Vacuolar Sulfate Transporters Are Essential Determinants Controlling Internal Distribution of Sulfate in Arabidopsis

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Uptake of external sulfate from the environment and use of internal vacuolar sulfate pools are two important aspects of the acquisition of sulfur for metabolism. In this study, we demonstrated that the vacuolar SULTR4-type sulfate transporter facilitates the efflux of sulfate from the vacuoles and plays critical roles in optimizing the internal distribution of sulfate in Arabidopsis thaliana. SULTR4;1-green fluorescent protein (GFP) and SULTR4;2-GFP fusion proteins were expressed under the control of their own promoters in transgenic Arabidopsis. The fusion proteins were accumulated specifically in the tonoplast membranes and were localized predominantly in the pericycle and xylem parenchyma cells of roots and hypocotyls. In roots, SULTR4;1 was constantly accumulated regardless of the changes of sulfur conditions, whereas SULTR4;2 became abundant by sulfur limitation. In shoots, both transporters were accumulated by sulfur limitation. Vacuoles isolated from callus of the sultr4;1 sultr4;2 double knockout showed excess accumulation of sulfate, which was substantially decreased by overexpression of SULTR4;1-GFP. In seedlings, the supplied [35S]sulfate was retained in the root tissue of the sultr4;1 sultr4;2 double knockout mutant. Comparison of the double and single knockouts suggested that SULTR4;1 plays a major role and SULTR4;2 has a supplementary function. Overexpression of SULTR4;1-GFP significantly decreased accumulation of [35S]sulfate in the root tissue, complementing the phenotype of the double mutant. These results suggested that SULTR4-type transporters, particularly SULTR4;1, actively mediate the efflux of sulfate from the vacuole lumen into the cytoplasm and influence the capacity for vacuolar storage of sulfate in the root tissue. The efflux function will promote rapid turnover of sulfate from the vacuoles particularly in the vasculature under conditions of low-sulfur supply, which will optimize the symplastic (cytoplasmic) flux of sulfate channeled toward the xylem vessels.

INTRODUCTION

Sulfur is one of the essential macronutrients for growth of higher plants (Marschner, 1995). After initial uptake of sulfate at the root epidermis, internal distribution through the vasculature and cell-to-cell symplastic movement are required to facilitate a continuous supply of sulfur to all organs and cell types. Multiple transport systems are activated to survive with a restricted sulfur source. First, plants use and induce high-affinity sulfate uptake systems in roots, facilitating absorption of a micromolar concentration of sulfate from the rhizosphere (Leggett and Epstein, 1956; Clarkson et al., 1983; Smith et al., 1995). Important additional regulated components are the internal transport systems. Sulfate taken up to the root tissue is transported to the aerial parts where reduction of sulfate and synthesis of sulfur–amino acids mainly take place (Leustek and Saito, 1999; Leustek et al., 2000; Saito, 2000). Transporters localized in the vasculature facilitate these transport processes. Responding to sulfur availability, a demand-driven regulation is believed to control the interorgan translocation of sulfate (Herschbach and Rennenberg, 1991; Lappartient and Touraine, 1996; Lappartient et al., 1999).

Recent studies have identified genes for several sulfate transporters responsible for the function of the primary sulfate uptake in the roots (reviewed in Hawkesford, 2003). The first plant sulfate transporter genes were isolated from Stylosanthes hamata through functional complementation of a sulfate transporter deletion mutant of yeast (Smith et al., 1995). The membrane proteins encoded by the isolated clones, SHST1 and SHST2, catalyzed sulfate uptake activities with micromolar Km values for sulfate that corresponded to the high-affinity sulfate uptake systems described in intact plant root (Leggett and Epstein, 1956). In addition, these transporters were expressed more abundantly in roots upon sulfur limitation. The sulfate transport activities of high-affinity transporters were dependent on the pH in the media, suggesting a function as proton/sulfate cotransporters (Lass and Ulrich-Eberius, 1984; Hawkesford et al., 1993; Smith et al., 1995). HVST1 from barley (Hordeum vulgare)
showed similar characteristics (Smith et al., 1997; Vidmar et al., 1999). More recently, Arabidopsis thaliana SULTR1;1 and SULTR1;2 (Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2003) were established to have functional properties similar to SHST1 and HVST1. Arabidopsis SULTR1;1 and SULTR1;2 were localized in the root hairs, epidermis, and cortex cells, and the respective abundances of mRNAs for these transporters increased upon sulfur starvation. Knockout of SULTR1;2 resulted in reduced sulfate uptake and growth, suggesting that this transporter plays a major role in the absorption of sulfate in the root (Shibagaki et al., 2002; Maruyama-Nakashita et al., 2003).

Vascular tissue-localizing sulfate transporters are suggested to play important roles in the internal transport of sulfate. Arabidopsis SULTR2;1 is a low-affinity sulfate transporter localized in the pericycle and vascular parenchyma cells (Takahashi et al., 1997, 2000). SULTR2;1 mRNA abundance significantly increased during sulfur starvation (Takahashi et al., 1997, 2000). Furthermore, the sultr2;2 mutant showed accumulation of SULTR2;1 mRNA (Maruyama-Nakashita et al., 2003), suggesting that the internal transport of sulfate was activated in response to the loss of sulfate uptake in the mutant. Further studies identified the role of SULTR1;3 as a functional sulfate transport system involved in phloem transport of sulfate (Yoshimoto et al., 2003). Taken together, the members of the sulfate transporter gene family appear to have distinctive cell type specificities and are regulated by sulfur availability to optimize both uptake and internal transport under varying sulfur availability. These mechanisms are suggested to be essential for optimization of sulfur supply during adaptation to sulfur deficiency.

