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Subcellular Location of Δ¹-Pyrroline-5-Carboxylate Reductase in Root/Nodule and Leaf of Soybean[1]

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ABSTRACT

The expression of Δ¹-pyrroline-5-carboxylate reductase (P5CR) gene was found to be higher in soybean root nodules than in leaves and roots, and its expression in roots appeared to be osmoregulated (AJ Delauney, DPS Verma [1990] Mol Gen Genet 221: 299–305). P5CR was purified to homogeneity as a monomeric protein of 29 kilodaltons by overexpression of a soybean P5CR cDNA clone in *Escherichia coli*. The pH optimum of the purified P5CR was altered by increasing the salt concentration, and maximum enzyme activity was attainable at a lower pH under high salt (0.2–1 molar NaCl). Kinetic studies of the purified enzyme suggested that nicotinamide adenine dinucleotide phosphate⁺ inhibited P5CR activity, whereas nicotinamide adenine dinucleotide⁺ did not. Subcellular fractionation and antibodies raised against purified soybean P5CR were used to investigate location of the enzyme in different parts of soybean as well as in leaves of transgenic tobacco plants synthesizing soybean P5CR. P5CR activity was present in cytoplasm of soybean roots and nodules as well as in leaves, but in leaves, about 15% of the activity was detected in the plastid fraction. The location of P5CR was further confirmed by western blot assay of the proteins from cytosol and plastid fractions of different parts of the plant. Expression of soybean nodule cytosolic P5CR in transgenic tobacco under the control of cauliflower mosaic virus 35S promoter led to the accumulation of this protein exclusively in the cytoplasm, suggesting that the chloroplastic activity may be due to the presence of a plastid form of the enzyme. The different locations of P5CR in root and leaf suggested that proline may be synthesized in different subcellular compartments in root and leaf. Proline concentration was not significantly increased in transgenic plants exhibiting high level P5CR activity, indicating that reduction of P5C is not a rate-limiting step in proline production.

Proline accumulation is one of the adaptations of plants to salinity and water deficit (4, 22, 34). The proline biosynthetic pathway in plants and its regulation in response to osmotic stress is, however, not well understood. A precise determination of the proline pathway and its compartmentalization in different tissues is essential not only for understanding the regulation of proline production, but also for establishing the role of this amino acid in conferring tolerance to salt and drought stresses. The proline pathway in plants has been proposed to be analogous to that in bacteria (7, 21), although proline can also be made from ornithine (1). In both pathways, a common intermediate, P5C[2], is reduced to proline by P5CR. P5CR catalyzes the last step of proline biosynthesis and has been partially purified from a number of plants (6, 16, 17, 23, 26, 27, 30). However, the activities of the first two enzymes in this pathway have not been detected in plants. We recently established the existence of these two enzyme activities in higher plants by molecular cloning of a cDNA that encodes a bifunctional enzyme catalyzing the first two steps of proline biosynthesis (see below). In addition, we have cloned OAT cDNA from the same tissue (A.J. Delauney, C.-A. Hu, D.P.S. Verma, unpublished data), which confirms that proline can be synthesized from arginine via ornithine or from ornithine made from glutamate.

P5CR activity was shown to be higher in soybean root nodules than in root (16). Using a soybean nodule cDNA library constructed in an expression vector (9), we have isolated cDNA clone encoding P5CR by functional complementation of an *Escherichia coli proC* mutant (9). Using the same approach, we recently isolated, from *Vigna aconitifolia* (mothbean) nodule cDNA library, a cDNA clone encoding a novel enzyme (P5C-synthetase) that complemented an *E. coli proB* and *proA* double mutant (14). This suggests that proline in plants is synthesized from glutamate by two gene products (P5CS and P5CR), making this pathway amenable to genetic manipulation. P5CR cDNA and genes have also been isolated from pea (R. Slochum, personal communication) and *Arabidopsis* (33).

