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Cloning and sequencing of protochlorophyllide reductase

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Putative protochlorophyllide reductase cDNA clones (252 and 113) were isolated from an etiolated-oat (*Avena sativa*) cDNA library. These were used to indirectly characterize a further clone, p127, isolated from a λ-phage gt11 cDNA library. The latter (1.15 kb in length) was sequenced, and the derived amino acid sequence was shown to be remarkably similar to that derived from chemical analysis of a CNBr-cleavage fragment of the purified reductase. p127 codes for more than 95% of the reductase protein.

INTRODUCTION

The study of enzymes has received a considerable boost recently as a consequence of developments in molecular genetics. In particular, work involving the techniques of enzyme overproduction and site-directed mutagenesis have culminated in detailed reaction schemes for many enzymes of both plant and animal origin. One particular plant enzyme we would like to study in detail is the light-requiring enzyme of chlorophyll biosynthesis, namely protochlorophyllide reductase (Griffiths, 1978). This catalyses the light-dependent transfer of reducing equivalents from NADPH to the porphyrin protochlorophyllide as the penultimate step of chlorophyll *a* biosynthesis by plants. Mechanistically, the enzyme, a membrane-associated protein of M_r approx. 36000, forms an enzyme–substrate complex with its substrates protochlorophyllide and NADPH. This is stable in darkness; however, the absorption of light induces hydrogen transfer from the coenzyme to the pigment, resulting in formation of chlorophyllide and $NADP^+$, which are finally released. Chemical-modification-type experiments performed previously have provided evidence for the involvement of certain groups on the protein with the enzyme's catalysis. Evidence was presented for the involvement of cysteine residues in substrate binding (Oliver & Griffiths, 1981), an anionic residue with NADPH binding (Griffiths, 1978) and an arginine residue as proton donor during the reaction (Oliver, 1981). In more recent work it has been shown that, mechanistically, hydride from the NADPH is delivered to the C-17 position of protochlorophyllide from the *pro-S* face of NADPH, whereas the C-18 position is protonated by water or an active-site (arginine?) residue (Begley & Young, 1989).

The significance of these studies would be considerably enhanced by structural information, particularly amino acid sequence, of the reductase and also by the availability of an abundant supply of the enzyme. An obvious start to try and achieve these ends is to prepare cDNA of the reductase mRNA. It should be mentioned that a barley (*Hordeum vulgare*) cDNA clone has already been extensively used to provide a probe for investigating the regulation of protochlorophyllide reductase formation (see review by Harpster & Apel, 1985). However, no structural information on this clone has been forthcoming, and as such it makes no contribution to the study of the nature of the reductase. Here we report on the isolation of a reductase cDNA clone prepared from oat (*Avena sativa*) polyadenylated [$poly(A)^+$] mRNA. Sequencing of the clone indicates that it codes for more than 95% of the mature protochlorophyllide reductase protein, thereby providing the first primary structure data for the enzyme.

MATERIALS AND METHODS

Plant material

Oat (*A. sativa* L., var Peniarth) seeds were purchased locally (British Seed Houses, Avonmouth, Bristol, U.K.). Seeds were grown on dampened Levington potting compost (Fison, Harston, Cambridge, U.K.) at 25 °C for 6 days in the dark as previously described (Mapleston & Griffiths, 1978). Illumination of the plants, when appropriate, was by an array of 40 W fluorescent daylight white tubes supplemented with 100 W tungsten lamps as described previously (Mapleston & Griffiths, 1978).

Enzymes and reagents

Restriction enzymes were purchased from U.K. sources (Boehringer Corp., P & S Biochemicals, Anglian Biotechnology Ltd. or Northumberland Biotechnology) and used as recommended by the suppliers. T4 DNA ligase and DNA polymerase I were from Amersham International. DNAase I was purchased from Boehringer.

Nucleotides and their derivatives were from Boehringer, and radiochemicals were from Amersham International. Transfer membranes, nylon, Hybond-N (pore size 0.45 μm) was purchased from Amersham International, and nitrocellulose (pore size 0.45 μm) was from Schleicher and Schuell (Dassel, Germany). The cloning vectors used (plasmids and phages) are well-described constructs, and these, together with the various

Abbreviation used: $poly(A)^+$, polyadenylated; ss, single-stranded; LHCP, light-harvesting chlorophyll *a*/*b* protein.

