Sulfate transporters in plants represent a family of proteins containing transmembrane domains that constitute the catalytic part of the protein, and a short linking region that joins this catalytic moiety with a carboxyl terminal STAS domain. The STAS domain resembles an anti-sigma factor antagonist of *Bacillus subtilis*, which is one distinguishing feature of the SLC26 transporter family; this family includes transporters for sulfate and other anions such as iodide and carbonate. Recent work has demonstrated that this domain is critical for the activity of *Arabidopsis thaliana* sulfate transporters, and specific lesions in this domain, or the exchange of STAS domains between different sulfate transporters, can severely impair transport activity. In this work we generated a *Saccharomyces cerevisiae* expression library of the *A. thaliana* Sultr1;2 gene with random mutations in the linking region-STAS domain, and identified STAS domain lesions that altered Sultr1;2 biogenesis and/or function. A number of mutations in the β-sheet that forms the core of the STAS domain prevented intracellular accumulation of Sultr1;2. In contrast, the linking region and one surface of the STAS domain containing amino termini of the first and second α-helices have a number of amino acids critical for the function of the protein; mutations in these regions still allow protein accumulation in the plasmamembrane, but the protein is no longer capable of efficiently transporting sulfate into cells. These results suggest that the STAS domain is critical for both the activity and biosynthesis/stability of the transporter, and that STAS sub-domains correlate with these specific functions.

Sulfate transporters, designated Sultr (*Sultr* for the gene) in plants, are part of the SLC26 family of anion transporters that also includes iodide, chloride and carbonate transporters and the motor protein prestin, which resides in the outer hair cells of the cochlea (1). SLC26 family proteins are predicted to have ten to fourteen transmembrane domains (TMDs) and a carboxyl (C)-terminal extension, designated STAS domain (2), that protrudes into the cytosol of the cell (3,4). The STAS domain is joined to the catalytic domain of transporters by a linking (L) region, varies in length, and is not highly conserved relative to the catalytic domain. Some characteristics that make SLC26 transporters distinct from each other are likely to reside within unique features of L-STAS domains.

Interestingly, the STAS domain of SLC26 transporter proteins resembles the bacterial anti-sigma factor antagonist SpoIIAA of *Bacillus* (2). SpoIIAA is a transcription factor that associates with the anti-sigma factor SpoIIAB; this association causes release of the sigma factor from SpoIIAB, triggering sporulation-specific transcription (5). SpoIIAB is also a kinase that can phosphorylate and inactivate SpoIIAA. Recently, STAS domains have been shown to be part of polypeptides other than anion transporters (6), suggesting that STAS sequences may serve a general regulatory function.

STAS domains of SLC26 transporters appear to play a role in the function/regulation of transport activity since mutations that alter this domain can cause loss of function resulting in the diseases diastrophic dysplasia, Pendred syndrome and congenital chloride diarrhea (7-12). Recent work, using a heterologous *Saccharomyces cerevisiae* system, has demonstrated that the STAS domain is critical for the activity of the *Arabidopsis thaliana* sulfate transporter Sultr1;2 (13,14). When the L-STAS region of the sulfate transporter was eliminated, most of the truncated protein either never reached the *S. cerevisiae*
plasmamembrane and was rapidly degraded, either en route to or once associated with the plasmamembrane. In contrast, if the STAS domain was deleted (L region maintained), the transporter integrated into the plasmamembrane, but the integrated protein had no detectable uptake activity. Studies of the DRA (down regulated in adenoma) Cl\(^{-}\)-HCO\(_3\)^{-} antiporter (SLC26A3) also demonstrated a STAS domain requirement for transport function (15). Furthermore, altering A. \textit{thaliana} Sultr1;2 for codons encoding two cysteines or a threonine of the STAS domain (the latter is analogous to the phosphorylated serine of SpoIIAA) resulted in a polypeptide no longer able to rescue the phenotype of a \textit{S. cerevisiae} sulfate transporter mutant (14). These results suggest that the STAS domain contributes to both the catalytic activity and biogenesis of Sultr1;2, and probably has analogous functions in other SLC26 proteins.

The control of ion homeostasis in cells is likely to involve interactions among transporters, ion channels, regulatory proteins, proteins involved in ion assimilation and sequestration, and concentrations of ions and perhaps other small molecules in the cytoplasm and/or extra-cellular environment. For SLC26 family proteins, the STAS domain appears to participate in some environment. For SLC26 family proteins, the STAS domain appears to participate in some aspects of homeostatic control. Recent studies with the DRA Cl\(^{-}\)-HCO\(_3\)^{-} antiporter have demonstrated the importance of STAS domain interactions with the regulatory sequence of the cystic fibrosis transmembrane conductance regulator, a cAMP-regulated Cl\(^{-}\) channel. This interaction modulates the activity of both the transporter and the channel (16).

To elucidate protein domain function, the generation and characterization of mutants can be invaluable. \textit{S. cerevisiae} has served as a heterologous expression system for many eukaryotic genes, and mutant strains have been used to study the catalytic activity and kinetic features of several plant sulfate transporters (13,14,17-20). In this study we generated a yeast expression library of the \textit{A. thaliana} Sultr1;2 gene that had random mutations in the L-STAS domain and selected individual, mutated \textit{Sultr1;2} genes by transforming them into CP154-7B, which is null for the two major \textit{S. cerevisiae} sulfate transporters (21). Transformants were analyzed for their ability to grow on SO\(_4\)^{2-} as a sole sulfur (S) source, their ability to transport SO\(_4\)^{2-}, and their capacity to accumulate the altered Sultr1;2 polypeptide and properly target it to the plasmamembrane. Lesions in the L-STAS encoding region of altered Sultr1;2 were characterized to determine which amino acids were critical to the functionality/biogenesis of the Sultr1;2 polypeptide. The consequences of mutations in the STAS domain are discussed and interpreted with the aid of a computer-derived structural model based on the crystal structure of SpoIIAA (14,22).

