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Plant members of a family of sulfate transporters reveal functional subtypes

(ion transport/sulfate uptake)

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ABSTRACT **Three plant sulfate transporter cDNAs have been isolated by complementation of a yeast mutant with a cDNA library derived from the tropical forage legume *Stylosanthes hamata*. Two of these cDNAs, *shst1* and *shst2*, encode high-affinity H^+/sulfate cotransporters that mediate the uptake of sulfate by plant roots from low concentrations of sulfate in the soil solution. The third, *shst3*, represents a different subtype encoding a lower affinity H^+/sulfate cotransporter, which may be involved in the internal transport of sulfate between cellular or subcellular compartments within the plant. The steady-state level of mRNA corresponding to both subtypes is subject to regulation by signals that ultimately respond to the external sulfate supply. These cDNAs represent the identification of plant members of a family of related sulfate transporter proteins whose sequences exhibit significant amino acid conservation in filamentous fungi, yeast, plants, and mammals.**

All plants need to absorb essential nutrient anions against large gradients of electrochemical potential; this is true for plants in both natural and agricultural environments. Evidently, transport mechanisms of high affinity have evolved, but their precise nature remains obscure, although they have been a major research topic for decades. Despite a wealth of physiological information, the molecular nature of the transporters and the way in which they are regulated are unknown.

Sulfate transport in plant roots or cultured cells has high- and low-affinity components (1); the former clearly respond to the sulfur-status of the organism, being strongly derepressed by sulfur-starvation and rapidly repressed by the restoration of a sufficient sulfur supply (2–4). High rates of sulfate uptake by previously sulfur-starved cells or roots appear to depend on protein synthesis; treatment with cycloheximide decreases sulfate influx with kinetics very similar to repression by sulfate (4, 5). Such results raised the question as to whether the control of transport activity was translational, or by posttranslational modification of the transporter, rather than by transcription of the genes that encode it. Here we present results demonstrating that changes in the level of the mRNA encoding the transporter are remarkably rapid and are quite compatible with changes in transport activity.

Membrane transport proteins from a wide variety of sources may be placed into distinct groups based upon primary sequence similarity or structural features (6, 7). Sequence homologies between the *Neurospora crassa* sulfate transporter (8) and a number of genes not previously associated with sulfate transport, including a human mucosa protein (9) and a nodule-specific protein (10), have been recognized recently (11). More recent additions to this group are a rat liver sulfate transporter (12), a human gene, *DTD*, a mutation in which results in diastrophic dysplasia (13), and the yeast high-affinity sulfate transporter (14). In this paper we report the cloning and analysis of plant members of this family, isolated from the tropical forage legume *Stylosanthes hamata* cv. Verano.

MATERIALS AND METHODS

Growth of *St. hamata*. RNA used in cDNA library construction and expression studies was derived from *St. hamata* cv. Verano. Plants were grown in 5-liter containers of the following nutrient solution at pH 6.0: 1000 μM $Ca(NO_3)_2$, 250 μM $MgSO_4$, 250 μM KNO_3, 20 μM KH_2PO_4, 18 μM NaFeEDTA, 46 μM H_3BO_3, 0.3 μM $CuCl_2$, 0.76 μM $ZnCl_2$, 4.5 μM $MnCl_2$, 0.015 μM $(NH_4)_6Mo_7O_{24}$. Sulfur limitation was introduced by transferring plants to medium in which the $MgSO_4$ was replaced by $MgCl_2$. Nutrient solutions were changed 7 days after planting and every 3 days thereafter. Plants were grown at 28°C in 75% relative humidity with a 16-hr light period of 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and harvested when 25 days old.

Microorganisms, Plasmids, and Media. *Saccharomyces cerevisiae* mutant YSD1 (yeast sulfate transport deletion mutant 1) was used for complementation of cDNAs. This mutant has been characterized by Smith *et al.* (14). A plasmid based on the yeast episomal shuttle vector pYES2 (Invitrogen) was used for cDNA library construction. A *Sal* I site was inserted into pYES2 in place of the *Kpn* I site to make pYES3, thus enabling directional cloning into the *Sal* I/*Not* I sites.

