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Update section

Short communication

Molecular cloning, nuclear gene structure, and developmental expression of NADPH: protochlorophyllide oxidoreductase in pea (*Pisum sativum* L.)

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Abstract

Complementary DNA clones and a corresponding nuclear gene (*lpcr*) encoding the NADPH-dependent protochlorophyllide oxidoreductase (pchlide reductase, EC 1.6.99.1) have been characterized from pea (*Pisum sativum* L.). The pea *lpcr* gene encodes a 43 118 Da precursor polypeptide comprised of a transit peptide of 64 amino acids and a mature protein of 336 amino acids. The coding portion of the gene is interrupted by four introns, two of which are located within the transit peptide coding portion of the gene. The deduced primary structure for the pea protein is similar to those reported for *Arabidopsis* and two monocot species. Northern blot analysis revealed little to no decrease in steady-state levels of mRNA encoding the enzyme in etiolated leaves illuminated with continuous white light for up to 48 h. In contrast, western blot analysis showed that the major immunoreactive species present in whole leaf extracts decreased to nearly undetectable levels during this same 48 h period. These results suggest that pchlide reductase activity in pea is primarily regulated post-transcriptionally, most likely at the level of translation initiation/elongation or protein turnover.

In most angiosperms the synthesis of chlorophyll is a light-dependent process regulated in part by the abundance and activity of NADPH-dependent protochlorophyllide oxidoreductase (pchlide reductase, EC 1.6.99.1) [2, 9]. Both pchlide reductase and its substrate protochlorophyllide accumulate to high levels in dark-grown tissues, but decrease dramatically upon exposure to light [17], due in part to a rapid proteolytic turnover of the enzyme [11, 13]. While a light-induced decline in pchlide reductase protein and activity appears to be common among angiosperms [7], the effect of

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X63060.

light on mRNA levels encoding the enzyme varies considerably [3, 7, 15]. We report here the cloning of cDNAs and a corresponding nuclear gene encoding the pchlide reductase from pea and the expression characteristics of this gene during light-induced development.

An expression library was constructed in λgt11 [12] with cDNAs synthesized from etiolated pea (*Pisum sativum* L. cv. Progress No. 9, Burpee Seed Co., Inc.) leaf poly(A)$^+$ RNA [10] and immunoscreened with polyclonal antibodies elicited against oat pchlide reductase [5]. A single immunoreactive phage (λPPCR700) was isolated, the 691 bp *Eco* RI insert sequenced [19], and found to encode a portion of the pea pchlide reductase. Additional overlapping phage (λPPCRzh3, λPPCRzh4, and λPPCRzh5) encoding the pchlide reductase were recovered by screening the library with ^{32}P-labelled [6] insert from pPPCR700 as probe. To isolate cDNAs (pPPCR205-5′ and pPPCR510-3′) containing the distal 5′ and 3′ sequences not present in our clones, a modified RACE strategy [8] was employed.

Subsequently, an unamplified pea genomic library, constructed in EMBL4 from *Eco* RI partial digestion fragments, was screened using ^{32}P-labelled pPPCR700 insert as probe and a single hybridizing phage (λPGEN2) containing 17 kb of genomic DNA was recovered. DNA restriction mapping and Southern hybridization analysis [16] localized the relevant coding and immediate flanking regions of the pea pchlide reductase gene (designated *lpcr* for light-dependent pchlide reductase) to an approximately 2.8 kb *Eco* RI-*Sac* I fragment. This fragment was subcloned (pPGEN2RS) and its nucleotide sequence determined [19].

The complete nucleotide sequence and predicted amino acid sequence of the pea *lpcr* gene is shown in Fig. 1. The gene encodes a 1427 nucleotide transcript containing 40 nucleotides of 5′-untranslated sequence, a single long open reading frame of 400 amino acids, and 185 nucleotides of 3′-untranslated sequence. Over the relevant coding and untranslated portions the pea *lpcr* gene encoded on λPGEN2 is identical to the composite cDNA sequence derived from the various cDNAs and PCR reaction products. Thirty-two nucleotides upstream from the end of the cDNA and presumptive start site of transcription is the sequence 'TTTATAAA' resembling the consensus TATA box.

The pea *lpcr* gene is interrupted by four introns, two within the portion of the gene encoding the transit peptide and two within the coding portion of the mature protein. The location of these introns was inferred by comparison to the cDNA sequence. The amino terminus of the mature pea protein was determined by limited Edman degradation analysis [22] and the resulting peptide sequence (underlined in Fig. 1) identified the N-terminal amino acid of the mature pea protein as Glu-65, further upstream than previously suggested [3, 20]. Based upon a processing site at Ala-64/Glu-65, the predicted 43 118 Da pea precursor polypeptide contains a 64 amino acid transit peptide and a mature protein of 336 amino acids. The predicted molecular mass of 36 398 Da for the mature pea protein is similar to the size estimates obtained by SDS-PAGE [7].