Amino acid biosynthetic pathways are generally compartmentalized in eucaryotes. P5CR activity has been detected in the chloroplast fraction of tobacco (27) and pea (29) leaves. In soybean, however, P5CR activity has been identified primarily in cytosol of root nodules (6, 16). In this report, we describe the purification and characterization of P5CR enzyme from *E. coli* expressing a soybean P5CR cDNA. Using subcellular fractionation and antibodies against soybean P5CR, we demonstrated that this enzyme is present in the cytosol of roots and root nodules as well as in leaves, but a small amount of the activity in leaves was found in the plastid fraction. The enzyme is found primarily in the cytosol of transgenic tobacco plants expressing soybean nodule P5CR

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[2] Abbreviations: D,L-P5C, D,L-Δ¹-pyrroline-5-carboxylate; P5CR, Δ¹-pyrroline-5-carboxylate reductase; P5CS, Δ¹-pyrroline-5-carboxylate synthetase; OAT, ornithine aminotransferase; TPDH, triose phosphate dehydrogenase; kan, kanamycin; CaMV, cauliflower mosaic virus; MS medium, Murashige and Skoog medium; IgG, immunoglobulin G.

constitutively. These data suggest that there are at least two isoenzymes of P5CR in plants (see also ref. 6), and their subcellular concentration may vary depending on metabolism and stress.

Earlier evidence suggested that proline accumulation in response to water or salt stress is due to elevated levels of P5CR (32). Salt treatment of seedlings increased the level of P5CR mRNA in soybean (9) and *Arabidopsis* (33). In tobacco, however, NaCl-induced proline accumulation did not appear to enhance P5CR activity, suggesting that P5CR may not be the rate-limiting enzyme in this pathway (19). Overexpression of soybean cytosolic P5CR in transgenic tobacco did not significantly change the level of proline, suggesting that the reduction of P5C is not the rate-limiting step in proline biosynthesis in plants, and the pathway may be regulated at an early step (see ref. 14), as in bacteria (7).

MATERIALS AND METHODS

Expression of Soybean P5CR in *E. coli* and Purification of Enzyme

The *E. coli* strain X342, harboring a pProC1 plasmid expressing soybean P5CR and complementing the mutation in this strain (9), was grown in Luria-Bertani medium containing ampicillin (50 μg/mL). The cells were harvested by centrifugation and resuspended (9 g wet weight/80 mL) in buffer A (100 mM potassium phosphate, pH 8.0, containing 10 mM β-mercaptoethanol). Following sonication (Benson Sonifier, Type 450) at 40 W, the slurry was centrifuged at 2600*g* for 25 min and the supernatant fractionated by ammonium sulfate precipitation. The 20 to 40% ammonium sulfate saturated fraction containing P5CR activity was dissolved in 22 mL of buffer B (20 mM Tris-HCl, pH 7.6, 2 mM β-mercaptoethanol) and dialyzed against the same buffer overnight at 4°C. Aliquots of the desalted fraction were loaded onto a Whatman DE-52 anion exchange column (16×200 mm), which was rinsed with 1 bed volume of buffer B and developed in a 0 to 0.4 M NaCl linear gradient. Fractions containing P5CR activity were pooled, precipitated with ammonium sulfate (50% saturation), dissolved in 6 mL of buffer B, and dialyzed against the same buffer. This fraction was further purified by affinity chromatography (17) on a Protrans Orange 3 (ICN Chemicals) column (10×25 mm). The bound proteins were eluted stepwise with 50, 150, and 500 mM NaCl in buffer B. P5CR activity was present in the 150 mM NaCl eluate, which was then desalted by dialysis against buffer B and concentrated using a Centriprep concentrator (Type 10) (Amicon, Inc.).

Preparation of Antibody Against Soybean P5CR

Affinity-purified enzyme fraction (200–300 μg of protein) was subjected to SDS-PAGE (12.5%), and protein bands were located by Coomassie blue staining. The major band corresponding to 29 kD was excised and ground in liquid nitrogen. The gel powder was suspended in 1 mL of 20 mM potassium phosphate (pH 8.0) mixed with an equal volume of Freund's adjuvant (GIBCO), and was used to immunize New Zealand White rabbits. The animals were boosted by subcutaneous injections after 3 weeks. Serum obtained 10 d after the third injection was stored at −20°C. For western blot assay, the serum was further fractionated by 30 to 50% saturated ammonium sulfate precipitation and passed through a Protein A column (Rainin Instruments), and the IgG fraction was eluted from the column with buffer C (100 mM glycine-HCl, pH 2.5, 400 mM NaCl). Following adjustment of the pH to 7.0, the IgG fraction was dialyzed against buffer D (10 mM sodium phosphate, pH 7.2, 0.9% NaCl) and stored at −20°C.

