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The role of 5′-adenylylsulfate reductase in the sulfur assimilation pathway of soybean: Molecular cloning, kinetic characterization, and gene expression

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Abstract

Soybean seeds are a major source of protein, but contain low levels of sulfur-containing amino acids. With the objective of studying the sulfur assimilation pathway of soybean, a full-length cDNA clone for 5′-adenylylsulfate reductase (APS reductase) was isolated and characterized. The cDNA clone contained an open reading frame of 1414 bp encoding a 52 kDa protein with a N-terminal chloroplast/plastid transit peptide. Southern blot analysis of genomic DNA indicated that the APS reductase in soybean is encoded by a small multigene family. Biochemical characterization of the heterologously expressed and purified protein shows that the clone encoded a functional APS reductase. Although expressed in tissues throughout the plant, these analyses established an abundant expression of the gene and activity of the encoded protein in the early developmental stages of soybean seed, which declined with seed maturity. Sulfur and phosphorus deprivation increased this expression level, while nitrogen starvation repressed APS reductase mRNA transcript and protein levels. Cold-treatment increased expression and the total activity of APS reductase in root tissues. This study provides insight into the sulfur assimilation pathway of this nutritionally important legume.

Keywords: *Glycine max*; *Leguminosae*; Legume; Soybean; Cloning; Kinetic analysis; Sulfur; 5′-Adenylyl reductase; APS reductase; Seed development; Nutrient stress; Cold stress

1. Introduction

Along with nitrogen and phosphorus, sulfur is an essential mineral nutrient required for plant growth and development (Hell, 1997). Sulfur is primarily available in the environment as sulfate (SO_4^{2-}), which following uptake into plants is reduced to sulfide through a series of enzymatic reactions (Schmidt and Jäger, 1992; Leustek and Saito, 1997; Bick and Leustek, 1998; Leustek et al., 2000; Hopkins et al., 2004). The first metabolic transformation in the sulfur assimilation pathway is catalyzed by ATP sulfurylase, which uses ATP and sulfate to generate

Abbreviations: APS, 5'-adenylylsulfate or adenosine 5'-phosphosulfate; APS reductase, 5'-adenylylsulfate reductase, EC 1.8.4.9 (also named adenosine 5'-phosphosulfate reductase); BSA, bovine serum albumin; DS, developmental stage; DTT, D/L-dithiothreitol; EST, expressed sequence tag; NTA, nitriloacetic acid; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; RT-PCR, reverse-transcript PCR.

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5′-adenylylsulfate (APS), a chemically activated form of sulfate. In the next step, plants directly metabolize APS to sulfite (SO_3^{2-}) through the action of 5′-adenylylsulfate reductase (APS reductase); this in contrast to microbes which convert APS to 3′-phosphoadenosine-5′-phosphosulfate (PAPS) for use as an activated sulfur-containing molecule (Setya et al., 1996; Gutierrez-Marcos et al., 1996). Reduction of sulfite to sulfide (S^{2-}) and its subsequent incorporation into cysteine then occurs. Cysteine serves as the precursor for all cellular compounds containing sulfur, including methionine, glutathione, proteins, and multiple natural products (Schmidt and Jäger, 1992; Hell, 1997; Leustek and Saito, 1997; Bick and Leustek, 1998; Leustek et al., 2000).

The relationship between sulfur assimilation and cysteine synthesis in plants is important for human and animal nutrition. Unlike plants and bacteria, humans and monogastric animals are unable to reduce sulfur and require dietary sources of the sulfur-containing amino acids cysteine and methionine (Schnug, 1997); however, certain crops like soybean (*Glycine max* L. Merr), which is a major source of vegetable protein worldwide in human and animal food, contain low-levels of the sulfur-containing amino acids (Burton et al., 1982; Burton, 1997). The lower than optimal sulfur amino acid content has driven efforts to increase cysteine and methionine levels in soybean and other crops. Owing to its rapid utilization, the flux for cysteine metabolism in plants is high (Giovanelli et al., 1980). During vegetative growth, sulfur moves from older to younger tissues in the form of sulfate, which is reduced to sulfide by the sulfur assimilation pathway for subsequent incorporation into cysteine (Schmutz and Brunold, 1982; von Arb and Brunold, 1986; Sunarpi and Anderson, 1996; Kopriva et al., 2001; Tabe and Droux, 2001). Attempts to introduce sulfur-rich proteins in developing seeds have resulted in reduction of endogenous sulfur-rich proteins (Tabe and Droux, 2001; Hofgen et al., 2001; Krishnan, 2005), which suggests that sulfur assimilation and cysteine biosynthesis do not supply sufficient metabolites in the engineered seeds to enhance sulfur-containing amino acid content.