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These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X17067.

Escherichia coli transformation strains, were obtained from sources within the University.

Standard chemicals and reagents were of the highest purity available purchased from BDH.

The reductase antibody used in this work was raised in rabbits from the SDS/PAGE-purified protein isolated from etioplasts of oats.

Methods

RNA extraction and purification. RNA was extracted from frozen (77 °K) leaf material (approx. 25 g) by grinding under N_2 in a sterile mortar, followed by extraction with 100 ml of a 2% (w/v) SDS buffer and finally phase separation with 100 ml of phenol/chloroform/3-methylbutan-1-ol (50:50:1, by vol.) by the method of Hall *et al.* (1978). Proteinase K (0.5 mg/ml) was added immediately before the initial extraction. RNA in the upper aqueous phase was ethanol-precipitated, and finally DNA was separated by precipitation of the RNA solution with LiCl. Poly(A)$^+$ mRNA was isolated from the total mRNA fraction by two cycles of chromatography on an oligo(dT)–cellulose column.

A protochlorophyllide reductase-enriched mRNA fraction was isolated on size fractionation of the poly(A)$^+$ mRNA by sucrose-density-gradient centrifugation, followed by assaying each fraction for reductase mRNA translatability using the wheat-germ translation system (see below) (Cashmore, 1979; Timko & Cashmore, 1983).

Translation *in vitro*. The wheat-germ system for translation *in vitro* of mRNA was prepared as described by Roman *et al.* (1976) from wheat germ provided by General Mills (Vallejo, CA, U.S.A.). Micrococcal nuclease (Sigma) treatment of the system was carried out as described by Pelham & Jackson (1976). Translation was measured as the incorporation of [^{35}S]methionine into acid-insoluble radioactivity using the trichloroacetic acid precipitation method of Mans & Novelli (1961). For immunoprecipitations, protein in translation mixtures was solubilized in SDS (Cuming & Bennett, 1981), followed by antibody addition and precipitation of the antibody–antigen complex with protein A–Sepharose (Batschauer *et al.*, 1986). Finally, the '*in vitro*' products were separated by SDS/PAGE using the gel system of Laemmli (1970). Peptide maps of '*in vitro*' products were obtained after partial proteolytic digestion with staphylococcal V8 proteinase as described by Cleveland *et al.* (1977).

Construction and screening of cDNA libraries. Plasmid cDNA libraries were constructed using cDNA prepared from dark-grown oat mRNA by reverse transcriptase (Dr. J. Beard, Life Science Inc.) using the procedure of Broglie *et al.* (1984). Homopolymer tailing of the cDNA by dCTP was followed by ligation into dG-tailed pAT153, which was used to transform *E. coli* strain HB101 essentially as described by Maniatis *et al.* (1982). Colonies of interest were selected as those showing preferential hybridization (see below) to ^{32}P-labelled ss cDNA synthesized from mRNA of dark- as opposed to light-grown plants (Apel *et al.*, 1983). Further screening of selected clones was by hybridization to labelled ss cDNA prepared from a sucrose-density-gradient-purified poly(A)$^+$ mRNA fraction enriched in translatable mRNA for the reductase. Finally, plasmids were isolated from the positive clones and the identity of the inserts confirmed as reductase-similar sequences during Northern-blot-hybridization and slot-blot analysis, hybrid-released translation and sequencing (see below). A cDNA library in the phage expression vector λgt11 was constructed using the Amersham cDNA synthesis and cloning kits. This was screened with the monospecific oat reductase antibody using the plaque-hybridization method of Young & Davies (1983). The most strongly hybridizing clones were rescreened to purity and inserts isolated by restriction-endonuclease-*Eco*RI digestion and agarose-gel electrophoresis. Clone p127 was selected for its insert size, 1.15 kb, which was re-ligated into the plasmid pEMBL12(+) with *Eco*RI linkers. It was characterized further by hybridization during Southern-blot analysis to the clone 252 identified earlier as a putative reductase clone and also by sequence analysis (see below).