Excess sulfate transported into cells accumulates mainly in the vacuoles and constitutes a large internal sulfur reserve (Kaiser et al., 1989; Martinoia et al., 2000). Apart from a function for storage, sulfate in the vacuoles may buffer or optimize the flux of sulfate transport through the plant. Despite the importance of reuse of vacuolar sulfate, pathways for sulfate transport at the tonoplast membranes are poorly understood at the molecular level. Physiological studies with isolated barley mesophyll vacuoles have reported that uptake of sulfate into the vacuole is stimulated by addition of MgATP (Kaiser et al., 1989). Proton-ATPase and proton-pyrophosphatase at the tonoplast membranes both generate a pH gradient (∆pH) and an inside positive membrane potential (∆Ψ) that serve as potential driving forces for anion influx across the tonoplast (Martinoia et al., 2000). Kaiser et al. (1989) reported that the uptake of sulfate into barley mesophyll vacuoles follows biphasic kinetics; transport of sulfate below 1 mM was saturable but was concentration dependent above 1 mM. These observations suggest that there is a transporter specific for import of sulfate into the vacuoles requiring ∆Ψ as the main driving force. Studies with Catharanthus roseus cell cultures indicated that uptake of phosphate into the vacuole was driven by ∆Ψ and was significantly inhibited in the presence of the sulfate analog chromate (Massonneau et al., 2000). Presumably, sulfate and phosphate are transported though a common or similar import mechanism; however, the exact system facilitating the influx has not been identified. In contrast with the cumulative evidence hypothesizing the existence of a ∆Ψ-dependent import of sulfate into the vacuole, a proton-coupled efflux transport activity resembling that suggested for nitrate efflux from tonoplast vesicles (Blumwald and Poole, 1985) has not been identified for sulfate.

This study demonstrates that the SULTR4-type vacuolar sulfate transporter facilitates the efflux function in Arabidopsis. The results suggested that the function of SULTR4;1 substantially contributes in preventing excessive accumulation of sulfate in the vacuoles of root xylem parenchyma cells. The second isoform, SULTR4;2, was suggested to have a similar but supplementary function. The data demonstrate a novel function of vacuolar ion transport systems as an essential component controlling the symplastic flow that delivers sulfate to the xylem vessels by balancing storage and turnover of sulfate in the root vacuoles.

RESULTS

Identification of Tonoplast-Localizing Sulfate Transporters

Two distinct tonoplast-localizing sulfate transporters, SULTR4;1 (At5g13550) and SULTR4;2 (At3g12520), have been identified in Arabidopsis (Figure 1). Localization of SULTR4;1 and SULTR4;2 transporters in the tonoplast was determined by expressing gene-green fluorescent protein (GFP) fusion constructs in Arabidopsis (Figure 1A). GFP was fused in frame to the C terminus of SULTR4;1 and SULTR4;2 and expressed in Arabidopsis under the control of the SULTR4;1 and SULTR4;2 5′-promoter sequences, respectively. The GFP-tagged proteins accumulated in membranous structures typical of tonoplasts in SULTR4;1-GFP and SULTR4;2-GFP plants (Figure 1). Fluorescence of GFP in the root tips of SULTR4;1-GFP (Figures 1B and 1C) and SULTR4;2-GFP plants (Figure 1D) presented clear views of tonoplast localization. Localization of SULTR4;1-GFP in the tonoplast was confirmed by merging the signals of GFP (Figure 1E) with a red fluorescent dye, FM4-64 (Figure 1F), which translocates to the tonoplast membrane by endocytosis. GFP (Figure 1E) and FM4-64 (Figure 1F) were colocalized in SULTR4;1-GFP plants, providing yellow staining of tonoplasts in the overlaid image (Figure 1G). In addition, GFP in the tonoplast was clearly distinguishable from the endocytotic vesicles (red dots in Figures 1F and 1G) where part of FM4-64 dye localizes during staining. SULTR4;2-GFP plants showed the same results indicating colocalization of the signals of GFP and FM4-64 on the tonoplasts (Figures 1H to 1J). In the shoots, there was specific localization of GFP in the tonoplast of the vascular parenchyma cells of hypocotyls in both plant lines (Figures 1K and 1L).

Previously, the N-terminal region of the Arabidopsis SULTR4;1 sulfate transporter was suggested to encode a chloroplastic transit peptide based on computer predictions and biolistic transformation (Takahashi et al., 1999). GFP fused to the N-terminal 99–amino acid residues or GFP replacing the C-terminal 13–amino acid residues of SULTR4;1 did not display tonoplast localization, suggesting an essential region within the C terminus of SULTR4;1 for proper assembly of transporters to

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the tonoplast membranes. A conserved amino acid sequence, EPLLS, was found in this particular region of SULTR4;1, SULTR4;2, and homologous ESTs from soybean (*Glycine max*) (accession number BE800569) and alfalfa (*Medicago sativa*) (accession number BF003985). Furthermore, pattern matching (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) indicated existence of this sequence within the C- and N-terminal hydrophilic regions of the NHX3 sodium/proton antiporter (At3g06370) (Yokoi et al., 2002) and an MRP-like ABC transporter (At3g13090) similar to the AtMRP4 vacuolar-localizing glutathione-conjugate transporter (Sánchez-Fernández et al., 2001; Martinoia et al., 2002), respectively.

**SULTR4;1 and SULTR4;2 Are Predominantly Localized in the Vasculatures of Root and Hypocotyl**

Cell type–specific expression of SULTR4;1 and SULTR4;2 was investigated in SULTR4;1-GFP and SULTR4;2-GFP transgenic Arabidopsis plants (Figure 2) expressing the gene-GFP fusion constructs (Figure 1). Both the SULTR4;1 and the SULTR4;2
promoters resulted in strong expression of the SULTR4;1-GFP and SULTR4;2-GFP fusion proteins, predominantly in the vascular tissues of roots and hypocotyls (Figure 2). In SULTR4;1-GFP plants, fluorescence of GFP was detected in roots and hypocotyls (Figure 2A), occurring from the root tip to the basal region. In roots, the SULTR4;1-GFP signal was predominantly present in the pericycle and parenchyma cells of the vascular tissues (Figures 2B and 2C). Epidermis, cortex, and endodermis of the mature part of root showed weak expression; however, the levels were not appreciable compared with those observed in the vascular tissues (Figure 2B). In hypocotyls, SULTR4;1-GFP was expressed strongly in the vasculature (Figures 2D and 2E).

