Plant Physiol. (1994) 105: 897-902

Cloning of a cDNA Encoding ATP Sulfurylase from *Arabidopsis thaliana* by Functional Expression in *Saccharomyces cerevisiae*[1]

Thomas Leustek*, Michael Murillo, and Miguel Cervantes

Center for Agricultural Molecular Biology, Rutgers University, New Brunswick, New Jersey 08903

ATP sulfurylase, the first enzyme in the sulfate assimilation pathway of plants, catalyzes the formation of adenosine phosphosulfate from ATP and sulfate. Here we report the cloning of a cDNA encoding ATP sulfurylase (*APS1*) from *Arabidopsis thaliana*. *APS1* was isolated by its ability to alleviate the methionine requirement of an ATP sulfurylase mutant strain of *Saccharomyces cerevisiae* (yeast). Expression of *APS1* correlated with the presence of ATP sulfurylase enzyme activity in cell extracts. *APS1* is a 1748-bp cDNA with an open reading frame predicted to encode a 463-amino acid, 51,372-D protein. The predicted amino acid sequence of *APS1* is similar to ATP sulfurylase of *S. cerevisiae*, with which it is 25% identical. Two lines of evidence indicate that *APS1* encodes a chloroplast form of ATP sulfurylase. Its predicted amino-terminal sequence resembles a chloroplast transit peptide; and the *APS1* polypeptide, synthesized in vitro, is capable of entering isolated intact chloroplasts. Several genomic DNA fragments that hybridize with the *APS1* probe were identified. The *APS1* cDNA hybridizes to three species of mRNA in leaves (1.85, 1.60, and 1.20 kb) and to a single species of mRNA in roots (1.85 kb).

Plants and microorganisms assimilate inorganic sulfate for the biosynthesis of various sulfur-containing compounds. ATP sulfurylase plays a key role in sulfate metabolism by catalyzing the formation of APS from ATP and sulfate. The phosphate-sulfate anhydride bond in APS activates sulfate for subsequent reactions. For example, sulfate can be further reduced to sulfide, the substrate for Cys biosynthesis. The sulfate in APS can also be transferred to a hydroxyl group to form a sulfate ester. Some proteins, peptides, oligosaccharides, and flavonoid compounds are sulfated on hydroxyl groups (Schmidt and Jäger, 1992). Activated sulfate can also be transferred to a carbon group to form sulfonic acid, as occurs in the biosynthesis of sulfolipids (Schmidt and Jäger, 1992). Sulfation of various molecules may play important regulatory functions; one example is a sulfated oligosaccharide produced by the nitrogen-fixing symbiont *Rhizobium meliloti*, which elicits root nodule formation in some leguminous plants (Lerouge et al., 1990).

In higher plants, all tissues are capable of sulfate assimilation although chloroplasts are the primary site for this process. Leaf cells contain two biochemically distinct forms of ATP sulfurylase in the cytosol and chloroplasts. The chloroplast enzyme is the predominant form in leaves (Lunn et al., 1990). In root cells the enzyme is localized in proplastids (Brunold and Suter, 1989). As yet, ATP sulfurylase has not been identified in higher plant mitochondria, although the finding by Li et al. (1991) that eukaryotic algae contain a mitochondrial form of the enzyme makes this a possibility.

ATP sulfurylase has been cloned from *Saccharomyces cerevisiae* (yeast), *Escherichia coli*, and *R. meliloti* (Cherest et al., 1987; Schwedock and Long, 1990; Leyh et al., 1992). In yeast a single gene, *MET3*, encodes cytoplasmically localized ATP sulfurylase. In *E. coli* and *R. meliloti* ATP sulfurylase is encoded by two genes, *cysD/cysN* and *nodP/nodQ*, respectively. The predicted amino acid sequences of the prokaryotic genes are very similar to each other but are highly divergent from the yeast enzyme.

Due to the central role that ATP sulfurylase plays in sulfur metabolism, we sought to clone and characterize the higher plant genes encoding this enzyme. Here we report the isolation of a cDNA from *Arabidopsis thaliana* encoding the chloroplast form of ATP sulfurylase.

MATERIALS AND METHODS

Strains and Media

A *Saccharomyces cerevisiae* strain carrying the *met3* and *ura3* mutations (strain S5) was constructed by crossing strain S30 with YPH499 (obtained from the Yeast Genetic Stock Center, University of California, Berkeley). A diploid derived from this cross was sporulated and a haploid strain (S5) was isolated with the genotype *MATα*, *met3*, *ura3-52*, *his3Δ*, *leu2-1* (or *leu2Δ*). Minimal medium for growth of yeast was prepared as described by Rose et al. (1990). Media containing Glc or Gal as sole carbon sources were supplemented with all compounds required by strain S5 for growth unless noted. Plasmids were prepared in *Escherichia coli* strain DH5α on Luria Broth medium with ampicillin (100 μg/mL).

cDNA Cloning

An *Arabidopsis thaliana* cDNA library, constructed in λYES, a dual *E. coli* and yeast expression vector, was obtained from Dr. Ron Davis (Stanford University, Stanford, CA) (Elledge

[1] This work was supported by The National Science Foundation (grant No. IBN-9301921), The New Jersey Agricultural Experiment Station, and The New Jersey Commission on Science and Technology.