DNA hybridizations. Probing of transformed colonies with labelled ss cDNA prepared from various mRNA samples was carried out essentially as described by Apel *et al.* (1983). 'Southern blotting' of restriction-enzyme digests of recombinant pUC plasmids was carried out as described by Southern (1975). Plasmid minipreps were prepared by the method of alkaline lysis as described by Maniatis *et al.* (1982).

RNA analysis. RNA dot- and slot-blot hybridizations were carried out on nitrocellulose, loading being achieved by the use of commercially available manifolds (Schleicher and Schuell). Quantification of the hybridization was by densitometric scanning of the developed autoradiographs using a computer-linked linear densitometer (Guildford Instruments). Northern-blot RNA samples were fractionated by electrophoresis in 1% formaldehyde/agarose gels, transferred to nitrocellulose and hybridized to ^{32}P-labelled [by nick translation (Amersham Kit)] probe of specific radioactivity $(1–5) \times 10^7$ c.p.m./μg of insert DNA as described by Maniatis *et al.* (1982). Autoradiography normally took up to 2 weeks, after which the film was developed in the usual manner.

DNA sequence analysis. This was carried out by the M13 cloning/chain-terminator sequencing method of Sanger *et al.* (1977) as described by Brown (1984).

Protein analysis. Purified reductase was extracted from sucrose-density-gradient-purified etioplast membranes (Beer & Griffiths, 1981). This was subjected to two cycles of SDS/PAGE. The electroeluted protein was dialysed extensively against distilled water and analysed for amino acid composition after acid hydrolysis, derivatization with phenyl isothiocyanate and separation by h.p.l.c. The analysis was kindly carried out by Dr. W. Mawby of this department. A purified sample of the protein was also subjected to cleavage by CNBr, followed once more by SDS/PAGE on 16%-acrylamide gels. The separated fragments were electroeluted, dialysed extensively against 1 mM-Na_2CO_3/0.05% (w/v) SDS and finally freeze-dried. Solid-phase sequencing of the samples was carried out at Science and Engineering Research Council (S.E.R.C.) Sequencing Facility at the University of Leeds.

RESULTS

The polypeptides synthesized *in vitro* under optimal conditions by the wheat-germ system primed with poly(A)$^+$ mRNA isolated from etiolated oats are shown in Fig. 1 (lane 1). Although somewhat overexposed, the Figure shows clearly that a larger number of polypeptides are made, ranging in M_r from approx. 5000 to 60000, with abundant species present at about 17000 and 41000. When the incubated mixture is challenged with a monospecific polyclonal antibody raised against purified protochlorophyllide reductase, a peptide of M_r approx. 41000 (P41) is specifically immunoprecipitated (Fig. 1, lane 2). An estimate of the M_r for this product as 41000 was made by comparing its mobility with that of phosphorylated pyruvate dehydrogenase (M_r 42000) (results not shown).

To investigate the relationship between the 41 kDa immunoprecipitated product and the native oat reductase, a partial proteolytic digest was performed on both proteins by using staphylococcal V8 proteinase and the products detected by fluorography or Coomassie Blue staining of the SDS/PAGE gels respectively, followed by densitometric scanning (Fig. 2). A number of co-migrating peptides are readily apparent among the hydrolysis products, in both cases implying that mature oat protochlorophyllide reductase and the P41 immunoprecipitated translation product share some related primary structure as well as immunological cross-reactivity. Despite a lower M_r than previously reported, i.e. 44000 (Apel, 1981), the implication is that P41 represents the *N*-terminally extended precursor form of the reductase. The effect of illumination on the oat mRNA was investigated by repeating the translation *in vitro* and immunoprecipitation experiments using poly(A)$^+$ mRNA isolated from etiolated oat plants illuminated for 0, 3, 6 or 15 h. Fig. 3(*a*) shows that the total polypeptide profiles of the 'de-etiolating' poly(A)$^+$ mRNAs are very similar, with most of the synthesized polypeptides present in all four samples at similar levels. An exception is the 41 kDa peptide, which declines rapidly on illumination and is barely visible among the total translation products after 15 h of illumination (Fig. 3*a*). This decline in the reductase mRNA appears more dramatic in the immunoprecipitates recovered from the various total translation products (Fig. 3*b*). On densitometric quantification of these data it can be shown that, after 15 h illumination of whole leaves, less than 15% of the original 'etiolated' reductase translatable mRNA is detectable by this method (results not shown). Fig. 4 shows the results obtained from extending this experiment by returning the 15 h-illuminated plants to darkness for a further 15 h. Under these conditions the light-induced decrease in translatable reductase mRNA is largely reversed. After 15 h re-darkening, this level returned to 73% of that of the original etiolated leaves, as estimated by densitometric scans of electrophoresed immunoprecipitates (Fig. 4).