In the sulfur assimilation pathway, APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP, using glutathione as the electron donor (Setya et al., 1996; Gutierrez-Marcos et al., 1996; Bick and Leustek, 1998; Bick et al., 1998). In plants, regulation of this enzyme is critical for controlling sulfur assimilation and it is localized in the chloroplast (Rotte and Leustek, 2000; Vauclare et al., 2002; Martin et al., 2005). Examination of APS reductase from *Arabidopsis thaliana* (thale cress) and *Lemna minor* indicated that the protein consists of an N-terminal reductase domain, which contains a catalytically essential $[4Fe–4S]^{2+}$ cluster, and a C-terminal glutaredoxin-like domain (Setya et al., 1996; Gutierrez-Marcos et al., 1996; Bick et al., 1998; Suter et al., 2000; Kopriva et al., 2001). Transcriptional and post-translational regulation of the *Arabidopsis* APS reductase in response to light, sulfur and nitrogen deprivation, glucose, and cytokinins has been described (Heiss et al., 1999; Yamaguchi et al., 1999; Koprivova et al., 2000; Bick et al., 2001; Ohkama et al., 2002; Hesse et al., 2003).

Although manipulation of key enzymes in thiol metabolism, such as APS reductase, could be used to increase the levels of cysteine and/or methionine (Tsakraklides et al., 2002), relatively little information about the enzymes of these pathways in crops or legumes is available (Chronis and Krishnan, 2003, 2004; Phartiyal et al., 2006). To better understand the sulfur assimilation pathway in soybean, the APS reductase from a cDNA library was cloned. Biochemical analysis of the heteologously expressed and purified protein shows that the clone encodes a functional APS reductase. Analysis of soybean tissues and seeds at different development stages shows elevated APS reductase expression and activity in young tissues, especially in the early stage seeds. Likewise, the effects of environmental and nutrient stresses on expression of APS reductase were examined. These studies provide information on the sulfur assimilation pathway of this nutritionally important legume.

2. Results and discussion

2.1. Isolation and sequence analysis of soybean APS reductase

Three positive APS reductase clones were recovered from the cDNA library screening. Two of these clones were similar in length, while the third was truncated (750 bp). The longest clone (1701 bp) contained a full-length open reading frame of 1414 bp, and was used for further characterization. The deduced 470 amino acid peptide chain encodes a 51.9 kDa protein, which based on sequence analysis using PSORT, PLOC, and ChloroP includes a chloroplast/plastid transit peptide sequence at the N-terminus (Nakai and Kanehisa, 1991; Emanuelsson et al., 1999; Park and Kanehisa, 2003). This is consistent with the known localization of the enzyme in *Arabidopsis* and the localization of the sulfate assimilation pathway to plastids (Schmidt and Jäger, 1992; Rotte and Leustek, 2000). The N-terminal domain contains a canonical APS/PAPS-reductase domain with a glutaredoxin-like domain at the C-terminus. Soybean APS reductase shares 61.5%, 70.3%, 62.3%, 45%, 53.5%, and 20.3% amino acid sequence identity with the enzymes from *A. thaliana, Brassica juncea* (Indian mustard), *Allium cepa* (onion), *Zea mays* (corn), *Enteromorpha intestinalis* (a green algae), and *Sinorhizobium meliloti* (a rhizosphere bacterium), respectively.

2.2. Genomic organization of soybean APS reductase

Southern blot analysis was performed to examine the gene or genes encoding APS reductase in soybean (Fig. 1). DNA was digested using DraI, EcoRI, EcoRV, HindIII, or XbaI, transferred to a nylon membrane, and

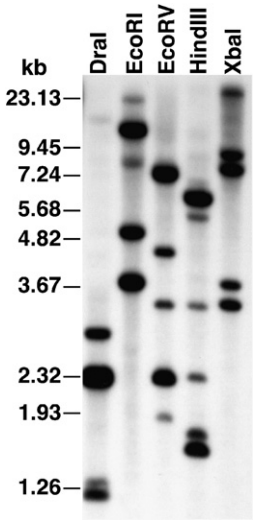

Fig. 1. Southern blot analysis of soybean genomic DNA. Eight micrograms of soybean genomic DNA was digested with DraI, EcoRI, EcoRV, HindIII and XbaI and fractionated on a 0.8% agarose gel. The gel was blotted to a nylon membrane followed by hybridization with [^{32}P]-labeled soybean APS reductase cDNA. The positions and sizes of the lambda-HindIII DNA markers are shown.

probed with ^{32}P-labeled APS reductase cDNA. The soybean APS reductase cDNA contains no internal sites for XbaI and HindIII. In each lane, hybridization to several DNA fragments was detected indicating that APS reductase is encoded by a multigene family in soybean. Our results are consistent with previous reports from other plant species (Gutierrez-Marcos et al., 1996; Kopriva and Koprivova, 2004).

