# Chapter 2

# Uptake, Distribution and Subcellular Transport of Sulfate

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## Summary

Sulfate transport in and out of the cell in all phototrophic organisms, and in addition in eukaryotes, influx and efflux from the vacuole, which acts as a cellular sulfate store, and into the plastid, the site of sulfate reduction, is facilitated by multiple transporters. In addition, in vascular plants these transport processes are coordinated to facilitate management of sulfur fluxes between organs, from roots to shoots and to generative tissues. In prokaryotes, uptake into the cell is driven predominantly by an ABC-transporter, which is the product of at least four genes and energized directly by ATP. In eukaryotes, a family of $H^+$-sulfate co-transporters (SulP) has been characterized which fulfills transport roles at least for uptake into the cell and efflux from the vacuole. In addition, differential expression of this gene family in vascular plants enables selective movement between tissues dependent on developmental cues and sulfate availability. A vital transport step is the transport into the plastid, for which a transporter has only been identified in algae and for which no vascular plant homologue is known. The expression of many sulfate transporters responds to availability and demand for sulfur, and transduction mechanisms which control this are beginning to be elucidated. The significance of a C-terminal STAS domain in SulP transporters is still unclear.

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## I. Introduction

The sulfur demands of phototrophic organisms including plants, algae and cyanobacteria are met primarily by the acquisition of sulfate from the environment. Specific transport systems have evolved to both take up and to fulfill internal requirements for the transport of sulfate. In addition, most phototrophic organisms including plants, have a wide range of transporters for organic molecules including sulfur-containing amino acids and in some instances these may contribute to the sulfur economy of the organism. In multi-cellular organisms such transporters are important for the distribution of reduced sulfur compounds such as glutathione (Rennenberg et al. 1979; Zhang et al. 2004) or S-methylmethionine (Bourgis et al. 1999), for example from sink to source tissues. Plants are also able to utilize atmospheric $H_2S$ absorbed via the leaves and assimilate this directly into cysteine, but this is seldom a major physiological consideration (De Kok et al. 2002). The importance of the transport steps is underlined by pathway analysis which indicates that sulfate uptake into the cell is the major regulated step (Vauclare et al. 2002).

Sulfate uptake into plants was determined to be an enzymatic process catalyzed by transporters, and was a saturable process with a high affinity for sulfate (19 μM), was pH dependent and was competitively inhibited by selenate (Leggett and Epstein 1956). The cloning of sulfate transporter genes and the subsequent detailed analyses of transport phenomena performed in the last decade, more than 40 years later, have verified the accuracy of this pioneering study.

Two major transport systems for sulfate have been described: the prokaryotic ABC-type transporter which includes a periplasmic sulfate binding protein and which occurs predominantly in prokaryotes including cyanobacteria, and the SulP family of $H^+$ -cotransporters which predominate in vascular plants. Genomic information has indicated that members of the SulP family also exist in prokaryotes transporting a diverse set of substrates. Although many ABC-type transporters exist in all eukaryotes, evidence for a role in sulfate transport in eukaryotes is restricted to one specific example in *Chlamydomonas reinhardtii*. The different transporters and their occurrence will be dealt with in turn.

*Abbreviations*: ABC–ATP-binding cassette; OAS–*O*-acetylserine; SLIM1–sulfur limitation 1 (transcription factor); STAS–sulfate transporter and anti-sigma factor antagonist; SURE–sulfur-responsive element

## II. Sulfate Transport in Cyanobacteria

The well-characterized sulfate permease of cyanobacteria, in common with other prokaryotic sulfate transporters (Kertesz 2001), belongs to the ABC-type (ATP-binding cassette) consisting of a multi-subunit transporter including a periplasmic substrate binding protein (probably present in substantial molar excess in the periplasmic space), two channel-forming membrane intrinsic proteins and a cytoplasm-located ATP-binding protein. The genes (see Table 1) that encode the proteins which are components of the complex are up-regulated under sulfur-deficient conditions, to aid in scavenging for available sulfur. Additionally, in an adaptation to low sulfur availability, the sbpA protein which is stoichiometrically the most abundant subunit, has no S-containing amino acid residues, as is the case for enteric bacteria. Although considerably enhancing sulfate uptake, sbpA is not absolutely essential for transport. In addition there is a regulatory gene, *cysR*, required for expression (Green et al. 1989; Kohn and Schumann 1993; Laudenbach and Grossman 1991). In eukaryotes, including *Arabidopsis*, a large family of ABC-transporter homologues with a wide substrate specificity (but as far as is known, not sulfate) exist as domains of single proteins rather than as separate proteins (Sanchez-Fernandez et al. 2001).

In addition, the availability of complete genomic sequences for a number of cyanobacteria indicates the existence of multiple transporters belonging to the SulP group (for detailed description of members of this group belonging to the vascular plants, see section IV.A) which could theoretically transport sulfate. Phylogenetic analysis indicates a distinct clustering of the cyanobacterial sequences and a divergence from the plant and other eukaryotic groups (Saier et al. 1999) which would be expected in these evolutionary distant organisms. Analysis of the prokaryotic sequences themselves, shows distinct clades which may be related to functionality.

*Table 1*. Gene designation of ABC-sulfate transporter subunits.

| Subunit | Cyanobacterial designation | Bacterial gene | *Chlamydomonas* chloroplast transporter | *Marchantia* chloroplast gene |
|---|---|---|---|---|
| Membrane pore | *cysT* | *cysT* | *SulP* | *mbpY* |
| Membrane pore | *cysW* | *cysW* | *SulP2* | |
| ATP binding protein | *cysA* | *cysA* | *Sabc* | *mbpX* (similar to *Arabidopsis NAP3* (Sanchez-Fernandez et al. 2001) |
| Periplasmic sulfate binding component | *sbpA* | *cysP* | *Sbp* (not periplasmic but associated with complex) | |

The cyanobacterial SulP sequences themselves form a diverse group, and for example one clade within the group seems to be involved in bicarbonate transport (Price et al. 2004) and another clade has one example, LtnT, found only in a few cyanobacteria, which is unable to transport sulfate but has a low affinity nitrate transporter activity (Maeda et al. 2006). Some bacterial homologues of the bicarbonate transporter group possess a C-terminal carbonic anhydrase domain (Felce and Saier 2004), which increases the inter-conversion of carbon dioxide and bicarbonate. No such fusion proteins appear in the cyanobacterial genomes studied to date.

