2;1* 3′-Downstream Region.

(A) Effects of the 3′-deletions of the *SULTR2;1* downstream region on Luc reporter activity under +S and –S conditions. Schematic representation of the 3′-deletions of the *SULTR2;1* downstream region fused to the *Luc* gene (left), relative Luc activities (middle), and the –S/+S ratios of Luc activities (right) are described. Error bars in the middle panel denote the SE of the mean. Asterisks indicate significant differences (Student's *t* test; $P < 0.05$) between 2;1PLT1077 and other lines. The –S/+S ratios of Luc activities in each independent transgenic line were calculated and are shown as open circles. The geometric means of the –S/+S ratios are shown on the right with lowercase letters representing the significant differences ($P < 0.05$).

(B) Deletion analysis of the region between +372 and +361 from the translational termination codon of *SULTR2;1*. The deletion constructs of the *SULTR2;1* downstream region (left) and the –S/+S ratios of Luc activities (right) are described as in **(A)**.

T_2 progenies of five to seven independent transgenic lines from each construct were grown for 10 d on agar medium containing 1500 μM (+S, open bars in **[A]**) or 15 μM (–S, closed bars in **[A]**) of sulfate. Luc activities of root tissues from 25 plantlets were assayed as described in the Methods.

reporter gene expression in roots in response to –S or repressing it on +S (Figure 2A). Removing the +372 to +360 region from 2;1PLT459 and 2;1PLT449 resulted in significantly lower –S/+S ratios of Luc activities in comparison to 2;1PLT372 and 2;1PLT360, which clearly indicated the importance of the nucleotide sequence from +372 to +360 in driving –S-responsive induction of gene expression in roots [Figure 2B; fusion constructs 2;1PLT459(-372/360) and 2;1PLT449(-372/360)]. In contrast, a moderate increment of Luc activity was observed in 2;1PLT449, 2;1PLT431, and 2;1PLT411 compared with 2;1PLT459 plants when they were grown on +S, indicating that the nucleotide sequence between +459 and +449 was involved in repression of *Luc* gene expression under +S and, thus, the absence of this region released the repression in the deletion lines (Figure 2A). Besides these two specific regions conferring significant +/–S responses, two additional regions from +460 to +663 and from +392 to +411 were found to affect *Luc* expression (Figure 2A). Deletion of a region from +460 to +663 reduced the Luc activity on –S; however, the –S/+S ratio of Luc activity was not altered to an extent that could be supported as statistically significant. Deletion of a region from +392 to +411 diminished the Luc activity on both +S and –S with a stronger reduction observed on –S; however, the resultant decrease in the –S/+S ratio of Luc activity was not statistically significant (Figure 2A).

To determine the core element within the sulfur-responsive region between +372 and +360 of the *SULTR2;1* 3′-downstream sequence, base substitution analysis was performed in transgenic Arabidopsis (Figure 3A). Six independent constructs, 2;1PLT372-bs1, 2;1PLT372-bs2, 2;1PLT372-bs3, 2;1PLT372-bs4, 2;1PLT372-bs5, and 2;1PLT372-bs6, representing successive 3-bp substitutions of nucleotide sequences from +372 to +360 in 2;1PLT372 were prepared and introduced into Arabidopsis. The T2 progeny of transgenic lines was grown for 10 d on +S and –S medium, and Luc activities in roots were

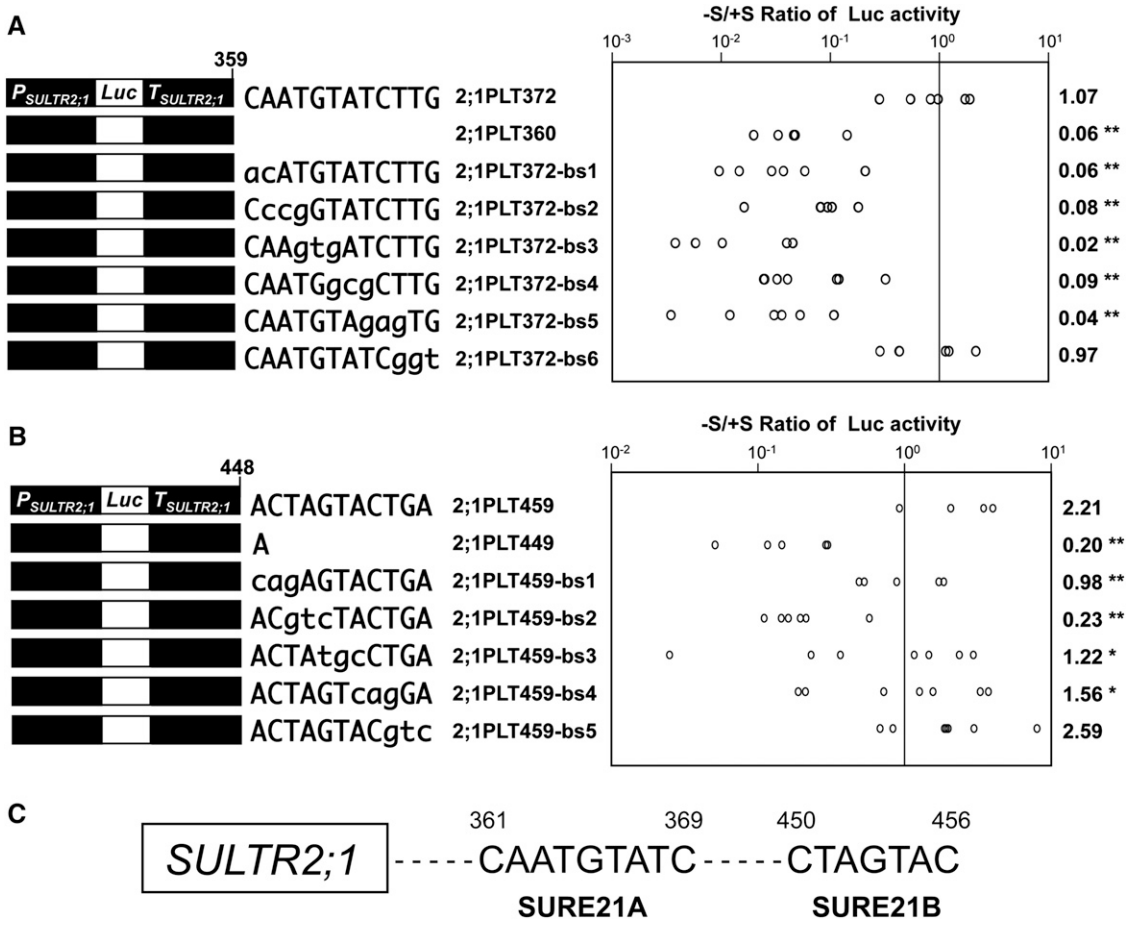

Figure 3. Base Substitution Analysis of the –S-Responsive Segments in the *SULTR2;1* 3′-Downstream Region.

(A) Base-substituted constructs from +372 to +361 of the *SULTR2;1* downstream region (left) and the –S/+S ratios of Luc activities (right).
(B) Base-substituted constructs from +449 to +459 of the *SULTR2;1* downstream region (left) and the –S/+S ratios of Luc activities (right).
In **(A)** and **(B)**, T2 progenies of five to seven independent transgenic lines from each construct were grown and analyzed as described in Figure 2. The –S/+S ratios of Luc activities in each independent transgenic line were calculated and are shown as open circles. The geometric means of the –S/+S ratios are shown on the right with asterisks representing the significant differences (*$0.05 \leq P < 0.1$ and **$P < 0.05$). Statistical analysis was performed using Student's *t* test compared with 2;1PLT372 **(A)** or 2;1PLT459 **(B)**.
(C) Sulfur-responsive elements determined in the 3′-downstream region of *SULTR2;1*. Two regions required for the –S-inducible expression of *SULTR2;1*, 5′-CAATGTATC-3′ between +361 and +369, and 5′-CTAGTAC-3′ between +450 and +456, were designated SURE21A and SURE21B, respectively.

determined in independent lines prepared for each construct (Figure 3A). Plants expressing 2;1PLT372-bs1, 2;1PLT372-bs2, 2;1PLT372-bs3, 2;1PLT372-bs4, and 2;1PLT372-bs5 showed –S/+S ratios of Luc activities from 0.02 to 0.09, comparable to the value in 2;1PLT360. In contrast, 2;1PLT372-bs6 plants showed an –S/+S ratio of 0.97, which was fairly consistent with the value in 2;1PLT372 (Figure 3A). These results indicated that the 9-bp sequence between +361 and +369, 5′-CAATGTATC-3′ (Figure 3A), was indispensable for driving –S-inducible expression of *SULTR2;1* in roots.

A similar base substitution analysis was performed with the +459 to +450 region as well (Figure 3B). Five independent constructs, 2;1PLT459-bs1, 2;1PLT459-bs2, 2;1PLT459-bs3, 2;1PLT459-bs4, and 2;1PLT459-bs5, representing 3-bp successive substitutions of nucleotide sequences from +449 to +459 region in the 2;1PLT459, were prepared and used for generating stable Arabidopsis transformants. The Luc activities in root tissues were determined in 10-d-old transgenic plants grown under +S and –S conditions (Figure 3B). Plants containing 2;1PLT459-bs5 showed almost the same level of induction of Luc activity as in 2;1PLT459. In contrast, other transgenic plants, 2;1PLT459-bs1, 2;1PLT459-bs2, 2;1PLT459-bs3, and 2;1PLT459-bs4, showed intermediate –S/+S ratios ranging from 0.23 to 1.56, but still lower than the values in 2;1PLT459 and 2;1PLT459-bs5. These results indicated that the 7-bp sequence between +450 and +456, 5′-CTAGTAC-3′ (Figure 3B), was necessary for the +S repression (Figure 2A) and consequently required for driving the –S-inducible gene expression of *SULTR2;1* in roots.

From these results, we designated the 9-bp sequence 5′-CAATGTATC-3′ between +361 and +369 as SURE21A and the 7-bp sequence 5′-CTAGTAC-3′ between +450 and +456 as SURE21B, representing the SURE of *SULTR2;1* (Figure 3C).

The SURE21A and SURE21B Enhancers Induce Gene Expression in Response to Sulfur Limitation

The 3′-untranslated region (UTR) controls mRNA stability and translational efficiency in plants (Gutiérrez et al., 1999; Fabian et al., 2010). The 3′UTR of *SULTR2;1* is reported to be 302 bp in length at the maximum length, according to the sequences deposited in the public database (TAIR; http://www.arabidopsis.org). Therefore, SURE21A and SURE21B are predicted to be located 60 to 150 bp downstream of the 3′UTR. To investigate whether the lack of sulfur nutrition alters the length of 3′UTR to incorporate SURE21A and SURE21B into the *SULTR2;1* transcripts, and whether the presence of these elements affects transcriptional termination in response to sulfur availabilities, the 3′-end nucleotide sequences of the *Luc*-$T_{SULTR2;1}$ fusion transcripts were determined in 2;1PLT1077 and 2;1PLT360 plants grown under +S and –S conditions. In all 20 independent clones tested in each condition, the *Luc*-$T_{SULTR2;1}$ transcripts carried a 241-bp *SULTR2;1* 3′UTR, which was identical to the native *SULTR2;1* transcripts found in wild-type plants (Supplemental Figure 1). These results showing the consistency in the length of the 3′UTR suggest that SURE21A and SURE21B are not likely to be associated with posttranscriptional mechanisms because these elements are located in the nontranscribed region and because they are neither incorporated into the 3′UTR nor do they alter the position of transcriptional termination in shoots and roots in response to sulfur nutrition.