2.3. Expression, purification, and biochemical analysis of soybean APS reductase

For *in vitro* characterization of the APS reductase from soybean, a bacterial expression construct was generated. This construct encodes an N-terminally hexahistidine-tagged protein lacking the putative chloroplast/plastid localization sequence. Based on comparison with the mature APS reductases from *L. minor* and *A. thaliana* (Setya et al., 1996; Gutierrez-Marcos et al., 1996; Kopriva et al., 2001), a truncated version of the soybean enzyme lacking the first 80 amino acids was used for protein expression, purification, and biochemical characterization. Recombinant APS reductase was expressed in *E. coli* and purified by nickel-affinity and size-exclusion chromatographic steps. The purified protein migrated as a 46 kDa species by SDS–PAGE (Fig. 2a), which corresponds to the predicted molecular weight for the heterologous protein lacking the chloroplast/plastic localization sequence. Based on gel-filtration chromatography, the soybean APS reductase eluted as a homodimeric protein.

To determine if the purified protein was functional, a radiochemical assay monitoring the release of [^{35}S]-sulfite from [^{35}S]-APS was used (Berendt et al., 1995). The specific

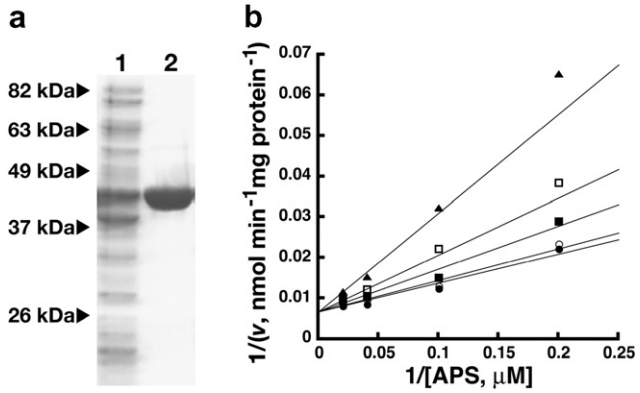

Fig. 2. Biochemical analysis of soybean APS reductase. (a) SDS–PAGE analysis of protein purification. Samples were stained for total protein using Coomassie Blue. Arrows correspond to molecular mass markers as indicated. Lane 1, 50 μg of sonicate. Lane 2, 5 μg of purified protein. (b) Product inhibition. Enzyme assays were performed as described in Section 3. Lines indicate the fit to the observed data. Double reciprocal plot of $1/v$ versus 1/[APS, μM] at 0, 0.1, 0.5, 1, 2.5 mM AMP (top to bottom).

activity of soybean APS reductase using APS as a substrate was 207 ± 31 nmol min^{-1} mg protein^{-1}. The steady-state kinetic parameters of the soybean enzyme are summarized in Table 1. Both glutathione and DTT were effective electron donors for the reaction catalyzed by APS reductase, but β-mercaptoethanol and cysteine were not. The heterologously expressed soybean enzyme displayed the same specificity for electron donors as the APS reductases from *Arabidopsis* and *Lemna* (Bick et al., 1998; Kopriva et al., 2001).

The effect of product inhibition on APS reductase was also examined. Sulfite (up to 100 mM) had no inhibitory effect on the enzyme; however, AMP was a competitive inhibitor with respect to APS with a $K_i = 1.0 \pm 0.2$ mM ($r^2 = 0.992$ for global fit of the data) (Fig. 2b). The product inhibition patterns are consistent with a rapid equilibrium ordered uni bi kinetic mechanism in which AMP and APS both bind the same enzyme form. Other potential kinetic mechanisms would display either a mixed-type or competitive inhibition for both products (Segal, 1975). This result is also consistent with two-step chemical mechanism for the enzyme in which the sulfonucleotide undergoes rapid nucleophilic attack (Carroll et al., 2005).

2.4. Expression profile of APS reductase in soybean tissues

To evaluate the expression of APS reductase in different tissues of soybean, total RNA extracted from hypocotyls,

Table 1
Steady-state kinetic parameters of soybean APS reductase

Varied substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
APS (10 mM DTT)	8.4 ± 0.7	11.2 ± 2.4	743,000
APS (10 mM GSH)	11.3 ± 1.7	17.4 ± 3.5	649,000
DTT (150 μM APS)	8.4 ± 0.3	495 ± 49	17,000
GSH (150 μM APS)	10.1 ± 0.2	595 ± 73	17,000

Reactions were performed as described in Section 3. The varied substrate is indicated and the concentration of fixed substrate is in parenthesis. All k_{cat} and K_m values are expressed as a mean ± SE for an $n = 3$.

