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Probing the Function of STAS Domains of the Arabidopsis Sulfate Transporters*

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Sulfate transporters in plants and animals are structurally conserved and have an amino-terminal domain that functions in transport and a carboxyl-terminal region that has been designated the STAS domain. The STAS domain in sulfate transporters has significant similarity to bacterial anti-sigma factor antagonists. To determine if the STAS domain has a role in controlling the activity of sulfate transporters, their stability, or their localization to the plasma membrane, we examined the effect of deleting or modifying the STAS domain of dominant sulfate transporters in roots of *Arabidopsis thaliana*. The *A. thaliana* Sultr1;2 and Sultr1;1 sulfate transporters rescue the methionine-dependent growth phenotype of the yeast sulfate transporter mutant strain CP154-7B. Constructs of Sultr1;2 in which the STAS domain was deleted (ΔSTAS) resulted in synthesis of a truncated polypeptide that was unable to rescue the CP154-7B phenotype. The inability of these constructs to rescue the mutant phenotype probably reflected both low level cellular accumulation of the transporter and the inability of the truncated protein to localize to the plasma membrane. Fusing the STAS domain from other sulfate transporters to Sultr1;2 ΔSTAS constructs restored elevated accumulation and plasma membrane localization, although the kinetics of sulfate uptake in the transformants were markedly altered with respect to transformants synthesizing wild-type Sultr1;2 protein. These results suggest that the STAS domain is essential, either directly or indirectly, for facilitating localization of the transporters to the plasma membrane, but it also appears to influence the kinetic properties of the catalytic domain of transporters.

Sulfate transporters constitute a large family of anion transporters (SLC26 or SulP family, transport commission no. 2.A.53) present in bacteria, fungi, plants, and mammals (1–3). These proteins function in the transport of anions such as sulfate, chloride, and carbonate, and their structure is highly conserved. They all have an amino-terminal region with ~12 transmembrane domains (TMDs)[1] followed by a linking region that connects to a carboxyl-terminal STAS (sulfate transporter and anti-sigma factor antagonist) domain; the STAS domain extends into the cytoplasm of the cell. Interestingly, this domain shares significant similarity with bacterial anti-sigma factor antagonists such as SpoIIAA of *Bacillus subtilis* (4). SpoIIAA is a small polypeptide that interacts with the anti-sigma factor SpoIIAB, freeing the sigma factor to function in directing RNA polymerase activity, which in turn facilitates sporulation (5). Although the exact function of the STAS domain associated with eukaryotic anion transporters has not been elucidated, mutations in STAS domains of members of the sulfate transporter family result in serious diseases, including diastrophic dysplasia, Pendred syndrome, and congenital chloride diarrhea. These findings suggest that the STAS domain contributes to the catalytic, biosynthetic, or regulatory aspects of anion transporters (6–11).

Although the function of the catalytic domain of anion transporters such as SHST1 from the legume *Styloxanthes hamata* has been probed over the last few years (12–14), few biochemical studies have been performed to evaluate STAS domain function. In one study of the *hDRA* gene (SLC26A3) of humans, which encodes an anion transporter that mediates Cl^--Cl^- and Cl^--HCO_3^- exchange in *Xenopus* oocytes, removal of the STAS domain abolished anion transport activity (15). Hence, whereas the STAS domain does seem to influence transport function, its exact role in modulating transport-associated events, whether catalytic or regulatory, is still not known.

Arabidopsis thaliana has 14 genes that encode five phylogenetic categories of putative sulfate transporters (2). All but two of these putative transporter proteins contain STAS domains. We have focused our studies on Sultr1;2, a dominant sulfate transporter in the roots of *A. thaliana*. Selenate-resistant mutants (*sel1*) of *A. thaliana* have been isolated that have lesions in the *Sultr1;2* gene (16). Of the mutant strains characterized, three out of five had changes in amino acids in the region of Sultr1;2 connecting the terminal transmembrane domain (TMD12) to the STAS domain (17). These results, plus the results that correlate STAS domain mutations with specific diseases in humans, suggest that the STAS domain and its orientation with respect to the catalytic domain may contribute to the activity/regulation associated with anion transporters.

To dissect the role of the STAS domain in sulfate transport function, we examined the activity of the *A. thaliana* sulfate transporter Sultr1;2, and modified versions of this transporter, in yeast cells null for sulfate transport activity (CP154-7B) (18). The STAS domains on Sultr1;2 were deleted or exchanged with STAS domains of other *A. thaliana* sulfate transporters, the chimeric proteins expressed in CP154-7B, and the ability of these proteins to rescue the mutant phenotype was evaluated. We also defined the kinetic characteristics of the modified

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[1] The abbreviations used are: TMD, transmembrane domain; STAS, sulfate transporter and anti-sigma factor antagonist; SD media, synthetic defined media; sGFP, synthetic green fluorescent protein; PVDF, polyvinylidene difluoride; Pma1p, H^+-ATPase1 protein; Dpm1p, dolichol phosphate mannose synthase 1 protein; ER, endoplasmic reticulum; HA, hemagglutinin.

transporters and determined their subcellular locations. These studies demonstrate that the STAS domain and the region that links the STAS domain to the catalytic domain are necessary, either directly or indirectly, for the correct and/or stable localization of the Sultr1;2 protein in the yeast plasma membrane, and that STAS domains from different sulfate transporter proteins can markedly affect the kinetic characteristics of transporter activity.

EXPERIMENTAL PROCEDURES

Yeast Strains—The strains of *Saccharomyces cerevisiae* used in this study were CP154-7B (*MATα*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *sul1*::*LEU2*, *sul2*::*URA3*), which is null for both the *Sul1* and *Sul2* sulfate transporter genes (18), and the parental strain W303 (*MATa*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*). Both strains were supplied by the Yeast Genetic Stock Center. Yeast cells were transformed by the lithium-acetate method described by Rose (19).

Growth of S. cerevisiae Cultures—*S. cerevisiae* cells used for subcellular fractionation, characterization of membrane polypeptides, and assays for quantification of cell growth rate and sulfate uptake activity were grown at 30 °C in synthetic defined (SD), liquid medium containing 50 μM $MgSO_4$, and supplemented with methionine, as indicated in the text. Cell growth was monitored by measuring the optical density of the cultures at 600 nm (A_{600}) in a DU640 spectrophotometer (Beckman Coulter, Palo Alto, CA).

Sulfate Uptake—The uptake of sulfate was assayed in exponentially growing cells ($A_{600} < 0.4$). Prior to the assay, cells were pelleted by centrifugation (2000 × *g*), washed twice with sulfate-free SD medium, re-suspended to an A_{600} of 1.6–2.0, and incubated at 30 °C with continuous shaking at 220 rpm. Two milliliters of cell suspension was transferred to a water-jacketed vessel maintained at 30 °C and continually stirred, and the uptake reaction was initiated with the addition of different concentrations of $^{35}SO_4^{2-}$ (0.2–100 μM) at a specific activity of 189 mCi/mol. Aliquots of 200 μl were removed from the reaction vessel at five different times between 10 and 120 s following the addition of $^{35}SO_4^{2-}$, and the cells in these aliquots were collected on 0.45-μm HA nitrocellulose membranes (Millipore, Bedford, MA) by vacuum filtration using an FH225V filter manifold (Amersham Biosciences) and immediately washed with 10 ml of ice-cold YPD medium (containing 1% yeast extract, 2% peptone, and 2% glucose). The filters were then immersed in a scintillation mixture (Ecolume, E&K, Campbell, CA) and radioactivity retained by the cells quantified in a Beckman-Coulter scintillation counter (Model LS6500).