To investigate the molecular mechanism of SURE21A and SURE21B controlling the –S-inducible transcript accumulation of *SULTR2;1* in roots, the effect of the *SULTR2;1* 3′-downstream intergenic region on *Luc* reporter gene expression was tested with combinations of several different promoters (Figure 4). The 2535-bp 5′-upstream region of *SULTR2;1* ($P_{SULTR2;1}$), the 2453-bp 5′-upstream region of *SULTR1;1* ($P_{SULTR1;1}$) lacking the sulfur-responsive region (Maruyama-Nakashita et al., 2005), and the cauliflower mosaic virus (CaMV) 35S promoter (P_{35S}) were used as promoters with the *SULTR2;1* 3′-downstream intergenic region ($T_{SULTR2;1}$). In addition to $P_{SULTR2;1}$:*Luc*:$T_{SULTR2;1}$ (2;1PLT, named 2;1PLT1077 in Figure 2) and $P_{SULTR1;1}$:*Luc*:T_{NOS} (1;1PLN; Maruyama-Nakashita et al., 2005) used in previous experiments, fusion gene constructs $P_{SULTR2;1}$:*Luc*:T_{NOS} (2;1PLN), $P_{SULTR1;1}$:*Luc*:$T_{SULTR2;1}$ (1;1PLT), P_{35S}:*Luc*:$T_{SULTR2;1}$ (35SLT), and P_{35S}:*Luc*:T_{NOS} (35SLN) were created and stably transformed into Arabidopsis plants by Agrobacterium infection. These transgenic plants were grown on +S and –S media for 10 d, and the Luc activities in root tissues were analyzed in independent lines prepared for each construct (Figure 4). In 2;1PLT plants, Luc activity was significantly higher (6.4-fold) under –S compared with +S. In contrast, in 2;1PLN plants, Luc activity was significantly lower (0.03-fold of +S) when grown under –S (Figure 4). These results were consistent with the observations when GFP was used as a reporter (Figure 1).

Analysis of 1;1PLT, 1;1PLN, 35SLT, and 35SLN plants revealed the ability of $T_{SULTR2;1}$ to induce Luc expression in response to –S (Figure 4). The Luc activity was 400-fold higher under –S compared with +S in 1;1PLT plants, whereas it significantly decreased under –S (0.19-fold of +S) in 1;1PLN plants (Figure 4). The *cis*-acting element for the –S-inducible expression of *SULTR1;1* is located between the 5′-upstream positions –2777 and –2762 of *SULTR1;1* (Maruyama-Nakashita et al., 2005). Since this element is absent from the 5′-region of *SULTR1;1* used in this experiment, the –S-responsive increase in the Luc activity observed in 1;1PLT plants is suggested to be dependent on the function of $T_{SULTR2;1}$. The positive effect of $T_{SULTR2;1}$ was also observed in a comparison between 35SLT and 35SLN plants. The 35SLT plants showed 84- and 1832-fold enhancement of Luc activities compared with the 35SLN plants under +S and –S conditions (Figure 4). The –S/+S ratios of Luc activities were 0.57 in 35SLT plants and 0.05 in 35SLN plants, indicating the presence of the –S-responsive region of $T_{SULTR2;1}$ gaining strong ability to increase the reporter gene expression driven by P_{35S} (Figure 4). These results suggest that the *SULTR2;1* 3′-region

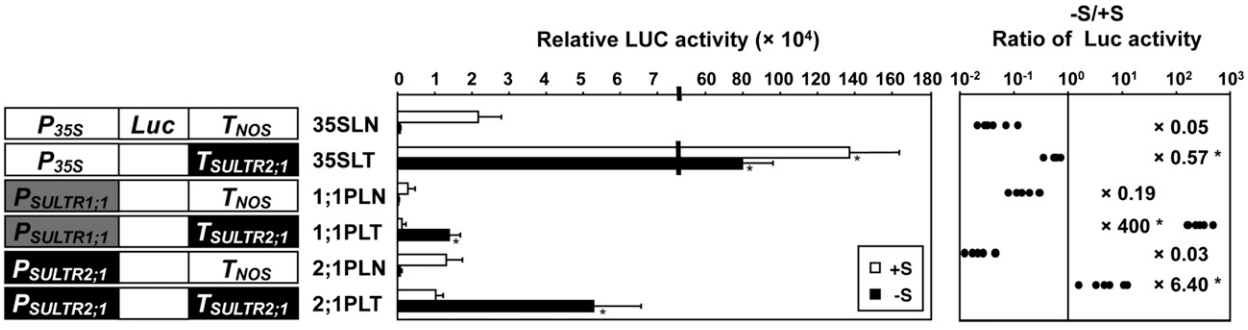

Figure 4. The *SULTR2;1* 3′-Region Controls –S-Inducible Expression Independent of the Promoter.

Schematic representation of the promoter-*Luc* constructs with or without *SULTR2;1* 3′-region (left), average values of relative Luc activities (middle), and the –S/+S ratios of Luc activities (right) are described. T2 progenies of five to six independent transgenic lines from each construct were grown and analyzed as described in Figure 2. Error bars in the middle panel denote SE. Asterisks in the middle panel indicate statistically significant differences ($P < 0.05$) between the plants harboring $T_{SULTR2;1}$ and T_{NOS} in the downstream of the same promoter. The –S/+S ratios of Luc activities in each independent transgenic line were calculated and are shown as closed circles. The geometric means of the –S/+S ratios are shown in the right graph with asterisks representing the significant differences ($P < 0.01$). P_{35S}, CaMV 35S promoter; $P_{SULTR1;1}$, 2453-bp upstream sequence from the translation initiation codon of *SULTR1;1*. Statistical analysis was performed using Student's *t* test.

($T_{SULTR2;1}$) positively controls reporter gene expression by acting as an enhancer to modulate the activity of the basic transcription machinery acting on the *SULTR2;1*, *SULTR1;1*, and CaMV 35S promoters.

We further tested whether the transposition of SURE21A and SURE21B to the vicinity of the promoter region and their placement in inverted orientations could still induce gene expression in response to –S (Figure 5). The –S-responsive 3′-region of *SULTR2;1* (from positions +332 to +459; *332-459*$T_{SULTR2;1}$) was relocated from the original position and placed in front of either the 2535-bp 5′-upstream region of *SULTR2;1* ($P_{SULTR2;1}$), the minimal promoter of *SULTR2;1* ($mP_{SULTR2;1}$), or a minimal 35S promoter (Benfey et al., 1989; $P_{35S\ minimal}$) in the sense or antisense orientation. These synthetic constructs were fused to the *Luc* reporter gene and introduced into Arabidopsis plants. The luciferase activities in roots were measured in 10-d-old transgenic plants grown under +S and –S (Figure 5). When sense and antisense *332-459*$T_{SULTR2;1}$ fragments were placed in front of $mP_{SULTR2;1}$ or $P_{35S\ minimal}$, the Luc activities of plants on –S were significantly higher than those on +S. In contrast, the Luc activities were not induced under –S when sense and antisense *332-459*$T_{SULTR2;1}$ were fused in front of the 2535-bp 5′-upstream region of *SULTR2;1* ($P_{SULTR2;1}$).

3′-Downstream Intergenic Region of *SULTR2;1* Induces Tissue-Specific Expression

With regard to tissue specificity, the 1077-bp 3′-downstream intergenic region of *SULTR2;1* ($T_{SULTR2;1}$) induced GFP accumulation under –S not only in vascular tissues but also in cortex

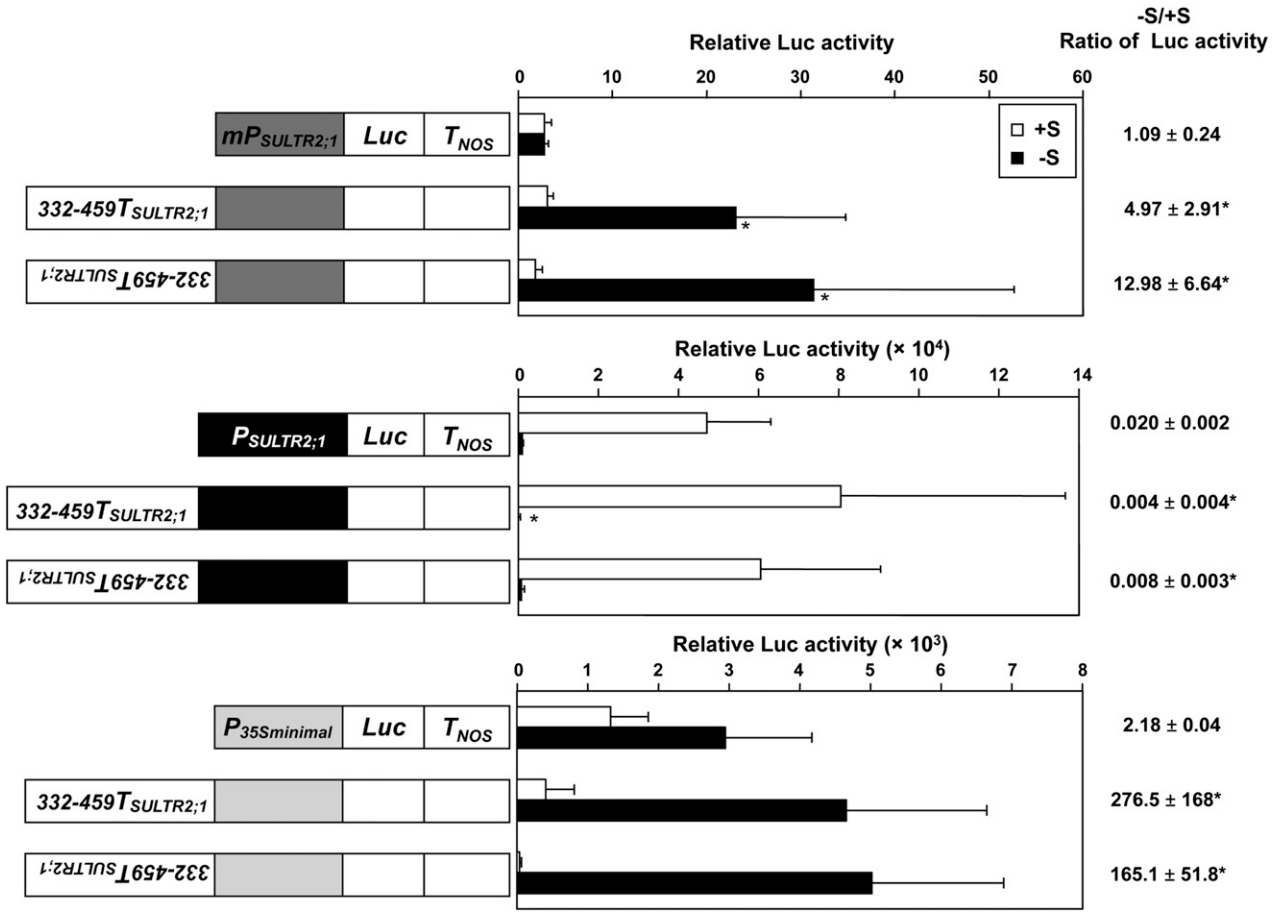

Figure 5. Transposition of *SULTR2;1* 3′-Region and the Complementary Sequence.