## III. Sulfate Transport in Algae and Aquatic Plants

Many of the characteristics of sulfate transport by higher plants were first described in studies on algae, partially due to their amenability for experimentation: *Lemna minor* (Neuenschwander et al. 1991; Thoiron et al. 1981), *Lemna paucicostata* (Datko and Mudd 1984a, b), *Lemna gibba* (Lass and Ullrich-Eberius 1984), *Hydrodictyon reticulatum* (Rybová et al. 1988), *Chlorella pyrenoidosa* (Vallee and Jeanjean 1968a, b), *Chlorella vulgaris* (Passera and Ferrari 1975), *Chlamydomonas reinhardtii* (Yildiz et al. 1994). Evidence for a $3H^+$-sulfate co-transport mechanism was obtained in *Lemna gibba* (Lass and Ullrich-Eberius 1984). The high affinity transport system was shown to require high proton concentrations suggesting a mechanism of a proton-sulfate co-transport system. This $H^+$-cotransport mechanism for sulfate transport was subsequently supported by studies on vascular plants which demonstrated a pH dependency of sulfate transport in *Brassica napus* vesicles (Hawkesford et al. 1993) and by the low pH stimulation of transport seen in yeast expressing the high affinity sulfate transporter from the legume, *Stylosanthes hamata* (Smith et al. 1995a).

Compartmental analysis in *Lemna minor* indicated the importance of the vacuole as an internal store of sulfate (Thoiron et al. 1981), although a store in which sulfate only turned over slowly (Datko and Mudd 1984b). An increased capacity for sulfate uptake under sulfur limiting conditions was reported in all instances, in common with the situation for higher plants (see below). It was suggested that both saturating high affinity and low affinity transport systems operated, but only the high affinity system was induced by sulfur limiting conditions (Datko and Mudd 1984b). An indication of the importance of the cysteine precursor, *O*-acetylserine (OAS) in determining sulfate transport expression (see section VII for a more detailed discussion and subsequent studies with vascular plants), in common with earlier bacterial studies (Kredich 1993), was provided in feeding experiments in *Lemna minor* (Neuenschwander et al. 1991).

In spite of these early studies and the clear ecological importance of algae in the aquatic environment (Norici et al. 2005) little further molecular characterization of the transporters has occurred with the exception of *Chlamydomonas rheinhardtii*. In this species a gene family of seven sulfate transporters of the SulP type has been identified (Pollock et al. 2005) but not further characterized, and there is a detailed characterization of a chloroplast transporter of the ABC-type (see section V). In addition, *Chlamydomonas rheinhardtii* has proved exceptionally useful for elucidation of genes involved in sensing and signaling sulfur status (see section VIII).

## IV. A Family of Sulfate Transporters in Higher Plants

### *A. The H⁺-Sulfate Co-Transporter Family (SulP)*

The first putative sulfate transporter sequence to be identified from plants was by homology to the *Neurospora crassa* sulfate transporter (Ketter and Marzluf 1988; Sandal and Marcker 1994). This was the soybean nodule-specific protein, encoded by the *GMAK170* gene, a protein of 486 amino acids, which was considerably shorter than the *Neurospora* sequence (788 amino acids). In this same report the human DRA gene (Hästbacka et al. 1994) was shown also to have homology to these sequences and this was subsequently characterized as a human $H^+$-sulfate transporter and part of a family of mammalian sulfate transporters, which were distinct from mammalian Na-coupled sulfate transporters (Bissig et al. 1994). No evidence has been forthcoming to demonstrate that the *GMAK170* gene product is able to function as a sulfate transporter.

The cloning of confirmed plant sulfate transporter cDNAs was achieved first by functional complementation of a sulfate transporter-deficient yeast mutant (Smith et al. 1995b) with plant cDNA libraries (Smith et al. 1995a; Smith et al. 1997; Takahashi et al. 1996). A homologous yeast sulfate transporter was also isolated using this mutant (Smith et al. 1995b). The first yeast sulfate transporter-deficient mutants were isolated by selection using selenate/chromate toxic analogues (Breton and Surdin-Kerjan 1977; Cherest et al. 1997; Smith et al. 1995b). Subsequently yeast deletion mutants have been essential tools for verification and functional characterization of cloned putative sulfate transporters. A similar selenate-resistance strategy was used to isolate transporter mutants of *Arabidopsis* (Shibagaki et al. 2002).

Initially three sulfate transporters were isolated from the tropical legume *Stylosanthes hamata*, showing either high or low affinity for sulfate in the yeast expression system. Subsequently a high affinity type was isolated in barley using an identical approach (Smith et al. 1997). In each case the cDNA libraries were made from mRNA isolated from root tissues of sulfur-starved plants, a condition known to induce maximal activity (see below). The availability of cDNA sequences facilitated the isolation and subsequent characterization of a number of cDNAs for sulfate transporters from *Arabidopsis* (Kataoka et al. 2004b; Shibagaki et al. 2002; Takahashi et al. 1996; Takahashi et al. 2000; Takahashi et al. 1997; Vidmar et al. 2000). Subsequently sulfate transporters have been cloned and analyzed from a range of plant species including maize (Bolchi et al. 1999; Hopkins et al. 2004), potato (Hopkins et al. 2005), tomato (Howarth et al. 2003), *Brassica* (Buchner et al. 2004b; Heiss et al. 1999), wheat (Buchner et al. 2004a), rice (Godwin et al. 2003) and *Sporobolus stapfianus* (Ng et al. 1996).