DNA Constructs and Plant Transformation

A full-length soybean P5CR cDNA was excised from pProC1 (9) by digestion with *Cla*I and *Apa*I, the ends filled with Klenow, and *Bam*HI linkers were attached. The fragment was cloned into the *Bam*HI site downstream of the CaMV-35S promoter in a vector pSN1 (pUC 18 containing the 35S promoter and nopaline synthetase 3′ end). This construct was inserted into the *Hind*III and *Eco*R I sites in pBIN 19. The resulting plasmid (pGM28) was introduced into *Agrobacterium tumefaciens* (LBA4404) by a modified freeze-thaw transformation procedure (3). Transformants selected on 50 μg/mL kan were used to infect leaf discs of *Nicotiana tobaccum* L. var Xanthi. The transformed tissue was selected on MS medium containing kan (100 μg/mL; ref. 8). Selected shoots were transferred to MS medium without plant hormones. After rooting, the transgenic plants were transferred to soil and grown in a controlled growth chamber. The primary transformants were allowed to self-fertilize. The seeds were collected and germinated on kan-containing agar with different concentrations of NaCl in a controlled growth chamber at 26°C.

Protein Extraction and Western Blotting

Leaves of soybean plants (2 weeks old) and soybean nodules (3 weeks old) were homogenized in liquid nitrogen and resuspended in buffer E (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF) containing 0.5 M sucrose. After passing through four layers of Miracloth (Calbiochem), the extract was centrifuged at 100,000*g* for 30 min, fractionated with ammonium sulfate, and dialyzed. Freshly harvested tobacco leaves from control and transgenic plants (5 g each) were ground in liquid nitrogen and extracted with 10 mL of 100 mM potassium phosphate (pH 8.0) at 4°C in a blender for 30 s. Cellular debris was removed by centrifugation at 2600*g* for 10 min, and the supernatant was recentrifuged at 70,000*g* for 10 min at 4°C. The supernatant was divided into two aliquots. β-Mercaptoethanol (5 mM) was added to the portion designated for enzyme activity assay (see below). For amino acid analysis, the supernatant was extracted three times with chloroform to remove proteins, lipids, and Chl, and the aqueous phase was used for chromatographic analysis. Protein concentration was determined with Bio-Rad reagent with BSA as the standard.

The chloroplast fraction was prepared as described by Schreier *et al.* (31). Samples were dissolved in SDS loading buffer and subjected to SDS-PAGE. Western blot analysis was performed as described by Burnette (5). P5CR protein was detected with the IgG fraction of the antibody raised against purified P5CR, followed by ECL western blot detection system (Amersham Corp.).

Enzyme Assays

P5CR activity was measured spectrophotometrically by monitoring the decrease in absorbance of NADPH at 340 nm. The reaction mixture contained 0.56 mM P5C, 0.12 mM NADPH, and 10 to 20 μL of enzyme in a total volume of 450 μL of buffer F (50 mM potassium phosphate, pH 7.2, 150 mM NaCl). Enzyme activity was measured on a HP8452A diode array spectrophotometer using the HP Kinetics program (Hewlett-Packard). One unit of P5CR activity was defined as the quantity of the enzyme catalyzing oxidation of 1 μmol/NADPH/min. Values for P5CR activity were corrected when the oxidation of NADH/NADPH exceeded 1% in the absence of substrate. The D,L-P5C stock solution was prepared by regenerating P5C from its 2,4-dinitrophenyl-hydrazine hydrochloride double salt (Sigma Chemical Co.) according to a modified method of Mezl and Knox (24). The hydrazone salt of P5C (17.5 mg) was mixed with 3.5 mL of 0.25 N HCl, and the suspension was extracted with the same volume of acetophenone by shaking for 30 min. The two phases were separated by centrifugation, and the lower, dark orange acetophenone phase containing the hydrazone was discarded. The upper, light yellow phase containing P5C was extracted twice with 3.5 mL of toluene to remove residual acetophenone. The acidic P5C solution was kept up to 1 week at 4°C and neutralized with NaOH solution before use. The concentration of P5C was determined with *o*-aminobenzaldehyde as described by Mezl and Knox (24).

The activity of $NADP^+$-dependent TPDH, a chloroplast marker enzyme, was measured as described by Leegood *et al.* (20).

Quantitation of Proline and P5C

Proline concentration was determined by modification of the method described by Gustavsson and Betner (11). For precolumn derivatization, 100 μL of freshly prepared 5 mM 9-fluorenylmethyl-*N*-succinimidyl carbonate in dimethylformamide was incubated with 100 μL of 100 mM sodium borate (pH 8.2) and 50 μL of plant extract for 5 min at room temperature. The reaction mixture was diluted immediately with the elution buffer to a suitable concentration for injection. Standard amino acid solutions of 5 μM each in buffer F were prepared as above. The sample (20 μL) was injected onto a 4.6×250 mm reverse phase column (Microsorb C18, particle size 5 μm, Rainin Instruments). The mobile phase was 47% acetonitrile and 53% aqueous solution (20% tetrahydrofuran and 80% 50 mM acetic acid, pH 4.0) at a flow rate of 0.8 mL/min. The amino acids were detected by a fluorescence detector (LDC Analytical Fluoro Monitor III with 254 nm excitation filter and 300–400 nm emission filter) linked to an integrator (Rainin Instruments).

