

Soybean ATP sulfurylase, a homodimeric enzyme involved in sulfur assimilation, is abundantly expressed in roots and induced by cold treatment

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Received 26 January 2006, and in revised form 28 March 2006

Available online 8 May 2006

Abstract

Soybeans are a rich source of protein and a key feed ingredient in livestock production, but lack sufficient levels of cysteine and methionine to meet the nutritional demands of swine or poultry as feed components. Although engineering the sulfur assimilatory pathway could lead to increased sulfur-containing amino acid content, little is known about this pathway in legumes. Here, we describe the cloning and characterization of soybean ATP sulfurylase (ATPS), which acts as the metabolic entry point into the sulfur assimilation pathway. Analysis of the ATPS clone isolated from a soybean seedling cDNA library revealed an open-reading frame, encoding a 52 kDa polypeptide with an N-terminal chloroplast/plastid transit peptide, which was related to the enzymes from *Arabidopsis*, potato, human, and yeast. Soybean ATP sulfurylase was expressed in *Escherichia coli* and purified to apparent homogeneity. Based on gel-filtration chromatography, the enzyme functions as a 100 kDa homodimer. Analysis of genomic DNA by Southern blotting revealed that multiple genes encode ATP sulfurylase in soybean. Analysis of the transcript profiles retrieved from a soybean EST database indicated that ATP sulfurylase mRNA was most abundant in root tissue. Cold treatment induced mRNA accumulation and enhanced the specific activity of ATP sulfurylase in root tissue. Northern blot analysis indicated a decline in the ATP sulfurylase transcript levels during seed development. Likewise, ATP sulfurylase specific activity also declined in the later stages of seed development. Increasing the expression levels of this key enzyme during soybean seed development could lead to an increase in the availability of sulfur amino acids, thereby enhancing the nutritional value of the crop.

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Keywords: ATP sulfurylase; Soybean; Sulfur assimilation; Seed development

Sulfur is an essential nutrient for plant life with numerous biological functions [1]. Plants assimilate inorganic sulfur as sulfate from the soil or as sulfur dioxide and hydrogen sulfide gases from the atmosphere [2]. Since inorganic sulfate is non-reactive, chemical activation of the molecule occurs through a series of enzymatic reactions yielding sulfide [3]. Subsequent incorporation of sulfide into cysteine provides a

metabolic precursor for all cellular components containing reduced sulfur, including methionine, glutathione, iron-sulfur clusters, vitamin cofactors like biotin and thiamin, and multiple secondary metabolites [4]. Ultimately, the amount of cysteine and methionine in edible plants influences their nutritional and economic value because monogastric animals, including humans, cannot reduce sulfur, and depend on dietary sources of this nutrient.

ATP sulfurylase (ATP:sulfate adenylyl transferase; EC 2.7.7.4) is the metabolic entry point into the sulfur assimilation pathway. This enzyme catalyzes the

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formation of adenosine-5'-phosphosulfate (APS)¹ and inorganic pyrophosphate (PP_i) from sulfate and ATP. This reaction yields a high-energy phosphoric-sulfuric acid anhydride bond that drives the sulfur assimilatory pathway. ATP sulfurylase activity in plants, first demonstrated by Asahi [5], occurs in multiple species, including maize, spinach, *Arabidopsis thaliana* (thale cress), soybean, potato, and tobacco [3]. Activity of the enzyme was found to be highest in young leaves and the elongation zone of roots in maize [6]. In response to sulfur deficiency, ATP sulfurylase activity in plant tissues increases [7,8]. Genes encoding chloroplastic, mitochondrial, and cytosolic ATP sulfurylase isoforms have been cloned from *Arabidopsis*, *Brassica juncea* (Indian mustard), and *Solanum tuberosum* (potato) [9–15]. Since chloroplasts are the primary site of reductive sulfate assimilation, the chloroplast isoform has been studied in detail. The respective role of cytosolic and mitochondrial isoenzymes in sulfate assimilation remains to be elucidated. In *Arabidopsis* leaves, the activity of the chloroplast isoform of ATP sulfurylase declines with the leaf age, while the activity of the cytosolic form increases during the same period, indicating that the function of cytosolic ATP sulfurylase may be unrelated to sulfate reduction [16]. In spite of its importance in sulfur assimilation, very little is known about this enzyme from legumes.

Although soybean (*Glycine max* L. Merr.) is a major source of vegetable protein worldwide in human and animal food with seeds that are ~40% protein on a dry weight basis, cysteine and methionine constitute less than 3.5% of total amino acid content [17,18]. Because this level does not fulfill the dietary requirements of animals, soybeans are considered low in sulfur-containing amino acids. This inadequacy has promoted efforts to increase the sulfur amino acid content of soybeans. In cases of engineered increases in soybean sulfur amino acid content, there is a concomitant decrease in endogenous sulfur-rich proteins [19,20]. This suggests that the supply of cysteine and methionine in developing seeds fails to meet the demand created by the introduction of sulfur-rich proteins.

Since manipulation of key enzymes involved in the sulfur assimilatory pathway could lead to an increase in the sulfur amino acid content of soybean [21], we initiated a study to characterize ATP sulfurylase in soybean. Here, we report the molecular cloning of ATP sulfurylase from a soybean cDNA library, the biochemical examination of heterologously expressed and purified protein, and the spatial and temporal characterization of mRNA and protein expression in developing soybean seeds and under normal and cold stress conditions.

Materials and methods

cDNA cloning of ATP sulfurylase from soybean

A 700 bp soybean expression sequence tag (EST), AW277946, showing similarity to ATP sulfurylases from other plant species was obtained from Genome systems (St. Louis, MO). The EST DNA was cloned in the *NotI* and *SalI* sites of the pSPORT vector (Invitrogen) and its nucleotide sequence determined (University of Missouri-Columbia DNA core facility). The vector was digested with the same enzymes and the gene insert gel-purified. One microgram of denatured, [³²P]-radiolabeled EST DNA was used to probe a soybean cv. Williams 82 seedling library (obtained from J. Polacco, University of Missouri). Three positive clones were obtained after performing plaque lifts. The phagemid containing the desired insert was excised from the ZAP vector (Stratagene) using the Rapid Excision Kit (Stratagene). Sequencing of the three positive clones revealed that they were identical. The sequence of the cDNA clone has been deposited in GenBank (AF452454).