The availability of the fully sequenced genomes of *Arabidopsis* and rice and indicated the existence around 14 genes sequences showing homology to the SulP sulfate transporters in each genome. Phylogenetic analysis of all known sequences clearly separates the plant, yeast, fungi and mammalian kingdoms (Saier et al. 1999), however analyses of sequence homology of the plant species alone, suggests at least five distinct clades within the plant family. A typical phylogenetic analysis of amino acid sequences of *Arabidopsis* and rice putative sulfate transporters is shown (Fig. 1). In most cases all species have a similar distribution of isoforms between the clades, although exactly corresponding homologues are only identifiable for closely related species (for example for *Arabidopsis* and *Brassica*, or for rice and wheat). In some species, for example wheat, not only does polyploidy increase the complexity of expressed isoforms, but also there have been recent duplication events resulting in additional very similar isoforms (Buchner et al. 2004a). There remains debate as to the significance of the multiple members of the family with regard to redundancy or individual specialization of isoforms (Hawkesford 2003).

The clade which forms Group 1 includes many well studied transporters and often comprises three genes, for example as found in *Arabidopsis* (*AtSULTR1;1–3*). Within Group 1, the monocotyledonous species are distinct from the *Arabidopsis* clade preventing the alignment to the direct corresponding homologues (Fig. 1, and wheat and maize data, not shown). Many Group 1 sulfate transporters have been expressed in yeast and most have high substrate affinities ($K_m$) for sulfate in this heterologous expression system, for

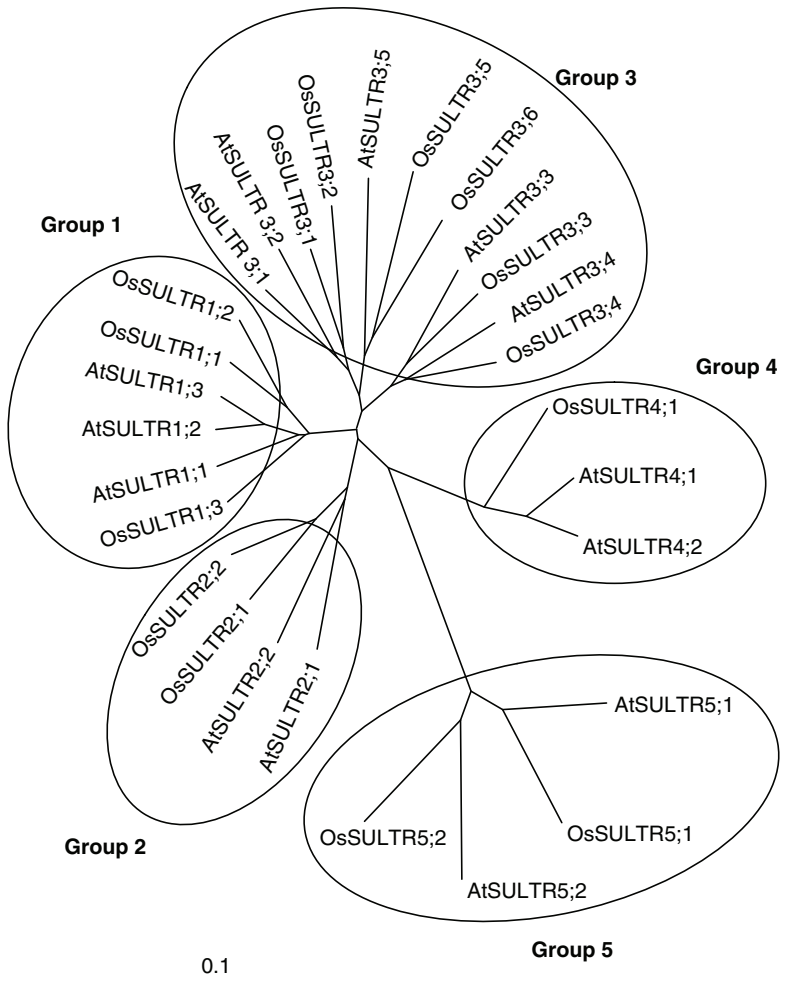


*Fig. 1*. Unrooted phylogenetic tree of the rice (*Oryza sativa*) and *Arabidopsis* members of the SulP sulfate transporter family. Phylogenetic representation of the plant sulfate transporter amino acid sequences showing subdivision into 5 putative Groups. Accession numbers: *Arabidopsis*: AtSULTR1;1, AB018695; AtSULTR1;2, AB042322; AtSULTR1;3, AB049624; AtSULTR2;1, AB003591; AtSULTR2;2, D85416; AtSULTR3;1, D89631; AtSULTR3;2, AB004060; AtSULTR3;3, AB023423; AtSULTR3;4, B054645; AtSULTR3;5, AB061739; AtSULTR4;1, AB008782; AtSULTR4;2, AB052775; AtSULTR5;1, NP_178147; AtSULTR5;2, NP_180139; rice: OsSULTR1;1, AF493790; OsSULTR1;2, AAN59764.1; OsSULTR1;3, BAC98594; OsSULTR2;1, AAN59769; OsSULTR2;2, AAN59770; OsSULTR3;1, NP_921514; OsSULTR3;2, AAN06871; OsSULTR3;3, AK104831; OsSULTR3;4, AK067270; OsSULTR3;5, NM_192602; OsSULTR3;6, NM_191791; OsSULTR4;1, AF493791; OsSULTR5;1, BAC05530; OsSULTR5;2, BAB03554. Alignments were performed using ClustalX program (Thompson et al. 1997) version 1.81 and the tree was drawn using the Treeview32 program (Page 1996).