* Corresponding author; fax 1-908-932-6535.

Abbreviation: APS, adenosine phosphosulfate.

et al., 1991). For library screening in yeast, λYES contains the *URA3* gene as a selectable marker for transformation, and expression of cDNA inserts is controlled by the *GAL1* promoter. The library was transformed into yeast strain S5 by electroporation (Becker and Guarente, 1991). Transformants were recovered on medium containing Glc and lacking uracil. The colonies were then replica plated onto medium containing Gal and lacking uracil to induce expression of cDNAs. After a 2-d incubation at 24°C, the transformants were replica plated onto Gal medium lacking uracil and Met and incubated at the same temperature to identify cDNAs that alleviate the Met requirement of strain S5.

DNA Sequencing

Positive cDNA clones were rescued into *E. coli* using the procedure of Strathern and Higgins (1991), and the cDNA insert was subcloned into the *Eco*RI site in pBluescript, SK(−) (Stratagene, La Jolla, CA). The dideoxy-chain termination method was used for sequence analysis (Sanger et al., 1977) using Sequenase (United States Biochemical). The DNA of both strands of *APS1* was completely sequenced using a combination of restriction fragment subclones and overlapping clones derived from exonuclease III deletions.

Growth of Yeast and Assay of ATP Sulfurylase

ATP sulfurylase activity was measured in extracts of yeast transformed with *APS1* in λYES. Cells were grown in liquid Glc or Gal media to an optical density (600 nm) of 1.0. The cells, harvested by centrifugation, were washed once in sterile water and then lysed in buffer containing 100 mM Tris-HCl (pH 8.2) using a Mini Bead Beater (Bio-Spec, Products, Bartlesville, OK) for 2 min. ATP sulfurylase activity was assayed by measurement of APS-dependent ATP synthesis (reverse reaction) as described by Renosto et al. (1991). Enzyme activity is reported in units (μmol of ATP formed in 1 min) per mg protein. The Bradford (1976) dye-binding assay was used to measure protein concentration using BSA as a standard.

Chloroplast Import Assay

APS1 was expressed by transcription and translation in vitro. APS1, cloned into the *Eco*RI site in pBluescript, was linearized with *Eco*RV that cuts once in the polylinker of the vector proximal to the 3′ end of the cDNA. The mRNA was transcribed with T3 RNA polymerase and then translated in a rabbit reticulocyte extract (Promega, Madison, WI) using L-[3,4,5-^{3}H]Leu (168 Ci/mmol) (New England Nuclear).

Import of the radioactively labeled *APS1* polypeptide into isolated pea chloroplasts was carried out as described by Cline et al. (1985) with minor modifications. In brief, the import reactions contained, in a volume of 300 μL, 50 mM Hepes-KOH (pH 8.0), 10 mM Mg-ATP, 330 mM sorbitol, 8 mM Leu, intact chloroplasts equal to 100 μg of Chl, and 50 μL of translation reaction equal to approximately 40,000 cpm, TCA-precipitable counts. For some reactions ATP was not added and hexokinase and Glc were added to final concentrations of 2 units/mL and 35 μM, respectively, to deplete ATP carried over in the translation mixture. Import assays were carried out in the dark at 25°C for 60 min in 1.5-mL microfuge tubes with occasional mixing. In some cases chloroplasts were treated, after import, with 50 μg of thermolysin for 30 min on ice. Prior to gel analysis, intact chloroplasts were reisolated by centrifugation through a 40% (v/v) Percoll cushion.

Nucleic Acid Blot Analysis

Total genomic DNA was isolated from young *A. thaliana* plants (Dellaporta et al., 1983). Approximately 2 μg of DNA was digested with restriction enzymes and the fragments were separated by electrophoresis through a 0.8% (w/v) agarose gel. The DNA fragments were nicked by exposure of the gel for 8 min to short-wavelength UV light and then denatured by soaking the gel for 8 min in a solution containing 0.4 N NaOH. The DNA fragments were transferred to nylon membrane (Zeta Probe, Bio-Rad) using a pressure blotter. The nylon membrane was treated with 50 mM Tris-HCl (pH 7.2) for 10 min and then prehybridized in a solution containing 0.5 M Na^+ (supplied as Na_2HPO_4, pH 7.2), 1 mM EDTA, and 7% (w/v) SDS at 65°C for 8 h. Hybridization was carried out in prehybridization buffer at 65°C for 16 h. A 1290-bp, *Xho*I DNA fragment, corresponding to the 5′ region of *APS1*, was used as a DNA probe. The probe was labeled by the random primer method (Feinberg and Vogelstein, 1983) using [α-^{32}P]dCTP (3000 Ci/mmol). The membrane was washed at maximum stringency in 100 mM Na^+ (pH 7.5, as described above), 1 mM EDTA, and 5% (w/v) SDS at 65°C.