RESULTS

Expression of Soybean P5CR in *E. coli*

We recently isolated a P5CR cDNA clone from a soybean root nodule cDNA expression library by complementation of an *E. coli pro* C^- strain (9). This enzyme was expressed as an unfused protein due to the presence of a termination codon upstream of the initiation codon. Translation of P5CR mRNA may have been reinitiated due to the presence of a Shine-Dalgarno-like sequence before the initiation codon (see ref. 9). Moreover, when placed in opposite orientation to the *lacZ* promoter, the P5CR cDNA sequence also functioned, indicating *lacZ*-independent transcription and translation (9). Expression of this unfused protein allowed us to purify soybean P5CR to homogeneity and determine its kinetic properties and subcellular location. P5CR enzyme was purified by ammonium sulfate fractionation and DEAE chromatography followed by affinity chromatography on Protrans Orange 3 (ICN Chemicals) column (Table I). After the last step, a homogeneous protein with an apparent molecular mass of 29 kD in SDS-PAGE (Fig. 1, lane 6) was obtained. This is consistent with the native enzyme recently purified from soybean root nodules (6). The final enzyme represents about 20-fold purification, suggesting that P5CR protein in *E. coli* constituted 4 to 5% of the total protein in the cell (see Fig. 1, lane 3). This made purification easier, even though 80% of the enzyme activity was not recovered during the purification. The resulting protein fraction was used for raising antibodies. The purified enzyme is stable for several weeks at −20°C in 40% glycerol containing 2% BSA and was used for kinetic studies.

Kinetic Properties and the Effect of Salt on P5CR Activity

P5CR is an NADPH/NADH-dependent reductase. The K_m values of purified soybean P5CR for the substrate (P5C) did not significantly differ with NADPH or NADH (0.24 mM) and were 0.212 and 0.179 mM, respectively (Fig. 2A). The K_m for NADH was much higher (0.385 mM) than for NADPH (0.031 mM) at a substrate concentration of 0.48 mM (Fig. 2B). The pH optima of soybean root/nodule P5CR appear to change with the salt concentration. At low salt (0–50 mM NaCl), the optimum pH was 8.5; increasing the salt concentration to 0.3 M NaCl broadened the pH optimum of the enzyme (Fig. 3). Higher salt concentration (1 M NaCl) decreased the enzyme activity, however. This suggests that P5CR may undergo conformational change under higher salt concentrations. The effect of various participating compounds in P5C enzymic reduction was also determined. As shown in Table II, NAD^+ had no inhibitory effect on P5CR activity, whereas $NADP^+$ inhibited the reaction significantly in the presence of either NADH or NADPH. The end product, proline, had no effect on the enzyme activity, as observed by Kohl *et al.* (16). The kinetic data from purified soybean P5CR was compared with the crude nodule enzyme (Table III). We

Table I. *Purification of Soybean P5CR from E. coli Expressing a P5CR-cDNA Clone*

Fraction	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*units*	*units/mg*	*-fold*	*%*
Crude extract	768	9600	12.5	1	100
$(NH_4)_2SO_4$ ppt. (20–40%)	125	4370	35.0	3	46
DEAE	24	3450	144.0	12	36
Affinity chromatography	8	1880	235.0	19	20

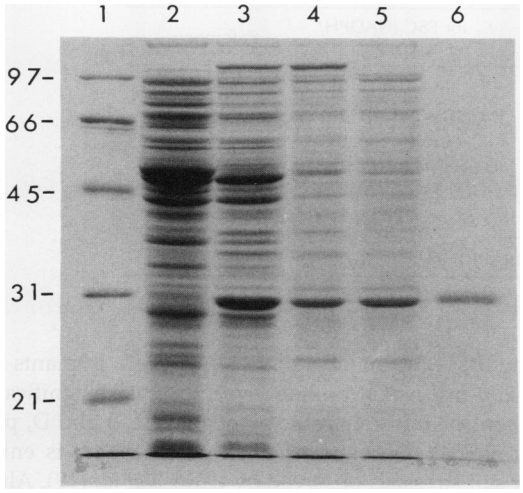

Figure 1. SDS-PAGE showing the protein patterns at different stages of purification of soybean P5CR expressed in *E. coli*. Lane 1, Molecular mass markers in kD; lane 2, crude extract (30 μg) of host *E. coli* cells; lane 3, crude extract (25 μg) of *E. coli* carrying soybean P5CR clone; lane 4, proteins (16 μg) from the 20 to 40% ammonium-sulfate precipitate; lane 5, active fraction (14 μg) from Whatman DE-52 column; lane 6, active fraction (4 μg) from Protrans Orange 3 affinity column.

