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Molecular Cloning and Evidence for Osmoregulation of the Δ^1-Pyrroline-5-Carboxylate Reductase (*proC*) Gene in Pea (*Pisum sativum* L.)[1,2]

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ABSTRACT

Several cDNA clones encoding Δ^1-pyrroline-5-carboxylate reductase (P5CR, L-proline:NAD[P]$^+$ 5-oxidoreductase, EC 1.5.1.2), which catalyzes the terminal step in proline biosynthesis, were isolated from a pea leaf library screened with a ^{32}P-labeled *AvaI* fragment of a soybean nodule P5CR cDNA (A.J. Delauney, D.P.S. Verma [1990] Mol Gen Genet 221: 299–305). DNA sequence analysis of one full-length 1.3-kb clone (pPPS3) indicated that the pea P5CR gene contains a single major open reading frame encoding a polypeptide of 28,242 Da. Genomic analysis suggested that two to three copies of the P5CR gene are present per haploid genome in pea. The primary structure of pea P5CR is 85% identical with that of soybean and exhibits significant homology to human, yeast, and *Escherichia coli* P5CR. The sequence of one of four highly conserved domains found in all prokaryotic and eukaryotic P5CRs is similar to the consensus sequence for the NAD(P)H-binding site of other enzymes. The pea P5CR cDNA hybridized to two transcripts, 1.3 and 1.1 kb in size, in polyadenylated RNA purified from leaf tissues of mature, light-grown plants (4 weeks old). Only the 1.3-kb transcript was detected in younger (1 week old) greened seedlings or in etiolated seedlings. In greened seedlings, steady-state levels of this 1.3-kb mRNA increased approximately 5-fold in root tissues within 6 h after plants were irrigated with 0.4 M NaCl, suggesting that expression of the P5CR gene is osmoregulated.

In a variety of monocot and dicot plant species, adaptation to an environmental stress is accompanied by the accumulation of amino acids and their derivatives (33). One of the best studied responses of this type involves the accumulation of proline under water or salt stress (22, 31). The accumulation of this amino acid results from an increased flux of glutamate to P5C[4] (2) and proline (27) in the proline biosynthetic pathway, as well as decreased rates of proline catabolism (32). In some tissues, proline levels may increase as much as 100-fold in response to stress. In corn roots grown at low water potentials, proline accumulation represented approximately 45% of the total osmotic adjustment (35).

In plants, the regulation of proline synthesis is poorly understood, in part because the proline biosynthetic pathway has not been completely characterized. On the basis of metabolic labeling studies, it appears that stress-induced proline synthesis is the result of a loss of feedback control in the proline pathway at the level of P5C formation (2). This situation is analogous to that in bacteria, in which proline synthesis is known to be regulated at the level of γ-glutamyl kinase via proline feedback inhibition (9). In plants, however, only the last enzyme in the proline pathway, P5CR, has been characterized (7, 19, 21). Evidence for the first two enzymes of this pathway, γ-glutamyl kinase and γ-glutamyl phosphate reductase, is not conclusive (21), and their role in the regulation of proline biosynthesis remains to be demonstrated.

In this context, it is interesting to note that increased P5CR activities and gene expression have been shown in a number of plants in which proline accumulation participates in the process of osmotic adjustment. For example, Triechel (34) found that P5CR activity increased 4-fold in response to NaCl adaptation in the halophyte *Mesembryanthemum nodiflorum*. Similarly, Laliberte and Hellebust (20) reported a 4-fold increase in P5CR activity accompanying salt adaptation in the halophytic alga *Chlorella autotrophica*. Recently, Delauney and Verma (12) cloned a soybean nodule P5CR gene and observed a 6-fold increase in P5CR mRNA levels in the roots of soybean seedlings in response to salinization.

The significance of this apparent osmoregulation of P5CR activity in these plants is unknown, especially because the activity of this enzyme is not modulated in response to osmotic adjustment in other plants (21). There are reports of kinetically distinguishable P5CR isozymes (18) and both cytosolic (18) and chloroplastic (26) forms of P5CR in plants. In view of the possibility that these different P5CRs might play fundamentally different roles in plant amino acid metabolism and stress adaptation, we conducted further studies of this enzyme.

We report here the isolation and characterization of a cDNA clone that encodes a pea leaf P5CR. The P5CR gene was expressed in roots and leaves of both etiolated and light-grown pea seedlings. Increased steady-state levels of P5CR mRNA were observed in the roots of salinized, light-grown seedlings, suggesting that expression of this gene is osmoregulated in these tissues.

[1] The nucleotide sequence for the pea Δ^1-pyrroline-5-carboxylate reductase gene has been deposited in the GenBank/EMBL data bases under accession No. X62842.