Construction of HA-tagged Polypeptides Encoding Sulfate Transporters—Plasmids for the expression of the carboxyl-terminal HA-tagged sulfate transporters were constructed in pYX222x (16). The plasmids expressing Sultr1;2-HA, Sultr1;1-HA, and Sultr3;1-HA were made by ligating the RT-PCR-amplified coding region of each gene into pYX222x yielding a carboxyl-terminal fusion of the HA epitope to the coding region of the transporter. The primers used were Sultr1;2-FEco (5′-atagaattcatgtcgtcaagagctcaccc-3′), Sultr1;2-RSal (5′-actaagtcgacttccgacctcgttggagag-3′), Sultr1;1-FEco (5′-ccggaattcatgtccgggactattaatccc-3′), Sultr1;1-RSal (5′-actaagtcgacaagtttgttgctcagccacttcc-3′), Sultr3;1-FEco (5′-agtgaattcatgggcacggaggactacac-3′), and Sultr3;1-RSal (5′-ataaagtcgacatacgttgttccaaggctcgtt-3′). The plasmid containing Sultr1;2-HA was digested to completion with SalI and partially digested with PstI, enabling us to replace the STAS domain of Sultr1;2 with the STAS domains of Sultr1;1 and Sultr3;1; the chimeric proteins generated were designated Sultr1;2–1;1 and Sultr1;2–3;1, respectively. The primers used to amplify the specific STAS domain encoding regions were Sultr1;1-STAS-FPst (5′-agaactgcagttctagggaagctgccaaa-3′), Sultr3;1-STAS-FPst (5′-aaaactgcagtgaagggaaacataccaaac-3′), and Sultr1;1-RSal and Sultr3;1-RSal, as shown above. Digestion of the vector containing Sultr1;2-HA with EcoRI and partial digestion with PstI allowed for the replacement of the region of Sultr1;2 containing the twelve predicted TMDs with the analogous region from Sultr1;1, yielding the plasmid containing Sultr1;1–1;2. The primers used were Sultr1;1-FEco (as shown above) and Sultr1;1–500TRPst (5′-attctgcagttcttggtctcgtcacttg-3′). A schematic representation of the different STAS domain constructs and the exact sites of fusion between the TMD domains of the transporters with the STAS domains are given in Fig. 1. For construction of Sultr1;2 fused to the synthetic green fluorescent protein (sGFP) and HA (Sultr1;2-sGFP), the DNA fragment for the entire coding region of Sultr1;2 was amplified by PCR with the primers, Sultr1;2-FEco (as shown above) and Sultr1;2-RNco10A (5′-aataaccatggcagcagcagcagcagcagcagcagcagcagcgacctcgttggattttgg-3′), digested with EcoRI and NcoI, and cloned into pTH2 (20), which generated Sultr1;2–10 alanine repeat (linker)-sGFP. The resulting plasmid served as template for PCR using the primer set Sultr1;2-FEco (as shown above) and sGFP-RSal (5′-actaagtcgacttgccttgtacagctcgtc-3′). The fragment was digested with EcoRI and SalI and cloned into pYX222x. All of the constructs were sequenced to ensure that no change was introduced in either the coding regions of the genes or the insert-vector junctions.

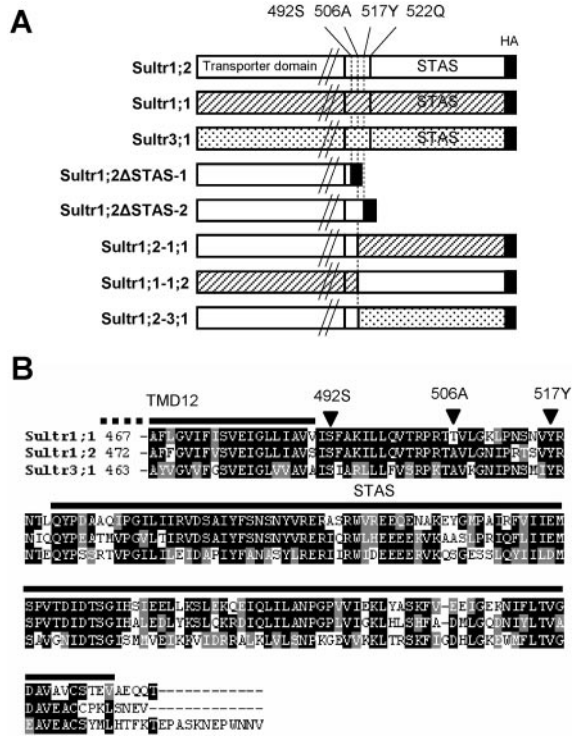

FIG. 1. *A*, schematic of the unmodified sulfate transporter proteins, the *Sultr1;2* gene deleted for the region encoding the STAS domain (Sultr1;2ΔSTAS), and constructs encoding the chimeric proteins composed of the catalytic domain of one sulfate transporter polypeptide (Sultr1;2 or Sultr1;1) and the STAS domain of another (Sultr1;2, Sultr1;1, or Sultr3;1). The Sultr1;2, Sultr1;1, and Sultr3;1 proteins are presented as *unshaded*, *lined*, and *stippled bars*, respectively. The *blackened regions* represent the HA tag. *B*, sequence alignment of the STAS domain of Sultr1;2, Sultr1;1, and Sultr3;1. Identical amino acids are *shaded in black*, and similar amino acids are *shaded in gray*. Amino acids at which Sultr1;2 is truncated in Sultr1;2ΔSTAS-1 or Sultr1;2ΔSTAS-2, or at which the catalytic and STAS domains of the chimeric proteins are fused, are indicated. Specifically, for Sultr1;2ΔSTAS-1 or Sultr1;2ΔSTAS-2, the truncated polypeptides terminate at serine 492 and tyrosine 517, respectively. The chimeric proteins Sultr1;2–1;1 and Sultr1;2–3;1 are composed of a catalytic transporter domain from Sultr1;2 (from methionine 1 to alanine 506) and a STAS domain from either Sultr1;1 (valine 502 to threonine 649) or Sultr3;1 (valine 498 to valine 658). The chimeric protein Sultr1;1–1;2 has a transporter domain from Sultr1;1 (from methionine 1 to threonine 500, which corresponds to threonine 505 in Sultr1;2) and a STAS domain from Sultr1;2 (alanine 506 to valine 653).

Microscopy—Strains expressing Sultr1;2-sGFP were grown in liquid medium to mid-logarithmic phase and mounted onto slides, and sGFP fluorescence was imaged using a Nikon TMD200 inverted fluorescence microscope equipped with a Nikon 60× 1.2-numerical aperture water immersion objective and a Bio-Rad MRC 1024 confocal head.

