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Cloning of a cDNA Encoded by a Member of the Arabidopsis thaliana ATP Sulfurylase Multigene Family

EXPRESSION STUDIES IN YEAST AND IN RELATION TO PLANT SULFUR NUTRITION*

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An Arabidopsis thaliana ATP sulfurylase cDNA (ASA1), encoding a putative chloroplastic isoform, has been cloned by functional complementation of a Saccharomyces cerevisiae (met3) ATP sulfurylase mutant which also has a poor sulfate transport capacity. Homologous complementation of the yeast mutant with the ATP sulfurylase gene restores both ATP sulfurylase function and sulfate transport. Heterologous complementation restores only ATP sulfurylase function as demonstrated by low (³⁵S) sulfate influx measurements and selenate resistance. A structural relationship between ATP sulfurylase and sulfate membrane transporters in yeast is proposed. The sequence of ASA1 is homologous to deduced plant and animal ATP sulfurylase sequences. Analyses indicate a potential tyrosine phosphorylation site which is unique to higher eukaryote sequences. ASA1 is specified by a single copy gene that is part of a multigene family in A. thaliana. At least two ASA1 copies are found in Brassica napus plants. ASA1 transcripts were found in all organs examined, with the highest transcript abundance and ATP sulfurylase activity in leaves or cotyledons. Absence of sulfate from culture media transiently increased B. napus transcript abundance, indicating that initially, the response to sulfate deprivation is transcriptionally regulated.

Sulfur is an essential mineral nutrient for plant and animal growth which, in its reduced form, is incorporated into sulfur amino acids, other sulfur-containing metabolites, and enzymes. In its oxidized form, it is incorporated into sulfolipids which are the major components of the chloroplast membrane (1). In both plants and microorganisms, active uptake of sulfate through specific transporters is followed by reduction to sulfide. As sulfate has a very low oxidation/reduction potential (1), transiently increased B. napus transcript abundance, indicating that initially, the response to sulfate deprivation is transcriptionally regulated.

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| The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U140715.
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1 The abbreviations used are: APS, adenosine 5′-phosphosulfate; EST, expressed sequence tag; bp, base pair(s); kb, kilobase pair(s); MOPS, 3-(N-morpholino)propanesulfonic acid.

EXPERIMENTAL PROCEDURES

Strains and Media—The following strains were used in this study, S. cerevisiae wild type W303-1A (MATα, his3, leu2, ade2, trp1, ura3) and the mutant strains CC371–4C (MATα, leu2, ura3, met3) and C155 (MATα, his3, leu2, ura3, met3-URA3), which were grown as described (24), unless otherwise stated. Plasmids were propagated in Escherichia coli.
ATP Sulfurylase from A. thaliana

col strain DH5α (Life Technologies, Inc.) in classical media selecting with ampicillin (25).

Plant Material—Oilseed rape (B. napus Metzger var Drakkar) seeds were grown as already described (26). The complete nutrient solution (+S) contains 2 mm MgSO₄ and the sulfate free medium (−S) was replaced by replacing MgSO₄ by the corresponding chloride salt. Organs were harvested after 8 days of growth. A. thaliana rosette seedlings were disinfected and sown in a sterile plastic box, on 3 layers of nylon mesh (1 mm) supported over the surface of a sterile nutrient +S solution identical with that used for B. napus, and aerated with filtered air. Growth conditions included a 16-h photoperiod, constant day and night temperature of 20°C ± 1°C, 70% relative humidity, and 120 μmol m⁻² s⁻¹ photosynthetically active radiation. After 3 weeks of sterile culture, plants were transferred either to fresh +S or −S nutrient media for 2 days, after which organs were harvested.