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[4] Abbreviations: P5C, pyrroline-5-carboxylate; P5CR, pyrroline-5-carboxylate reductase; poly(A), polyadenylation; SSC, standard sodium citrate; pfu, plaque-forming unit; ORF, open reading frame.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli strain PLK-F′ (*recA*, *hsdR*$^-$*M*$^+$, *mcrA*$^-$, *mcrB*$^-$, *lac*$^-$, *supE*, *gal*$^-$, [*F*′, *proAB*, *lacI*^{Q}, *lacZ*Δ*M15*, *Tn10*]) was used for cDNA library construction. *E. coli* strain XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*($r_k^-m_k^+$), *supE44*, *relA1*, *lac*$^-$, [*F*′, *proAB*, *lacI*^{Q}, *lacZ*Δ*M15*, *Tn10*]) was used to plate the cDNA library for screening and for rescue of recombinant pBluescript phagemids from the λZAP II vector (Stratagene, La Jolla, CA).

Construction of the Pea Leaf cDNA Library

Total RNA was extracted from 4-week-old pea (*Pisum sativum* L. cv Wando) leaf tissue using the guanidinium isothiocyanate method, and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography, as previously described (30). cDNA was synthesized using the ZAP-cDNA kit (Stratagene), according to the manufacturer's instructions. After the poly(A)$^+$ RNA (5 μg) was denatured with 10 mM methyl mercury hydroxide, first-strand cDNA was synthesized using Moloney-Murine leukemia virus reverse transcriptase and a poly(T) adapter primer containing an *Xho*I site. After second-strand cDNA synthesis, double-stranded cDNAs were ligated to *Eco*RI adapters, digested with *Eco*RI and *Xho*I, and size fractionated on a Sepharose CL-4B minicolumn.

cDNAs between 400 and 5000 bp in size were collected and ligated to prepared UniZap XR (λZap II) arms. The ligated DNA was packaged in vitro (Gigapak II Gold, Stratagene) and used to transfect *E. coli* PLK-F′ cells. Plaques from 43 individual confluent plates (150 mm) were collected into buffer (1.0 M Tris [pH 7.5], 0.5 M NaCl, 0.15 M $MgSO_4$, 0.01% gelatin). Aliquots from each plate were combined to form a complete library, which contained 1.9×10^6 recombinants.

Isolation of P5CR cDNA Clones

The pea cDNA library was plated with XL1-Blue cells on NZY plates at approximately 2.5×10^4 pfu/plate. Plaques were lifted onto nylon filters (MagnaGraph; Micron Separations, Westboro, MA). Plaque DNA was denatured and UV cross-linked to the filter, which was baked in an 80°C vacuum oven for 1 h. Filters were preincubated in hybridization solution (5× SSPE [0.75 M NaCl, 0.05 M NaH_2PO_4, 6 mM EDTA (pH 7.4)], 2× Denhardt's solution, 50% formamide, 0.1% SDS, 150 μg of salmon sperm DNA) at 40°C for 2 h. Fifty nanograms of a 607-bp *Ava*I fragment of a cDNA encoding soybean nodule P5CR (pProC1, ref. 12) was labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) to a specific activity of 2 to 5×10^8 dpm/μg using a randomly primed DNA labeling kit (United States Biochemical, Cleveland, OH). The probe was denatured and added to fresh hybridization solution, and the filters were incubated in this solution for 18 h at 40°C. Hybridized filters were washed twice at room temperature with 1× SSC (0.15 M NaCl, 15 mM sodium citrate [pH 7.4]) containing 0.1% SDS and then once at 42°C and exposed to film. Putative P5CR clones were identified on autoradiograms, and the clones were plaque purified. Recombinant pBluescript SK$^-$ phagemids were excised in vivo from the λZap II vector by infecting XL1-Blue cells with 200 μL of purified phage (3×10^9 pfu/mL) and 1.2 μL of R408 helper phage (1×10^{11} pfu/ml), according to the manufacturer's instructions (Stratagene). Small-scale preparations of phagemid DNA (16) were used for restriction mapping and sequencing.

DNA Sequencing

Double-stranded phagemid DNA was denatured in 0.2 M NaOH, 0.2 mM EDTA, neutralized by addition of 0.3 M sodium acetate (pH 5), precipitated with ethanol, collected by centrifugation, and dissolved in H_2O. The DNA (3 μg) was sequenced using a Sequenase version 2.0 kit (United States Biochemical). Several restriction fragments of the cDNA insert were subcloned into plasmid pTZ18U to facilitate sequencing. Both strands of the cDNA insert were completely sequenced. Sequence analyses were carried out using the GeneJockey version 1.2 software package (Biosoft, Cambridge, UK) run on a MacIntosh SE computer.

Southern Blot Analysis

Genomic DNA was isolated from pea leaves as described by Richards (28), using the modification of Murray and Thompson (25) in which polysaccharides are removed from the sample by complexation with 1% cetyltrimethylammonium bromide in the presence of 0.7 M NaCl before chloroform extraction and precipitation of DNA. DNA samples were digested with restriction enzymes, and 10 μg of DNA was electrophoresed through an 0.8% agarose gel. The DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min and then by neutralization in 0.5 M Tris (pH 7.5), 1.5 M NaCl. DNA was transferred to a nylon membrane (Magnagraph) by capillary blotting in 10× SSC and fixed to the membrane by UV cross-linking and baking in an 80°C vacuum oven.