Sa. cerevisiae was transformed by a LiCl/PEG method (15). The procedures and defined medium used to grow organisms and to select *Sa. cerevisiae* transformants involved in sulfate transport were similar to those used to isolate the high-affinity sulfate transporter from *Sa. cerevisiae* (14).

Extraction of DNA and Total RNA. Genomic DNA was extracted from *St. hamata* by a method involving buoyant density centrifugation in CsCl (16). Plasmid DNA was extracted from *Sa. cerevisiae* by vigorously shaking the cells with glass beads in buffer, detergents, and phenol/chloroform/3-methyl-1-butanol (17).

Total RNA was extracted from *St. hamata* roots with guanidinium thiocyanate. Root extracts for cDNA library construction were purified with chloroform/3-methyl-1-butanol (24:1, vol/vol); the RNA was precipitated with 2-propanol, dissolved again in 10 mM Tris, pH 7.6/1 mM EDTA, and precipitated overnight in 2 M LiCl at 4°C. Extracts of root and leaf RNA for Northern blot analyses were purified through a 5.7 M CsCl pad (18).

cDNA Library Construction. Poly(A)$^+$ RNA was extracted from total RNA using the Promega PolyATtract system.

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Abbreviations: MSD, membrane-spanning domain; YSD, yeast sulfate transport deletion mutant; SHST, *Stylosanthes hamata* sulfate transporter.

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§The nucleotide sequences of the cDNAs that encode SHST1, SHST2, and SHST3 have been deposited in the GenBank data base (accession nos. X82255, X82256, and X82454).

cDNA was prepared from 5 μg of poly(A)$^+$ RNA using the SuperScript lambda system (GIBCO/BRL) and directionally cloned into pYES3. This library was electroporated into *Escherichia coli* strain DH10B and yielded ≈2 × 10^6 transformants of which ≈90% were recombinants.

Sulfate Uptake Assays. Sulfate uptake by *Sa. cerevisiae* was measured by suspending cells in medium containing ^{35}S-labeled SO_4^{2-}. Uptake was terminated by centrifuging 100 μl of cell suspension through 100 μl of silicon oil (Dow–Corning 550)/dinonylphthalate (BDH–Merck) (1:1, vol/vol) into 2 μl of 40% (vol/vol) perchloric acid in the bottom of 400-μl microcentrifuge tubes. The tips of these tubes were then cut off into 3 ml of scintillant (Optifluor, Packard), and the radioactivity was assayed. Uptake rates were measured over the 1-min uptake period between 30 and 90 s after adding the cells to ^{35}S-labeled SO_4^{2-} medium.

Southern Blots. Restriction digests of 10 μg of genomic DNA were separated by overnight electrophoresis in 0.8% agarose. DNA was transferred to a Hybond N$^+$ nylon membrane (Amersham) by capillary blotting (19) and fixed by UV cross-linking. Membranes were prehybridized with 0.25 M Na_2HPO_4 (pH 7.2) in 7% SDS for 10 min at 55°C and then hybridized with ^{32}P-labeled probe (prepared with Amersham Megaprime) for at least 12 hr at 55°C. After removal of the hybridization solution, membranes were washed twice with 2× SSC in 0.1% SDS at room temperature and then for 30 min with 2× SSC in 0.1% SDS at 60°C for a medium-stringency wash or for 30 min with 0.1× SSC in 0.1% SDS at 65°C for a high-stringency wash.

Northern Blots. Thirty micrograms of total RNA was separated by overnight electrophoresis at 1.6 V·cm^{-1} in 1.2% agarose containing 2.2 M formaldehyde (19). The gel was then equilibrated with 0.5× TBE, and RNA was electrophoretically transferred to a Zeta-Probe GT membrane (Bio-Rad) using the Bio-Rad Trans-Blot SD procedure. RNA was fixed to the membranes by UV cross-linking and probed using similar hybridization and washing procedures to those used for Southern blots.

