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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 coenzymes. 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 relative to available cellular reductants, the primary step in assimilation requires its activation via an ATP-dependent reaction (2). This reaction is catalyzed by ATP sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4) and leads to the formation of adenosine 5′-phosphosulfate (APS)[1] (1). The

equilibrium for the formation of this product is thermodynamically unfavorable and, as the efficiency of APS removal by subsequent reactions seems insufficient for energetic compensation, shift of the thermodynamic balance through subcellular compartmentalization or substrate channeling may occur (2).

ATP sulfurylase has been purified from a wide range of sources and characterized extensively at the biochemical level from plants (3–5), animals (6, 7), and fungi (8, 9). In plants, leaves are considered to be the main site of sulfur assimilation with ATP sulfurylase activity found predominantly in chloroplasts and at low levels in the cytosol (4, 5). All the enzyme activities involved in assimilatory sulfate reduction have also been detected in plant root proplastids (10). Although ATP sulfurylase isoforms with different biochemical properties have been purified from higher plants, no specific cellular function has been attributed to any of them.

An ATP sulfurylase gene was first cloned from *Saccharomyces cerevisiae* (11). Genes have subsequently been cloned from prokaryotes (12–14) and another lower eukaryote (15), and several cDNAs have been cloned from plants (16–19) and animals (20, 21). The prokaryotic enzymes are heterodimers with a catalytic subunit and a subunit that acts as a stimulatory GTPase (2). The yeast and plant enzymes are homooligomers, dimeric and tetrameric (5, 11, 19), and do not respond to GTP (15). In animals, ATP sulfurylase and APS kinase reside on a single bifunctional protein (7, 20, 21).

Using an *Arabidopsis thaliana* cDNA library, we have functionally complemented an ATP sulfurylase-defective yeast mutant. We report the isolation of the corresponding ATP sulfurylase cDNA clone that restores yeast methionine heterotrophy and ATP sulfurylase activity. The clone was sequenced and found to be identical with one of the three previously cloned *A. thaliana* ATP sulfurylases (18, 19), except for differences in the 3′ sequence. Comparison of yeast mutants complemented by the heterologous cDNA and homologous gene led us to propose the existence of a structural relationship between ATP sulfurylase and membrane sulfate transporters in yeast. ATP sulfurylase activity has been shown to increase under sulfur limiting conditions in plants (22, 23), but the mechanism by which this response is induced has not been determined. We have carried out ATP sulfurylase expression studies in relation to sulfur availability in *A. thaliana* and *Brassica napus.*

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*

[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.

coli 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 MgSO$_4$ and the sulfate free medium (−S) was prepared by replacing MgSO$_4$ by the corresponding chloride salt. Organs were harvested after 8 days of culture. *A. thaliana* (L.) Heynh (mutant type *erecta* of the ecotype Landsberg) seeds were disinfected and sown in a sterile plastic box, on 3 layers of nylon mesh (1 mm^2) 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 20 °C temperature and 70% humidity, and 250 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 p*M3-4* (24) was in plasmid pEMBLYe23 (p*M3-32*), 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 with *Not*I and subcloned into pBluescript SK+ (Stratagene).

Isolation of an Arabidopsis cDNA Encoding an ATP Sulfurylase—The yeast mutant CC371-4C was transformed with 5 μg of the *Arabidopsis* cDNA library (6 × 10^7 cells μg^{-1}) by the spheroplast protocol (28). The transformation mixture was plated on 2% Bacto-agar selective Difco yeast nitrogen base supplemented with 0.1 mM homocysteine, then incubated at 28 °C for several days. The Ura$^+$ colonies were pooled, plated on yeast nitrogen base, which contains 25 mM ammonium sulfate as the sole sulfur source, and incubated at 28 °C for several days. Complementation of both the *ura3* and *met3* functions were confirmed using 5-fluoro-orotic acid (29) and appropriate selective culture media. Plasmid DNA was recovered from complemented yeast transformants as described (30). The plasmid pFL61-*ASA1* was reintroduced into the ATP sulfurylase-defective *S. cerevisiae* strains CC371-4C 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). Electrophoreses 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 (GCG, Madison, WI), NCBI BLAST (GenBank™, R90; EMBL, R39; Swiss-Prot, R30, PIR, R45) (32, 33) and Prosite (34).