The blot was preincubated in hybridization solution (5× SSPE, 5× Denhardt's solution, 50% formamide, 0.5% SDS, 200 μg of salmon sperm DNA) at 42°C for 2 h. A ^{32}P-labeled 830-bp *Eco*RI/*Bam*HI fragment of pea clone pPPS1, encompassing nearly all of the P5CR ORF, was prepared as described above (specific activity of 8.7×10^8 dpm/μg). The probe was denatured and added to fresh hybridization solution, and the blots were incubated for 16 h at 42°C. The blots were then washed twice each with 5× SSC, 0.5% SDS at room temperature and then with 1× SSC, 0.5% SDS and 0.2× SSC, 0.5% SDS, both at 37°C, and exposed to film.

Northern Blot Analysis

Large-scale purification of total RNA from 4-week-old green pea leaf tissues and fractionation of poly(A)$^+$ RNA and poly(A)$^-$ RNA was carried out as previously described (30). Small-scale isolation of total RNA from 1-week-old pea seedlings used in salt-stress studies (see below) was performed using the method of Chomczynski and Sacchi (8). RNA samples were electrophoresed in a 1% agarose-0.6 M formaldehyde gel and transferred to a nylon membrane (Magnagraph) in 10× SSC. ^{32}P-Labeling of the pea P5CR probe and

northern hybridization were carried out as described above for the Southern analysis. Changes in steady-state levels of P5CR mRNA in response to salt stress were quantitated from autoradiograms using a scanning densitometer (Ultroscan XL; Pharmacia LKB Biotechnology, Piscataway, NJ).

Plant Materials

Pea (*Pisum sativum* L. cv Wando) seeds were planted in washed vermiculite moistened with tap water and grown in darkness or in illuminated growth chambers (16 h of light/8 h of dark) at 22°C for 4 d.

Salinization of Pea Seedlings

Seedlings were irrigated with either tap water or 0.4 M NaCl solutions and grown for an additional 3 d. During this treatment period, root and leaf tissues from both control and salt-treated plants were harvested at 6, 24, and 72 h. Harvested tissues were immediately weighed, quick frozen in liquid N_2, and stored at −80°C before proline determinations or protein and RNA extraction. All manipulations of etiolated seedlings were carried out under green safelights.

Determination of Tissue Proline Contents

The proline content of pea tissues was determined according to the method of Bates et al. (1). Briefly, liquid N_2-frozen tissues were homogenized directly in 3% (w/v) sulfosalicylic acid on ice. Homogenates were centrifuged at 14,000*g* for 20 min at 4°C, and the supernatants were transferred to clean tubes for quantitation of proline. Tissue precipitates were saved for protein analyses (see below). Proline in the 3% sulfosalicylic supernatants was derivatized with ninhydrin and quantitated by A_{520} and comparison with a proline standard curve.

Extraction and Quantitation of Tissue Proteins

Proteins in the 3% sulfosalicylic acid precipitates were solubilized in 1.0 N KOH on ice and the potassium sulfosalicylic acid precipitate was removed by brief centrifugation. Aliquots of the soluble tissue proteins were immediately added to SDS-PAGE sample buffer and boiled to denature the proteins. Protein contents in these samples were determined using the method of Bradford (3).

SDS-PAGE Immunoblot Analysis P5CR Protein Levels in Pea Tissues

SDS-PAGE separations of total soluble proteins from pea tissues, electroblotting onto a nitrocellulose membrane, and immunodetection were carried out as described by Slocum et al. (30) using a rabbit polyclonal antibody produced against SDS-PAGE-denatured soybean nodule P5CR protein, which had been overexpressed in *E. coli* (D.P.S. Verma, personal communication).

RESULTS

Characterization of P5CR Clones

Three cDNA clones, of approximately 150,000 screened from a pea leaf cDNA library, hybridized to a ^{32}P-labeled *Ava*I fragment of the soybean P5CR cDNA pProC1 (12). The putative P5CR clones were characterized by restriction mapping, and one clone (pPPS1) was selected for sequencing. pPPS1 was found to be truncated only four amino acids downstream from the presumed N terminus, based on additional sequence data for the 5′ end of the P5CR gene that was later obtained from two full-length clones, pPPS2 and pPPS3.

P5CR clone pPPS3 contained a 1269-bp insert whose nucleotide sequence is shown in Figure 1. This cDNA comprised 37 bp of 5′-untranslated sequence, a single major ORF of 819 bp initiated by an ATG codon and terminated by the stop codon TAA (OCH), and 391 bp of 3′-untranslated sequence followed by a poly(A) tract. The ORF encoded a polypeptide of 273 amino acids with a predicted molecular mass of 28,242 Da. Codon usage in the pea P5CR gene was not significantly different from that reported for other dicot genes (24).