The expression of SULTR4;2-GFP was observed predominantly in the mature part of roots, close to the shoot/root junction, and in the hypocotyls (Figure 2). SULTR4;2-GFP plants presented strong fluorescence when grown on a low sulfur medium (see also Figures 3 and 4). In roots, the signals of SULTR4;2-GFP were detected in the pericycles and parenchyma cells within the vascular tissues (Figures 2B and 2C), which resembles the cell-type specificity of SULTR4;1-GFP. Within the hypocotyls, SULTR4;2-GFP was expressed abundantly in the vasculature and epidermis but was expressed also in the cortical cells (Figures 2D and 2E).

Regulation of SULTR4;1 and SULTR4;2 by Sulfur Limitation

The effect of sulfur availability on expression of SULTR4;1 and SULTR4;2 was determined (Figure 3). The mRNA contents of both transporters in Arabidopsis were estimated by quantitative real-time RT-PCR. When plants were grown on sulfur-replete medium, both SULTR4;1 and SULTR4;2 mRNAs were relatively abundant in roots compared with shoots (Figures 3A and 3B). SULTR4;1 was expressed in roots both under sulfur-sufficient and -deficient conditions; however, the mRNA contents in shoots increased upon sulfur limitation (Figure 3A). SULTR4;2 mRNA was highly inducible by sulfur limitation both in shoots and roots (Figure 3B). The response of SULTR4;2 to sulfur limitation was seen also in the GFP fusion gene expression systems (right-hand panel of Figure 4A). Transgenic plants with the gene-GFP fusion constructs (Figure 1) were used for the analysis. The accumulation of transporter-GFP fusion protein in the roots and hypocotyls of SULTR4;2-GFP plants corresponded with the observed patterns of mRNA expression (Figure 3B). Fluorescence of GFP was increased approximately threefold to fivefold in the roots of sulfur-starved plants (−S) compared with the control plants (+S) grown with an adequate sulfur supply (Figure 4B). Three independent transgenic lines with homozygous single T-DNA insertions showed the same response. The SULTR4;1-GFP fusion proteins were accumulated both under +S and −S conditions (left-hand panel of Figure 4A); however, the levels were not remarkably modulated by sulfur limitation in the roots (Figure 4B), as expected from the quantification of mRNA contents in the RT-PCR analysis (Figure 3A).

The sultr4;1 sultr4;2 Double Knockout

To investigate the function of SULTR4;1 and SULTR4;2 transporters in planta, knockout mutants were isolated from the pools...
of Arabidopsis T-DNA insertion lines (Arabidopsis Knockout Facility at the University of Wisconsin Biotech Center, http://www.biotech.wisc.edu/Arabidopsis/) (Krysan et al., 1999). Mutants were isolated with T-DNAs integrated in the 7th intron of SULTR4;1 and 17th exon of SULTR4;2, respectively (Figure 5A). Homozygous progenies with single T-DNA insertions were selected by PCR and DNA gel blot hybridization analysis. The sultr4;1 and sultr4;2 mutants were crossed, and the double knockout was selected from the F2 population. RT-PCR analysis indicated that SULTR4;1 and SULTR4;2 mRNAs translatable to functional proteins were not detected in the T-DNA insertion lines (Figure 5B). To confirm the function of SULTR4;1 transporter, SULTR4;1-GFP (Figure 1) was introduced into the sultr4;1 sultr4;2 double mutant by crossing. Two independent lines, #2c and #42a, containing triple homozygous insertions of sultr4;1::T-DNA, sultr4;2::T-DNA, and SULTR4;1-GFP were selected by PCR analysis and GFP imaging as described in Methods. No accumulation of the endogenous SULTR4;1 and SULTR4;2 transcripts was observed in #2c and #42a plants; however, SULTR4;1-GFP mRNA was expressed from the SULTR4;1-GFP construct (Figure 5B). DNA gel blot hybridization indicated that a single copy of SULTR4;1-GFP was inserted in #2c and #42a plants (data not shown). The SULTR4;1-GFP mRNA in #2c and #42a was expressed approximately threefold higher than the SULTR4;1 mRNA in the wild type (Figure 5B), presumably because of the position effect of the T-DNA insertion sites. In addition, SULTR4;1-GFP was accumulated by sulfur limitation in #2c and #42a plants (Figure 5B), showing approximately threefold to twofold increase in shoots and roots, respectively. The response of SULTR4;1-GFP mRNA in #2c and #42a corresponded to the changes of native SULTR4;1 mRNA in the wild-type plants (Figures 3 and 5B). Furthermore, the mutants and SULTR4;1-GFP plants showed no morphological phenotypes both under sulfur replete and sulfur limited conditions. These plants were used for further experiments.

![Figure 3](image.png)

**Figure 3.** Effects of Sulfur Limitation on SULTR4;1 and SULTR4;2 Transcripts.

Real-time RT-PCR analysis was performed on 2-week-old plants continuously grown on MS media containing 1500, 800, 400, 200, 100, and 50 μM of sulfate. The mRNA contents of SULTR4;1 (A) and SULTR4;2 (B) in shoot (open bars) and root (closed bars) were determined by real-time PCR. Means of independent triplicate samples and SD values (n = 3) are indicated.

