Plastidic Pathway of Serine Biosynthesis

MOLECULAR CLONING AND EXPRESSION OF 3-PHOSPHOSERINE PHOSPHATASE FROM ARABIDOPSIS THALIANA*

In plants, Ser is biosynthesized by two different pathways: a photorespiratory pathway via Gly and a plastidic pathway via the phosphorylated metabolites from 3-phosphoglycerate. In contrast to the better characterization of the photorespiratory pathway at a molecular level, the molecular regulation and significance of the plastidic pathway are not yet well understood. An Arabidopsis thaliana cDNA encoding 3-phosphoserine phosphatase, the enzyme that is responsible for the conversion of 3-phosphoserine to Ser in the final step of the plastidic pathway of Ser biosynthesis, was cloned by functional complementation of an Escherichia coli serB mutant. The 1.1-kilobase pair full-length cDNA, encoding 295 amino acids in its open reading frame, contains a putative organelle targeting presequence. Chloroplastic targeting has been demonstrated by particle gun bombardment using an N-terminal 60-amino acid green fluorescence protein fusion protein. Southern hybridization suggested the existence of a single-copy gene that mapped to chromosome 1. 3-Phosphoserine phosphatase enzyme activity was detected in vitro in the overexpressed protein in E. coli. Northern analysis revealed preferential gene expression in leaf and root tissues of light-grown plants with an 1.5-fold abundance in the root compared with the leaf tissues. This indicates the possible role of the plastidic pathway in supplying Ser to non-photosynthetic tissues, in contrast to the function of the photorespiratory pathway in photosynthetic tissues. This work completes the molecular cloning and characterization of the three genes involved in the plastidic pathway of Ser biosynthesis in higher plants.

Multiple pathways are responsible for Ser biosynthesis in plant cells. The oxidative photosynthetic carbon cycle constitutes the principal biochemical pathway in photorespiration. Gly and Ser are intermediates in the photorespiratory metabolism of glycolate to 3-phosphoglycerate (3-PGA) in leaves (Fig. 1) (1). Ser biosynthesis via this pathway has received much attention and is well documented (2). The glycine decoxylase multienzyme complex (GDC) (3) and serine hydroxymethyltransferase (SHMT) (4) are responsible for the photorespiratory conversion of Gly to Ser in plant mitochondria. Servaites and Orgen (5) demonstrated that 50% of the initial Ser synthesis remained after chemical inhibition of the photorespiratory pathway, suggesting that a second pathway is activated for Ser formation. In the phosphorylated pathway, an alternative pathway of Ser biosynthesis that takes place in the plastids, Ser is synthesized from 3-PGA via a series of reactions catalyzed by 3-phosphoglycerate dehydrogenase (PGDH), 3-phosphoserine aminotransferase (PSAT), and 3-phosphoserine phosphatase (PSP) (Fig. 1). This pathway is proposed to be important in supplying Ser to non-photosynthetic tissues (6) and fast proliferating tissues (7) and in the dark (8–10) when the photorespiratory rate is low.

A non-phosphorylated pathway of Ser biosynthesis via glyceraldehyde and hydroxyxypyruvate (11) was also proposed to take place in plant tissues in the dark. In animals, however, this pathway is assumed to be involved in Ser catabolism (12). This could also be the case in plant cells because glyceraldehyde dehydrogenase, which catalyzes glyceraldehyde to hydroxyxypyruvate, was found to be tissue-specific and strongly expressed in light-grown leaves (13). Actually, it is unknown to what extent glyceral can be converted to Ser in plant cells.