Two putative poly(A) signals, ATTATA and AAGCTT, were located 34 and 27 bp upstream from the poly(A) tract, respectively (Fig. 1). A third poly(A) signal ATAAAT and a GTTTTCTGCT sequence, similar to the GGUUUUCGCU motif believed to play a role in efficient poly(A) of pea *rbcS* mRNA (23), were located 206 and 290 bp upstream from the poly(A) addition site, respectively.

All three P5CR clones were found to differ from each other slightly over a short stretch of sequence at the extreme ends of their 3′-untranslated regions, just before the poly(A) site. The longest 3′ end was found in pPPS3 (Fig. 1). Nucleotides 1206 to 1214 were missing in pPPS2, and nucleotides 1202 to 1214 were deleted in pPPS1.

Comparison of the pea and soybean P5CR amino acid sequences (data not shown) indicates that the pea sequence was 85% identical with the soybean sequence and, if one takes conservative substitutions into account (Fig. 2), exhibited 94% close homology. This is not surprising because both genes are from leguminous species. In contrast, the pea P5CR sequence was only 34% identical with that of the *E. coli* enzyme (13), 27% identical with yeast P5CR (4), and 36% identical with human P5CR (14).

Although overall amino acid sequence homology among the plant P5CR and other prokaryotic and eukaryotic P5CR was low, several domains within these enzymes were highly conserved. In Figure 2, these conserved sequences are compared, along with the corresponding sequences for P5CR from *Pseudomonas aeruginosa* (29) and the archaebacterium *Methanobrevibacter smithii* (15). Within these domains, sequence identity with pea P5CR ranged from 18% for *M. smithii* to 94% for the soybean enzyme.

Southern and Northern Analyses

Southern blot analysis of pea leaf genomic DNA hybridized to a ^{32}P-labeled *Eco*RI/*Bam*HI fragment of clone pPPS1, encompassing nearly all of the P5CR ORF, indicated that there

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 -36                                AAACAACACCAGCATTTCATCTGTCACGTAACTAAAC

      Met Glu Ile Leu Pro Ile Leu Ser Asp Ser Tyr Thr Leu Gly Phe Ile Gly     17
    1 ATG GAA ATC CTT CCC ATT CTC TCC GAT TCC TAC ACT CTA GGT TTC ATC GGC

      Ala Gly Lys Met Ala Glu Ser Ile Ala Lys Gly Ala Ser Arg Ser Gly Val     34
   53 GCC GGA AAA ATG GCC GAA AGC ATT GCC AAA GGC GCC TCC CGC TCC GGC GTG

      Leu Pro Ser Ser Arg Ile Val Thr Ala His Ser Asn Pro Ser Arg Arg Ala     51
  104 TTA CCC TCT TCC CGC ATC GTA ACC GCT CAC TCT AAC CCC TCT CGT CGA GCC

      Ala Phe Glu Ser Ile Gly Ile Thr Val Leu Ser Ser Asn Asp Asp Val Val     68
  155 GCC TTC GAG TCC ATC GGC ATC ACC GTC CTT TCT TCC AAC GAC GAT GTC GTT

      Arg Ala Ser Asn Val Val Val Phe Ser Val Lys Pro Gln Leu Val Lys Asp     85
  206 CGT GCC AGC AAC GTC GTC GTT TTC TCG GTT AAA CCT CAA TTA GTG AAG GAC

      Val Val Leu Lys Leu Lys Pro Leu Leu Thr Lys Asp Lys Leu Leu Val Ser    102
  257 GTT GTG TTG AAA TTG AAG CCG CTC CTA ACT AAG GAC AAG CTT TTG GTT TCT

      Val Ala Ala Gly Ile Lys Leu Lys Asp Leu Gln Glu Trp Ala Gly His Glu    119
  308 GTT GCT GCT GGA ATC AAG TTG AAA GAT CTT CAG GAA TGG GCT GGC CAC GAA

      Arg Phe Ile Arg Val Met Pro Asn Thr Pro Ala Ala Val Gly Gln Ala Ala    136
  359 AGA TTT ATA AGG GTA ATG CCA AAT ACA CCT GCT GCA GTT GGC CAG GCG GCA

      Ser Val Met Ser Leu Gly Gly Ala Ala Thr Glu Glu Asp Ala Asn Leu Ile    153
  410 TCA GTT ATG AGC TTG GGA GGA GCT GCA ACA GAA GAA GAT GCA AAT CTT ATA

      Ser Gln Leu Phe Gly Ser Ile Gly Lys Ile Trp Lys Ala Asp Asp Lys Phe    170
  461 TCC CAA TTA TTT GGG TCA ATT GGC AAA ATA TGG AAA GCT GAT GAT AAG TTT