Polypeptide Analyses—Cells in mid-logarithmic phase of growth (A_{600} = 0.2–0.3) were chilled at 4 °C, harvested by centrifugation (2000 × *g*, 5 min), washed once in ice-cold wash solution (5 mM Tris-HCl, pH 7.5, 10 mM sodium azide, 10 mM potassium fluoride), resuspended in cold TE-buffered 20% sucrose containing a protease inhibitor mixture (Sigma, P8215), and then disrupted by agitation with glass beads (425–600 μm). The protein concentration of the lysate was determined by the BCA assay (Pierce, Rockford, IL). Ten μg of protein was used for analyses of total cellular protein by SDS-PAGE (see below). To fractionate lysed cells into different subcellular compartments, lysates containing 600 μg of total protein was loaded onto a 5-ml, 30–60% sucrose gradient and centrifuged at 4 °C for 19 h at 31,000 rpm in the SW-60 rotor (Beckman Coulter). The gradient was separated into 13

fractions of equal volume, and an equal volume of loading buffer (6.25 mM Tris-HCl, pH 6.8, 5% SDS, 6 M urea, 500 mM dithiothreitol, 10% glycerol, and 0.002% bromphenol blue) was added to both total cell lysate and to each of the fractions from the sucrose gradient. The samples were heated at 37 °C for 20 min and centrifuged at full speed in an Eppendorf microcentrifuge for 2 min to remove insoluble material, and then the solubilized polypeptides were resolved on a 10 or 12% polyacrylamide gel by SDS-PAGE (21). Resolved polypeptides were transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer method (Bio-Rad, Hercules, CA), and the polypeptides with a carboxyl-terminal HA tag were immunodetected by reacting the membranes with anti-HA antibodies (Roche Applied Science, Mannheim, Germany), followed by treatment with horseradish peroxidase-conjugated rabbit anti-rat IgG (Sigma). Peroxidase activity was detected using an enhanced chemiluminescence assay (Amersham Biosciences). Specific antibodies raised to H^+ ATPase1 (Pma1p), a plasma membrane marker, were obtained from Dr. Carolyn W. Slayman, Yale University, whereas antibodies against the dolichol phosphate mannose synthase 1 (Dpm1p), an endoplasmic reticulum membrane (ER) marker, were purchased from Molecular Probes (Eugene, OR). Signal strength was quantified using Scion Image software (Scion Corp., Frederick, MD).

RESULTS

Rescue of Yeast sul1 sul2 Mutant by A. thaliana Sulfate Transporter Genes and Requirement for the STAS Domain—A *S. cerevisiae* mutant null for *Sul1* and *Sul2* sulfate transporter genes, designated CP154-7B, is unable grow on medium containing sulfate as the sole sulfur source, but grows rapidly in medium supplemented with methionine (18). A number of plant sulfate transporter genes have been shown to rescue this mutant to sulfate prototrophy (22, 23), suggesting that the sulfate transporters of plants can be synthesized in yeast cells and localized to their site of function (2, 22–24). Previous work demonstrated that the *A. thaliana* sulfate transporters Sultr1;2 and Sultr1;1 were able to complement the yeast CP154-7B mutant, and this heterologous system was used to determine the kinetic characteristics associated with this and other transporters (22, 23).

To evaluate the role of the STAS domain of *A. thaliana* sulfate transporters on transporter activity, abundance and subcellular location in the heterologous yeast system, we modified the *Sultr1;2* gene in various ways and transformed it into CP154-7B cells. Most of our work focused on Sultr1;2, because it is a major transporter that functions in the roots of *A. thaliana*, and several mutations that alter the activity of this protein have been previously identified (16, 17). We also chose to use the STAS domains of Sultr1;1, a sulfate transporter in the same group as Sultr1;2, and Sultr3;1, a transporter in a different group, to determine if the STAS domain of the different transporters influence transporter activity or localization within the cell. Sultr1;2 and Sultr1;1 are high affinity, closely related transporters with an amino acid similarity (identity) of 80% (69%). In contrast, the amino acid similarity (identity) between Sultr3;1 and Sultr1;2/Sultr1;1 is 69% (51%)/67% (49%). Furthermore, Sultr1;1 and Sultr1;2 are co-expressed in root cortex cells while Sultr3;1 is expressed specifically in leaves (22, 25). Although Sultr1;2 is expressed constitutively, with some increase in the transcript level in response to sulfur deprivation, Sultr1;1 expression dramatically increases when plants are deprived of sulfate (22).

The coding regions of the sulfate transporter genes, derived from specific cDNAs, were separately cloned into the yeast expression vector, pYX222x (16), which was then used to transform CP154-7B. pYX222x is a high copy number plasmid vector with the 2-μm origin of replication and the *HIS3* gene as a selection marker. Within this vector the inserted gene was fused at the carboxyl-terminal to the HA epitope tag. As shown qualitatively in Fig. 2, the HA-tagged Sultr1;1 and Sultr1;2, but not Sultr3;1, rescued the mutant phenotype allowing it to grow on medium in

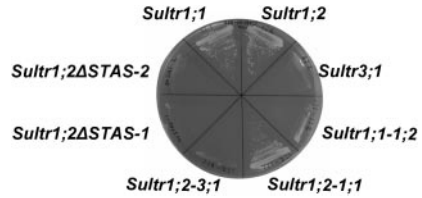

FIG. 2. **The growth of CP154-7B cells on plates expressing unmodified, truncated, and chimeric sulfate transporters.** Transformants were grown at 30 °C for 3 days on solid SD (-histidine-methionine drop out) medium containing 50 μM sulfate.

which sulfate was the sole sulfur source. A quantitative evaluation of the growth of the rescued strain on sulfate-containing, liquid medium, either with or without methionine supplementation, is shown in Fig. 3. Table I shows the doubling time of the different strains and transformants. In medium supplemented with methionine, transformants harboring the cDNAs for the three different transporters (Sultr1;2, Sultr1;1, and Sultr3;1) grew at rates that ranged from 65–75% of that of the parental strain (W303; the strain used to generate CP154-7B, carrying pYX222x to complement histidine auxotrophy) and attained a cell density of ~50% that of the parental strain (Fig. 3, *lower panel*, and Table I). In contrast, in medium devoid of methionine, the CP154-7B mutant was rescued by introduction of the *Sultr1;2* or *Sultr1;1* genes, but not by introduction of the *Sultr3;1* gene (Fig. 3, *upper panel*, and Table I). Those transformants harboring the *Sultr1*;2 or *Sultr1;1* genes grew on medium with sulfate as the sole sulfur source at rates of ~50% of that of W303; generally, CP154-7B harboring *Sultr1;2* grew slightly faster than CP154-7B harboring *Sultr1;1*.