Transcript profiles of ATP sulfurylase using soybean EST database

The nucleotide sequence of ATP sulfurylase was utilized to search the soybean expressed sequence tags (ESTs) database (http://www.tigr.org/tigrscripts/tgi/T_index.cgi?species=soybean) by BLASTn analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>). ESTs showing significant homology to ATP sulfurylase were separated according to the tissue type from which they were isolated and the frequency of their occurrence tabulated.

Isolation of soybean genomic DNA and Southern blot analysis

Total genomic DNA was isolated from leaf tissue of soybean cv. Williams 82 using the CTAB method [22]. Eight micrograms of genomic DNA were digested with different restriction enzymes and size-fractionated by electrophoresis on a 0.8% agarose gel. Digested DNA samples were blotted to nylon membranes by capillary transfer. The 1.4 kb cDNA insert of ATP sulfurylase was released from the vector by *EcoRI* digestion and used as a probe after labeling with [³²P]-dCTP using the Ladderman kit (Takara Bio Inc.). Pre-hybridization (10 h, 65 °C) and subsequent hybridization (12 h, 65 °C) reactions were carried out in 6 × SSPE buffer (1 × SSPE is 0.1 M NaCl, 0.01 NaH₂PO₄, and 0.001 M EDTA), 5 × Denhardt's solution, 0.5% SDS, and 50 μg ml⁻¹ sheared and denatured salmon sperm DNA. After hybridization, the membrane was washed three times in 2 × SSPE and 0.5% SDS at room temperature for 10 min, two times in 0.1 × SSPE and 0.1% SDS at 65 °C for 30 min, and then exposed to X-ray films at -80 °C.

¹ Abbreviations used: APS, adenosine-5'-phosphosulfate; ATP sulfurylase, ATP:sulfate adenylyl transferase; EST, expressed sequence tag; PP_i, inorganic pyrophosphate.

Generation of expression vectors

An expression construct for N-terminally hexahistidine-tagged soybean ATP sulfurylase lacking the chloroplast transit peptide (GmATPS Δ 48) was generated. Primers for the amplification of GmATPS Δ 48 were 5'-dTTTTGCTAGCGCGCTGATCGAACAGATGG-3' (forward primer, the *NheI* site is underlined) and 5'-dTTTTGATCCTTAAGCTGGAACAGCTTCCTGCAC-3' (reverse primer, the *Bam*HI site is underlined and the stop codon is in bold). PCR products were amplified from plasmid templates using Pfx Platinum polymerase (Invitrogen), digested with *NheI* and *Bam*HI, and ligated into *NheI/Bam*HI-digested pET28a vector. Automated nucleotide sequencing of the pET28a-GmATPS Δ 48 vector confirmed the fidelity of the PCR product (Washington University Sequencing Facility, St. Louis, MO).

For use in the ATP sulfurylase assays, an expression construct of the *Arabidopsis thaliana* APS kinase (GenBank U05238) with a truncated N-terminus was generated by PCR using an *Arabidopsis* cDNA library as template. Primers for the amplification of AtAPSK Δ 77 were 5'-dTTATGGCTAGCAACTCGACAAATATAAAGTGGC-3' (forward primer, the *NheI* site is underlined) and 5'-dTTTGAATTCTGTTATGCTTGAAGATAACCCCTTGTTATC-3' (reverse primer, the *Eco*RI site is underlined, and the stop codon is in bold). A 700 bp PCR product was amplified from the flower cDNA library using Pfx Platinum polymerase (Invitrogen), digested with *NheI* and *Eco*RI, and ligated into *NheI/Eco*RI-digested pET28a. Automated nucleotide sequencing of the pET28a-AtAPSK Δ 77 vector was performed as above.

Expression in *E. coli* and protein purification

Expression constructs were transformed into *Escherichia coli* Rosetta (DE3) cells. Transformed *E. coli* were grown at 37 °C in Terrific broth containing kanamycin (50 μ g mL⁻¹) and chloramphenicol (34 μ g mL⁻¹) until $A_{600\text{nm}} = 0.8$ –1.0. After induction with 1 mM isopropyl 1-thio- β -D-galactopyranoside, the cultures were grown at 20 °C for 4–6 h. Cells were pelleted by centrifugation and resuspended in lysis buffer (50 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol, and 1% (v/v) Tween 20). After sonication and centrifugation, the supernatant was passed over a Ni²⁺-NTA column previously equilibrated with lysis buffer. Unbound protein was washed out using wash buffer (lysis buffer without Tween 20). His-tagged protein was eluted with elution buffer (wash buffer containing 250 mM imidazole), and then loaded onto a Superdex-200 FPLC column equilibrated with 25 mM Hepes (pH 7.5) and 150 mM NaCl. Fractions containing protein were eluted and stored at -80 °C. Antibodies to recombinant soybean ATP sulfurylase were raised in a white New Zealand female rabbit at the University of Missouri as described previously [23].