      Phe Asp Ala Ile Thr Gly Leu Ser Gly Ser Gly Pro Ala Tyr Ile Tyr Leu    187
  512 TTT GAT GCA ATA ACT GGT CTA AGT GGC AGT GGT CCT GCT TAT ATT TAT TTA

      Ala Ile Glu Ala Leu Ala Asp Gly Gly Val Ala Ala Gly Leu Pro Arg Asp    204
  563 GCA ATA GAG GCT CTG GCT GAT GGA GGA GTA GCA GCT GGT CTA CCG CGT GAT

      Leu Ala Leu Ser Leu Ala Ser Gln Thr Val Leu Gly Ala Ala Ser Met Ala    221
  614 CTT GCA TTA AGT CTA GCT TCT CAG ACT GTA CTA GGA GCA GCA TCA ATG GCA

      Thr Leu Ser Gly Lys His Pro Gly Gln Leu Lys Asp Asp Val Thr Ser Pro    238
  665 ACC CTT AGT GGG AAG CAT CCA GGA CAG CTC AAG GAT GAT GTC ACT TCT CCT

      Gly Gly Thr Thr Ile Ala Gly Val His Glu Leu Glu Lys Gly Gly Phe Arg    255
  716 GGC GGG ACA ACA ATT GCA GGC GTT CAT GAG TTG GAA AAA GGG GGT TTC CGT

      Gly Thr Leu Met Asn Ala Val Val Ala Ala Ala Lys Arg Ser Arg Glu Leu    272
  767 GGA ACA CTG ATG AAT GCT GTT GTT GCT GCC GCT AAA CGC AGC CGA GAG CTT

      Ser Stop                                                               273
  818 TCC TAA ACCTATAGGTGAGGATCCTATAGCAGCAGAGAGCTTTCCTAAATGGGCGAGGATCCTAT
  883 TATTTCTAAGAGAAAACTTTGATTTCAGTAGAGTTTTCTGCTTACATATAGTATGTCAAAGTAGCCT
  950 TCTTTGAGATGAGTAGTAGTTGCGGAAATGTTTAGTCTTAGAAACTTCTTTTATAAATGCTCTACTA
 1017 GTATAGACGATTTCAGCCAAGTAGCTCAGAATAACAGCCAGTCCCGCTTATTGTTGCTTGCCGGGGC
 1084 TTGACGTTTCTTTACTGAAGACGATCATTAAGAACAATTTGGCCACTAGACATGGATTCACATGTAC
 1151 ATGCAGTTATGCATTTTGTCTCTAATTATATCAAGCTTATTTCGTTTTGCCTTTGAAAGGTTCCAAA
 1218 AAAAAAAAAAAAAAA
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Figure 1. Nucleotide sequence of clone pPPS3 and deduced amino acid sequence for pea P5CR. Putative 3′-end poly(A) sequences are indicated by underlining. The numbering system refers to the DNA sequence starting at the first ATG as +1.

are approximately two to three copies of the P5CR gene per haploid genome (Fig. 3). Northern blot analyses of poly(A)$^+$ RNA isolated from leaf tissues of mature greened seedlings showed two distinct transcripts 1.3 and 1.1 kb in size (Fig. 4). The 1.1-kb transcript was not detected in total RNA extracted from leaves or roots of younger (1 week old) greened or etiolated seedlings (Fig. 5), but low levels of this transcript were observed in total RNA from leaves of 3-week-old light-grown seedlings (data not shown).

Effect of Salt Stress on Levels of P5CR mRNA and Protein and Proline in Leaf and Root Tissues

In greened pea seedlings, steady-state levels of the 1.3-kb mRNA in leaf tissues were approximately 5-fold higher than in root tissues in control plants (Fig. 5). Irrigation of these seedlings with 0.4 M NaCl resulted in a 5-fold increase in levels of the 1.3-kb P5CR mRNA in root tissues within 6 h, as compared with control seedlings (Fig. 5). Root P5CR mRNA levels remained 4- and 3-fold higher in salt-stressed plants at 24 and 72 h, respectively. In contrast, there was no significant influence of salinization on levels of the 1.3-kb transcript in leaf tissues of these seedlings.

Similarly, salt treatment had no effect on levels of the 1.3-kb mRNA in either the roots or shoots of etiolated seedlings (Fig. 5). However, in the etiolated seedlings, transcript levels were 5-fold higher in roots than in leaves. The 1.3-kb mRNA also increased approximately 6-fold in roots of control seedlings during the 3-d course of the experiment.

Changes in P5CR protein levels in these seedlings were estimated by immunoblotting using a soybean nodule P5CR antibody, which weakly recognized an M_r 28,000 protein in greened and etiolated tissues. In all tissues, levels of the presumed P5CR protein were poorly correlated with levels of 1.3-kb mRNA in control or salinized seedlings, remaining essentially constant (data not shown).