Whether the placement of the introns within the *lpcr* gene coincides with a division of the pchlide reductase into functional domains remains to be elucidated. However, the location of intron II eleven amino acids from the cleavage site in the pchlide reductase precursor is consistent with the division of transit peptide from mature protein observed in other nuclear-encoded chloroplast proteins. Of yet unresolved significance is the occurrence of two introns within the transit peptide-coding sequences of the pea *lpcr* gene. Such an arrangement could allow for differential splicing, giving rise to proteins with transit peptides of different lengths that alter the intra-organellar targeting of the protein during plastid development.

The pea pchlide reductase is 82.7% identical with barley [20], 85.4% identical with oat [5], and 86.9% identical with *Arabidopsis* [3] at the amino acid level. Thus, while monocot species share considerable similarity (barley versus oat is 96.8% identical), the pea enzyme is only slightly more similar to the dicot *Arabidopsis* than it is to either of the monocot species. Barley [20], oat

Fig. 1. Nucleotide and predicted amino acid sequence of pea pchlide reductase cDNA and gene. The nucleotide sequence of the pea *lpcr* gene is shown along with the predicted amino acid sequence of the encoded protein. The start site of transcription and initiating nucleotide of the cDNA (pPPCR 205-5′) has been designated as position + 1. The beginning of the composite cDNA sequence is indicated by an asterisk. The apparent polyadenylation signal used in the gene is underlined and the polyadenylation site and end of the composite cDNA are indicated by the '> polyA^{+}'. Numbering of the predicted amino acid sequence is given to the right beginning with MET at position 1. The amino terminus of the protein (designated by the bold underline) was determined by automated Edman degradation performed on a Model 473 Protein Sequencer (Applied Biosystems Inc.) using standard sequencing programs provided by the manufacturer. The complete nucleotide sequence of both the antisense and sense strands was determined for all clones [19]. With the exception of a C→T substitution at nucleotide 770 that results in a change from Val→Ala in pPPCR205-5′, the sequences of the various cDNAs and PCR amplification products were identical in all overlapping regions.

[5], and *Arabidopsis* [3] each contain four cysteine residues, the positions of which are identical in the various proteins. The mature pea protein contains only three of these conserved cysteine residues, the fourth cysteine being replaced by asparagine (Asp-171). Since the exact number of cysteine residues required for binding and photoreduction of photochlorophyllide or for maintaining the three dimensional structure of the protein [5] is not known, we can only speculate that either all four residues are not necessary, or the pea enzyme has subtly different structural and/or functional activities.

The greatest differences in primary structure among the various pchlide reductases occur within the transit peptide and immediate amino-

terminal regions (i.e., residues 1–70) of the protein. Differences in absolute length, composition, and hydropathy distinguish the precursors from the various plants. However, despite such differences, the overall distribution of charged amino acids and position of conserved proline residues in the various precursors conform to the general framework for chloroplast transit peptides [14].

Although the deduced amino acid sequences of the pchlide reductases from different species are highly conserved, considerable divergence exists in the nucleotide sequences encoding these proteins. Unlike the cDNAs encoding the oat and barley pchlide reductases where the nucleotide sequences are approximately 92% identical within the portions encoding the mature protein, the pea and *Arabidopsis* sequences are only 68% and 58% identical [3], respectively, when compared to the monocot sequences. This observed high degree of similarity in deduced amino acid sequence and substantially lower conservation in nucleotide sequence among *lpcr* genes suggests that direct selection may be operating to actively maintain protein structure/conformation.

To study the effects of light on pchlide reductase levels in pea, 7–10-day old etiolated seedlings were illuminated with continuous white light (150 μW/cm^2; 8000 lux) for various lengths of time and a series of northern blot and western blot analyses were performed. Little or no change in the steady-state levels of pchlide reductase mRNA was observed in expanding primary leaves during the initial 48 hrs of greening (Fig. 2). Low levels of pchlide reductase mRNA were detected in stems and, only after prolonged exposure of the filters, in the roots of dark-grown plants. These results differ significantly from previous studies in monocots where a dramatic drop in steady-state levels of mRNA encoding pchlide reductase has been observed following illumination [1, 18], but more closely resemble the lack of light-induced decrease in pchlide reductase mRNA found in cress [15]. *Arabidopsis*, with a moderate reduction in steady state pchlide reductase mRNA levels upon greening, appears to be intermediate in its response [3].