To determine if the STAS domain of Sultr1;2 was essential for rescuing the CP154-7B mutant phenotype, we prepared constructs encoding truncated Sultr1;2 polypeptides devoid of the STAS domain (Fig. 1). The limits of the STAS domain were defined by homology to the anti-sigma factor antagonist SpoIIAA (4). The most carboxyl-terminal TMD of Sultr1;2 (TMD12) is predicted to end at serine 490 (490S) based on MEMSAT2 (26), and homology to SpoIIAA starts at glutamine (522Q) and ends at leucine (649L), as defined by PROSITE (PS50801) (Fig. 1*B*). Two different "Sultr1;2ΔSTAS" constructs were tested for their ability to complement the CP154-7B mutant; in "Sultr1;2ΔSTAS-1" and "Sultr1;2ΔSTAS-2" the constructs encode truncated Sultr1;2 proteins that terminate at serine (492S) and tyrosine (517Y), respectively (Fig. 1). The Sultr1;2ΔSTAS-1 protein lacks the entire STAS domain as well as the amino acids that comprise the region that links the TMDs of the transporter to the STAS domain, whereas the Sultr1;2ΔSTAS-2 protein lacks the STAS domain but contains nearly all of the linking region. Neither of these constructs was able to rescue the CP154-7B mutant phenotype in medium containing sulfate as the sole sulfur source (Figs. 2 and 3, *upper panel*). However, in methionine-supplemented medium, CP154-7B transformed with these constructs grew essentially as well as CP154-7B transformed with the entire *Sultr1;2* gene or empty vector (Fig. 3, *lower panel*). These results raised the possibilities that the truncated protein products were nonfunctional, could not localize to their site of action or were more unstable than the full-length Sultr1;2 protein.

To test if the STAS domain from one of the sulfate transporters could functionally substitute for that of another, we constructed three chimeric genes in which STAS domains among the different sulfate transport polypeptides were exchanged (see Fig. 1). Truncated Sultr1;2 (at alanine 506) was fused with the STAS domain from Sultr1;1 (at valine 502 to threonine 649) and Sultr3;1 (valine 498 to valine 658) to yield Sultr1;2–1;1 and

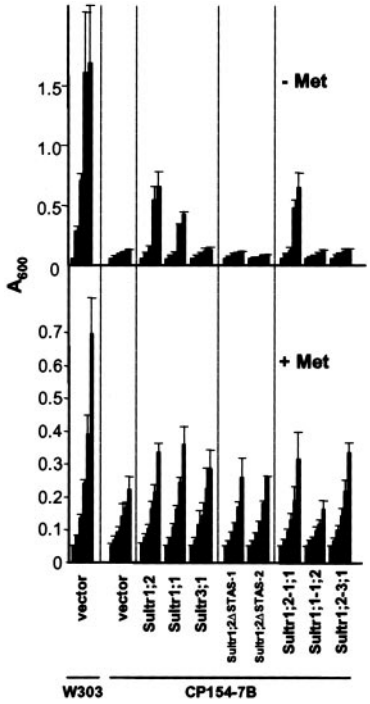

FIG. 3. **Growth of the parental W303 strains, CP154-7B cells and CP154-7B cells expressing the various transporter constructs.** Cells were grown to mid-logarithmic phase, washed, and diluted to an A_{600} of 0.04 in histidine-deficient SD medium containing 50 μM sulfate and no methionine (−Met), or 50 μM sulfate and 400 μM methionine (+Met), and then grown with shaking at 260 rpm at 30 °C for various times. In the *upper panel* (−Met), each *bar* represents the A_{600} measured at 3, 9, 13, 24, and 27 h after the cells were diluted into minus methionine medium. In the *lower panel* (+Met), each *bar* represents the A_{600} measured at 3, 4.5, 6, 7.5, 9, and 11 h after dilution into methionine-containing medium.

TABLE I

Growth rates (doubling time in hour, measured as a change in A_{600}) of the parental S. cerevisiae strain and CP154-7B transformed with genes encoding various A. thaliana sulfate transporters, truncated Sultr1;2, and chimeric sulfate transporter polypeptides

The values shown in parenthesis represent standard deviations.

		Doubling time	
		Unsupplemented	Methionine 400 μM
		h	
W303	Vector	2.6 (0.3)	2.1 (0.1)
CP154-7B	Sultr1;2	6.3 (0.4)	2.9 (0.2)
	Sultr1;1	7.6 (0.1)	2.8 (0.2)
	Sultr3;1	20.9 (5.0)	3.1 (0.5)
	Vector	20.2 (4.5)	3.5 (0.5)
	Sultr1;2ΔSTAS-1	23.7 (4.0)	3.5 (0.5)
	Sultr1;2ΔSTAS-2	22.0 (5.0)	3.2 (0.2)
	Sultr1;2–1;1	6.4 (0.5)	3.2 (0.4)
	Sultr1;1–1;2	20.1 (4.9)	5.2 (1.1)
	Sultr1;2–3;1	20.6 (4.0)	3.4 (1.0)

Sultr1;2–3;1. Additionally, the truncated Sultr1;1 polypeptide (at threonine 500) was fused to the STAS domain of Sultr1;2 (alanine 506 to valine 653) to yield Sultr1;1–1;2 (Fig. 1). All the chimeric proteins were HA-tagged at their carboxyl termini. The yeast cells expressing Sultr1;2–1;1 grew as well as those expressing the full-length Sultr1;2 polypeptide in medium lacking methionine, while there was essentially no growth of cells expressing Sultr1;1–1;2 or Sultr1;2–3;1 (Fig. 3, *upper panel*). All of the strains with the chimeric constructs grew in methionine-supplemented medium at rates comparable to that of CP154-7B transformant with the entire *Sultr1;2* gene or the empty vector (Fig. 3, *lower panel*).

Sulfate Transport Kinetics in Yeast Transformants—We performed sulfate uptake assays with parental W303 cells carrying the plasmid pYX222x and with the CP154-7B mutant harboring the Sultr1;2, Sultr1;1, Sultr1;2ΔSTAS-1/-2 and the chimeric constructs (Fig. 4). The K_m and V_{max} for sulfate uptake of W303 (which contains both the *Sul1* and *Sul2* genes) was ~12 μM and 33 of pmol sulfate/s/mg dry weight (DW), respectively (Table II). Cells harboring constructs encoding Sultr1;2 and Sultr1;1 showed high affinity sulfate transport with a K_m of 1.1 and 1.0, and a V_{max} of 4.4 and 3.2 pmol of sulfate/s/mg DW (Table II). CP154-7B cells and CP154-7B cells expressing Sultr3;1, Sultr1;2ΔSTAS-1, or Sultr1;2ΔSTAS-2 constructs failed to show detectable sulfate transport activity (data not shown). Mutant cells expressing the chimeric protein Sultr1;2–1;1 exhibited a 4-fold lower affinity for sulfate and a V_{max} that was higher than that of cells harboring unmodified Sultr1;2 (Fig. 4 and Table II). Cells harboring constructs for the chimeric proteins Sultr1;1–1;2 and Sultr1;2–3;1 showed little sulfate transport activity, as indicated by their low V_{max} for sulfate uptake (Fig. 4 and Table II), which was expected based on their inability to grow in sulfate-containing medium that was not supplemented with methionine.