Sequence Analysis. Sequences were aligned using PILEUP from the Wisconsin Genetics Computer Group package. Phylogenetic distances were calculated and plotted as a phylogenetic tree using PHYLIP (20).

RESULTS AND DISCUSSION

Isolation of cDNAs Encoding Sulfate Transporters. cDNAs that encode sulfate transporters were cloned from *St. hamata* by complementation of *Sa. cerevisiae* sulfate transport mutant YSD1 (14). YSD1 was transformed with a cDNA library derived from the roots of *St. hamata* plants that had been deprived of an external sulfate supply for 72 hr. Screening for the ability of transformants to grow on medium containing 100 μM sulfate resulted in the isolation of eight functional clones. Restriction enzyme analyses and partial sequencing of these clones revealed that they represented three different classes of cDNAs: *shst1, shst2*, and *shst3* (for *St. hamata* sulfate transporter).

The functions of the proteins encoded by *shst1, shst2*, and *shst3* were verified by measuring sulfate uptake in complemented yeast mutant YSD1 (Table 1). Expression of *shst1, shst2*, and *shst3* induced by D-galactose allowed the YSD1 mutant to transport sulfate into the cells. Growth on D-glucose abolished this expression, and no sulfate was transported into the cells.

Cotransporter Activity. Anions are thought to be cotransported with H$^+$ (or OH$^-$) ions across the plasma membrane (21); accumulation of the anion is driven by transmembrane H$^+$ gradients generated by proton pumps. Dissipation of these gradients by increasing the external pH or by high concentrations of external buffer has been shown to severely diminish anion transport by plant roots (22). When the polypeptides SHST1, SHST2, and SHST3 were expressed in the yeast mutant YSD1, sulfate transport into the cells was markedly lowered by increasing the external pH from 5.6 to 7.6 (Fig. 1). In other experiments in which the external pH was kept constant but the concentration of external buffer was increased, sulfate transport also decreased considerably (data not shown). These results suggest that the polypeptides function as H$^+$/sulfate cotransporters and agree with characteristics previously identified in transport assays with isolated plasma membrane vesicles from plant roots (23). Rates of sulfate transport via SHST3 were more sensitive to higher external pH (Fig. 1), which, together with results on the expression of SHST3 (see below), suggests that SHST3 may be adapted for sulfate transport from environments such as the xylem sap or the vacuole, which are generally quite tightly regulated in the pH range 5.0–5.5.

Table 1. Characteristics of *shst1, shst2*, and *shst3* when expressed in yeast mutant YSD1

Insert	Number of amino acids	M_r × 10^{-3}	K_m*, μM	SO_4^{2-} uptake rate†, nmol of SO_4^{2-} per mg of cells per min: Glucose	Galactose
None				0.001 ± 0.002	0.001 ± 0.001
shst1	667	73.1	10.0 ± 0.6	0.008 ± 0.003	0.554 ± 0.025
shst2	662	72.7	11.2 ± 0.5	0.010 ± 0.003	0.882 ± 0.031
shst3	644	69.5	99.2 ± 4.8	0.002 ± 0.000	0.274 ± 0.018

*K_m was determined by expressing cDNA inserts in yeast mutant YSD1 grown on medium containing D-galactose and homocysteine thiolactone. K_m was derived by fitting the first-order kinetic equation $y = V_m x/(K_m + x)$ to sulfate uptake rates measured between 30 and 90 sec after transferring cells to medium containing ^{35}S-labeled $MgSO_4$.

†Expression was measured as the rate of uptake of ^{35}S-labeled sulfate from medium containing 50 μM sulfate.

