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Isolation and characterization of a cDNA encoding a sulfate transporter from *Arabidopsis thaliana*

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Abstract A cDNA encoding a sulfate transporter was isolated from *Arabidopsis thaliana*. The isolated clone contained an open reading frame encoding a polypeptide of 658 amino acids, exhibiting the highest similarity (62%) with the sequence of the low-affinity sulfate transporter of a tropical legume *Stylosanthes hamata*. Northern blot analysis indicated the constitutive accumulation of a 2.6 kb length transcript in leaves and roots of seedlings. We also propose that *A. thaliana* contains three sulfate transporter genes which are expressed as 3.0, 2.7 and 2.6 kb length transcripts, respectively, in an organ-specific manner.

Key words: *Arabadopsis thaliana*; cDNA cloning; Expressed sequence tag; Sulfate transporter

1. Introduction

Plants and microorganisms assimilate inorganic sulfate into sulfur-containing amino acids for their own protein synthesis. Therefore, uptake of sulfate is considered to be an indispensable step in the maintenance of their viability. This crucial step to acquire the essential nutrient source is carried out by the proton/sulfate co-transporters in the plasma membranes [1].

Recently, cDNA clones encoding sulfate transporter proteins have been isolated from a tropical leguminous plant *Stylosanthes hamata*. Characterization of sulfate transport activities in a gene-disrupted yeast mutant [2] demonstrated the presence of two different transporters, the high- and low-affinity types toward sulfate, according to kinetic characteristics [3]. Changes in mRNA levels, induced by sulfate starvation, corresponded with changes of the transportation activities determined physiologically [4–7]. In the filamentous fungus, *Neurospora crassa*, expression of the *cys14* gene encoding sulfate permease II was activated by sulfur limitation [8,9]. The positive acting regulatory protein, Cys3, which binds to specific sites of the 5′-untranslated region of *cys14*, was required for its expression [9,10]. It is possible that higher plants also adapt to fluctuation in environmental sulfur availability, by modulating the expression of genes encoding sulfate transporters.

To investigate the regulation of sulfur uptake and subsequent sulfur metabolism in higher plants, we have initiated molecular cloning of sulfate transporter genes from *Arabidopsis thaliana*. The present study is the first report on the isolation and characterization of a cDNA clone encoding the sulfate transporter from *A. thaliana*.

2. Materials and methods

2.1. cDNA cloning

A cDNA library was constructed from poly(A)$^+$ RNA of 3-week-old whole plants of *A. thaliana* Columbia in the λgt11 vector. Approx. 5.0×10^5 amplified plaques were screened with the ^{32}P-labeled cDNA insert of the *Arabidopsis* EST, No. 130L5T7 (accession no. T44718) [11]. Hybridization of the membranes (Hybond N+, Amersham) was carried out at 65°C in 5×SSPE (0.9 M NaCl, 0.05 M sodium phosphate pH 7.7, 5 mM EDTA), 0.5% SDS, 5×Denhardt's solution and 25 mg ml^{-1} salmon sperm DNA. Final washing of the membranes was conducted at 65°C in 0.1×SSPE and 0.1% SDS [12].

2.2. DNA sequencing

The cDNA inserts of the isolated positive clones were subcloned in the *Bam*HI site of pBluescript II SK− (Stratagene). The full length DNA sequence was determined from both strands using a series of overlapping exonuclease III digested clones created by an Exo/Mung deletion kit (Stratagene). Sequencing was carried out by dideoxy-chain termination methods with Thermo Sequenase (Amersham) using a Shimadzu DNA sequencer model DSQ1000.

2.3. DNA and RNA blot hybridization analyses

Arabidopsis plants for DNA and RNA preparation were grown on GM agar plates [13] under continuous light at 23°C. Genomic DNA was isolated from the leaves of 3-week-old seedlings as described in [14]. For Southern hybridization analysis, genomic DNA (5 μg) was digested with restriction enzymes, separated in a 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham). Total RNA was isolated from the leaves and roots of 3-week-old seedlings by a phenol/SDS method and precipitated by LiCl as described elsewhere [12]. For Northern hybridization analysis, total RNA (20 μg) was separated under denaturing conditions in a 1.2% agarose gel containing formaldehyde and transferred to a Hybond N+ membrane (Amersham). DNA and RNA blots were probed with the ^{32}P-labeled cDNA insert fragment of the isolated clone. To verify equivalent loadings of RNA on blots, membranes were probed with a ^{32}P-labeled rice rDNA (pRR217) [15]. Hybridization and washing of the membranes were carried out as described for library screening. Hybridization signals were detected by a BAS-2000 image analyzer (Fuji Film).