Proline contents of the pea seedlings are shown in Figure 6. In the leaves and roots of untreated greened seedlings, proline levels reflected 1.3-kb mRNA levels, being approximately 5-fold higher in leaves. In salinized seedlings, proline accumulated to the same extent in both leaves and roots, again reflecting the increase in root 1.3-kb mRNA to levels approximating those of leaf tissues (Fig. 5). Proline levels in salt-stressed roots, in which there was a rapid increase in 1.3-kb mRNA levels, increased 12-fold over controls.

In etiolated seedlings, levels of 1.3-kb mRNA and proline accumulation were poorly correlated. Proline levels in the leaf were 2-fold higher than in roots, despite the fact that 1.3-kb mRNA levels were 5-fold higher in the latter tissues. Salt-induced proline accumulation was approximately equal in leaf and root tissues, although the magnitude of the response was higher in the root.

DISCUSSION

The pea P5CR gene characterized in this study exhibits a single major ORF that encodes a protein containing 273

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pea            (98-106)   KLLVSVAAG   (122-130)   IRVMPNTPA
                          |||||||||               |||||||||
soybean        (99-107)   KLLVSVAAG   (123-131)   IRVMPNTPA
                             : |: :               || |:|||
human          (88-96)    DRHIVVSCA   (118-126)   IRCMTNTPV
                          |||:|:|||                |||:||||
yeast          (109-117)  KLLISLAAG   (131-139)   CRVMTNTPA
                           |:||:|||               || ||||||
E. coli        (91-99)    SLVVSIAAG   (116-124)   IRAMPNTPA
                           |:||:|||               :| ||||||
P. aeruginosa  (91-99)    QLIVSIAAG   (116-124)   VRCMPNTPA
                            ::   ||               |    :
M. smithii     (90-98)    TQIIHTCAG   (115-123)   ISSTYDEDN

pea            (172-185)  DAITGLSGSGPAYI   (227-246)  HPGQLKDDVTSPGGTTIAGV
                          |||||||||||||:              |||||||||||||||||:|:
soybean        (173-186)  DAITGLSGSGPAYV   (228-247)  HPGQLKDDVTSPGGTTITGI
                          ||:||||||||||               |||||||:|:||||:|| ::
human          (168-181)  DAVTGLSGSGPAYA   (223-242)  HPGQLKDNVSSPGGATIHAL
                          || |:| |||||::              ||: || :| :||||||||:
yeast          (181-194)  DAATALVGSGPAFV   (236-255)  HPSVLKHQVCTPGGTTIAGL
                           :: |:|||:|||:              ||| ||| | ||||||| :|
E. coli        (166-179)  HPVVGVSGSSPAYV   (221-240)  HPGALKDMVCSPGGTTIEAV
                          ||:|::|||||||                |::|:  |||| ||| |::
P. aeruginosa  (167-180)  DAVTAVSGSGPAYF   (222-241)  EPAELRRRVTSPNGTTEAAI
                          :  |  ::  ||::               | :| : |::  | |  |:
M. smithii     (166-179)  EIATIAASCMPAFI   (221-240)  SPDELINKVATKNGITQKGL
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Figure 2. Conserved sequences among prokaryotic and eukaryotic P5CRs and comparison with the pea P5CR amino acid sequence. Residues with a line above indicate identity with pea residues; conservative substitutions are indicated with two dots over the residue. Conservative substitutions are defined as: (a) C; (b) S, T, P, A, G; (c) N, D, E, Q; (d) H, R, K; (e) M, I, L, V; (f) F, Y, W (10). The underlined pea sequence is similar to the GTGIAP motif occurring within the consensus sequence for the putative NAD(P)H-ribose-binding site of several enzymes (6).

amino acid residues, assuming that the first ATG is the translational start codon (Fig. 1). Several lines of evidence support this assumption. First, use of this codon preserves amino acid sequence homology between the pea and soybean P5CR enzymes. Direct amino acid sequence data for N-terminal (residues 1–15) and internal peptide fragments (residues 221–241) from purified soybean nodule P5CR (7) confirm the translated sequences. Second, the major ORF of the pea P5CR gene encodes a polypeptide with a predicted M_r of 28,242, and the soybean nodule P5CR antibody recognizes an M_r 28,000 polypeptide from pea tissues on SDS-PAGE immunoblots. Finally, the predicted size for the pea enzyme is also similar to that for P5CRs purified from other plant species (7, 19) and from *E. coli* (13).

Unlike P5CRs characterized from other organisms, neither the pea nor soybean enzyme contains any Cys residues.

The functions of the highly conserved domains within P5CR proteins (Fig. 2) are unknown. However, one of these domains, spanning residues 235 to 266 of the pea enzyme, may be involved in binding the NAD(P)H cofactor. Its sequence fits the overall consensus sequence characteristic of the $\beta\alpha\beta$-fold involved in binding the ADP moiety of flavin adenine dinucleotide/NAD cofactors (37). The occurrence of a hydrophobic Ala residue at position 266, in place of the acidic residues Asp or Glu, further suggests that the pea P5CR encoded by clone pPPS3 would utilize NADPH rather than NADH as a cofactor (37). This domain also contains a GTTIAG sequence (Fig. 2), which is similar to the GTGIAP motif found within the NAD(P)H-ribose-binding site of nitric oxide synthase and several other enzymes utilizing this cofactor (6).