Schematic representation of the gene constructs of *SULTR2;1* 3′-region and the complementary sequence fused to the 5′-upstream region of *SULTR2;1* ($P_{SULTR2;1}$), minimal promoter of *SULTR2;1* ($mP_{SULTR2;1}$) or CaMV 35S minimal promoter ($P_{35S\ minimal}$) and *Luc* (left), relative Luc activities (middle), and the geometric means ± SE of the –S/+S ratios of Luc activities (right) are described. T2 progenies of five to seven independent transgenic lines from each construct were grown and analyzed. Error bars in the middle panel denote SE. Significant differences ($P < 0.05$) between the transgenic plants harboring the *SULTR2;1* 3′-region and the corresponding control are shown as asterisks. Statistical analysis was performed using Student's *t* test. $P_{SULTR2;1}$ and $mP_{SULTR2;1}$ represent the 2535- and 142-bp upstream sequences from the translational initiation codon of *SULTR2;1*. *332-459*$T_{SULTR2;1}$ indicates the 127-bp sequence between +332 and +459 downstream of the translational termination codon of *SULTR2;1*. The inverted description of *332-459*$T_{SULTR2;1}$ indicates the complementary sequence of *332-459*$T_{SULTR2;1}$.

and epidermis in 2;1PGT plants (Figure 1C). We further analyzed the effect of the $T_{SULTR2;1}$ on tissue-specific distribution of GFP expression in combination with other –S-noninducible promoters in transgenic Arabidopsis to demonstrate whether the elements in the 3′-region alone were able to influence gene expression in other root cell types while still retaining the –S inducibility (Figure 6). For this purpose, the *SULTR2;1* 3′-region ($T_{SULTR2;1}$) was fused to GFP reporter and placed downstream of either $P_{35S\ minimal}$ (Benfey et al., 1989) or 2453-bp 5′-upstream region of *SULTR1;1* promoter [$P_{SULTR1;1(2453)}$] to generate $P_{35S\ minimal}$:*GFP*:$T_{SULTR2;1}$ (35mPGT) and $P_{SULTR1;1(2453)}$:*GFP*:$T_{SULTR2;1}$ (1;1P2453GT). These fusion gene constructs were introduced into Arabidopsis plants by Agrobacterium infection, and independent transgenic lines were obtained for microscopic analysis. 2;1PGT (Figure 1) and $P_{SULTR1;1(3995)}$:*GFP*:T_{NOS} (1;1P3995GN; Maruyama-Nakashita et al., 2004a) were used as positive control lines to monitor the –S-inducible expression in roots. 35mPGT, 1;1P2453GT, and the control lines were grown on +S and –S media for 9 d, and the GFP in root tissues was observed by fluorescence microscopy (Figure 6).

In 2;1PGT plants, GFP accumulation was more significant in the basal region than in apical region of roots in both +S and –S conditions (Figure 6). The –S-induced GFP accumulation in 2;1PGT roots was mainly observed in vascular tissues, but minor fluorescence was also detected in the cortex and epidermis (Figures 1C and 6A). In 35mPGT plants, GFP fluorescence was not detected under +S conditions. However, under –S conditions, 35mPGT plants showed strong GFP accumulation in roots, and GFP levels were much higher in the basal region than in apical region of roots (Figure 6B). GFP fluorescence was mainly distributed in the cortex and epidermis including root hairs, and minor fluorescence was detected in vascular tissues (Figures 6B and 6F).

SULTR1;1 is a sulfate transporter that facilitates sulfate uptake in roots, and it is localized in root epidermis including root hairs (Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2007). The –S-response of *SULTR1;1* is dependent on the sulfur-responsive *cis*-acting element (SURE11) located at positions –2777 to –2762 of the 5′-upstream region of *SULTR1;1* (Maruyama-Nakashita et al., 2005). In 1;1P3995GN plants carrying the transgene construct with SURE11, GFP fluorescence was greatly induced under –S conditions mainly in the epidermal cells of the apical zone with a high density of root hairs observed near the root tip (Figures 6C and 6G; Maruyama-Nakashita et al., 2004a). In contrast, in 1;1P2453GT plants carrying the construct missing SURE11 from the promoter region but

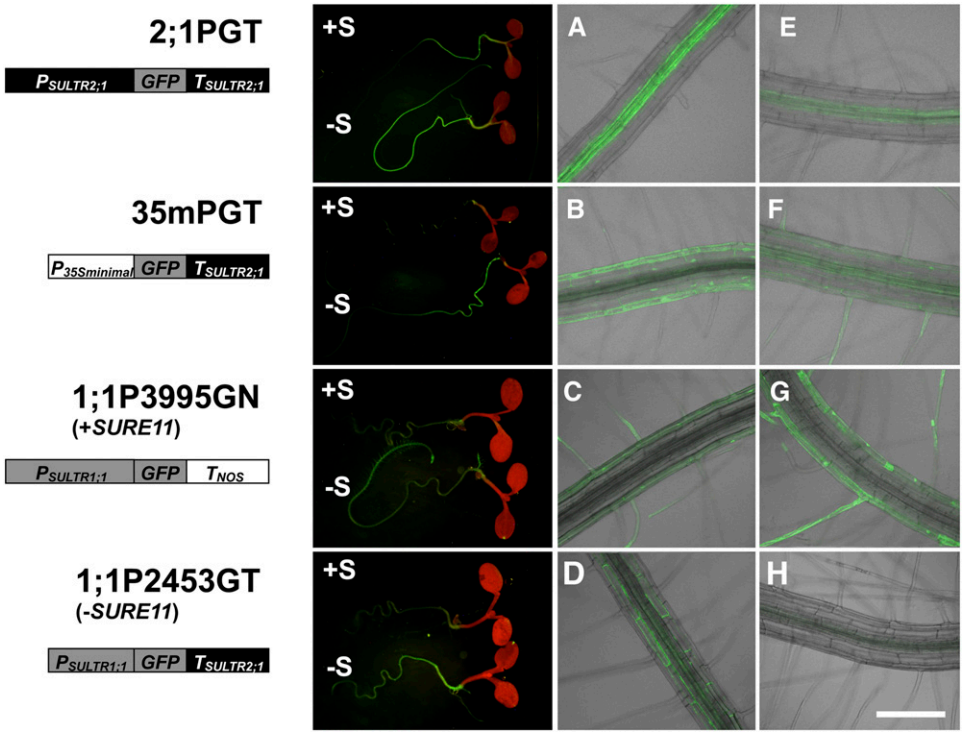

Figure 6. Tissue Specificity of –S-Inducible Expression Driven by the *SULTR2;1* 3′-Region.

Schematic representation of the promoter-*GFP* constructs with or without the *SULTR2;1* 3′-region (left), GFP accumulation in transgenic lines grown under +S or –S condition (middle), and the close-up images of basal (**[A]** to **[D]**) and apical (**[E]** to **[H]**) regions of root tissues grown under –S condition (right) are shown. T2 transformants carrying each construct were grown for 10 d on +S and –S medium. $P_{35S\ minimal}$, CaMV 35S minimal promoter; $P_{SULTR1;1}$, 5′-upstream sequence from the translation initiation codon of *SULTR1;1*. The –S-responsive element, SURE11, is present in the 5′-upstream sequence of *SULTR1;1* in 1;1P3995GN and absent from 1;1P2453GT, as indicated in the diagram. Bar = 200 μm.

containing SURE21A and SURE21B in the 3′-region, GFP was predominantly localized in the basal part of roots under –S, mainly in the cortex (Figure 6D) with slightly moderate but significant levels of signals observed in vascular tissues (Figures 6D and 6H), suggesting that –S-induced GFP accumulations in these cell types and tissues were due to the presence of $T_{SULTR2;1}$.

These results indicated that $T_{SULTR2;1}$ was able to induce gene expression preferentially in the basal region of roots. Although the precise distributions of GFP fluorescence through the radial axis of roots were not identical among the lines made with different fusion constructs, the induction of GFP accumulation was dependent on $T_{SULTR2;1}$ and was observed in the basal regions of roots in all cases tested in this study (Figures 6A, 6B, and 6D). Therefore, it is reasonable to assume that $T_{SULTR2;1}$ is able to induce gene expression in response to –S regardless of the promoter sequences of *SULTR2;1* and *SULTR1;1* determining tissue specificities (Figures 4 and 6). Furthermore, results obtained with the 35mPGT plants suggest that $T_{SULTR2;1}$ has enhancer activity to induce gene expression in response to –S specifically in roots.

Induction of SULTR2;1 in Roots Contributes to Both Sulfate Uptake and Internal Translocation

To elucidate the physiological meaning of –S-inducible *SULTR2;1* expression in roots, Arabidopsis mutant lines containing T-DNA insertions between the end of *SULTR2;1* 3′UTR and SURE21A or SURE21B in the 3′-nontranscribed intergenic region were searched in the database, and SAIL_363_C06 in Col-0 background and FLAG_373B04 in Wassilewskija (Ws) background (Samson et al., 2002; Sessions et al., 2002; T-DNA Express, http://signal.salk.edu/cgi-bin/tdnaexpress) were identified as candidates. These T-DNA insertion lines were selected as homozygous lines by PCR-based screening, and the T-DNA insertions of 306 and 415 bp downstream of *SULTR2;1* in SAIL_363_C06 and FLAG_373B04, respectively, were confirmed by sequencing. These lines were named tKO (Figure 7A) as they contained T-DNA insertions in the 3′-nontranscribed region.

The tKO and the wild-type plants (Col-0 and Ws) were grown under +S and –S conditions, and the *SULTR2;1* transcript levels were analyzed by quantitative RT-PCR (Figures 7B and 7C). In wild-type plants, the transcript levels of *SULTR2;1* were higher in roots grown on –S compared with those on +S, while they were reversed in shoots, i.e., lower on –S compared with +S, as reported previously (Takahashi et al., 2000; Kataoka et al., 2004b). In roots grown under +S conditions, the transcript levels of *SULTR2;1* were not significantly different between the wild type and tKO. However, in roots grown under –S conditions, the *SULTR2;1* transcript accumulation was observed only in the wild type but not in the tKO (Figure 7C). Furthermore, the transcript levels of *SULTR2;1* in the tKO roots were significantly lower on –S compared with those on +S. These data demonstrated the essential roles of SURE21A and SURE21B in the induction and maintenance of *SULTR2;1* expression in roots grown under –S conditions.

In shoots, by contrast, *SULTR2;1* was partially suppressed in the tKO lines under both +S and –S conditions. The –S-responsive repression of *SULTR2;1* transcript was reproducible in the tKO lines as those in the wild-type plants, although the transcript levels of *SULTR2;1* were consistently lower in tKO than in the wild-type plants under both +S and –S conditions (Figure 7C). Our results further suggested that *SULTR2;1* repression in shoots was more significant in tKO, as indicated by the +S/–S ratios of *SULTR2;1* transcript levels exhibiting an increase in the magnitude of repression from 2.46 in Col-0 to 5.23 in Col-tKO or from 2.07 in Ws to 4.58 in Ws-tKO. To investigate whether the T-DNA insertions in tKOs have any significant effects on the *SULTR2;1* transcript terminations as those were confirmed in 2;1PLT1077 and 2;1PLT360, the 3′-end nucleotide sequences of the *SULTR2;1* transcripts were determined in tKO and wild-type plants grown under +S and –S conditions. The length of 3′UTR was 241 bp, which was mostly consistent and conserved in shoots and roots of the wild type and tKOs under both +S and –S growth conditions, with a few exceptions showing a length of 254 bp in roots of –S-grown tKOs (Supplemental Figure 1).