Plasmids and cDNA Library—The A. thaliana cDNA library was constructed in the yeast shuttle expression vector pFL61 which bears the yeast URA3 marker and the constitutive phosphoglycerate kinase promoter (27). This library was prepared from mRNA extracted from complete two-leaf stage seedlings, including roots. The yeast ATP sulfurylase gene which had been subcloned from pM3-4 (24) was in plasmid pMELY23 (pM3-32), which bears the yeast URA3 gene. Plasmids were prepared using Qiagen kits according to the manufacturer’s instructions (Qiagen Inc.). For sequencing, the insert was excised from pFL61 and subcloned into plasmid SK (+). Sulfurylase gene which had been subcloned from pFL61-ASA1 and C155 using a lithium chloride transformation procedure (31).

DNA Sequencing—The sequence of the ASA1 cDNA was determined from both strands by the dideoxy chain termination method with a double-stranded DNA template and the Taq dye primer cycle sequencing kit (Applied Biosystems) according to the manufacturer’s instructions (Part No. 901482). Electrophoresis were performed using 6% (w/v) denaturing acrylamide gels in the Model 373A DNA Sequencer Applied Biosystems. The sequence was analyzed and compared using sequence analysis software packages from Genetic Computer Group (GCC, Madison, WI). NCBI BLAST (GenBank™; R90; EMBL; R30; Swiss-Prot; R30) and Prosite (32) libraries were accessed.

Nucleic Acid Hybridization Analysis—For Southern blot hybridization, genomic DNA was prepared from B. napus (cotedyledons from 8-day-old seedlings) or A. thaliana (germinated 4-day-old seedlings) as described (35). DNA was digested with restriction enzymes according to the manufacturer’s instructions, and the resulting fragments were separated by electrophoresis in a 0.8% (w/v) agarose gel followed by denaturation of DNA and transfer to BA85 nitrocellulose filters (Schleicher & Schuell) (25). The filter was then hybridized at 42°C with ASA1 cDNA fragments random primer-labeled (T, Quickprime kit, Pharmacia) with [α-32P]dCTP (3000 Ci mmol⁻¹) (ICN Biomedicals) at 1.25 × 10⁵ dpm μl⁻¹ for 16–24 h. Fornamide (50% v/v) containing prehybridization and hybridization solutions were prepared as described (25), with T, coli tRNA (100 μg ml⁻¹) used as a blocking agent. Filters were washed twice at room temperature for 5 min and twice at 42°C for 15 min in 30 μl NaCl, 3 μl trisodium citrate, pH 7.0, 0.1% (w/v) SDS, to remove nonspecific hybridization. Blots were then autoradiographed for 12–110 h at −80°C with intensifying screens.

Protein Purification—Total hybridization, total protein purification, and separation of total organs using the guanidinium thiocyanate/phenol/chloroform extraction method (36). Approximately 10 μg of total RNA were denatured and separated in a 0.66 M formaldehyde, agarose (1.2% w/v) gel in MOPS buffer (25). The gel was then washed twice for 20 min in 3 μl NaCl, 0.3 μl trisodium citrate, pH 7.0, before transfer of RNA to nitrocellulose and hybridizations as described above. For slot blot analysis, approximately 1 μg of total RNA per slot was denatured and applied to nitrocellulose (Sartorius) using the Minifold II slot-blot manifold (Schleicher & Schuell) as described (37); duplicates of each sample and nonspecific controls of 50 μg of E. coli tRNA were loaded. After hybridization, as described above, blots were exposed to preflashed film (38) for 50–70 h at −80°C with intensifying screens. Quantification of Northern blots was carried out using the Image system (Appligene) in conjunction with the National Institutes of Health Image program (39).

Plant ATP Sulfurylase Assay—Soluble protein extracts were obtained by grinding the organs to a fine powder in liquid N₂, then adding 4 ml of ice-cold extraction buffer per g fresh weight of tissue, 100 μm Tris-HCl, pH 8.0, 10 μm EDTA, 2 μm DTT followed by centrifugation at 4°C for 10 min at 7,800 g. ATP sulfurylase activity measurements were carried out on sulfate-free dialyzed fractions (8) by molybdenum (3). The pyrophosphate released from ATP was determined according to Ref. 40.