Properties of *shst1, shst2*, and *shst3*. Measurements of the K_m for sulfate of SHST1 and SHST2 when expressed in the yeast mutant indicate that they are high-affinity sulfate transporters (Table 1). These K_m values are very similar to that measured for sulfate absorption by barley roots in the classical experiments of Leggett and Epstein (1). SHST1 and SHST2 are similar to one another in size and in their deduced amino acid sequences (Fig. 2) (97% similar and 95.3% identical). Southern

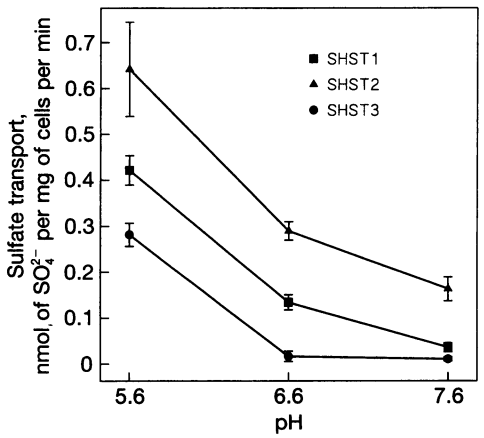

FIG. 1. Effect of external pH on the transport of sulfate into cells of yeast mutant YSD1 complemented with clone *shst1, shst2*, or *shst3*. Cells were grown on medium containing homocysteine thiolactone and D-galactose in order to express the polypeptide SHST1, SHST2, or SHST3. Sulfate transport was measured in similar medium containing 50 μM $^{35}SO_4^{2-}$. pH was adjusted by adding 0.1 M NaOH. (Error bars indicate the SD.)