Total RNA was isolated from axenically grown leaves or roots of *A. thaliana* using guanidinium thiocyanate (Chomczynski and Sacchi, 1987). Approximately 10 μg of RNA was separated by electrophoresis through a 1% (w/v) agarose gel containing formaldehyde (Lehrach et al., 1977). The gel was rinsed several times in water and then treated for 15 min with a solution containing 0.05 N NaOH and 10 mM NaCl. RNA was transferred to nylon membrane and the membrane was processed as described above. Hybridization was with the 1290-bp, random primer-labeled *Xho*I DNA fragment. The membrane was washed at maximum stringency in 25 mM Na^+ (pH 7.5 as described above), 1 mM EDTA, and 5% (w/v) SDS at 65°C.

RESULTS

Isolation of a cDNA Clone Encoding ATP Sulfurylase, *APS1*

To identify cDNA clones encoding ATP sulfurylase, an *A. thaliana* cDNA library, constructed in λYES, a yeast expression vector (Elledge et al., 1991), was screened for clones that alleviate the Met requirement of an ATP sulfurylase mutant yeast strain (S5). Expression of cDNAs cloned into λYES is under control of the yeast *GAL1* promoter. Clones that permit the growth of S5 on medium lacking Met were identified at a frequency of 1 for every 17,000 transformants. Figure 1 shows the growth of S5 transformed with one of the complementary clones, *APS1*, or with λYES. Cells transformed with either plasmid are able to grow on medium containing Met,

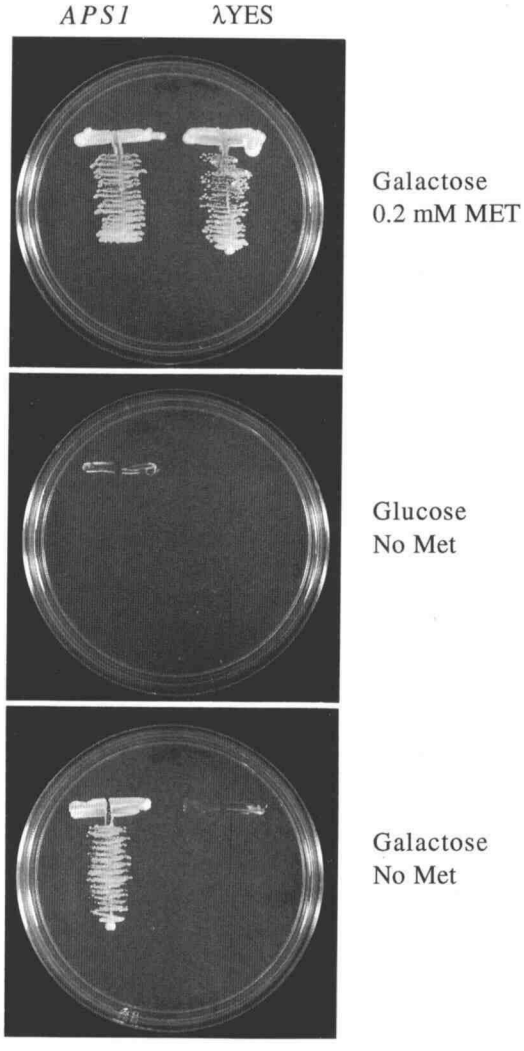

Figure 1. Growth assay of an ATP sulfurylase mutant yeast containing *APS1* or λYES. Cells were streaked on media containing Gal with Met, Gal without Met, or Glc without Met. The cultures were incubated at 24°C for 5 d. The growth rate of *met3* yeast transformed with *ATS1* was similar to that of yeast containing the wild-type *MET3* gene on medium with Gal as the carbon source.

but neither strain is able to grow on medium lacking Met if Glc is provided as the carbon source. In contrast, only cells carrying *APS1* are able to grow on medium lacking Met if Gal is the carbon source. This result is consistent with the Gal-dependent expression of cDNAs in the λYES vector under transcriptional control of the *GAL1* promoter. To confirm the authenticity of *APS1*, the plasmid was recovered by transformation of *E. coli* and then reintroduced into S5. All the transformants resulting from reintroduction of *APS1* were able to grow on Gal medium lacking Met. These results indicate that *APS1* likely encodes ATP sulfurylase.