Yeast ATP Sulfurylase Assay—Yeast cells were grown at 28°C in appropriate culture solutions to an absorbance of 1.0 measured at 650 nm. Extraction was essentially as described (41) using cell breakage with glass beads and a modified extraction buffer, 100 μm Tris-HCl, pH 7.5, 0.2 μm EDTA, 1 μm phenylmethylsulfonfluoride, 10% (w/v) glycerol. The supernatant was desalted on a Sephadex G-25 M column (Pharmacia PD-10) equilibrated with extraction buffer minus phenylmethylsulfonfluoride. ATP sulfurylase activity was measured according to Ref. 42.

Protein Assays—Protein content of extracts were measured by the method of Lowry using bovine serum albumin as a standard.

RESULTS

Isolation of an A. thaliana ATP Sulfurylase cDNA—Yeast strains CC371-4C and C155 have both been shown to be defective in ATP sulfurylase activity (24) as a result of point or insertion mutations, respectively, and are unable to grow on media where sulfate is the only sulfur source. Heterologous complementation using an A. thaliana cDNA library in a yeast expression vector (27) was employed to obtain an ATP sulfurylase clone using strain CC371-4C. Initial transformation with selection for uracil auxotrophies resulted in only 500 complemented colonies. These were recovered for secondary screening and replated on media containing sulfate as the sole sulfur source; 4 positive clones were obtained and analyzed further. Using 5-fluoro-otic acid, uracil and methionine auxotrophs were confirmed to arise from plasmid complementation and not recombination (29). Plasmids were recovered from yeast into E. coli and amplified. Restriction analysis of the four plasmids indicated that they were identical, thus one representative was taken. The strain CC371-4C was retransformed with this plasmid and complementation of methionine and uracil auxotrophies was confirmed. The cDNA clone was termed ASA1 (ATP sulfurylase Arabidopsis 1).

Sequence Analysis of cDNA and Comparison of Deduced Amino Acid Sequence with Other ATP Sulfurylases—Analysis of the insert from pFL61-ASA1 indicated a 1431-bp open reading frame in the 1662-bp cDNA which would encode a polypeptide of 476 amino acids with a calculated molecular mass of 53,604 Da (Fig. 1). The predicted polypeptide is encoded by bases 59 to 1489 with the 3' untranslated region containing a putative polyadenylation signal AATAAT (46) at base 1643.
ASA1 is homologous to the sequence AtMet3-1 (18) and APS2 (19). ASA1 has a 5'-leader sequence 40 bp longer than that of AtMet3-1 (the data base accession for APS2 contains only 3' sequence at present) and the 3' sequences differ apparently through polyadenylation in response to an earlier signal in ASA1. The proposed amino acid sequence of mature ASA1 shows lower homology to the other A. thaliana ATP sulfurylase cDNA sequences APS1 (17) and APS3 (19) (67% identity) than they exhibit to each other (89% identity). Comparison to Solanum tuberosum S.T.S-1 and S.T.S-2 sequences showed slightly higher identity (74% and 69%, respectively) (16). Nonetheless, these values are higher than those obtained with ATP sulfurylase cDNAs recently cloned from animals, Mus musculus (21) and Urechis caupo (21) (59% identity).

**Features of Deduced Amino Acid Sequence**—The first 62 amino acids of the ASA1 open reading frame have features which correspond to a translational start, also suggested for APS2 (19), as this region is rich in hydroxylated amino acids, contains no acidic amino acids, and has a hydrophobic valine residue at position 59 in agreement with the chloroplast transit peptide cleavage-site motif (47, 48). Additionally, the N-terminal sequence of a chloroplast ATP sulfurylase isomerase purified from spinach (SLIDPDSGLSLDLSQVTKPHN) (5) is identical for the first 8 amino acids to ASA1 amino acids 63–70 (underlined in Fig. 1). Three blocks of homology with functional features have been proposed for ATP sulfurylase sequences. Two conserved blocks (Fig. 1, blocks I and IV) could be involved in sulfate binding (15). The third block contains part of the sequence GXXXG which corresponds to a phosphate-binding loop motif and may be an ATP-binding region (14) (Fig. 1, block III). In addition to these blocks, the ASA1 coding region contains a putative tyrosine phosphorylation site at amino acid 286, asterisked in Fig. 1, block II. This site conforms to the consensus motif (R/K)XX(D/E)XXX (Prosite accession number PS00007) and is also present in the other higher eukaryote ATP sulfurylase sequences (Fig. 1, block II).

