Plant Molecular Biology **30**: 15–37, 1996.

The *pc-1* phenotype of *Chlamydomonas reinhardtii* results from a deletion mutation in the nuclear gene for NADPH:protochlorophyllide oxidoreductase

Jianming Li[1] and Michael P. Timko*
*Department of Biology, University of Virginia, Charlottesville, VA 22903, USA (*author for correspondence);*
[1] *Present address: Department of Plant Biology, The Salk Institute, San Diego, CA 92186*

Received 17 July 1995; accepted in revised form 20 September 1995

Key words: *Chlamydomonas*, chlorophyll biosynthesis, gene expression, protochlorophyllide reductase

Abstract

The *pc-1* mutant of *Chlamydomonas reinhardtii* has been shown to be incapable of protochlorophyllide photoconversion *in vivo* and is thought to be defective in light-dependent NADPH:protochlorophyllide oxidoreductase activity. We have isolated and characterized the nuclear genes encoding this enzyme from wild-type and *pc-1* mutant *Chlamydomonas* cells. The wild-type *CRlpcr-1* gene encodes a 397 amino acid polypeptide of which the N-terminal 57 residues comprise the chloroplast transit sequence. The *Chlamydomonas* protochlorophyllide reductase has 66–70% identity (79–82% similarity) to the higher plant enzymes. Transcripts encoding protochlorophyllide reductase are abundant in dark-grown wild-type cells, but absent or at very low levels in cells grown in the light. Similarily, immunoreactive protochlorophyllide reductase protein is also present to a greater extent in dark- versus light-grown wild-type cells. Both *pc-1* and *pc-1 y-7* cells lack *CRlpcr-1* mRNA and the major (36 kDa) immunodetectable form of protochlorophyllide reductase consistent with their inability to photoreduce protochlorophyllide. DNA sequence analysis revealed that the *lpcr* gene in *pc-1 y-7* cells contains a two-nucleotide deletion within the fourth and fifth codons of the protochlorophyllide reductase precursor that causes a shift in the reading frame and results in premature termination of translation. The absence of protochlorophyllide reductase message in *pc-1* and *pc-1 y-7* cells is likely the consequence of this frameshift mutation in the *lpcr* gene. Introduction of the *CRlpcr-1* gene into *pc-1 y-7* cells by nuclear transformation was sufficient to restore the wild-type phenotype. Transformants contained both protochlorophyllide reductase mRNA and immunodetectable enzyme protein. These studies demonstrate that *pc-1* was in fact a defect in protochlorophyllide reductase activity and provide the first *in vivo* molecular evidence that the *lpcr* gene product is essential for light-dependent protochlorophyllide reduction.

Introduction

The reduction of protochlorophyllide (pchlide) to chlorophyllide is a key regulatory step in chlorophyll formation and the overall processes of chloroplast development and photomorphogenesis. In most higher plants and some algae the reduction of pchlide is a light-dependent reaction catalyzed

The nucleotide sequences data reported will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U36752.

by a nuclear-encoded, cytoplasmically synthesized enzyme known as NADPH:protochlorophyllide oxidoreductase (pchlide reductase; EC 1.6.99.1) [2, 30]. The structure of this enzyme and the gene(s) that encode it (designated *lpcr*) have been characterized from a variety of higher plants, including both angiosperms [8, 14, 37, 74, 78, 82] and gymnosperms [24, 79]. A considerable amount of information is present in the literature describing the manner in which the synthesis, abundance, and activity of this enzyme are regulated by such factors as light, cell type, and plant developmental age [35, 73].

In addition to this light-dependent mechanism for pchlide reduction, photosynthetic bacteria, cyanobacteria, and some green algae and higher plants are capable of synthesizing significant amounts of chlorophyll in the dark [6]. Although the phenomenon of dark chlorophyll synthesis has been described for decades [10], the biochemistry of this process (i.e., the mechanism for light-independent pchlide reduction) and its regulation remain largely unknown. Those plant and algal species examined to date that are capable of dark chlorophyll formation appear to uniformly contain within their chloroplast genomes three genes, designated *chlL* (or *gidA*), *chlN* (or *frxC*), and *chlB* (or ORF510 or ORF563) [5], whose participation has been implicated in this process. For example, in *Chlamydomonas reinhardtii* it has been demonstrated that mutations in any one of these three loci (e.g., *chlL*, *chlN*, or *chlB*) results in the loss of chlorophyll formation in the dark and the accumulation of the biosynthetic intermediate ppchlide within the cells [11, 50, 51, 70, 80]. Although supporting biochemical data are lacking at this time, these mutagenesis data have been taken as evidence for a catalytic role for the products of these genes in this crucial step of chlorophyll synthesis.

There is also evidence indicating that in addition to these three chloroplast genes, nuclear-encoded factors may also be involved in light-independent chlorophyll formation [6]. At least seven independent loci have been mapped in *C. reinhardtii* that affect light-independent pchlide reduction [19, 20, 71]. These mutations cause the so-called 'yellow-in-the-dark' or *y* phenotype in which cells lack the capacity for chlorophyll formation in the dark, but are still capable of photoreducing pchlide and synthesizing chlorophyll in the light. The role of the products of the *y* loci in light-independent pchlide reduction is presently unknown. There is, however, ample evidence that nuclear-encoded factors are involved in various steps of chloroplast gene expression (e.g., *trans* splicing, mRNA stability, and translation), assembly of plastid-localized complexes, or cofactor formation [68].

Mutagenesis of *y-1–4*, a temperature-sensitive allele of the *C. reinhardtii* yellow mutant *y-1*, led to the identification of strains that were defective in pchlide photoconversion *in vivo* [21]. The mutations present in these strains all mapped to a single locus that was designated *pc-1*. Although defective in light-dependent pchlide reduction, *pc-1* cells retain the capacity for light-independent chlorophyll formation and synthesize about 52% of wild-type levels of chlorophyll in the dark and ca. 36% of the wild-type chlorophyll levels in the light. The molecular nature of the *pc-1* mutation is not known but has been suggested to be a defect in the pchlide reductase [22]. Only one other mutant in light-dependent pchlide reduction has been previously reported, the *L-6* mutant of *Arabidopsis* [67], although the nature of the defect in this organism was never characterized.

In the past several years there have been an increasing number of reports describing the successful identification of nuclear genes in *Chlamydomonas* using mutant complementation by nuclear transformation [17, 58, 65, 66, 76]. In this study we report the isolation and characterization of the nuclear gene encoding the light-dependent pchlide reductase from *C. reinhardtii*. We present the expression characteristics of this gene in light- and dark-grown wild-type and mutant cellular backgrounds. We have also determined the molecular basis for the *pc-1* phenotype and demonstrated that this mutation can be complemented by expression of the wild-type gene in the mutant background. Our results are discussed in relation to the mechanism by which the expression of the

light-dependent and light-independent pchlide reductase activities are integrated and regulated.

Materials and methods

Algal strains and cell culture conditions

Wild-type *C. reinhardtii* cells, strain c137, were obtained from Michel Goldschmidt-Clermont (University of Geneva, Switzerland); *y-7* and *pc-1* were obtained from Elizabeth Harris (The *Chlamydomonas* Genetic Stock Center, Duke University, Durham, NC); and *pc-1 y-7* was provided by Wei-yei Wang (University of Iowa, Iowa City). Cells were grown either in Tris-acetate phosphate (TAP) medium [33] at 28 °C on a gyratory shaker at 200 rpm under constant light, or on agar-solidified TAP medium plates as described in Li *et al.* [50]. For the preparation of dark-grown wild-type and mutant cells, 50–100 ml of TAP medium was inoculated with light-grown cells from TAP agar plates. The cultures were grown for 2 days in the light and then wrapped with two layers of aluminum foil and grown in darkness for 6 days. The cells were harvested by centrifugation at $8000 \times g$. When necessary, dark-grown cultures were handled under dim green safelights.

Genomic phage isolation and characterization

About ten genome equivalents (10^5 phage) of an amplified *C. reinhardtii* λ-EMBL3 genomic DNA library prepared as five independent sublibraries [28] were screened by plaque hybridization [72] using a random-primed [18] ^{32}P-labeled fragment from pWPnPCR-901, a cDNA that encodes the C-terminal portion of the white pine pchlide reductase [79]. For plaque screening, nylon filters were prehybridized in buffer [13] containing 500 mM sodium phosphate buffer pH 7.2, 1 mM Na_2-EDTA, 7.0% (w/v) SDS, and 1.0% (w/v) BSA, and hybridized in the same buffer with $1–2 \times 10^6$ cpm/ml of radiolabeled probe for 16–24 h. The filters were washed twice for 30 min in $0.1 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 6.8), 0.1% (w/v) SDS at 23° C, blotted dry, wrapped in saran wrap, and exposed to X-ray film with a Cronex intensifying screen at −80° C for 24–36 h.

Several hybridizing phage were isolated from each sublibrary and further purified by two additional rounds of screening. DNA was prepared from five independent phage, one derived from each of the five sublibraries, and subjected to restriction mapping and DNA blot hybridization analyses [72] as shown in Fig. 1. One phage (φCG3b) exhibited only weak hybridization signals and was not further characterized; the remaining four phage (φCG1b, φCG2b, φCG4b, and φCG5b) were used in our subsequent studies.

The relevant coding and immediate flanking regions of the *C. reinhardtii lpcr* gene were localized in each phage and these regions were subcloned into the appropriate sites within the polylinker region of pBluescriptKS(−) plasmid vector: the 2.0 kb *Hind* III-*Sal* I fragment of φCG1b; the 2.0 kb *Sal* I-*Xho* I fragment of φCG2b; the 3.5 kb *Hind III-Sal* I fragment of φCG4b; and the 2.5 kb *Sal* I-*Xho* I fragment of φCG5b. The subcloned DNA fragments were either completely or partially sequenced to confirm that these phage contained identical *lpcr* coding sequences. The 8.0 kb *Nhe* I-*Sal* I fragment of φCG4b was subcloned into *Pst* I-*Sal* I digested pBluescriptKS(−) vector and used to determine the nucleotide sequence of shown in Fig. 2 and for nuclear transformation experiments involving *pc-1 y-7* (see below).

Genomic DNA gel blot analysis

Total genomic DNA was isolated from *C. reinhardtii* c137 cells and purified by CsCl gradient ultracentrifugation [50, 69] with minor modifications. DNA gel blot analyses was performed as described by Li *et al.* [50]. About 10 μg of total genomic DNA were digested with various restriction enzymes, the digested DNA fragments were separated by electrophoresis on a 0.8% agarose