We further tested *APS1* by measuring ATP sulfurylase activity in extracts of strain S5 carrying this cDNA clone grown on medium with Glc or Gal. ATP sulfurylase activity was not detected in cells grown on Glc but was detected in cells grown on Gal medium with or without Met (Table I). This confirms that enzyme activity occurs under conditions that promote *APS1* expression. An extract from cells grown on medium containing Gal and Met was assayed to rule out the possibility that ATP sulfurylase activity results from reversion of the *met3* mutant gene. If so, the expression of ATP sulfurylase would be expected to be repressed by Met, as has been shown for a number of genes encoding Met biosynthetic enzymes (Cherest et al., 1987). The minor difference in ATP sulfurylase activity detected in cells grown on Gal medium with and without Met indicates that the activity is not due to *MET3* expression.

Sequence Analysis of *APS1*

The nucleotide sequence and deduced amino acid sequence of *APS1* are shown in Figure 2. *APS1* is a 1748-bp cDNA with an open reading frame predicted to encode a 463-amino acid, 51,372-D protein. The codon bias of *APS1* is similar to that of genes from *A. thaliana* (Wada et al., 1992). The amino acid sequence of *APS1* did not show significant similarity with any sequence in the GenBank data base. Therefore, we compared the sequence of *APS1* with that of ATP sulfurylase genes from yeast, *E. coli*, and *R. meliloti*. The best homology was found with ATP sulfurylase from yeast, with which it was 25% identical and 47% similar. The alignment of *APS1* with the yeast sequence is shown in Figure 3. The similarity of *APS1* with ATP sulfurylase from *E. coli* and *R. meliloti* was much lower, approximately 15% identity. There are no strong local regions of similarity shared by ATP sulfurylases from *A. thaliana*, yeast, and prokaryotes.

Sequence analysis of *APS1* indicated the presence of a 49-residue amino-terminal sequence that is not found in the yeast ATP sulfurylase (underlined in Fig. 3). This amino-terminal extension is rich in hydroxylated and small hydrophobic amino acids and has a net positive charge. These characteristics are similar to those of transit peptides that target proteins to chloroplasts (Keegstra et al., 1989). Two potential transit peptide cleavage sites can be predicted at positions 43 or 48 based on the presence of Arg at positions 42 or 47. Arg is commonly found one residue removed from the cleavage site in chloroplast transit peptides (Gavel and von Heijne, 1990). Also, cleavage at either site would generate an amino terminus on the mature protein that is similar to the terminus of yeast ATP sulfurylase.

Table I. *ATP sulfurylase activity in yeast transformed with APS1*

The minimum, clearly detectable level of activity in this assay is 0.001. The experiment reported here was performed once. It should be noted that yeast carrying the mutant *met3* locus has previously been reported to be devoid of ATP sulfurylase activity (Cherest et al., 1987). ATP sulfurylase activity was assayed by measuring the APS-dependent formation of ATP. Enzyme activity is expressed in units (μmol ATP formed per min) per mg protein.