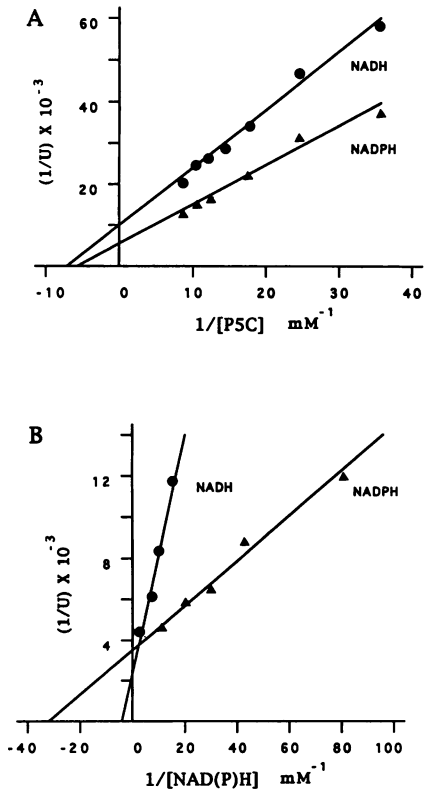

Figure 2. Lineweaver-Burk plots: A, Of substrate (P5C) at 0.24 mM NADH and NADPH. B, Of NADPH/NADH at a substrate concentration of 0.48 mM. The enzyme reaction was carried out at pH 7.2.

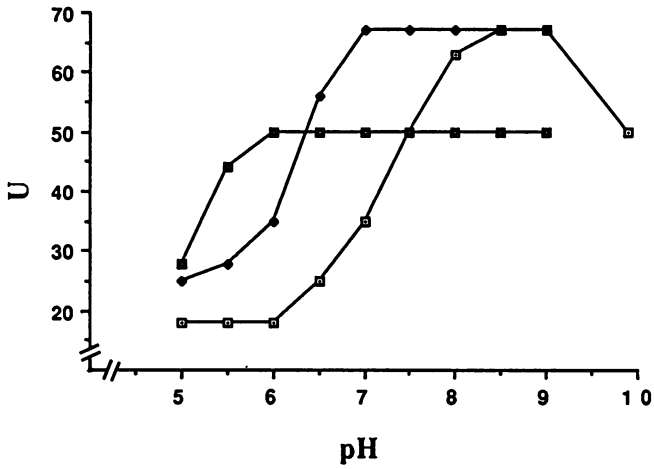

Figure 3. Effect of salt on the pH optima of soybean P5CR. The enzyme activity was measured as described in "Materials and Methods" at different pH in 20 mM phosphate buffer containing different amounts of NaCl: Control (□); 0.3 M NaCl (■); 0.5 M NaCl (■).

also assayed for the possibility of reverse reaction (proline dehydrogenase activity) by P5CR at different pHs (6.0–10.5) in the presence of $NAD(P)^+$ (0.24 mM) and proline (0.6 mM). No significant activity was found in these assay conditions. In a different buffer system at a higher pH (160 mM Na-glycine, pH 10.4), some activity (approximately 20%) was detected (data not shown); however, this pH may not be physiologically significant.

Effect of Salt Concentration on Proline Production in Transgenic Tobacco Plants Overproducing P5CR

To determine the role of P5CR in enhancing proline production, soybean P5CR cDNA was placed under the control

Table II. *Effect of the Participating Compounds in the P5C Enzymic Reduction*[a]

Coenzyme	Compound	Concentration	Activity
		mM	%
NADH (0.12 mM)	None		100
	NAD^+	0.03	100
		0.12	100
		1.00	100
	$NADP^+$	0.03	84
		0.24	30
		1.00	28
	L-proline	1.00	100
	NADPH	0.03	116
		0.12	130
		0.36	81
NADPH (0.12 mM)	None		100
	NAD^+	1.00	100
	$NADP^+$	0.03	100
		0.36	75
		1.00	61
	L-proline	1.00	100
		9.60	100

[a] Substrate concentration in all assays was 0.48 mM P5C at pH 7.2.