Enzyme assays

Initial velocities were measured using a Beckman DU800 UV/Vis spectrophotometer by observing the rate of change in absorbance of pyridine nucleotide at 340 nm ($\epsilon = 6270 \text{ M}^{-1} \text{ cm}^{-1}$) in 0.5 mL systems at 25 °C. The specific activity of APS kinase (1.5 μ mol min⁻¹ mg⁻¹) was determined spectrophotometrically [24]. ATP sulfurylase activity was monitored for the forward (APS synthesis) and reverse (ATP synthesis) reactions using assay conditions described earlier [8,11]. For APS synthesis, assays contained 50 mM Tris (pH 8.0), 15 mM MgCl₂, 100 mM NaCl, 0.2 mM NADH, 0.05 U of APS kinase, 20 U of pyruvate kinase, and 30 U of lactate dehydrogenase. Kinetic constants were determined using 20 mM Na₂SO₄ with varied ATP (0–10 mM) or using 10 mM ATP with varied Na₂SO₄ (0–10 mM). For ATP synthesis, assays contained 50 mM Tris (pH 8.0), 5 mM MgCl₂, 1 mM NADP⁺, 1 mM glucose, 2 U of hexokinase, and 1 U of glucose-6-phosphate dehydrogenase. Kinetic constants were determined using 1 mM sodium pyrophosphate with varied APS (0–100 μ M) or using 50 μ M APS with varied sodium pyrophosphate (0–1 mM). All reactions were initiated by addition of enzyme and were corrected for non-enzymatic rates. Calculation of k_{cat} and K_{m} values used Kaleidagraph (Synergy Software) to fit the untransformed data to $v = k_{\text{cat}}[S]/(K_{\text{m}} + [S])$.

Seeds and plant tissue

Soybean seeds from the cultivar Williams 82 were sterilized and germinated in the dark for 3 days on 1% agar (v/v) plates at 30 °C. Seedlings were transferred to pots containing perlite and grown in the greenhouse. Each pot was inserted into a larger pot containing nutrient solution (2 mM CaCl₂, 0.5 mM MgSO₄, 0.63 mM K₂SO₄, 0.5 mM K₂HPO₄, 0.25 mM NH₄NO₃, 0.025 mM FeC₆H₅O₇, 2.3 μ M H₃BO₃, 0.9 μ M MnSO₄, 0.6 μ M ZnSO₄, 0.1 μ M NaMoO₄, 0.11 μ M NiCl₂, 0.01 μ M CoCl₂, 0.15 μ M CuSO₄, pH 5–6). Growing plants had access to the nutrient solution through a piece of cheesecloth immersed half in the nutrient solution and half in the perlite. Plants were grown at 22–26 °C under natural light.

For analysis of ATP sulfurylase expression at the different seed developmental stages, seeds were harvested from field-grown soybean plants at weeklong intervals, starting 22 days after anthesis, frozen in liquid nitrogen, and sorted into different development stages. Developing soybean seeds weighing between 20 to 30, 80 to 100, 200 to 220, and 300 to 350 mg represented stages 1 through 4, respectively. For all stages, seeds contained the embryo and seed coat but were separated from the pod walls.

For cold treatment studies, pots containing one-week old soybean seedlings were transferred to a refrigerated chamber maintained at 10 °C. After four days of cold treatment, the plants were removed from the refrigerated chamber and allowed to recover at 26 °C. Tissue samples collected at different time points during the cold and

recovery phases were frozen in liquid nitrogen and stored at -80°C .

Northern blot analysis

For northern hybridization, total RNA from seeds, leaves, stem, and roots was isolated using Trizol reagent (Invitrogen). Equal amounts of RNA (10 μg) were electrophoresed in a 1.2% formaldehyde gel, transferred to nylon membranes and immobilized by UV cross-linking. Probe isolation and labeling were as described for Southern hybridization. Prehybridization (10 h, 65°C) and hybridization (30 h, 55°C) were carried out in 7% SDS (w/v), 0.191 M Na_2HPO_4 , 0.058 M NaH_2PO_4 , 1% (w/v) BSA, and 100 $\mu\text{g ml}^{-1}$ salmon sperm DNA. After hybridization, the membrane was washed three times for 10 min at room temperature in $2 \times \text{SSC}$ and 0.5% SDS, and then exposed to X-ray film at -80°C .

Western blot analysis

Total protein extracts from soybean roots were fractionated by SDS-PAGE. Proteins from the gels were transferred to a nitrocellulose membrane in electrode buffer (25 mM Tris (pH 8.3) 192 mM glycine, 20% methanol). The membrane was washed with TBS (80 mM Tris, 200 mM NaCl, pH 7.5) and incubated with TBS containing 5% (w/v) non-fat milk. Following this, membranes were incubated in 1:5000 dilution of antibodies raised against recombinant ATP sulfurylase. Reactive proteins were identified using a 1:3000 dilution of affinity-purified goat anti-rabbit IgG-horseradish peroxidase conjugate antibody (Bio-Rad) with color detection by enhanced chemiluminescent substrate according to the manufacturer's protocol (Pierce).

Results

Isolation of a cDNA encoding ATP sulfurylase (ATPS) from soybean

Three identical clones for ATP sulfurylase were obtained by screening a soybean cDNA library using a 0.7 kb soybean ATP sulfurylase EST clone insert as a probe. The full-length cDNA sequence of this clone was 1661 bp long with a complete open reading frame of 1386 bp. The nucleotide sequence showed similarity with known ATP sulfurylase sequences. The cDNA encoded a 462 amino acid protein with a predicted molecular weight of 51.8 kDa and a $pI=7.3$. Analysis of the protein sequence using PSORT, PLOC, and ChloroP indicated that the protein has a chloroplast/plastid transit peptide sequence in its N-terminal region [25–27]. Processing of the transit peptide will release a mature protein having an apparent molecular weight of 46.4 kDa and a pI of 6.26. The protein shares 77% (461 residues overlap) 83% (417 residues overlap), 62% (390 residues overlap) and 29% (354 residues overlap) amino acid sequence identity with the enzymes from *Arabidopsis*

thaliana, *Solanum tuberosum* (potato), *Homo sapiens* (human), and *Saccharomyces cerevisiae* (yeast), respectively (Fig. 1). The plant enzymes only contain the ATP sulfurylase domain, unlike the human and yeast enzymes, which include an APS kinase domain located at the N- or C-terminal regions, respectively [28,29].