![Figure 4](image.png)

**Figure 4.** SULTR4;2-GFP Fusion Protein Accumulates by Sulfur Limitation.

(A) SULTR4;1-GFP (line 4;1-6d) and SULTR4;2-GFP (line 4;2-7e) plants were grown vertically on MS medium containing 1500 μM sulfate (+S) or 15 μM sulfate (−S) for 8 d. (B) Quantification of GFP in three independent transgenic lines from each construct. Intensities of GFP in the roots of −S plants (closed bars for SULTR4;1-GFP; hatched bars for SULTR4;2-GFP) were calculated as relative values compared with the +S conditions (open bars). Means ± SD (n = 8).
The Efflux Function of SULTR4 Controls Sulfate Contents in the Vacuoles

To determine the function of SULTR4 vacuolar transporters, intact vacuoles were isolated from the wild type, sultr4;1 sultr4;2 double knockout, and SULTR4;1-GFP lines #2c and #42a. Callus was generated from the hypocotyls of etiolated seedlings and propagated for vacuole isolation. As shown in Figure 6A, neutral red staining ensured the integrity of the tonoplast and intactness of the vacuoles isolated from the callus. Fluorescence of GFP was observed in the tonoplasts of the vacuoles isolated from #2c- and #42a-derived callus (Figure 6B). Measurements of ion contents indicated increased accumulation of sulfate in the vacuoles of the double knockout compared with the wild type (Figure 6C). The amount of sulfate was standardized with the

Figure 5. The sultr4;1 sultr4;2 Double Knockout.

(A) T-DNA insertions in SULTR4;1 and SULTR4;2 genes and structure of SULTR4;1-GFP. Closed bars, exons; open bars, 5’- and 3’-untranslated regions; hatched bar, GFP fused to the C terminus of SULTR4;1; dashed horizontal lines, positions of the start and the end of SULTR4;1 coding region. Positions of the primers used for RT-PCR analysis in (B) are indicated with arrowheads. T-DNAS are not described in actual sizes.

(B) RT-PCR analysis of wild-type (Wassilewskija [Ws]), sultr4;1 knockout, sultr4;2 knockout, sultr4;1 sultr4;2 double knockout, and SULTR4;1-GFP lines (#2c and #42a) in sultr4;1 sultr4;2 background. Plants were grown on low-sulfur medium (-S; 50 μM sulfate) or with adequate sulfur (+S; 1550 μM sulfate) for 10 days and subjected to RT-PCR. Positions of the primer pairs are indicated in (A). α-Tubulin (Ludwig et al., 1987) is a constitutive control. The primer sequences are described in Methods.

Figure 6. Sulfate Accumulates in the Vacuoles of the sultr4;1 sultr4;2 Knockout.

(A) Neutral red staining of the vacuoles isolated from callus. Bar = 50 μm.

(B) Localization of GFP fluorescence in the vacuoles of SULTR4;1-GFP lines. Differential interference contrast images are presented in the right-hand panels. Bars = 10 μm.

(C) Ion analysis of the isolated vacuoles of the wild type (Ws), sultr4;1 sultr4;2 knockout, and SULTR4;1-GFP lines (#2c and #42a). The peaks of sulfate, sodium, and calcium are indicated on the electropherograms of capillary electrophoresis.

(D) Sulfate contents of the vacuoles of the wild type (Ws), sultr4;1 sultr4;2 knockout, and SULTR4;1-GFP lines (#2c and #42a). Sulfate and calcium contents were determined by capillary electrophoresis. Relative values were calculated using the amount of calcium as an internal control. Statistically significant difference from the wild type (Ws) is indicated by an asterisk (P < 0.05). Means ± se (n = 8 for the wild type and sultr4;1 sultr4;2; n = 3 for the SULTR4;1-GFP lines #2c and #42a)).
calcium content that showed a constant level of accumulation between the wild type and knockout. The sulfate content in the vacuole was estimated as \( \sim 1.7 \)-fold higher in the double knockout than in the wild type (Figure 6D). In addition, the sulfate content of the vacuole was significantly decreased by expressing the SULTR4;1-GFP construct in the double knockout (#2c and #42a) (Figures 6C and 6D). It is suggested that the function of SULTR4;1 is linked to the efflux of sulfate from the vacuoles and that SULTR4;1-GFP fusion protein functions to complement the double knockout, releasing sulfate from the vacuoles. These results provided functional evidence for the role of SULTR4;1 vacuolar sulfate transporter, facilitating the efflux of sulfate from the vacuoles.

SULTR4 Vacuolar Transporters Control Storage of Sulfate in Roots

The roles of SULTR4;1 and SULTR4;2 transporters were determined by feeding \( ^{35} \text{S} \) sulfate to the roots and measuring distribution in the seedlings (Figure 7). Plants were grown under low-sulfur conditions (50 \( \mu \)M sulfate) to maximize SULTR4;1 and SULTR4;2 expression. The results indicated that the activities of SULTR4 transporters modulate the storage of sulfate in the roots. More \( ^{35} \text{S} \) sulfate was accumulated in the roots when the function of SULTR4;1 was absent in the sultr4;1 mutant (Figure 7A). The function of SULTR4;1 was confirmed in sultr4;1 sultr4;2 double knockout plants overexpressing SULTR4;1-GFP (#2c and #42a) (Figure 7B). The content of radioactive sulfate was significantly decreased in the roots of #2c and #42a as compared with the double knockout. The results indicated that the capacity of vacuolar storage of sulfate decreased significantly in the roots of #2c and #42a plants. This is suggested to be the consequence of abundant expression of SULTR4;1-GFP in the roots of #2c and #42a (Figure 5B) that allows rapid release of sulfate from the vacuoles in the root tissues. These lines were even more efficient at releasing sulfate than the wild type (Figure 7B). The results of #2c and #42a showed clear genetic complementation of the double mutant by overexpression of SULTR4;1-GFP.