3. Results and discussion

3.1. Identification of ESTs encoding Arabidopsis sulfate transporters

In order to isolate cDNAs encoding the sulfate transporter from *Arabidopsis*, we first searched for EST clones homologous with the sulfate transporters of *S. hamata* [3], *Saccharomyces cerevisiae* [2] and *N. crassa* [9]. EST clones 76E7T7 (accession no. T21459), 130L5T7 (accession no. T44718) and 142F20T7 (accession no. T76088) [11] were selected as candi-

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Abbreviations: AST, *Arabidopsis thaliana* sulfate transporter; EST, expressed sequence tag; MSD, membrane-spanning domain

The sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank data base (accession no. D85416).

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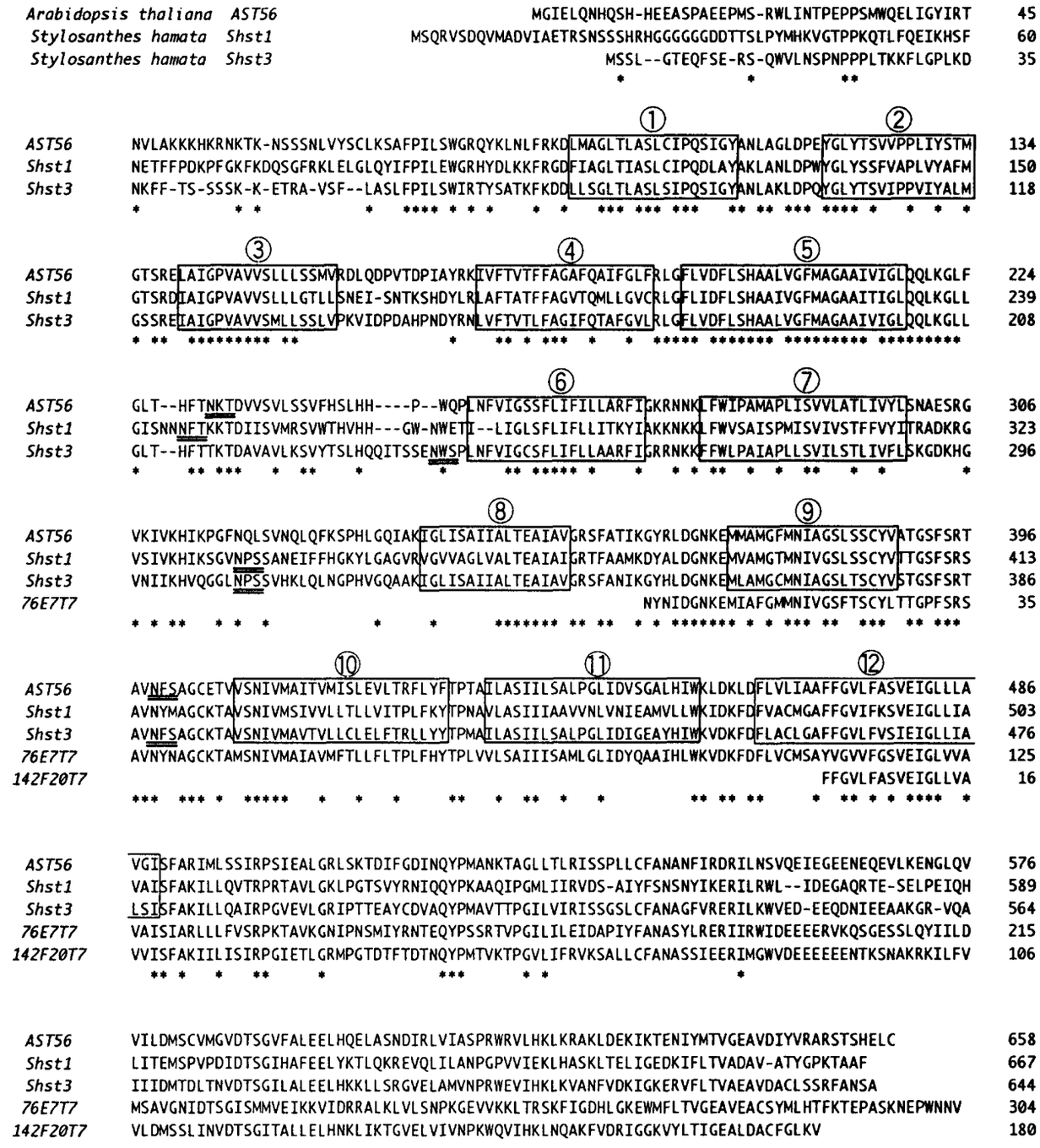