Expression, purification, and kinetic analysis of soybean ATP sulfurylase (ATPS)

To characterize the enzymatic activity of the protein encoded by the cDNA, a bacterial expression construct was generated and used to transform *E. coli* cells. When the full-length cDNA, which included the chloroplast/plastid localization sequence, was used to express soybean ATP sulfurylase with an N-terminal His-tag, the protein was insoluble. Based on comparison to the N-terminal sequence of the mature ATP sulfurylase isoform purified from spinach chloroplasts [8], a truncated version of soybean ATP sulfurylase lacking the first 48 amino acids was generated for protein expression and purification. Removal of the putative localization sequence improved the yield of the N-terminally hexahistidine-tagged protein in *E. coli*. The protein was purified by nickel affinity and size-exclusion chromatographies (Fig. 2A). The purified protein migrated as a 48 kDa species by SDS-PAGE, which corresponds with the predicted molecular weight of the His-tagged GmATPS Δ 48 protein, and eluted from the size-exclusion chromatography column as sharp peak corresponding to a 100 kDa molecule, indicating that the enzyme is active as a homodimer (Fig. 2B).

The specific activities of the purified protein for APS and ATP synthesis were 2.2 and 26.7 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$, respectively. These specific activities are comparable to those reported for the *Arabidopsis* enzyme, which were 2.9 and 48.7 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ for APS and ATP synthesis, respectively [11]. The steady-state kinetic parameters of soybean ATP sulfurylase for the forward reaction (APS synthesis) and the reverse reaction (ATP synthesis) are summarized in Table 1. In the forward reaction, the determined K_m values of the soybean enzyme fall within the range of reported K_m values of the ATP sulfurylases from spinach, fungi, and humans, which are 46–240 μM for ATP and 160–870 μM for sulfate [8,30,31]. Likewise, in the reverse reaction, the K_m values of the soybean ATP sulfurylase are similar to those observed with the spinach, fungal, and human enzymes for PP_i (6.5–35 μM) and APS (0.4–30 μM) [8,30,31]. In addition, the soybean ATP sulfurylase, like the *Arabidopsis*, spinach, fungal, and human enzymes [8,11,30,31], favors the reverse reaction.

ATP sulfurylase activity in soybean tissues

Measurement of ATP sulfurylase activity from leaves, stems, roots, and seeds (developmental stage 5) indicated similar levels of specific activity in seeds and root tissue

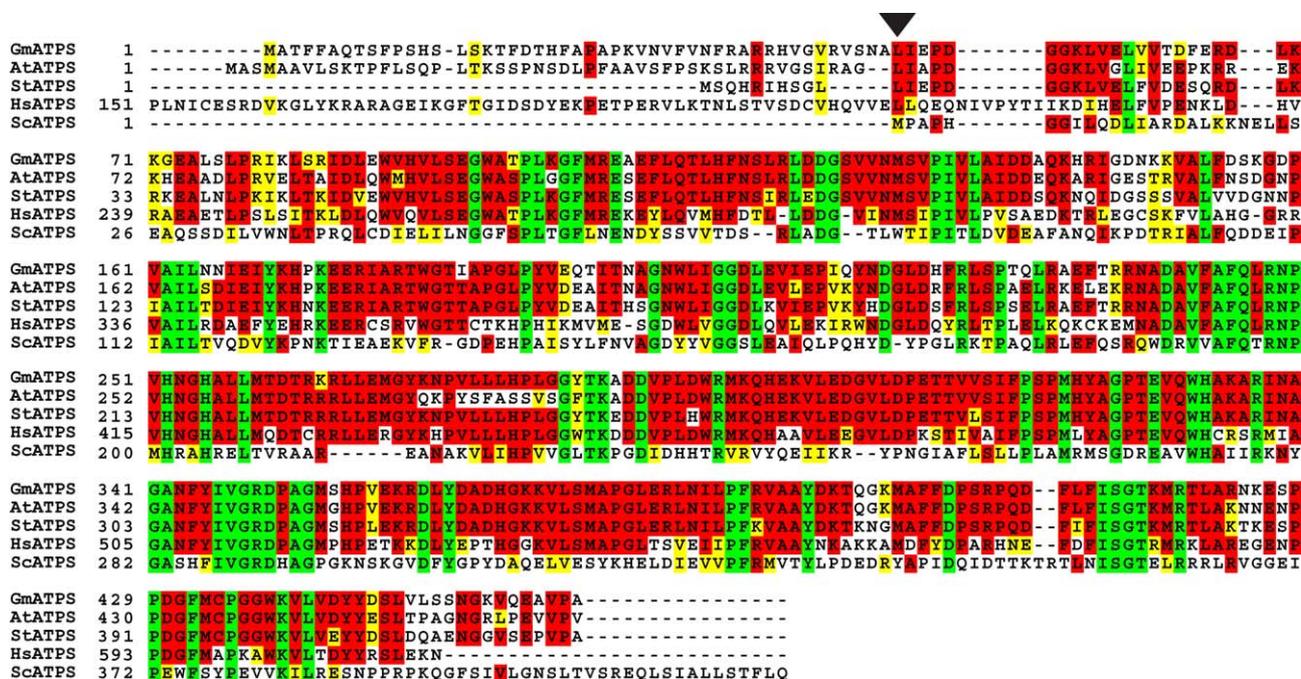


Fig. 1. Multiple sequence alignment of ATP sulfurylases from different organisms. The sequences of the ATP sulfurylases from soybean (*Glycine max*, GmATPS, and AF452454), *Arabidopsis thaliana* (AtATPS, GenBank: AAA21570.1), potato (*Solanum tuberosum*, StATPS, and EMB:CAA52953.1), human (HsATPS, GenBank: AAC64583.1), and *Saccharomyces cerevisiae* (ScATPS, EMB:CAA42726.1) are shown. Numbers indicate the residue number of the amino acid at the beginning of the line. Dashes indicate gaps to facilitate best alignment. Green shading indicates conserved residues; red shading indicates residues showing more than 60% identity; yellow shading indicates those residues showing more than 60% similarity. The black arrow indicates the first amino acid of soybean ATP sulfurylase used in the pET28a-GmATPSΔ48 expression construct. Residues 1–150 HsATPS and residues 422–521 of ScATPS, which correspond to the APS kinase domains of each protein, are not shown.