roots, leaves, flowers, and seeds at mid-developmental stages and analyzed by RT-PCR (Fig. 3a). RT-PCR product was detected in all the tissues analyzed with leaf, flower, and nodule tissues showing slightly higher mRNA levels than hypocotyls and roots. A 1.4 kb RT-PCR product was detected, as would be expected from the length of the cloned gene. Western blot analysis using protein extracted from the above-mentioned tissues showed a similar trend in the levels of APS reductase protein (Fig. 3b); however, the APS reductase protein levels are elevated in the nodules compared to other soybean tissues. APS reductase activity in stem, root, leaf, and seed tissues was also confirmed by a radiochemical assay (Fig. 3c). Similar specific activities for APS reductase were observed in stem, root, and mature leaf tissues. As observed in *Arabidopsis* (Schmutz and Brunold, 1982; von Arb and Brunold, 1986; Sunarpi and Anderson, 1996; Hartmann et al., 2000; Kopriva et al., 2001; Tabe and Droux, 2001), the activity of APS reductase in young leaves displayed more than a 7-fold higher activity compared to mature leaves. This is consistent with the demand for sulfur assimilation and cysteine biosynthesis in developing tissues with reduction of sulfate occurring at target tissues. Interestingly, APS reductase activity in late development stage (DS6) seeds was 8-fold higher than in mature stem, root, and leaf tissues.

For further examination of the developmental regulation on APS reductase in soybean seeds, the levels of mRNA transcript, protein expression, and enzymatic activity at different growth stages (DS1-6) of seed were measured (Fig. 4). The highest transcript level was detected in the youngest stage (DS1) with a steady decline in mRNA levels observed as the seeds matured (Fig. 4a). The same trend was observed for protein levels. Western blots show high expression of APS reductase in the younger seed (DS1-2) stages (Fig. 4b), despite the fact that the total protein content was relatively low in the younger seed stages and the increased with age, due to accumulation of storage proteins. Levels of APS reductase transcript and protein were lowest in older stage (DS5 and DS6) seeds. Likewise, total specific activity declined up to 8-fold as the seeds matured (Fig. 4c). Strikingly, APS reductase activity in young seeds (DS1-2) was 30–40-fold greater than that observed in stems, roots, and mature leaves.

Previous studies of ATP sulfurylase and the two enzymes of cysteine biosynthesis (serine acetyltransferase and *O*-acetylserine sulfhydrylase) in soybean, also show

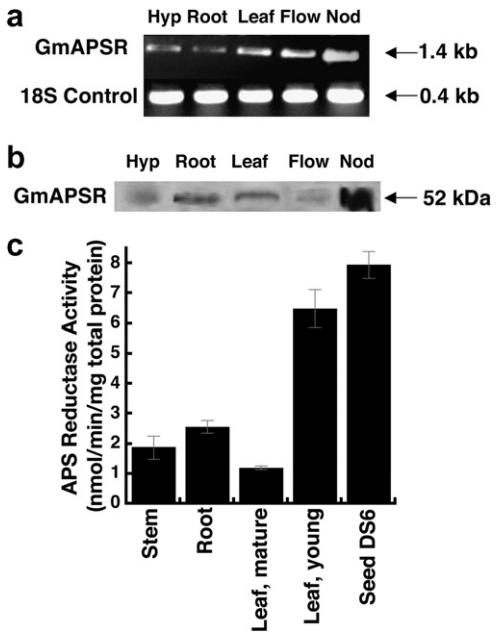

Fig. 3. Expression of APS reductase in different soybean tissues. (a) Total RNA (1 μg) from hypocotyl (Hyp), root, leaf, flower (Flow), and nodule (Nod) was analyzed by RT-PCR for expression of APS reductase. The upper panel shows the RT-PCR product for soybean APS reductase (GmAPSR) and the lower panel shows the RT-PCR product for the 18S ribosomal RNA (control). (b) Western blot analysis of APS reductase expression in hypocotyl (Hyp), root, leaf, flower (Flow), and nodule (Nod) of soybean. Total protein extracts (20 μg) were resolved by SDS–PAGE and were electrophoretically transferred to a nitrocellulose membrane and probed with antibodies raised against soybean APS reductase. (c) APS reductase activity in soybean tissues. Protein extraction and enzyme assays were as described in Section 3. Bars represent the standard error of the mean ($n = 3$).