In previous attempts at cloning the reductase (see the Materials and methods section) the procedure involving homopolymer tailing (Deng & Wu, 1981) of cDNA into the vector pAT 153 proved the most successful, producing a library of approx. 7000 recombinant colonies selected on the basis of correct antibiotic-resistance phenotype. Of these clones, 164 were selected during a primary screening as showing preferential hybridization to [^{32}P]cDNA synthesized from etiolated as opposed to light-grown oat mRNA. A subsequent screening, probing

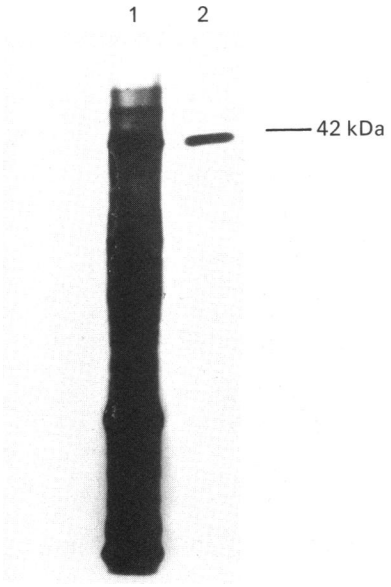

Fig. 1. Translation products synthesized *in vitro* from poly(A)$^+$ mRNA of etiolated oats

The translation assay, comprising the wheat-germ system and [^{35}S]methionine, was programmed with poly(A)$^+$ mRNA from etiolated oats as described in the text. After incubation an aliquot was taken for analysis by SDS/15%-PAGE and the labelled polypeptides were revealed by fluorography (lane 1). A second aliquot was immunoprecipitated with antibody raised against protochlorophyllide reductase from oat and the resulting precipitate analysed as described above (lane 2).

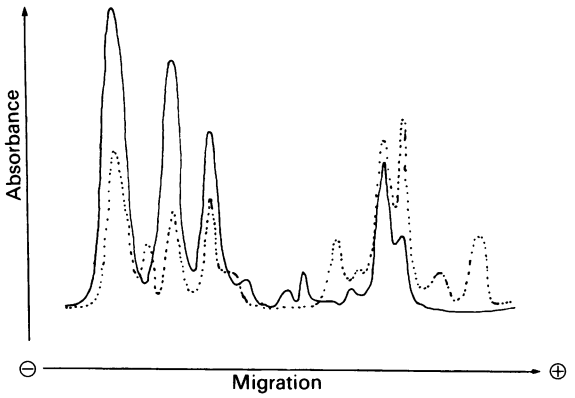

Fig. 2. Comparison of the partial proteolytic digestion products of protochlorophyllide reductase and the product P41 formed *in vitro*

Aliquots of the reductase purified from etiolated oats and an excised gel band containing '*in-vitro*'-labelled P41 (see Fig. 1) were partially digested with staphylococcal V8 proteinase and the products separated on SDS/20%-PAGE. The products were revealed by Coomassie Blue staining (——) for the mature reductase and fluorography (·····) for the P41. Both profiles are presented as their respective densitometric scans.