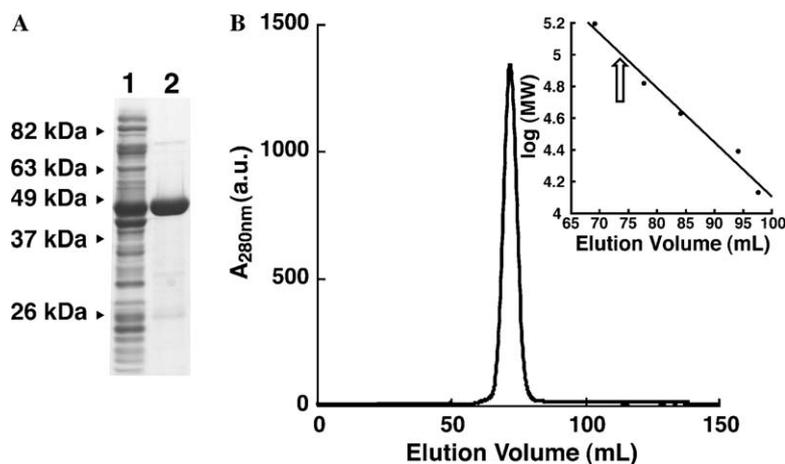


Fig. 2. Expression and purification of soybean ATP sulfurylase. (A) SDS-PAGE analysis of protein purification. Samples were stained for total protein using Coomassie Blue. Arrows correspond to molecular mass markers as indicated. Lane 1, 75 µg of sonicate. Lane 2, 5 µg of purified protein. (B) Size-exclusion chromatography of soybean ATP sulfurylase. Purified soybean ATP sulfurylase was chromatographed on a Superdex-200 16/60 FPLC column in 25 mM HEPES (pH 7.5) and 150 mM NaCl. Inset: molecular weight calibration of the size-exclusion column. The following standards were used to calibrate the column under the same buffer conditions: ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), albumin (67.0 kDa), and aldolase (158 kDa). The elution volume of soybean ATP sulfurylase is indicated by the arrow.

(Fig. 3A). The specific activities of ATP sulfurylase in stem and leaf tissue were comparable but lower than activities in seeds and roots. Young leaves exhibited higher enzyme activity than mature leaves (Fig. 3A). We also examined the ATP sulfurylase activity in soybean seeds from different developmental stages. Seeds

harvested between stages 1 to 4 had significantly higher levels of ATP sulfurylase activity, with the highest activity detected in seeds at developmental stage 2 (Fig. 3B). The specific activity of ATP sulfurylase in young seeds was several-fold greater than any other tissue examined (Fig. 3B).

Table 1
Steady-state kinetic parameters of soybean ATP sulfurylase

	Forward reaction		Reverse reaction	
	ATP	SO ₄ ²⁻	MgPPi	APS
k_{cat} (s ⁻¹)	1.49 ± 0.04	2.11 ± 0.10	19.1 ± 0.5	24.5 ± 1.3
K_{m} (μM)	195 ± 22	364 ± 66	24.4 ± 2.5	14.6 ± 2.1
$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	7,640	5,800	783,000	1,680,000

Reactions were performed spectrophotometrically as described in the Materials and methods. All k_{cat} and K_{m} values are expressed as a mean ± SE for an $n = 3$.

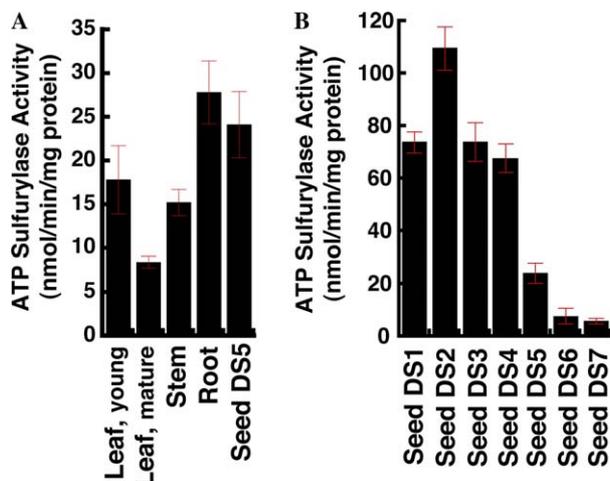


Fig. 3. ATP sulfurylase activity in different tissues. Activity of ATP sulfurylase was measured in protein extracts from roots, stems, leaves and seeds (A). ATP sulfurylase activity of soybean seed samples collected at different developmental stages (B). Bars represent the standard error of the mean ($n = 3$).

Genomic organization of soybean ATP sulfurylase

Southern blot analysis was performed using soybean genomic DNA to examine the gene(s) encoding ATP sulfurylase (Fig. 4). DNA was digested using *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, or *Xba*I, transferred to a nylon membrane, and probed with [³²P]-labeled ATP sulfurylase cDNA. The soybean ATP sulfurylase cDNA isolated contains no internal sites for any of the restriction enzymes used. Two to four genomic fragments hybridized to the full-length cDNA probe in each digested sample, suggesting that a multigene family in soybean encodes different isoforms of ATP sulfurylase.