Nucleic Acid Hybridization Analysis—For Southern blot hybridization, genomic DNA was prepared from *B. napus* (cotyledons from 8-day-old seedlings) or *A. thaliana* (pregerminated 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 prime-labeled (T$_7$ Quickprime kit, Pharmacia) with [α-^{32}P]dCTP (3000 Ci·mmol^{-1}) (ICN Biomedicals) at 1.25–2 × 10^6 dpm·ml^{-1} for 16–24 h. Formamide (50% v/v) containing prehybridization and hybridization solutions were prepared as described (25), with *E. 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 mM NaCl, 3 mM 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.

For Northern blot hybridization, total RNA was isolated from frozen 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 M NaCl, 0.3 M 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 Imager system (Appligene) in conjunction with the National Institutes of Health Image program (39).

Plant ATP Sulfurylase Assay—Soluble protein extracts were obtained from 5 g fresh weight of each *B. napus* organ essentially as described (3) except for initial extraction. Crude extracts were obtained by grinding the organs to a fine powder in liquid N$_2$, then adding 4 ml of ice-cold extraction buffer per g fresh weight of tissue, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 mM 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 molybdolysis (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 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% (w/v) glycerol. The supernatant was desalted on a Sephadex G-25 M column (Pharmacia PD-1O) equilibrated with extraction buffer minus phenylmethylsulfonyl fluoride. ATP sulfurylase activity was measured according to Ref. 42.

Yeast [^{35}S]Sulfate Transport Assay—Yeast were grown at 28 °C in the absence of sulfate, in a synthetic glucose minimal liquid solution (43) with the required auxotrophy factors and 0.2 mM DL-homocysteine as the sole sulfur source. [^{35}S]Sulfate uptake assays were performed as described (44), at 0.1 mM Na$_2$SO$_4$ final concentration, and was initiated by addition of 0.33 μCi of carrier-free [^{35}S]Na$_2$SO$_4$ (ICN Biomedicals) to each uptake measurement tube. Uptake duration was between 30 s and 3 min, where uptake is known to be linear. Sulfate uptake was stopped by isotopic dilution with ice-cold 0.5 mM MgSO$_4$, vacuum filtration through a glass microfiber filter (GF/C, Whatman) applied to a prechilled stainless steel filtration device, and radioactivity retained on the filters was counted.

Protein Assays—Protein content of extracts were measured by the modified (45) method of Lowry using bovine serum albumin as a standard.

Reagents—Chemicals were purchased from Sigma or Merck and were "AnalaR" grade or the highest grade available. Restriction and modifying enzymes were obtained from Life Technologies, Inc.

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-orotic acid, uracil and methionine auxotrophies 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* (<u>A</u>TP sulfurylase <u>A</u>rabidopsis <u>1</u>).

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.