**EXPERIMENTAL PROCEDURES**

\textbf{Yeast strains -} The \textit{S. cerevisiae} strain used in this study was CP154-7B (MAT\(\alpha\), ade2, his3, leu2, trp1, ura3, sul1::LEU2, sul2::URA3), which is null for both the \textit{Sul1} and \textit{Sul2} sulfate transporter genes (21).

\textbf{Construction and screening of a library of Sultr1;2 containing random mutations in the STAS domain -}

1) \textit{Intermediate construct (pIMRNDM):} Plasmids used for expression of the C-terminal, hemagglutinin (HA)-tagged sulfate transporters were constructed in pYX222x (13) (Fig. 1A). This plasmid (a) was digested with EcoRI and BamHI and the resulting linearized vector (a') in which the region encoding the catalytic domain plus part of the region encoding the L-STAS domain (to the BamHI site) was deleted. The deleted plasmid was ligated with a PCR-amplified EcoRI-BamHI fragment containing the sequence encoding Sultr1;2, extending from the initiator Met1 to Phe478 (the end of the catalytic region; this fragment is designated 'b' in Fig. 1A). The primer set used to create b is forward 5' atg tcg gaa ttc atg tca aga gag ctc gtg gac 3' and reverse 5' ata eco ri tcc aat 3'. The resulting intermediate construct, pIMRNDM, was used to clone a randomly mutagenized L-STAS encoding region. For this purpose, we introduced a BamHI site at nucleotides 1495-1500 in the Sultr1;2 cDNA (GenBank accession no.AB042322), which resulted in a V479G substitution in TMD12 of the catalytic region of the transporter. This amino acid is not conserved among \textit{S. cerevisiae}, plant and human SLC26 family members (it could be V, L, G, A, I, or F), and this region of the C-terminal TMD does not appear to directly influence substrate transport.
2) Construction of Sultr1;2 library with mutated STAS domains: During the amplification of the Sultr1;2 gene, we identified an allele of the Sultr1;2 gene with the missense mutation I553V (a to g at position 1717) that arose as a consequence of the natural error rate of Taq DNA polymerase. This mutation eliminated the BamHI site in the Sultr1;2 cDNA. The CP154-7B strain transformed with this allele (WT) was rescued for the methionine auxotrophy phenotype and exhibited \( \text{SO}_4^{2-} \) transport activity similar to that of CP154-7B harboring the unaltered Sultr1;2 gene (WT; Fig. 1B). The Sultr1;2 cDNA encoding the I553V lesion was used as a template for PCR-random mutagenesis; this enabled us to exploit the BamHI sites at the amino (N)-terminal end of the L-STAS domain for digesting (with BamHI/SalI) and cloning of PCR fragments into pMRNDM deleted for the L-STAS region of Sultr1;2. Random mutagenesis of the L-STAS encoding region was performed by PCR amplification of the cDNA using the DiversityKit (Clontech, Mountain View, CA) and the primer set RNDMFB 5' atc gga tcc gta ctt ctt att gcc gtc tcg 3' and RNDMRS 5' act aag tct ccc gac ctt gtt gga gag ttt tgg acg gca 3'. Two conditions with different MnSO\(_4\) concentrations (320 \( \mu \)M and 640 \( \mu \)M, for 'mild' and 'severe' mutagenesis, respectively) were used to generate the mutagenized libraries. The mildly mutagenized library had 2-3 mutation per 1,000 bp while the severely mutagenized library had 4-6 mutations per 1,000 bp. The PCR fragment of the mutated L-STAS region (c in Fig. 1A) was digested with BamHI and SalI and cloned into pMRNDM digested with the same enzymes (c' in Fig. 1A).

3) Selection and growth of transformants: The cDNA L-STAS mutagenized library was transformed into CP154-7B by the lithium acetate method (23) and transformants were selected for histidine auxotrophy. Each transformant was streaked onto low \( \text{SO}_4^{2-} \) SD medium (500 \( \mu \)M \( \text{SO}_4^{2-} \), -met -his) and high \( \text{SO}_4^{2-} \) SD medium (40 mM \( \text{SO}_4^{2-} \), 143 \( \mu \)M methionine, -his) to test for \( \text{SO}_4^{2-} \)-dependent growth. Growth of the transformants was monitored at 30°C for 2-3 d. DNA fragments coding for the L-STAS domains of the various Sultr1;2 alleles were amplified by colony PCR using a primer set that annealed to sequences bordering the L-STAS encoding region (forward 5' c atc ctc gca get atc atc at 3' and reverse 5' ctc agt tag cta get gag 3') and the amplified fragment was sequenced.

**RESULTS**

Generating mutagenized L-STAS of Sultr1;2 - Random mutations were introduced into the Sultr1;2 sequence encoding the L-STAS domain (from the end of TMD12 to the end of the
polypeptide; described in EXPERIMENTAL PROCEDURES). The mutant Sultr1;2 cDNA library was transformed into S. cerevisiae CP154-7B (21) and transformants tested for methionine-independent, SO$_4$$^{2-}$-dependent growth on solid medium (Fig. 2A). This assay provided an evaluation of the effectiveness of the introduced Sultr1;2 gene in rescuing the methionine auxotrophy phenotype of CP154-7B. Sultr1;2 alleles carried by transformants unable to grow in the absence of methionine were mostly from the 'mild condition' mutagenized library (except for alleles 'Y517C+V616E', 'N545I' and 'S588R') since the 'severe condition' mutagenized library often gave multiple mutations, making it difficult to interpret the phenotypes; these alleles were categorized as 'nonfunctional'. We also identified 'functional' alleles in transformants for which the mutated Sultr1;2 was able to rescue the methionine auxotrophy phenotype of CP154-7B (Fig. 2A). These functional alleles were isolated using the severe condition mutagenized library since the majority of changes in functional alleles identified from the mild mutagenesis library were silent (the amino acid sequence was not altered). Colony PCR was performed to amplify DNA fragments encoding the L-STA domain of Sultr1;2 in transformants, and amplified fragments were directly sequenced to identify mutated bases. For nonfunctional mutants, only alleles containing a single missense mutation were chosen for further analyses, except for the allele with both Y517C and V616E substitutions. Table 1 shows specific changes in STAS domain sequences from transformants expressing functional or nonfunctional Sultr1;2, provides a qualitative view of accumulation of Sultr1;2 in cells, and indicates whether or not the transporter localizes to the plasmamembrane (see below). A DNA fragment encoding the L-STA peptide from each mutant allele was re-cloned into plMRNDM to generate full-length Sultr1;2, the ligated insert was sequenced to insure that no modification occurred during the cloning procedure, and the plasmid with the recombinant gene was transformed into CP154-7B; re-transformation was performed to confirm that the phenotype was solely a consequence of a lesion in the L-STA region of the polypeptide.