Expression profile of ATP sulfurylase in soybean

To determine the distribution of ATP sulfurylase in different tissues, we examined the soybean UniGene sequences which align with this protein. The nucleotide sequence of soybean ATP sulfurylase (AF452454) was used in BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST/>) searches. A total of 123 ESTs (UniGene Gma 2313) representing cDNA libraries constructed with mRNA isolated

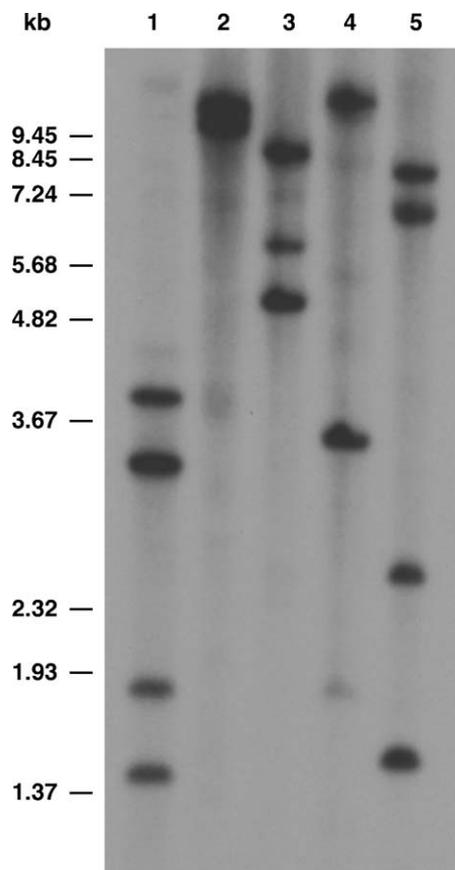


Fig. 4. Southern blot analysis of soybean genomic DNA. Eight μg of soybean genomic DNA was restricted with *Dra*I (lane 1), *Eco*RI (lane 2), *Eco*RV (lane 3), *Hind*III (lane 4), and *Xba*I (lane 5) and resolved on a 0.8% agarose gel. The gel was blotted to nitrocellulose membrane followed by hybridization with [³²P]-labeled soybean ATP sulfurylase cDNA. The positions and sizes of the lambda-*Hind*III DNA marker are indicated.

from different tissues and some subjected to different treatments showed significant homology to soybean ATP sulfurylase cDNA clone. The average length of the ESTs was approximately 400 bp and a majority of the ESTs lacked the amino terminal portion of the protein. Sequences matching ATP sulfurylase were found in various tissue types, but occurred most frequently in the roots. We conducted a similar analysis using the TIGR Soybean (*Glycine max*) Gene Index (<http://tigrblast.tigr.org/tgi/data/tblastn-soybean>). This analysis identified four EST contigs (TC204560, TC208153, TC218491, and TC213685) showing sequence similarity to ATP sulfurylase. The ATP sulfurylase identified in this study belongs to the TC204560 contig and includes 88 ESTs. Even though ATP sulfurylase ESTs are distributed in a wide variety of tissue types, it is expressed most abundantly in the root tissue followed by stem and seedlings (data not shown). There were 8 ESTs representing each of the TC208153 and TC218491 contigs and 2 ESTs in the TC213685 contig which presumably represent members of different ATP sulfurylase genes. Similarly, the occurrence of multiple copies of ATP sulfurylase genes has been reported in other plant species [9,10,13,16].

Spatial and temporal expression of ATP sulfurylase in soybean

To determine the expression of ATP sulfurylase in different tissues of the soybean plant, total RNA was extracted from roots, leaves, and seeds at mid-developmental stages. Northern blot analysis revealed that the ATPS probe hybridized to a 1.4 kb RNA transcript in the root and leaf tissues although the intensity of hybridization signal varied significantly depending on the tissue used (Fig. 5A). For comparison of ATP sulfurylase mRNA expression during seed development, we analyzed seeds from different developmental stages using Northern blots (Fig. 5B). The mRNA transcript levels, which were at their highest levels in young seeds (DS1, DS2), declined with seed development (DS3, DS4).

Cold treatment induces the expression of ATP sulfurylase in soybean

Previous studies have established the involvement of glutathione in the protection of plants from low temperature [32]. Because the sulfur assimilatory pathway is involved in the synthesis of glutathione, we suspected that cold-treatment would influence the expression of ATP sulfurylase. To test this possibility, soybean seedlings were subjected to cold stress and the levels of ATP sulfurylase mRNA in stem and root tissues were examined by Northern blot analysis (Fig. 6). Overall, cold-treatment induced expression of ATP sulfurylase with increased transcription observed after 8 h. In stem tissue, the highest mRNA levels were observed at 48–72 h (Fig. 6A). In roots, increased gene expression occurred between 12 and 72 h (Fig. 6B). ATP sulfurylase transcript levels in both stems and roots declined rapidly within 8 h of the beginning of the recovery period. We also monitored ATP sulfurylase levels and enzyme activity in root tissue to determine whether they were altered as a consequence of cold treatment. Western blot analysis revealed that ATP sulfurylase levels increased during cold treatment and decreased when the plants were

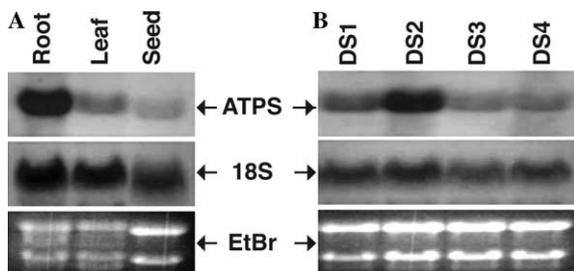


Fig. 5. Expression of ATP sulfurylase in different tissues. Total RNA was extracted from roots, leaves, and seeds, resolved on a 1.2% formaldehyde gel, and probed with [³²P]-labeled ATP sulfurylase cDNA clone (A). Expression of ATP sulfurylase in developing soybean seeds, which were grouped into developmental stages based on size is shown in (B). Hybridization with a soybean 18S rRNA probe was employed as a control for equal loading. The bottom lane in (A and B) visualizes the ethidium bromide-stained gel revealing uniform loading of RNA samples.