FIG. 1. **Alignment of *A. thaliana* ASA1 with other eukaryotic ATP sulfurylase amino acid sequences.** The amino acids of the open reading frame encoded by the *A. thaliana ASA1* cDNA are compared to the ATP sulfurylase sequences *APS1* (17) and *APS3* (*A. thaliana*) (19), *StMet3-1* and *StMet3-2* (*S. tuberosum*) (16), *MET3* (*S. cerevisiae*) (11), and *APS* (*P. chrysogenum*) (15), and *U. caupo* (21) represents the sequence of bifunctional ATP sulfurylase/APS kinase sequence. The alignments were obtained using the PILEUP GCG program. The full sequence for *ASA1* is shown, but for the other clones only the sequence containing the homology blocks, discussed in the text, are shown. *Roman numerals* refer to blocks of homology. *Boxed areas* represent regions where all but one of the sequences are identical or all the sequences show similarity. Positions where all residues are identical are *shaded*. Numbers at the ends of lines indicate the position of the most 3′ amino acid relative to the start of the protein. * indicates the position of putative tyrosine phosphorylation and ↓ indicates the *ASA1* putative transit peptide cleavage site. Residues *underlined* correspond to those identical with the N-terminal sequence from purified spinach ATP sulfurylase (5). *Dashes* indicate gaps in the sequence to yield the best alignment.

```
ASA1                                                        M S L M I R S S Y V S H I T L F Q P R N S K P S S F T   27
                                                                                            ▼
ASA1      N Q I S F L S S S N N N P F L N L V Y K R N L T M Q S V S K M T V K S S L I D P D G G E L V E L I V   77
ASA1      P E T E I G V K K A E S E T M P K V K L N Q I D L E W V H V I S E G W A S P L K G F M R E D E Y L Q   127
ASA1      S L H F N S L R L K N G T F V N M S L P I V L A I D D D T K E Q I G S S E N V A L V C P Q G D I I G   177
ASA1      S L R S V E I Y K H N K E E R I A R T W G T T S P G L P Y V E E Y I T P S G N W L I G G D L E V F E   227

ASA1      P I K Y N D G L D H Y R L S P K Q L R E E F D N R Q A D A V F A F Q L R N P V H N G H A L L M N D T   277
APS1      P V K Y N D G L D R F R L S P A E L R K E L E K R N A D A V F A F Q L R N P V H N G H A L L M T D T   263
APS3      P V K Y N D G L D R F R L S P F E L R K E L E K R G A D A V F A F Q L R N P V H N G H A L L M T D T   265
StMet3-2  P I K Y H D G L D R F R L S P A E L R D E F T R R N A D A V F A F Q L R N P V H N G H A L L M T D T   263
StMet3-1  P V K Y H D G L D S F R L S P S E L R A E F T R R N A D A V F A F Q L R N P V H N G H A L L M T D T   224
U. caupo  R I T W N D G L D E Y R L T P N E L R A K F R A L N A D A V F A F Q L R N P V H N G H A L L M T D T   420
MET3      L P Q H Y D - Y P G L R K T P A Q L R L E F Q S R Q W D R V V A F Q T R N P M H R A H R E L T V R A   211
APS       K L N H Y D - Y V A L R Y T P A E L R V H F D K L G W S R V V A F Q T R N P M H R A H R E L T V R A   213

ASA1      R K R L L E M G Y K N P V L L L H P L G G F T K A D D V P L D V R M E Q H S K V L E D G V L D P K T   327
APS1      R R R L L E M G Y Q K P Y S F A S S V S G F T K A D D V P L D W R M K Q H E K V L E D G V L D P E T   313
APS3      R R R L L E M G Y K N P I L L L H P L G G F T K A D D V P L S W R M K Q H E K V L E D G V L D P E T   315
StMet3-2  R R R L L E M G Y K N P V L L L H P L G G Y T K A D D V P L E W R M K Q H E M V L E D G V L D P E T   313
StMet3-1  R R R L L E M G Y K N P V L L L H P L G G Y T K E D D V P L H W R M K Q H E K V L E D G V L D P E T   274
U. caupo  R R R L T E R G Y K K P V L L L H P L G G W T K D D D V P L A W R M K Q H Q A I L D E K V L D P D Y   470
MET3      A R E - - - - - - A N A K V L I H P V V G L T K P G D I D H H T R V R V Y Q E I I K R - - Y P N G I   253