The previously characterized A. thaliana sell-3, sell-7 and sell-8 mutants have lesions in the L-encoding region of Sultr1;2 (G509E, P503L, 1511T, respectively); these lesions make the plant resistant to selenate (27). SO$_4$$^{2-}$ uptake rates in the sell-8 mutant of A. thaliana were reported to be lower than in wild-type plants (27). None of these altered Sultr1;2 alleles could rescue the methionine auxotrophy phenotype of CP154-7B, as shown in Fig. 2A (compare sulfate 40 mM with sulfate 500 µM, top row of each grid). These mutant alleles do not have the lesions incorporated into the constructs for cloning purposes (V479G and I553V).

Phenotypic analysis of S. cerevisiae cells expressing Sultr1;2 - SO$_4$$^{2-}$-dependent growth characteristics of strains newly transformed with plasmids containing WT, WT' and the randomly mutagenized Sultr1;2 alleles were monitored for growth in high and low SO$_4$$^{2-}$ medium. CP154-7B transformed with unaltered Sultr1;2 or with Sultr1;2 allele with the V479G and I553V changes doubled in approximately 2.8 h in SD–his medium containing 40 mM SO$_4$$^{2-}$ (high SO$_4$$^{2-}$ medium, Fig. 2B, left panel, WT and WT'), which is close to that of wild-type S. cerevisiae cells (W303 cells, not shown). All nonfunctional transformants grew in high SO$_4$$^{2-}$ medium with doubling times from 3-4 h (not shown). In most cases, growth rates were only slightly slower than that of cells harboring unaltered Sultr1;2. While the doubling time of CP154-7B with unaltered Sultr1;2 on low SO$_4$$^{2-}$ medium was ~5 h (Fig. 2B, right panel, WT and WT'), all nonfunctional transformants showed no, or extremely slow growth in low SO$_4$$^{2-}$ liquid medium (not shown). In contrast, doubling times of CP154-7B with functional Sultr1;2 alleles ranged from 5 to nearly 30 h on low SO$_4$$^{2-}$ medium (Fig. 2B, right panel), and from 3-4 h on high SO$_4$$^{2-}$ medium (Fig. 2B, left panel).

Accumulation of nonfunctional Sultr1;2 - Sultr1;2 protein accumulation in nonfunctional transformants was evaluated in whole cell extracts by western blot analyses using antibodies specific for the HA tag (positioned at the C-terminus of the Sultr1;2 protein). The fusion of the HA epitope to the C-terminus of the introduced protein does not affect the ability of Sultr1;2 to rescue the CP154-7B mutant phenotype (13). An antibody against Pma1 was used as a control to identify the plasmamembrane fraction and to normalize the HA signal. The level of the HA signal relative to
that of Pma1 is shown in Fig. 3A and quantified in Fig. 3B. Some transformants with nonfunctional Sultr1:2 had significantly less Sultr1:2 protein than cells transformed with WT’ Sultr1:2. Transformants for which the ratio of Sultr1:2 to Pma1 signal was 20% or less relative to that measured in transformants harboring the WT’ Sultr1:2 construct were S546P, Q573P, I577N, E578K and L607W. Low level protein accumulation in these strains is likely to explain their inability to rescue SO4\(^{2-}\) uptake; its Vmax is 10% of WT’, while protein accumulation of Sultr1:2 in these strains and their ability to take up SO4\(^{2-}\) are shown in Fig. 5A and B, respectively. We also calculated a Km and Vmax for the uptake of SO4\(^{2-}\) by each strain, as shown in Table 2. Growth rates of individual strains varied and generally showed a positive correlation with the Vmax for SO4\(^{2-}\) uptake (Table 2; growth rate data are from Fig. 2B); the growth of E560G, which has a large standard deviation, is the only exception. The decreased rate of SO4\(^{2-}\) uptake in many of the strains harboring functional Sultr1:2 mutant alleles was mostly explained by decreased protein accumulation, with a few exceptions. T527A+L615M+T637A and A540S+V549I+I608N show decreased SO4\(^{2-}\) uptake relative to WT’ (Fig. 5B) while protein accumulation (Fig. 5A) and distribution in plasmamembrane is comparable to that of WT’ (data not shown). V549F also shows very limited uptake; its Vmax is 10% of WT’, while protein accumulation is approximately half of WT’.

**DISCUSSION**

This study focused on determining residues of the L-STAS domain critical for biogenesis and functionality of Sultr1:2. We performed random mutagenesis of the Sultr1:2 sequence encoding the L-STAS domain, replaced the unaltered L-STAS encoding region with a population of mutagenized L-STAS encoding regions and identified lesions that resulted in both functional and nonfunctional Sultr1:2 polypeptides (Table 1, Fig. 6). Among the alleles encoding nonfunctional Sultr1:2, some accumulated little protein (Class I), most of which was unable to properly integrate into the plasmamembrane, and others accumulated normal protein levels with seemingly proper
plasmamembrane localization (Class II). We also identified alleles with lesions in the L-STAS domain that could complement the CP154-7B phenotype (Class III), although usually not fully restore the growth rate in low SO4\textsuperscript{2−} medium.

To help interpret the effect of amino acid substitutions in mutated Sultr1;2, we used a STAS domain model, based on the SpoIIAA crystal structure (14). The STAS domain is thought to consist of four β-strands, forming a β-sheet, surrounded by five α-helices, as shown in Fig. 7. The β-sheet in association with hydrophobic surfaces of the α-helices, forms a hydrophobic core that is not readily accessible to the external medium. In contrast, the externally exposed surfaces of the α-helices, and the loops between α-helices and β-strands are predicted to be exposed and available for interactions with molecules in the solution environment. Although the L region was shown to be of importance for transporter function, it could not be modeled since it is not very similar to other known structures in the Protein Data Base (PDB).