**Genomic Organization of ASA1**—Southern blot analyses of A. thaliana and B. napus genomic DNA digested with four restriction enzymes were carried out in order to estimate the copy number of ASA1 in their respective genomes. The ASA1 probe hybridized to 2 fragments in the A. thaliana EcoRV and BamHI digested DNA, which both have a restriction site in the cDNA, and to one fragment in both the EcoRI- and HindIII-digested DNA (Fig. 2). This indicates that ASA1 is encoded by a single copy gene in A. thaliana. Three to five genomic DNA fragments hybridized to the ASA1 probe in B. napus at high stringency (Fig. 2), suggesting that there are at least two or three copies of ASA1 homologous genes present in its genome. Properties of Yeast Expressing pASA1—Yeast expressing the yeast ATP sulfurylase, in the vector pEMBL3 (pEcoRI- or HindIII-digested; 37) containing the gene CC371-4C rescued by pFL61-ASA1, although restored in media containing sulfate as the sole sulfur source, had a rate that was 2.8 × lower than that of wild type, with a doubling time of ~440 min (Fig. 3). No growth was observed for the mutant containing the vector pFL61 alone. The same mutant expressing the gene encoding the yeast ATP sulfurylase in the vector pEMBL3-Eye23 (pM3-32), showed essentially wild type growth kinetics. The ability of these strains to grow on sulfate containing media correlated with ATP sulfurylase activities measured in their extracts (Table I). The activities measured for both Met3-1 strains (CC371-4C and CCI55) containing pFL61-ASA1 were 66% higher than those measured from extracts of CC371-4C complemented by pM3-32, but 37% lower than those of the wild type (W303-1A). When ASA1 was expressed in the Met3-1 strain, the ATP sulfurylase activity was 30% lower than when it was expressed in the met3 mutants and 50% of that in the noncomplemented Met3-1 strain (Table I).

In addition to the lack of ATP sulfurylase activity, yeast strains which have the met3 mutation have been shown to be defective in sulfate transport (44). In order to ascertain the effect of complementation on sulfate transport, we used sodium, as a toxic structural analogue of sulfate, in drop assays. When the strains were grown on sulfateless media (containing
formed with pFL61-M3–32 and pFL61-M3–32e, separated by agarose gel (0.8%) electrophoresis, transferred onto nitrocellulose, and then hybridized with 32P-labeled ASA1 DNA fragments.

**Fig. 2. Southern blot analysis of the genomic DNA from A. thaliana and B. napus.** The genomic DNA (10 μg) was digested by restriction enzymes (E, EcoRI; H, HindIII; B, BamHI; RV, EcoRV), separated by agarose gel (0.8%) electrophoresis, transferred onto nitrocellulose, and then hybridized with 32P-labeled ASA1 DNA fragments.

**Fig. 3. Growth curve of yeast strains transformed with ASA1 or MET3.** Yeast were grown from 50 milliunits initial absorbance, measured at 650 nm, in synthetic media supplemented with 0.4 mM sodium selenate, only the wild-type W303-1A and the mutant W303–1A 538 46.0