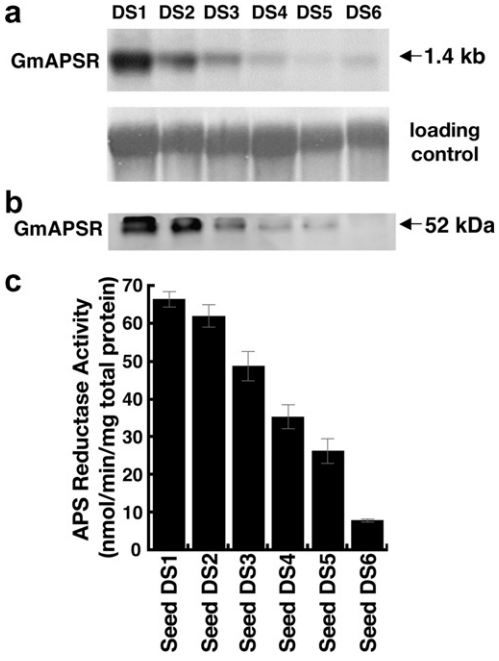

Fig. 4. Developmental regulation of APS reductase in soybean seeds. (a) Northern blot analysis showing the APS reductase mRNA transcript levels (upper panel) at different stages of seed development. Total RNA extracted from seeds was resolved on a formaldehyde gel and probed with [^{32}P]-labeled APS reductase cDNA clone. The membrane was stained with methylene blue to monitor the quality and quantity of the RNA transferred (loading control; lower panel). (b) Western blot analysis of APS reductase expression at different developmental stages. Total protein extracts (20 μg) were resolved by SDS–PAGE and were electrophoretically transferred to a nitrocellulose membrane and probed with antibodies raised against soybean APS reductase. (c) APS reductase activity in soybean seeds. Protein extraction and enzyme assays were as described in Section 3. Bars represent the standard error of the mean ($n = 3$).

similar patterns of high expression in young seeds (Chronis and Krishnan, 2003,2004; Phartiyal et al., 2006). Given the high protein content in soybean seeds, and that sulfur is transported to growing tissues in the form of sulfate (Setya et al., 1996; Leustek and Saito, 1997; Bick et al., 1998; Kopriva et al., 2001), these results suggest that seeds require significantly higher sulfur assimilation activity than other tissues to meet the demand for protein synthesis during soybean seed development.

2.5. Regulation of APS reductase expression under nutrient and cold stress conditions

Because sulfur assimilation is the first step in the processes leading to metabolic utilization of this mineral nutrient, which include responses to abiotic and biotic stresses, we examined how APS reductase is regulated under nutrient deprivation and cold stress conditions. Sulfur assimilatory enzymes have shown to be regulated by the availability of specific nutrients in the medium (Reuveny et al., 1980; Sunarpi and Anderson, 1996; Takahashi et al., 1997; Yamaguchi et al., 1999; Koprivova et al., 2000; Hawkesford, 2000; Kopriva et al., 2001). For deprivation of nitrogen, phosphorus, and sulfur, soybean plants were grown hydroponically in conditions lacking each nutrient. For additional sulfur stress conditions, cotyledons were removed from the plants.

The absence of sulfur (–S1) results in increased APS reductase transcript levels with even higher expression observed under severe sulfur deprivation conditions (–S2) (Fig. 5a). Exposure time of the northern blot was adjusted to keep the –S2 sample from overwhelming the adjacent lanes. In contrast, during nitrogen starvation (–N), mRNA levels were drastically reduced (Fig. 5a). As observed in other plants (Reuveny et al., 1980; Sunarpi and Anderson, 1996; Takahashi et al., 1997; Yamaguchi et al., 1999; Koprivova et al., 2000; Hawkesford, 2000; Kopriva et al., 2002), the use of nitrogen and sulfur are coordinately regulated because both are integral for protein synthesis. In the absence of adequate nitrogen content in the plant, the utilization of cysteine for protein synthesis is reduced, which coincides with down-regulation of enzymes involved in sulfur assimilation and cysteine synthesis. Interestingly, deprivation of phosphorus (–P) from the media also increased expression of APS reductase (Fig. 5a). Sulfur assimilation utilizes ATP, a phosphate-containing molecule, during the transport process from soil into the plant and for production of APS (Takahashi et al., 1997; Schnug, 1997). Low phosphorus conditions would limit the amount of available ATP, which would reduce uptake and assimilation of sulfur. Lower sulfur levels would in turn increase expression of APS reductase. Also, reduced phosphate availability leads to an increase in the amount of sulfolipids and a concomitant decrease in phospholipids (Essigmann et al., 1998). UDP-sulfoquinovose synthase (SQD1), a sulfolipid biosynthesis protein utilizes sulfite as S donor. Since sulfite is a product of APS reductase, it is not surprising