example AtSULTR1;1 has a $K_m$ of 1.5–3.6 µM (Takahashi et al. 2000; Vidmar et al. 2000) and AtSULTR 1;2 a $K_m$ of 6.9 µM (Yoshimoto et al. 2002). Many of the transporters in this Group are highly expressed in the root tissues, and furthermore are highly regulated by sulfur supply, with massively increased transcript abundance when plants are sulfur-deficient, for example (Buchner et al. 2004b). Studies of cellular expression patterns indicate expression in root tips, root hairs, exodermal, cortical and endodermal layers with less expression in the central vascular region (Rae and Smith 2002; Shibagaki et al. 2002; Takahashi et al. 2000; Yoshimoto et al. 2002). A notable exception is AtSULTR1;3 whose expression appears to be specific to the phloem

in both root and cotyledons (Yoshimoto et al. 2003). In tomato contrasting expression occurs for the two isoforms reported, with one isoform showing general expression, highest in exodermis and endodermis and the other with primarily exodermal expression (Howarth et al. 2003). Sulfate uptake, but not transfer to the shoot was impaired in selenate resistant mutants with a lesions in *AtSULTR1;2* (Shibagaki et al. 2002); only mutations of *AtSULTR1;2* confer selenate resistance in *Arabidopsis* (El Kassis et al. 2007). Taken together, it is clear that Group 1 transporters are responsible for primary sulfate acquisition in plant roots.

Typically two sulfate transporter isoforms for any individual species are found in Group 2 (Fig. 1). One of the original *Stylosanthes hamata* isoforms belongs to this group (Smith et al. 1995a). Affinities ($K_m$) for sulfate are generally lower than for Group 1: ShST3, 99 µM (Smith et al. 1995a); AtSULTR2;1, 0.41 mM (Takahashi et al. 2000) although one report indicates 5 µM (Vidmar et al. 2000); AtSULTR2;2, > 1.2 mM (Takahashi et al. 2000). Group 2 sulfate transporters are regulated by sulfur nutrition, although not generally so dramatically as for the Group 1 sulfate transporters. One or the other isoforms is generally more strongly regulated, but not in a consistent pattern: in root tissues AtSULTR2;2 is more regulated than 2;1 (Takahashi et al. 2000), however the pattern for the closely homologous *Brassica* isoforms is reversed (Buchner et al. 2004b). Both isoforms are usually expressed throughout the plant (Buchner et al. 2004b) but tend to be localized to vascular tissues (Takahashi et al. 2000). It is likely that this Group of transporters contribute to translocation of sulfate within the plant vascular systems. Variation between species, for example as quoted above, may reflect different developmental stages or a need for more precision in localization of analysis.

The Group 3 clade is relatively large and diverse with five *Arabidopsis* and six rice sulfate transporters (Fig. 1). At least three and possibly four sub-clades are apparent, each containing both rice and *Arabidopsis* examples, indicating relatively ancient gene duplications. Only one isoform, AtSULTR3;5 has been successfully expressed and characterized in yeast, however transport was only observed when co-expressed with AtSULTR2;1. The observed $K_m$ for sulfate was 503 µM in the co-expression system compared to 545 µM for AtSULTR2;1 alone, along with an approximate threefold increase in $V_{max}$, (Kataoka et al. 2004a). It is proposed that a hetero-dimer is required for activity of AtSULTR3;5 and for maximal activity of AtSULTR2;1. *In planta* AtSULTR2;1 and 3;5 are co-expressed in the root xylem parenchyma and pericycle cells, and although AtSULTR3;5 is constitutively expressed with no regulation by sulfur nutrition, a role for the dimer in enhancing uptake during deficiency via interaction with the inducible SULTR2;1 is suggested. In *Lotus japonicus* root nodules, the 3;5 homologue (SST1), which complements a yeast sulfate transporter deficient mutant for growth on sulfate media, functions as a sulfate transporter across the symbiosome membrane and mutant analysis indicates that this sulfate import is crucial for nitrogen fixation (Krusell et al. 2005). None of the five *Brassica* Group 3 transporters are sulfur-regulated although tissue specificity of isoform expression varies greatly (Buchner et al. 2004b).

In *Arabidopsis* and *Brassica* there are two Group 4 isoforms (Buchner et al. 2004b; Kataoka et al. 2004b), however in rice and wheat there only appears to be one (Buchner and Hawkesford, unpublished). Early localization data entailing identification of putative transit sequence and utilizing a partial sulfate transporter-green fluorescent protein fusion protein, indicated a chloroplast membrane localization (Takahashi et al. 1999). There is an absolute requirement to transport sulfate into the chloroplast, the site of reduction and the mechanism for this transport has never been confirmed. Subsequent additional analysis with full length sulfate transporter:green fluorescent protein constructs showed a more conclusive tonoplast membrane localisation for the *Arabidopsis* Group 4 sulfate transporters (Kataoka et al. 2004b). Expression was highest in roots tissues in both *Arabidopsis* and *Brassica* (Buchner et al. 2004b; Kataoka et al. 2004b), was induced by sulfur-deficiency (particularly SULTR4;2) and at least in the case of *Arabidopsis* was localized to pericycle and xylem parenchyma cells. Analysis of *Arabidopsis* double knockout plants, and critically of vacuoles isolated from these plants indicated a role in sulfate efflux from the vacuole tissue. For example, vacuoles isolated from the double knockout (4;1 and 4:2) line contained more sulfate than the wild-type, and this was decreased in lines over-expressing a 4;1

construct. However, no direct demonstration of transport, for example in yeast mutants, has been achieved. Irrespective of specific sulfate transport function, *in planta* studies indicated that sulfate efflux from the root vacuoles was dependent on the presence of functional Group 4 proteins. It is hypothesized that this would optimize channeling of sulfate toward the xylem vessels and thus expression of the Group 4 transporters in roots would have a potential role in regulating root to shoot transport (Kataoka et al. 2004b).