**Table I**

<table>
<thead>
<tr>
<th>Yeast strainsa</th>
<th>ATP sulfurylase activity</th>
<th>[35S]Sulfate influx measurements in yeast</th>
<th>Activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1A</td>
<td>538</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>W303-1A + pM3–32a</td>
<td>2329</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>W303-1A + pFL61-ASA1a</td>
<td>243</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CC371–4C</td>
<td>356</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>CC371–4C + pFL61-ASA1</td>
<td>113</td>
<td>178.5</td>
<td></td>
</tr>
<tr>
<td>C155</td>
<td>3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C155 + pFL61-ASA1</td>
<td>317</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a Relevant strain phenotypes: W303-1A (MET3a, ura3), CC371–4C (met3, ura3), C155 (met3:URA3).
b ATP sulfurylase activity and [35S]sulfate influx are the mean of three or four independent experiments, respectively. The results are all expressed as nmol · min−1 · mg−1 protein. ND indicates not determined.
c ATP sulfurylase activity was measured in the forward direction by molybdoysis.
d Unidirectional [35S]sulfate influxes were measured for 3 min in 0.1 mM MgSO4 at 30 °C.
e pM3–32 and pFL61-ASA1 represent plasmids containing the yeast gene and the plant cDNA ATP sulfurylase genes, respectively.

**DISCUSSION**

In this paper we report the cloning, by functional complementation of a yeast mutant, of a cDNA which encodes an A. thaliana ATP sulfurylase (ASA1). The sequence of this clone is identical with the previously reported AtMet3-1 (18) and APS2 cDNAs (19), except that the latter part of its 3′-untranslated sequence is different. Heterogeneity at the 3′ ends of mRNA encoded by a single plant gene has been shown to result from polyadenylation of the transcripts at multiple sites (46). The pol(A) of the two ASA1 homologous sequences and an homologous EST (accession number Z26572) are located downstream of the ASA1 pol(A) which seems to agree with the observed

homocysteine as the sole sulfur source) in the presence of 4 mM sodium selenate, only the wild-type W303-1A and the mutant complemented by the yeast gene (CC371–4C + pM3–32) were unable to grow, whereas the mutant complemented by the A. thaliana ATP sulfurylase cDNA (CC371–4C + pFL61-ASA1) grew as well as the noncomplemented Met3+ mutant (Fig. 4). To investigate whether the selenate growth effect was related to sulfate transport, [35S]sulfate influxes were measured (Table 1). The heterologous expression of pFL61-ASA1 partly restored the sulfate uptake function to the mutant, but to less than 20% of the sulfate uptake capacity of the yeast control. When the mutant was complemented by the homologous MET3 ATP sulfurylase gene, the [35S]sulfate influx was almost 4-fold higher than in the yeast control.

**Fig. 4. Test for selenate resistance.** Yeast were grown in sulfur-free synthetic liquid media supplemented with 0.1 mM homocysteine as the sole sulfur source and the required auxotrophy factors. Yeast were washed and 30 μl (at ~0.8 milliunit absorbance at 650 nm) were dropped onto agarose sulfate-free media supplemented with 0.1 mM homocysteine which contained either no selenate (~Se) or 4 mM sodium selenate (~Se). WT corresponds to W303-1A strain which has a wild type yeast MET3 gene. CC371–4C is a met3 mutant and is either not complemented (Mut), transformed with a plasmid containing the yeast MET3 gene (Met3-2), or transformed with a plasmid containing the A. thaliana cDNA ASA1 (ASA1-1).

Sulfur Starvation Effects on the Abundance of ASA1 Transcripts in Roots and Leaves—Northern blots of total RNA from A. thaliana leaves and roots identified an ASA1 hybridizing transcript of approximately 1.9 kb (Fig. 5). This transcript was most abundant in leaves, and on sulfur starvation the transcript abundance increased, but only in roots (1.8-fold). As it is difficult to grow A. thaliana in the hydroponic conditions required to impose short term sulfur starvation, B. napus, a related Brassicaceae with high sulfate requirements, was compared to A. thaliana for its suitability in further studies. Northern blots of B. napus total RNA hybridized to an ASA1 probe also revealed a transcript of ~1.9 kb (Fig. 5). An increase in transcript abundance was observed in both cotyledons and roots on sulfur deprivation, 1.3-fold and 1.5-fold, respectively.