**Mutations that affect biogenesis of Sultr1;2**

β-sheet - Class I mutations diminished Sultr1;2 protein accumulation in the plasmamembrane (surrounded by blue boxes in Fig. 6) and some Class III mutations decreased protein accumulation, with some activity still retained (surrounded by black boxes in Fig. 6). Interestingly, most of the lesions for which the protein shows little accumulation are in or immediately contiguous to β-strands 3 and 4 that are part of the β-sheet structure (Figs. 6 and 7); the mutation in or immediately adjacent to β1 and β2 were nonfunctional, but the protein accumulated in the plasmamembrane.

The Q573P, I577N and E578K substitutions in β3 all alter the charge/hydrophobic character of the domain (Pro potentially causes a kink in the polypeptide); polypeptides with these amino acid substitutions do not accumulate in CP154-7B (Fig. 3). As shown in the logo diagram (Fig. 6), I577 and E578 are highly conserved while Q573 is not. Substitution of the conserved, hydrophobic L607 to W in β4 also caused marked reduction in polypeptide accumulation (Fig. 3).

Some mutations predicted to be in the β-sheet vicinity, as shown in Fig. 7, also make the polypeptide accumulate to a lesser extent than WT Sultr1;2. Sultr1;2 with N611K or N611S (end of β4) substitution cannot rescue the CP154-7B phenotype; these strains exhibit a significant amount of protein accumulation (considered to be Class II), but lower than in WT, suggesting that the N611K/S lesions negatively affect protein biogenesis as well as transport function. N611 is analogous to N81 in SpoIIAA of *B. sphaericus*. The side chain of N81 forms a hydrogen bond with A102 in SpoIIAA, extending the β-sheet structure (22). Thus, N611 of Sultr1;2 STAS may form an analogous hydrogen bond with L636 (corresponds to A102 in SpoIIAA) and thereby help stabilize the β-sheet structure of the polypeptide. CP154-7B transformed with Sultr1;2 with the G630V substitution (between α3 and α4, Class III), predicted to be in the region corresponding to β5 in SpoIIAA, exhibited reduced SO4\textsuperscript{2−} uptake activity (Fig. 5B), which also correlated its reduced protein accumulation (Fig. 5A and Table 2), suggesting that the G630V substitution (colored in grey in Fig. 7) also affects protein biogenesis/turnover rather than function.

In addition, the V537A and V537D mutations, considered Class II, are in the C-terminal region of β2 (Fig. 6, surrounded by purple boxes); the former lesion did cause significant reduction in polypeptide accumulation while the polypeptide level in the latter remained high (Fig. 3). The V537D substitution appears to result in a defect in plasmamembrane localization of Sultr1;2 (Fig. 4). These results suggest that V537 may be involved in the biogenesis of Sultr1;2 as well as transport function. Next to V537, a D538G substitution generated a Class III mutant; the Sultr1;2 protein accumulates to low levels in CP154-7B with low SO4\textsuperscript{2−} transport activity. However, this allele has two other lesions, R550G (α1) and I617M (α3). The Sultr1;2 protein with a single I617M substitution accumulated in CP154-7B transformants to a level comparable to that of the wild type (Fig. 4). These results suggest that I617M substitution accumulated in CP154-7B transformants to a level comparable to that of the unaltered protein and exhibited near normal levels of SO4\textsuperscript{2−} transport activity. Thus D538G and/or R550G are likely to be responsible for the defect in protein biogenesis.

Together, these findings demonstrate that lesions within or close to the STAS domain β-sheet of Sultr1;2 can significantly change protein stability or biogenesis; this appears to be
especially true when the lesions are in β3 and β4. The β-sheet seems to serve as a core structure of the STAS domain and lesions within this structure may disrupt proper STAS packaging, which could destabilize the entire Sultr1;2 polypeptide.

Other destabilizing lesions - While most modifications of the L region do not significantly alter protein accumulation, the I496N lesion is a notable exception; this mutant polypeptide accumulates to approximately 25% of the level of unaltered Sultr1;2 (the lowest level among Class II mutant alleles), with some being localized to the plasmamembrane (Figs. 3 and 4). Hence, the linker region may play some role in the biogenesis/stabilization of Sultr1;2. Recently, the motor protein prestin (SLC26A5), altered at a position corresponding to I496 in Sultr1;2 (V499G in prestin), with an additional Y501H change, exhibited normal protein accumulation with diminished functionality (29). Position I496 is represented by hydrophobic amino acids in most sulfate transporters (Fig. 6; I496N mutations is surrounded by a purple box).

The S546P substitution is the only Class I modification characterized that is in an α helix (Table 1). Introduction of a Pro residue in this α helix may cause extreme conformation perturbation by introducing steric constraints into the protein and disrupting the α1 structure, which in turn could lead to a marked decrease in protein accumulation. Furthermore, CP154-7B expressing Sultr1;2 with a E560G lesion exhibits slow growth and low rates of SO₄²⁻ uptake, which correlates with reduced protein accumulation (Table 2). These results suggest a potential role of E560 in protein biogenesis/stability rather than in function (it is colored in grey in Fig. 7). E560 is highly conserved among A. thaliana Sultr polypeptides and part of an acidic cluster located at the C-terminal end of α1, or possibly at the beginning of the variable loop (2).

Mutations that affect activity of Sultr1;2 but not biogenesis

Class II mutations are defined as lesions that eliminate the functionality of Sultr1;2 although the aberrant polypeptide accumulates to relatively normal levels in the plasmamembrane (surrounded by red boxes in Fig. 6). These lesions are mostly in the L region and the protein surface formed by the N termini of α-helices and some contiguous sequences. These subdomains appear to be crucial for maintaining transporter activity.