Group 5 is quite distinct from the other isoforms (sequences are quite dissimilar to other sulfate transporters) and typically contains two isoforms for any given species, and the two isoforms are also quite dissimilar to one another. The most striking observation is that Group 5 sulfate transporters are truncated sequences and possess little N or C-terminal regions beyond the transmembrane domain. Some secondary structure predictions suggest fewer membrane spanning helices although there is no reason to suspect such a divergence from the rest of the family. Green fluorescent protein fusion constructs localize Group 5 members to internal membranes and it would be tempting to speculate a role in vacuolar loading, although studies with knock out mutants have failed to give a clear phenotype (Buchner, Takahashi and Hawkesford, unpublished). There are no reports indicating sulfate transport, either *in planta* or in expression systems such as yeast. The possibility remains that these transporters have a substrate other than sulfate.

### *B. Structure*

Predicted protein sizes for eukaryotic members of this family are in the range 500–700 amino acids and although predictions of secondary structure vary widely depending upon prediction method used and sequences analyzed, a consensus of 10–12 transmembrane spanning helices is predicted. One possible model is shown in Fig. 2.

Structure–function relationships have been examined by site-directed mutagenesis of the *Stylosanthes* ShST1 transporter and subsequent analysis of localization and function in yeast mutants (Howitt 2005; Khurana et al. 2000; Loughlin et al. 2002; Shelden et al. 2001, 2003). Mutations in the human DTDST transporter, known to be responsible for diastrophic dysplasia disease (Hästbacka et al. 1994), and involving conserved residues in transmembrane helices 9 and 11 when introduced into ShST1 affect either transport activity or trafficking to the plasma membrane (Khurana et al. 2000). Similarly three semi-conserved proline residues, unusually predicted to be in transmembrane helices 1–3 (notable for short extra membrane sequence linking loops) were also critical for transporter function (Shelden et al. 2001). Charged residues, which may influence topology, or be involved in ion binding or in ion channel function, have been mutated systematically: evidence was obtained for pairing of residues which would indicate tertiary structure arrangements of the transmembrane regions (Shelden et al. 2003). ShST1 contains five cysteine residues (non-conserved) and a cysteine-less variant was shown to have transport characteristics indistinguishable from the wild type (Howitt 2005); this variant will be useful for future topology analysis using combinations of mutagenesis introducing cysteine residues and probing with sulfhydryl reagents.

Homology of the carboxyl terminal region of most eukaryotic SulP transporters to bacterial anti-sigma factor antagonists, for example the *Bacillus subtilis* SPOIIAA, has defined this region as the STAS (sulfate transporter and anti-sigma factor antagonist) domain (Aravind and Koonin 2000). SpoIIAA protein is involved in nutrient regulation of sporulation; dephosphorylation of a serine activates the protein, enabling interaction with a second protein with the net result of the release of the sigma factor which induces sporulation. In the DRA transporter, the STAS domain is involved in a protein-protein interaction with the cystic fibrosis transmembrane regulator resulting in a mutual activation (Ko et al. 2004). All plant sulfate transporters examined with the notable exception of the Group 5 transporters possess this domain. To examine and test the possibility that the STAS domain may have a regulatory role in plants or may be involved in trafficking to or stability in the plasma membrane, both chimaeric and deletion constructs (Shibagaki and Grossman 2004) together with site-directed mutagenesis (Rouached et al. 2005) have been performed. Deletion of the STAS domain prevented trafficking to the plasma membrane, and heterologous chimaeras had a deleterious effect on transport kinetics (Shibagaki and Grossman 2005). Mutations