We further analyzed sulfate uptake and translocation in tKO plants to assess the contribution of the –S-inducible expression of *SULTR2;1* in roots (Figure 8). Ten-day-old plants grown under +S and –S conditions were incubated for 1 h with +S medium containing the [^{35}S] sulfate. Sulfate uptake activity was calculated by dividing the total [^{35}S] radioisotope accumulated in the seedlings with the fresh weight of the root (Figure 8A). The sulfate translocation activity was calculated as a shoot-to-root ratio of distribution of [^{35}S] sulfate in the seedlings (Figure 8B). Under +S conditions, both sulfate uptake and translocation activity did not differ between wild-type and tKO plants (Figure 8). These results indicated that the decrease in *SULTR2;1* transcripts in the foliar part of tKO did not modulate the sulfate uptake activity and sulfate distribution in 10-d-old plants. In contrast, sulfate uptake and translocation activity were significantly lower in tKO than in the wild-type plants grown under –S conditions (Figure 8). These results provided evidence that induction of *SULTR2;1* expression in roots through the function of the 3′-nontranscribed region was important for controlling both the sulfate uptake and translocation under –S conditions.

DISCUSSION

The 3′-Nontranscribed Intergenic Region Is Responsible for the Induction of *SULTR2;1* Gene Expression in Arabidopsis Roots under Sulfur-Limited Conditions

An increase of *SULTR2;1* transcript levels in Arabidopsis roots is a typical –S response (Takahashi et al., 2000; Hirai et al., 2003; Maruyama-Nakashita et al., 2003, 2005; Nikiforova et al., 2003; Iyer-Pascuzzi et al., 2011). However, unlike the other –S-inducible isoforms of the *SULTR* family members, *SULTR2;1* does not require the function of SLIM1 transcription factor to induce its expression in roots under S-limited conditions (Maruyama-Nakashita et al., 2006). The APS reductase gene family is also induced by –S in a SLIM1-independent manner; however, the molecular mechanisms underlying their –S-responsive expression have not been elucidated (Maruyama-Nakashita et al., 2006; Davidian and Kopriva, 2010). In this study, we analyzed the regulation of *SULTR2;1* with a particular focus on the

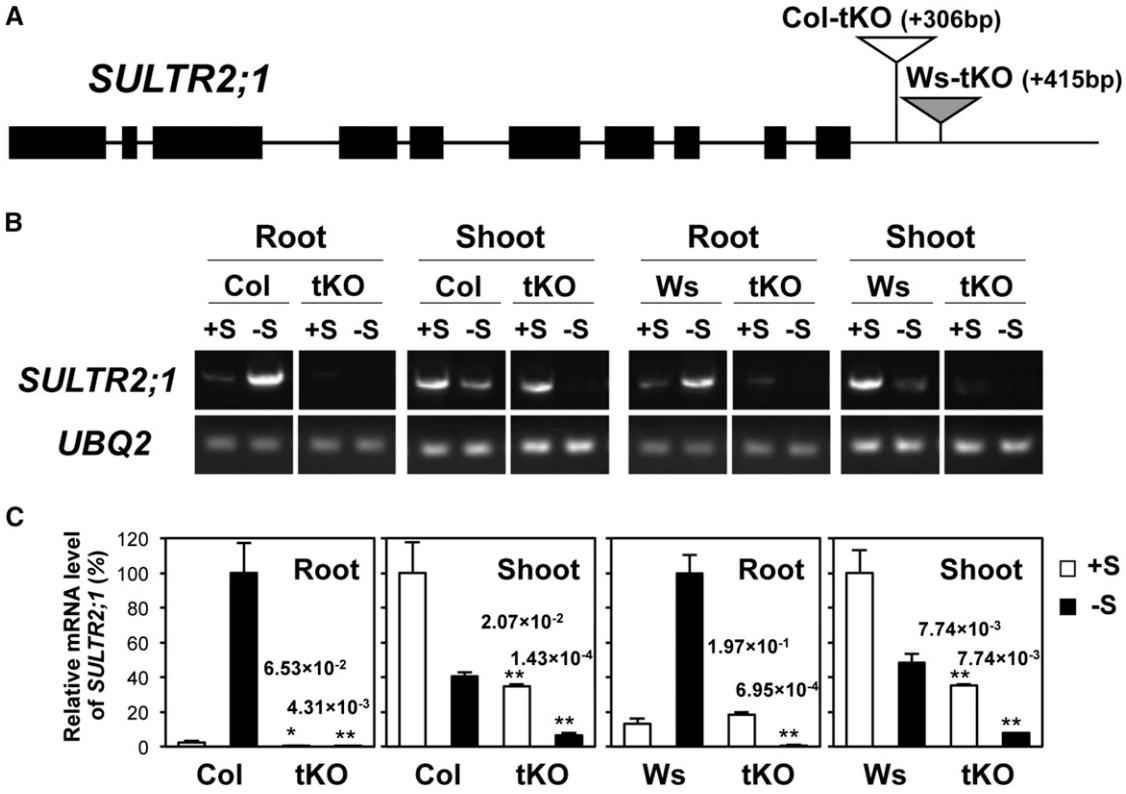

Figure 7. T-DNA Insertion between Stop Codon and SURE21A/SURE21B Disrupts –S-Inducible Expression of *SULTR2;1* in Roots.

(A) Structure of *SULTR2;1* genomic region and T-DNA insertion sites (triangles) in the knockout (tKO) mutants. Thick black bars represent exons. In the tKO mutants in Col-0 and Ws backgrounds, T-DNAs are inserted in positions +306 and +415 bp from the translational termination codon of *SULTR2;1*, as indicated by white and gray triangles, respectively.

(B) Gel images of real-time RT-PCR products showing *SULTR2;1* and *UBQ2* (*UBIQUITIN2*) mRNA accumulations. The wild type (Col-0 and Ws) and tKO mutants were grown for 10 d on +S and –S media.

(C) Relative transcript levels of *SULTR2;1* quantified by real-time RT-PCR. *UBQ2* transcript levels were used for normalization. *SULTR2;1* transcript levels in wild-type roots under –S and wild-type shoots under +S are shown as 100%. Error bars denote SE ($n = 3$). P values of the Student's *t* tests of the comparisons between the wild-type and tKO plants on either +S or –S conditions are indicated above the tKO data with asterisks (*$0.05 \leq P < 0.1$ and **$P < 0.05$).

–S induction of its transcript accumulation in Arabidopsis roots and identified the sulfur-responsive *cis*-acting elements, SURE21A and SURE21B, located at the positions +361/+369 and +450/+456, respectively, in the 3′-nontranscribed intergenic region (Figures 1 to 3).

The distinct function of both these elements in the –S-inducible expression of *SULTR2;1* is suggested by the deletion study (Figure 2). Deletion of +450/+459 region of *SULTR2;1* significantly enhances the Luc activity under +S, indicating that SURE21B, 5′-CTAGTAC-3′, contributes to the repression of *SULTR2;1* expression under +S. In contrast, deletion of +361/+372 region of *SULTR2;1* significantly diminishes the Luc activity under –S, indicating that SURE21A, 5′-CAATGTATC-3′, contributes to the induction of *SULTR2;1* gene expression under –S. These results suggest that the –S-dependent upregulation of *SULTR2;1* occurs due to a combinatorial effect of two elements operating in favor of releasing the repression and stimulating the induction of gene expression, allowing a strict control of *SULTR2;1* expression possibly under two different signals derived from changes in the sulfur status. In addition to these two core elements, +460/+663 and +392/+411 regions are suggested to be partly required for maintenance of *SULTR2;1* transcript levels although their roles in controlling the +/–S responses appear subsidiary (Figure 2).

Several lines of evidence indicate that *SULTR2;1* transcripts accumulate in roots in response to –S due to a transcriptional mechanism, despite the presence of two *cis*-acting elements, SURE21A and SURE21B, identified in the 3′-downstream intergenic region. Because of this unusual location of *cis*-acting elements, posttranscriptional regulation of mRNA stability associated with the nucleotide sequences of 3′UTR was suspected as a mechanism underlying *SULTR2;1* transcript modulation. However, since SURE21A and SURE21B are located downstream of the 3′UTR, and since the length of the 3′UTRs of *SULTR2;1* mRNA in tKO or *Luc*-fusion mRNA in reporter lines were unchanged regardless of sulfur conditions or of the presence of these –S-responsive elements (Supplemental Figure 1), a posttranscriptional regulation of the *SULTR2;1* mRNA stability through the function of 3′UTR seems an unlikely

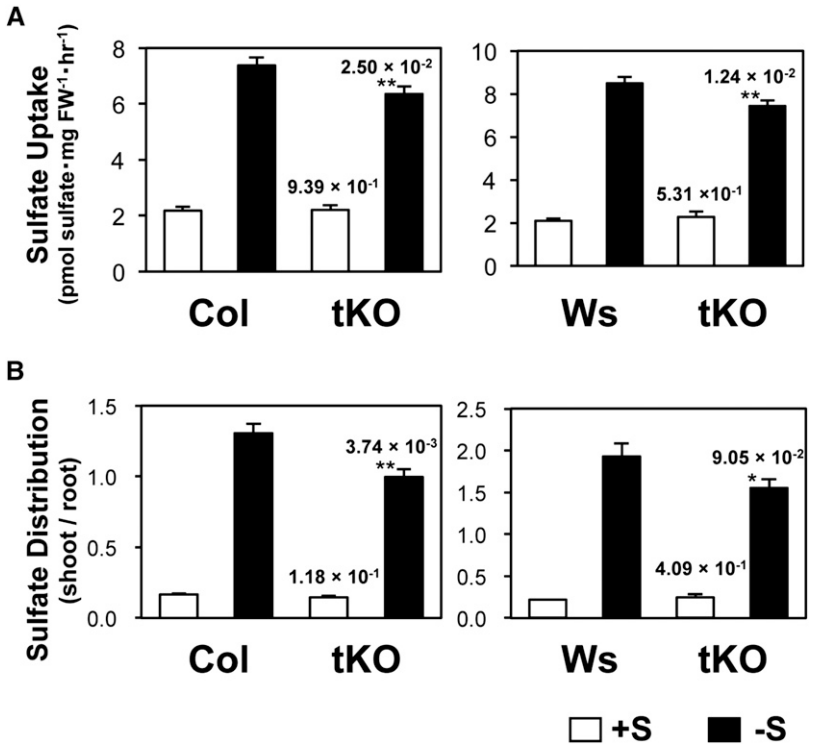

Figure 8. Sulfate Uptake Activity and Sulfate Distribution in Wild-Type and tKO Plants Grown under +S and –S Conditions.

(A) Sulfate uptake activity of the wild type and tKO mutant. Ten-day-old plants grown under +S and –S conditions were used for the analysis. The absolute values of [^{35}S] sulfate uptake rates are indicated.