Similarly, \( ^{35} \text{S} \) sulfate was accumulated in the roots of the sultr4;2 knockout (340 \( \pm \) 22 pmol/mg fresh weight [FW]), showing statistically significant differences from the wild type (268 \( \pm \) 13 pmol/mg FW). This was also evident from the comparison between the sultr4;1 sultr4;2 (868 \( \pm \) 54 pmol/mg FW) and sultr4;1 knockouts (710 \( \pm \) 32 pmol/mg FW) (Figure 7A). The increase of \( ^{35} \text{S} \) from the wild type was relatively smaller in sultr4;2 than in sultr4;1. These results suggested that SULTR4;1 is the main transporter facilitating unloading of vacuolar sulfate reserve in the roots, and SULTR4;2 may play similar and supplementary roles supporting the SULTR4;1 function at the tonoplast. Contribution of SULTR4;2 was estimated \( \sim 15\% \) of the SULTR4;1 function. Although the function has not been confirmed by reintroduction of SULTR4;2 in the knockout, the results suggested that this second isoform may additionally contribute in controlling the efflux of sulfate from the vacuoles in roots. In contrast with the changes in roots, the amount of \( ^{35} \text{S} \) sulfate transported to shoot was not drastically changed in the knockouts and in the overexpressors (Figures 7A and 7B). It is suggested that loading of sulfate into the xylem is homeostatically controlled to maintain a constant level translocated to the shoots.

**DISCUSSION**

The sequencing of the Arabidopsis genome identified more than 600 predicted open reading frames that encode potential
membrane-bound transporters (Arabidopsis Genome Initiative, 2000). They were classified into families of transporters playing specific roles in various cell types and developmental stages: uptake of nutrients, distribution of metabolites, sequestration of toxic compounds, generation of membrane potentials, and control of turgor pressure. For the uptake and assimilation of inorganic nutrients, higher plants have developed multiple plasma membrane-bound transporters that are responsible for the delivery of inorganic ions to the metabolic pathways. In general, they are regulated for adaptation to fluctuations of nutrient availability. Movement of ions through consecutive cell layers requires a concerted action of several cell type–specific transporters. To adapt to nutrient stress, plants need transporters with different kinetic properties and the ability to control expression in response to nutrient availabilities. Consequently, transport of nutrients is a complex phenomenon consisting of multiple components. Through the aid of Arabidopsis genomics tools, recent characterization of the T-DNA knockouts provided concrete evidence for the roles of plasma membrane–localizing solute transporters in the initial uptake and internal transport of the major nutrients in Arabidopsis. The process of nutrient uptake and translocation could be attributed to the functions of transporters in the plasma membrane. By contrast, the functions of vacuolar transporters have not been resolved at the molecular level, even though their critical contribution to plant nutrition has been postulated for nutrient storage in the vacuoles and remobilization.

After acquisition from the soil at the epidermis and cortex, sulfate moves toward the stele and is delivered to the various organs through vascular tissues. As with nitrate and phosphate (Martinoia et al., 1981; Blumwald and Poole, 1985; Mimura et al., 1990; Massonneau et al., 2000), sulfate accumulates mostly in vacuoles (Kaiser et al., 1989). In general, an inside-positive membrane potential across the tonoplast enables accumulation of negatively charged ions in the vacuole lumen (Martinoia et al., 2000). According to the earlier physiological studies using barley mesophyll vacuoles, it is conceivable that $\Delta \psi$ drives the influx of sulfate to the vacuoles (Kaiser et al., 1989). By contrast, the inside-acidic pH gradient favors the action of the proton/sulfate cotransporter to function for efflux. In this study, we demonstrated vacuolar localization of SULTR4;1 and SULTR4;2 sulfate transporters (Figure 1) and presented genetic evidence that supports their essential function in the efflux of sulfate from the vacuole lumen. The measurements of sulfate contents in the isolated intact vacuoles clearly indicated that the sultr4;1 sultr4;2 double knockout accumulates sulfate in the vacuoles (Figure 6). Our results further indicated that expression of SULTR4;1-GFP functionally complements this phenotype, reducing sulfate contents back to the wild-type level. These results strongly suggested that SULTR4-type sulfate transporters mediate the efflux of sulfate from the vacuoles, releasing storage of inorganic sulfate to the cytoplasm.

Judging from the close sequence similarities with other functional sulfate transporters in plants (Smith et al., 1995, 1997; Takahashi et al., 1997, 2000; Vidmar et al., 1999, 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002, 2003; Howarth et al., 2003), it is suggested that SULTR4;1 and SULTR4;2 belong to the proton/sulfate cotransporter family and may use $\Delta p$H across the tonoplast as a motive force for the efflux of sulfate. Database searches found several other SULTR4 transporters in various plant species. cDNAs from rice (Oryza sativa) (accession number AF493793) and Brassica napus (accession number AJ416461) and EST sequences from soybean (accession numbers AI965651 and BE800569) and alfalfa (accession number BF003989) showed substantial similarities to Arabidopsis SULTR4;1 and SULTR4;2. Phylogenetic analysis indicated that these SULTR4-related transporters are clustered into a distinct group in the plant sulfate transporter gene family, referred to as Group 4 (reviewed in Hawkesford, 2003). It is suggested that SULTR4 transporters are found ubiquitously in the plant kingdom and may have similar functions as vacuolar sulfate transporters. An interesting feature of SULTR4;1 and SULTR4;2 was the observed tissue-specific expression. SULTR4;1-GFP and SULTR4;2-GFP plants showed localization of GFP predominantly in the vacuoles of pericycle and xylem parenchyma cells of roots and hypocotyl (Figure 2). These specific cell types in the vasculatures potentially function for loading of inorganic...
nutrients to the xylem stream in roots. These observations prompted us to postulate that the functions of SULTR4;1 and SULTR4;2 vacuolar sulfate transporters are expected to control the flux of sulfate at xylem parenchyma cells. As shown in Figure 7A, the sultr4;1 sultr4;2 double knockout was unable to turnover sulfate pools in the root tissues efficiently. SULTR4;1 was suggested to be the major facilitator fulfilling this function. By contrast, SULTR4;2 showed relatively small contribution in remobilizing the sulfate reserve in the root vacuoles (Figure 7A). Taken together with the function of SULTR4;1-GFP determined from the measurements of sulfate in the isolated vacuoles (Figure 6), it is suggested that sulfate is deposited in the vacuoles of the double knockout, which may constitute a buildup of sulfate pool in the root tissues (Figure 7). The function of SULTR4;1 in facilitating the turnover of the root vacuolar sulfate pool was evident from the results of SULTR4;1-GFP plants, displaying a mirror image of the knockout phenotype (Figure 7B).