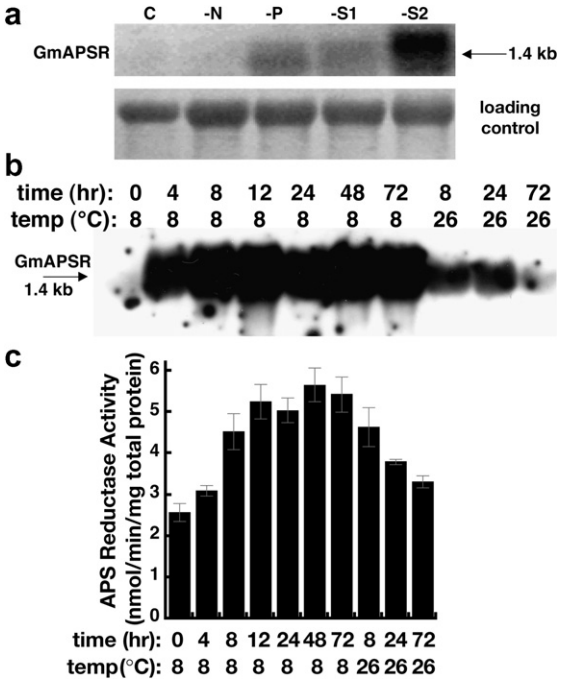

Fig. 5. Effect of stress on APS reductase levels in soybean roots. (a) Northern blot analysis of APS reductase transcript levels in roots of control plants (C) and plants grown in the absence of nitrogen (–N), phosphorus (–P), or sulfur (–S1 and –S2) (upper panel). The –S2 plants were sulfur starved and had half of both cotyedons removed after 7 days of plant growth. Total RNA extracted from seeds was resolved on a formaldehyde gel and probed with [^{32}P]-labeled APS reductase cDNA clone. The membrane was stained with methylene blue to monitor the quality and quantity of the RNA transferred (loading control; lower panel). (b) Northern blot analysis of APS reductase mRNA levels in plants treated at the indicated temperature and time. (c) APS reductase activity was measured in protein extracts from root samples collected at different time points during cold stress and recovery phases. Bars represent the standard error of the mean ($n = 3$).

that phosphate starvation also enhances the expression of APS reductase. Further examination of the link between sulfur and phosphorus metabolism may establish overlapping regulatory processes involved in sensing internal and external nutrient levels (Schachtman and Shin, 2007).

In addition to nutrient deprivation conditions, we examined the effect of cold stress on expression of APS reductase. Previously, we showed that cold-treatment of soybean plants results in increased expression and activity of the sulfur assimilation enzyme ATP sulfurylase (Phartiyal et al., 2006). To determine if this also occurs with APS reductase, soybean plants were placed at 8 °C. Cold-treatment (0–12 h) results in higher transcript levels of APS reductase (Fig. 5b), which are stable for 24–72 h. Following a return to higher temperature (26 °C), mRNA levels decrease to a pre-cold treatment levels. Measurement of APS reductase activity shows a similar 3-fold increase in specific activity that plateaus between 24 and 72 h (Fig. 5c). Following a return to normal temperature conditions, total activity in root extracts returned to pre-cold treatment levels. Since sulfur assimilation and cysteine biosynthesis feed into the production of glutathione, which is

an essential regulator of intracellular redox environment under multiple biotic and abiotic stress conditions, including low temperatures (Kocsy et al., 2000,2001), increased expression of APS reductase would enhance the capacity for sulfur assimilation.

2.6. Conclusions

Although protein-rich soybeans are an important component in human and animal diets worldwide, they contain relatively low-levels of cysteine and methionine. Developing soybean seeds act as a nutrient sink during the production of seed storage proteins (Sunarpi and Anderson, 1996; Sexton and Shibles, 1999). Understanding the metabolic pathways involved in nutrient utilization and the regulation of those pathways is important for exploring possible strategies for improving sulfur-containing amino acid content in soybean seeds. Here we have described the cloning and biochemical characterization of APS reductase, a key enzyme of sulfur assimilation, from soybean. Examination of the tissue distribution and expression of APS reductase in developing seeds demonstrates that young soybean seeds are major sites of sulfur reduction. Together with earlier studies (Chronis and Krishnan, 2003,2004; Phartiyal et al., 2006), these results suggest that augmenting expression of sulfur assimilation and cysteine biosynthesis genes throughout seed development may allow for continued production of sulfur-containing amino acids for incorporation into seed storage proteins. In addition, modification of sulfur assimilation may also be important for responding to cold stress because APS reductase expression and activity increases following temperature stress. Further exploration of this new aspect of sulfur metabolism is in progress.