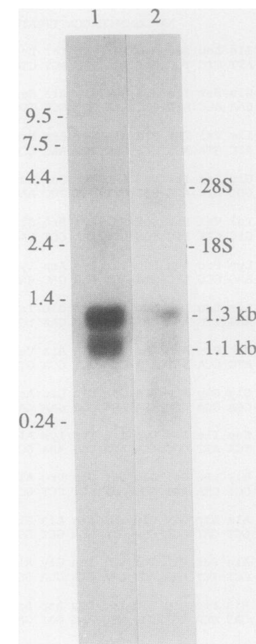

Figure 4. Northern blot analysis of pea leaf poly(A)$^+$ RNA (5 μg; lane 1) and poly(A)$^-$ RNA (10 μg; lane 2) hybridized with a ^{32}P-labeled 832-bp *Eco*RI/*Bam*HI fragment of clone pPPS1. Positions of the 28S and 18S rRNA bands and RNA size markers (kb) are indicated.

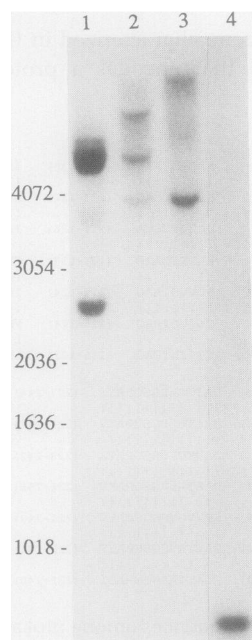

Figure 3. Southern blot analysis of pea leaf genomic DNA hybridized with a ^{32}P-labeled 832-bp *Eco*RI/*Bam*HI fragment of clone pPPS1. DNA (10 μg) was digested with *Eco*RI (lane 1), *Kpn*I (lane 2), or *Sac*I (lane 3), which do not cut at any sites within the clone. Reconstruction lane 4 contains two gene copy equivalents of probe DNA. DNA size markers (kb) are indicated.

To date, all of the P5CR clones that we have characterized appear to encode the same cytosolic enzyme, represented by the single 1.3-kb transcript seen on northern blots. The N-terminal sequence for the pea P5CR polypeptide does not exhibit the general characteristics of presequences associated with proteins imported into mitochondria or chloroplasts, such as an abundance of hydrophobic residues and a net positive charge (36). Soybean nodule (18) and yeast (5) P5CRs have also been reported to be cytosolic proteins. However, chloroplast-localized P5CR activities have been reported for both greened and etiolated pea tissues (26).

Northern analysis of pea leaf poly(A)$^+$ RNA from mature (4 week old) leaf tissue indicates that two distinct P5CR transcripts, 1.3 and 1.1 kb in size, occur in this tissue. In younger pea and soybean (12) seedlings, only the 1.3-kb transcript is expressed. The occurrence of two different transcripts in mature seedlings may result from expression of different P5CR genes, differential splicing of transcripts, or the use of different poly(A) sites during transcript processing. Dougherty et al. (14) speculated that differential splicing of the P5CR transcript in human hepatoma cells, in which there appears to be a single copy of the P5CR gene, might give rise to the kinetically distinguishable P5CR isozymes that have been characterized in different animal tissues.

Multiple poly(A) patterns have been shown for several plant genes (11), and analysis of pea P5CR clone pPPS3 (Fig. 1) suggests that the two pea transcripts may result from the use of different poly(A) signals. Unlike the soybean P5CR