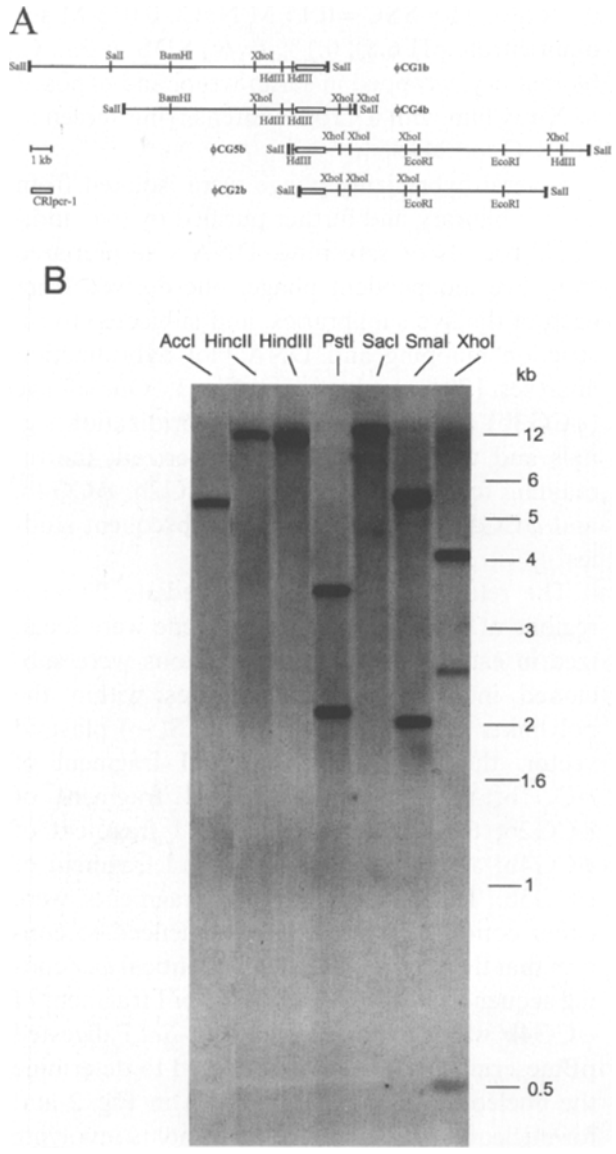

Fig. 1. Structure and organization of the *CRlpcr-1* locus in *Chlamydomonas reinhardtii*. A. Restriction maps of the four phage isolated from a screen of the *C. reinhardtii* genomic library. The maps show the location of the *CRlpcr-1*-coding sequences. The *Sal* I sites at both ends of each insert are from the cloning sites of the *Bam* HI-digested λEMBL3 vector. The open boxes denote the coding region including the two introns of the gene. The placement of restriction sites was accomplished by the analysis of DNA fragments generated from single and/or multiple restriction enzyme digests as well as hybridization patterns of these restriction fragments with the ^{32}P-labeled pine *lpcr* cDNA probe. The DNA fragments are aligned to emphasize the overlapping nature of each phage clone. B. DNA blot analysis of wild-type *C. reinhardtii* genomic DNA. Two μg of total genomic DNA was cut with each of the following restriction enzymes: *Acc* I, *Hind* III, *Sma* I, *Sst* I, *Pst* I, and *Xho* I. The resulting DNA restriction fragments were separated on a 0.8% agarose-TBE gel, transferred to a GeneScreen *Plus* nylon membrane, and hybridized to a ^{32}P-labeled 2.5 kb *Hinc*II-*Sal* I probe derived from φCG4b. Molecular weight markers are shown to the right.

gel, and transferred onto GeneScreen Plus nylon membranes (NEN-Dupont). The nylon membranes were then hybridized with a ^{32}P-labeled 2.5 kb *Hinc*II-*Sal* I fragment derived from φCG4b.

DNA sequence analyses

Dideoxynucleotide sequencing was performed on double-stranded DNA templates using Sequenase Version 2.0 according to the manufacturer's protocol (United States Biochemical). Sequencing templates consisted of subcloned restriction fragments and/or nested Exonuclease III deletion products. Most sequencing reactions were carried out with [α-^{35}S]-dATP (Amersham) and both dGTP and 7-deaza-dGTP to resolve the commonly observed compression in *C. reinhardtii* nuclear DNA. Both strands of the 4.2 kb *Kpn* I fragment of pNS8k were completely sequenced. The DNA and deduced amino acid sequences were analyzed using the GCG Sequence Analysis Software Package, Version 7.2 ([16]; Genetic Computer Group, University of Wisconsin Biotechnology Center, Madison, WI).

RNA gel blot analysis

Cells from 100 ml of culture were collected by centrifugation at 6000 × ***g*** for 5 min and immediately resuspended in 5 ml lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 15 mM EDTA, 2% w/v SDS and 40 μg/ml proteinase K). The suspension was shaken gently at room temperature for 20 min to lyse the cells and total RNA released by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1 v/v) followed by one extraction with chloroform/

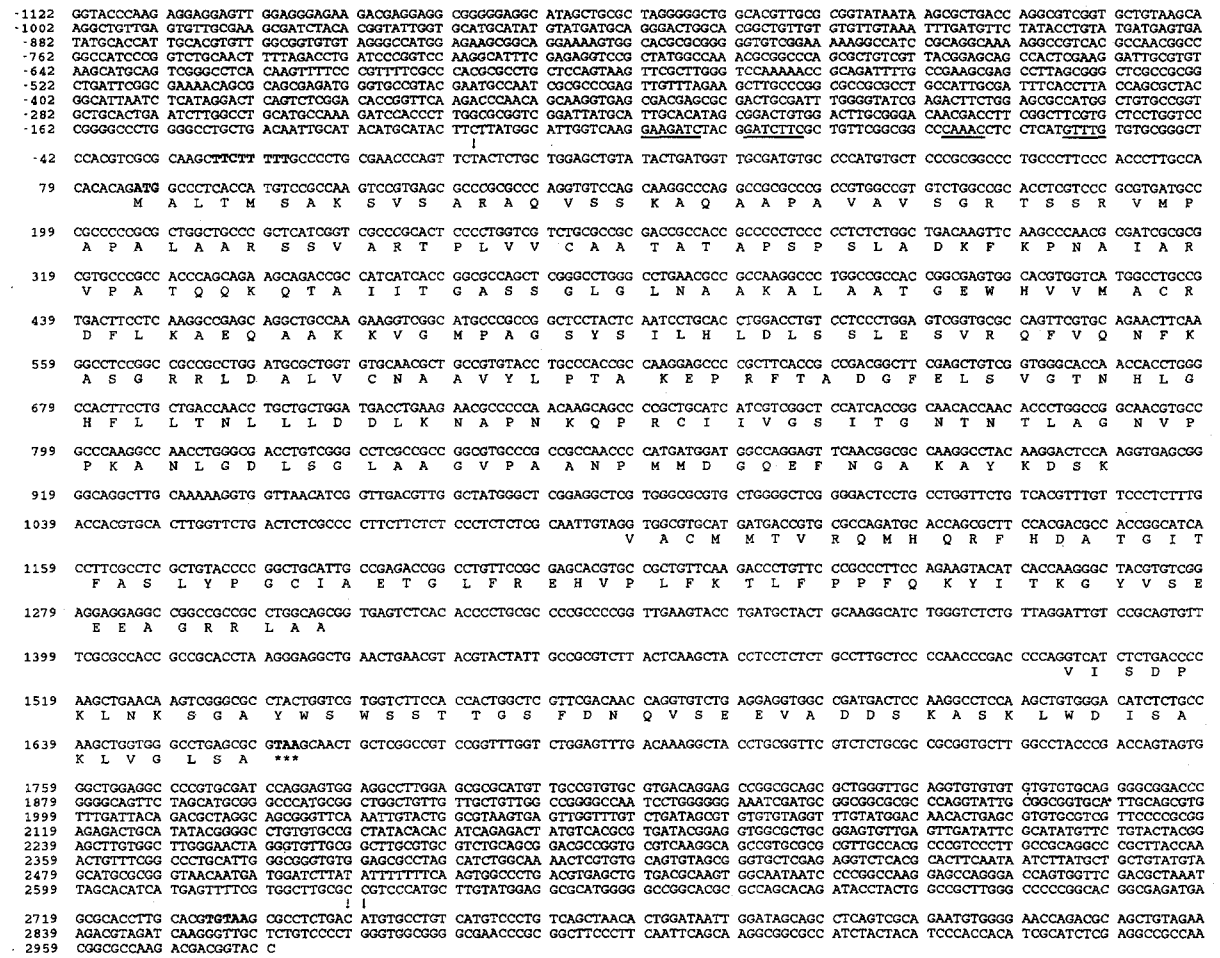

Fig. 2. Nucleotide sequence and the deduced amino acid sequence of the *C. reinhardtii CRlpcr-1* gene. Complete nucleotide sequence of the 4.2 kb *Kpn* I fragment containing the *CRlpcr-1* gene along with the deduced amino acid sequence of its encoded protein. The positions of introns were determined by codon preference analysis using the GCG Sequence Analysis Program [16] and by comparison to protein sequences previously reported for various higher plant enzymes. The numbers on the left side refer to nucleotide positions relative cap site designated as + 1 and indicated by the arrow. The numbers on the right side refer to amino acid positions relative to the assigned initiation methionine residue. Translational start and stop codons are in boldfaced type. The location of the TATA box (TTCTTTTT) and polyadenylation signals (TGTAA) are given boldfaced; the polyadenylation site is indicated by the double arrow. Two inverted repeats in the 5′-flanking region of the putative 'TATA box' are underlined.

isoamyl alcohol (24:1). The RNA was precipitated overnight at −20 °C with 2.5 volume of ethanol, pelleted by centrifugation, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. RNA was further purified by precipitation with LiCl [72]. The final RNA precipitate was resuspended in a minimal volume of DEPC-treated water and its concentration determined spectrophotometrically. RNA gel blot analysis was carried out as described by Li *et al.* [50].

Mapping the 5′ and 3′ ends of the CRlpcr-1 *transcript*

The 5′ end of the *CRlpcr-1* transcript was determined by primer extension analysis using an oligonucleotide primer (K1310R, 5′-ATCA-CGCGGGACGAGGTG-3′) complementary to nucleotides 178 to 195 that was radiolabeled with ^{32}P-ATP in the presence of T4 polynucleotide kinase (Gibco-BRL). The primer extension reac-

tion contained 5 ng of the purified radiolabeled primer, 10 μg of DNase-treated total cellular RNA in Moloney murine leukemia virus reverse transcriptase reaction buffer (Gibco-BRL) in a total volume of 8.5 μl. The mixture was heated at 90 °C for 2 min, followed by 10 min at 65 °C, and then slowly cooled to 37 °C within 60 min. The annealing reaction was mixed with 4 μl of solution containing 2.5 mM each dNTP, 1.5 μl of 100 mM DTT, and 200 units of SuperScript RNaseH-reverse transcriptase (Gibco-BRL), and the reaction mixture was incubated at 42 °C for 60 min. EDTA and RNase A were added at final concentrations of 20 mM and 20 μg/ml, respectively, and the reaction was stopped by incubating the reaction mixture at 37 °C for 30 min. The reaction products were extracted with one volume of phenol/chloroform (1:1) followed by one volume of chloroform, and precipitated with two volume of ethanol at −20 °C overnight. One half of the extension products was analyzed on a 6% polyacrylamide sequencing gel and visualized by autoradiography. A sequencing reaction using the unlabelled extension primer was run in parallel as a reference to determine the size of the extended product.

For determination of the 3′ end of the *CRlpcr-1* transcript, rapid amplification of cDNA ends (RACE) [26] was performed using the RACE System (Gibco-BRL, Gaithersburg, MD). First-strand cDNA synthesis was carried out in a reaction cocktail containing 10 μg of total cellular RNA isolated from dark-grown cells, 5 ng of poly-d(T)-adapter primer [5′-GGTCGACGC-GGCCGCTCTAGA$(T)_{17}$-3′] (Gibco-BRL), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 400 μM each of dNTP, and 8 units of SuperScript II reverse transcriptase. The reaction was allowed to procede for 30 min at 30 °C and the reaction products were then purified on a GlassMAX DNA isolation spin cartridge. The purified first-strand cDNA was diluted 20-fold and 1 μl of the diluted cDNA was used in amplification reactions containing *Taq* Extender buffer (Stratagene), 200 μM dNTPs, 1.25–2.5% (v/v) DMSO, 2.5 units of *Taq* polymerase (Boehringer Mannheim), 2.5 units of *Taq* Extender (Stratagene) and 100 ng each of the poly(dT)-adapter primer (Gibco-BRL) and a gene-specific primer (K3000F, 5′-GACCCGG-GGCAGTTCTA-3′). The amplification conditions for the first round of the PCR were 10 min denaturation at 95 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 45 s, ending with a 10 min extension at 72 °C. One microliter of a 50-fold dilution of the amplification products were reamplified as described above except that (K3640, 5′-AGTGGCCCTGACGTGAGC-3′) was used as the gene-specific primer and the annealing temperature was raised to 60 °C. Following treatment with the Klenow fragment of DNA polymerase I, the PCR reaction products were separated on a 1% low-melting-temperature agarose gel (FMC BioProducts) and the major band at ca. 300 bp was excised and subcloned into the *Eco* RV site of pBluescriptKS(−). Individual clones were isolated and sequenced as described above.