3. Experimental

3.1. Cloning of APS reductase from soybean

A 433 bp soybean expressed sequence tag (EST), BE058404, showing similarity to APS reductases from other species, was obtained from Genome Systems (St. Louis, MO). Fifty ng of DNA was α-^{32}P-labeled using DNA labeling beads (Amersham). Unincorporated nucleotides were removed from the probe by passing it through Bio-Gel P-60 gel column (Bio-Rad). The radiolabeled EST was used to probe a soybean seedling cDNA library (provided by Dr. Joseph Polacco, University of Missouri, Columbia). Recombinant λ phage in the library were used to infect *Escherichia coli* Y1090 cells and the plaques were blotted onto nitrocellulose membranes and screened for positives. Orienting the plates with the hybridization signals on X-ray film identified positive plaques. The phagemid sequences from the phage DNA were excised using the Rapid Excision Kit (Stratagene). Bacterial cells containing the phagemids were obtained using selective antibiotic markers. After isolation of phagemids from the bacterial cells, the DNA was sequenced from both strands (DNA core facility, University of Missouri, Columbia). The sequence of the cDNA clone has been deposited in GenBank (accession number: AF452450). Southern blot analysis was performed as described earlier (Phartiyal et al., 2006).

3.2. Protein expression and purification

A bacterial expression construct for N-terminally hexahistidine-tagged *Glycine max* APS reductase lacking the chloroplast transit peptide (GmAPSRΔ80) was generated by PCR. The PCR product was amplified from the plasmid template using Pfx Platinum polymerase (Invitrogen), digested with NheI and BamHI, and ligated into NheI/BamHI-digested pET28a (Novagen) to generate the pET28a-GmAPSRΔ80 expression construct. Automated nucleotide sequencing confirmed the fidelity of the construct. Expression constructs were transformed into *E. coli* Rosetta (DE3) (Novagen) cells. Transformed *E. coli* were grown at 37 °C in Terrific broth containing 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol until $A_{600\ nm} = 0.8$–1.0. After induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside, the cultures were grown at 20 °C for 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 Ni^{2+}-nitriloacetic acid (NTA) agarose (Qiagen) column. The column was washed with lysis buffer without Tween-20 and the His-tagged protein eluted with elution buffer (wash buffer containing 250 mM imidazole). Size-exclusion chromatography was performed using a Superdex-200 16/20 FPLC column (Amersham Biosciences) equilibrated with 25 mM HEPES (pH 7.5) and 150 mM NaCl.

3.3. Enzyme assays

Enzymatic activity of APS reductase was measured using a radiometric assay that monitors formation of acid volatile [^{35}S]-sulfite (Berendt et al., 1995). Radiolabeled APS was prepared from [^{35}S]-PAPS (1.145 Ci/mmol; Perkin–Elmer Life Sciences) by addition of nuclease P1 (Setya et al., 1996). Standard assay conditions were 100 mM Tris–HCl (pH 8.5), 500 mM Na_2SO_4, 1 mM EDTA, 125 μM APS (250 nCi [^{35}S]-APS), and 10 mM D/L-dithiothreitol (DTT) in a 100 μL reaction volume. Assays were performed at 30 °C and were initiated by addition of 0.5 μg protein. Reactions were quenched after 20 min by addition of 5 μL 0.84 M Na_2SO_3, then addition of 45 μL of sulfuric acid. Reaction rates were linear from 5 to 30 min. Uncapped reaction tubes were placed into 6 mL scintillation vials containing 1 mL trioctylamine. The scintillation vial was capped and incubated overnight. Reaction tubes were removed from the vials, 3 mL Ecolume scintillation fluid was added, and the radioactivity counted by scintilla-

tion counting. All reaction rates were corrected for non-enzymatic rates. Steady-state kinetic constants were determined under standard assay conditions using DTT (10 mM) or glutathione (10 mM) with varied APS (0–150 μM) or using 150 μM APS with varied DTT or glutathione (0.1–10 mM). Calculation of k_{cat} and K_m values used Kaleidagraph (Synergy Software) to fit the untransformed data to $v = k_{cat}[S]/(K_m + [S])$. Reaction rates in the presence of AMP (0–2.5 mM) and sulfite (0–50 mM) were determined at varied substrate concentrations. Data from these experiments were fitted to the equation for competitive inhibition, $v = (V_{max}[S])/((K_m(1 + [I]/K_{is}) + [S])$, by global fitting analysis in SigmaPlot (Systat Software, Inc.) (Cleland, 1979).

Assays of APS reductase in extracts of soybean tissues were performed using the standard assay system. Tissue extracts were prepared in 100 mM Tris (pH 8.5), 2 mM DTT, 1 mM EDTA. Homogenates were centrifuged at 4 °C and the supernatant collected. Assays contained 10–100 μg total protein and were quenched after 30 min, as described above.