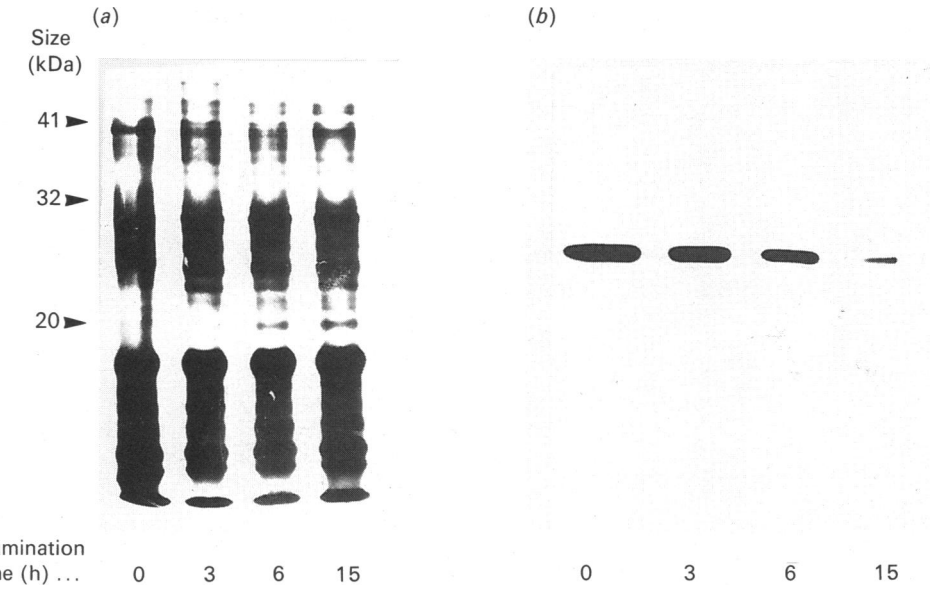

Fig. 3. Effect of illumination of etiolated oats on the '*in-vitro*' translation products of the corresponding poly(A)$^+$ mRNA

Etiolated oat seedlings, 6 days old, were illuminated, then poly(A)$^+$ mRNA was isolated at the times indicated as described in the text. Equal amounts of the RNA were translated, and then (*a*) 2 μl aliquots were taken for analysis of the total products by SDS/PAGE and fluorography as described in Fig. 1; (*b*) 50 μl aliquots were immunoprecipitated with anti-(oat reductase) IgG and the immunocomplexes analysed by SDS/PAGE and fluorography as described in Fig. 1.

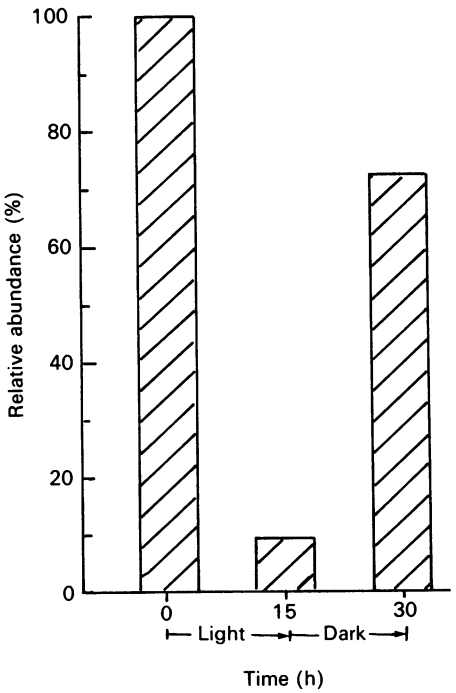

Fig. 4. Re-accumulation of translatable mRNA encoding protochlorophyllide reductase on redarkening of illuminated oats

Etiolated oat plants were illuminated for 15 h, followed by 15 h in darkness. Poly(A)$^+$ mRNA was isolated from the plants at the times indicated. Each RNA sample was translated and reductase mRNA translatability was quantified by immunoprecipitation, SDS/PAGE, fluorography and densitometry as described in Fig. 3. The results are presented as percentages of the value for etiolated plants.