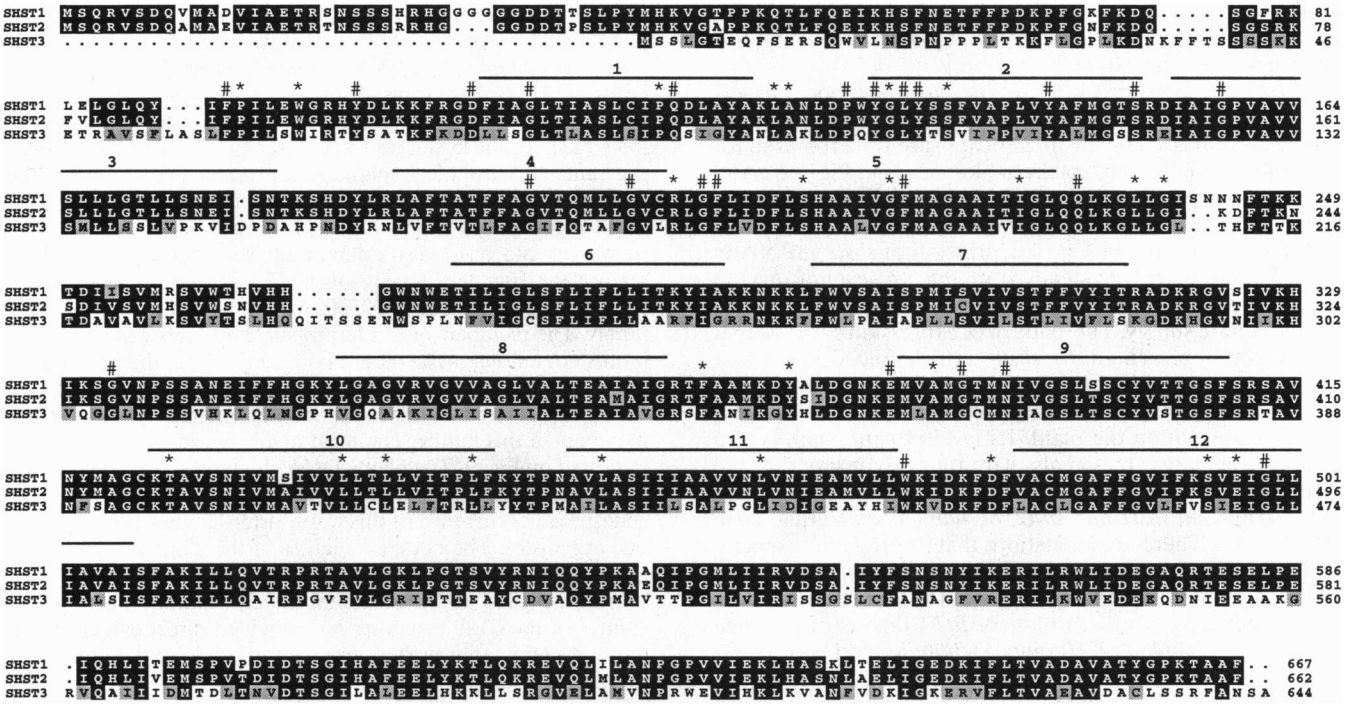

FIG. 2. Alignment of deduced amino acid sequences of *St. hamata* sulfate transporters SHST1, SHST2, and SHST3. Perfect homology is indicated by a black background, and conserved substitutions are indicated by shaded boxes. Marked amino acid residues are conserved across all (#) or all except one (*) of the 10 published sequences in this family of sulfate transporters. Horizontal, numbered bars indicate the location within the *St. hamata* sequences of the consensus membrane-spanning domains, as predicted by MEMSAT (24). The 10 published full-length sequences in this family of eukaryotic sulfate transporters are *N. crassa cys-14* (8), yeast *sul1* (14), soybean nodule-specific *GmAK170* (10), *Stylosanthes shst1, shst2* and *shst3*, rat liver *sat-1* (12), mouse sulfate transporter (accession no. D42049), human diastrophic dysplasia (DTD) (13), and human colon mucosa (DRA) (9). In addition, there are two fragments in the sequence data bases that belong to this family, rice expressed sequence tag fragment (accession No. D25000) and *A. thaliana* expressed sequence tag fragment (accession No. T21459).

blot analyses of genomic DNA from *St. hamata* digested with *Kpn* I, *Bcl* I, or *Hpa* I and hybridized with a combined *shst1/shst2* probe indicated that these genes are members of a small family (data not shown). Southern blots at reduced hybridization stringency revealed that related members of this subtype also occur in other plant species including *Arabidopsis thaliana* and *Lycopersicon esculentum.*

The deduced amino acid sequence of SHST3 is 74% similar and 52% identical to those of SHST1 and SHST2. The amino terminus of SHST3 is truncated by 40 residues compared to SHST1, and the amino- and carboxyl-terminal regions show considerable sequence divergence (Fig. 2). This subtype has a lower affinity for sulfate, as indicated by the higher K_m, when expressed in yeast (Table 1) and is clearly dissimilar to the subtype represented by SHST1 and SHST2. A *shst3* probe did not cross-hybridize with *shst1* or *shst2* at reduced stringency (2× SSC/0.1% SDS at 60°C). Southern blot analysis indicated that *shst3* was also represented by a small gene family in *St. hamata* (data not shown). Cross-hybridization experiments indicated that the genome of *A. thaliana* also contains members of the subtype represented by *shst3* (data not shown).

Expression of the *shst1, shst2*, and *shst3* Genes. The two subtypes of plant sulfate transporters show distinct patterns of gene expression in *St. hamata.* Expression of mRNA corresponding to *shst1* and *shst2* could not be detected in leaves by Northern blot analysis (Fig. 3 *Upper*). These mRNAs were expressed at moderate abundance in the roots of plants adequately supplied with external sulfate, but their abundance was markedly increased when the roots were deprived of an external sulfate supply for 72 hr. When sulfate was resupplied for 24 hr, mRNA decreased to levels found in plants grown continuously on external sulfate (Fig. 3 *Lower*). The expression patterns of these genes in response to external sulfate supply mirror changes in the rates of sulfate uptake measured in another tropical forage legume, *Macroptilium atropurpureum* (3). These data suggest that the *shst1/shst2* gene products are high-affinity, repressible H^+/sulfate cotransporters that mediate the uptake of sulfate by