Analysis of the Effect of Sulfur Starvation on ATP Sulfurylase Expression in B. napus—Analyses of both relative RNA abundance and ATP sulfurylase activity were carried out on organs of 8-day-old B. napus seedlings grown in sulfate-limiting conditions for 2 and 4 days. Total RNA extracts from hypocotyls also contain ASA1 hybridizing transcripts of ~1.9 kb (Fig. 6, upper panel). The patterns of relative ASA1 RNA abundance and ATP sulfurylase activity up to 2 days sulfate starvation are comparable in roots and cotyledons, highest in cotyledons, and the largest relative increase in roots (Fig. 6, A, B, and C). However, after 4 days of sulfate deprivation, although the ATP sulfurylase activity had increased, the ASA1 RNA abundance decreased in both cotyledons and roots. Hypocotyls showed different expression profiles on sulfur starvation; no significant changes in the relative transcript abundance was observed, and a specific activity decrease after 2 days was followed by an increase.

**DISCUSSION**

In this paper we report the cloning, by functional complementation of a yeast mutant, of a cDNA which encodes an A. thaliana ATP sulfurylase (ASA1). The sequence of this clone is identical with the previously reported AtMet3-1 (18) and APS2 cDNAs (19), except that the latter part of its 3′-untranslated sequence is different. Heterogeneity at the 3′ ends of mRNA encoded by a single plant gene has been shown to result from polyadenylation of the transcripts at multiple sites (46). The pol(A) of the two ASA1 homologous sequences and an homologous EST (accession number Z26572) are located downstream of the ASA1 pol(A) which seems to agree with the observed
preference for polyadenylation in response to the second site roots (B. napus activity, suggesting that more activity than required for growth be any growth limitation due to insufficient ATP sulfurylase similar to that of the wild type (Fig. 3) there does not seem to reduced the activity of the noncomplemented Met3 compared to when it was expressed in the yeast and the plant proteins form chimeric structures with reduced or no ATP sulfurylase activity. In contrast, the activity measured for pM3-32 in the Met3− strain was 20-fold higher than that when the plasmid complemented the mutant strains and 4-fold greater than the noncomplemented Met3− strain which supports the suggestion that, in the wild type strain, ATP sulfurylase activity levels are not growth-limiting. Yeast strains which have the met3 mutation have been shown to be defective in sulfate transport as well as in ATP sulfurylase activity (44). These mutants, unlike wild type strains, can grow on media containing selenate, a toxic analogue of sulfate (Fig. 4). Comparison of strains, expressing either the homologous or heterologous ATP sulfurylases, by growth on selenate-containing media showed that only the strain expressing the homologous protein was able to grow (Fig. 4). This was confirmed to result from reduced [35S]sulfate transport capacity (Table I) and demonstrates that ASA1 does not fully complement the met3 mutant phenotype. This suggests that the observed differences in growth kinetics (Fig. 3) probably result from the low sulfate uptake rate in the pFL61-ASA1 complemented mutant rather than differences in expression level or structure. The loss of sulfate transport when the ATP sulfurylase gene is mutated indicates that structural interactions, which are essential for efficient sulfate uptake, may exist in yeast between some sulfate transporters and ATP sulfurylase. In plants, such an interaction between the sulfate transporter and ATP sulfurylase may not exist or may depend on specific recognition domains which are absent or different from those of yeast. Alternatively, the presence of the transit peptide may affect protein conformation and this interaction. Interestingly, a yeast mutant defective in sulfate transport exhibits normal ATP sulfurylase activity (49). Interaction between the sulfate transporter and the first enzyme in the sulfate metabolic pathway may be advantageous as it could result in a degree of sulfate channeling from the transporter into the sulfate binding and activation site of the ATP sulfurylase. This could push the unfavorable thermodynamic equilibrium of the ATP sulfurylase reaction toward forming the APS product. Such a channeling mechanism has been suggested in plants for the cysteine synthase complex (50, 51) and for the animal bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase (7, 20, 21).