L region - Most of lesions in the L region of Sultr1;2, including P503L, G509E, I511T, I511N, Y517C+V616E and R518S, caused little change in either protein abundance or plasmamembrane localization, suggesting an involvement of the L region in sulfate transporter function and not biogenesis. All of these positions are highly conserved among SLC26 family members. P503 is in a predicted loop that may separate two β-strands or a β-strand and α-helix, based on various algorithms used for protein structure predictions [PELE Protein Structure Prediction at http://workbench.sdsc.edu], and is fully conserved in SLC26 family members (Fig. 6). This Pro may undergo cis-trans isomerization, which may specify the physical distance between the catalytic moiety of the transporter and the STAS domain to, in some unknown way, regulate transport activity; substitution by a Leu would markedly change this distance.

Specific STAS surface critical for activity - Sultr1;2 with individual Y542C, F543Y or N545I substitutions appeared completely nonfunctional even though there was normal accumulation of the polypeptide in the plasmamembrane (Figs. 2, 3 and 4). These residues are located in the N-terminus of α1 and the loop adjacent to α1 (Figs. 6 and 7), regions that are well conserved in transporters of the SLC26 family (Fig. 6). It is noteworthy that a Cys is observed at the position analogous to Y542 of Sultr1;2 only in low affinity type sulfate transporters (Sultr2;1 and Sultr2;2), and a Tyr at the position analogous to F543 of Sultr1;2 in human SLC26A proteins, but not in those of plants (Fig. 7). This suggests strict Tyr and Phe requirements at these positions in some SLC26A transporter sub-types. In addition, Sultr1;2 with three amino acid substitutions, A540S, V549I and I608V, accumulates to normal levels in transformed CP154-7B, but has reduced SO₄²⁻ uptake activity (Fig. 5 and Table 2, Class III). Although it is not known which among the three substitutions leads to reduced transport activity, A540S is most likely to have the greatest effect on the structure/function of the polypeptide since the V549I and I608V substitutions are likely to have little effect on protein structure/activity because of the highly similar character of V and I (therefore, a red box was placed around A540S in
Fig. 6). Position 540 is occupied by a non-polar amino acid (Pro, Ala, Leu) in most SLC26A family members (Fig. 6). Substitution by Ser, which is polar, may disturb molecular interactions required for function. The results presented above suggest that the N-terminus of α1 plus the adjacent loop are critical for maintaining Sultr1;2 function (not accumulation or localization). This conclusion is also supported by the finding that the I544N allele of human DRA (HCO3−-Cl− exchanger) is functionally inactive and causes congenital chloride diarrhea (15,30); I544 in human DRA corresponds to I541 in Sultr1;2.

The polypeptide surface defined by the N terminus of α1 can be extended toward the top end of the β-sheet structure and the N-terminus of α2; these regions also contain Class II lesions and the side-chains of all of these residues are in close proximity in the 3D structural model (delimited by dotted lines in Fig. 7). The Q522K and Y523H substitutions, at the N-terminus of β1 (Fig. 6), result in accumulation of nonfunctional Sultr1;2 in the plasmamembrane (Figs. 3 and 4). Sultr1;2 with V537A/D (C-terminal end of β2) or S588R mutations (N-terminal end of α2) are also non-functional; these mutant proteins accumulate in cells to a somewhat lower level than the WT’ protein (Figs. 3 and 4). A T587A substitution (potentially a phosphorylation site) was previously shown to eliminate Sultr1;2 activity and is a potential site of phosphorylation (14). The S588, T587 and D586 side chains have the potential to form charge-dipoles, similar to the interactions demonstrated for SpoIIAA (22).

Overall consideration of the polypeptide structure predicts that residues Q522, Y523, V537, A540, Y542, F543, N545 T587, S588 are located on the same STAS surface (see Fig. 7). Hence, lesions on this surface appear to generate a protein with severely compromised function. This STAS surface is analogous to a region of SpoIIAA that allows SpoIIAB binding and subsequent SpoIIAA phosphorylation (31). While there is no evidence of phosphorylation of STAS domains of SLC26A family proteins, accumulation of critical amino acid residues along the STAS surface associated with protein interactions in SpoIIAA raises the possibility of molecular interactions that affect sulfate transporter activity in this analogous STAS sub-domain. Recently preliminary results have demonstrated an interaction between L-STAS regions (these regions appear to form a homomeric oligomer), and that both the L and the STAS domains are involved in this interaction; more work is required to determine the precise requirements for this interaction.

Furthermore, there seems to be a requirement for a Val at position 549 of Sultr1;2 since the V549F substitution (toward the middle of α1) shows only residual transporter activity, although the protein accumulates to nearly the same extent as the WT’ protein (Fig. 5) and is localized in plasmamembrane (data not shown). A substitution by Phe, another hydrophobic amino acid, but with a bulky side chain, causes a loss of activity. Interestingly, Phe is present at this position in some human but not in plant SLC26A proteins (Fig. 6). Examination of the predicted 3D structure of the Sultr1;2 STAS domain suggests that V549 is either part of or contiguous to the putative interacting surface discussed above (see Fig. 7) and that a V549F substitution may distort the surface, compromising specific interactions.

The results for some mutated proteins are more difficult to interpret because of the presence of multiple lesions. The T527A(β1) + L615M(α3) +T637A (between α3 and α4) strain accumulates Sultr1;2 to approximately the same level as that of WT’, but has approximately 30% lower SO42− uptake activity (Fig. 5), suggesting that none of these changes significantly alters Sultr1;2 biogenesis, but that they have some effect on transport function.

Lesions that do not affect biogenesis or function

Although ClassIII mutations were identified in order to find residues of the STAS domain that were not essential residues for Sultr1;2 activity, most mutations in this class decreased protein accumulation and/or function to some extent, except for Q521L and I617M. Sultr1;2 with a Q521L or I617M substitution rescued the CP154-7B growth (Fig. 2) and SO42− transport (Fig. 5B and Table 2) phenotypes to the same extent as WT’. Q521 is at the most C-terminal residue of the L region, I617 is in α3, and neither of them is highly conserved among family members (Fig. 6; these mutations are surrounded by black boxes). These results suggest that thre is no absolute requirement for Gln and Ile at these positions. Other mutations identified within α3 include P614S, L615M, and V616E (Fig. 6). Since the
alleles with these mutations all contain other lesions, definitive conclusions on the importance of α3 are difficult to draw at this time.