The molecular studies on the phosphorylated Ser biosynthetic pathway in plastids started with the cDNA cloning of spinach PSAT (8). The first molecular cloning and characterization of Arabidopsis PGDH (9) and PSAT (10), the first two enzymes in this pathway, have recently been carried out in our laboratory. Both Arabidopsis PGDH and PSAT are plastidic proteins, and their catalytic activities are not sensitive to Ser inhibition. The genes encoding Arabidopsis PGDH and PSAT have a common feature in their mRNA expression patterns, in which they demonstrated root-preferential expression (9, 10). These results suggested that the phosphorylated pathway in plastids is regulated at the mRNA expression level, so as to ensure Ser formation in non-photosynthetic tissues, in which photorespiratory Ser biosynthesis is suppressed. To confirm this hypothesis, it is necessary to isolate the cDNA and gene encoding PSP, the terminal enzyme in the plastidic pathway, and to examine its biochemical properties and mRNA expression pattern.

In this study, we describe the successful molecular cloning and characterization of PSP, the enzyme that is responsible for the dephosphorylation of 3-phosphoserine to Ser in the phos-
3-Phosphoserine Phosphatase from Arabidopsis

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—Arabidopsis thaliana ecotype Columbia seeds were germinated and grown on germination medium (14) agar plates under 16/8-h light and dark cycles at 22 °C for 3 weeks. For the dark-treated seedlings that were used for Northern analyses, 2-week-old seedlings were wrapped in aluminum foil and subsequently grown for another 1 week before RNA extraction was carried out.

**Functional Complementation of Escherichia coli Mutants**—cDNA cloning of PSP was carried out by functional complementation of E. coli SK472 (CSGC 6102) (8, relA1, spoT1, thi-1, zji-202; :Tn10, serB22), which was obtained from the E. coli Genetic Stock Center (Department of Biology, Yale University, New Haven, CT). Approximately 1 × 10^6 cells were transformed with plasmid DNA recovered from the AYES A. thaliana cDNA expression library (15) by both heat shock and the electroporation transformation method (17), plated on M9 minimal medium with 50 μg/ml ampicillin, and then incubated at 37 °C for 1 week.

**Isolation of cDNA and Genomic Clones**—To isolate a longer cDNA clone, FPSP4, the cDNA clone that was isolated by functional complementation of E. coli serB^-^ mutant SK472, was used as probe to screen 5 × 10^5 amplified plaques.

Hybridization of the membranes (Hybond N^+^, Amersham Pharmacia Biotech) was carried out at 65 °C in 5 × SSPE (0.9 M NaCl, 0.05 M sodium phosphate (pH 7.7), and 5 mM EDTA), 0.5% SDS, 5 × Denhardt’s solution, and 25 μg/ml salmon sperm DNA. Membranes were washed at maximum stringency in 1 × SSPE and 0.1% SDS for 10 min twice with final washing conducted at 65 °C in 0.1 × SSPE and 0.1% SDS for 10 min and were then exposed to Fuji x-ray film.

Genomic cloning of PSP was carried out by polymerase chain reaction amplification using primers 5^-^CGCGGATCCTATAGAGGCTTTTAGTCCAAT-3^9 and 5^-^CGCGGATCCTATAGAGGCTTTTAGTCCAAT-3^9. Autosequencing was conducted by dye-exchange chain termination methods with Thermo Sequenase (Amersham Pharmacia Biotech) using a Shimadzu Model DSQ1000 DNA sequencer.

**Nucleic Acid Preparation and Blot Analyses**—Genomic DNA was extracted from the leaves of 3-week-old seedlings as described (16). Total RNA was isolated from the leaves and roots of 3-week-old seedlings by a modified guanidine HCl method as described (17). RNA blots were probed with 32P-labeled probe synthesized from the cDNA clones CPSP-1 and CPGDH-5 (10) and CPSAT-5 (9). To investigate the mRNA expression levels of GDC and SHMT, 32P-labeled probes synthesized to clone, FPSP4, the cDNA clone that was isolated by functional complementation of E. coli serB^-^ mutant SK472, was used as probe to screen 5 × 10^5 amplified plaques.