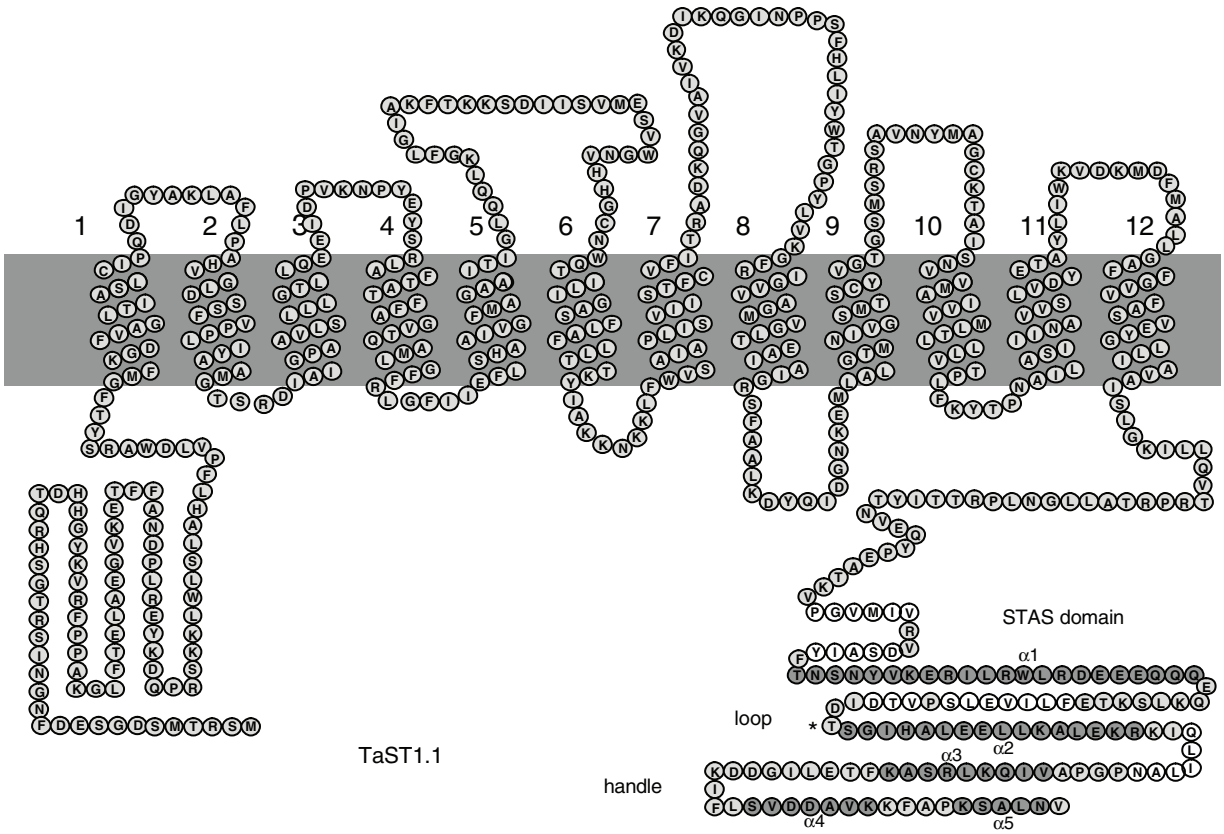


*Fig. 2*. Possible topology of a wheat sulfate transporter. The suggested topology of a sulfate transporter (TaST-B1.1b, AJ512820 (Buchner et al. 2004a)) from *Triticum aestivum*. Consensus positions of 12 trans-membrane helices were determined from an alignment of six plant sulfate transporters (wheat (Q8H0K4, Q9XGB6, Q8H2D7), maize (Q9AT12), barley (Q40008) and *Stylosanthes* (P53391)) using the Aramemmon programme (http://aramemnon.botanik.uni-koeln.de/). Arrangements of extra-membrane loops are arbitrary although the STAS domain is depicted schematically. Within the STAS domain, alpha helices (α1–5) are shaded dark grey and beta sheet structures have no shading. A conserved phosphorylatable threonine in the loop region is indicated by an asterisk.

of the equivalent phosphorylatable residue (compared to the SpoIIAA protein) in the plant sulfate transporter (a threonine, marked with an asterisk in Fig. 2) resulted in a complete loss of activity of sulfate transport (Rouached et al. 2005). It is hypothesized that there are protein:protein interactions, mediated via the STAS domain, which are required for sulfate transporter function in plants.

## V. Transport into the Chloroplast

There is an absolute requirement for transport of sulfate across the chloroplast (or plastid in the root) inner membrane into the stroma, the site for reduction and assimilation of sulfate into cysteine. Sulfate uptake into isolated chloroplasts has saturable kinetics with a $K_m$ of around 2.5–3 mM and with a $V_{max}$ of 0.7–13 µmol per mg Chl per hour (Gross et al. 1990; Mourioux and Douce 1979). Furthermore transport was competitively inhibited by phosphate, and therefore it was suggested that the triose-phosphate/phosphate translocator was responsible for sulfate uptake in addition to its primary phosphate transport function. The rates were much lower than for phosphate reflecting the greater requirement for phosphate in $CO_2$ fixation. The triose-phosphate/phosphate translocator has been cloned (Flügge et al. 1989) but no conclusive evidence has been presented that this transporter is responsible for sulfate uptake into the chloroplast *in vivo*.

A multi-subunit ABC-type transporter has been shown to be present in the chloroplast membrane of *Chlamydomonas reinhardtii* (Chen and Melis 2004; Chen et al. 2005; Chen et al. 2003; Melis and Chen 2005). It is proposed that the transporter comprises a heterodimer of transmembrane proteins constituting the 'pore', two associated cytosolic sulfate binding proteins and two stroma located ATP binding subunits attached to each of the membrane subunits (Melis and Chen 2005). Homologues to the nuclear gene for the membrane pore proteins (termed confusingly *SulP* but with no relation to the SulP family) have been identified in many prokaryotes, including cyanobacteria (homologous to the *cysT* gene) and in some eukaryotes, for example the liverwort, *Marchantia polmorpha*, in which it is a chloroplast gene. Corresponding nuclear genes have been identified for the other subunits in *Chlamydomonas*, designated *SulP2, Sbp* and *Sabc*, respectively (Table 1). Available evidence supports a role for the *SulP* gene product in chloroplast sulfate transport in *Chlamydomonas* as, for example, it is induced by sulfur-deficiency and antisense transformants are impaired in sulfate transport (Chen and Melis 2004; Chen et al. 2005). No such genes have been found in vascular plants (Chen et al. 2003).

It has been suggested that one of the subtypes of the SulP family (Group 4, see above for discussion on this topic) may be responsible for plastid sulfate influx (Takahashi et al. 1999), but this transporter has subsequently been shown to be a tonoplast membrane protein (Kataoka et al. 2004b).

Proteome analysis of the plastid membrane of *Arabidopsis* indicates the occurrence of 19 ABC-type transporters, any of which theoretically may be responsible for sulfate uptake into higher plant plastids (Weber et al. 2005).