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**FOOTNOTES**

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1The abbreviations used are: TMD, trans-membrane domain; C-terminal, carboxyl-terminal; L region, linking region; DRA, down regulated in adenoma; S, sulfur; HA, hemagglutinin; PCR, polymerase chain reaction; DTDST, diastrophic dysplasia sulfate transporter; N-terminal, amino-terminal; OD600, optical density at 600 nm; Pma1, plasmamembrane H+-ATPase 1; PDB, Protein Data Base; DW, dry weight.

**FIGURE and TABLE LEGENDS**

**Table 1. Summary of mutations analyzed in this work.** *Class I* includes alleles of the mutated Sultr1;2 gene for which the protein accumulates to significantly lower levels (less than 20%) than that of the unaltered Sultr1;2 polypeptide, but does not rescue the methionine auxotroph phenotype of CP154-7B. *Class II* includes alleles of mutated Sultr1;2 gene for which the protein is expressed and accumulates in the plasmamembrane, but does not rescue the methionine auxotroph phenotype of CP154-7B. *Class III* includes alleles of mutated Sultr1;2 gene for which the protein confers varying SO$_4^{2-}$ uptake activity to CP154-7B, but the transformants may not grow as rapidly in low SO$_4^{2-}$ medium as CP154-7B transformed with the unaltered Sultr1;2 gene. Complementation of the growth phenotype of CP154-7B on SO$_4^{2-}$ is based on data shown in Fig. 2: -, no growth on SO$_4^{2-}$; + and ++, slow growth on SO$_4^{2-}$ (+ doubling time >10 h; ++ 6-10 h in low SO$_4^{2-}$ medium); ++++, growth on SO$_4^{2-}$ is comparable to that of CP154-7B transformed with the unaltered Sultr1;2 gene. Protein accumulation measurements are based on immunological signals from the fused C-terminal HA epitope relative to the Pma1 signal, as shown in Fig. 3: +, lower than 20 % of wild type; ++, between 20% and 70 % of wild type; ++++, higher than 70 % of wild type. Accumulation in the plasmamembrane is based on data presented in Fig. 4.

**Table 2. Phenotypes associated with Class III Sultr1;2 alleles.** Vmax and Km are based on data shown in Fig. 5B, relative protein accumulation is from Fig. 5A, and doubling time in low SO$_4^{2-}$ liquid medium is from Fig. 2B. Standard deviations are in parenthesis.

**Fig. 1. A,** Schematic of steps in the construction of the full-length Sultr1;2 gene with random mutations in the L-STAS-encoding region. (a) unaltered Sultr1;2 cDNA in the vector pYX222x. (a') vector fragment from (a) after digestion with *Eco*RI and *Bam*HI. (b) PCR amplified portion of Sultr1;2 cDNA encoding Met1 to Phe478 (catalytic domain) digested with *Eco*RI and *Bam*HI. (c) pIMRNDM, intermediate plasmid generated by the ligation of (a') and (b). (c') vector fragment from (e) after digested with *Bam*HI and *Sal*I. (d) same as (a) except for mutation from a to c at 1717 (GenBank accession no. AB042322); this mutation eliminated a *Bam*HI site in the STAS domain coding sequence. (e) PCR amplified fragment encoding the L-STAS domain (480S to 653V) with random mutations. Fragments (c') and (e) were ligated to generate
the library of \textit{Sultr1;2} cDNA with random mutations in the L-STAS-encoding region. \textbf{B}, \textit{SO}_4^{2-} uptake rate in CP154-7B cells expressing wild-type Sultr1;2 (WT) and Sultr1;2 with V479G and I553V mutations (WT'). Assays were performed as described in \textbf{EXPERIMENTAL PROCEDURES} and uptake rates were normalized to dry weight (DW).

\textbf{Fig. 2.} Growth of CP154-7B harboring the unaltered \textit{Sultr1;2} gene or \textit{Sultr1;2} with random mutations in the L-STAS domain. \textbf{A}, CP154-7B transformed with constructs harboring the unaltered \textit{Sultr1;2} gene (WT), \textit{Sultr1;2} with two mutations introduced for cloning purposes (WT'), mutated versions of \textit{Sultr1;2}, and an empty vector plasmid (vector). The descriptions of amino acid changes caused by the introduced mutations are used as allele names. Transformed lines were grown on solid SD -his medium containing 40 mM \textit{SO}_4^{2-} (left panel) or SD -his -met medium containing 500 \( \mu \text{M} \textit{SO}_4^{2-} \) (right panel), at 30\( ^\circ \text{C} \) for 2 d. \textbf{B}, Doubling times of CP154-7B expressing functional Sultr1;2 in liquid medium containing 40 mM and 500 \( \mu \text{M} \textit{SO}_4^{2-} \). The bars, representing the doubling time in wild-type and mutant strains, are marked with 'a' (at end of bar) when the growth rates were significantly different from those of WT' (p<0.02, two-tailed Student's t-test). The doubling time of each transformant is an average value obtained from at least three separate experiments.

\textbf{Fig. 3.} Sultr1;2 protein levels in strains carrying mutant alleles of \textit{Sultr1;2}. \textbf{A}, Lysates were prepared from CP154-7B expressing various mutated forms of Sultr1;2. The polypeptides of the lysates were resolved by SDS-PAGE, transferred to PVDF membranes, and the Sultr1;2 and Pma1 proteins were detected using antibodies, as described in \textbf{EXPERIMENTAL PROCEDURES}. \textbf{B}, Signal strengths of antibody reactions were quantified using Image J (http://rsb.info.nih.gov/ij/) and those for Sultr1;2 (anti-HA antibodies) were normalized with respect to those for Pma1 (anti-Pma1 antibodies). *Allele “Y517C” also has a V616E substitution.