Table III. *Comparison of Soybean P5CR Kinetic Data*

Enzyme Source	K_m for NADPH/NADH	K_m for P5C (NADPH/NADH)	Reference
Soybean leaf	0.05/0.10 (10 mM P5C)[a]	0.154 (0.1 mM NAD[P]H)	26
Soybean nodule	0.06/1.55 (0.5 mM P5C)	0.12/0.20 (0.68 mM NAD[P]H)	16
Expressed in *E. coli* (soybean nodule)	0.031/0.385 (0.48 mM P5C)	0.212/0.179 (0.24 mM NAD[P]H)	Present work

[a] Values in parentheses are substrate and cofactor concentrations.

of CaMV-35S promoter and introduced into tobacco plants via *A. tumefaciens*-mediated transformation. Northern blot analysis of tissue from transgenic plants confirmed that the soybean P5CR mRNA was expressed in these plants (data not shown); a functional enzyme was also produced in these plants. The control and transgenic plants expressing P5CR at levels 50 times higher than in control plants (0.36 *versus* 0.06 enzyme units/mg protein, in transgenic and control plants, respectively) were assayed for the level of proline and P5C after growing in 200 mM NaCl. As shown in Figure 4, transgenic tobacco plants (panel C) contained the same level of free proline as the control tobacco (panel A) despite this vast increase in P5CR activity in the transgenic plants, suggesting that P5C may be limited in the cell. In plants treated with 200 mM NaCl, proline levels increased significantly in both control and transgenic plants (Fig. 4, B and D, peak 3), confirming that proline production in tobacco is enhanced under salt stress, as observed by LaRosa *et al.* (19). Although the transgenic plants showed slightly higher levels of proline than control plants after treatment with NaCl (see Fig. 4, B and D), these differences are not significant in multiple sample analysis. Therefore, salt treatment may stimulate the production of P5C, which is rapidly converted to proline by P5CR. The amount of P5CR normally present in the plant appears to be sufficient for the observed high level of proline synthesis, because increased P5CR activity in transgenic tobacco did not significantly increase proline accumulation. No accumulation of P5C was found in salt-treated plants. Based on these data, we concluded that P5C reduction is not the rate-limiting step in proline synthesis.

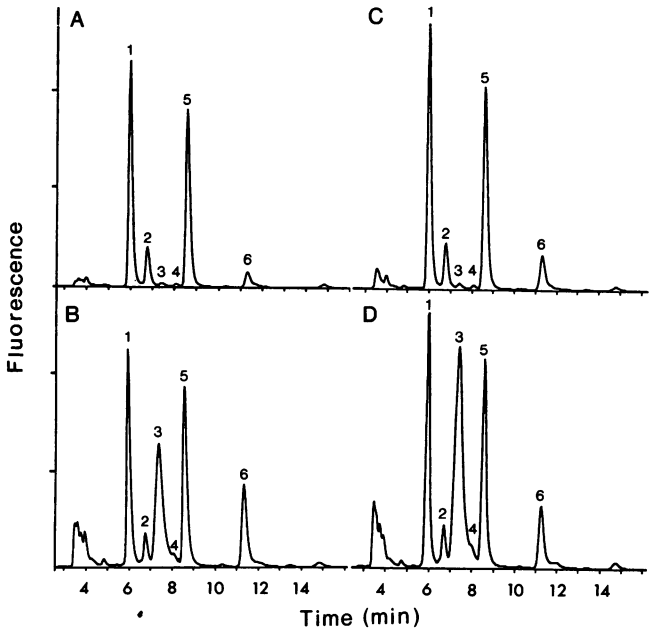

Figure 4. Amino acid fluorescence chromatograms of leaf extracts from wild type (A, B) and transgenic tobacco plants (C, D). The plants were grown without salt (A, C) or in 200 mM NaCl (B, D). Peaks were identified using known compounds. Peak 1, Unreacted 9-fluorenylmethyl-*N*-succinimidyl carbonate; peak 2, byproduct fluorenyl-methoxy-carbonate; peak 3, proline; peak 4, by-product fluorenemethanol; peak 5, P5C; peak 6, unknown. The elution buffer contained 47% acetonitrite and 53% aqueous solution (20% tetrahydrofuran and 80% 50 mM acetic acid, pH 4.0) at a flow rate of 0.8 mL/min.