(B) Sulfate distribution in the wild type and tKO mutant shown as shoot/root ratios of [^{35}S] sulfate accumulations. Error bars denote SE (n = 8). P values of the Student's t tests of the comparisons between the wild-type and tKO plants on either +S or –S conditions are indicated above the tKO data with asterisks (*$0.05 \leq P < 0.1$ and **$P < 0.05$).

scenario. Our results rather strongly support the involvement of SURE21A and SURE21B as enhancers involved in transcriptional mechanisms. SURE21A and SURE21B effectively enhanced reporter gene expression under –S when they were relocated to the vicinity of promoter sequence in the 5′-upstream region (Figure 5). In addition, they enhanced reporter gene expression in combination with several different promoters lacking the ability to respond to –S (Figures 4 and 6). The SURE21A/SURE21B-dependent activation of the reporter activity is detected under –S even in combination with the 35S minimal promoter (Figures 5 and 6). These results indicate that SURE21A and SURE21B function sufficiently as *cis*-acting elements that may act on the basic transcription machinery, possibly by direct or indirect interactions with the preinitiation complex of general transcription factors. Nevertheless their placement at a distance from the reporter gene toward the 5′ direction appears to limit their proper function. The T-DNA insertions between the *SULTR2;1* coding sequence and SURE21 block –S induction of *SULTR2;1* in roots (Figure 7), suggesting that distances toward the 3′-direction also affect their function as enhancers.

Several *cis*-acting elements or regions responsive to –S, such as SURE11, UPE-box, –S-responsive region of *NIT3*, and β-subunit gene promoter of β-conglycinin, have been previously reported (Awazuhara et al., 2002; Kutz et al., 2002; Maruyama-Nakashita et al., 2005; Davidian and Kopriva, 2010; Wawrzyńska et al., 2010). Neither SURE21A nor SURE21B shows any sequence similarity to these –S-responsive sequences. We further searched for SURE21A and SURE21B in the 500-bp 5′-upstream and 3′-downstream sequences of the 469 –S-regulated genes previously selected from microarray analysis (Table 1; Maruyama-Nakashita et al., 2006). SURE21A (5′-CAATGTATC-3′) and SURE21B (5′-CTAGTAC-3′) or their complementary sequences were found in 11 and 25 genes, respectively (Table 1). Among them, four of the –S-upregulated genes, *SULTR2;1* (AT5G10180), γ-glutamyl cyclotransferase 2;1 (*GGCT2;1*; AT5G26220; Paulose et al., 2013), *LSU1* (AT3G49580; Wawrzyńska et al., 2010; Lewandowska et al., 2010), and nuclear transport factor 2 (*NTF2*) family protein (AT3G09250), contained both SURE21A and SURE21B. In contrast, none of the –S-downregulated genes contained sequences that matched with both the *cis*-acting elements. *GGCT2;1* and *LSU1* carried SURE21A or its complementary sequence in their upstream region and the complementary sequence of SURE21B in their downstream region. Other than *SULTR2;1*, only the *NTF2* family protein carried both sequences in their downstream region, but they were found on the complementary strand. These results indicate that SURE21A and SURE21B may be present as *cis*-acting elements in –S-upregulated genes, but their combinations and presence in the 3′-region may be specific to *SULTR2;1* and *NTF2*.

Induction of *SULTR2;1* Expression in Roots Contributes to –S-Induced Root-to-Shoot Transport and Uptake of Sulfate

SULTR2;1 is considered to be responsible for retrieval of apoplasmic sulfate in root vasculature (Takahashi et al., 1997, 2000; Kataoka et al., 2004b). It is suggested that efficient retrieval of sulfate into xylem parenchyma cells would help the loading of sulfate to the xylem stream under –S. A recent study using a *sultr2;1*-null knockout line suggests that the disruption of *SULTR2;1* diminishes root-to-shoot transport of sulfate in plants grown under +S but not under –S (Kawashima et al., 2011). In contrast, a decrease in the root-to-shoot translocation of sulfate specifically under –S conditions was observed in the mutants containing T-DNA insertions in the 3′-nontranscribed region of *SULTR2;1* (tKO) in this study (Figure 8). Since the major impact of the T-DNA insertion mutations on *SULTR2;1* expression in tKO lines is observed in roots under –S conditions (Figure 7), the phenotype of root-to-shoot sulfate transport is suggested to be relevant to the function of SULTR2;1 in roots. The results presented in this study therefore provide a strong evidence for the root-specific function of SULTR2;1, increasing the root-to-shoot transport of sulfate under –S. Our results support the previously suggested roles of SULTR2;1 in Arabidopsis roots (Takahashi et al., 2000; Kataoka et al., 2004b) and explain the relevance of its –S-responsive induction of gene expression led by a transcriptional control mechanism requiring the *cis*-acting elements, SURE21A and SURE21B.

Table 1. SURE21A (CAATGTATC), SURE21B (CTAGTAC), and the Complementary Sequences, GATACATTG and GTACTAG, in the Upstream or Downstream Regions of –S-Responsive Genes

−S Response	Sequence	Affymetrix ID	AGI Code	Gene Name	Position
Upregulated	**SURE21A**	**250475_at**	**AT5G10180**	**Sulfate transporter, SULTR2;1**	**+361**
	5′-CAATGTATC-3′	254113_at	AT4G24900	Titan-like, TTL (nuclear C2H2 domain-containing protein)	+322
		258151_at	AT3G18080	B-S Glucosidase 44, BGLU44	+4
		246884_at	**AT5G26220**	**γ-Glutamyl cyclotransferase 2;1 (GGCT2;1)**	**-305**
	5′-GATACATTG-3′	**259039_at**	**AT3G09250**	**Nuclear transport factor 2 (NTF2) family protein**	**+72**
		252269_at	**AT3G49580**	**Response to low sulfur 1, LSU1**	**-131**
		249752_at	AT5G24660	Response to low sulfur 2, LSU2	−159
	SURE21B	266709_at	AT2G03120	Signal peptide peptidase family protein, ATSPP	+476
	5′-CTAGTAC-3′	**250475_at**	**AT5G10180**	**Sulfate transporter, SULTR2;1**	**+450**
		251928_at	AT3G53980	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	+263
		260064_at	AT1G73730	Sulfur limitation 1/EIL3	+149
	5′-GTACTAG-3′	**246884_at**	**AT5G26220**	**γ-Glutamyl cyclotransferase 2;1 (GGCT2;1)**	**+221**
		252269_at	**AT3G49580**	**Response to low sulfur 1, LSU1**	**+190**
		260196_at	AT1G67570	Protein of unknown function (DUF3537)	+188
		259039_at	**AT3G09250**	**Nuclear transport factor 2 (NTF2) family protein**	**+184**
		263297_at	AT2G15310	ADP-ribosylation factor B1A, ATARFB1A	+65
		259476_at	AT1G19000	Homeodomain-like superfamily protein	+61
Downregulated	**SURE21A**	255773_at	AT1G18590	Sulfotransferase, SOT17	+476
	5′-CAATGTATC-3′	252612_at	AT3G45160	Putative membrane lipoprotein	+433
		260902_at	AT1G21440	Phosphoenolpyruvate carboxylase family protein	+38
	5′-GATACATTG-3′	263477_at	AT2G31790	UDP-glycosyltransferase superfamily protein	+204
	SURE21B	260745_at	AT1G78370	Glutathione *S*-transferase, ATGSTU20	+240
	5′-CTAGTAC-3′	255711_at	AT4G00090	Transducin/WD40 repeat-like superfamily protein	+185
		250226_at	AT5G13780	Acyl-CoA N-acyltransferase (NAT) superfamily protein	+52
		261536_at	AT1G01790	K^+ efflux antiporter, KEA1	−85
		262774_at	AT1G13230	Leucine-rich repeat protein pii-2	−402
		247193_at	AT5G65380	MATE efflux family protein	−488
	5′-GTACTAG-3′	250226_at	AT5G13780	Acyl-CoA N-acyltransferase (NAT) superfamily protein	+362
		260557_at	AT2G43610	Glycoside hydrolase family 19, similar to chitinase	+362
		250032_at	AT5G18170	Glutamate dehydrogenase 1, GDH1	+135
		250438_at	AT5G10580	Protein of unknown function	+111
		259264_at	AT3G01260	Aldose 1-epimerase family protein	+105
		258977_s_at	AT3G02020	Aspartate kinase 3, AK3	+31
		262064_at	AT1G56070	Low expression of osmotically Responsive genes 1, LOS1	+15
		249025_at	AT5G44720	Molybdenum cofactor sulfurase family protein	−347
		259813_at	AT1G49860	Glutathione *S*-transferase 14, ATGSTF14	−362

Positions of SURE21A and SURE21B between the 1- to 500-bp upstream or downstream regions of −S-responsive genes are indicated. Genes having both SURE21A and SURE21B are shown in bold.

Results shown in this study further indicate the partial contribution of SULTR2;1 to the uptake of sulfate in roots (Figure 8). Previous studies indicate that the –S-inducible sulfate uptake activity is mainly attributed to the function of the two sulfate transporter genes, *SULTR1;1* and *SULTR1;2*, in Arabidopsis (Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Maruyama-Nakashita et al., 2003; Yoshimoto et al., 2002, 2007). Gene expression studies using promoter-reporter constructs further indicate a strong regulation of both *SULTR1;1* and *SULTR1;2* dependent on their –S-responsive promoters (Maruyama-Nakashita et al., 2004a, 2004b, 2005). Thus, SULTR1;1 and SULTR1;2 are considered as major components of the –S-inducible sulfate uptake activity in Arabidopsis roots. However, the double T-DNA knockout plant of *SULTR1;1* and *SULTR1;2* still can increase sulfate uptake activity in response to –S (Yoshimoto et al., 2007), suggesting that another transport system in roots induced by –S remains unidentified. Reduction of sulfate uptake activity shown by T-DNA insertions disrupting SURE21A and SURE21B functions suggests that SULTR2;1 is a potential candidate for this alternative –S-inducible component (Figure 8). The localization of *SULTR2;1* gene expression in cortex cells (Figures 1 and 6) in addition to xylem parenchyma and pericycle cells (Takahashi et al., 1997, 2000) also supports the functionality of SULTR2;1 mediating sulfate uptake in roots.

The *SULTR2;1* 3′-Nontranscribed Region Is Also Effective for the Maintenance of Basal Levels of Gene Expression

Besides controlling –S responses, the 3′-nontranscribed region of *SULTR2;1* also seems to be responsible for maintaining the basal levels of gene expression in both roots and shoots (Figures 2, 4, and 7). Luc activities of 2;1PLT391 roots were 17- and 40-fold lower than those of 2;1PLT411 roots under +S and –S, respectively (Figure 2). This suggests that the nucleotide sequence between +392 and +411, 5′-TATTTGGTGAATCAAATTAG-3′, contains information required for the maintenance of *SULTR2;1* basal expression levels in roots independent of the functions of SURE21A and SURE21B in controlling the –S responses. The Luc activity driven by CaMV 35S promoter with *SULTR2;1* 3′-region (35SLT) is 26-fold higher than with NOS terminator (35SLN) even under +S (Figure 4). In contrast, the Luc activity driven by *SULTR1;1* promoter lacking SURE11 but with *SULTR2;1* 3′-region (1;1PLT) is 2.7-fold lower than with the NOS terminator (1;1PLN) under +S (Figure 4). These results indicate that *SULTR2;1* 3′-region may enhance expression levels of genes located nearby, although the effect seems restricted by the promoter sequences (Figures 2 and 4).