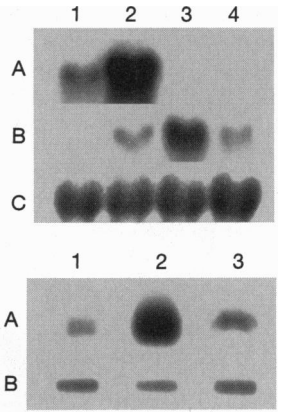

FIG. 3. Northern blot analysis of expression of the sulfate transporter genes in *St. hamata.* (*Upper*) Total root RNA (lanes 1 and 2) and total leaf RNA (lanes 3 and 4) from *St. hamata* plants grown under normal sulfur nutrition (lanes 1 and 3) or deprived of sulfate for 72 hr (lanes 2 and 4). RNA was hybridized with a probe from either *shst1/shst2* combined (row A), *shst3* (row B), or rDNA (row C). Filters were washed at high stringency and exposed to x-ray film for 24 hr (row A), 6 days (row B), or 2 hr (row C). (*Lower*) Total root RNA from *St. hamata* plants grown continuously with 250 μM sulfate (lane 1), deprived of sulfate for 72 hr (lane 2), or deprived of sulfate for 72 hr and then resupplied with 250 μM sulfate for 24 hr (lane 3). RNA was hybridized with a probe from *shst1* (row A) or rDNA (row B). Filters were washed at high stringency and exposed to x-ray film for 6 hr (row A) or 1 hr (row B).

plant roots and that the rate of sulfate uptake is regulated at the level of gene expression by signals that ultimately respond to the external sulfate supply.

The mRNA corresponding to the *shst3* subtype was expressed at much lower levels than those of the *shst1/shst2* subtype, as indicated by the longer exposure time required for measurable signals from Northern blot analysis (Fig. 3 *Upper*). There were slightly enhanced levels of expression in roots deprived of sulfate for 72 hr. However, in contrast to *shst1/shst2*, there was a high level of expression of *shst3* mRNA in leaves. Furthermore, the steady-state mRNA level of *shst3* in leaves was decreased when the roots were deprived of an external sulfate supply. This pattern of expression suggests that the subtype represented by *shst3*, which encodes a lower affinity H^+/sulfate cotransporter, may be involved in the internal transport of sulfate between cellular or subcellular compartments within the plant. Its level of expression is also subject to regulation by signals that ultimately respond to the external sulfate supply.

The Origins of *shst1* and *shst2*. *St. hamata* cv. Verano is an allotetraploid. There are indications that its progenitor species may be the diploid species *St. hamata* and *Stylosanthes humilis* (25). The origins of the genes for *shst1* and *shst2* were investigated using Southern blots of *Bcl* I digests of genomic DNA from *St. hamata* cv. Verano, *St. humilis*, and two closely related genotypes of diploid *St. hamata* (Fig. 4). The existence of two internal *Bcl* I sites in the *shst1* cDNA allows us to predict that the 1.5-kb fragment in lanes 1 and 4 must correspond to this gene. Because one *Bcl* I site is very close to the 5′ end of the *shst1* cDNA (≈50 bp), the 5′-flanking genomic fragment would not be expected to hybridize appreciably. This is consistent with the other fragment in lane 1 corresponding to the 3′-flanking fragment associated with this gene. The *Bcl* I restriction digest of the *shst2* cDNA allows the possibility that this gene could reside on a single hybridizing restriction fragment, which we propose is the larger of the two fragments ≈4.4 kb in length (lanes 2–4). These data provide preliminary evidence that *shst1* and *shst2* may represent homoeologous genes from the two different progenitor genomes, *shst1* originating from *St. humilis* and *shst2* from diploid *St. hamata*.