Protein extraction and immunoblot analysis

Cell cultures were grown to mid-logarithmic phase (approximately $2–6 \times 10^6$ cells/ml), collected by centrifugation at $7500 \times g$ for 5 min at 4 °C, and resuspended in 50 mM Tris-HCl pH 6.8, 2% (w/v) SDS. The cell suspension was disrupted by sonication (3×15 s at 70% maximum power) with a Sonifier cell disrupter (Model W185, Heat System-Ultrasonic). The homogenate was clarified by centrifugation at $10000 \times g$ for 10 min at 4 °C, and the resulting supernatant was collected as total cellular protein extract. The protein concentration of the supernatant was measured using bicinchoninic acid (BCA) method (Pierce Chemicals).

Aliquots of the supernatant were mixed with an equal volume of gel loading buffer (50 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.005% (w/v) Bromophenol Blue), boiled for 5 min, and centrifuged for 5 min at $14000 \times g$ to pellet any undissolved materials. Protein samples were then separated on

12% (w/v) SDS-polyacrylamide gels, electroblotted to nitrocellulose membranes (Schleicher & Schuell) [35] and the membranes blocked in a solution of 5% (w/v) Carnation non-fat dry milk, 0.01% (v/v) Tween-20 in Tris-buffered saline (TBS is 20 mM Tris-HCl pH 7.5, 500 mM NaCl) for 1 h at room temperature. After blocking, the membranes were washed three times with 0.01% (v/v) Tween-20 in TBS and incubated for 10 hours at room temperature with polyclonal antibodies raised against the purified wheat pchlide reductase (obtained from W. Trevor Griffiths, University of Bristol, UK) in TBS containing 5% (w/v) carnation non-fat dry milk and 0.01% (v/v) Tween-20. The membranes were then washed three times in TBS containing 0.01% (v/v) Tween-20 and incubated overnight at 4 °C with horseradish peroxidase-conjugated goat antirabbit-IgG secondary antibody (Amersham) in TBS containing 5% (w/v) carnation non-fat dry milk and 0.01% (v/v) Tween-20. Following three additional washes as described above, antigen-antibody complexes were detected using the Enhanced Chemiluminescence Detection kit (Amersham).

Transformation of pc-1 y-7 *cells*

Glass-bead transformation of *pc-1 y-7* was carried out essentially as described by Kindle [44]. Liquid cultures (100 ml) of *pc-1 y-7* were grown in TAP medium in total darkness to a cell density of ca. $1-2 \times 10^6$ cells/ml. The cells were collected by centrifugation at $3000 \times g$, washed once with fresh TAP medium, and resuspended to a density of 7×10^7 cells/ml with TAP medium. The cell suspension (0.5 ml) was transferred to a 15 ml conical tube containing 0.5 g of sterile acid-washed glass beads (710–1180 μm diameter; Sigma). A 5 μg portion of pNS8k DNA linearized by digestion with *Xba* I was added to the cell suspension followed by the addition of 65 μl of 40% (w/v) polyethylene glycol 8000 (PEG 8000). The cells were then mixed using a Vortex Genie II at maximum speed for 30–60 seconds and then spread onto Petri plates containing TAP medium solidified with agar. Cells were grown under dim white light and independent transformants selected by their green-in-the-light phenotypes and ability to grow in the light. Individual colonies were streaked on fresh TAP agar plates, single colonies were picked and restreaked in the same manner. After four rounds of subculturing, single colonies were selected and used for the various analyses described below. All transformants are maintained on TAP agar plates under room light.

Characterization of the lpcr *gene from* pc-1 y-7 *cells*

The complete *lpcr* gene in *pc-1 y-7* cells was isolated by PCR amplification of four overlapping fragments of genomic DNA. The following primers were used in the amplification reactions: K001F, 5′-GTGGTACCCAAGAGAGGAGG-3′; K1310R, 5′-ATCACGCGGGACGAGG-TG-3′; K1120F, 5′-TCTACTCTGCTGGAG-CTG-3′; K2160R, 5′-AACCAAGTGCACG-TGGTC-3′; K1990F, 5′-GGATGGCCAGGA-GTTCAA-3′; K3260R, 5′-GTGGTATAGCG-GCACACAGG-3′; K2690F, 5′-TCGTTCGA-CAACCAGGTGTC-3′; K3890R, 5′-GGA-CATGACAGGCACATG-3′. Amplification reactions contained ca. 50–100 ng total *pc-1 y-7* genomic DNA, in *Taq* Extender buffer (Stratagene) with 200 μM of each dNTP, 125 ng of each forward and reverse primers, 1.25–2.5% (v/v) DMSO, 2.5 units of *Taq* polymerase (Boehringer Mannheim) and 2.5 units of *Taq* Extender (Stratagene). The amplification conditions were: 10 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 90 s, and a final extension period of 10 min at 72 °C. The amplification products were treated with the Klenow fragment of DNA polymerase I (Boehringer Mannheim), purified by agarose gel electrophoresis, and ligated into the *Eco* RV site of pBluescript KS(−). In order to minimize any errors in nucleotide sequence resulting from misincorporation mutations during the amplification reactions, plasmid DNA was prepared from sev-

eral independent transformants representing each genomic fragment amplified, and these templates were used in subsequent DNA sequence analysis as described above.

Hybridization screening of a partial library of pc-1 y-7 *genomic DNA*

Total Genomic DNA was isolated and purified from *pc-1 y-7* as described above, digested to completion with *Xho* I and *Cla* I and fractionated on a 1% (w/v) low melt agarose gel. The fraction of *pc-1 y-7* DNA with the same mobility as the 4.5 kb *Xho* I-*Cla* I fragment of pNS8k was excised, eluted from the gel, and ligated into pBluescript KS(−) vector previously digested with *Xho* I and *Cla* I. The ligation mixture was transformed into competent DH5α cells (Gibco-BRL) and the resulting transformants screened by colony hybridization [72] using the ^{32}P-labeled 4.5 kb *Xho* I-*Cla* I fragment of pNS8k as probe. Plasmids containing the *pc-1 y-7 lpcr* coding sequences were subjected to DNA sequence analysis as described above.

Results

Structure of the nuclear gene for protochlorophyllide reductase in wild-type Chlamydomonas

A *C. reinhardtii* genomic DNA library constructed in λ-EMBL3 was screened by plaque hybridization using a random-primed ^{32}P-labeled cDNA (pWPnPCR901) encoding the light-dependent pchlide reductase from white pine, *Pinus strobus* [79], as probe. Four phage, designated φCG1b, φCG2b, φCG4b, and φCG5b, were isolated and shown by restriction mapping and DNA gel blot analysis to contain nuclear genomic sequences encoding pchlide reductase. As shown in Fig. 1A, the DNA fragments contained in the four phage were partially overlapping and contiguous over an ca. 40 kb region of the genome. Consistent with our analysis of the phage DNA, genomic DNA gel blot analysis, shown in Fig. 1B, revealed that only a single gene (which we designated *CRlpcr-1*) encodes the pchlide reductase in *Chlamydomonas*. An 8.0 kb *Nhe* I-*Sal* I fragment from φCG4b containing the entire *CRlpcr-1* gene was subcloned into the pBluescriptKS(−) plasmid vector yielding plasmid pNS8k and this plasmid was subsequently used in the analyses described below.

The nucleotide sequences of the relevant coding and non-coding portions of the *C. reinhardtii CRlpcr-1* gene contained on the 4.2 kb *Kpn* I fragment of pNS8k were determined and are presented in Fig. 2 along with the deduced amino acid sequence of the encoded protein. *CRlpcr-1* encodes a 397 amino acid polypeptide (M_r ca. 42000) of which the first 57 amino acids of the deduced protein sequence comprise the chloroplast transit peptide required for import of the cytoplasmically synthesized precursor into the developing plastid. The transit peptide of pchlide reductase shares several features in common with transit peptides of other nuclear-encoded precursors of *Chlamydomonas* chloroplast proteins including a short uncharged segment at its amino-terminus, a central region rich in hydroxy-groups, small hydrophobic residues, and positively charged amino acids, a carboxy-terminal region that may form an amphiphilic β-strand and a tetrapeptide, V-C-A-A, that matches the loosely conserved consensus cleavage site (V/I)-X-(A/I)↓A [25]. Based on the assigned transit peptide cleavage site, the mature *C. reinhardtii* pchlide reductase protein contains 337 amino acids, has a M_r of ca. 36000 and a pI of 9.4, similar to values reported for the mature pchlide reductase protein of higher plants.

The results of pair-wise comparison of the deduced amino acid sequence of the *C. reinhardtii* pchlide reductase with those of higher plants (e.g., *Arabidopsis* [8], barley [74], oat [14], pea [78], loblolly pine [79] and wheat [82]) and the cyanobacterium *Synechocystis* [81] are presented in Table 1. A high degree of sequence conservation exists between the mature algal enzyme and those of higher plants with values for amino acid sequence identity and similarity of 65–70% and 79–82%, respectively. Less homology is observed

Table 1. Identity (%) and similarity (%) among protochlorophyllide reductases from various organisms.

	Chlamydomonas	*Synechocystis*	*H. vulgare*	*T. aestivum*	*A. sativa*	*P. sativum*	*A. thaliana*	*P. taeda*
Chlamydomonas	100 (100)	52 (72)	65 (79)	65 (79)	66 (80)	69 (82)	70 (82)	70 (82)
Synechocystis		100 (100)	54 (74)	53 (72)	52 (73)	56 (74)	56 (74)	54 (74)
Hordeum vulgare			100 (100)	99 (99)	97 (98)	84 (91)	82 (91)	79 (89)
Triticum aestivum				100 (100)	97 (98)	85 (93)	82 (91)	80 (90)
Avena sativa					100 (100)	85 (93)	83 (92)	80 (90)
Pisum sativum						100 (100)	89 (95)	86 (95)
Arabidopsis thaliana							100 (100)	85 (94)
Pinus taeda								100 (100)

The deduced amino acid sequence of the mature protochlorophyllide reductase protein encocded by *CRlpcr-1* was compared with those of higher plants. Sequence alignment and analysis was performed using the PILEUP program in the GCG Sequence Analysis package [16]. Given are the percentages of identical and similar (in parenthesis) residues over the mature protein. Protein sequences used in the analysis correspond to the following GenBank accession numbers: *Synechocystis*, L37783; pea, X63060; loblolly pine (P. *taeda*), X66727; *Arabidopsis*, ATTS1128; barley, X15869; oat, X17067; wheat, TAPWR5PI.

with the cyanobacterial enzyme (i.e., 52% identity and 72% similarity). Very low or no sequence similarities were observed in the transit peptide and the very N-terminal regions of the mature protein when the *C. reinhardtii* and higher plant pchlide reductases were compared. Like its higher-plant counterparts, the algal pchlide reductase has a large number of basic amino acids and a relatively high proportion of hydrophobic residues. The four cysteine residues almost uniformly conserved in the higher plant and cyanobacterial enzymes [73, 78, 81] are also present in the *C. reinhardtii* enzyme, as are the tyrosine and lysine residues recently defined as part of the active site in the enzyme [85].

The mature protein-coding region of the gene is interrupted by two introns that are 179 and 196 nucleotides (nt) in length, respectively. The first intron (Intron I) occurs between codons 275 and 276 and the second (Intron II) between codons 345 and 346. The nucleotide sequences at the exon/intron boundaries conform closely with the consensus (XG¦¦GTG(A/C)G(T/C)---intron---CAG¦¦GC) *C. reinhardtii* splice junctions [86]. While the location of the Intron I is not conserved with respect to those found in the higher plant *lpcr* genes, the placement of Intron II appears to be conserved in all plant and algal species thus far examined, including angiosperms and gymnosperms.

The 5′ end of the mRNA transcribed from the *CRlpcr-1* gene was determined by primer extension analysis using total RNA isolated from dark-grown wild-type cells and an ^{32}P-labeled oligonucleotide primer complementary to nucleotides 178–195. As shown in Fig. 3A, only one major extension product was generated and this product terminated at a T residue 85 nt 5′ to the assigned initiation ATG codon. A potential TATA box [29, 38] followed by a G + C-rich segment can be found 27 nt upstream of the start site of the *CRlpcr-1* transcript (see Fig. 2). Upstream of the TATA box are two inverted repeats at positions −102 to −85 (5′-GAAGATC....G-ATCTTC-3′) and −70 to −53 (5′-CAAA-C....GTTTG-3′) that are similar to two inverted repeats (GAAGTC....GACTTC and CAAAC....GTTTG) found at similar positions in the promoter of pea *lpcr* gene [78]. A functional role for these sequences in the regulation of the *CRlpcr-1* expression remains to be investigated.