Sequence analysis indicates that ASA1 has a 62-amino acid putative transit peptide. Homology of the proposed mature ASA1 protein to N-terminal sequence of a spinach chloroplast isozyme (5) suggests its chloroplastic location. In addition to blocks of homology common to all eukaryotic ATP sulfurylase sequences, a putative tyrosine phosphorylation site was identified (Fig. 1, block II) which is conserved only in the higher eukaryote sequences. This site is situated between the conserved block proposed to form part of the catalytic site (Fig. 1, block I) (15) and a region of homology including the putative phosphate binding loop sequence (Fig. 1, block III) (14). Translational control of enzyme activity through tyrosine phosphorylation has been demonstrated extensively in animals. In plants, a tyrosine kinase has only recently been cloned (52), and the regulation of a plant enzyme by tyrosine phosphorylation has just been demonstrated in vivo (53). Prokaryotic ATP sulfurylases have been found to contain a GTP binding motif and to be stimulated by GTP (2). GTP has little effect on the activity of plant, yeast, and fungal enzymes (15) and, in common with other eukaryotic ATP sulfurylase sequences, no GTP
binding motif is found in the ASA1 sequence. Labeling of cysteine residues in P. chrysogenum has identified two buried cysteine residues which may have important structural functions, one of which is in a region whose sequence is conserved in A. nidulans and S. cerevisiae (15). Although this cysteine is not conserved in the plant sequences, there is a conserved cysteine in the plant ATP sulfurylases which belongs to a highly conserved block at the 3' end (Fig. 1, block V).

Genomic Southern analysis (Fig. 2) demonstrated that ASA1 is encoded by a unique gene in agreement with the results of Murillo and Leustek (19) for APS2. Under low stringency conditions, an additional 2–3 fragments were detected in each A. thaliana genomic DNA digest (not shown) indicating at least 2 related ATP sulfurylase genes. This agrees with the cloning of two different full-length A. thaliana ATP sulfurylase cDNAs, APS1 and APS3 (17, 19). Southern blots probed with these cDNAs indicate that they are also single copy genes, suggesting that the ATP sulfurylase gene family in A. thaliana has just these three members (19). Our data base searches have, however, identified six additional ASA1 homologous A. thaliana ESTs (accession numbers T21966, T24953, R29819, T88260, T45338, and T21042) which appear to encode one or several related ATP sulfurylases. Analyses using the deduced protein sequence from the 6 combined sequences showed 62, 68, and 72% identity, respectively, to ASA1, APS1, and APS3. These values are sufficiently different to suggest a fourth ATP sulfurylase gene in A. thaliana which may not cross-hybridize, even under low stringency conditions. As the six ESTs were all identified from the same systematic sequencing program (54), they may represent an organ or treatment-specific ATP sulfurylase form. In A. thaliana, the existence of a fifth ATP sulfurylase gene expressed in the cytosol might be expected since cell fractionation and enzyme activity studies on spinach revealed a cytosolic form (4). In addition, a cDNA without a transit peptide has been cloned from S. tuberosum (16). Therefore, the A. thaliana ATP sulfurylase family probably consists of at least four chloroplastic and perhaps one cytosolic isoform. The identification of at least 2 ASA1-like gene copies in B. napus is consistent with the allotetraploid state of this species (55).

Northern blot analyses of A. thaliana and B. napus identified a transcript of ~1.9 kb in all organs examined, with highest expression in leaves (Fig. 5). An APS1 probe also identified a transcript (1.85 kb) in leaf and root total RNA (17) which was most abundant in leaves. An increase in transcript abundance in roots of both A. thaliana and B. napus was observed on sulfur deprivation, 1.8-fold and 1.5-fold, respectively, indicating that the expression of the ASA1 gene responds to the availability of sulfate. The apparent absence of response in A. thaliana leaves to sulfate starvation seems in contrast to the 1.3-fold increase observed in B. napus. This can be attributed to the difference in the age of the organs used, 9-day-old cotyledons compared to 3-week-old leaves, and is more likely to reflect the higher sulfur requirement of the young organs. ATP sulfurylase specific activity has been shown to be high in young leaves, decreasing as the leaves mature (23, 56) indicating that the sulfur requirement of mature leaves is low.