APS       A R S - - - - - - R Q A N V L I H P V V G L T K P G D I D H F T R V R A Y Q A L L P R - - Y P N G M   255

ASA1      T I V S I F P S P M H Y A G P T E V Q W H A K A R I N A G A N F Y I V G R D P A G M G H P T E K R D   377
APS1      T V V S I F P S P M H Y A G P T E V Q W H A K A R I N A G A N F Y I V G R D P A G M G H P V E K R D   363
APS3      T V V S I F P S P M L Y A G P T E V Q W H A K A R I N A G A N F Y I V G R D P A G M G H P V E K R D   365
StMet3-2  T V V S I F P S P M H Y A G P T E V Q W H A K A R I N A G A N F Y I V G R D P A G M G H P L E K R D   363
StMet3-1  T V L S I F P S P M H Y A G P T E V Q W H A K A R I N A G A N F Y I V G R D P A G M S H P L E K R D   324
U. caupo  T V M A I F P S P M M Y A G P T E V Q W H A K A R M S T G A N F Y I V G R D P A G M G H P H P E T K Q D   520
MET3      A F L S L L P L A M R M S G D R E A V W H A I I R K N Y G A S H F I V G R D H A G P G K N S K G V D   303
APS       A V L G L L G L A M R M G G R R E A I W H A I I R K N H G A T H F I V G R D H A G P G S N S K G V D   305

ASA1      L Y D P D H G K R V L S M A P G L E K L N I L P F R V A A Y D T I E K K M A F F D P S R A K E F - -   425
APS1      L Y D A D H G K K V L S M A P G L E R L N I L P F R V A A Y D K T Q G K M A F F D P S R P Q D F - -   411
APS3      L Y D A D H G K K V L S M A P G L E R L N I L P F R V A A Y D K T Q G K M A F F D P S R A Q D F - -   413
StMet3-2  L Y D A D H G K K V L S M A P G L E R L N I L P F K V A A Y D K T Q N G M A F F D P S R P Q D F - -   411
StMet3-1  L Y D A D H G K K V L S M A P G L E R L N I L P F K V A A Y D K T K N G M A F F D P S R P Q D F - -   472
U. caupo  L Y N A T H G A K V L T M A P G L T Q L E I V P F R V A A Y N K T K S O M D F Y D P E R H D E F - -   568
MET3      F Y G P Y D A Q E L V E S Y K H E L D I E V V P F R M V T Y L P D E D R Y A P I D Q I D T T K T R T   353
APS       F Y G P Y D A Q H A V E K Y K D E L G I E V V E F Q M V T Y L P D T D E Y R P V D Q V P A G - V K T   354

ASA1      L F I S G T K M R T Y A R T G E N P P D G F M C P S G W N V L V K Y Y E S L Q E S E A K Q Q A V V S A   476
APS1      L F I S G T K M R T L A K N N E N P P D G F M C P G G W K V L V D Y Y E S L T P A G N G R L P E V V P   463
APS3      L F I S G T K M R A L A K N R E N P P D G F M C P G G W K V L V D Y Y D S L T L T G N T K L P E K I P   465
StMet3-2  L F I S G T K M R A L A K T K E N P P D G F M C P G G W K V L V E Y Y D S L T P S E N G R V S E P V P   463
StMet3-1  I F I S G T K M R T L A K T K E S P P D G F M C P G G W K V L V E Y Y D S L D Q A E N G G V S E P V P   424
U. caupo  M F I S G T K M R A R A G E T P P N G F M A P S A W K I M V K Y Y K T K A Q Q S                         610
MET3      L N I S G T E L R R R L R V G G E I P E W F S Y P E V V K I L R E S N P P R P K Q G F S I V L G N S L   405::521
APS       L N I S G T E L R R R L R - S A H I P E W F S Y P E V V K I L R E S N P P R A T Q G F T I F L T G Y M   405::572
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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* StMet3-1 and StMet3-2 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* (20) 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 transit peptide, 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 isoform purified from spinach (SLIDPDGGSLIDLIVPENER) (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 G*XX*K*X*G 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)*XXX*(D/E)*XX*Y (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 Eco*RV and *Bam*HI digested DNA, which both have a restriction site in the cDNA, and to one fragment in both the *Eco*RI- or *Hin*dIII-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—The growth of yeast 