The *CRlpcr-1* transcript contains an exceptionally long 3′-UTR extending approximately 1106 nts from the stop codon. As shown in Fig. 3B, the polyadenylation site is located 11–12 nt downstream of a concensus polyadenylation signal, TGTAA [75]. Note that the first A residue of the poly(A)-tail may be encoded by the gene or added by polyadenylation. Long 3′-UTR have been previously noted in a number of *Chlamydomonas* nuclear transcripts [15, 29, 36, 56] although their importance in influencing

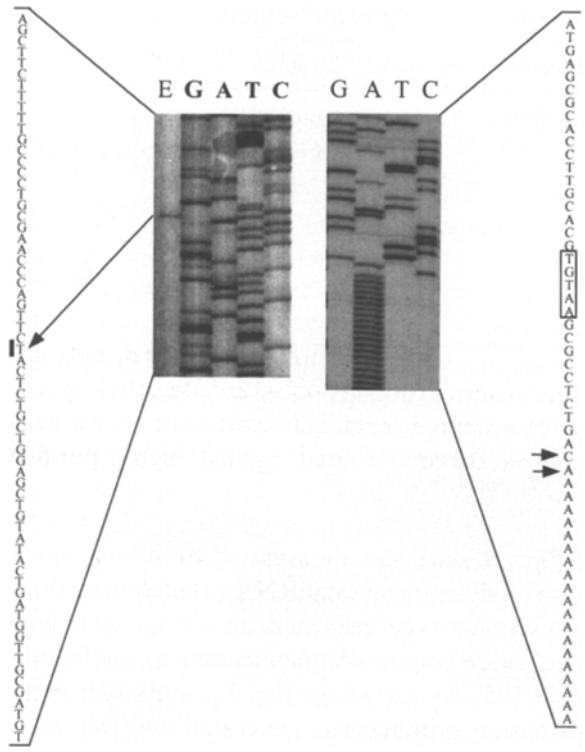

Fig. 3. Mapping of the 5′ and 3′ ends of the *CRlpcr-1* transcript. A. The products of primer extension analysis used to determine the 5′ end of the *CRlpcr-1* transcript. Total RNA isolated from dark-grown wild-type cells was annealed to a ^{32}P-labeled oligonucleotide complementary to nucleotides 178–195 (see Fig. 1) and the primer was extended with reverse transcriptase. The extension products (lane E) were analyzed on a 6% polyacrylamide sequencing gel using DNA sequencing reactions (lanes G, A, T, and C) of the plasmid pNS8k with the same primer as molecular weight standards to determine the exact size of the extended products. The sequence shown to the left corresponds the displayed portion of the pNS8k sequence reactions and the T residue marked by a short vertical bar and an arrow corresponds to the extension product. The boxed 8 nucleotides (TTCTTTTT) comprise the proposed TATA box. B. 3′ RACE analysis was carried out as described in Materials and methods. The specific products were subcloned into the *Eco* RV site of pBluescriptKS(−) vector and their nucleotide sequences determined. Shown is autoradiograph of the nucleotide sequence at the 3′ end of one cDNA spanning the polyadenylation site. The nucleotide ladder shown is from *CRlpcr-1* and the arrows indicate the proposed 3′ end of the *CRlpcr-1* transcript. The proposed polyadenylation signal (TGTAA) is boxed.

rRNA stability is still unknown. No sequences capable of forming extended secondary structures (e.g., stem-loops) are readily apparent in the 3′-UTR of the *CRlpcr-1* transcript and codon-preference analysis did not identify any open reading frames in either the sense or anti-sense orientations within this region of the transcript. The 3′-UTR of the *CRlpcr-1* transcript does, however, contain seven GT repeats located 189 bp downstream of the TAA stop codon. Such GT repeats have been recognized in the 3′-UTRs of other *C. reinhardtii* nuclear transcripts (e.g., in the *rbcS1* [29] and *CAH1* genes [27]) and are thought to have potential regulatory properties.

Abundance of protochlorophyllide reductase mRNA and protein in wild-type and mutant cells

The steady-state levels of mRNA encoding the pchlide reductase were examined in dark- and light-grown wild-type and mutant *C. reinhardtii* cells by RNA gel blot analysis using a random-primed ^{32}P-labeled 1.7 kb *Sac* II fragment containing the entire coding sequence of the *CRlpcr-1* gene as probe. As shown in Fig. 4A, a ca. 2.3 kb transcript, corresponding to the predicted size of the *CRlpcr-1* transcript, is easily detected in total RNA extracted from dark-grown cells, whereas little or no *CRlpcr-1* mRNA is detectable in total RNA prepared from cells grown in the light. The negative effect of light on steady-state *CRlpcr-1* transcript levels is similar to that observed in some monocot species where a dramatic reduction in *lpcr* transcript levels has been shown to occur upon illumination of etiolated leaf tissues with light [1, 4, 23, 57].

The *pc-1* mutation has been proposed to result from a structural defect in light-dependent pchlide reductase activity since cells harboring this mutation fail to reduce pchlide in the light, even though they reportedly assemble pigment-protein complexes normally associated with pchlide photoconversion activities [22]. The effects of *pc-1* are best observed in cells containing a *y* mutation (e.g., *pc-1 y-7*), since these strains are incapable of reducing pchlide independent of light availability, and therefore appear yellow in the dark, and die upon exposure to light [22].

To further characterize the nature of the defect

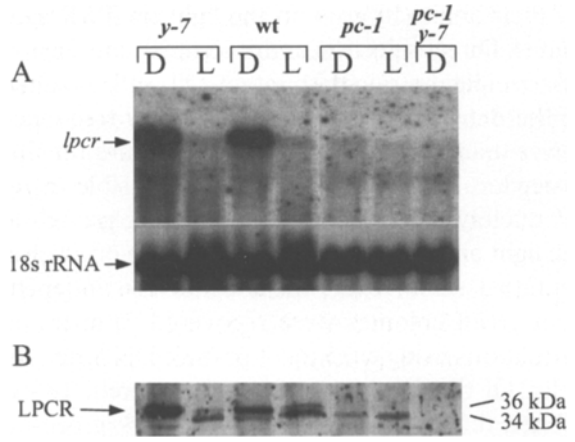

Fig. 4. Analysis of protochlorophyllide reductase message and protein levels in wild-type and mutant cells. A. Results of RNA blot analysis of total RNA isolated from dark- and light-grown wild-type and *y-7*, *pc-1*, and *pc-1 y-7 C. reinhardtii* cells. Ca. 30 μg of total RNA per sample was separated on a 1% agarose-formaldehyde gel, blotted to nitrocellulose filters, and hybridized with a ^{32}P-labeled 1.7 kb *Sac* II fragment from pNS8k. Approximate sizes in kb are given on the left. After removal of the *lpcr* probe, the same filter was hybridized with a ^{32}P-labeled 18S rRNA to demonstrate that equal amount of total RNA was loaded into each lane. B. Shown are the results of immunoblot analysis of protochlorophyllide reductase protein levels in dark- and light- grown wild-type and mutant cells. Total cellular proteins were extracted from dark- (D) and light-grown (L) cells, and equivalent amounts (ca. 100 μg) were fractionated on a 12% (w/v) SDS-polyacrylamide gel, electrophoretically transferred to a nitrocellulose filter, and reacted with antiserum to the wheat protochlorophyllide reductase. The antigen-antibody complexes were reacted with anti-rabbit secondary antibodies conjugated with horseradish peroxidase, and visualized by chemiluminesence as described in the Materials and methods. The immunoreactive 36 kDa and 34 kDa polypeptides are indicated by arrows. The size of the immunoreactive proteins was measured against known standard proteins of 18 kDa, 29 kDa, and 43 kDa.

associated with the *pc-1* genotype, total RNA was isolated from either dark- or light-grown *pc-1*, *y-7*, and *pc-1 y-7* cells and pchlide reductase message levels were analyzed by RNA gel blot analysis as described above. Dark-grown *y-7* cells accumulated approximately wild-type levels of pchlide reductase mRNA, whereas dark-grown *pc-1* and *pc-1 y-7* cells contained either no detectable pchlide reductase transcripts or extremely low levels of transcript detectable only after prolonged autoradiography (Fig. 4A). Like wild-type cells, *y-7* and *pc-1* cells do not accumulate pchlide reductase mRNA when grown in the light. Since the *pc-1 y-7* double mutant dies upon exposure to light it was only possible to measure transcript levels in the dark for this strain.

In contrast to the dramatic difference in transcript abundance observed between dark- and light-grown wild-type *C. reinhardtii* cells, the effects of light on pchlide reductase protein levels are more subtle. As shown in Fig. 4B, two immunoreactive polypeptides are detected in extracts prepared from light- and dark-grown cells using antiserum elicited against highly purified wheat pchlide reductase: a 36 kDa protein, which corresponds to the predicted size of the mature pchlide reductase encoded in *CRlpcr-1*, and a smaller 34 kDa protein. The immunoreactive 36 kDa form of the protein accumulated to high levels in dark-grown cells and its abundance was significantly reduced in response to growth in the light. On the other hand, the amount of the 34 kDa polypeptide was low in dark-grown cells and its levels increased slightly upon transfer of the cells to the light. No 36 kDa pchlide reductase protein could be immunodetected in either dark- or light-grown *pc-1* cells or in *pc-1 y-7* cells grown in the dark. *pc-1* cells did, however, contain very low levels of the immunoreactive 34 kDa polypeptide and, like wild-type cells, its abundance appeared to be slightly enhanced by growth in the light. Similar to wild-type cells, *y-7* cells accumulated both the immunoreactive 36 kDa and 34 kDa polypeptides when grown in the light. Dark-grown *y-7* cells, however, showed substantially higher levels of the 36 kDa polypeptide relative to that found in wild-type cells and little or no detectable 34 kDa protein. The greater accumulation of the 36 kDa polypeptide in dark-grown *y-7* cells compared to wild-type cells could result in part from of an increased availability of pchlide for the formation of ternary complexes by the light-dependent enzyme (i.e., enzyme, NADPH, and pchlide) [61] as a consequence of the failure of *y-7* cells to utilize pchlide in light-independent reduction reactions [19, 20].

The lack of any immunodetectable pchlide re-

ductase protein in *pc-1 y-7* is not consistent with the results of the cell fractionation studies of Ford *et al.* [22] which suggested that inactive pchlide reductase protein was present in these cells. Our results showing the presence of small amounts of the 34 kDa protein in *pc-1* cells but not *pc-1 y-7* cells raises the question of the nature and origin of this immunoreactive polypeptide both in the mutant and in wild-type cells. Since genomic DNA gel blot analysis clearly showed that only a single gene encodes the pchlide reductase in the *C. reinhardtii* genome, several explanations are possible. One explanation is that the 34 kDa polypeptide may represent a size variant derived by either differential processing during or after import of the pchlide reductase precursor into the plastid, secondary modification of the mature protein, or proteolysis during extraction. Alternatively, the immunoreactive 34 kDa polypeptide may not be pchlide reductase, but rather a structurally related protein capable of cross-reacting with the anti-wheat pchlide reductase polyclonal antibodies.

The pc-1 *phenotype can be rescued by transformation of* pc-1 y-7 *with the* CRlpcr-1 *gene*

To determine whether the *pc-1* phenotype resulted solely from a structural defect in the gene encoding pchlide reductase, we tested whether our cloned wild-type *CRlpcr-1* gene could function in a *pc-1* background and was sufficient to rescue the mutant phenotype. Since *pc-1 y-7* cells lack both light-dependent and light-independent pchlide reduction activities, are phenotypically yellow-in-the-dark and incapable of growth in the light [21], we reasoned that working in this double mutant background would facilitate the direct selection of transformants. Successful complementation of the *pc-1* mutation would be expected to yield transformants which remain yellow-in-the-dark (by virtue of the presence of *y-7*) but green upon transfer to the light.