From these results, we propose a hypothetical model for the roles of SULTR4;1 and SULTR4;2 tonoplast-localizing sulfate transporters in Arabidopsis (Figure 8). In accordance with this model, the sultr4;1 sultr4;2 double knockout accumulated [35S]sulfate in roots, suggesting a deposition of sulfate in the vacuoles before the step of xylem loading. The wild type as well as the reintroduction of a functional SULTR4;1 transporter in the double knockout, in the form of the SULTR4;1-GFP fusion protein, prevents accumulation of sulfate in the vacuole by constitutive efflux. Consequently, sulfate entering the vascular tissue is channeled directly through the cytoplasm of xylem parenchyma cells to reach the xylem vessels. SULTR4;1 was present both under sulfur-sufficient and -deficient conditions in roots (Figures 3 and 4), suggesting its constitutive contribution in maintaining this symplastic flux connected to the process of xylem loading. When the supply of sulfate is suboptimal, induction of SULTR1 high-affinity transport systems will increase the initial uptake capacities at the root surface (Takahashi et al., 2000; Yoshimoto et al., 2002) and provide sulfate to the vasculature. Subsequently, upregulation of SULTR4;2 (Figures 3 and 4) may reinforce the vacuolar efflux mechanism, minimizing unnecessary accumulation of sulfate in the vacuoles of vascular tissues. These circumstances will provide continuous and direct symplastic flux of sulfate toward the xylem stream, supporting efficient transfer of sulfur source to the aerial parts in sulfur-starved plants. SULTR4;1 and SULTR4;2 expressed in the vasculature of hypocotyls would additionally contribute in maintaining the continuous flow of root-to-shoot transfer of sulfate, minimizing recapturing of sulfate to the vacuoles in the conducting tissues.

In general, the vacuoles of cortical cells retain a large volume of storage capacity in the root tissue, and nutrient ions including sulfate may accumulate there subsequently after the uptake from the soil solution. However, the functions of vascular transport systems controlling accumulation of sulfate in the cortical cells are not defined. As shown in this study, SULTR4;1-GFP expressed in the root tissue was far more abundant in the vasculature than in the cortex (Figure 2). From these observations, contribution of SULTR4;1 in modulating vascular storage of sulfate in the cortical cell is suggested to be limited. Besides the direct effect of the knockout of SULTR4;1 transporters in the vasculature, the uptake of sulfate to roots may increase to maintain the flux for xylem loading in the knockout mutant. Sulfate incorporated excessively to the root tissue may be allocated to the vacuoles of cortical cells and may eventually form a large storage of sulfate before entering the vasculature.

The characterization of the SULTR4-type vascular transporters demonstrated here provides a new scheme of internal balancing mechanisms of nutrient transport in the vasculature. The observed uniformity of the [35S] transfer to the shoot (Figure 7) may suggest that the step of xylem loading is additionally controlled to retain a tight control of long-distance translocation of sulfate particularly under conditions of limited sulfur supply; however, sulfate transporters facilitating the loading step have not been identified. In addition, for the complete understanding of the vascular sulfate transport systems, identification of influx facilitators capable of transporting sulfate into the vacuoles will be required.

METHODS

Plant Materials

Arabidopsis thaliana plants were grown under 16-h-light/8-h-dark cycles at 22°C and 60% relative humidity. GM medium (Valvekens et al., 1988) containing 1× MS salts (Wako Pure Chemical, Osaka, Japan) and 1% sucrose constituted the control condition. Sulfate-deficient medium was prepared by replacing sulfate salts in MS salts with equivalent chloride salts as described previously (Takahashi et al., 2000).

The sultr4;1 and sultr4;2 knockouts were obtained from the T-DNA insertion lines of the Arabidopsis Knockout Facility at the University of Wisconsin Biotech Center (http://www.biotech.wisc.edu/Arabidopsis/) (Krysan et al., 1999). Pooled DNA of T-DNA lines were screened by PCR using gene-specific primers: 4:1-F (5’-ATCATGTCCTACCGATCTCT-CAGCCTCAA-3’) and 4:1-R (5’-TCTCCACTGATAAAGCTGCTT-CAAC-3’) for SULTR4;1 and 4:2-F (5’-ATCTGCAACCCGCTTCA-TCTTCATCCTAC-3’) and 4:2-R (5’-TCGATACATCTACGTAGTGC-GTGAG-3’) for SULTR4;2. Integration of T-DNAs was confirmed by sequencing the PCR product of the T-DNA flanking regions. Single insertion of T-DNA in the mutant was confirmed by DNA gel blot hybridization. The sultr4;1 and sultr4;2 mutants were crossed to generate the sultr4;1 sultr4;2 double knockout. The F1 plants were self-fertilized, and the double mutant containing homozygous insertion of both T-DNAs was selected from the F2 population by PCR.