3.4. Plant culture

Soybean (*Glycine max* (L.) Merr., cv Williams 82) seeds were germinated in the dark for 3 days on 1% water agar (v/v) plates at 30 °C. Seedlings were planted in pots (3 plants/pot) containing perlite and were grown hydroponically in nutrient solution (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.63 mM K_2SO_4, 0.5 mM K_2HPO_4, 0.25 mM NH_4NO_3, 0.025 mM $FeC_6H_5O_7$, 2.3 μM H_3BO_3, 0.9 μM $MnSO_4$, 0.6 μM $ZnSO_4$, 0.1 μM $NaMoO_4$, 0.11 μM $NiCl_2$, 0.01 μM $CoCl_2$ and 0.15 μM $CuSO_4$). Plants were grown in the green house under 12 h of light and constant temperature (20–25 °C) for 2 weeks. To starve the plants of nitrogen (–N), NH_4NO_3 was omitted from the media, for phosphorus deprivation (–P), K_2HPO_4 was replaced with KCl and for sulfur starvation (–S1), K_2SO_4, $MgSO_4$ and $ZnSO_4$ were substituted with KCl, $MgCl_2$ and $ZnCl_2$, respectively. Because cotyledons are the major source of sulfur for the growing plant, an additional experiment (–S2) was set up in which half of both the cotyledon leaves was removed a week after the plants were transferred to the medium lacking sulfur. Nutrient solution was replenished once every week. After 15 days of growth, roots were harvested, frozen in liquid nitrogen and stored at −80 °C until used. Each treatment included three replications and the samples from each replication were pooled for analysis. The experiment was repeated once. Developing seeds were collected from the field at one-week interval and were divided into six developmental stages (DS1-6) based on seed size.

3.5. Reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from different tissues (200–500 mg) using Trizol reagent (Invitrogen). For RT-PCR, 1 μg of total RNA was used as template and gene-specific primers, designed form either end of the full-length cDNA, were used for amplification. Any contaminant DNA from the template was digested by DNase treatment (Invitrogen) followed by reverse transcription and PCR using the One-Step RT-PCR kit (Qiagen). Oligonucleotides designed for a conserved region of 18S ribosomal RNA of 400 bp were used as controls.

3.6. Northern analysis

RNA was isolated as described above. Equal amounts of total RNA (10 μg) were electrophoresed in a 1.5% (w/v) agarose-formaldehyde gel, transferred by capillary blotting to a Hybond-N^+ charged nylon membrane (Amersham Life Sciences), and fixed to the membrane by UV cross-linking. Blots were stained using a methylene blue stain solution (Herrin and Schmidt, 1988). For hybridization, the cDNA probes (50–100 ng) were [^{32}P]-labeled using DNA-labeling beads and purified through a Micro Bio-Spin Chromatography column (Bio-Rad). For all northern blotting, full-length cDNA was used as the probe. Probes were denatured before being added to the hybridization solution. Pre-hybridization (over-night) and hybridization (16 h) were carried out at 65 °C in 7% (w/v) SDS, 0.191 M Na_2HPO_4, 0.058 M NaH_2PO_4, 1% (w/v) BSA and 100 μg mL^{-1} denatured Salmon sperm DNA. Following hybridization, membranes were washed three times at 65 °C in 2× SSC, 0.5% (w/v) SDS, and then exposed to X-ray film at −80 °C for 24 h.

3.7. Western blot analysis

Antibodies to soybean APS reductase were generated using gel-purified protein. For the first injection, the antigen (500 μg) was emulsified with Freund's complete adjuvant and injected into a female New Zealand albino rabbit. The second protein injection (300 μg) was emulsified with incomplete adjuvant and given a month after the first injection. The first titer was collected 40 d after the first injection and the final sample 10 d thereafter.

Plant tissues were homogenized in liq. N_2 using a pestle and mortar. Equal amounts of tissue (100 mg, fresh weight) were extracted with 500 μL of sample buffer (60 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.03 mM bromophenol blue). Protein samples (20 μg total protein) were electrophoresed on a 10% SDS–PAGE gel, and then were transferred to nitrocellulose membranes. After transfer, the membrane was blocked with TBS (10 mM Tris–HCl (pH 7.5), 500 mM NaCl) containing 5% (w/v) non-fat dry milk and then incubated with 1:1500 diluted antibody in the blocking buffer (TBS containing 5% (w/v) non-fat dry milk). Affinity-purified goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad) was used as the secondary antibody at a 1:3000 dilution in TBS-T (TBS with 0.05% (v/v) Tween-20) containing 5% non-fat dry milk. The membrane

was washed four times in TBST for 10 min before and after incubation in secondary antibody. Immuno-reactive polypeptides were identified either with HRP color development (Bio-Rad) or by an enhanced chemiluminescent substrate for detection of HRP (Pierce).

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