Localization of Sultr1;2, Sultr1;2ΔSTAS, and Chimeric Transport Proteins—To examine the subcellular abundance and distribution of the introduced transporters, we quantified the HA-tagged proteins in total cellular extracts and in cellular membranes of CP154-7B, and CP154-7B transformed with the various constructs after fractionation on sucrose gradients. An equal amount of total cellular protein from each strain was resolved by SDS-PAGE, transferred to PVDF membranes, and, as shown in Fig. 5, the HA-tagged transporters were detected immunologically. The levels of the truncated STAS proteins (ΔSTAS) in the cell were low relative to the levels observed in transformants expressing Sultr1;2 or Sultr1;1 (Fig. 5, compare *lanes b* and *c* with *d* and *e*); the Sultr1;2ΔSTAS-1 polypeptide was nearly undetectable. In contrast, the levels of Sultr1;2ΔSTAS-1, Sultr1;2ΔSTAS-2, and Sultr1;2 transcripts in the transformants were nearly identical (determined by RNA blot hybridizations; data not shown), suggesting that the differences in protein levels were a consequence of post-translational events.

In strains expressing Sultr1;2 and Sultr1;1, multiple polypeptides of different molecular masses were observed. The product with the greatest apparent molecular mass, and the most abundant product, was ~70 kDa, which is the expected mass of the full-length polypeptide, whereas a second antigenic polypeptide had an apparent molecular mass of ~55 kDa; other less abundant, lower molecular mass antigenic species were also observed. Substantial levels of apparent full-length Sultr3;1, Sultr1;1–1;2, and Sultr1;2–3;1 polypeptides were also observed in whole cell preparations (Fig. 5, *lanes f*, *h*, and *i*), although transformants synthesizing these polypeptides were unable to grow in medium with sulfate as the sole sulfur source (Fig. 3). These results suggest that the Sultr3;1, Sultr1;1–1;2, and Sultr1;2–3;1 polypeptides were either not properly localized to the cytoplasmic membranes or that they were properly localized but unable to function in transporting sulfate into cells.

Fractionation of the membranes of cells harboring Sultr1;1, Sultr1;2, and Sultr3;1 constructs, presented in Fig. 6*A*, shows that much of the full-length protein in the transformants was present in the plasma membrane, co-sedimenting at the bottom of the sucrose gradient with the marker for the plasma membrane ATPase (Pma1p). As shown in Fig. 6*B*, between 40 and 70% of these transporters is present in the plasma membrane fraction (fractions 11–13 of the gradient). These results suggest

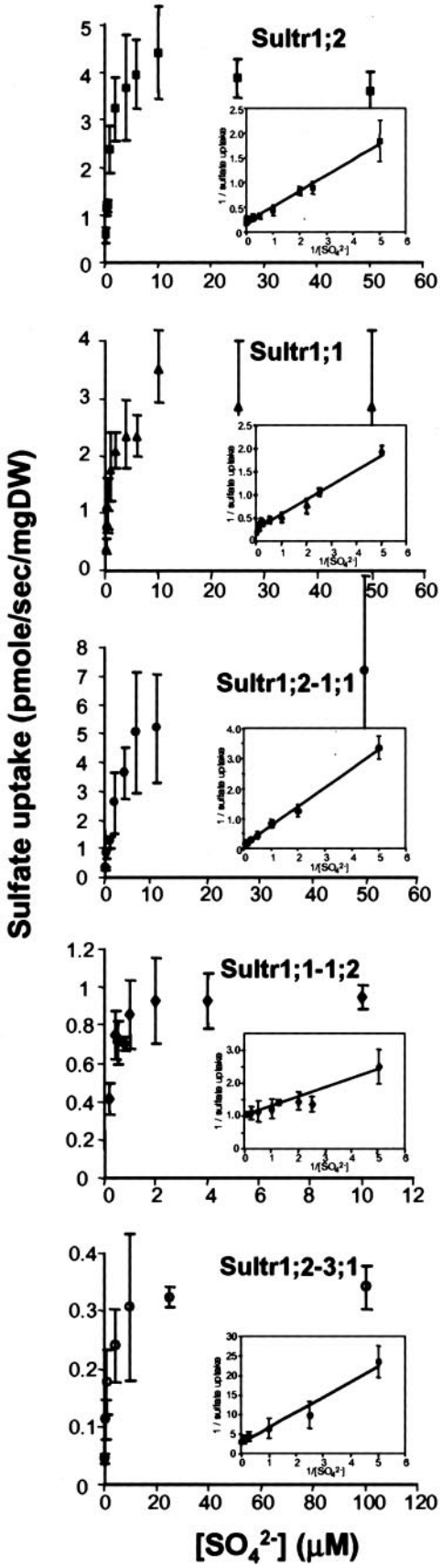

FIG. 4. **Sulfate uptake kinetics for CP154-7B transformants harboring the various constructs.** Uptake of radioactive sulfate was determined by measuring accumulation of radioactivity in the cells at five different times following the introduction of various concentrations of sulfate, as indicated on the *x*-axis. Each value represents a mean, ± S.D., determined from at least three independent experiments.

TABLE II
Kinetic properties of modified and unmodified sulfate transporter polypeptides in CP154-7B

K_m and V_{max} values were calculated from measurements of sulfate uptake as shown in Fig. 4 and based on the single ligand binding equation, $y = V_{max}/(x + K_m)$. The K_m is expressed as micromolar, whereas the V_{max} is picomoles of sulfate/s/mg dry weight. The values shown in parenthesis represent standard deviations.

		V_{max}	K_m
		pmol sulfate/s/mg	*μM*
W303	vector	33 (2.4)	12 (2.1)
CP154-7B	Sultr1;2	4.4 (0.2)	1.1 (0.2)
	Sultr1;1	3.2 (0.2)	1.0 (0.3)
	Sultr1;2–1;1	7.8 (0.9)	4.3 (1.4)
	Sultr1;1–1;2	1.0 (0.1)	0.2 (0.1)
	Sultr1;2–3;1	0.3 (0.02)	1.0 (0.4)

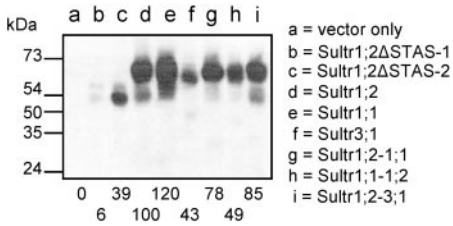

FIG. 5. **The levels of sulfate transporter proteins observed in whole cell *S. cerevisiae* extracts.** CP154-7B cells expressing wild-type, STAS domain-deleted, and chimeric sulfate transporters tagged with HA were harvested at an A_{600} of between 0.2 and 0.3 and lysed by agitation with glass beads, and the proteins in the lysates were denatured in the loading dye at 37 °C for 20 min. The protein in the lysate (10 μg) was separated by SDS-PAGE, transferred to PVDF membranes, and the sulfate transporter proteins were detected using anti-HA antibody. *Numbers underneath each lane* give the relative amount of transporter protein, as detected by HA antibody.