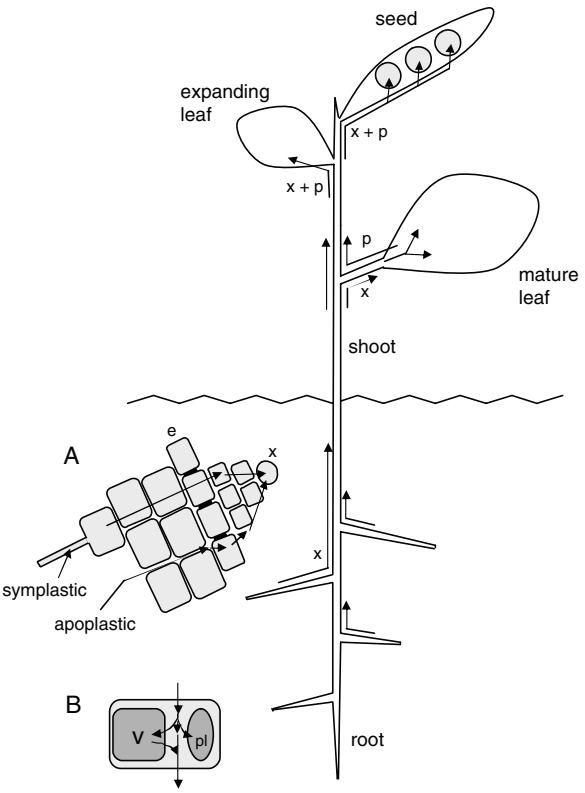


*Fig. 3*. Routes of sulfate movements *in planta*. Schematic representation of major fluxes of sulfate from the soil solution to the root xylem (x) and pathways to various sink tissues. Fluxes around the plant in xylem or phloem (p) are indicated by arrows. The inset (A) shows possible symplastic and apoplastic fluxes from cell to cell within the root cross section. The endodermal layer (e) is shown with the Casparian barrier. Inset B indicates theoretical fluxes of sulfate across individual membranes in a typical cell: initially into the cell, both into and subsequently out of the vacuole (v) as a temporary store, into the plastid (pl) for reduction, or exported from the cell.

## VI. Fluxes of Sulfate around the Plant

Fluxes of sulfate into and around a stylized vascular plant are shown in Fig. 3. Initial sulfate uptake into the symplast may be at several sites, potentially occurring through root hairs, at the root periphery or having passed through the cell walls of the cortex (apoplastic route), at a site near to the endodermis which acts as an apoplastic barrier. Within the root symplasm there may be cell to cell transfer via plasmodesmata (symplastic route), however at least one efflux step is required prior to xylem loading. Vascular plants distribute sulfur around the plant, regulated in response to changing demands; this is at least partially achieved by the flexibility provided by the gene family of transporters (SulP family) which have different kinetic properties and different patterns of expression in response to tissue, developmental and environmental cues. Distribution and redistribution of sulfur pools during development in both vegetative and generative tissues have been described in soybean and wheat. Initial distribution occurs via the xylem, however sulfate is preferentially distributed to young expanding

leaves. Sulfate is redistributed from mature leaves to roots (Bell et al. 1995; Rennenberg et al. 1979), younger leaves (Sunarpi and Anderson 1996) or generative sinks (seeds). As redistribution occurs during development, patterns of expression are modified, in some cases only in specific cells, for example to facilitate xylem to phloem transfer enabling preferential flux to young leaves rather than mature leaves (Anderson 2005). Redistribution is an important process during grain filling in cereals and coincides with redistribution of resources (N, C and S) occurring during leaf senescence. Sulfate in the vacuoles of mature leaves is an important store of sulfur and redistribution is particularly important under sulfur-limiting conditions, although the efficiency for this may vary between species. For example in *Macroptilium atropurpureum* sulfate pools were only slowly redistributed from mature leaves (Clarkson et al. 1983) and in *Brassica napus* there was a time delay before sulfate was redistributed from mature to young leaves (Blake-Kalff et al. 1998). It has been suggested that remobilization from the vacuole (the major internal store of sulfate) may be limiting (Bell et al. 1994). In response to sulfur-limitation, transporters involved in primary uptake in the root (BSULTR1;1 and 1;2), those involved in low affinity internal transport (2;1) and in vacuolar efflux (4;1) show increased transcript abundance. Similarly in stem tissues BSULTR2;1 shows increased expression in response to sulfur-limitation, as does BSULTR1;1 and 1;2, and in the leaves BSULTR1;1, 1;2, 4;1 and 4;2 increase in expression. Increased expression of the vacuolar efflux transporters correlates with unloading tissue sulfate during sulfur-stress, that is utilizing stored reserves, whereas the increased expression of BSULTR1;1 and 1;2 observed in leaf tissue may reflect a localized requirement for enhanced uptake into a cell, for example in young tissues.

Most research has focused on uptake into cells but as outlined above, there is also a requirement for efflux. There is little data to indicate how this is achieved at the molecular level, whether involving discrete transporters, specific or non-specific ion channels (Frachisse et al. 1999; Roberts 2006) or whether the SulP family catalyzes these effluxes. This is an area requiring further examination.

## VII. Regulation by Availability and Demand

Increased capacity for sulfate uptake has already being described for many eukaryotic algae (see above). A marked induction of sulfate transport capacity under sulfur insufficient conditions is also seen in cell cultures (Smith 1975), in plant roots including barley (Lee 1982) and *Macroptilium atropurpureum* (Clarkson et al. 1983) and in isolated *Brassica napus* vesicles (Hawkesford et al. 1993).

Uptake into barley increases at least 10–15-fold, and this appears to be an increase in $V_{max}$ rather than an effect on $K_m$. This increase is interpreted as increased transporter abundance rather than modification of transporter kinetics. Increased transporter capacity in isolated vesicles also indicated an increase specifically in a membrane component as being responsible for the increased transport capacity (Hawkesford et al. 1993). Transcript abundance has been determined with the availability of gene probes and in most cases a massive increase in transcript abundance upon sulfur-limitation has been observed (Smith et al. 1995a; Smith et al. 1997). Upon sulfur re-supply the abundance of transcripts reduced rapidly (usually within a few hours) indicating a rapid sensing and transduction pathway for the repression (Buchner et al. 2004b; Smith et al. 1997). Using an antibody to a synthesized polypeptide, plasma membrane-located sulfate transporter protein was detected and seen to change in abundance in parallel with changes in whole plant sulfate uptake capacity (Hawkesford and Wray 2000; Hopkins et al. 2005). A notable exception to this simple regulatory model was noted in potato (Hopkins et al. 2005), where only a small transient increase in uptake capacity was seen in spite of a large change in specific mRNA abundance. In all cases the change in transcript abundance appears to be very great in contrast to the measured changes in transporter activity. This may indicate additional post transcriptional levels of control.