The effect of the *SULTR2;1* 3′-region on basal gene expression is also suggested by comparisons between tKOs and wild types in both Col-0 and Ws backgrounds. *SULTR2;1* transcript levels in shoots were 3- and 6-fold lower in tKOs than in wild types under +S and –S, respectively (Figure 7). On the other hand, *SULTR2;1* transcript levels in roots did not significantly differ between tKO and wild-type plants under +S. These results indicate that T-DNA insertions near SURE21A and SURE21B in the *SULTR2;1* 3′-region may have additional negative impacts on *SULTR2;1* expression in shoots under both +S and –S conditions. Recent studies using the *sultr2;1* null-KO plants suggest that SULTR2;1 contributes to the translocation of sulfate or sulfur-containing metabolites from old to young leaves possibly through phloem (Liang et al., 2010; Kawashima et al., 2011). Partial suppression of *SULTR2;1* expression due to T-DNA insertions in the 3′-region may also affect the distribution of sulfur in the aerial tissues of tKO. How this gene expression mechanism driven by the *SULTR2;1* 3′-region can be controlled in parallel with miR395 regulating the *SULTR2;1* transcript levels in shoots remains to be investigated.

Prediction of SURE21A and SURE21B Binding Proteins

Unraveling the molecular machinery of –S-inducible gene expression of *SULTR2;1* will likely involve the identification of *trans*-acting factors binding to SURE21A and SURE21B. The *trans*-factors expected to be involved in this mechanism would be repressed or activated under –S by binding to or dissociating from the *cis*-elements, thereby influencing the basic machinery of transcription, and ultimately would enhance expression of *SULTR2;1*. SURE21 consists of two elements, SURE21A and SURE21B, which are located 80 bp apart, suggesting that the two kinds of DNA binding proteins may bind to each element and cooperatively stimulate *SULTR2;1* transcription under –S.

Database searches on PLACE (Higo et al., 1999) revealed sequence similarity between SURE21A and SURE21B and several *cis*-acting elements reported in plants. SURE21A, 5′-CAATGTATC-3′, includes the CAAT and GATA motifs. CAAT has been reported as a tissue-specific sequence of a pea (*Pisum sativum*) legumin gene that is functional in tobacco (*Nicotiana tabacum*; Shirsat et al., 1989); however, its binding protein has not been found yet. The plant GATA motif was first identified in the light-responsive *Cab* promoters in tobacco (Lam and Chua, 1989). Subsequently, it was also found in the promoter of spinach (*Spinacia oleracea*) nitrite reductase gene, whose expression is controlled by the availability of ammonium (Rastogi et al., 1997). The *Arabidopsis* genome has 29 GATA family transcription factors, classified into four subfamilies that are characterized by a single zinc finger domain with 18 or 20 residues in the zinc finger loop (Reyes et al., 2004). Several GATA transcription factors are reported to have biological functions in plant growth and development, including cell elongation, cell division, chloroplast development through chlorophyll synthesis, and gibberellin signaling (Shikata et al., 2004; Bi et al., 2005; Richter et al., 2010; Hudson et al., 2011; Chiang et al., 2012). SURE21B, 5′-CTAGTAC-3′, includes YATC and GTAC motifs. YATC has been reported as a *cis*-regulatory element for mesophyll-specific expression of phosphoenolpyruvate carboxylase in the C4 plant *Flaveria trinevia* (Gowik et al., 2004). However, its binding protein has not been reported. Arabidopsis also has 16 members of the SQUAMOSA-promoter binding protein-like (SPL) family of GTAC binding transcription factors (Cardon et al., 1999; Guo et al., 2008; Yang et al., 2008). SPL is a plant-specific transcription factor, with a highly conserved region of 76 amino acids named the SBP-box, that is responsible for both the interaction with DNA and for nuclear import (Klein et al., 1996; Birkenbihl et al., 2005; Yang et al., 2008). The SPL family members play diverse roles in developmental processes and stress responses in plants (Yamasaki et al., 2009; Preston and Hileman, 2013).

Transcript levels of Arabidopsis GATA and SPL family transcription factors in +S and –S roots are summarized in

Supplemental Tables 1 and 2. GATA4, GATA18, SPL2, and SPL15 are repressed under –S, but none of them is induced under the same condition (Supplemental Tables 1 and 2). Our deletion study suggested that SURE21A including a GATA motif is involved in the induction of transcription in response to –S (Figure 2). If any of the GATA transcription factors function as a repressor, decreased levels of GATA4 and GATA18 may trigger an increase in *SULTR2;1* expression under –S. The deletion study also suggested that SURE21B, which includes a GTAC motif, is involved in the repression of transcription under +S (Figure 2). None of the SPL transcription factors has been reported as a transcriptional repressor; however, it can be hypothesized that the binding of these SPL proteins inhibits *SULTR2;1* expression under +S, while their repression leads to an induction of *SULTR2;1* expression under –S. A number of questions and possibilities still remain as to whether and how the binding of these two transcription factors to SURE21A and SURE21B and their interactions with each other would induce *SULTR2;1* expression. The existence of novel proteins that bind to SURE21A and SURE21B would be another possibility. Identification of other SURE21A and SURE21B binding proteins and clarification of their functions need to be investigated for understanding of this transcriptional control machinery.

In summary, in this study, we identified two novel sulfur-responsive elements, SURE21A and SURE21B, located in the 3′-nontranscribed region as *cis*-acting elements indispensable for inducing *SULTR2;1* expression in roots in response to –S. The function of SULTR2;1 in the uptake and translocation of sulfate under –S in the roots was revealed from the experiments using tKO mutants containing T-DNA insertions in the 3′-nontranscribed region of *SULTR2;1*. This work provides evidence for a molecular mechanism underlying –S-induced expression of *SULTR2;1* in Arabidopsis roots and explains the physiological meaning of the induction of *SULTR2;1* expression with relevance to the presence of the two possible enhancers, SURE21A and SURE21B. The ability of the *SULTR2;1* 3′-nontranscribed region to control the –S-responsive and the tissue-specific gene expressions of *SULTR2;1* demonstrates another regulatory mechanism of sulfate uptake and translocation in plants. The mechanism demonstrated in this study represents a part of the transcriptional machinery, which is under control of signal transduction pathways operating in response to changes in sulfur conditions, but is distinct from the SLIM1-dependent regulatory pathway. It is suggested that SURE21A/SURE21B-dependent transcriptional induction and miR395-dependent posttranscriptional regulation allow fine-tuning of the *SULTR2;1* transcript levels in roots. These multiple mechanisms are probably controlled by signals carrying spatio-temporal and conditional information on sulfur status to make timely and flexible responses to deficiency of such an essential macronutrient as sulfur, which needs to be acquired from the soil environment by the expression of functional sulfate transporters in roots.

METHODS

Plant Growth

Arabidopsis thaliana plants were grown at 22°C under 16-h-light/8-h-dark long-day cycles. Plants were grown on mineral nutrient media (Hirai et al., 1995) containing 1% sucrose. For preparation of agar medium, agar was washed twice with 1 liter of deionized water and vacuum filtered to remove sulfate. +S agar medium was supplied with 1500 μM $MgSO_4$. –S agar medium was supplied with 15 μM $MgSO_4$, and Mg concentration was adjusted to 1500 μM by adding $MgCl_2$.

Transgenic Plants

The chimeric constructs are shown schematically in Figures 1 to 6. The primers used are listed in Supplemental Table 3.

The synthesis of $P_{SULTR2;1}$:*GFP*:$T_{SULTR2;1}$, $P_{SULTR2;1}$:*Luc*:$T_{SULTR2;1}$, $P_{SULTR2;1}$:*GFP*:T_{NOS}, and $P_{SULTR2;1}$:*Luc*:T_{NOS} fusion gene constructs (Figures 1, 2, 5, and 6) was performed as follows: The *SULTR2;1* 5′region ($P_{SULTR2;1}$) 2535 bp upstream of the translational initiation site of *SULTR2;1* was amplified from genomic DNA of Arabidopsis (ecotype Col-0) by PCR using *Pfu* turbo DNA polymerase (Stratagene-Agilent) and SULTR2;1PFSpe and SULTR2;1PR-Bam primers. The amplified PCR fragment containing the *Spe*I and *Bam*HI sites on the ends was cloned into pCR-BluntII-TOPO (Invitrogen) and sequenced. Similarly, the *SULTR2;1* 3′-region ($T_{SULTR2;1}$) 1077 bp downstream of the translational termination site of *SULTR2;1* was amplified using SULTR2;1TerFNot and SULTR2;1TerREco primers, cloned, and sequenced. Then, $T_{SULTR2;1}$ as the *Not*I-*Eco*RI-ended fragment was cloned between the *Not*I and *Eco*RI sites of pTH2 or pTHLuc, replacing the nopaline synthase terminator region (T_{NOS}) in these vectors. pTHLuc is a plasmid made by substitution of the *Nco*I-*Not*I GFP region in pTH2 (Chiu et al., 1996) by the Luc coding sequence (Promega). From these plasmids, either in pCR-BluntII-TOPO, pTH2, or pTHLuc vector backgrounds, $P_{SULTR2;1}$ as the *Spe*I-*Bam*HI fragment, and *GFP*:$T_{SULTR2;1}$, *Luc*:$T_{SULTR2;1}$, *GFP*:T_{NOS}, and *Luc*:T_{NOS} as the *Bam*HI-*Eco*RI fragments were isolated and cloned between the *Xba*I and *Eco*RI sites of pBI101 (Clontech), replacing the *GUS*:T_{NOS} region in this binary vector to obtain the promoter:reporter:terminator fusion constructs.

For the synthesis of P_{35S}:*Luc*:$T_{SULTR2;1}$ and P_{35S}:*Luc*:T_{NOS} fusion gene constructs (Figure 4), *Luc*:$T_{SULTR2;1}$ or *Luc*:T_{NOS} as the *Bam*HI-*Eco*RI fragments were cloned between the *Bam*HI and *Eco*RI sites of pBI121 (Clontech), replacing the *GUS*-T_{NOS} region. For the synthesis of $P_{SULTR1;1}$:*Luc*:$T_{SULTR2;1}$ and $P_{SULTR1;1}$:*Luc*:T_{NOS} fusion gene constructs (Figure 4), the *SULTR1;1* 5′region ($P_{SULTR1;1}$) 2453 bp upstream of the translation initiation site of *SULTR1;1*, which lacks the sulfur-responsive element of the *SULTR1;1* promoter (SURE11; Maruyama-Nakashita et al., 2005), was obtained as the *Sal*I-*Bam*HI fragment, ligated with the *Luc*:$T_{SULTR2;1}$ or *Luc*:T_{NOS} *Bam*HI-*Eco*RI fragments, and cloned between the *Sal*I and *Eco*RI sites of pBI101, replacing the *GUS*:T_{NOS} region.

For the deletion analysis of $T_{SULTR2;1}$ (Figure 2A), the regions starting from the translational termination site of *SULTR2;1* and ending at the positions +663, +459, +449, +431, +411, +391, +372, +360, +352, +332, and +267 were amplified from the plasmid containing the 1077-bp $T_{SULTR2;1}$ fragment by PCR using *Pfu* turbo DNA polymerase. PCR was performed using SULTR2;1TerFNot as a forward primer and SULTR2;1TerREco663, 459, 449, 431, 411, 391, 372, 360, 352, 332, or 267 as reverse primers. The amplified PCR fragments containing the *Not*I and *Eco*RI sites on ends were cloned and sequenced. These $T_{SULTR2;1}$ deletion fragments were then cloned between the *Not*I and *Eco*RI sites of pTHLuc to be fused to *Luc* using *Not*I. The *Luc*:$T_{SULTR2;1}$ fusions including $T_{SULTR2;1}$ deletions were isolated as the *Bam*HI-*Eco*RI-ended fragments and cloned into the *Xba*I-*Eco*RI sites of pBI101 together with the $P_{SULTR2;1}$ *Spe*I-*Bam*HI fragment.