that all three of the full-length sulfate transporter proteins localize to the plasma membrane and that even though Sultr3;1 appears correctly localized, it is not effective at facilitating the transport of sulfate into yeast cells. Furthermore, either none or little of the Sultr1;2ΔSTAS-1 protein is localized to the plasma membranes, with the majority being in the ER fraction; it mostly co-localized with the ER marker dolichol phosphate mannose synthase (Dpm1p). In the case of Sultr1;2ΔSTAS-2, much of the small amount of protein that accumulates in these cells co-sediments with the plasma membrane marker. This suggests that the linker region is important for localizing the protein to the plasma membrane or a membrane fraction that co-sediments with the plasma membrane in the gradient used for fractionation in these experiments; in another fractionation experiment, Sultr1;2ΔSTAS-2 does not exactly co-sediment with the plasma membrane (see "Discussion"). Furthermore, the linking region either directly or indirectly affects the abundance of the protein in the cell. In contrast, all of the chimeric polypeptides (Sultr1;2–1;1, Sultr1;1–1;2, and Sultr1;2–3;1) synthesized in *S. cerevisiae* transformants were synthesized at levels comparable to that of the unmodified transporters, and all showed abundant, prominent peaks in the plasma membrane fraction, with some co-sedimentation with ER membranes. These results suggest that placing a heterologous STAS domain at the carboxyl terminus of either Sultr1;2 or Sultr1;1 allows for increased accumulation and proper targeting of the polypeptide, although the chimeric polypeptides, with the exception of Sultr1;2–1;1, do not appear to function in sulfate transport (Fig. 4) or enable cells to grow on sulfate as the sole sulfur source (Fig. 3).

We also constructed an in-frame fusion between Sultr1;2 and sGFP, with a 10-alanine repeat linking Sultr1;2 to sGFP. In

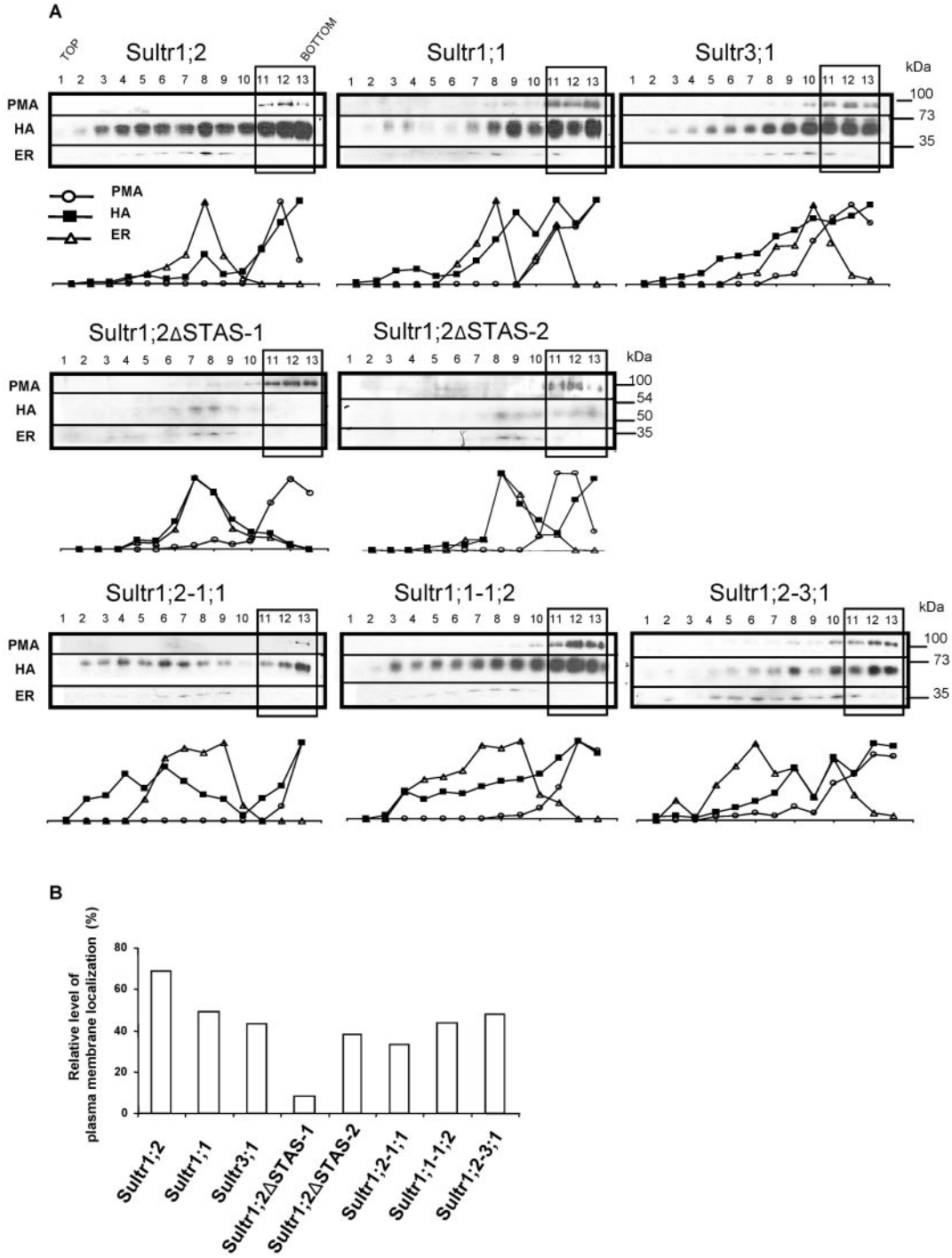

FIG. 6. **Subcellular localization of sulfate transporter proteins in *S. cerevisiae* cells.** A, the lysates, as shown in Fig. 5 were fractionated in a 30–60% sucrose gradient, the fractions were analyzed by SDS-PAGE, and the sulfate transporter was detected by immunoreaction with the anti-HA antibodies. The distribution of the plasma membrane marker Pma1p, and the ER marker Dpm1p, was visualized by Western blot analyses using monospecific antibodies. The fractions, *boxed* by a *black solid line* (*fractions 11–13*), represent those fractions containing the plasma membrane. The relative signal strengths along the gradient of the marker polypeptides and the HA-tagged sulfate transporters were quantified by Image software (Scion Corp., Frederick, MD) and presented as a *graph* underneath each of the Western blots. *B*, the relative level, as a percentage of plasma membrane-localized polypeptides to total HA-tagged sulfate transporter polypeptide in the gradient.

cells harboring this construct we can visualize localization of the transporter in *S. cerevisiae* cells. As shown in Fig. 7*B*, in cells expressing sGFP from the plasmid pYX222x, the fluorescence signal appeared to be present throughout the cytoplasm of the cell; the exclusion of signal in the center of the cell is the consequence of the large vacuole present in the *S. cerevisiae* cell. In contrast, in CP154-7B cells harboring *Sultr1;2* fused to sGFP, much of the sGFP appeared localized to the cytoplasmic membranes, which is visualized as a more even distribution of sGFP fluorescence over the entire cell surface (Fig. 7*A*). Some of the sGFP may be concentrated in the cytosol in the region around the nucleus, typically representing ER, and in the cytosol near the cell surface, which may represent endosomes and cytoplasmic membrane polypeptides that are being resorbed by the cell and degraded (Fig. 7*A*). Transformants expressing Sultr1;2-sGFP were able to grow in medium containing sulfate as the sole sulfur source, at a rate similar to that of CP154-7B transformed with *Sultr1;2* (data not shown).