Linearized pNS8k DNA containing the complete *CRlpcr-1* gene was transformed into *pc-1 y-7* cells [44] and transformed cells were selected for by their ability to grow in the light on TAP agar plates. Direct selection in this manner and the use of a recipient strain with intact cell walls resulted in the detection of transformants at a frequency lower than that reported in the literature for this procedure [44]. However, it was possible to reproducibly recover colonies exhibiting growth in the light and a green phenotype, and in one representative set of experiments, nineteen independent green colonies were recovered from transformations using ca. 5 μg of pNS8k DNA per 10^7 cells. Of the 19 transformants recovered, 18 exhibited the expected yellow-in-the-dark/green-in-the-light phenotype. One strain, Tm208, was found to be able to green in the dark suggesting that it was likely the result of either a reversion or suppression of the *y-7* mutation which restored light-independent pchlide reduction and dark chlorophyll formation to the *pc-1 y-7* double mutant. Control transformations using pBluescript KS(–) vector DNA alone, pSH3k DNA (a plasmid containing a promoterless *CRlpcr-1* gene), or no DNA in the transformation resulted in the recovery of only a single green colony. Like Tm208, this strain also displayed a green-in-the-dark phenotype suggesting that it most likely arose by reversion or suppression of the *y-7* mutation.

Since it is possible to obtain a yellow-in-the-dark/green-in-the-light phenotype by a number of mechanisms (e.g., complementation, reversion, intragenic second-site mutation, or extragenic suppression of the original *pc-1* mutation) we examined the genomic DNA from the various transformants by DNA gel blot analysis for the presence of extra copies of the *lpcr* gene in their genome. As shown in Fig. 5, DNA from wild-type, *y-7*, *pc-1*, and *pc-1 y-7* contain three prominent *Pst* I fragments that hybridize with a ^{32}P-labeled 4.2 kb *Kpn* I fragment derived from pNS8k. The largest *Pst* I fragment contains the entire amino acid coding region, ca. 1.1 kb of the 5′-flanking region, and half of the 3′-untranslated region of the wild-type *lpcr* gene, while the 2.0 kb *Pst* I fragment contains the other half of the 3′-untranslated region of the gene. Genomic DNA isolated from 12 of the transformed strains (Tm202, Tm203, Tm206, Tm207, Tm209, Tm210,

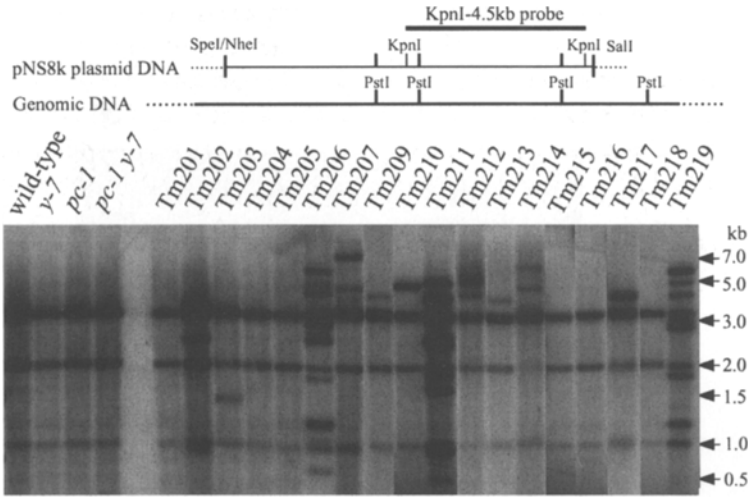

Fig. 5. Genomic DNA blot analysis of the pNS8k transformed strains. Shown are the results of gel blot analysis of total genomic DNA isolated from wild-type, *y-7*, *pc-1*, *pc-1 y-7*, and 18 different potential transformed strains exhiniting a yellow-in-the-dark/green-in-the-light phenotype. Total genomic DNA (ca. 10 μg) was digested with *Pst* I, fractionated by electrophoresis on agarose, and blotted to nylon membranes. The membrane was then hybridized with a ^{32}P-labeled 4.2 kb *Kpn* I fragment isolated from pNS8k. Three *Pst* I fragments (indicated by arrows) are detected in wild-type, *y-7*, *pc-1*, or *pc-1 y-7* cells. Transformants are designated as Tm201–219.

Tm211, Tm212, Tm213, Tm214, Tm217 and Tm219) clearly contained *Pst* I band(s) in addition to the three bands associated with the wild-type genome. Some transformed strains, such as Tm206, Tm211, and Tm219, contained six or more extra *Pst* I fragments, suggesting that multiple copies of the wild-type *CRlpcr-1* gene were inserted into their genomes. No additional hybridizing bands were detected in the other 6 transformants (Tm201, Tm204, Tm205, Tm215, Tm216, and Tm218). Although it is possible that these transformants arose via homologous recombination at the *pc-1* locus, the frequency of such events are expected to be extremely low relative to random insertion events using this transformation method [44, 77]. More likely, these cell lines arose by intragenic second-site mutation, reversion, or extragenic suppression of the original *pc-1* mutation.

Protochlorophyllide reductase message and protein levels in transformed cells

Since no mRNA encoding pchlide reductase is detectable in dark-grown *pc-1* and *pc-1 y-7* cells, we wanted to determine whether the introduction of one or more copies of the *CRlpcr-1* and recovery of the green-in-the-light phenotype was correlated with the accumulation of pchlide reductase mRNA in the transformed strains. Total RNA was isolated from dark-grown cells of several randomly selected transformants that contained one or more introduced copy of the *CRlpcr-1* gene in their genomes and the presence of pchlide reductase message was analyzed by RNA gel blot analysis using a ^{32}P-labeled 1.7 kb *Sac* II fragment from pNS8k as probe. As shown in Fig. 6A, each of the transformants tested (e.g., Tm206, Tm210, and Tm219) contained the 2.3 kb pchlide reductase message, albeit at levels consistently lower than that observed in either dark-grown wild-type or *y-7* cells. Based on a limited analyses, it did not appear that message abundance in the transformants was correlated to the number of introduced gene copies, since transformants such as Tm206 and Tm219 that contained multiple copies of the introduced *CRlpcr-1* gene have levels of pchlide reductase mRNA similar to that found in Tm210 which appears to contain only a single introduced copy of the gene. Copy-number-independent expression of reintro-

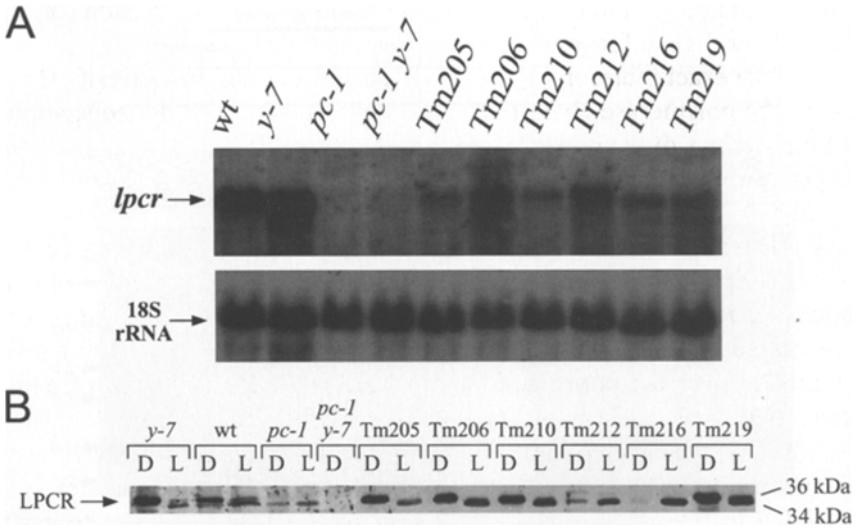

Fig. 6. Analysis of *CRlpcr-1* expression in transform cell line. A. Total RNA (ca. 30 μg) isolated from randomly selected transformants was fractionated on a 1.0% agarose-formaldehyde gel, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled 1.7 kb *Sac* II fragment isolated from pNS8k. Total RNA isolated from wild-type and *y-7* cells serve as a positive control in the experiment, whereas total RNA from *pc-1* and *pc-1 y-7* are used as negative controls. The *lpcr* probe was stripped from the filter and the filter was hybridized with a ^{32}P-labeled 18S rRNA probe to ensure equal amount of sample loading. B. Shown are the results of immunoblot analysis of protochlorophyllide reductase protein levels in various randomly selected transformed cells. Total cellular proteins were extracted from dark- (D) and light-grown (L) cells, and equivalent amounts (ca. 100 μg) were fractionated on a 12% (w/v) SDS-polyacrylamide gel, electrophoretically transferred to a nitrocellulose filter, and reacted with antiserum to the wheat protochlorophyllide reductase. The antigen-antibody complexes were reacted with anti-rabbit secondary antibodies conjugated with horseradish peroxidase, and visualized by chemiluminesence as described in the Materials and methods. The immunoreactive 36 kDa and 34 kDa polypeptides are indicated by arrows. The size of the immunoreactive proteins was measured against known standard proteins of 18 kDa, 29 kDa, and 43 kDa.

duced *C. reinhardtii* nuclear genes has been noted by others [9, 45, 55].

If the introduced *CRlpcr-1* gene(s) were functioning normally, we would expect to see accumulation of the 36 kDa pchlide reductase protein in the transformed cells. As shown in Fig. 6B, immunoreactive 36 kDa pchlide reductase protein is found in all of the extracts prepared from dark-grown transformed cells. The level of immunoreactive protein in three of the transformants was similar to that found in dark-grown *y-7* cells, whereas Tm212 had much lower levels of enzyme protein in the dark. While all of the transformants analyzed appeared similar to *y-7* and contained no immunodetectable 34 kDa polypeptide in the dark, the transformants all showed substantially higher levels of accumulation of this polypeptide in the light than previously observed in *y-7*. In one of the transformants, Tm219, high levels of both the 36 kDa and 34 kDa polypeptides are observed in the light.

It is worth noting that all six strains which exhibited wild-type phenotypes in the light but showed no evidence of introduced copies of the *CRlpcr-1* gene within their genomes contained both pchlide reductase message and immunodetectible pchlide reductase protein as shown for Tm205 and Tm216 in Figs. 6A and 6B, respectively.

Molecular basis of the pc-1 *mutation*

The accumulation of pchlide reductase mRNA and protein in *pc-1 y-7* cells following the introduction of wild-type copies of the *CRlpcr-1* gene

strongly suggested that the *pc-1* mutation did in fact lie within the structural gene for pchlide reductase. To determine the exact molecular nature of the *pc-1* mutation, the polymerase chain reaction was used to amplify four overlapping DNA fragments covering the entire coding region and a portion of the 5′- and 3′-flanking region of the *lpcr* gene in *pc-1 y-7*. The amplification products were cloned into BluescriptKS(−) plasmid vector, their nucleotide sequence determined, and compared those generated from analysis of either wild-type or *pc-1* DNA. Over the entire coding portion of the gene only one discrepancy was found. As shown in Fig. 7, two nucleotides, a C and an A residue at position +12 and +13, respectively, were deleted in the *pc-1* mutant *lpcr* gene. This two nucleotide deletion causes a frame-shift at the junction between the fourth and fifth codons in the wild-type transcript and results in a message whose translation terminates prematurely after formation of a 67 amino acid polypeptide.

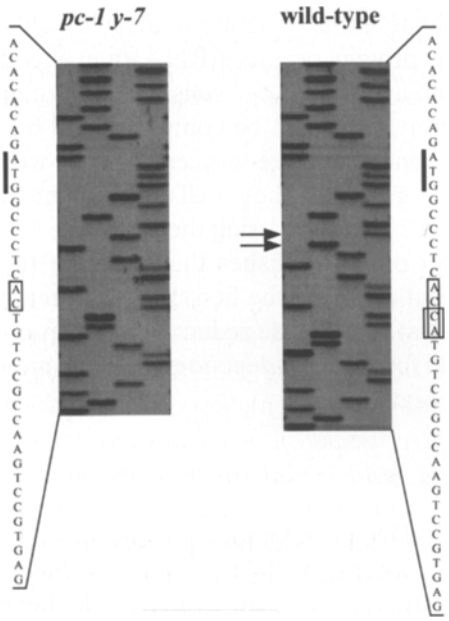

Fig. 7. Analysis of the nucleotide sequence of the *lpcr* gene in *pc-1 y-7* cells. Shown are the nucleotide sequences of the *CRlpcr-1* gene from wild-type cells and the analogous region of the gene isolated from *pc-1 y-7* cells. The deleted two nucleotides are indicated by *arrowheads* on sequencing diagram and double boxed in the wild-type DNA sequence. The assigned translational initiation codon (ATG) is indicated by a vertical bar.