Homology with Other Eukaryotic Sulfate Transporters. The sequences of the *St. hamata* sulfate transporter cDNAs show strong similarity to several recently cloned sequences that form a transporter family (11, 13). The family shows no significant homologies with other reported transporter families and superfamilies (6, 7) or to Na^+/sulfate cotransporters from rat renal tissue (26) and ileal tissue (27) (GenBank accession nos. L19102 and U08031, respectively). The identified members of the sulfate transporter family are the *Neurospora crassa cys-14* gene (8); a *Sa. cerevisiae* H^+/sulfate transporter (14); a soybean nodule-specific gene product, *GmAK170* (10); a rat liver H^+/sulfate transporter, *sat-1* (12); a mouse sulfate transporter (accession no. D42049); the human diastrophic dysplasia gene product, DTD (13); and the human colon mucosa *DRA* gene product, whose expression is down-regulated in adenomas and adenocarcinomas (9). The sequence data bases also contain an *Arabidopsis* expressed sequence tag fragment (accession no. T21459) and a rice cDNA fragment (accession no. D25000) that show homology to this family. The members of this family share a number of conserved motifs containing invariant amino acid residues, the presence of which in the deduced amino acid sequences of *shst1, shst2*, and *shst3* (Fig. 2) clearly establishes these *St. hamata* clones as plant members of this family. The most highly conserved residues are indicated on Fig. 2. The plant cDNA clones and the cDNA clone of the yeast high-affinity sulfate transporter (14) demonstrate the widespread occurrence of this transporter family among eukaryotic organisms. The detailed analysis of the plant and yeast (14) gene products verifies the function of these transporters. Expression of the plant and yeast clones in yeast mutant YSD1 under control of the *GAL* promoter has provided direct evidence that these proteins transport sulfate across biological membranes (Table 1).

The addition of the plant members to this family of sulfate transporters now reveals that these transporters have been conserved across eukaryotic organisms ranging from filamentous fungi, yeasts, and plants to mammals and humans. A comprehensive alignment of all the presently known protein sequences in this sulfate transporter family was made, and phylogenetic relationships were derived (Fig. 5). The mammalian, the yeast and fungal, and the plant members of the family all fall into distinct groups. The high similarity of certain members is evident—for example, the human DTD and mouse sequences and the SHST1 and SHST2 sequences. SHST3 falls into a quite distinct branch of the tree to the other *St. hamata* sequences. Sequences of the rice and *Arabidopsis* fragments are not of sufficient length to correctly infer phylogenetic relationships.

Topology of a Family of Sulfate Transporters. Many cation/solute symporters examined to date have been suggested to contain 12 membrane-spanning domains (MSDs) (see ref. 7). Predictions of 12 MSDs were made using the MEMSAT program (24) on all of the sequences known to be members of this family. Analysis of the positions of the predicted helices on the aligned sequences, together with an analysis of the hydropathy profiles, indicated a consensus location for 12 MSDs. These

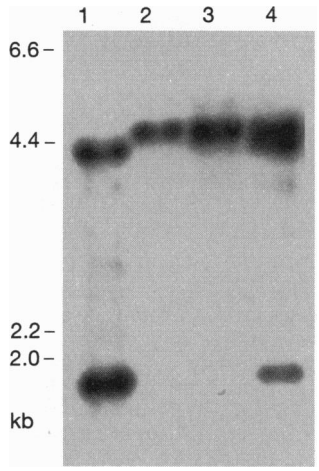

FIG. 4. Southern blot analysis with *Bcl* I digests of allotetraploid *St. hamata* cv. Verano (lane 4) and its putative diploid progenitors, *St. humilis* cv. Paterson (lane 1) and two closely related genotypes of diploid *St. hamata* CPI33231 (lane 2) and CPI56211 (lane 3). Blots were hybridized with probes from *shst1/shst2* combined.