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 pEMBLYe23 (p*M3-32*), 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⁻* strains (CC371-4C and C155) containing pFL61-*ASA1* were 66% higher than those measured from extracts of CC371-4C complemented by p*M3-32*, but 37% lower than those of the wild type (W303-1A). When *ASA1* was expressed in the Met3⁺ 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⁺ 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 selenate, as a toxic structural analogue of sulfate, in drop assays. When the strains were grown on sulfateless media (containing
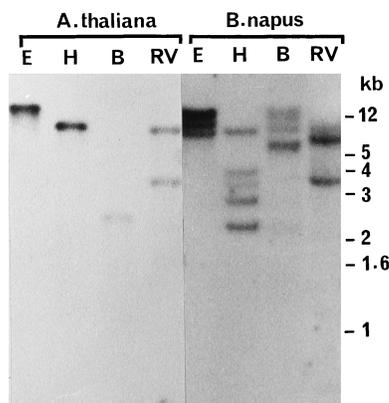
FIG. 2. **Southern blot analysis of the genomic DNA from *A. thaliana* and *B. napus*.** The genomic DNA (10 μg) was digested by restriction enzymes (*E, Eco*RI; *H, Hin*dIII; *B, Bam*HI; *RV, Eco*RV), separated by agarose gel (0.8%) electrophoresis, transferred onto nitrocellulose, and then hybridized with ^{32}P-labeled *ASA1* DNA fragments.
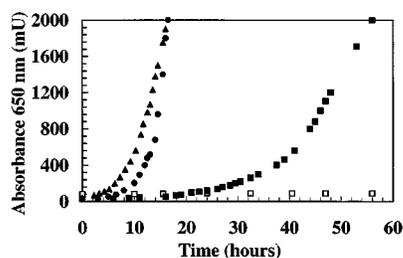

FIG. 3. **Growth curve of yeast strains transformed with *ASA1* or *MET3*.** Yeast were grown from 50 milliunits initial absorbance, measured at 650 nm, in synthetic media supplemented with 0.4 mM sodium sulfate as the sole sulfur source and the required auxotrophy factors. *Symbols* correspond as follows: ●, Met3$^+$ strain W303-1A; □, *met3* mutant strain CC371-4C; ■, *met3* mutant strain CC371-4C transformed with pFL61-*ASA1*; ▲, *met3* mutant strain CC371-4C transformed with p*M3-32*.

TABLE I
ATP sulfurylase activity and [^{35}S]sulfate influx measurements in yeast

Yeast strains[a]	Activity[b]	
	ATP sulfurylase[c]	[^{35}S]Sulfate influx[d]
W303–1A	538	46.0
W303–1A + p*M3-32*[e]	2329	ND
W303–1A + pFL61-*ASA1*[e]	243	ND
CC371–4C	0	0
CC371–4C + pFL61-*ASA1*	356	7.9
CC371–4C + p*M3-32*	113	178.5
C155	3	ND
C155 + pFL61-*ASA1*	317	ND

[a] Relevant strain phenotypes: W303–1A (*MET3*$^+$, *ura3*), CC371–4C (*met3*, *ura3*), C155 (*met3::URA3*).
[b] The ATP sulfurylase activity and [^{35}S]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 molybdolysis.
[d] Unidirectional [^{35}S]sulfate influxes were measured for 3 min in 0.1 mM MgSO$_4$ at 30 °C.
[e] p*M3-32* and pFL61-*ASA1* represent plasmids containing the yeast gene and the plant cDNA ATP sulfurylase clones, respectively.

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 + p*M3-32*) 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, [^{35}S]sulfate influxes were measured (Table
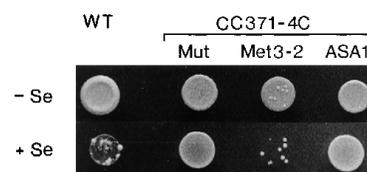

FIG. 4. **Test for selenate resistance.** Yeast were grown in sulfur-free synthetic liquid media supplemented with 0.1 mM homocysteine as the sole sulfur source and the required auxotrophy factors. Yeast were washed and 30 μl (at ~0.8 milliunit absorbance at 650 nm) were dropped onto agarose sulfate-free 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*).

I). 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 [^{35}S]sulfate influx was almost 4-fold higher than in the yeast control.

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 transcript relative 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 poly(A) of the two *ASA1* homologous sequences and an homologous EST (accession number Z26572) are located downstream of the *ASA1* poly(A) which seems to agree with the observed
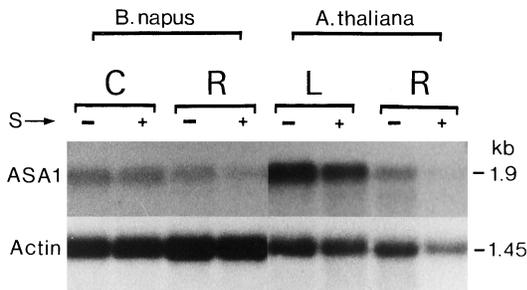
FIG. 5. **Northern blot analysis of total RNA from *A. thaliana* and *B. napus* grown with and without sulfate.** Total RNA (10 μg) from plants grown in the presence (+) or absence (−) of sulfate (*S*) was isolated from leaves (*L*), cotyledons (*C*), or roots (*R*) and electrophoresed on an agarose (1.2%) gel, transferred onto a nitrocellulose filter, and then hybridized with ^{32}P-labeled DNA fragments either *ASA1* or actin.