In order to further verify that the 2 nucleotide deletion identified by sequencing of the PCR fragments represented the true molecular nature of the *pc-1* mutation in the *pc-1 y-7* double mutant, a partial genomic library was constructed in pBluescriptKS(−) by cloning 4.0–4.5 kb fragments of *Xho* I and *Cla* I double-digested genomic DNA from *pc-1 y-7*. The partial library was screened by colony hybridization using a ^{32}P-labeled 4.5 kb *Xho* I-*Cla* I fragment of pNS8k as probe. Several positive clones were identified and the resulting plasmid DNAs were sequenced. The same two-nucleotide deletion present in the PCR-generated fragments was found in all of the positive clones identified in the library screen, thereby confirming the nature of the mutation in *pc-1*.

The two-nucleotide deletion in *pc-1* eliminated an *Hph* I restriction site (dTCACCA→TCAC) that is present in the wild-type *CRlpcr-1* gene. Therefore, an *Hph* I restriction fragment length polymorphism (RFLP) should exist between the wild-type and *pc-1* or *pc-1 y-7* genomes. As shown in Fig. 8, gel blot analysis of total DNA isolated from wild-type and *y-7* cells (lanes 1 and 2, respectively) that was digested with *Hph* I and hybridized with a ^{32}P-labeled 440 bp *Hind* III fragment from pNS8k contained a strongly hybridizing 440 bp *Hph* I fragment. Consistent with the predicted *Hph* I RFLP, this 440 bp fragment is absent in *pc-1* and *pc-1 y-7* genomic DNA (lanes 3 and 4, respectively), but has been replaced by a strongly hybridizing 700 bp band instead. Genomic DNA isolated from the various transformants containing introduced copies of *CRlpcr-1* in their genome (e.g., Tm202, Tm206, Tm219) showed the presence of both the 440 bp and 700 bp fragments indicating that in fact wild-type copies (containing the *Hph* I site) were now present in the genome. Strains which were phenotypically recovered to wild-type (e.g., Tm205, Tm216, Tm218) but failed to show evidence of introduced copies of *CRlpcr-1* in their genomes (cf. Fig. 5) also failed to exhibit the 440 bp fragment associated with the wild-type *CRlpcr-1* gene.

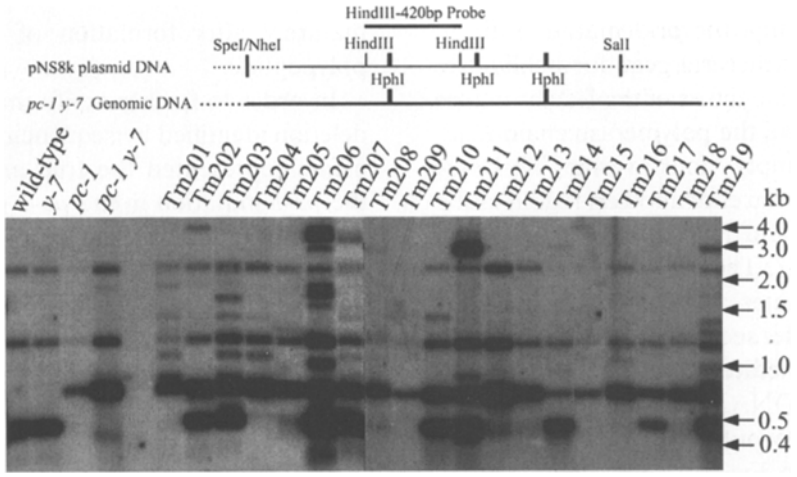

Fig. 8. Restriction fragment length polymorphism analyses of DNA in wild-type, *y-7*, *pc-1*, and *pc-1 y-7* cells. Total genomic DNA, isolated from each of the various transformed strains (Tm201 through Tm219), was digested with *Hph* I, fractionated on a 1.0% agarose gel, and transferred onto nylon membranes. The membranes were then hybridized with a ^{32}P-labeled 440 bp *Hind* III fragment from pNS8k. Based on DNA sequence analysis, the probe should recognize a 440 bp *Hph* I fragment DNA isolated from wild-type and *y-7* cells and a 770 bp *Hph* I fragment in DNA prepared from *pc-1* and *pc-1 y-7* cells. The position of molecular weight markers are given to the right.

Discussion

The *pc-1* mutant of *C. reinhardtii* was originally isolated by Ford *et al.* [21] in a mutagenesis screen of the temperature-sensitive yellow-in-the-dark mutant *y-1–4* and shown to be defective in pchlide photoconversion *in vivo*. Based upon the results of cell fractionation experiments with *y-7* and *pc-1 y-7* cells, it was proposed that *pc-1* likely encoded a structural defect in pchlide reductase, and although the enzyme was present in the mutant cells and capable of binding pchlide, it was inactive [22]. Consistent with this earlier prediction we demonstrate in this paper that *pc-1* does in fact encode a defect in pchlide reductase activity. We have determined that the molecular basis for this defect is a two nucleotide deletion within the sequences encoding the precursor to the pchlide reductase protein. The consequence of this deletion is a shift in the reading frame in mutant transcript that results in a premature termination of translation and the formation of a truncated pchlide reductase polypeptide of about 67 amino acids. In contrast to the results of Ford *et al.* [22], we found that *pc-1* and *pc-1 y-7* cells failed to accumulate either immunodetectable pchlide reductase protein or the mRNA that encodes it. The inability of *pc-1 y-7* cells to catalyze pchlide photoconversion can be complemented by introducing one or more copies of the wild-type *CRlpcr-1* gene into these cells. Complementation of the *pc-1* mutation using the wild-type *CRlpcr-1* gene not only establishes the nature of the *pc-1* locus, but provides the first direct molecular evidence that the pchlide reductase protein is solely required for the light-dependent pchlide reduction in this organism.

Gene organization and protein structure

Cloned cDNAs or genomic sequences encoding light-dependent pchlide reductases have been previously reported from a variety of higher plants [8, 14, 24, 74, 78, 79, 82] and the cyanobacterium *Synechocystis* [81]. This is the first description of the structure and expression characteristics of an *lpcr* gene in a green algal species. A strong conservation in gene organization has previously been noted in comparisons of *lpcr* genes from other higher plants including a very conserved place-

ment of introns within the various genes [79]. In contrast, little overall conservation is found in comparisons of the structure of the *CRlpcr-1* gene with those reported from pea [78] and pine [79]. The *CRlpcr-1* gene lacks introns within its transit peptide and extreme amino terminus typical for higher plants. Only the placement of Intron II appears to be conserved in all plant and algal species thus far examined. The importance of this placement with respect to the evolution of functional domains predicted to be involved in substrate-binding (e.g., pchlide and NADPH) and catalytic activity of the enzyme [85] is still a matter of speculation.

The previously noted high degree of conservation in primary protein structure observed among light-dependent pchlide reductases can now be extended to include *C. reinhardtii*. As previously noted [79], the degree of similarity among pchlide reductases varies depending upon the species' phylogenetic relationship. Therefore, it is not surprising that the mature form of the *C. reinhardtii* pchlide reductase has greater homology (65–70% identical and 79–82% similar) to those found in higher plants then it does to its cyanobacterial counterpart (52% identical and 72% similar).

Several highly conserved domains have now been identified within the pchlide reductases [48, 85] and based upon both primary and tertiary-structure comparisons it has been proposed that the pchlide reductases belong to a superfamily of related enzymes referred to as the RED family of proteins [48]. Among the important features now recognized are a conserved nucleotide binding domain located near the N-terminus of the mature protein (amino acid residues 115 to 147; 24 of 33 residues identical). Included within this region are conserved residues thought to be involved in the electrostatic interaction of the protein with the 2′-phosphate group in NADPH [61, 82] and cofactor specificity [82, 85]. Like the higher-plant and cyanobacterial enzymes, the *Chlamydomonas* pchlide reductase contains four cysteine residues whose location within the protein is absolutely conserved. Derivatization experiments with [^{3}H] N-phenylmaleimide have previously implicated one or more of these residues in substrate (pchlide) binding [61]. Recent experiments in our laboratory with the pea enzyme suggest that at least one of these cysteine residues (corresponding to Cys-305 in the *C. reinhardtii* enzyme) is absolutely essential for function (H.M. Wilks and M.P. Timko, unpublished observations).

A conserved stretch of 11 amino acids (corresponding to residues 294 through 305 in the algal enzyme) is thought to comprise part of the active site the enzyme. This region, G-A-K-A-Y-K-D-S-K-V-A-C, includes the conserved tyrosine (Tyr-298) and a lysine (Lys-302) residues present in all RED family members and recently demonstrated to be essential for catalysis in the pea pchlide reductase [48, 85]. Interestingly, two features of the active site domain in the algal enzyme distinguish it from those present in the higher plant and cyanobacterial enzymes. In the *C. reinhardtii* enzyme, an additional alanine residue, Ala-304, is present in the domain and, in contrast to the high degree of conservation observed in the regions immediately flanking the active site in the higher plant and cyanobacterial proteins, little similarity is observed over the same region in the algal enzyme. For example, only 4 (M, D, G, and F) out of 15 amino acids on the amino-side and 5 (M, Q, H, R, and H) out of 15 residues on the carboxyl side of the active site are conserved when comparing the *C. reinhardtii* protein with those of higher plants, whereas 14 out of 15 amino acid residues are identical on the carboxy side of the active site among all characterized higher-plant enzymes. The functional or structural significance of these alterations remain to be determined.

Regulation of CRlpcr-1 *expression in wild-type and mutant cells*

The negative effect of light on steady-state levels of pchlide reductase mRNA in *C. reinhardtii* is similar to that observed in some monocot species where it has been demonstrated that a dramatic reduction in pchlide reductase transcript levels occurs upon exposure of etiolated tissues to light [1, 4, 14, 34]. This result contrasts with the observations made in some dicot and gymnosperm

species where little or no effect of light is observed on *lpcr* message levels [8, 23, 46, 78, 79] or where the effects of light are conditioned by the developmental growth stage [35, 79]. In barley, the light-induced decline in steady-state pchlide reductase message levels has been shown to be the result of a phytochrome-mediated reduction of transcription rates from the *lpcr* gene(s) [59]. To what extent the light-induced decline in steady-state levels of pchlide reductase mRNA in *C. reinhardtii* reflects changes in the rate of *CRlpcr-1* transcription is not known.

The expression patterns of a number of *C. reinhardtii* nuclear genes encoding chloroplast-localized proteins, including several enzymes involved in chlorophyll biosynthesis, have been examined and shown to be regulated by light [29, 38, 39, 40, 53, 54]. For at least one of these genes, *cabII-1*, run-on transcription assays in isolated nuclei indicate that the rapid light-induced increase in mRNA abundance is primarily due to regulation at the transcriptional level [40]. Although the exact mechanism mediating the light regulation of gene expression in *C. reinhardtii* has yet to be determined, several studies strongly suggest the involvement of a blue-light receptor either operating at the level of transcription or through one or more post-transcriptional processes [42, 43, 53, 54].

It is also possible that some or all of the light-induced loss of pchlide reductase message in dark-grown *C. reinhardtii* cultures is the result of changes in mRNA stability or rates of mRNA turnover. There is now considerable evidence indicating that specific sequences within the 5′-leader and/or 3′-untranslated regions (UTRs) of individual transcripts, a variety of *trans*-acting factors, as well as components of the translational apparatus are involved in regulating mRNA stability [7, 62, 63]. For example, a variety of nuclear genes have now been characterized in *C. reinhardtii* that influence either directly or indirectly the stability of specific chloroplast transcripts through interactions at either the 3′ or 5′ UTR [68]. Considerably less is known about the mechanism(s) controlling stability or turnover of nuclear transcripts in this organism.