As effects of sulfate starvation on the relative abundance of ASA1 hybridizing transcripts in B. napus were similar to those observed in A. thaliana, although the cDNA probe was heterologous, the use of B. napus in these studies was considered appropriate. ATP sulfurylase activity and Northern analysis carried out on the same B. napus organs showed similar patterns up to two days of sulfate starvation with the largest relative increase in roots and highest in cotyledons (Fig. 6, A and C). Specific activities increased in all organs with sulfur starvation, although in hypocotyls after 2 days they decreased then increased. This indicates that in these organs initial changes in enzyme activity on sulfur starvation are probably transcriptionally regulated. Increased ATP sulfurylase activity in response to the absence of sulfate in the external media has previously been observed in higher plants (22, 23). In M. atropurpureum, this difference was also found to be largest in roots, with only a slight initial activity increase in leaves. Large increases in relative transcript abundance for the recently cloned plant sulfate transporters have also been observed for roots (49). Sulfate translocation studies have demonstrated that roots are the predominant sulfur sink during sulfate deprivation (23). These results, together might indicate that roots have priority for sulfate utilization. Such a priority could be envisaged to improve plant survival under sulfate limiting conditions by augmenting the sulfate foraging ability of roots.

After 4 days of sulfate deprivation, although the enzyme activity had increased, the RNA abundance decreased in both cotyledons and roots. A difference between relative ATP sulfurylase activity and RNA abundance profiles was also observed for hypocotyls (Fig. 6, A and B). B. napus probably expresses multiple forms of ATP sulfurylases whose combined enzyme activities might differ, on sulfur starvation, from the profile of one transcript type. The observed differences could, however, result from translational control, but as the observed increases in activity, observed on deprivation, are greater than increases in ASA1 RNA abundance, simultaneous increases in expression of several transcript types seems likely.

The increase in transcript abundance observed in B. napus leaves suggests that the absence of sulfate in the root external media is perceived by leaves. Sulfate uptake and transport from roots to shoots have been shown to be inhibited by glutathione (57) which has been proposed to act as a quantitative signal informing the plant of its sulfur status (58). Glutathione levels have been found to decrease on sulfate starvation, and could induce the changes in transcript abundance on sulfate starvation.

CONCLUSIONS

We have cloned a cDNA which encodes an A. thaliana ATP sulfurylase (ASA1) with a putative chloroplast transit peptide. ASA1 is encoded by a single copy gene that is part of a multigene family in A. thaliana, probably consisting of at least four members. Yeast mutants deficient in ATP sulfurylase also lack sulfate uptake capacity. Comparison of these mutants transformed with the heterologous ASA1 plant cDNA to those transformed with the homologous yeast MET3 ATP sulfurylase gene showed that, although ATP sulfurylase activity is completely restored, the sulfate uptake ability is not fully complemented. We propose a model involving structural interaction between the yeast plasma membrane sulfate carrier and the cytosolic ATP sulfurylase. We plan to verify this model in yeast and examine the possibility of such an interaction in plants.

A putative tyrosine phosphorylation site was found in the ASA1 sequence which is conserved, but only in higher eukaryote ATP sulfurylases. This tyrosine is situated between the catalytic site and bind ATP (14, 15). Investigation of this region with regard to possible regulation and catalytic functions should be informative. Northern blot analysis showed that ASA1 is expressed in all A. thaliana and B. napus organs examined with highest expression in leaves or cotyledons, respectively. The relative RNA abundance and ATP sulfurylase activity in B. napus were found to increase in cotyledons, and to

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a greater degree in roots, after 2 days of sulfate starvation. This indicates that the initial response to sulfate starvation is probably at the transcriptional level. Subsequently relative ASA1 RNA abundance decreases whereas ATP sulfurylase activity continues to increase, this could be as a result of translational control or other members of the ATP sulfurylase gene family having different response profiles to sulfate starvation.

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