FIG. 6. **Northern blot analysis and ATP sulfurylase activity of *B. napus* organs grown with and without sulfate.** Total RNA or soluble protein was isolated from cotyledons (*Co*), hypocotyls (*H*), and roots (*R*) after growth in the presence (+4) or absence of sulfate for 2 (−2) or 4 (−4) days. RNA (10 μg) was electrophoresed on an agarose (1.2%) gel, transferred onto a nitrocellulose filter, and then hybridized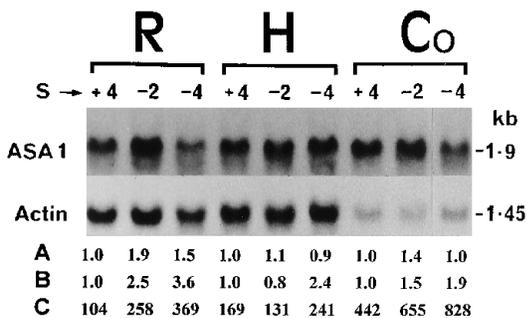 with ^{32}P-labeled DNA fragments either *ASA1* or actin. *A*, the relative abundances of *ASA1* hybridizing transcripts in these organs, determined from RNA slot-blot analysis, standardized to actin hybridization, and compared for each organ to the sulfate (+) value. Duplicate slots were carried out for each sample. *B*, the relative ATP sulfurylase specific activities in these organs compared for each organ to the sulfate + value. *C*, the ATP sulfurylase specific activities measured in these organs, expressed as nmol·min^{-1}·mg^{-1} of protein, are the mean of three replicates.

preference for polyadenylation in response to the second site after the open reading frame (46).

Yeast *met3* mutants complemented with *ASA1* had a lower growth rate than wild type or the mutant complemented by the homologous gene. This result may reflect differences between constitutive expression of *ASA1* by the phosphoglycerate kinase promoter and expression of the yeast ATP sulfurylase gene by its own promoter. Alternatively, the presence of a chloroplast transit peptide or differences in primary sequence between the yeast and *ASA1* could significantly alter the activity of the plant enzyme. Putative cytosolic and chloroplastic forms of ATP sulfurylase have been cloned from potato by functional complementation (16); however, only the activity of the cytosolic form expressed in yeast was presented. Activity measurements for all complemented *met3* mutants were lower than for the noncomplemented Met3$^+$ strain, but as the growth curve of the mutant complemented with the homologous gene is similar to that of the wild type (Fig. 3) there does not seem to be any growth limitation due to insufficient ATP sulfurylase activity, suggesting that more activity than required for growth is expressed in the wild type strain. Expression of *ASA1* in the Met3$^+$ strain reduced the ATP sulfurylase activity by 30% compared to when it was expressed in the *met3* mutants and reduced the activity of the noncomplemented Met3$^+$ strain by 50% (Table I). This result implies that the plant ATP sulfur-

ylase protein or cDNA has an effect on either the yeast protein activity or the yeast gene expression. For example, ATP sulfurylase is a homomultimeric protein in plants (5, 19) and yeast (9), and it is possible that in the non-mutant Met3$^+$ strain the co-expressed yeast and plant proteins form chimeric structures with reduced or no ATP sulfurylase activity. In contrast, the activity measured for p*M3-32* 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 heterologous protein was able to grow (Fig. 4). This was confirmed to result from reduced [^{35}S]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, T42953, 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,[2] and this 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 two homology blocks proposed to correspond to part of 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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[2] C. Deswarte, A. Borchers, H. Logan, and J.-C. Davidian, unpublished results.

a greater degree in roots, after 2 days of sulfur 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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