Hybridization of the membranes was carried out at 65 °C in 5 × SSPE, 0.1% SDS for 10 min twice with final washing conducted at 65 °C in 0.1 × SSPE and 0.1% SDS for 10 min and were then exposed to Fuji x-ray film.

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Relative values of mRNA transcripts were calculated based on the hybridization intensities of specific signals on the blots quantified by a Fuji BAS-2000 image analyzer.

**Restriction fragment length polymorphism mapping** was carried out with 30 recombinant inbred lines (19). Hybridization and washing were carried out as described above, except the final washing condition was reduced to 0.5 × SSPE and 0.1% SDS for 10 min at 65 °C. The map distances were kindly calculated by Dr. M. Anderson (Nottingham Arabidopsis Stock Center) based on the restriction fragment length polymorphism profiles generated by Hha1.

**Overexpression of Recombinant Enzyme**—We followed a general method of DNA engineering according to Sambrook et al. (17). Primers AB5 (5^-^CGGATCCCTGAAACCATTACTCAGGTACG-3^9) and AB6 (5^-^CGCGATCTGGAGGTTTCATCGAGTGC-3^9) were used to create a NeoI site at the 5^-^and a BamHI site at the 3^-^end of the open reading frame of PSP. The engineered DNA fragments were inserted into the appropriate sites of pET3d (Novagen), in which the cDNA was placed under a strong δ10 promoter in both sense and antisense orientations.
tations. The plasmids were then introduced into E. coli BL21(DE3) pLysS, in which the gene for lysogenic T7 RNA polymerase, under the lacUV5 promoter, is induced by isopropyl-1-thio-D-galactopyranoside.

Enzyme Assay—PSP activity was determined by measuring the phosphate release from 3-phosphoserine at 30 °C in a volume of 0.5 ml of reaction mixture containing 80 mM Tris-HCl (pH 7.5), 10 mM 3-phosphoserine, 3 mM MgCl2, and 20 μl of extract to start the reaction (20).

The rate was linear for at least 15 min. After 10 min, 0.25 ml of 15% trichloroacetic acid was added to stop the reaction. Free phosphate was determined (21), and corrections were made for zero time blank “rates.” Blanks without enzyme were also run. One unit of enzyme is the amount that catalyzes the conversion of 1 μmol of substrate/min under the stated condition.

Subcellular Organelle Localization by GFP—To verify the subcellular localization of PSP in plant cells, primers 59-CTCCAGTCGACATGGAAAGCATTAACTACTT-39 and 59-CCTGGCCATGGCTCGTGAGGCACAGAA-39 were used to amplify a 180-base pair fragment of the 59-end of PSP for subcloning into plasmid CaMV35S-sGFP(S65T)-NOS3 (22) with a cauliflower mosaic virus 35S-labeled promoter at its 59-end, GFP as reporter protein, and nopaline synthetase terminator as transcription terminator. The plasmid construct 35S-VP-sGFP(S65T) (22), carrying transit peptide sequence obtained from the ribulose-1,5-bisphosphate carboxylase small subunit polypeptide of Arabidopsis (23), was used as a positive control.

These plasmids were used for subsequent particle gun bombardment. Particle gun bombardment was carried out using the Helios Gene-Gun system (Bio-Rad) following the standard protocol provided by the supplier. Individual leaves were viewed with a fluorescent microscope (BX50-FLA, Olympus) using a Chroma dual band filter, FITC, and TRITC (Olympus). Photographs were taken using Fujichrome ISO 100 film.

RISCES—DNA subcloning and sequencing, SDS-polyacrylamide gel electrophoresis, and protein quantitation were carried out as described (17). Plasmid recovery of DNA from the AYES A. thaliana cDNA expression library was carried out as described (15).