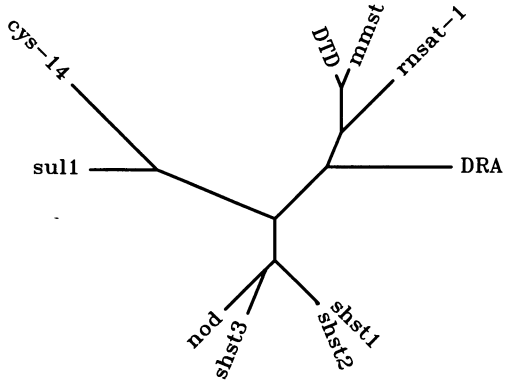

FIG. 5. Phylogenetic relationships between members of a family of eukaryotic sulfate transporters. The 10 full-length deduced amino acid sequences identified in the legend to Fig. 2 were aligned using PILEUP, and phylogenetic distances were calculated using PHYLIP and plotted as a phylogenetic tree.

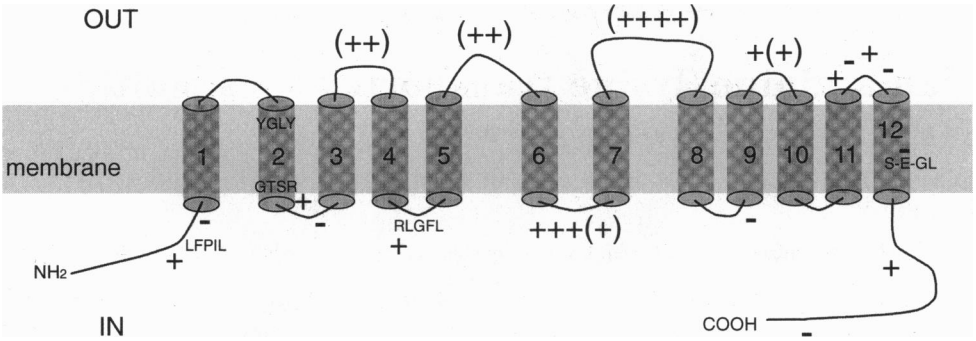

FIG. 6. Consensus model for a eukaryotic sulfate transporter based on a comparison of the presently known members of the family identified in the legend to Fig. 2. Approximate locations of selected conserved motifs and charged residues are marked. At other locations there are clusters of positively charged residues that are not strictly conserved (indicated in parentheses).

proposed MSDs are marked on the alignment of the *St. hamata* sequences (Fig. 2). The positions of the tentative hydrophobic MSDs indicated agree closely with those proposed by Hästbacka *et al.* (13) for the rat liver sulfate transporter (*sat-1*) and the human *DTD* and *DRA* gene products. Hästbacka *et al.* (13) also propose a carboxyl-terminal membrane-associated region. The potential glycosylation site present in all of the mammalian sequences between helices 3 and 4 is not present in any of the plant or fungal sequences, and the alignments introduce a gap in this region.

The topology diagram (Fig. 6) indicates the relative distribution of the helices within the aligned sequences. The location of conserved charged residues and clusters of charged residues are also indicated together with the location of a number of the distinctive conserved motifs. The clusters of extracellular basic residues seen in all members of this family are atypical for membrane proteins (28), and along with the presence of at least two charged residues buried within MSDs, may have important functional consequences for these transporters. A role in anion binding or channeling might be expected for these basic residues. Clarkson *et al.* (5) demonstrated the sensitivity of the barley sulfate transport system to a membrane-impermeant arginine-specific reagent, hydroxyphenylglyoxal, indicating the presence of essential extracellular accessible arginine residue(s). The predicted model includes a number of extracellular arginine residues in the *St. hamata* sequences, only one of which, between MSDs 9 and 10, is conserved in all three sequences.

The H^+/sulfate cotransporter family represents an additional membrane cotransporter superfamily. Structural and functional analysis of the H^+/sulfate cotransporter family will facilitate a wider understanding of general ion transport phenomena.

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