\textbf{Fig. 4.} Subcellular localization of mutant Sultr1;2 polypeptides. Cell lysates (see \textbf{EXPERIMENTAL PROCEDURES}) were fractionated on 30-60% sucrose gradients, fractions were separated by SDS-PAGE, and Sultr1;2 immuno-detected by anti-HA antibodies (HA). Distributions of the plasmamembrane marker Pma1 (PM) and the ER marker Dpm1 (ER) were visualized by western blot analyses using monoclonal antibodies. The plasmamembrane was mainly in fractions 10-13. The *Y517 allele also has a V616E mutation. A ‘L’ indicates low accumulation of Sultr1;2 in whole cell extracts while *** indicates that no Sultr1;2 was detected in the plasmamembrane.

\textbf{Fig. 5.} Analysis of strains expressing functional Sultr1;2 in CP154-7B. \textbf{A}, Sultr1;2 accumulation was determined in the same manner as described for \textbf{Fig. 3}. \textbf{B}, \textit{SO}_4^{2-} uptake rates (see \textbf{EXPERIMENTAL PROCEDURES}) of CP154-7B expressing mutant and wild type (WT') Sultr1;2 were determined. The uptake assay was performed with cells grown in SD -his -met medium containing 500 \( \mu \text{M} \textit{SO}_4^{2-} \) at an OD\textsubscript{600}=0.1-0.3. Standard deviation for uptake values for all mutants was small, except for the Q521L strain, for which standard deviations are shown. T527A* represents Sultr1;2 with the substitutions T527A, L615M and T637A. Other alleles of \textit{Sultr1;2} with multiple mutations are A540S* (A540S, V549I and I608V); E560K* (E560K, I608V and P614S), and D538G* (D538G, R550G and I617M).

\textbf{Fig. 6.} Sequence logos representing residue frequencies. Frequencies were determined using twelve \textit{A. thaliana} sulfate transporters, five human SLC26 family members and 1h4z (SpoIIAA). Features of the presentation were previously described (24; http://weblogo.berkeley.edu/). The Y axis indicates how often a particular amino acid is present at the location. Basic amino acids (K, R and H) are in blue, acidic amino acids (D and E) in red, polar amino acids (G,S,T,Y,C) in green, N,Q in purple and hydrophobic amino acids (A,V,L,I,P,W,F,M) in black. Only amino acids with a certain degree of conservation are included in the output of the logo diagram. Unconserved amino acids do not appear in the output. The amino acid sequence of Sultr1;2 is below the sequence logos and specific mutations analyzed are indicated below the
Sultr1;2 sequence. Blue boxes mark nonfunctional Class I substitutions that cause a decrease in protein accumulation. Red boxes mark nonfunctional Class II substitutions that allow for high level protein accumulation, and proper localization of the protein to the plasmamembrane; exceptions are I496N and V537A/D (surrounded by purple boxes), which affect protein accumulation more severely than the other Class II lesions (see text). Black boxes mark Class III mutations, which often result in reduced protein accumulation, but the Sultr1;2 that remains confers some uptake activity to transformants. Asterisks indicate alleles with multiple mutations. Allele V616E* has Y517C, allele T527A* has L615M and T637A, allele D538G* has R550G and I617M, allele P614S* has E560K and I608V, and allele I608V* has A540S and V549I. Two mutations introduced for cloning purposes during the construction of mutagenized library are also indicated (V479G and I553V).