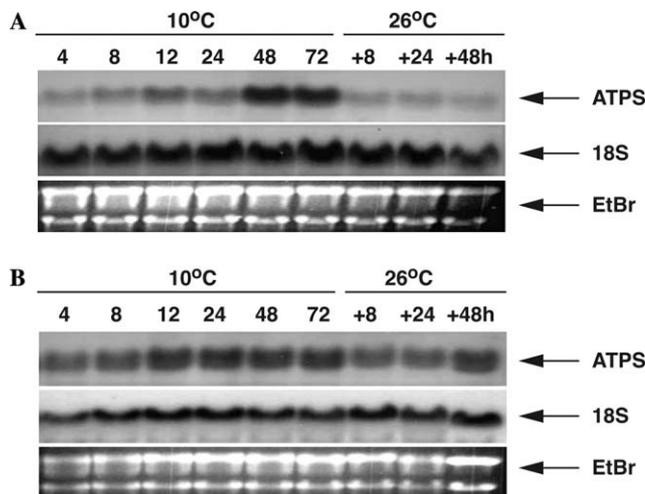


Fig. 6. Induction of ATP sulfurylase expression by cold treatment. Total RNA extracted from stem (A) and roots (B) of plants exposed to cold treatment was resolved on a 1.2% formaldehyde gel and probed with [³²P]-labeled ATP sulfurylase cDNA clone. The numbers at the top of the figure represent the time-period (in hours) at which the samples were harvested during the cold treatment or the recovery phase. Hybridization with a soybean 18S rRNA probe was employed as a control for equal loading. The bottom lane in (A and B) visualizes the ethidium bromide-stained gel revealing uniform loading of RNA samples.

returned to a normal growth temperature (Fig. 7). The effect of cold treatment on ATP sulfurylase was clearly seen on enzyme activity. When the plants were subjected to cold

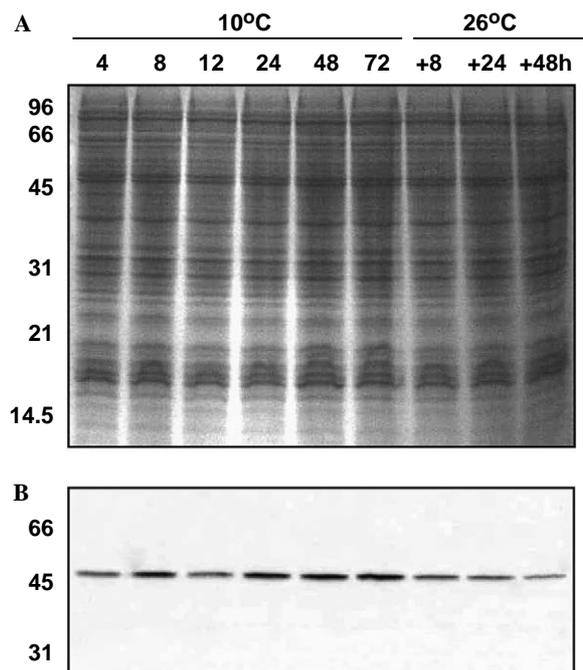


Fig. 7. Immunoblot analysis of ATP sulfurylase in soybean roots. Total root proteins collected at different time points during the cold and recovery phases were resolved on a 12.5% SDS-PAGE and were stained with Coomassie brilliant blue (A) or electrophoretically transferred to a nitrocellulose membrane and probed with antibodies raised against soybean ATP sulfurylase (B). Molecular mass marker sizes (in kDa) are shown to the left of the figure.

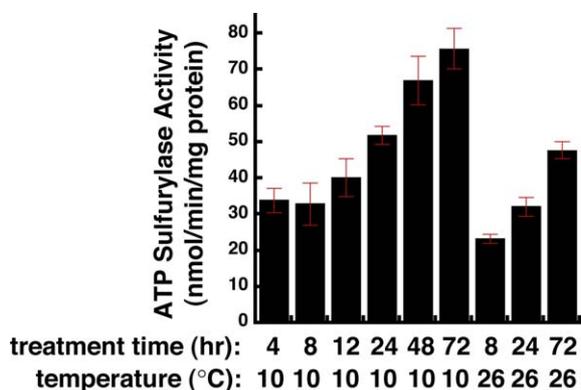


Fig. 8. ATP sulfurylase activity in cold treated soybean roots. ATP sulfurylase activity was measured in protein extracts from root samples collected at different time points during the cold and recovery phases. Bars represent the standard error of the mean ($n = 3$).

treatment ATP sulfurylase activity gradually increased. After 72 h at 10°C, ATP sulfurylase activity was 2.2-fold greater than that observed in roots at the beginning of the treatment. Within 8 h after returning to the 26°C, there was 3.2-fold decrease in ATP sulfurylase activity (Fig. 8).

Discussion

Sulfate activation and its reduction to sulfide are key steps for providing plants with cysteine, methionine, glutathione, iron-sulfur clusters, vitamin cofactors like biotin and thiamin, and multiple secondary metabolites. Because many animals, including humans, cannot reduce sulfur and depend on dietary sources for this nutrient, cysteine and methionine levels in crops influences their nutritional and economic value. In soybean, efforts to engineer increases in cysteine and methionine content results in a decrease in endogenous sulfur-rich proteins [19], suggesting that the sulfur supply in developing seeds is limiting. Although manipulation of key enzymes involved in the sulfur assimilatory pathway could lead to increased cysteine and methionine content in soybeans [21], little is known about this pathway in legumes.

Biochemical characterization of soybean ATP sulfurylase demonstrates that the steady-state kinetic properties of this enzyme (Table 1) are similar to those determined for the proteins isolated from *Arabidopsis*, spinach, fungi, and humans [8,11,30,31]. Contrary to earlier reports, which suggest that ATP sulfurylase from spinach and *Arabidopsis* are homotetramers [8,11], the soybean enzyme migrated as a homodimer using size-exclusion chromatography (Fig. 2). The earlier purifications used gel filtration columns with a broader molecular weight resolution than the column used in this study, which may have resulted in an overestimation of native weight. A homodimeric structure for the plant enzymes is also consistent with the architecture of the ATP sulfurylases from humans and a marine bacterium [33,34].