A model for repression of transporter activity by sulfate or reduced sulfur compounds and de-repression under sulfur limiting conditions when these compounds are depleted has been suggested. This is based on evidence of external supply of sulfate or cysteine inhibiting sulfate uptake in cultured tobacco cells (Smith 1976)

or of methionine inhibiting sulfate uptake in *Chlorella* (Passera and Ferrari 1975), or glutathione inhibiting uptake in tobacco cells or roots (Herschbach and Rennenberg 1994; Rennenberg et al. 1988; Rennenberg et al. 1989). Analysis of mRNA abundance indicates that substantial regulation is at the level of transcription (Smith et al. 1997) and efforts have been made to determine the identity of the regulatory molecule more precisely. For example, feeding experiments on sulfur-depleted maize seedlings showed that cysteine but not glutathione repressed sulfate transporter expression in the presence of the glutathione synthesis inhibitor butathionine sulphoximine (Bolchi et al. 1999). Similar experiments in *Arabidopsis*, again using butathionine sulphoximine, indicated, in contrast, that glutathione synthesis was required for repression (Lappartient et al. 1999) and furthermore glutathione in the phloem acted as a shoot to root signal indicating shoot demand for sulfur (Lappartient and Touraine 1996). A refinement of the model includes OAS, the precursor for cysteine and the direct link to C/N metabolism, as a positive effector of sulfate transporter gene expression (Neuenschwander et al. 1991; Smith 1977; Smith et al. 1997). This dual model is based on the proposed regulatory model for the cysteine regulon in *Escherichia coli* (Kredich 1993) and has been adopted as a basic working model in plants (Hawkesford et al. 2003), although no homologues of the substrate-interacting *trans*-acting factors found in *E.coli* are known in plants. External supply of OAS to plant roots resulted in increased sulfate transporter transcript abundance in parallel with increased tissue cysteine and glutathione, suggestive of a dominant influence of OAS (Smith et al. 1997). Whilst over-expression of enzymes leading to OAS accumulation in leaf tissues resulted in sulfate transporter transcript induction in the roots, measurements in the same study of bulk tissue OAS accumulation after prolonged sulfur-limitation did not correlate with transcript abundance or uptake activity (Hopkins et al. 2005). The role of OAS in regulatory mechanisms remains to be confirmed.

## VIII. Signal Transduction Pathway

Expression of many components of the sulfate transport system in cyanobacteria, algae and plants respond to availability of or demand for sulfur. The sensing may be via metabolic intermediates which accumulate or are depleted (section VII) and these levels must then be transduced to changes in gene expression via *trans*-acting factors in a manner similar to that found in bacteria (Kredich 1993) or yeast (Thomas and Surdin-Kerjan 1997). Three mutants (*sac1, 2, 3*) affecting responses to sulfur supply were identified in *Chlamydomonas* (Davies et al. 1994). Sac1 is a membrane protein and may be involved in sensing and is critical in the down-regulation of photosynthesis during sulfur limitation (Davies et al. 1996) and up-regulation of ATP sulfurylase (Yildiz et al. 1996); no close homologue has been found in vascular plants. *Sac2* mutants also have weakened induction of sulfur-deficiency induced genes, but the nature of the *sac2* gene is unknown. The sac3 protein is a Snf1-like protein kinase and the mutant lacks any control of the arylsulfatase gene and fails to up-regulate high affinity sulfate transport in response to sulfur-limitation (Davies et al. 1999). The closest plant homologue is SNRK2.3 and mutations in this gene in *Arabidopsis* reduced induction of the ATSULTR2;2 gene in sulfur-limited conditions (Kimura et al. 2006). Cytokinins may be independently involved in the regulation of the transporters as cytokinin treatment down-regulated AtSULTR1;1 and 1;2, and was dependent on the CRE1/WOL/AHK4 receptor (Maruyama-Nakashita et al. 2004b). A seven base-pair *cis*-acting element, termed SURE (sulfur-responsive element), has been identified which occurs in the promoter region of many sulfur responsive genes, including the transporters (Maruyama-Nakashita et al. 2005) but also in non-responsive gene promoters; therefore additional, as yet unidentified *cis*-elements must be required to confer specificity. A transcription factor, SLIM1, identified in a screen for sulfur deficiency-non-responsive mutants, belonging to the EIL family (ethylene-insensitive-like), has been shown to be required for induction of *SULTR1;2* and other sulfate transporter gene expression under low sulfur conditions (Maruyama-Nakashita et al. 2006). Interestingly, although also involved in activating expression of glucosinolate degradation pathway genes, sulfur-regulation of APS reductase gene expression appears to be independent of SLIM1. A specific requirement for a protein phosphatase as an upstream regulatory factor for *SULTR;1*

induction of expression by sulfur deficiency has also been proposed (Maruyama-Nakashita et al. 2004a). All of these observations have yet to be integrated into a coherent model of a signal transduction pathway.

## IX. Perspective

Since the early descriptions of sulfate transport in cereals and in algae, substantial progress has been made in elucidating the complexity of sulfate transport systems. A major task remains to understand regulation of expression of isoforms in relation to changing availability and demand, particularly in complex vascular plants. In vascular plants the roles for many of the SulP isoforms are elusive and a focus for continuing research. Elucidation of transporter structure may provide insights into regulation and selectivity, for example between sulfate and selenate. An intriguing area concerns the significance of the STAS domain and possible interactions with other proteins in the cell. With the predominant emphasis having been on uptake, and to some extent subcellular transport, the molecular basis of efflux mechanisms, which may be catalyzed by channels or members of the SulP family, and which are responsible for cell to cell transfer and xylem loading are virtually unknown.

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