Growth Medium	ATP Sulfurylase Activity
	units/mg protein
Glc + Met	N.D.
Gal − Met	0.023
Gal + Met	0.012

```
CTACGTCAGGGCAAATCTCTATCTCTCTCCATTAGACCTTGAAGCAGCCATAGCCTAACAAAACCTTCAA 70

CAATGGCTTCAATGGCTGCCGTCTTAAGCAAAACTCCATTCCTCTCTCAACCACTAACCAAATCATCTCC 140
  M  A  S  M  A  A  V  L  S  K  T  P  F  L  S  Q  P  L  T  K  S  S  P

AAACTCCGATCTCCCCTTCGCCGCGGTTTCCTTCCCTTCCAAATCCCTACGCCGCCGCGTAGGATCAATC 210
  N  S  D  L  P  F  A  A  V  S  F  P  S  K  S  L  R  R  R  V  G  S  I

CGAGCCGGATTAATCGCTCCCGACGGTGGTAAGCTTGTAGGGCTCATCGTGGAAGAGCCAAAGCGGCGAG 280
R  A  G  L  I  A  P  D  G  G  K  L  V  G  L  I  V  E  E  P  K  R  R  E

AGAAGAAACACGAGGCGGCGGATTTGCCACGTGTTGAGCTGACGGCGATTGACTTGCAATGGATGCATGT 350
  K  K  H  E  A  A  D  L  P  R  V  E  L  T  A  I  D  L  Q  W  M  H  V

ATTAAGCGAAGGCTGGGCAAGTCCACTCGGAGGTTTCATGAGAGAATCCGAGTTCCTCCAAACTCTTCAT 420
  L  S  E  G  W  A  S  P  L  G  G  F  M  R  E  S  E  F  L  Q  T  L  H

TTTAACTCGCTACGTCTTGACGACGGCTCCGTCGTTAACATGTCCGTGCCTATTGTTCTCGCTATTGACG 490
F  N  S  L  R  L  D  D  G  S  V  V  N  M  S  V  P  I  V  L  A  I  D  D

ATGAACAAAAAGCACGTATCGGCGAGTCTACACGTGTCGCTCTTTTCAATTCCGATGGTAACCCCGTCGC 560
  E  Q  K  A  R  I  G  E  S  T  R  V  A  L  F  N  S  D  G  N  P  V  A

TATCCTCAGCGATATTGAGATTTATAAGCATCCAAAGGAAGAAAGGATAGCTAGAACATGGGGTACGACG 630
  I  L  S  D  I  E  I  Y  K  H  P  K  E  E  R  I  A  R  T  W  G  T  T

GCTCCAGGTTTGCCTTACGTAGACGAGGCGATAACTAATGCTGGAAACTGGCTCATTGGGGGTGATCTTG 700
A  P  G  L  P  Y  V  D  E  A  I  T  N  A  G  N  W  L  I  G  G  D  L  E

AGGTTCTTGAGCCAGTGAAGTACAATGATGGGCTTGATCGTTTCAGGCTTTCGCCTGCTGAGTTACGTAA 770
  V  L  E  P  V  K  Y  N  D  G  L  D  R  F  R  L  S  P  A  E  L  R  K

AGAGTTGGAGAAGCGTAATGCGGATGCGGTGTTTGCTTTCCAGCTGAGGAATCCTGTTCATAATGGTCAT 840
  E  L  E  K  R  N  A  D  A  V  F  A  F  Q  L  R  N  P  V  H  N  G  H

GCTCTTCTTATGACTGATACTCGTAGGAGACTTCTTGAGATGGGTTACCAAAAACCCTATTCTTTTGCTT 910
A  L  L  M  T  D  T  R  R  R  L  L  E  M  G  Y  Q  K  P  Y  S  F  A  S

CATCCGTTAGTGGGTTTACAAAGGCTGATGATGTTCCTTTAGATTGGAGGATGAAGCAACACGAGAAGGT 980
  S  V  S  G  F  T  K  A  D  D  V  P  L  D  W  R  M  K  Q  H  E  K  V

TCTAGAGGATGGTGTTCTCGATCCGGAGACTACAGTGGTTTCGATATTCCCGTCACCTATGCATTACGCT 1050
  L  E  D  G  V  L  D  P  E  T  T  V  V  S  I  F  P  S  P  M  H  Y  A

GGTCCAACCGAAGTGCAGTGGCACGCAAAGGCTAGAATCAATGCTGGTGCTAACTTTTACATTGTGGGTC 1120
G  P  T  E  V  Q  W  H  A  K  A  R  I  N  A  G  A  N  F  Y  I  V  G  R

GTGATCCTGCTGGGATGGGTCATCCAGTAGAGAAACGTGATCTTTACGATGCTGATCATGGAAAGAAAGT 1190
  D  P  A  G  M  G  H  P  V  E  K  R  D  L  Y  D  A  D  H  G  K  K  V

ACTAAGCATGGCACCAGGACTCGAACGACTCAACATCCTTCCTTTCAGGGTTGCTGCATATGACAAGACG 1260
  L  S  M  A  P  G  L  E  R  L  N  I  L  P  F  R  V  A  A  Y  D  K  T

CAAGGCAAGATGGCTTTCTTCGATCCCTCGAGGCCTCAAGATTTCTTGTTCATCTCCGGCACTAAGATGC 1330
Q  G  K  M  A  F  F  D  P  S  R  P  Q  D  F  L  F  I  S  G  T  K  M  R

GCACATTGGCAAAGAACAACGAAAACCCGCCAGACGGTTTTATGTGCCCAGGTGGATGGAAAGTTCTGGT 1400
  T  L  A  K  N  N  E  N  P  P  D  G  F  M  C  P  G  G  W  K  V  L  V

GGATTACTATGAGAGCTTGACTCCGGCGGGTAATGGTAGACTACCAGAAGTGGTTCCGGTGTAAGACAAA 1490
  D  Y  Y  E  S  L  T  P  A  G  N  G  R  L  P  E  V  V  P  V  *

ACTGTTCGTTTCAAATTGTAACGTTTGTGTTGTGAAGCCTTGTAGCAACAATCATTGTTGTATTGGGAGA 1470
GAAGCCTATGTATAATCTGGCTTGACCTTTTTCCAAATAAAATACAGAAGAAAAAAGACTGTTTTCGTTT 1540
GCAAGATAATTTACGAACTTGTAATATTTGTGCTCAACTTGTACATATATGAACGATGTTTACAAAAAAA 1610
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1678
```