Subcellular Location of P5CR in Soybean Root/Nodule and Leaf Tissues

P5CR activity has been localized in chloroplasts in tobacco and pea (27, 29). Analysis of the amino acid sequence of soybean P5CR deduced from the cDNA clone did not reveal any putative transit peptide, consistent with the location of this enzyme in the cytoplasm. Western blot analysis of cytosolic proteins from root and root nodule showed a 29-kD protein band that is specifically recognized by the antibody against P5CR (Fig. 5, lanes 2 and 3). The location of P5CR in leaves was determined in soybean and transgenic tobacco

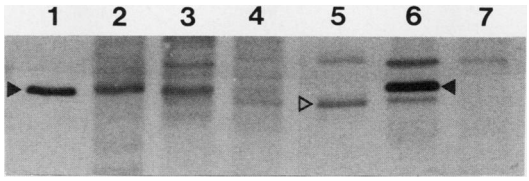

Figure 5. Localization of P5CR in soybean and transgenic tobacco constitutively expressing soybean P5CR. Western blot of protein extracts: Lane 1, Purified soybean P5CR fraction from lane 6 of Figure 1; lane 2, soybean root supernatant; lane 3, supernatant from soybean nodule; lane 4, supernatant of soybean leaf; lane 5, chloroplast pellet; lane 6, supernatant of transgenic leaves; lane 7, control (untransformed) leaves. A closed arrow indicates the cytosolic form of P5CR, and the open arrow points to the chloroplastic form of the enzyme.

plants expressing high levels of soybean P5CR. P5CR activity assay (Table IV) and western blot analysis of soluble and organelle proteins from transgenic tobacco using antibody against purified P5CR (Fig. 5, lane 6) showed that soybean nodule P5CR is located exclusively in the cytosol of transgenic tobacco. The P5CR synthesized in cytosol of transgenic tobacco did not enter the plastids. Analysis of P5CR activity in soybean leaves further revealed that the bulk of this activity is present in the cytosolic fraction (Table IV), with about 15% of P5CR activity detected in the chloroplast fraction. The activity of a known chloroplast marker enzyme, TPDH, was found exclusively in the chloroplast preparation (Table IV), indicating that subcellular fractionation did not damage the chloroplasts.

Western blot analysis of soluble and organelle proteins using P5CR antibody identified an immunoreactive band of smaller size (26 kD) in the chloroplast-enriched fraction (Fig. 5, lane 5) and a 29-kD protein in the cytosol fraction of leaves (lane 4), identical to that detected in root and nodules (Fig. 5, lanes 1 and 2). Preimmune serum did not react with the band corresponding to P5CR (data not shown). The difference in the size of bands reacting with P5CR in the cytosol and chloroplast fractions could be due to the removal of a chloroplast targeting sequence. The amount of protein cross-reactive with P5CR in the chloroplastic fraction (lane 5), furthermore, does not directly coincide with the level of enzyme activity in this fraction. Thus, it appears that there is more than one P5CR isoform in soybean (see also ref. 6). This observation is supported by genomic Southern blot analysis indicating that there is more than one P5CR gene in soybean (9). P5CR isoforms located in cytosol of root and nodule, but also in plastids of leaf, suggest that proline biosynthesis takes place in different cellular compartments in photosynthetic and nonphotosynthetic tissues. The activity of these enzymes may change with the availability of nitrogen and under water stress.

DISCUSSION

Proline in plants can be made from glutamate, as in procaryotes, or from ornithine and/or arginine. P5CR activity has been shown to increase under salt stress (2, 18, 32) as proline accumulates (10, 19). By isolating genes encoding enzymes involved in biosynthesis, we have demonstrated that both routes are operational in higher plants (14). We have recently demonstrated that treatment of soybean roots with 0.2 M NaCl enhances expression of the P5CR gene (9). Expression of *Arabidopsis* P5CR gene also seems to be regulated by salt stress (33). In light of these observations, we expected that enhancing the P5CR level in plants would increase proline production. Consequently, we introduced soybean P5CR cDNA under the control of CaMV-35S (constitutive) promoter in tobacco. This resulted in highly elevated levels of P5CR activity (up to 50-fold) in the cytoplasmic fraction of transgenic tobacco plants, which confirmed that in root and nodule tissues, P5CR is located in this subcellular compartment. Analysis of control and transgenic plants for proline and P5C content under salt stress did not reveal any significant differences, however, indicating that P5CR activity may not be the rate-limiting factor in proline production. Similar findings were reported by LaRosa *et al.* (19) by assaying P5CR activity in salt-treated suspension cultures. Salt-enhanced proline accumulation may, therefore, be due to increased production of P5C, which is then converted to proline by normal or enhanced levels of P5CR.