RESULTS

cDNA Cloning of Arabidopsis PSP by Functional Complementation of an E. coli Mutant—The E. coli serB- mutant strain SK472 is deficient in PSP activity and is unable to grow on M9 minimal medium plates without Ser supplementation. Complemented cells that were ampicillin-resistant were obtained at a frequency of 50 from 1 × 105 cells transformed with a total of 16 μg of plasmid DNA recovered from the AYES Arabidopsis cDNA expression library (15). The cDNA clone obtained (FPSP4) was ~1 kilobase pairs in length, and it starts immediately from the ATG translation codon.

To obtain a longer cDNA clone, 4.5 × 105 phage plaques produced from an Arabidopsis whole plant cDNA library were screened with the cDNA clone (FPSP4) isolated by functional complementation, and only two positive clones were isolated. Of the two positive clones selected for further studies, CPSP-1, which contains the largest cDNA insert (1.1 kilobase pairs),
A 180-base pair polymerase chain reaction-amplified fragment was subcloned into plasmid CaMV35S-sGFP(S65T)-NOS3, encoding the N-terminal 60 amino acids of PSP from seedlings. A 180-base pair polymerase chain reaction-amplified fragment from the leaves of 3-week-old seedlings. For every lane, Arabidopsis Gene-Gun system at a pressure of 100 p.s.i. on 3-week-old protein. Particle gun bombardment was carried out using the Helios Biochemical Characterization of Arabidopsis PSP—Recombinant fusion protein CPSP-GFP, containing the 60 amino acids at the N terminus of CPSP-1 fused to jellyfish GFP, could be detected in intact tissues after delivering the constructs into Arabidopsis leaves by particle gun bombardment. The observed signals in the construct containing the predicted transit peptide of PSP from Arabidopsis (Fig. 4b) were similar to those exhibited by the transit peptide of the ribulose-1,5-bisphosphate carboxylase small subunit polypeptide of Arabidopsis (23), was used as a positive control. α, fluorescence signal pattern exhibited by 35S-TP-sGFP(S65T), which was known to be targeted to chloroplasts (22). β, fluorescence signal pattern exhibited by CPSP-GFP of Arabidopsis (this study). Bar = 100 μm.

Fig. 3. Southern blot analysis of PSP. Genomic DNA was extracted from the leaves of 3-week-old seedlings. For every lane, ~5 μg of genomic DNA was digested with restriction enzyme, separated by electrophoresis through a 0.8% (w/v) agarose gel, transferred to a Hybond N* membrane, and then hybridized with 32P-labeled CPSAT-5. The final washing was performed in 0.1× SSPE and 0.1% SDS at 65 °C for 10 min. kb, kilobase pairs.

Fig. 4. Organelle localization of PSP using GFP as reporter protein. Particle gun bombardment was carried out using the Helios Gene-Gun system at a pressure of 100 p.s.i. on 3-week-old Arabidopsis seedlings. A 180-base pair polymerase chain reaction-amplified fragment encoding the N-terminal 60 amino acids of PSP from Arabidopsis was subcloned into plasmid CaMV35S-sGFP(S65T)-NOS3. The 35S-TP-sGFP(S65T) construct, carrying transit peptide sequence obtained from the ribulose-1,5-bisphosphate carboxylase small subunit polypeptide of Arabidopsis (23), was used as a positive control. α, fluorescence signal pattern exhibited by 35S-TP-sGFP(S65T), which was known to be targeted to chloroplasts (22); β, fluorescence signal pattern exhibited by CPSP-GFP of Arabidopsis (this study). Bar = 100 μm.

of genomic and cDNA sequences revealed the presence of six short introns, splitting the open reading frame into seven exons.

The Southern blot results (Fig. 3) demonstrate a single major fragment being hybridized upon digestion with BamHI, BgIII, EcoRI, SacI, and XbaI. Two bands were observed upon digestion with EcoRV due to the presence of restriction sites for this endonuclease in the genomic structure. The results suggested that Arabidopsis PSP is a single-copy gene, and it was mapped to chromosome 1, between the markers g3786 and g3829.