Figure 2. Nucleotide sequence and deduced amino acid sequence of *APS1*. The nucleotides are numbered and the termination codon is indicated with an asterisk.

```
                                                                    
MASMAAVLSKTPFLSQPLTKSSPNSDLPFAAVSFPSKSLRRRVGSIRAGL   50 APS1
                                                    :
                                                    M    1 MET3

IAPDGGKLVGLIVEEPKRREKKHEAADLPRV...ELTAIDLQWMHVLSEG  97 APS1
:|| || |  ||: :: ::     |   :    ||:  |: : ::  |
PAPHGGILQDLIARDALKKNELLSEAQSSDILVWNLTPRQLCDIELILNG  51 MET3

WASPLGGFMRESEFLQTLHFNSLRLDDGSVVNMSVPIVLAIDDEQKARIG 147 APS1
 :|||:||: |::  : :  :  || ||::  :::|| | :|:     |
GFSPLTGFLNENDYSSVV..TDSRLADGTL..WTIPITLDVDEAFANQIK  97 MET3

ESTRVALFNSDGNPVAILSDIEIYKHPKEERIARTWGTTAPGLPYVDEAI 197 APS1
  ||:|||: |  |:|||:  ::||  |   : : :  : |  | :  ::
PDTRIALFQDDEIPIAILTVQDVYKPNKTIEAEKVF.RGDPEHPAISYLF 146 MET3

TNAGNWLIGGDLEVLEPVKYNDGLDRFRLSPAELRKELEKRNADAVFAFQ 247 APS1
: ||   :|| ||:: ::  :|    :| :|| || |:  |::| |:|||
NVAGDYYVGGSLEAIQLPQHYD.YPGLRKTPAQLRLEFQSRQWDRVVAFQ 195 MET3

LRNPVHNGHALLMTDTRRRLLEMGYQKPYSFASSVSGFTKADDVPLDWRM 297 APS1
 |||:|  |  |     |         :  ::  | |:||: |:    |:
TRNPMHRAHRELTVRAARE......ANAKVLIHPVVGLTKPGDIDHHTRV 239 MET3

KQHEKVLEDGVLDPETTVVSIFPSPMHYAGPTEVQWHAKARINAGANFYI 347 APS1
:   ::          ::|::| :|:  |  |: ||| :| | ||:  |
RVYQEIIKR..YPNGIAFLSLLPLAMRMSGDREAVWHAIIRKNYGASHFI 287 MET3

VGRDPAGMGHPVEKRDLYDADHGKKVLSMAPGLERLNILPFRVAAYDKTQ 397 APS1
|||| ||:|:     |:| :     ::          : ::|||:: |
VGRDHAGPGKNSKGVDFYGPYDAQELVESYKHELDIEVVPFRMVTYLPDE 337 MET3

GKMAFFDPSRPQD..FLFISGTKMRTLAKNNENPPDGFMCPGGWKVLVDY 445 APS1
 : |::|   :   | |||| :|  :: :  :|: | :|  :|:|
DRYAPIDQIDTTKTRTLNISGTELRRRLRVGGEIPEWFSYPEVVKILR.. 385 MET3

YESLTPAGNGRLPEVVPV                                  463 APS1
 ||  |  : :  |:
.ESNPPRPKQGFSIVLGNSLTVSREQLSIALLSTFLQFGGGRYYKIFEHN 434 MET3
```

Figure 3. Amino acid sequence alignment of *APS1* and *MET3*. The Genetics Computer Group program BESTFIT was used to generate the alignment, and spaces, indicated by dots in the sequence, were introduced to optimize the alignment. The identity and numbering of the sequences is shown on the right. Only 434 amino acids of the 522 in the *MET3* are shown. Identical amino acids are indicated with solid lines and conservative substitutions are indicated with colons. The amino-terminal extension of *APS1* is underlined. Above the transit peptide, hydroxylated amino acids are indicated by solid circles and charged amino acids are indicated by positive or negative signs.

Import of the *APS1* Polypeptide into Chloroplasts

The presence of a possible chloroplast transit peptide suggested that *APS1* may encode the chloroplast form of ATP sulfurylase. We tested whether the *APS1* translation product was able to enter chloroplasts by conducting in vitro import assays (Fig. 4). A full-length *APS1* polypeptide was synthesized in vitro with [^{3}H]Leu to generate a translation product estimated to be 52 kD (lane 1), close to that of the predicted amino acid sequence of *APS1*. One major smaller product evident in lane 1 may result from either internal initiation or termination of translation. After incubation with isolated chloroplasts, a smaller polypeptide of 47 kD associated with the chloroplasts (lane 2). This smaller polypeptide was resistant to degradation by the protease thermolysin (lane 3), indicating that it was localized within chloroplasts. If ATP was eliminated from the import reaction buffer, the *APS1* polypeptide could not enter the chloroplasts (lane 4).