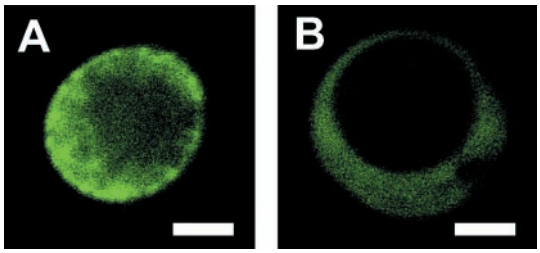

FIG. 7. **Confocal image of CP154-7B cells expressing the fusion protein Sultr1;2-sGFP (*A*) or free sGFP (*B*).** The *bar* on the image represents 3 μm.

DISCUSSION

To gain insights into the function of the STAS domain, which exists in almost all members of the sulfate transporter family of proteins, we generated *A. thaliana* sulfate transporters that were modified for the STAS domain. These modified proteins were HA-tagged, allowing us to monitor their accumulation and subcellular localizations in *S. cerevisiae*. In all cases, when constructs encoding untagged transporters were introduced into the *S. cerevisiae* mutant CP154-7B, which lacks both of the endogenous sulfate transporters, the growth phenotype was indistinguishable from that of the strain harboring the HA-tagged transporters (data not shown).

The A. thaliana Sulfate Transporters Sultr1;2, Sultr1;1, and Sultr3;1—With respect to sulfate uptake activity, Sultr1;2 and Sultr1;1 showed high affinity uptake in *S. cerevisiae* cells, with a K_m of ~1.0 μM for both. This is a lower value than was previously reported (K_m = 6.9 μM for Sultr1;2 and 3.6 μM for Sultr1;1 (22, 23)) and may reflect the difference in assay methods used for the measurements. *Sultr1;2* transformants had a significantly higher V_{max} for sulfate than *Sultr1;1* transformants (Table II), which may be the reason for the difference in the observed growth rates between these two strains in medium containing sulfate as the sole sulfur source; these strains grow at essentially the same rates in methionine-supplemented medium (Fig. 3 and Table I).

Among CP154-7B transformants harboring the *A. thaliana* sulfate transporter genes, transformants expressing Sultr1;2 grew most rapidly in medium containing sulfate as its sole sulfur source. However, this strain still had a doubling time that was only ~50% of that of the parental W303 strain, which contains the native Sul1 and Sul2 transporter proteins. This difference in growth rate is probably a consequence of the markedly different V_{max} values for sulfate transport among the strains; the V_{max} for sulfate transport in W303 is ~8 times higher than that of *Sultr1;2* transformants (although the K_m is much lower in the strain harboring *Sultr1;2*). The relatively low V_{max} for sulfate transport in the transformants may be a consequence of relatively low levels of the heterologous transport proteins in the plasma membrane and/or a decreased transport activity because of aberrant regulation of these transporters in *S. cerevisiae*. For example, there may be a positive regulator of sulfate transporter activity that functions in *A. thaliana* but is not present in *S. cerevisiae*; such regulatory factors are required for complementation of *S. cerevisiae* with the plant plasma membrane H^+-ATPase (27). Furthermore, the transformants grow more slowly than the W303 parental strain even in methionine-supplemented medium, a condition reported to suppress accumulation of *Sul1* and *Sul2* mRNA (18). These results suggest that the loss of *Sul1* and *Sul2* expression in W303 is not complete in methionine-supplemented medium and that under such conditions the ability of W303 to accumulate more sulfate than *Sultr1;2* transformants allows for more rapid growth.

The expression of the *Sultr1;2* or *Sultr1;1* genes in *S. cerevisiae* from a high copy number plasmid under the control of the active promoter from the triose phosphate isomerase gene may lead to high level of accumulation of these transporter proteins in the plasma membrane. However, as demonstrated, in addition to apparent full-length protein products, there are truncated polypeptides for all introduced *A. thaliana* sulfate transporter; these are likely to be degradation products, because the amount of the smaller polypeptides parallel the accumulation of the full-length protein. The truncated products of the *A. thaliana* transporters were not detected in the *S. cerevisiae* plasma membrane fraction, suggesting that these products are either never localized to the plasma membrane or are rapidly degraded once they associate with the plasma membrane.

To determine the percentage of expressed *A. thaliana* sulfate transporters that co-migrated with *S. cerevisiae* plasma membranes, sucrose gradients were used to resolve the plasma membrane from the other membrane systems of the cell and the level of the transporter was evaluated immunologically (Fig. 5). Although total accumulation of Sultr1;2 and Sultr1;1 polypeptides was about the same in the transformed lines, ~69% of total Sultr1;2 and ~49% of total Sultr1;1 accumulated in the plasma membrane fraction, suggesting a preferential localization of Sultr1;2 to the plasma membrane of *S. cerevisiae*. This may partly explain the difference in V_{max} and growth rates observed for *Sultr1;2* relative to *Sultr1;1* transformants.

The relatively high level of the heterologous transporter polypeptides associated with the *S. cerevisiae* ER fraction suggests that a significant proportion of these transporters is misfolded and/or aggregated and may be unable to exit the ER. Generally, high level expression of heterologous proteins in *S. cerevisiae* can result in significantly more misfolding than is observed for endogenous proteins.

The distribution of Sultr1;2 in *S. cerevisiae* membranes as determined by subcellular fractionation studies agrees with localization of Sultr1;2-sGFP, as determined by confocal fluorescence microscopy. In CP154-7B cells expressing Sultr1;2-sGFP, much of the fluorescent signal appeared associated with the plasma membrane, the cytosol adjacent to the cell surface (in which endosomes are trafficking to the plasma membrane, and plasma membrane proteins are being resorbed and degraded) and the perinuclear region, indicative of ER localization (Fig. 7*A*). Expression of free sGFP in CP154-7B was observed to be equally spread over the cytosol of the cell (Fig. 7*B*).

Of the wild-type transporter genes of *A. thaliana* used in these studies, *Sultr3;1* was unable to rescue CP154-7B; transformants showed neither growth in the absence of methionine nor a significant level of sulfate uptake activity. However, a relatively high amount of the Sultr3;1 polypeptide was present in the plasma membrane fraction of the transformants (43% of the total), suggesting that the Sultr3;1 protein that does associate with the plasma membrane is not active; it may require some post-translational modification to elicit high level activity. Many proteins synthesized in the ER are post-translationally modified prior to localization at their site of function. Alternative, it is possible that Sultr3;1 is activated in *A. thaliana* (but not in *S. cerevisiae*) after insertion into the plasma membrane or is involved in the export rather than the import of sulfate into plant cells.