The fragments having deletions of +361/+372 region with or without +449/+459 region of $T_{SULTR2;1}$ (Figure 2B) were made by overlap extension and amplified by two-step PCR. In the first PCR, a segment from the translational termination site of *SULTR2;1* to the position +360 and two other segments from +373 to either +449 or +459 were amplified from the plasmid containing the 1077-bp $T_{SULTR2;1}$ fragment using high-fidelity KOD-Plus DNA polymerase (Toyobo) and the primer sets, SULTR2;1TerFNot and 2;1T(360/372)R, and 2;1T(360/372)F and either 2;1TerREco449 or 2;1TerREco459. The resultant fragments were mixed to make overlaps

and used as templates for the second PCR, which was performed using SULTR2;1TerFNot and either 2;1TerREco449 or 2;1TerREco459.

The mutated fragments of the +449/+459 region (Figure 3) were amplified from the plasmid containing the 459-bp $T_{SULTR2;1}$ fragment using 2;1TerFNot as the forward primer and 2;1T459Rbs-1, -2, -3, -4, or -5 as reverse primers. The mutated fragments of the +361/+372 region (Figure 3) were made by overlap extension and amplified by a two-step PCR. In the first PCR, the regions from the translation termination site of *SULTR2;1* to the mutated position, and the regions from mutated position to +459 were amplified from the plasmid containing the 459-bp $T_{SULTR2;1}$ fragment, using the primer pairs between the forward primer 2;1TerFNot and either of the reverse primers 2;1T(360-372)Rbs-1, -2, -3, -4, -5, or -6, and the primer pairs between either of the forward primers 2;1T(360-372)Fbs-1, -2, -3, -4, -5, or -6 and the reverse primer 2;1TerREco459. The resultant fragments containing the same mutations were mixed to make overlaps and used as templates for the second PCR, which was performed using SULTR2;1-TerFNot and 2;1TerREco459. The amplified PCR fragments containing the *Not*I and *Eco*RI sites on ends were cloned into pCR-BluntII-TOPO and sequenced. The *Not*I-*Eco*RI-ended fragments with mutations were cloned between the *Not*I and *Eco*RI sites of pTHLuc to be fused to *Luc* using *Not*I. From these plasmids, the *Luc*:$T_{SULTR2;1}$ fusions with mutations were isolated as *Bam*HI-*Eco*RI fragments and cloned between the *Xba*I and *Eco*RI sites of pBI101, together with the $P_{SULTR2;1}$ *Spe*I-*Bam*HI fragment.

Construction of *332-459*$T_{SULTR2;1}$(*sense/antisense*):$P_{SULTR2;1}$:*Luc*:T_{NOS}, *332-459*$T_{SULTR2;1}$(*sense/antisense*):$mP_{SULTR2;1}$:*Luc*:T_{NOS}, and *332-459*$T_{SULTR2;1}$(*sense/antisense*):$P_{35S\ minimal}$:*Luc*:T_{NOS} fusion genes was performed as follows: The +332 to +459 region of $T_{SULTR2;1}$ in sense or antisense orientations [*332-459*$T_{SULTR2;1}$(*sense*) or *332-459*$T_{SULTR2;1}$(*antisense*)] were created by annealing the two sets of complementary synthetic oligomers, 2;1TerFHindXba(sense) and 2;1TerRHindXba (sense), and 2;1TerFHindXba(antisense) and 2;1TerRHindXba(antisense), to have *Hin*dIII and *Xba*I sites on ends. As $mP_{SULTR2;1}$, a DNA fragment covering 142 bp upstream of the translation start site of SULTR2;1 was created by annealing the two complementary synthetic oligomers, 2;1miniProFXbaBam and 2;1miniProRXbaBam. As $P_{35S\ minimal}$, the –46/0 region of the CaMV 35S promoter was created by annealing the two complementary synthetic oligomers, 35SminiF-Xba and 35SminiR-Bam. These minimal promoter fragments were cloned between the *Xba*I and *Bam*HI sites of pBI101-Luc (Maruyama-Nakashita et al., 2005). The resultant plasmid containing the $mP_{SULTR2;1}$:*Luc*:T_{NOS} or $P_{35S\ minimal}$:*Luc*:T_{NOS} fusion gene was used to clone the *Hin*dIII-*Xba*I-ended *332-459*$T_{SULTR2;1}$ (*sense*) or *332-459*$T_{SULTR2;1}$(*antisense*) fragments in front of $mP_{SULTR2;1}$ or $P_{35S\ minimal}$. $P_{SULTR2;1}$:*Luc*:T_{NOS} fusion construct made in pBI101 was also used for cloning these sense or antisense fragments in front of the 2535-bp $P_{SULTR2;1}$ fragment.

For the construction of $P_{35S\ minimal}$:*GFP*:$T_{SULTR2;1}$ (35mPGT in Figure 6), the –46/0 region of the CaMV 35S promoter was created by annealing the two complementary synthetic oligomers 35SminiF-Sal and 35SminiR-Bam, and the resultant fragment was cloned between the *Sal*I and *Bam*HI site in front of the *GFP*:$T_{SULTR2;1}$ fusion construct made in pBI101. For the construction of $P_{SULTR1;1(2453)}$:*GFP*:$T_{SULTR2;1}$ fusion gene (1;1P2453GT in Figure 6), the 2453-bp *SULTR1;1* 5′-region was isolated as a *Sal*I-*Bam*HI fragment from the plasmid created previously (Maruyama-Nakashita et al., 2004a) and cloned between the *Sal*I and *Bam*HI site in front of the *GFP*:$T_{SULTR2;1}$ fusion construct made in pBI101. $P_{SULTR1;1(3995)}$:*GFP*:T_{NOS} was described previously (Maruyama-Nakashita et al., 2004a; 1;1P3995GT in Figure 6).

The resultant binary plasmids were transferred to *Agrobacterium tumefaciens* GV3101 (pMP90) (Koncz and Schell, 1986) by freeze and thaw methods. Arabidopsis plants were transformed according to the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on GM media (Valvekens et al., 1988) containing 50 mg L^{-1} kanamycin sulfate. Kanamycin-resistant T2 progenies were used for the analysis.

Imaging of GFP Expression

Expression of GFP in whole intact Arabidopsis seedlings was visualized using a FluorImager 595 image analyzer under 488-nm laser excitation (Molecular Dynamics-GE Healthcare). Fluorescence of GFP was detected using a 515- to 545-nm band-pass filter. Autofluorescence derived from chlorophyll was scanned in parallel using a 610-nm long-pass filter.

Tissue-specific expression of GFP in transgenic plants was visualized using a FluoView 500 confocal laser scanning microscopy system equipped with a 505- to 525-nm band-pass filter (Olympus).

Luciferase Assay

Luciferase activity was determined using a Mithras LB 940 multilabel reader (Berthold Technologies) following the manufacturer's instructions. Protein extraction and luciferase assay were performed using the Luciferase Assay System (Promega) as described previously (Maruyama-Nakashita et al., 2005). The luciferase activities were expressed as relative luminescence units per milligram of protein. Protein concentration was determined with a Bio-Rad protein assay kit according to Bradford (1976) using BSA as a standard protein.

Prediction of SURE21A and SURE21B in Up- and Downstream Sequences of –S-Regulated Genes

Subio Platform programs were used for data analysis. To identify the SURE21A (5′-CAATGTATC-3′) and SURE21B (5′-CTAGTAC-3′), and their complementarily sequences in upstream and downstream sequences of –S-regulated genes, the list of 469 –S-regulated genes selected from microarray analysis was imported (Maruyama-Nakashita et al., 2006). Among the 469 genes, –S-upregulated and -downregulated genes were separated by their fold change of mRNA levels in –S compared with +S. A group of 238 genes with –S/+S fold-change values of >1 was categorized as –S-upregulated, and the remaining 231 genes with –S/+S fold-change values of <1 were categorized as –S-downregulated genes. The presence and the positions of SURE21A and SURE21B were searched using Genomic Analysis Plug-In (Subio).

Isolation of T-DNA Insertion Mutants for 3′-Nontranscribed Region of *SULTR2;1*

Homozygous knockout mutants for SAIL_363_C06 (Sessions et al., 2002; Col-0 background) and FLAG_373B04 (Samson et al., 2002; Ws background) containing T-DNA insertions in the 3′-nontranscribed region of *SULTR2;1* were obtained by a screening based on PCR analysis. Confirmation of the T-DNA insertion in SAIL_363_C06 was done by PCR on genomic DNA using T-DNA left border primer SAIL-LB3 (Sessions et al., 2002) and *SULTR2;1*-specific primers, SULTR2;1TerFNot or 2;1T656R. The T-DNA insertion in FLAG_373B04 was confirmed by PCR on genomic DNA using T-DNA left border primer FLAG-LB4 (Samson et al., 2002) and *SULTR2;1*-specific primers. The primers used are listed in Supplemental Table 4.

Real-Time RT-PCR Analysis of *SULTR2;1*

RNA preparation and reverse transcription were performed as reported previously (Maruyama-Nakashita et al., 2004a). Real-time PCR was performed using SYBR Green Perfect real-time kit (Takara Bio) and Thermal Cycler Dice real-time system (Takara Bio) using the gene-specific primers for *SULTR2;1* (SULTR2;1-1648F, 5′-ATGACGGTTAAGACTCCCGGA-3′, and SULTR2;1-1750R, 5′-CGACCCATCCCATAATCCTTT-3′) and for *UBIQUITIN2* (UBQ2-144F, 5′-CCAAGATCCAGGACAAAGAAGGA-3′, and UBQ2-372R, 5′-TGGAGACGAGCATAACACTTGC-3′). The relative mRNA levels were calculated using *UBIQUITIN2* as an internal standard.

3′-Rapid Amplification of cDNA Ends

RNA preparation was performed as reported previously (Maruyama-Nakashita et al., 2004a). Reverse transcription and RT-PCR was performed using Smart RACE cDNA amplification kit according to the user's manual (Clontech-Takara Bio). Following the reverse transcription using 3′-RACE CDS primer, the first PCR was performed using Universal Primer A mix (Short) and the gene-specific primers SULTR2;1-3′RACE(1781F), 5′-GCAATGCCAAGAGAAAGATCCTCTTTGTAGT-3′, for amplification of the 3′UTR of *SULTR2;1* in Col-0, tKO-Col, Ws, and tKO-Ws, and Luc-3′RACE-1: 5′-CATCTTCGACGCAGGTGTCGCAGGT-3′, for amplification of 3′UTR of *Luc* in 2;1PLT1077 and 2;1PLT360 plants. The reaction mixtures of the first PCR were used as the templates for the nested PCR. The nested PCR were performed using Nested Universal Primer A and the gene-specific primers SULTR2;1-3′RACE(1938F), 5′-CAAGCTGAAT-CAAGCAAAGTTCGTCGACAGA-3′, for amplification of 3′UTR of *SULTR2;1*, and Luc-3′RACE-2: 5′-GAGATCGTGGATTACGTCGCCAGT-3′, for amplification of 3′UTR of *Luc*. The amplified fragments were cloned into pGEM-T Easy Vectors (Promega) and sequenced.