Subcellular Localization of Arabidopsis PSP—The recombinant fusion protein CPSP-GFP, containing the 60 amino acids at the N terminus of CPSP-1 fused to jellyfish GFP, could be detected in intact tissues after delivering the constructs into Arabidopsis leaves by particle gun bombardment. The observed signals in the construct containing the predicted transit peptide of PSP from Arabidopsis (Fig. 4b) were similar to those exhibited by the transit peptide of the ribulose-1,5-bisphosphate carboxylase small subunit polypeptide of Arabidopsis (23) (Fig. 4a), which was already known to be sufficient for translocation to chloroplasts (22). The expression pattern of the plastidic proteins was distinct from the signals observed in the cytosol and nucleus of the empty vector, sGFP(S65T), without a transit peptide (25). These results confirmed that the N-terminal sequence of Arabidopsis PSP is sufficient for the translocation of a passenger protein into chloroplasts. Thus, Arabidopsis PSP is a plastidic protein.
FIG. 6. Gene expression of serine biosynthetic genes from Arabidopsis.
Ten μg of total RNA was separated under denaturing conditions on a 1.2% agarose gel containing formaldehyde, transferred to a Hybond N+ membrane, and then probed with 32P-labeled cDNA clone. The final washing was performed in 0.1× SSPE and 0.1% SDS at 65 °C for 10 min. The probes used were all from A. thaliana. A, PGDH (10); B, PSAT (9); C, PSP (this study); D, H-protein (3); E, SHMT (DDBJ/GenBankTM/EBI accession number T42313).

**Gene Expression of Arabidopsis PSP—**The mRNA expression level of PSP was examined in leaf and root tissues of both light-grown and dark-treated plants. Northern analyses were also carried out with two other enzymes, PGDH and PSAT, in the plastidic pathway and with two enzymes, H-protein (a subunit of GDC) and SHMT, in the photorespiratory pathway for comparison with PSP expression. The highest level of PSP mRNA expression was observed in light-grown root tissues (Fig. 6C). It is ~1.5-fold higher than the mRNA expression in light-grown leaf tissues. Lower mRNA expression levels were detected in the dark-grown leaf and root tissues. The level in dark-grown roots was still higher than in dark-grown shoots. These trends of expression were also similar for PGDH (Fig. 6A) and PSAT (Fig. 6B); in contrast, the mRNAs of H-protein (Fig. 6D) and SHMT (Fig. 6E) accumulated almost absolutely in the light-grown leaves.

**DISCUSSION**

In this study, the first molecular cloning of PSP from the plant kingdom was successfully achieved from Arabidopsis by functional complementation of E. coli serB mutant SK472. In the deduced amino acid sequence, two highly conserved motifs containing aspartate residues are found in PSP: DXDST and (T/V) motif. Both these domains have high similarity to the consensus phosphorylation site of ATPases of the Na+/K+ -ATPase family, suggesting that there may be functional homology between PSP and ATPases (26). Replacement of the first aspartate of the DXDST motif by asparagine or glutamate resulted in complete inactivation of the enzyme, suggesting that the first aspartate of this motif is the phosphorylated residue in human PSP (26). PSP belongs to a new phosphotransferase family with an amino-terminal DXD(T/V) motif that serves as an intermediate phosphoryl acceptor (26).

Arabidopsis PSP is a single-copy gene located at chromosome 1,~2.2 centimorgans below marker g3786 and 0.1 centimorgan above the gene for PGDH, which catalyzes the first step of the phosphorylated pathway of Ser biosynthesis. The actual significance of their proximity to the regulation of Ser biosynthesis remains a question that needs further investigation.

The $K_m$ value for PSP in the chloroplast extract of spinach was reported to be 1.1 mM for 3-phosphoserine, and the activity was Mg2+-dependent at optimum pH 7 (20). Arabidopsis recombinant PSP exhibited a higher $K_m$ value of 3.2 mM for 3-phosphoserine.