Complementation was performed by crossing the sultr4;1 sultr4;2 double knockout with the SULTR4;1-GFP transgenic plant line described below. Triple homozygous T-DNA insertions of sultr4;1::T-DNA; sultr4;2::T-DNA; and SULTR4;1-GFP were confirmed by PCR and the analysis of segregation of GFP fluorescence. The F2 plants containing homozygous T-DNA insertions in SULTR4;1 and SULTR4;2 were selected by PCR using gene-specific primers. Plants containing the SULTR4;1-GFP construct were selected by scanning the plants on a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA). Finally, lines #2c and #42a, showing fluorescence of GFP in all F4 progenies in the double knockout background, were selected for analysis.

Gene-GFP Fusion Constructs

The Arabidopsis SULTR4;1 genomic region that starts from position −2080 bp upstream of the transcription initiation site and terminates at the end of the coding sequence was amplified from ecotype Columbia genomic DNA by PCR. Amplification was performed using high-fidelity KOD plus DNA polymerase (Toyobo, Tokyo, Japan), and the nucleotide
sequence was checked to confirm the identity with the corresponding Arabidopsis genomic sequence (Arabidopsis Genome Initiative, 2000). Gene-specific primers 41-F-SalI (5' -GTGCACTCCACAGCCTGCCT-CACATACGTAG-3') and 41-R-BglII (5' -AGATTTTCTCCACTGTA-TAACATGGTCTTCTTCA-3') were used to create SalI and BglII sites on both ends. The SalI-BglII fragment of SULTR4;1 and the BamHI-EcoRI fragment of 3SS-1-sGFP (S65T) (Chiu et al., 1996) containing the GFP coding sequence and the nopaline synthase terminator were ligated and inserted between the SalI and EcoRI sites of pBI101 (Clontech, Palo Alto, CA), replacing β-glucuronidase and the nopaline synthase terminator. In the case of the SULTR4;2-GFP fusion, the SULTR4;2 genomic region that starts from the position –1983 bp upstream of the translation initiation site and terminates at the end of the coding sequence was amplified by PCR. Gene-specific primers 42-F-Spel (5' -ACTAGTAGATACAT-CAATGGCTGGCCAGACTGATC-3') and 42-R-NcoI (5' -CCATGG-I(5'-CATGGTAG-TATTTCTCTCTTGACAACTAGTTCTCTCTA-3') were used to create Spel and NcoI sites on both ends. The Spel-NcoI fragment of SULTR4;2 and the NcoI-EcoRI fragment of 3SS-1-sGFP (S65T) containing the GFP coding sequence and the nopaline synthase terminator were ligated and inserted between the XbaI and EcoRI sites of pBI101, as in the case of the SULTR4;1-GFP fusion. The binary plasmids were transferred to Agrobacterium tumefaciens GV3101 (pMP90) (Koncz and Schell, 1986) by the freeze-thaw method (Höfgen and Willmitzer, 1988). Arabidopsis plants were transformed according to the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on GM media (Valvekens et al., 1998). Transgenic plants were obtained in parallel using a 610-nm long-pass filter. Cell fluorescence of GFP in transgenic plants was observed under a BX61 microscope after staining with neutral red.

Microscopy and Imaging of GFP

Fluorescence of GFP in transgenic plants was observed under a BX61 microscope equipped with a FV500 confocal laser scanning system (Olympus, Tokyo) and a 505- to 525-nm band-pass filter (Olympus). Cell walls were stained by propidium iodide (Sigma, St. Louis, MO), submerging plant materials in 10 μg mL−1 solution for 1 min. For the staining of tonoplasts, plants were submersed in 20 μM FM4-64 (Molecular Probes, Eugene, OR) for 5 min, washed three times in water, and incubated for 18 h on the medium under dark conditions to let the dye sequester to the tonoplast. The fluorescence of propidium iodide and FM4-64 was observed under a confocal laser scanning microscope with a 560-nm long-pass filter (Olympus). Cross sections were prepared from root tissues embedded in 5% agar. Tissues were cut into 125-μm sections with a DTK-1000 microlicer (Dosaka, Kyoto, Japan) and observed under a confocal laser scanning microscope (Olympus). For in vivo quantification of GFP, SULTR4;1-GFP and SULTR4;2-GFP plants were scanned in a FluorImager M595 equipped with a 515- to 545-nm band-pass filter. Fluorescence of chlorophyll was scanned in parallel using a 610-nm long-pass filter.

Vacuole Isolation and Sulfate Measurement

Callus was isolated from the hypocotyls of etiolated seedlings of the knockout, wild-type, and SULTR4;1-GFP plants. The hypocotyl explants from etiolated seedlings were incubated on CIM medium (Valvekens et al., 1988) at 22°C under dark conditions, and the calli initiated were propagated on CIM medium under the same conditions. Protoplasts and vacuoles were isolated by modifying the methods described by Massonneau et al. (2000) and Shimaoka et al. (2004). Approximately 2 g of calli were incubated in 60 mL of enzyme solution containing MS salts, 600 mM sorbitol, and 10 mM Mes adjusted to pH 6.0 with Tris, 1 mM CaCl2, 1% cellulase Y-C (Kyowa Chemical Products, Kyoto, Japan), 2% cellulase ONOZUKA RS (Yakult, Tokyo), and 0.2% pectolyase Y-23 (Yakult). Enzymatic digestion was performed for 4.5 h at 32°C under continuous shaking at 100 rpm. After digestion, protoplasts were filtered once through a 1-mm mesh to remove cell debris. Purification of protoplasts and vacuoles was performed using medium B (30 mM Hepes adjusted to pH 7.2 with Tris, 30 mM potassium gluconate, 2 mM MgCl2, and 2 mM EGTA) (Shimaoka et al., 2004) on a basic sucrose/sorbitol gradient as follows: the filtrate of crude protoplasts was transferred to a 50-mL tube and underlaid with P1 medium (medium B supplemented with 400 mM sorbitol and 50% Percoll). After centrifugation at 180g for 10 min, the protoplasts above the P1 medium layer were collected to a new test tube. P1 medium was added to the protoplasts to a total volume of 6 mL and mixed gently. This was overlaid with 2 mL of P2 medium (medium B supplemented with 400 mM sucrose) and 2 mL of P3 medium (medium B supplemented with 133 mM sucrose and 267 mM sorbitol) and centrifuged first at 180g for 2 min and then at 1600g for 8 min. The protoplasts having the same gravity were collected from the interface between P2 and P3. One volume of purified protoplasts was mixed with the same volume of medium B and incubated on ice for 5 min to release vacuoles by hypotonic shock. Four milliliters of P2 medium was added to the resultant mixture of protoplasts and vacuoles, and then 1 mL of P3 medium, 1.5 mL of P4 medium (medium B supplemented with 100 mM sucrose and 300 mM sorbitol), and 0.5 mL of P5 medium (medium B supplemented with 400 mM sorbitol) were sequentially overlaid. The gradient was centrifuged first at 180g for 2 min and then at 1600g for 8 min. The purified vacuoles were obtained on the interface between P4 and P5. To confirm the intactness, purified vacuoles were observed under a microscope after staining with neutral red.