Sulfate Uptake

Plants were grown vertically for 10 d on +S (1500 μM sulfate) or –S (15 μM sulfate) media. The roots were submerged in +S nutrient solution containing 15 μM [^{35}S] sodium sulfate (Amersham Biosciences) and incubated for 1 h. Washing and measurement were performed as described previously (Kataoka et al., 2004b; Maruyama-Nakashita et al., 2004b).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers and AGI numbers: SULTR2;1 (At5g10180), SULTR1;1 (At4g08620), and UBQ2 (At2g36170).

Supplemental Data

Supplemental Figure 1. Length of 3′-Untranslated Region Determined by 3′-RACE.

Supplemental Table 1. Transcript Levels of GATA Transcription Factor Genes in Plant Roots under Sulfur-Sufficient and -Deficient Conditions.

Supplemental Table 2. Transcript Levels of SPL Transcription Factor Genes in Plant Roots under Sulfur-Sufficient and -Deficient Conditions.

Supplemental Table 3. Oligonucleotides Used for the Construction of Plant Transformation Vectors.

Supplemental Table 4. Primers Used for the Isolation of T-DNA Insertion Mutants.

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AUTHOR CONTRIBUTIONS

A.M.-N. designed the research. A.M.-N., A.W.-T., and E.I. performed research. A.M.-N., A.W.-T., E.I., T.Y., K.S., and H.T. analyzed data. A.M.-N and H.T. wrote the article.

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REFERENCES

Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121:** 207–221.

Awazuhara, M., Kim, H., Goto, D.B., Matsui, A., Hayashi, H., Chino, M., Kim, S.-G., Naito, S., and Fujiwara, T. (2002). A 235-bp region from a nutritionally regulated soybean seed-specific gene promoter can confer its sulfur and nitrogen response to a constitutive promoter in aerial tissues of *Arabidopsis thaliana.* Plant Sci. **163:** 75–82.

Benfey, P.N., Ren, L., and Chua, N.H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. **8:** 2195–2202.

Bi, Y.M., Zhang, Y., Signorelli, T., Zhao, R., Zhu, T., and Rothstein, S. (2005). Genetic analysis of Arabidopsis GATA transcription factor gene family reveals a nitrate-inducible member important for chlorophyll synthesis and glucose sensitivity. Plant J. **44:** 680–692.

Birkenbihl, R.P., Jach, G., Saedler, H., and Huijser, P. (2005). Functional dissection of the plant-specific SBP-domain: overlap of the DNA-binding and nuclear localization domains. J. Mol. Biol. **352:** 585–596.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterisation of the *Arabidopsis* SBP-box genes. Gene **237:** 91–104.

Chiang, Y.H., Zubo, Y.O., Tapken, W., Kim, H.J., Lavanway, A.M., Howard, L., Pilon, M., Kieber, J.J., and Schaller, G.E. (2012). Functional characterization of the GATA transcription factors GNC and CGA1 reveals their key role in chloroplast development, growth, and division in Arabidopsis. Plant Physiol. **160:** 332–348.

Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996). Engineered GFP as a vital reporter in plants. Curr. Biol. **6:** 325–330.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J. **16:** 735–743.

Davidian, J.C., and Kopriva, S. (2010). Regulation of sulfate uptake and assimilation—the same or not the same? Mol. Plant **3:** 314–325.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. **79:** 351–379.

Gowik, U., Burscheidt, J., Akyildiz, M., Schlue, U., Koczor, M., Streubel, M., and Westhoff, P. (2004). *cis*-Regulatory elements for mesophyll-specific gene expression in the C4 plant *Flaveria trinervia*, the promoter of the C4 phosphoenolpyruvate carboxylase gene. Plant Cell **16:** 1077–1090.

Gutiérrez, R.A., MacIntosh, G.C., and Green, P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. **4:** 429–438.

Guo, A.-Y., Zhu, Q.-H., Gu, X., Ge, S., Yang, J., and Luo, J. (2008). Genome-wide identification and evolutionary analysis of the plant specific SBP-box transcription factor family. Gene **418:** 1–8.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res. **27:** 297–300.

Hudson, D., Guevara, D., Yaish, M.W., Hannam, C., Long, N., Clarke, J.D., Bi, Y.-M., and Rothstein, S.J. (2011). GNC and CGA1 modulate chlorophyll biosynthesis and glutamate synthase (GLU1/Fd-GOGAT) expression in Arabidopsis. PLoS ONE **6:** e26765.

Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M., and Saito, K. (2003). Global expression profiling of sulfur-starved *Arabidopsis* by DNA macroarray reveals the role of *O*-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J. **33:** 651–663.

Hirai, M.Y., Fujiwara, T., Chino, M., and Naito, S. (1995). Effects of sulfate concentrations on the expression of a soybean seed storage protein gene and its reversibility in transgenic *Arabidopsis thaliana.* Plant Cell Physiol. **36:** 1331–1339.

Iyer-Pascuzzi, A.S., Jackson, T., Cui, H., Petricka, J.J., Busch, W., Tsukagoshi, H., and Benfey, P.N. (2011). Cell identity regulators link development and stress responses in the *Arabidopsis* root. Dev. Cell **21:** 770–782.

Jones-Rhoades, M.W., and Bartel, D.P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol. Cell **14:** 787–799.

Kataoka, T., Watanabe-Takahashi, A., Hayashi, N., Ohnishi, M., Mimura, T., Buchner, P., Hawkesford, M.J., Yamaya, T., and Takahashi, H. (2004a). Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in *Arabidopsis.* Plant Cell **16:** 2693–2704.

Kataoka, T., Hayashi, N., Yamaya, T., and Takahashi, H. (2004b). 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.

Kawashima, C.G., Yoshimoto, N., Maruyama-Nakashita, A., Tsuchiya, Y.N., Saito, K., Takahashi, H., and Dalmay, T. (2009). Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. Plant J. **57:** 313–321.

Kawashima, C.G., et al. (2011). Interplay of SLIM1 and miR395 in the regulation of sulfate assimilation in *Arabidopsis.* Plant J. **66:** 863–876.

Klein, J., Saedler, H., and Huijser, P. (1996). A new family of DNA binding proteins includes putative transcriptional regulators of the *Antirrhinum majus* floral meristem identity gene *SQUAMOSA.* Mol. Gen. Genet. **250:** 7–16.

Koncz, C., and Schell, J. (1986). The promoter of T-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel types of *Agrobacterium* binary vector. Mol. Gen. Genet. **204:** 383–396.

Kutz, A., Müller, A., Hennig, P., Kaiser, W.M., Piotrowski, M., and Weiler, E.W. (2002). A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana.* Plant J. **30:** 95–106.

Lam, E., and Chua, N.H. (1989). ASF-2: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in Cab promoters. Plant Cell **1:** 1147–1156.

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

Lewandowska, M., Wawrzynska, A., Moniuszko, G., Lukomska, J., Zientara, K., Piecho, M., Hodurek, P., Zhukov, I., Liszewska, F., Nikiforova, V., and Sirko, A. (2010). A contribution to identification of novel regulators of plant response to sulfur deficiency: characteristics of a tobacco gene UP9C, its protein product and the effects of UP9C silencing. Mol. Plant **3:** 347–360.

Liang, G., Yang, F., and Yu, D. (2010). MicroRNA395 mediates regulation of sulfate accumulation and allocation in *Arabidopsis thaliana.* Plant J. **62:** 1046–1057.

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

Nikiforova, V., Freitag, J., Kempa, S., Adamik, M., Hesse, H., and Hoefgen, R. (2003). Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J. **33:** 633–650.

Paulose, B., Chhikara, S., Coomey, J., Jung, H.I., Vatamaniuk, O., and Dhankher, O.P. (2013). A γ-glutamyl cyclotransferase protects *Arabidopsis* plants from heavy metal toxicity by recycling glutamate to maintain glutathione homeostasis. Plant Cell **25:** 4580–4595.

Preston, J.C., and Hileman, L.C. (2013). Functional evolution in the plant SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) gene family. Front. Plant Sci. **4:** 80.

Rastogi, R., Bate, N.J., Sivasankar, S., and Rothstein, S.J. (1997). Footprinting of the spinach nitrite reductase gene promoter reveals the preservation of nitrate regulatory elements between fungi and higher plants. Plant Mol. Biol. **34:** 465–476.

Reyes, J.C., Muro-Pastor, M.I., and Florencio, F.J. (2004). The GATA family of transcription factors in *Arabidopsis* and rice. Plant Physiol. **134:** 1718–1732.

Richter, R., Behringer, C., Müller, I.K., and Schwechheimer, C. (2010). The GATA-type transcription factors GNC and GNL/CGA1 repress gibberellin signaling downstream from DELLA proteins and PHYTOCHROME-INTERACTING FACTORS. Genes Dev. **24:** 2093–2104.

Saito, K. (2004). Sulfur assimilatory metabolism. The long and smelling road. Plant Physiol. **136:** 2443–2450.

Samson, F., Brunaud, V., Balzergue, S., Dubreucq, B., Lepiniec, L., Pelletier, G., Caboche, M., and Lecharny, A. (2002). FLAGdb/FST: a database of mapped flanking insertion sites (FSTs) of *Arabidopsis thaliana* T-DNA transformants. Nucleic Acids Res. **30:** 94–97.

Sessions, A., et al. (2002). A high-throughput *Arabidopsis* reverse genetics system. Plant Cell **14:** 2985–2994.

Shibagaki, N., Rose, A., McDermott, J.P., Fujiwara, T., Hayashi, H., Yoneyama, T., and Davies, J.P. (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.

Shikata, M., Matsuda, Y., Ando, K., Nishii, A., Takemura, M., Yokota, A., and Kohchi, T. (2004). Characterization of *Arabidopsis* ZIM, a member of a novel plant-specific GATA factor gene family. J. Exp. Bot. **55:** 631–639.

Shirsat, A., Wilford, N., Croy, R., and Boulter, D. (1989). Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. Mol. Gen. Genet. **215:** 326–331.

Takahashi, H., Buchner, P., Yoshimoto, N., Hawkesford, M.J., and Shiu, S.H. (2012). Evolutionary relationships and functional diversity of plant sulfate transporters. Front. Plant Sci. **2:** 119.

Takahashi, H., Kopriva, S., Giordano, M., Saito, K., and Hell, R. (2011). Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu. Rev. Plant Biol. **62:** 157–184.

Takahashi, H., Watanabe-Takahashi, A., Smith, F.W., Blake-Kalff, M., Hawkesford, M.J., and 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, J.A., Engler, G., Van Montagu, M., and 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.

Valvekens, D., Montagu, M.V., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA **85:** 5536–5540.

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

Wawrzyńska, A., Lewandowska, M., and Sirko, A. (2010). Nicotiana tabacum EIL2 directly regulates expression of at least one tobacco gene induced by sulphur starvation. J. Exp. Bot. **61:** 889–900.

Yamasaki, H., Hayashi, M., Fukazawa, M., Kobayashi, Y., and Shikanai, T. (2009). SQUAMOSA promoter binding protein–like7 is a central regulator for copper homeostasis in *Arabidopsis.* Plant Cell **21:** 347–361.

Yang, Z., Wang, X., Gu, S., Hu, Z., Xu, H., and Xu, C. (2008). Comparative study of SBP-box gene family in *Arabidopsis* and rice. Gene **407:** 1–11.

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

Yoshimoto, N., Inoue, E., Saito, K., Yamaya, T., and Takahashi, H. (2003). Phloem-localizing sulfate transporter, Sultr1;3, mediates redistribution of sulfur from source to sink organs in *Arabidopsis.* Plant Physiol. **131:** 1511–1517.

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