In E. coli, PSP was inhibited by Ser (27). The inhibition is neither the competitive nor the non-competitive type with respect to Ser. The equilibrium positions of PSAT and PGDH will favor 3-PGA when PSP is inhibited, thereby ensuring that the pathway as a whole is effectively controlled. The phosphorylated pathway is controlled primarily by the demand for Ser rather than the supply of the precursor, 3-PGA (27). Approximately 60% of Arabidopsis PSP activity is inhibited at 10 mM Ser. This finding is consistent with the report of Larsson and Albertsson (20) that the spinach PSP activity was reduced rather than eliminated completely at high Ser concentration. Analysis of the control of mammalian Ser biosynthesis also revealed product inhibition of PSP activity, the final step of the pathway (28).

In bacteria and animals, Ser is mainly synthesized via the phosphorylated pathway (29), using 3-PGA that was derived from glycolysis or from the oxidative or reductive pentose phosphate pathway. The situation is much more complicated in photosynthetic plant tissues since Ser biosynthesis can proceed via the photorespiratory pathway (30). There are two pools of Ser biosynthesized de novo in photosynthetic cells (Fig. 1): one in the mitochondria, where Ser is synthesized via the photorespiratory pathway, and the other in the chloroplasts, where the phosphorylated pathway is present. Larsson and Albertsson (20) suggested a chloroplast localization of plant PSP based on their physiological investigation. In the present study, particle gun bombardment experiments using the recombinant protein CPSP-GFP confirmed that Arabidopsis PSP is a plastidic protein, indicating there is a pathway of Ser formation in the chloroplasts/plastids.

Ser production in the mitochondria, catalyzed by the series of reactions of GDC and SHMT, is conceived to be the major source of Ser supply in light-grown plants. However, the significant gene expression level of Ser-biosynthesizing enzymes in the phosphorylated pathway in the leaf tissues of light-grown plants (Fig. 6, A-C) indicates the coexistence of a source of Ser in the chloroplasts. It is not surprising since 3-PGA, the precursor of this pathway, is generated in a large amount in chloroplasts via the carboxylation of ribulose-1,5-bisphosphate during photosynthesis.

The preferential mRNA expression pattern of PSP is consist-
ent with that of PGDH and PSAT, the initial two enzymes in the phosphorylated pathway of Ser biosynthesis. These three genes were preferentially expressed in the root tissues of light-grown plants (Fig. 6, A-C). 3-PGA generated through the oxidative pentose phosphate pathway may serve as the precursor of Ser biosynthesis in the plastids of root tissues (Fig. 1).

The phosphorylated pathway of Ser biosynthesis is also proposed to be an important Ser supply in the dark when the photorespiratory rate is low. The above supposition is supported by the fact that both GDC and SHMT were hardly detected in both leaf and root tissues of the dark-treated seedling (Fig. 6, D and E). Moreover, the amount of PGDH and PSP mRNAs detected in the root tissues was significantly higher than in the leaf tissues from dark-treated plants, revealing a root-preferential expression of these genes, irrespective of their light dependence.

The high expression level of PGDH, PSAT, and PSP in the root tissues of light-grown plants could be due to the high demand of Ser or the availability of 3-PGA during photosynthesis. High enzyme activities of PSAT in fast proliferating tissues, e.g. root meristems and apical tissues, etc., where there is a high Ser requirement was reported (6). Therefore, the highest expression level of these genes in the root tissues of light-grown plants is needed for high demand of Ser, suggesting that these genes are light-regulated.

In conclusion, there are two Ser biosynthesis pathways in plants: one is mitochondrially located and related to photosphere, and the other takes place in the plastids. In root tissues, the plastidic pathway most probably is the major route of Ser biosynthesis, whereas in leaf tissues, where there are two Ser biosynthetic pathways, the plastidic pathway may become the main supply of Ser in non-photosynthetic tissues or in the dark.

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