Effects of the pc-1 *mutation of on* CRlpcr-1 *transcript levels*

The absence or greatly reduced level of the pchlide reductase transcript in *pc-1* and *pc-1 y-7* could be the result of either decreased transcription or increased mRNA turnover mediated by one or more posttranscriptional process altered by the *pc-1* mutation. The molecular nature of the mutation in the *pc-1* locus suggests one possible mechanism to explain the absence of pchlide reductase mRNA in *pc-1* and *pc-1 y-7* cells, as well as account for the lack of immunoreactive pchlide reductase protein(s) in these cells. The presence of a premature termination codon into the pchlide reductase mRNA as a consequence of the two nucleotide deletion in *pc-1* could result in the formation of a mRNA with substantially decreased stability due to the premature termination of translation of that message. Decreased message stability (or accelerated mRNA decay) caused by premature translational termination or reduced translation efficiency has been observed in a variety of prokaryotic and eukaryotic organisms [12, 62, 63] including higher plants [41, 84] and most recently *C. reinhardtii* [66]. In this latter case, the plastocyanin-deficient phenotype associated with the *C. reinhardtii* mutant *ac-208* was shown to be the result of a frameshift mutation that introduces a premature termination codon in the wild-type plastocyanin mRNA. As a consequence, translation of the *ac-208* message terminates prematurely leading to an apparent destabilization of the message since *ac-208* cells contain only 3% of wild-type message levels and fail to accumulate plastocyanin [66].

The mechanism(s) of by which nonsense mutations lead to mRNA destabilization is not presently known. Both *cis*-acting sequences and *trans*-acting factors involved in the process have been identified [32, 49, 64] and some studies have suggested that the location of the termination codon within the body of the mutant transcript can influence the severity of the destabilization effect [52, 60, 64, 83], with nonsense mutations located more proximal to the 5′ end of the transcript resulting in greater destabilization than those lo-

cated near the 3′ end [32, 52, 64]. The presence of a premature termination codon within the first ca. 200 nucleotides of the 2.3 kb pchlide reductase message might therefore be expected to have a strong destabilizing effect, consistent with our observation that pchlide reductase transcripts are absent or present at only extremely low levels in *pc-1* and *pc-1 y-7* cells.

Multiple forms of protochlorophyllide reductase protein

Two immunodetectable forms of pchlide reductase have been reported in a number of different higher plant species including *Arabidopsis* [3], barley [37], oat [14, 61], pea [35, 78], and pine [24]. Recent studies in *Arabidopsis* [3], barley [37], and pine [24, 79] indicate that these different size variants are the products of separate genes under distinct photoregulatory programs. Genomic DNA gel blot analysis clearly showed that that pchlide reductase is encoded by only a single gene in the *C. reinhardtii* genome, however, extracts from wild-type cells consistently were found to contain two immunoreactive polypeptides of 36 kDa and 34 kDa, respectively. The 36 kDa protein, which predominates in dark-grown cells, is most likely the mature form of the pchlide reductase protein. Its disappearance in the light is consistent with the light-induced breakdown of the protein in the absence of its substrate that has been well documented in other systems [23, 34]. Consistent with their failure to accummulate pchlide reductase mRNA, *pc-1 y-7* cells also failed to accumulate any immunodetectable 36 kDa pchlide reductase protein. The presence of small amounts of the immunoreactive 34 kDa protein in dark- and light-grown *pc-1* cells is quite unexpected and raises questions about the nature and origin of this protein.

A trivial explanation for its origin is that the 34 kDa polypeptide is not pchlide reductase, but rather a structurally-related protein capable of cross-reacting immunologically with the anti-wheat pchlide reductase polyclonal serum. Although we cannot rule this out, the 34 kDa polypeptide was also detected in wild-type and *pc-1* mutant cells when antibodies prepared against the pea pchlide reductase were used in our assays (data not shown). The 34 kDa polypeptide may also represent a size variant of the mature pchlide reductase derived by either secondary modification or differential processing during or after import of the pchlide reductase precursor into the plastid. The difference in relative abundance of the two immunoreactive polypeptides in light- and dark-grown wild-type *C. reinhardtii*, (i.e., the 36 kDa polypeptide is more abundant in dark-grown cells than in light-grown cells, whereas the inverse situation exists for the 34 kDa polypeptide), suggests that their accumulation may be dependent upon factors associated with the stage of plastid differentiation. The observation that the 34 kDa polypeptide is not present in either dark-grown *y-7* or *pc-1 y-7* cells is consistent with this hypothesis and indicates that its accumulation may in fact require the presence of a functional chloroplast.

An alternative possibility is that the 34 kDa polypeptide arises through the use of an alternative initiation codon. Based on comparisons with other initiation codons [47], the assigned translation start site for the pchlide reductase message is not a strong initiator and, therefore, it is possible that some of the ribosomes upon which the message assembles bypass the first ATG and initiate translation at the second ATG codon located 12 nucleotides downstream. The consequence of this faulty initiation in wild-type cells would be the formation of a precursor with a slightly truncated chloroplast transit peptide (i.e., missing five residues at the extreme N-terminus) and perhaps a subsequently altered processing site. In *pc-1* or *pc-1 y-7* mutant cells, the frameshift mutation eliminates this second ATG codon thus precluding the possibility for initiation at the second ATG. If in fact translation failed to initiate at the first ATG start codon, the next downstream ATG codon in the correct reading frame in the mutant transcript does not occur until nucleotides 109 to 111. Ribosomes initiating at this position could synthesize a polypeptide of 361 amino acids with a substantially truncated

chloroplast transit peptide. Based upon our current understanding of the requirements for transport into the chloroplast, such a truncation would likely preclude proper import and processing of this precursor.

Our studies described here have provided information on the structure and expression characteristics of an important nuclear gene for chlorophyll formation in wild-type and mutant cellular backgrounds. These studies should allow for the design of experiments aimed at a better understanding of the relationship between the light-dependent and light-independent mechanisms for pchlide reduction. The *pc-1* mutant might also be an excellent experimental system to study factors involved in nonsense-mediated mRNA destabilization in plants as well as assist in the identification of gene products involved in the normal stabilization or turnover of plant mRNAs.

Acknowledgements

The authors wish to express their thanks to Michel Goldschmidt-Clermont (University of Geneva) for providing the EMBL3 genomic library and various strains used in this study; to Trevor Griffiths for providing antiserum to the wheat pchlide reductase; to Andy Wang for making the *pc-1 y-7* strain available; and to Elizabeth Harris and The *Chlamydomonas* Genetic Stock Center for providing various strains and technical advice. This work was supported in part by a National Science Foundation grant awarded to M.P.T.

References

1. Apel K: The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Phytochrome-induced decrease in translatable mRNA coding for the NADPH:protochlorophyllide oxidoreductase. Eur J Biochem 120: 89–93 (1981).
2. Apel K, Santel J-J, Redlinger TE, Falk H: The protochorophyllide holochrome of barley (*Hordeum vulgare* L.). Isolation and characterization of the NADPH protochlorophyllide oxidoreductase. Eur J Biochem 111: 251–258 (1980).
3. Armstrong GA, Runge S, Frick G, Sperling U, Apel K: Identification of NADPH:protochlorophyllide oxidoreductases A and B: A branched pathway for light-dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. Plant Physiol 108: 1505–1517 (1995).
4. Batschauer A, Apel K: An inverse control by phytochrome of the expression of two nuclear genes in barley (*Hordeum vulgare* L.). Eur J Biochem 143: 593–597 (1984).
5. Bauer CE, Bollivar DW, Suzuki JY: Genetic analysis of photopigment biosynthesis in eubacteria: A guiding light for algae and plants. J Bact 175: 3919–3925 (1993).
6. Beale SI, Weinstein JD: Tetrapyrrole metabolism in photosynthetic organisms. In: Dailey HM (ed) Biosynthesis of Heme and Chlorophyll, pp. 297–391. McGraw-Hill, New York (1990)
7. Beelman CA, Parker R: Degradation of mRNA in eukaryotes. Cell 81: 179–183 (1995).
8. Benli M, Schulz R, Apel K: Effect of light on the NADPH-protochlorophyllide oxidoreducatase of *Arabidopsis thaliana*. Plant Mol Biol 16: 615–625 (1991).
9. Blankenship JE, Kindle KL: Expression of chimeric genes by the light-regulated *cabII-1* promoter in *Chlamydomonas reinhardtii*: a *cabII-1/nit1* gene functions as a dominant selectable marker in a *nit1*$^-$ *nit2*$^-$ strain. Mol Cell Biol 12: 5268–5279 (1992).
10. Bogorad L: Factors associated with the synthesis of chlorophyll in the dark in seedlings of *Pinus jeffreyi*. Bot Gaz 111: 221–241 (1950).
11. Choquet Y, Rahire M, Girard-Bascou J, Erickson J, Rochaix, J-D: A chloroplast gene is required for the light-independent accumulation of chlorophyll in *Chlamydomonas reinhardtii*. EMBO J 11: 1697–1704 (1992).
12. Cheng J, Maquat LE: Nonsense codons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half-life of cytoplasmic mRNA. Mol Cell Biol 13: 1892–1902 (1993).
13. Church GM, Gilbert W: Genomic sequencing. Proc Natl Acad Sci USA 81: 1991–1995 (1984).
14. Darrah PM, Kay SA, Teakle GR, Griffiths WT: Cloning and sequencing of protochlorophyllide reductase. Biochem J 265: 789–798 (1990).
15. de Hostos EL, Schilling J, Grossman AR: Structure and expression of the gene encoding the periplasmic arylsulfatase of *Chlamydomonas reinhardtii*. Mol Gen Genet 218: 229–239 (1989).
16. Devereux J, Haeberli P, Smithies O: A comprehensive set of sequence analysis programs for the VAX. Nucl Acids Res 12: 387–395 (1984).
17. Diener DR, Curry AM, Johnson KA, Williams BD, Lefebvre PA, Kindle KL, Rosenbaum JL: Rescue of a paralyzed-flagella mutant of *Chlamydomonas* by transformation. Proc Natl Acad Sci USA 87: 5739–5743 (1990).
18. Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13 (1983).