Fig. 1. Amino acid sequence alignment of *A. thaliana AST56* with *S. hamata* sulfate transporters, *Shst1* and *Shst3*, and *A. thaliana* ESTs, 76E7T7 and 142F20T7. Asterisks indicate consensus amino acids. Boxes indicate the 12 putative membrane-spanning domains (MSD). Amino acids doubly underlined indicate the putative *N*-glycosylation motifs.

dates for library screening. The EST clones were sequenced and the deduced amino acid sequences were aligned with the reported sequences of sulfate transporters [2,3,9]. As shown in Fig. 1, the *Arabidopsis* EST clones exhibited around 50–60% homology with the carboxyl-terminal region of the sulfate transporters, *Shst1*, *Shst2* and *Shst3*, of *S. hamata* [3]. Several highly conserved motifs identical with them were also identified in these sequences.

3.2. *Isolation and sequencing of a cDNA encoding the sulfate transporter, AST56, from* Arabidopsis

A ^{32}P-labeled cDNA insert of 130L5T7 was used for screening an *Arabidopsis* λgt11 cDNA library. The final washing of the membranes was carried out under high stringency conditions (65°C in 0.1×SSPE and 0.1% SDS). Five positive clones were isolated from 5.0×10^5 amplified plaques. With respect to these positive clones, no cross-hybridization with the ESTs,

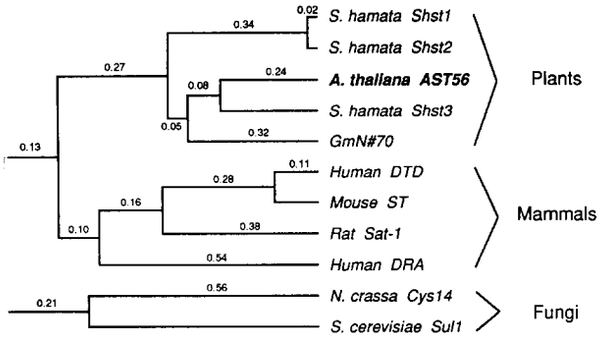

Fig. 2. Molecular phylogenic tree of the eukaryotic sulfate transporters. Numbers indicate branch length as proportional genetic divergence.

76E7T7 and 142F20T7, occurred under these washing conditions. Among them, *AST56* containing a cDNA insert of approx. 2.1 kb was chosen for further analyses. The nucleotide sequence of *AST56* was fully sequenced from both strands. The *AST56* insert was 2114 bp in length and contained an open reading frame of 1974 bp encoding a polypeptide of 658 amino acids (Fig. 1). The calculated molecular mass of *AST56* was 72 342 Da, which was in good agreement with those of the sulfate transporter proteins of *S. hamata* [3].

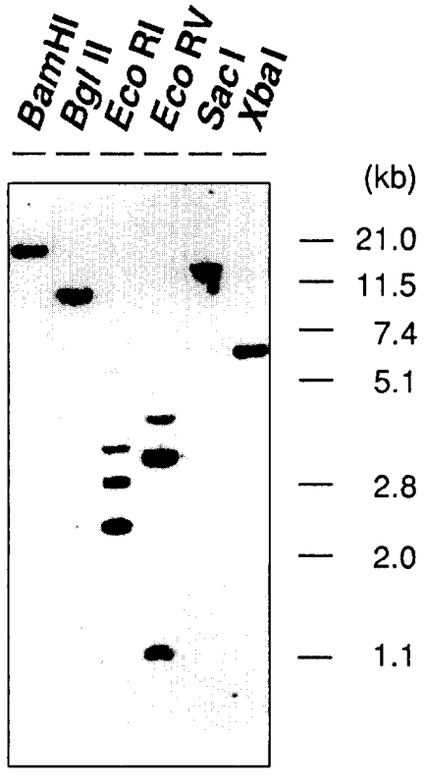

Fig. 3. Southern blot analysis of genomic DNA of *A. thaliana*. Genomic DNAs (5 μg) digested with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Sac*I and *Xba*I were separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with the ^{32}P-labeled cDNA insert of *AST56*. The membrane was washed under high stringency conditions and exposed to the imaging plate of the image analyzer BAS-2000 for 12 h.