**Fig. 7. 3D structural model of STAS domain of Sultr1;2 with mutations that affect its function.** These figures were generated using the program PyMol (http://pymol.sourceforge.net/), showing the 3D structure of STAS domain based on the 3D model reported by Rouached et al (2005) from two different angles. β strands are colored yellow. The structures show side chains of the original residues substituted in mutant Sultr1;2 polypeptides. Coloring of the residues indicate the consequences of the substitution, as in **Fig. 6.** Note that E560G and G630V (substitutions in grey) cause decreased accumulation of the Sultr1;2 polypeptide, but the remaining protein exhibits some activity (suggesting that E560 and G630 are important in Sultr1;2 biogenesis). The mutations that allow Sultr1;2 protein accumulation but diminish its function are mostly clustered on the STAS surface, which is delimited in both STAS domain orientations shown by dotted lines. This surface includes A540, Y542, F543 and N545 which are in or contiguous to the N-terminus of α1, T587 and S588 in the N-terminal end of α2, Q522 and Y523 in the N-terminal end of β1 and V537 in the C-terminal end of β2.
| Phenotype                          | Mutation class | Codon change | Amino acid change | Location in STAS | Rescue of CP154-7B growth | Protein accumulation | Localization to plasmamembrane |
|-----------------------------------|----------------|--------------|-------------------|------------------|---------------------------|---------------------|--------------------------|
| I                                 | 1696 CA→CGA   | G546P        | α1                | -                | +                         | -                   | -                        |
|                                   | 1777 CA→CGC   | Q577P        | β3                | -                | +                         | -                   | -                        |
|                                   | 1791 AT→ACA   | E577N        | β3                | +                | -                         | -                   | +                        |
|                                   | 1792 GAA→AAA, 1774 ATT→ATA (572E-1) | E578K | β3 | - | + | - |
|                                   | 1879 TGG→TGG  | L607W        | β4 | + | - | - |
| II                                | 1546 AGA→ACC | AP66N       | L region         | -                | ++                       | +                   | +                        |
|                                   | 1567 CCT→CTT  | P583L        | L region         | -                | ++                       | +                   | +                        |
|                                   | 1585 GGA→GAA  | Q509E        | L region         | -                | ++                       | +                   | +                        |
|                                   | 1591 AAT→AAT  | I511T        | L region         | -                | ++                       | +                   | +                        |
|                                   | 1609 TAC→TGT, 1653 ATT→ATC(535I), 1723 AGA→GAG(555R), G106 GTG→GAG | Y517C, V516E | L region and α3 | - | ++ | + |
|                                   | 1612 AGA→AGA  | R518S        | L region         | -                | ++                       | +                   | +                        |
|                                   | 1624 CAG→CAG  | Q522K        | β1 | - | ++ | + |
|                                   | 1627 TAT→CAT  | Y523H        | β1 | - | ++ | + |
|                                   | 1669 GTT→GCT  | V537A        | β2 | - | ++ | + |
|                                   | 1669 GTT→GAT  | V537D        | β2 | - | ++ | + |
|                                   | 1684 TAC→TCT  | V542C        | α1 | - | ++ | + |
|                                   | 1687 TTC→TAC  | F543Y        | α1 | - | ++ | + |
|                                   | 1693 AGA→ATC, 1801 CCT→CCT(P581P), 1906 CGG→CGG(P614P) | N545I | α1 | - | +++ | + |
|                                   | 1822 AGT→AGT  | S588R        | α2 | - | ++ | + |
|                                   | 1891 AAT→AAA  | N611K        | between β4 and α3 | - | ++ | + |
|                                   | 1891 AAT→AGT  | N611S        | between β4 and α3 | - | ++ | + |
| III                               | 1621 CAA→CTA  | Q521L        | L region         | +++ | ++ | + |
|                                   | 1785 GTT→ATT  | V549F        | α1 | + | ++ | + |
|                                   | 1788 CAA→CGA  | E560G        | α1 | + | ++ | + |
|                                   | 1909 ATG→ATG  | B617M        | α3 | +++ | ++ | + |
|                                   | 1909 ATG→ATG  | B617M        | α3 | +++ | ++ | + |
|                                   | 1672 GAC→TGC, 1708 AGA→GQA, 1909 ATG→ATT | G630V | between α3 and α4 | + | ++ | + |
|                                   | 1639 ACT→GCT, 1903 TGG→ATG, 1969 ACC→GCC(609A) | D538R, R550L, I617M | + | ++ | + |
|                                   | 1758 CAC→AAA, 1883 ATT→ATT, 1889 CCG→CCG | E560K, I608V, P614S | + | ++ | + |
|                                   | 1678 GCC→CTC, 1762 GTT→ATT, 1892 ATT→ATT | A540S, V549L, I608V | + | ++ | + |
| Amino acid change                  | Km (µM) | Vmax (pmole/sec/mgDW) | Relative protein accumulation | doubling time (hr) |
|-----------------------------------|---------|-----------------------|------------------------------|-------------------|
| WT'                               | 2.7 (0.6) | 11.5 (0.8)            | 1.0 (0.1)                    | 5.6 (0.5)         |
| I617M                             | 2.4 (0.3) | 10.8 (0.7)            | 1.1 (0.2)                    | 6.5 (1.0)         |
| Q521L                             | 1.7 (0.5) | 8.3 (2.1)             | 1.1 (0.3)                    | 5.8 (0.6)         |
| T527A, L615M, T637A               | 2.8 (0.3) | 6.7 (2.4)             | 1.3 (0.6)                    | 9.1 (2.0)         |
| A540S, V549I, I608V               | 1.5 (0.7) | 6.1 (1.9)             | 0.6 (0.1)                    | 8.7 (2.5)         |
| E560K, I608V, P614S               | 1.8 (0.4) | 4.1 (2.0)             | 0.7 (0.3)                    | 12.5 (3.2)        |
| E560G                             | 1.7 (0.8) | 4.3 (0.5)             | 0.7 (0.2)                    | 21.3 (9.3)        |
| D538G,R550G,I617M                 | 0.6 (0.1) | 2.7 (0.2)             | 0.6 (0.2)                    | 12.2 (2.5)        |
| V549F                             | 1.5 (0.1) | 1.2 (0.2)             | 0.6 (0.2)                    | 25.2 (6.6)        |
| G630V                             | 1.6 (0.4) | 1.2 (0.1)             | 0.2 (0.1)                    | 28.6 (9.8)        |
**Figure 1**

### A

- **(a)** PCR, Digest with EcoRI/BamHI
- **(b)** Digest with EcoRI/BamHI
- **(c)** pIMRNDM
- **(c')** Digest with BamHI/SalI
- **(d)** template DNA
- **(e)** PCR mutagenesis, digest with BamHI/SalI
- **(a')** ligate (a') and (b)
- **(c')** ligate (c') and (e)

### B

**SO₄²⁻ uptake rate (pmole/sec/mgDW)**

- O WT
- WT'(V479G, I553V)

**[SO₄²⁻] (µM)**

- 0
- 2
- 4
- 6
- 8

*Figure 1 shows the schematic representation of the experimental procedures. The procedures include PCR followed by digestion with EcoRI/BamHI, ligation of fragments, and PCR mutagenesis with digestion using BamHI/SalI. The diagram also includes a graph representing the SO₄²⁻ uptake rate with different concentrations of [SO₄²⁻].*
Figure 2

![Image of Figure 2 showing graphs and data](http://www.jbc.org/Downloadedfrom)
Figure 3

A

\[ \text{\(\alpha\)Pma1} \]
\[ \text{\(\alpha\)HA} \]

\[ \text{\(\alpha\)Pma1} \]
\[ \text{\(\alpha\)HA} \]

---

B

Relative signal strength of HA to Pma1

Linking region

\[ \beta_1 \] \( \beta_2 \) \( \alpha_1 \) \( \beta_3 \) \( \beta_4 \) \( \beta_{x=0.3} \)
Figure 4

[Diagram showing PM fractions with markers for WT, PM (Pma1), HA (Sultr1;2), and ER (Dpm1). Various mutants are labeled with their respective amino acid changes, such as G509E, I511T, Q522K, Y517C*, R518S, I496N, P503L, G509E, I511T, I511N, Y517C*, R518S, Q522K, Y523H, V527A, V537D, Y542C, F543Y, N545I, S546P, Q573P, I577N, E578K, S588R, L607W, N611K, N611S.]
Figure 5

A

\[ \text{Relative signal strength of } \alpha \text{Pma1 to } \alpha \text{HA} \]

B

\[ \text{SO}_4^{2-} \text{-uptake rate (pmole/sec/mgDW)} \]

\[ [\text{SO}_4^{2-}] (\mu \text{M}) \]
Figure 7
The role of the stas domain in the function and biogenesis of a sulfate transporter as probed by random mutagenesis
Nakako Shibagaki and Arthur R. Grossman

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