P5CR appears to occur in both cytoplasm and chloroplast in soybean leaves (see also ref. 25). This is consistent with studies on barley and tobacco (27). In pea, however, this enzyme is primarily located in chloroplasts of leaves (29). The presence of an immunoreactive peptide against the P5CR antibody in the chloroplastic fraction of soybean (Fig. 5, lane 5) suggests that this may be the chloroplastic form of the enzyme. Plastid P5CR appears to be smaller than the cytosolic enzyme (Fig. 5, *cf.* lanes 4 and 5). In order for plastid targeting to occur, the plastid P5CR must contain a transit peptide. More than one copy of the P5CR gene occurs in soybean (9), and the presence of different isozymes of P5CR has been suggested even in nodule tissue (6). We have isolated one of these genes from a soybean genomic library (15). Two genes encoding P5CR have recently been isolated from *Arabidopsis*. The *Arabidopsis* genes share over 90% nucleotide sequence homology (33), but whether they encode cytosolic or plastid P5CR is unknown. In the location of this enzyme in two different subcellular compartments, P5CR is similar to glutamine synthetase, which occurs in a cytosolic form in root and nodule and a chloroplastic form in leaves (13, 25). Preliminary results (data not shown) suggest that water deficit increases P5CR activity more in chloroplasts than in cytosol. This may be due to the participation of proline in the transfer of hydride ions between cytosol and chloroplast, similar to transfer between cytosol and mitochondria in animals (28), and as has been proposed between host cell cytosol and bacteroids (16).

Soybean leaf P5CR has been previously purified, but no molecular data are available (26). Kinetically, this enzyme is similar to the cytosolic form discussed here and that reported by Kohl *et al.* (16). Soybean P5CR *in vitro* uses both NADPH and NADH as cofactors and is inhibited by $NADP^+$. Its K_m value for NADH is about 10-fold higher than for NADPH and is consistent with results obtained by Kohl *et al.* (16). The role of P5CR in NADPH oxidation may be more important than proline production in nodules, as suggested by Kohl *et al.* (16).

Proline biosynthesis in plants appears to be regulated at both transcriptional and enzymic levels. P5CR mRNA increases in salt-treated soybean seedlings (9), and the enzyme

Table IV. *Distribution of P5CR and TPDH Activities in Cytosol and Chloroplast Fractions of Soybean Leaves*

Subcellular Fraction	Total Protein	P5CR	TPDH
	mg	*mU/mg*[a]	*mU/mg*[a]
Cytosol	29.0	13.6 + 0.4	1.3 + 0.2
Chloroplast	7.8	8.6 + 0.7	75.9 + 2.9

[a] One unit of P5CR and TPDH activity was defined as 1 μmol $NADP^+$ produced·min mg protein^{-1}. Two grams of leaf tissue were fractionated to obtain chloroplast fraction, and enzyme activities were determined as described in "Materials and Methods."

seems to undergo conformational change under high salt concentration, resulting in increased activity at lower pH and broadening of the pH optimum (Fig. 3). The fact that P5CR activity was less affected by pH changes under high salt may be physiologically significant and may help plants to adapt to stress conditions. Because the reduction of P5C is not a rate-limiting step in proline production, this pathway may be controlled at an earlier step, as it is in procaryotes. We have recently isolated a *Vigna* cDNA clone that encodes a fused protein homologous to the bacterial ProB and ProA proteins. Enzyme assays showed that it is a bifunctional enzyme (P5C synthetase [P5CS] having activities of both γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase) and is feedback regulated in a manner analogous to that in bacteria (14). Because the primary control mechanism for most amino acid biosynthesis pathways is regulated by feedback inhibition of the first enzyme in the pathway by end products (12), P5CS appears to be a key enzyme in the control of proline biosynthesis. In plants, moreover, P5C is also produced from ornithine by OAT, and we have isolated a *Vigna* clone encoding this enzyme (A.J. Delauney, C.-A. Hu, D.P.S. Verma, unpublished data). Thus, the regulation of proline biosynthesis in plants may depend upon the source of the precursor and the nitrogen status of the cell. The subcellular location of proline biosynthesis enzymes in roots and leaves of soybean corresponds with the presence of glutamine synthetase, which may help provide substrate for proline production in the respective compartments. Understanding the regulation of this pathway is vital for the success of genetically engineering drought and salinity tolerance in crop plants.

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