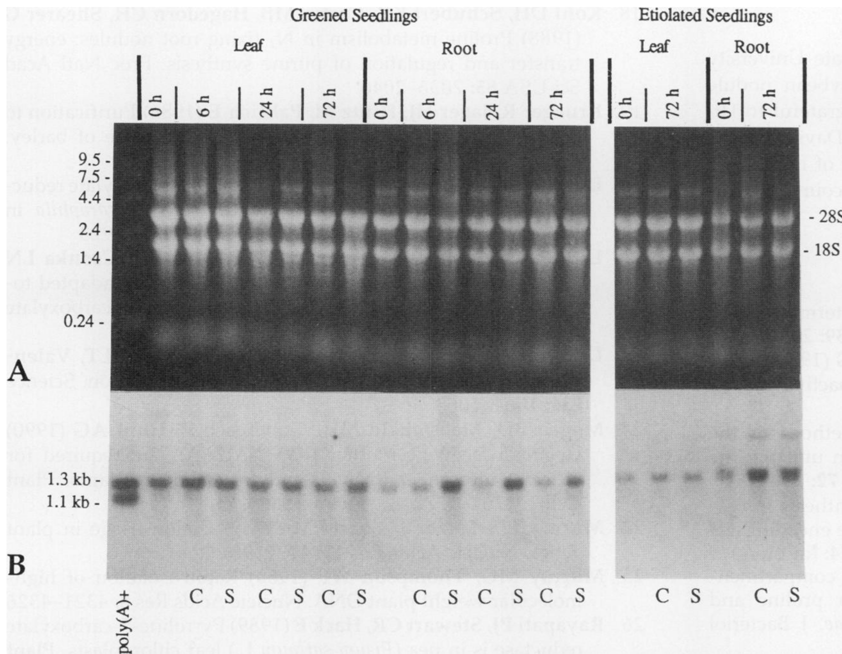

Figure 5. Northern analysis of changes in steady-state levels of the P5CR 1.3-kb transcript in response to salinization in etiolated and greened pea seedlings. Total RNA (20 μg/lane) was isolated from leaf and root tissues 6, 24, or 72 h after irrigation with 0.4 M NaCl. The positions of the 28S and 18S rRNA bands and RNA size markers (kb) are indicated (A). The corresponding northern blot (B), probed with a ^{32}P-labeled *EcoRI/BamHI* fragment of clone pPPS1, shows the 1.3- and 1.1-kb P5CR transcripts in the poly(A)$^+$ RNA lane (5 μg) and the 1.3-kb transcript in control (C) and salt-treated (S) seedlings.

gene, which appears to use the canonical AATAAA poly(A) signal characteristic of animal genes, the pea P5CR gene resembles most other plant genes that have been studied in that it uses poly(A) signals that have diverged significantly from the AATAAA motif (11, 17). The putative poly(A) signals in the pea P5CR gene are clustered at two positions that would be expected to produce transcripts of approximately 1.3 and 1.1 kb in size. Even within the 1.3-kb transcript, however, the actual site of poly(A) differs among the three P5CR clones that we have characterized, occurring at three discrete loci over a span of 13 nucleotides. This may be attributable to the fact that two different putative poly(A) signals are located proximal to one another about 30 bp upstream from the poly(A) tract in the pea P5CR gene.

Steady-state levels of the 1.3-kb transcript increase in the roots of light-grown seedlings upon salt treatment, suggesting that P5CR gene expression is osmoregulated in these tissues. In contrast to the previous report that salt-inducible P5CR expression occurred in the roots of etiolated soybean seedlings (12), we found no evidence for this in either roots or leaves of etiolated pea seedlings. The reason for this discrepancy is unknown, but the results of our studies suggest that patterns of P5CR expression are influenced by light. Tissue-specific differences in P5CR expression may reflect fundamental changes in the dynamics of proline metabolism in root and leaf tissues in response to light. Furthermore, the appearance of the 1.1-kb mRNA only in older light-grown seedlings suggests that the expression of this P5CR transcript may be developmentally regulated in light-grown plants.

The significance of the observed salt-inducible expression of the P5CR gene, with regard to a possible role for P5CR in the regulation of proline synthesis, is unclear. Changes in P5CR transcript levels, in response to salinization, are not seen in etiolated seedlings or in leaves of greened seedlings, although proline accumulation is observed in all salt-stressed tissues. In the roots of greened seedlings, increased transcript levels do approximately parallel a marked increase in proline levels. In all tissues, however, P5CR protein levels appear to remain more or less constant, irrespective of changes in 1.3-kb mRNA levels. It is possible that salt stress simply induces the synthesis and/or stabilization of P5CR mRNA without leading to increased synthesis or accumulation of P5CR protein. We did not measure P5CR activities in the present study, but if one assumes that activity levels reflect P5CR protein levels in the pea tissues, then it seems reasonable to conclude that proline accumulation in the salt-stressed seedlings is not modulated by changes in P5CR activity but is regulated at the level of P5C formation, as has been suggested by previous workers.

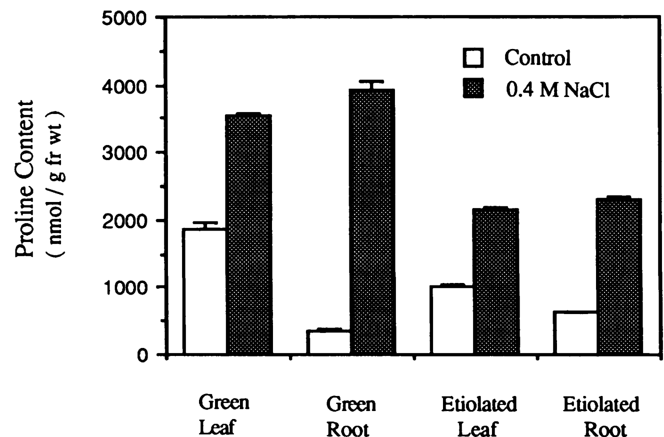

Figure 6. Proline contents of leaf and root tissues of pea seedlings 72 h after irrigation with 0.4 M NaCl. Data represent three replicates. SE bars are indicated. fr wt, Fresh weight.

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