While light has little influence on pchlide reductase mRNA levels in pea, the effect on enzyme protein is quite different (Fig. 3). Under continuous illumination the concentration of the major immunoreactive 36–38 kDa pchlide reductase protein found in etiolated leaves declines dramatically. However, even as late as 48 h after illumination trace amounts of the protein are detectable. A second weaker immunoreactive band of approximately 34 kDa is also present in the pea leaf extracts. The abundance of this immunoreactive species does not appear to change during light-induced development. This minor band was

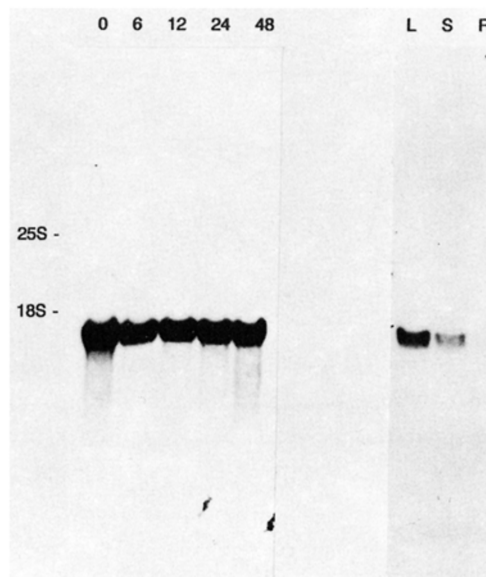

Fig. 2. Northern blot analysis of pchlide reductase expression in various tissues during light induced development. Left panel shows analysis of RNAs prepared from pea leaf tissues receiving: Lane 0, 0 h of illumination; Lane 6, 6 h of illumination; Lane 12, 12 h of illumination; Lane 24, 24 h of illumination; Lane 48, 48 h of illumination; Lane L, dark-grown leaf tissues; Lane S, dark-grown stem; Lane R, roots of dark-grown seedlings. Equivalent amounts of total RNA (50 μg/sample) [4] were fractionated on formaldehyde-agarose gels, transferred to nitrocellulose membranes [16] and hybridized with $1-2 \times 10^6$ cpm/ml of ^{32}P-labelled insert from pPPCR700 in 5 × SSPE, 50% (v/v) deionized formamide, 5 × Denhardt's solution, 0.1% (w/v)) SDS and 100 μg/ml denatured salmon sperm at 42 °C for 12 to 16 h. The filters were washed twice for 15 min in 0.1 × SSC, 0.1% (w/v) SDS at 23 °C.

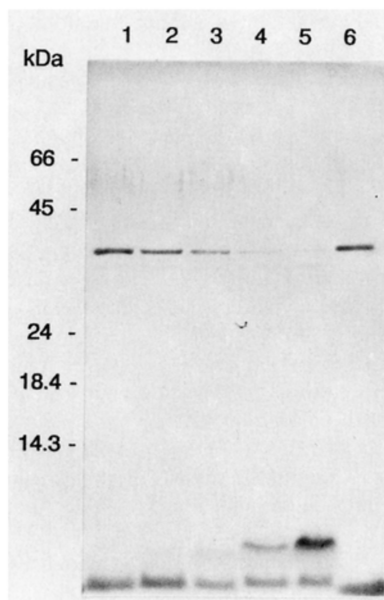

Fig. 3. Western blot analysis of pchlide reductase levels in etiolated and greening leaves. Equivalent amounts of total leaf protein from pea (100 μg) or oat (25 μg) were electrophoresed through a 12% (w/v) SDS-polyacrylamide gel, transferred to nitrocellulose and reacted with the oat anti-Pchlide reductase antibody [5] as previously described [21]. Lanes 1–5 are pea leaf extracts: Lane 1, 0 h of illumination; Lane 2, 6 h of illumination; Lane 3, 12 h of illumination; Lane 4, 24 h of illumination; Lane 5, 48 h of illumination. Lane 6 is etiolated oat. Molecular weight markers are as follows: 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 24 kDa, trypsinogen; 18.4 kDa, β-lactoglobulin; 14.3 kDa, lysozyme. Dark-staining material at the electrophoretic front are free chlorophyll micelles and pyronin Y tracking dye (lowest band). Whole-cell extracts were prepared by grinding tissue in buffer containing 40 mM TRIS-HCl pH 8.0, 2.0% (w/v) SDS. The homogenate was clarified by centrifugation (10 min, 10000 × *g*), the resulting supernatant was made 5.0% (v/v)) with 2-mercaptoethanol and heated for 3 min at 100 °C.

not observed in our preparations of etioplast membranes from pea, but is only present in whole leaf extracts. Whether this protein is in fact a different gene product, a differentially processed form, or a proteolytic degradation product of the major pchlide reductase requires further verification.

The results presented above further demonstrate that while the light-dependent turnover of pchlide reductase appears to be universal among higher plants, the extent and rapidity of the decline in mRNA levels encoding the protein differs significantly between monocots and dicots [3, 7] and to a lesser extent among individual dicot species as well [3, 15, and this study]. The rapid decline in pchlide reductase protein levels and lack of a concomitant decrease in mRNA observed in pea suggests that, unlike most monocot species where regulation has been demonstrated to take place at the level of transcription [18], pchlide reductase levels in this species may be regulated by one or more post-transcriptional mechanisms. The most likely candidates are at the level of translation (initiation/elongation) and/or protein turnover. Clearly, additional studies are necessary to resolve this issue and determine the significance of differences in levels of control among various plant species.

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