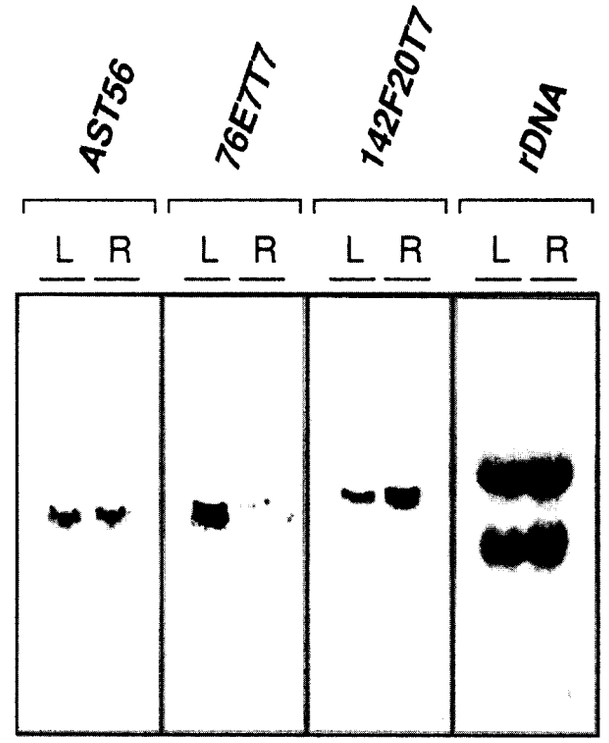

Fig. 4. Northern blot analysis of total RNA of *A. thaliana*. Total RNAs (20 μg) of leaves (L) and roots (R) were separated by agarose-formaldehyde gel electrophoresis, transferred to nylon membranes and hybridized with ^{32}P-labeled probes. The membranes were washed under the high stringency condition and exposed to the imaging plate of the image analyzer BAS-2000 for 18 h (AST56), 2 h (76E7T7, 142F20T7) and 0.5 h (rDNA).

3.3. Comparison of the deduced amino acid sequence with the eukaryotic sulfate transporter

The deduced amino acid sequence of *AST56* showed strong similarity to the sulfate transporters of the tropical leguminous plant *S. hamata* (*Shst1*, *Shst2* and *Shst3*) [3], as shown in Fig. 1. The 12 putative membrane-spanning domains (MSD) predicted by the TopPredII program [16] were also conserved in the deduced amino acid sequence of *AST56*. These results indicated that *AST56* is a member of the membrane-bound sulfate transporters of *A. thaliana*. Two *N*-glycosylation motifs (Asn-X-Ser, Asn-X-Thr) were found between the MSDs. The Asn-Phe-Ser motif between the MSDs 9 and 10 was identical with that of *Shst3*.

Similarities of the amino acid sequence with other eukaryotic sulfate transporters were found as follows: *S. hamata Shst1*, 50% (accession no. X82255) [3]; *S. hamata Shst2*, 48% (accession no. X82256) [3]; *S. hamata Shst3*, 62% (accession no. X82254) [3]; soybean nodule *GmN#70*, 52% (accession no. D13505) [17]; *S. cerevisiae Sul1*, 27% (accession no. X82013) [2]; *N. crassa Cys14*, 37% (accession no. M59167) [9]; rat *Sat-1*, 26% (accession no. L23413) [18]; mouse, 26% (accession no. D42049); human *DTD*, 28% (accession no. U14528) [19]; human *DRA*, 27% (accession no. L02785) [20]. Phylogenic relationships of these amino acid sequences indicated the existence of three individual groups comprised of the higher plant, mammalian, and yeast and fungal members (Fig. 2). The isolated *Arabidopsis* clone, *AST56*, belonged in the branch of the low-affinity members in the higher plant