These results demonstrate that the *APS1* polypeptide is

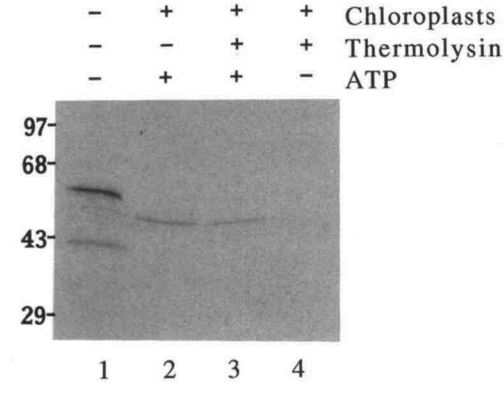

Figure 4. Import of *APS1* polypeptide into isolated chloroplasts. *APS1* polypeptide was synthesized in vitro in a rabbit reticulocyte extract with [^{3}H]Leu. Radioactively labeled protein was analyzed by electrophoresis on a 10% (w/v) polyacrylamide gel and autoradiography. *APS1* polypeptide is shown in lane 1; *APS1* polypeptide incubated with isolated chloroplasts is shown in lane 2; treatment of the chloroplasts with thermolysin is shown in lane 3; and the import reaction carried out without ATP followed by thermolysin treatment is shown in lane 4. The positions of molecular mass markers are shown on the left in kD. The various treatments are shown above the figure.

capable of entering isolated, intact chloroplasts in an ATP-dependent manner. Import of proteins into chloroplasts is known to be ATP dependent (Keegstra et al., 1989). Furthermore, the imported *APS1* polypeptide is smaller than the initial translation product, consistent with proteolytic cleavage of the transit peptide after import. The size of the transit peptide is 5 kD based on this analysis, in agreement with the predicted size of the transit peptide shown in Figure 3.

Analysis of DNA for Sequences Homologous with *APS1*

DNA blot analysis was used to examine the gene or genes encoding ATP sulfurylase in *A. thaliana* (Fig. 5). The figure shows that in each lane several DNA fragments hybridize either strongly or weakly with the *APS1* probe. These DNA fragments may contain different ATP sulfurylase genes or they may represent separate exons of a single gene.

In Vivo Expression of ATP Sulfurylase mRNA

We used the *APS1* cDNA as probe on RNA blot experiments to determine the pattern of ATP sulfurylase mRNA expression in leaves and roots of *A. thaliana* (Fig. 6). The *APS1* probe detected three transcripts in leaves (1.85, 1.60, and 1.20 kb; lane 1). A single mRNA species of 1.85 kb was detected in roots (lane 2).

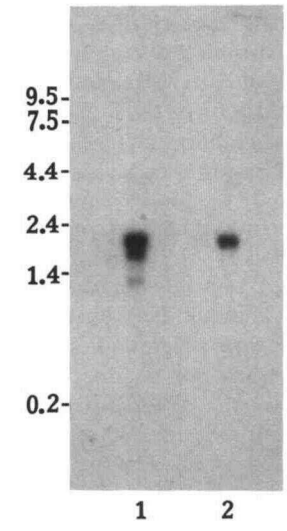

Figure 6. Blot analysis of total RNA from *A. thaliana* using *APS1* as a probe. Ten micrograms of RNA from leaves (lane 1) or roots (lane 2) were separated by electrophoresis on a formaldehyde gel, blotted onto a nylon membrane, and hybridized to a 1290-bp *Xho*I fragment from *APS1*. The positions of RNA size markers are shown on the left in kb.

DISCUSSION

In this paper we present evidence that the cDNA *APS1* encodes the chloroplast form of ATP sulfurylase from *A. thaliana*. The cloning strategy employed to isolate *APS1* (functional complementation of the yeast *met3* mutation) depended on expression of a functional enzyme capable of replacing the endogenous yeast enzyme. Cells carrying *APS1* in λYES are able to grow and form active ATP sulfurylase when grown on medium with Gal as a carbon source, a condition that is expected to induce *APS1* expression, but are unable to grow or form an active enzyme if Glc is provided, a condition that is expected to repress its expression (Fig. 1; Table I).

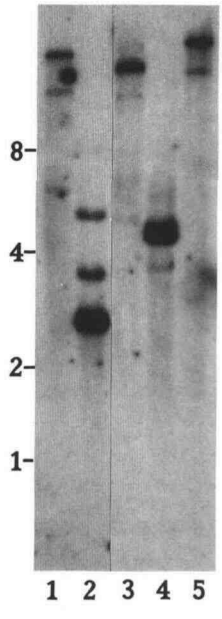

Figure 5. Blot analysis of DNA from *A. thaliana* using *APS1* as a probe. Approximately 2 μg of DNA was digested with a restriction enzyme, separated by electrophoresis through a 0.8% (w/v) agarose gel, transferred to Zeta-probe membrane, and hybridized to a 1290-bp fragment from the 5′ region of *APS1*. The restriction enzymes used included *Eco*RI (lane 1); *Hin*dIII (lane 2); *Nco*I (lane 3); *Sst*I (lane 4); and *Xho*I (lane 5). The positions of DNA size markers are shown on the left in kb.