19. Ford C, Wang W-Y: Three new *yellow* loci in *Chlamydomonas reinhardtii*. Mol Gen Genet 179: 259–263 (1980).
20. Ford C, Wang W-Y: Temperature sensitive *yellow* mutants of *Chlamydomonas reinhardtii*. Mol Gen Genet 180: 5–10 (1980).
21. Ford C, Mitchell S, Wang W-Y: Protochlorophyllide photoconversion mutants of *Chlamydomonas reinhardtii*. Mol Gen Genet 184: 460–464 (1981).
22. Ford C, Mitchell S, Wang W-Y: Characterization of NADPH:protochlorophyllide photoconversion in the *y-7* and *pc-1 y-7* mutants of *Chlamydomonas reinhardtii*. Mol Gen Genet 194: 290–292 (1983).
23. Forreiter C, van Cleve B, Schmidt A, Apel K: Evidence for a general light-dependent negative control of NADPH-protochlorophyllide oxidoreductase in angiosperms. Planta 183: 126–132 (1990).
24. Forreiter C, Apel K: Light-independent and light-dependent protochlorophyllide-reducing activities and two distinct NADPH-prototochlorophyllide oxidoreductase polypeptides in mountain pine (*Pinus mungo*). Planta 190: 536–545 (1993).
25. Franzén LG: Analysis of chloroplast and mitochondrial targeting sequences from the green alga *Chlamydomonas reinhardtii*. Biol Membr 11: 304–309 (1994).
26. Frohman MA, Dush MK, Martin GR: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85: 8998–9002 (1988).
27. Fujiwara S, Fukuzawa H, Tachiki A, Miyachi S: Structural and differential expression of two genes encoding carbonic anhydrase in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 87: 9779–9783 (1990).
28. Goldschmidt-Clermont M: The two genes for the small subunit of RuBP carboxylase/oxygenase are closely linked in *Chlamydomonas reinhardtii*. Plant Mol Biol 6: 13–21 (1986).
29. Goldschmidt-Clermont M, Rahire M: Sequence, evolution and differential expression of the two genes encoding variant small subunits of ribulose bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. J Mol Biol 191: 421–432 (1986).
30. Griffiths WT: Protochlorophyll and protochlorophyllide as precursors for chlorophyll synthesis *in vitro*. FEBS Lett 49: 196–200 (1974).
31. Gromoff ED, Treier U, Beck CF: Three light inducible heat shock genes of *Chlamydomonas reinhardtii*. Mol Cell Biol 15: 3911–3918 (1989).
32. Hagan KW, Ruiz-Echevarria MJ, Quan Y, Peltz SW: Characterization of *cis*-acting sequences and decay intermediates involved in nonsense-mediated mRNA turnover. Mol Cell Biol 15: 809–823 (1995).
33. Harris EH: The Chlamydomonas Source Book: A Comprehensive Guide to Biology and Laboratory Use, Academic Press, San Diego (1989).
34. Hauser I, Dehesh K, Apel K: Proteolytic degraadation *in vitro* of the NADPH-protochlorophyllide reductase of barley (*Hordeum vulgare* L.). Arch Biochem Biophys 228: 577–586 (1984).
35. He Z, Li J, Sundqvist C, Timko MP: Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.). Plant Physiol 106: 537–546 (1994).
36. Hill KL, Li HH, Singer J, Merchant S: Isolation and structural characterization of the *Chlamydomonas reinhardtii* gene for cytochrome c_6: analysis of kinetics and metal specificity of its copper-responsive expression. J Biol Chem 266: 15060–15067 (1991).
37. Holtorf H, Reinbothe S, Reinbothe C, Bereza B, Apel K: Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.) Proc Natl Acad Sci USA 92: 3254–3258 (1995).
38. Imbault P, Wittemer C, Johanningmeier U, Jacobs JD, Howell SH: Structure of the *Chlamydomonas reinhardtii cabII-1* gene encoding a chlorophyll-a/b-binding protein. Gene 73: 397–407 (1988).
39. Ish-Shalom D, Kloppstech K, Ohad I: Light-regulation of the 22 kd heat shock gene transcription and its translation product accumulation in *Chlamydomonas reinhardtii*. EMBO J 9: 2657–2661 (1990).
40. Jasper F, Quednau B, Kortenjann M, Johanningmeier U: Control of *cab* gene expression in synchronized *Chlamydomonas reinhardtii* cells. J Photochem Photobiol B: Biol 11: 139–150 (1991).
41. Jofuku KD, Schipper RD, Goldberg RB: A frameshift mutation prevents Kunitz tripsin inhibitor mRNA accumulation in soybean embryos. Plant Cell 1: 427–435 (1989).
42. Johanningenmeier U, Howell SH: Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in *Chlamydomonas reinhardtii*. Possible involvement of chlorophyll synthesis precursors. J Biol Chem 259: 13541–13549 (1984).
43. Kindle KL: Expression of a gene for light-harvesting chlorophyll *a/b* binding protein in *Chlamydomonas reinhardtii*: effect of light and acetate. Plant Mol Biol 9: 547–563 (1987).
44. Kindle KL: High-frequency nuclear transformation of *Chlamydomonas reinhardtii* Proc Natl Acad Sci USA 87: 1228–1232 (1990).
45. Kindle KL, Schnell RA, Fernandez E, Lefebvre PA: Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J Cell Biol 109: 2589–2601 (1989).
46. Kittsteiner U, Paulsen H, Schendel R, Rudiger W: Lack of light regulation of NADPH:protochlorophyllide oxidoreductase mRNA in cress seedling (*Lepidium sativum* L.). Z Naturforsch 45C: 1077–1079 (1990).
47. Kozak M: The scanning model for translation: an update. J Cell Biol 108: 229–241 (1989).
48. Labesse G, Vidal-Cros A, Chomilier J, Gaudry M, Mornon J-P: Structural comparisons lead to the definition of a new superfamily of NAD(P)(H)-accepting oxi-

doreductases: the single-domain reductases/epimerases/dehydrogenases (the 'Red' family). Biochem J 304: 95–99 (1994).
49. Leeds P, Peltz SW, Jacobson A, Culbertson MR: The product of the yeast *UPF1* gene is required for rapid turnover of mRNAs containing a premature translational termination codon. Genes Devel 5: 2303–2314 (1991).
50. Li J, Goldschmidt-Clermont M, Timko MP: Chloroplast-encoded *chlB* is required for light-independent protochlorophyllide reductase activity in *Chlamydomonas reinhardtii*. Plant Cell 5: 1817–1829 (1993).
51. Liu X-Q, Xu H, Huang C: Chloroplast *chlB* gene is required for light-independent chlorophyll accumulation in *Chlamydomonas reinhardtii*. Plant Mol Biol 23: 297–308 (1993).
52. Losson R, Lacroute F: Interference of nonsense mutations with eukaryotic messenger RNA stability. Proc Natl Acad Sci USA 76: 5134–5137 (1979).
53. Matters GL, Beale SI: Structural and light-regulated expression of the *gsa* gene encoding the chlorophyll biosynthetic enzyme, glutamate 1-semialdehyde aminotransferase, in *Chlamydomonas reinhardtii*. Plant Mol Biol 24: 617–629 (1994).
54. Matters GL, Beale SI: Structure and expression of the *Chlamydomonas reinhardtii alad* gene encoding the chlorophyll biosynthetic enzyme, δ-aminolevulinic acid dehydratase (porphobilinogen synthase). Plant Mol Biol 27: 607–617 (1995).
55. Mayfield SP: Overexpression of the oxygen-evolving enhancer 1 protein and its consequences on photosystem II accumulation. Planta 185: 105–110 (1991).
56. Mayfield SP, Schirmer-Rahire M, Frank G, Zuber H, Rochaix J-D: Analysis of the genes of the OEE1 and OEE3 proteins of the photosystem II complex from *Chlamydomonas reinhardtii*. Plant Mol Biol 12: 683–693 (1989).
57. Meyer G, Bliedung H, Kloppstech K: NADPH-protochlorophyllide oxidoreductase: reciprocal regulation in mono- and dicotyledonean plants. Plant Cell Rep 2: 26–29 (1983).
58. Mitchell DR, Kang Y: Identification of *oda6* as a *Chlamydomonas* dynein mutant by rescue with the wild-type gene. J Cell Biol. 113: 835–842 (1991).
59. Mosinger E, Batschauer A, Schafer E, Apel K: Phytochrome control of *in vitro* transcription of specific genes in isolated nuclei from barley (*Hordeum vulgare* L.). Eur J Biochem 147: 137–142 (1985).
60. Nilsson G, Belasco JG, Cohen SN, von Gabain A: Effect of premature termination of translation on mRNA stability depends on the site of ribosome release. Proc Natl Acad Sci USA 84: 4890–4894 (1987).
61. Oliver RP, Griffiths WT: Covalent labeling of the NADPH-protochlorophyllide oxidoreductase from etioplast membranes with (^{3}H)N-phenylmaleimide. Biochem J 195: 93–101 (1981).
62. Peltz SW, Brewer G, Bernstein P, Ross J: Regulation of mRNA turnover in eukaryotic cells. Crit Rev Eukar Gene Exp 1: 99–126 (1991).
63. Peltz SW, Jacobson A: mRNA stability: in *trans*-it. Cur Opin Cell Biol 4: 979–983 (1992).
64. Peltz SW, Brown AH, Jacobson A: mRNA destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and one *trans*-acting factor. Genes Devel 7: 1737–1754 (1993).
65. Quesada R, Galvan A, Fernandez E: Identification of nitrate transporter genes in *Chlamydomonas reinhardtii*. Plant J 5: 407–419 (1994).
66. Quinn J, Li HH, Singer J, Morimoto B, Mets L, Kindle K, Merchant S: The plastocyanin-deficient phenotype of *Chlamydomonas reinhardtii Ac-208* results from a frameshift mutation in the nuclear gene encoding preapoplastocyanin. J Biol Chem 268: 7832–7841 (1993).
67. Röbbeln G: Über die Protochlorophyllreduktion in einer Mutant von *Arabidopsis thaliana (L) Heynh.* Planta 47: 532 (1956).
68. Rochaix J-D: Posttranscriptional steps in the expression of chloroplast genes. Annu Rev Cell Biol 8: 1–28 (1992).
69. Rochaix JD, Mayfield S, Goldschmidt-Clermont M, Erikson J: Molecular Biology of *Chlamydomonas*. In: Shaw CH (ed) Plant Molecular Biology: A Practical Approach, pp. 253–275. IRL Press, Oxford (1988).
70. Roitgrund C, Mets LJ: Localization of two novel chloroplast genome functions: *trans*-splicing of RNA and protochlorophyllide reduction. Curr Genet 17: 147–153 (1990).
71. Sager R: Inheritance in the green alga *Chlamydomonas reinhardtii*. Genetics 40: 476–489 (1955).
72. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
73. Schulz R, Senger H: Protochlorophyllide reductase: a key enzyme in the greening process. In: Sundqvist C, Ryberg M (eds) Pigment-Protein Complexes in Plastids: Synthesis and Assembly, pp. 179–218., Academic Press, New York (1993).
74. Schulz R, Steinmuller K, Klaas M, Forreiter C, Rasmussen S, Hiller C, Apel K: Nucleotide sequence of a cDNA coding for the NADPH-protochlorophyllide oxidoreductase (PCR) of barley *(Hordeum vulgare* L.) and its expression in *Escherichia coli*. Mol Gen Genet 217: 355–361 (1989).
75. Silflow CD, Chisholm RL, Conner TW, Ranum LPW: The two α-tubulin genes of *Chlamydomonas reinhardtii* code for slightly different proteins. Mol Cell Biol 5: 2389–2398 (1985).
76. Smart EJ, Selman BR: Complementation of a *Chlamydomonas reinhardtii* mutant defective in the nuclear gene encoding the chloroplast coupling factor (CF1) γ-subunit (*atpC*). J Bioenerg Biomemb 3: 275–284 (1993).
77. Soeinde OA, Kindle KL: Homologous recombination in the nuclear genome of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 90: 9199–9203 (1993).

78. Spano AJ, He Z, Michel H, Hunt DF, Timko MP: Molecular cloning, nuclear gene structure, and developmental expression of NADPH-protochlorophyllide oxidoreductase in pea (*Pisum sativum* L.). Plant Mol Biol 18: 967–972 (1992).
79. Spano AJ, He Z, Timko MP: NADPH:protochlorophyllide oxidoreductases in white pine (*Pinus strobus*) and loblolly pine (*Pinus taeda*). Evidence for light and developmental regulation of expression and conservation in gene organization and protein structure between angiosperms and gymnosperms. Mol Gen Genet 236: 86–95 (1992).
80. Suzuki JY, Bauer CE: Light-independent chlorophyll biosynthesis: Involvement of the chloroplast gene *chlL* (*frxC*). Plant Cell 4: 929–940 (1992).
81. Suzuki JY, Bauer CE: A prokaryotic origin for light-dependent chlorophyll biosynthesis of plants. Proc Natl Acad Sci USA 92: 3749–3753 (1995).
82. Teakle GR, Griffiths WT: Cloning, characterization and import studies on protochlorophyllide reductase from wheat (*Triticum aestivum*). Biochem J 196: 225–230 (1993).
83. Urlaub G, Mitchell PJ, Ciudad CJ, Chasin LA: Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. Mol Cell Biol 9: 2868–2880 (1989).
84. Voelker TA, Moreno J, Crispeels MJ: Expression analysis of a pseudogene in transgenic tobacco: a frameshift mutation prevent mRNA accumulation. Plant Cell 2: 255–261 (1990).
85. Wilks HM, Timko MP: A light-dependent complementation system for analysis of NADPH:protochlorophyllide oxidoreductase. Identification and mutagenesis of two conserved residues that are essential for enzyme activity. Proc Natl Acad Sci USA 92: 724–728 (1995).
86. Zimmer WE, Schloss JA, Silflow CD, Youngblom J, Watterson DM: Structural organization, DNA sequence, and expression of the calmodulin gene. J Biol Chem 263: 19370–19383 (1988).