The Sultr1;2ΔSTAS Constructs—The Sultr1;2 polypeptides deleted for the STAS domain were not able to rescue the sulfate transporter phenotype of CP154-7B, suggesting that the STAS domain serves an essential role for the biosynthesis of an active sulfate transporter. The Sultr1;2ΔSTAS constructs do not accumulate to high levels in transformants; this is especially true of Sultr1;2ΔSTAS-1, which is lacking both the STAS and TMD-

contiguous linker domain. This truncated polypeptide is either not made efficiently in cells, which is unlikely, or is unstable and degraded in the ER, where essentially all of the truncated protein is localized. In contrast, transformants expressing Sultr1;2ΔSTAS-2, in which just the STAS domain has been eliminated from the protein (it retains the linker polypeptide), appear to accumulate a significant amount of the truncated transporter, although still low, in the cytoplasmic membrane fraction. These results suggest that addition of the linker region has some stabilizing effect on the protein (*e.g.* allowing for folding to a more stable conformation). However, in another experiment, we observed that Sultr1;2ΔSTAS-2 sedimented along the sucrose gradient mainly in fractions lying between those containing the ER and plasma membrane; this fraction may contain vacuolar membranes. All of the other transporter polypeptides showed consistent co-sedimentation with the plasma membrane fraction, as in Fig. 6 (28) (data not shown). Therefore, like Sultr1;2ΔSTAS-1, Sultr1;2ΔSTAS-2 may be misfolded and targeted for degradation either prior to or soon after association with the plasma membrane. Together, the subcellular localization experiments suggest that the STAS domain is important for moving polypeptides to the plasma membrane or for maintaining stability of the transporter polypeptides during their biogenesis. A similar situation may exist for the human SLC26A3 anion exchanger in which elimination of the carboxyl-terminal cytoplasmic extension contiguous to the TMD region of the polypeptide leads to a significant decrease in protein accumulation (15).

Analyses of Sulfate Transporters with Heterologous STAS Domains—If the STAS domain is important for proper folding and/or stabilization of transporters, constructing chimeric sulfate transporter proteins with heterologous STAS domains may allow for their accumulation in the plasma membrane. The Sultr1;2–1;1, Sultr1;1–1;2, and Sultr1;2–3;1 chimeric proteins were able to accumulate in *S. cerevisiae* (Fig. 5) and exhibited significant localization to the plasma membrane (Fig. 6). However, the sulfate uptake properties of CB154–7B harboring these chimeric proteins were altered relative to transformants with the native proteins from which the catalytic domains were derived (Fig. 4). Of the chimeric proteins generated, only Sultr1;2–1;1 was able to rescue the CP154-7B mutant phenotype; transformants expressing this protein grew at essentially the same rate in medium containing sulfate as a sole sulfur source as those expressing the unmodified Sultr1;2 polypeptide (Figs. 2 and 3). However, these transformants exhibited an affinity for sulfate that was reduced by ~4-fold and a capacity for sulfate uptake that was ~80% more than that of transformants expressing Sultr1;2 with its native STAS domain (Table II). The Sultr1;1–1;2 polypeptide accumulated to a lesser extent in transformed CP154-7B cells than the unmodified Sultr1;1 polypeptide (Fig. 5), which probably reflects the markedly reduced V_{max} for sulfate transport observed in transformants expressing the chimeric protein (although the ratio of plasma membrane localized to total protein was about the same for the two strains). Sultr1;2–3;1 accumulated to a comparable level to that of cells expressing unmodified Sultr1;2, with strong localization to the plasma membrane fraction, although the V_{max} for sulfate uptake was extremely low. This suggests that the chimeric protein is strongly reduced in its capacity to transport the substrate, or that most of the transporter molecules present in the plasma membrane were not active.

As noted above, growth of CP154-7B harboring any of the sulfate transporter constructs used in this study on methionine-supplemented medium was slower than that of the parental W303 strain. The capacity for sulfate transport in the parental strain is ~5-fold more than that of the transformants with the highest V_{max} for sulfate uptake (Sultr1;2–1;1 and Sultr1;2). Hence, reduced sulfate uptake in the transformants may limit their growth, even in the presence of another sulfur source (sulfate itself is needed for optimal growth, because it is essential for the sulfation of macromolecules and/or maintenance of ion homeostasis, and not enough is acquired via the heterologous transporters under low sulfate conditions). This is likely to be the case, because the CP154-7B mutant harboring an empty vector divides considerably more slowly than the parental W303 strain when grown on methionine with a low level of sulfate (Table I). Furthermore, with methionine supplementation, the mutant strains expressing functional transporter grow a little faster than the empty vector control, but significantly slower than W303. The strains expressing transporter constructs that don't provide the cell with much sulfate uptake capacity grow at approximately the same rate as the empty vector control (except for Sultr1;1–1;2, which grows even more slowly). There still is the possibility that overexpression of sulfate transporters in *S. cerevisiae* cells and overpopulation of the cytoplasmic membranes with these integral membrane proteins disrupt, to some extent, ion homeostasis and/or physiological activities associated with the plasma membrane; such an effect could antagonize the effect of having increased sulfate transport in some of the transgenic strains. Fusing the transporter genes to inducible promoters may help us to distinguish between these possibilities.

Taken together, the data obtained for the truncated and chimeric constructs suggest that the STAS domain and the region that links the STAS domain to the catalytic domain of the sulfate transporters are important for accumulation of the sulfate transporters in the plasma membrane. The results also suggest that the STAS domain is tailored to a specific transporter domain, and that swapping STAS domains can significantly influence transporter activity. This may be accomplished by interactions of the STAS domain with the catalytic region of the transporter, or through associations with other proteins in the cell. Such interactions are likely to be sequence-specific, because STAS domains do not appear to be interchangeable, which may be reflected in the significant differences among the STAS domains of the different *A. thaliana* sulfate transporters. The amino acid similarity (identity) between STAS domains (from 520I of Sultr1;2) is 80 (62)% for Sultr1;2 and Sultr1;1, 72 (46)% for Sultr1;2 and Sultr3;1, and 66 (38)% for Sultr3;1 and Sultr1;1.

A number of proteins with transport function are known to have cytosolic extensions that modulate transport activity through intra- or intermolecular interaction with proteins or cellular metabolites. For example, the activities of cyclic nucleotide-gated ion channels (29), H^+-ATPases (27, 30, 31), and Na^+/H^+ antiporter (32–34) may be modulated by carboxyl-terminal extensions that are not part of the channel-forming domain of the polypeptide. The activity of the mammalian Na^+/H^+ antiporter, NHE1, is regulated negatively by a domain within its carboxyl-terminal cytosolic extension, probably through intramolecular interactions (32), and positively by the binding of a calcineurin B-like protein to the cytosolic extension (33). In a recent study, the Cos3p polypeptide of *S. cerevisiae* was found to interact with the carboxyl-terminal, juxtamembrane domain of the Na^+/H^+ exchanger Nha1, possibly increasing the activity of this antiporter and enhancing the salinity resistance of cells (34). The STAS domains and linker regions of the sulfate transporters are thought to be important for modulating transporter activity, because *sel1–3*, *sel1–7*, and *sel1–8* mutations in the linker region between TMD12 and the STAS domain of Sultr1;2 (17) abolish sulfate transport activity with-

out interrupting membrane localization of the protein,[2] and mutations within the STAS domains of mammalian sulfate transporter family members cause severe disease phenotypes (6, 8, 10, 11). Furthermore, experiments currently being performed have shown that the STAS domains along with the linker regions of Sultr1;2 and Sultr3;1, but not Sultr1;1, show strong homologous associations, suggesting a specificity of interactions among sulfate transporter polypeptides.[2] Systematic analyses of the STAS domain and linker regions of the sulfate transporters with respect to catalytic function and interactions with cellular metabolites and proteins are likely to reveal important mechanisms involved in nutrient acquisition in both plants and animals.

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