Our analysis of *APS1* revealed that it likely encodes the chloroplast form of ATP sulfurylase based on the similarity of its predicted amino-terminal sequence with chloroplast transit peptides and on its ability to enter isolated intact chloroplasts in vitro (Figs. 3 and 4). Chloroplast ATP sulfurylase is known to be the predominant form of the enzyme in leaves of several higher plants (Schmidt and Jäger, 1992) and it constitutes 84% of the total ATP sulfurylase enzyme activity in leaves of spinach (Lunn et al., 1990). ATP sulfurylase is also present in the cytosol of leaves (Lunn et al., 1990) and in roots, where a single form of ATP sulfurylase is localized exclusively in proplastids (Brunold and Suter, 1989).

Since higher plants are known to contain multiple forms of ATP sulfurylase, we studied the occurrence of DNA fragments and mRNA transcripts that may encode these isoenzymes. Multiple DNA fragments were found that hybridize with the *APS1* cDNA (Fig. 5). RNA blot analysis showed that leaves contain three transcripts of 1.85, 1.60, and 1.20 kb, whereas roots contain a single transcript of 1.85 kb (Fig. 6). These results support the hypothesis that ATP sulfurylase is encoded by a gene family in *A. thaliana*. Recently, we have isolated two additional cDNAs encoding ATP sulfurylase from *A. thaliana* that are different in sequence from *APS1* (T. Leustek, unpublished data), lending further support to this

hypothesis. If this is correct, then the RNA blot analysis shown in Figure 6 indicates that some of these genes are expressed in an organ-specific manner. Because roots contain a single, proplastid form of ATP sulfurylase (Brunoldt and Suter, 1989), it is tempting to speculate that *APS1* may be encoded by the 1.85-kb transcript expressed in both leaves and roots (Fig. 6).

There are other interpretations of the results from DNA and RNA blot analysis. First, the multiple DNA fragments hybridizing with *APS1* could represent separate exons of a single gene. Also, the multiple RNA transcripts could result from a single gene with several different transcriptional start sites or a single transcript that is differentially processed. However, based on the correlation of our study with biochemical analyses indicating the existence of multiple ATP sulfurylase enzymes, we consider the alternative explanations of our data unlikely. Our continuing studies on ATP sulfurylase genes of *A. thaliana* will further elucidate the physiological role of these isoenzymes in the sulfate assimilation pathway.

ACKNOWLEDGMENTS

We wish to thank Dr. Irwin Segel for conducting the ATP sulfurylase enzyme assays and for helpful discussions. We also wish to thank Drs. Peter Day and Barbara Zilinskas for their comments on the manuscript.

Received November 26, 1993; accepted March 15, 1994.

The GenBank accession number of the sequence reported in this article is U05218.

LITERATURE CITED

Becker DM, Guarente L (1991) High-efficiency transformation of yeast by electroporation. Methods Enzymol **194:** 182–186

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brunold C, Suter M (1989) Localization of enzymes of assimilatory sulfate reduction in pea roots. Planta **179:** 228–234

Cherest H, Kerjan P, Surdin-Kerjan Y (1987) The *Saccharomyces cerevisiae MET3* gene: nucleotide sequence and relationship of the 5′-non-coding region to that of *MET25*. Mol Gen Genet **210:** 307–313

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162:** 156–159

Cline K, Werner-Washburne M, Lubben TH, Keegstra K (1985) Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. J Biol Chem **260:** 3691–3696

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–22

Elledge SJ, Mulligan JT, Ramer SW, Spottswood M, Davis RW (1991) λYES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc Natl Acad Sci USA **88:** 1731–1735

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett **261:** 455–458

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplast precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol **40:** 471–501

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions: a critical reexamination. Biochemistry **16:** 4743

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé JC, Dénarié J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulfated and acylated glucosamine oligosaccharide signal. Nature **344:** 781–784

Leyh TS, Vogt TF, Suo Y (1992) The DNA sequence of the sulfate activation locus from *Escherichia coli* K-12. J Biol Chem **267:** 10405–10410

Li J, Saidha T, Schiff JA (1991) Purification and properties of two forms of ATP sulfurylase from *Euglena*. Biochim Biophys Acta **1078:** 68–76

Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP sulfurylase and O-acetylserine(thio)lyase in spinach leaves. Plant Physiol **94:** 1345–1352

Renosto F, Martin RL, Borrel JL, Nelson DC, Segel IH (1991) ATP sulfurylase from trophosome tissue of *Riftia pachyptila* (hydrothermal vent tube worm). Arch Biochem Biophys **290:** 66–78

Rose MD, Winston F, Hieter P (1990) Methods in Yeast Genetics. A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 177–179

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Schmidt A, Jäger K (1992) Open questions about sulfur metabolism in plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 325–349

Schwedock J, Long SR (1990) ATP sulfurylase activity of the nodP and nodQ gene products of *Rhizobium meliloti*. Nature **348:** 644–647

Strathern JN, Higgins DR (1991) Recovery of plasmids from yeast into *Escherichia coli*: shuttle vectors. Methods Enzymol **194:** 319–328

Wada K, Wada Y, Ishibashi F, Gojobori T, Ikemura T (1992) Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res **20:** 2111–2118