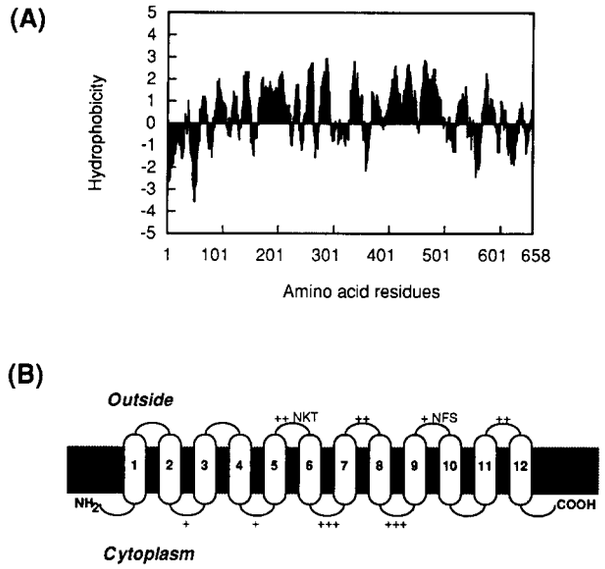

Fig. 5. Computer analysis of the protein structure of *AST56*. (A) Hydropathy plot by the method of Kyte and Doolittle [21]. (B) Prediction of the topology by the TopPred II program [16]. Conserved positively charged amino acids (+) and the putative *N*-glycosylation sites (NKT and NFS) are described between the MSDs. Numbers indicate the 12 putative MSDs corresponding to Fig. 1.

group, whereas it was highly divergent from the mammalian and fungal groups.

3.4. Hybridization analyses

Southern blot analysis indicated the presence of a single copy of *AST56* in the *Arabidopsis* genome (Fig. 3). Since *AST56* cDNA contains an *Eco*RI site and 3 *Eco*RV sites, several distinct hybridization signals were obtained in the case of digestion with these restriction enzymes.

Expression of *AST56* in seedlings was analyzed by RNA blot hybridization in comparison with the two homologous ESTs (76E7T7 and 142F20T7) (Fig. 4). The length of the detected hybridization signal for *AST56* (2.6 kb) was in good agreement with that of the isolated cDNA, considering the attachment of an additional 5′-untranslated region and a poly(A) tail. The transcript sizes of 76E7T7 and 142F20T7 were estimated to be 2.7 and 3.0 kb, respectively, closely matching that of *AST56* (2.6 kb). Taking account of the sequence similarities, the two ESTs and *AST56* were assumed to represent the three different members of the sulfate transporter family in *A. thaliana*.

The *AST56* transcripts accumulated constitutively in both leaves and roots of the light-grown green seedlings of *A. thaliana*. However, the transcript of 76E7T7 was accumulated almost exclusively in leaves; in contrast, the transcript of 142F20T7 was preferentially detected in roots. Furthermore, the mRNA level of *AST56* was roughly estimated to be 1/10–1/20 of the other two homologues, resembling the relatively lower expression level of *Shst3* in *S. hamata* [3]. These results suggested that *AST56* is presumably a member of the low-affinity sulfate transporter in *A. thaliana*. Although cloning and sequencing of the full-length clones corresponding to the other two ESTs are required, the present data strongly suggested the existence of three different sulfate transporters expressed in an organ-specific manner in *A. thaliana*.

3.5. Structural features of the AST56 polypeptide

The hydropathy profile of *AST56* polypeptide by the method of Kyte and Doolittle [21] showed the presence of 12–16 hydrophobic clusters, indicating the typical characteristic of membrane-bound proteins (Fig. 5A). Prediction of the topology using the TopPredII program by Claros and Von Heijne [16] also indicated the presence of 12–16 putative hydrophobic segments for MSDs. Based on the calculation by this program, the most probable predicted structure is shown in Fig. 5B. This model showed a well-conserved location of 12 putative MSDs, resembling those of the known eukaryotic sulfate transporters (Figs. 1 and 5B). According to this model, the N-terminus and the C-terminus of the polypeptide were assumed to be located in the cytoplasm, while the two putative *N*-glycosylation sites were identified on the extracellular side, showing the characteristics for the membrane protein. Positively charged amino acids (Lys, Arg) were assumed to appear on both sides of the membranes. These basic amino acid residues located externally may have some function for SO_4^{2-} binding and channeling through the membranes as proposed by Clarkson et al. [22]. According to their prediction [3], Arg-395 of *AST56* identical with Arg-385 of *Shst3* between the MSDs 9 and 10, was assumed to be the probable extracellular basic residue for SO_4^{2-} binding. Further investigation should be performed in order to explain the individual roles of these specific residues and functional motifs.

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