Comparison of soybean ATP sulfurylase with homologs from different plant species reveals high sequence similarity

[9–13,15]. Sequence alignment with the enzymes from other organisms, including yeast and human (Fig. 1), shows significantly lower amino acid identity. In bacteria, mammals, yeast, fungi, and plants, the domain structure of ATP sulfurylases varies. ATP sulfurylases from prokaryotes, like *E. coli*, are heterodimeric proteins in which a GTPase subunit activates the catalytic subunit by allosteric interactions [35]. In yeast and fungi, the enzyme is a homohexamer with each monomer containing an N-terminal ATP sulfurylase domain and a C-terminal APS kinase domain, which functions as an allosteric regulatory site [29,36]. Interestingly, the human enzyme functions as a homodimeric protein with the ATP sulfurylase/APS kinase domain order reversed compared to the yeast enzyme [33]. The oligomeric structure of the ATP sulfurylases from soybean and other plants may resemble the homodimeric structure of the enzyme from a symbiotic bacterium from a marine tubeworm [34], which also lacks the APS kinase domain. Currently, no structural information is available for an ATP sulfurylase from any plant species.

Higher plants contain multiple isoforms of ATP sulfurylase encoded by gene families, as shown in *Arabidopsis*, *Brassica napus*, and potato [9,10,13]. Our results indicate a gene family in soybean also encodes the enzyme because multiple DNA fragments hybridize to the ATP sulfurylase cDNA during Southern blot analysis (Fig. 4). The presence of a gene family suggests that different isoforms will have varied tissue and organelle distribution patterns. This observation is supported by the distribution of ATP sulfurylase ESTs in the database. It is interesting to note that the expression of ATP sulfurylase as revealed by the EST database is most abundant in soybean root tissues. Our Northern blot analysis corroborates that the mRNA encoding ATP sulfurylase is most abundant in the root tissue. Sequence analysis of the soybean ATP sulfurylase clone described here indicates the presence of a chloroplast/plastid localization signal at the N-terminal of the protein similar to that described in other plant species [9,11–13]. In addition, our results suggest that the soybean ATP sulfurylase characterized here is likely a proplastid-localized isoform, since higher expression was observed in roots than leaves (Fig. 5). Typically, analysis of ATP sulfurylase activity and gene expression in various plant tissues shows that the gene is highly expressed in leaves [10,13,37]. Studies in spinach, potato, and *Arabidopsis* also describe the presence of ATP sulfurylase in tissues other than leaves, like the proplastids of roots [8,10,15,38]. In roots, ATP sulfurylase activity increases in the elongation zone, where growing cells require greater nutrient levels [6]. Moreover, roots are a priority for sulfur utilization and act as sulfur sinks for long-term storage. For example, in *Brassica napus*, ATP sulfurylase expression increases in roots and cotyledons during sulfur starvation [13].

Induction or de-repression of ATP sulfurylase activity and expression levels occurs in tobacco cells, *Arabidopsis*, *B. napus*, and corn under sulfur starvation [13,39–43]. Our results demonstrate that cold treatment also enhances

expression of ATP sulfurylase (Fig. 6). An increase in cysteine synthesis occurs when plants are subjected to stress conditions. Glutathione, which requires cysteine for its synthesis, has been shown to be involved in protection of plants from low temperature [32]. Since ATP sulfurylase is the key rate-limiting enzyme controlling the sulfur assimilatory pathway [44], it is reasonable to expect that cold treatment would enhance the expression of this enzyme. The role of sulfur metabolites in cold response is further strengthened by earlier reports in which increased APS reductase activity was detected in maize stressed by cold temperatures [32,45].

Since seeds are prime sites for cysteine and methionine synthesis, seed development places a significant demand on sulfur allocation [46,47]. Transcript levels of ATP sulfurylase and total enzyme activity steadily decrease with soybean seed age (Figs. 3B and 5), suggesting an increased need for the sulfur assimilation pathway during early developmental stages. This parallels earlier observations that ATP sulfurylase activity and transcript levels decrease with leaf age [16,48,49]. ATP sulfurylase expression at different seed development stages also corresponds with previously reported expression patterns of the cysteine biosynthesis enzymes, i.e., *O*-acetylserine sulfhydrylase and serine acetyltransferase [50,51], which is consistent given the metabolic connection between sulfur assimilation and cysteine biosynthesis.

Accumulation of seed storage proteins in soybean is regulated by sulfur and nitrogen supply. Under excess nitrogen levels, β -conglycinin production is enhanced and glycinin synthesis is inhibited [18,52]. Reversing the decline in ATP sulfurylase, *O*-acetylserine sulfurylase, and serine acetyltransferase level may facilitate the synthesis of sulfur rich amino acids for subsequent incorporation into soybean seed storage proteins [50,51]. Because the bulk of seed storage proteins are synthesized during the mid-stage of seed development, it would be desirable to have a sufficient supply of sulfur and cysteine in this period.

Acknowledgments

The authors wish to thank Dr. Ahmed Mahmoud for Southern blot analysis, Dr. Joe Polacco (University of Missouri-Columbia) for providing the soybean seedling cDNA library, and Dr. Edgar Cahoon (Danforth Plant Science Center) for providing the *Arabidopsis thaliana* cDNA library. This work was funded in part by an U.S. Department of Agriculture grant (NRI-2005-02518) to J.M.J. and an Illinois-Missouri Biotechnology Alliance grant to H.B.K. and J.M.J. Product names are necessary to report factually on available data; however, the University of Missouri, Danforth Plant Science Center and the USDA neither guarantees nor warrants the standard of product, and the use of the name by the University of Missouri and the USDA implies no approval of the product to the exclusion of others that may be suitable.

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