The isolated vacuoles were disrupted by ultrasonication and filtered through an Ultrafree-MC 5000 NMWL filter unit (Millipore, Bedford, MA) at 10,000g. Quantification of sulfate and calcium was performed by a HP30 capillary electrophoresis system (Agilent Technologies, Palo Alto, CA).

[35S]Sulfate Transport

The sulfate uptake experiments were performed according to the methods described by Maryama-Nakashita et al. (2004). Plants were grown on low sulfate MS agar medium containing 50 μM sulfate as a sulfur source and 1% sucrose. Arabidopsis seeds were germinated on 300-μm nylon mesh embedded vertically on the agar plates. After 10 d of culture, nylon mesh was pulled out from the agar plates and fixed on a square plastic frame placed on a Petri dish containing 50 mL of the MS liquid medium without sulfate. The roots were submerged in the liquid medium. Labelling was started by the addition of 50 μM (1.23 MBq/L) [35S] sodium sulfate (Amersham Biosciences, Piscataway, NJ). After 1 h of labeling, the roots were rinsed twice in 60 mL of nonradioactive MS liquid medium containing 50 μM sulfate. The roots were further incubated for 4 h in 75 mL of nonradioactive medium. The shoots and roots were excised and digested in 1 mL of 100 mM HCl for 1 h. Four milliliters of Ultima Gold scintillation cocktail (Perkin-Elmer, Boston, MA) was added, and the radioactivity was measured in a scintillation counter (Aloka, Tokyo).

Real-Time RT-PCR

The mRNA contents of SULTR4;1 and SULTR4;2 were determined by RT-PCR using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and the GeneAmp 5700 System (Applied Biosystems). Total RNA was extracted from Arabidopsis plants using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed on 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Specific amplification of SULTR4;1 and SULTR4;2 was performed using gene-specific primers 41-1-F (5′-TGCACACCTCCAAGACTGCT-CTCTTCTC-3′) and 41-1-R (5′-GATTGCGAGGCCACGATTTGGTTCCTCACT-3′) for SULTR4;1 and 42-2-F (5′-GATTCCACCTCTTCTGTACACTCACT-3′) for SULTR4;2.
and 4:2-1R (5'-TATGGGTAAGCCAGCTAATCC-3') for SULTR4;2. The mRNA contents were calibrated using purified SULTR4;1 and SULTR4;2 cDNAs as standards. Equality of RNA preparation was confirmed by constitutive expression of ubiquitin (accession number J05508) as described previously (Maruyama-Nakashita et al., 2004).

RT-PCR for Determination of Knockouts

Knockout of SULTR4;1 in the double knockout and in the complemented lines #2c and #42a was confirmed by RT-PCR using specific primers, 4:1-F (5'-AACGGATTCTCTTAAAGGTGCAAGGGTG-3') and 4:1-R (5'-TGTGATACAGATGATATGTTGATCT-3'), designed on the 5'- and 3'-untranslated regions of SULTR4;1. The endogenous SULTR4;1 mRNA was specifically amplified with these primer pairs and can be distinguished from the SULTR4;1-GFP transgene expression. Designed on both ends of the coding region, 4:1-F (5'-CACTGATCCAAGGGTGCAAGGGTG-3') and 4:1-R (5'-TGTGATACAGATGATATGTTGATCT-3') were used to compare the contents of SULTR4;1-GFP mRNA in #2c and #42a and SULTR4;1 mRNA in the wild type. To determine the knockout of SULTR4;2 mRNA, 4:2-F (5'-ATCTCTCCACCGCTTACACCCTCTCTCTCTCTCTCTC-3') and 4:2-R (5'-TGCATTCAACCTTCAGTTAGGTGCATGAG-3') were used. Equality of RNA preparation was confirmed by constitutive expression of α-tubulin using gene-specific primers, TUB-F29 (5'-TCGAAAATTAGGTTCTTCTAGGAAAC-3') and TUB-R29 (5'-CCAAGACATATTTCAGGATTAAACA-3'), as described previously (Yoshimoto et al., 2002). PCR was performed by ExTaq DNA polymerase (Takara, Tokyo) for 24 cycles where the cDNAs are exponentially amplified. PCR products were stained with SYBR green II (Takara) and detected using a FluorImager 595 with a 515- to 545-nm band-pass filter (Molecular Dynamics). Signals obtained by this method (Figure 5) were fairly comparable with the mRNA contents determined by real-time PCR (Figure 3).

Sequence data from this work have been deposited with the EMBL/GenBank data libraries under accession numbers AB008782, AB052775, BE800569, BF003985, AF493793, AJ416461, J05508, and A965651.

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