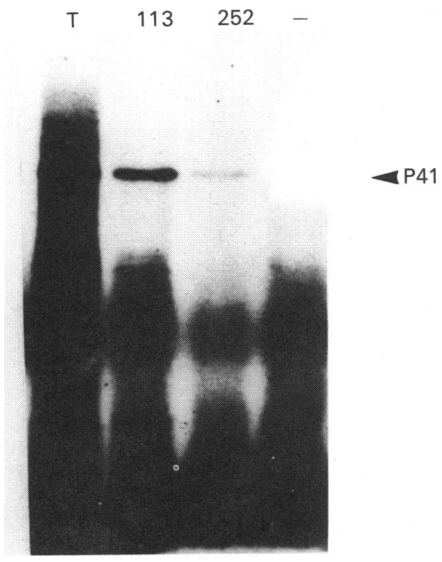

Fig. 5. Hybrid-released translation of clones 113 and 252 immobilized on diazobenzyloxymethyl-paper

Recombinant plasmids 113 and 252 were linearized with *Eco*RI, ethanol-precipitated, denatured by heating in dimethyl sulphoxide, cooled, and added to 0.5 cm squares of freshly prepared diazobenzyloxymethyl-paper as described by Broglie *et al.* (1984). After prehybridization, poly(A)$^+$ mRNA from dark-grown oats was added under hybridizing conditions, followed by washing and finally elution of the bound RNA, which was translated in the wheat-germ system as in Fig. 1. Tracks of both total RNA (T) and no RNA (–) are also shown.

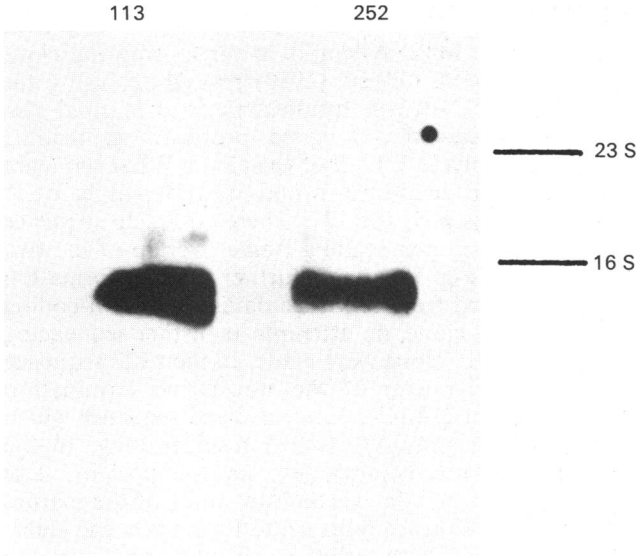

Fig. 6. 'Northern-blot' hybridization of ^{32}P-labelled 113 and 252 to etiolated-oat poly(A)$^+$ mRNA

Etiolated-oat poly(A)$^+$ mRNA (1 μg) was fractionated in formaldehyde/agarose gels and transferred to nitrocellulose as described in the text. The two strips were separately hybridized with ^{32}P-labelled either 113 or 252 plasmid DNA. *E. coli* rRNA markers were run at the same time and stained with ethidium bromide as shown.

with labelled cDNA prepared from reductase-enriched mRNA purified by sucrose-density-gradient centrifugation, resulted in 16 positive clones, from which two putative reductase clones, 113 and 252, were recovered after further selection by hybrid-released translation (Fig. 5). The plasmids from both 113 and 252 contained DNA sequences which cross-hybridized during Southern-blot analysis (P. M. Darrah, unpublished work), while hybridizing during Northern-blot analysis (Fig. 6) to a single mRNA species of approx. 1600 bases among etiolated-oat mRNA in accordance with the expected size of the P41 message. Further, when poly(A)$^+$ mRNA samples, isolated from illuminated and re-darkened etiolated oat plants, were probed quantitatively in a 'dot-blot' apparatus with ^{32}P-labelled clone 113, the expected pattern of hybridization was observed (Fig. 7), with a dramatic decrease in hybridization on illumination followed by a recovery on subsequent re-darkening. Analysis of the insert length excised from each clone by endonuclease-*Pst*I digestion showed clone 252 to contain an insert of approx. 300 bp and clone 113 one of approx. 600 bp (results not shown).

When the small sizes of the inserts in the two clones were realized, a new attempt to reclone the reductase was undertaken. A new strategy involving the cloning of cDNA made, as described above, from poly(A)$^+$ mRNA of oat seedlings into the λ-phage expression vector gt11 was employed using the monospecific polyclonal antibody raised against oat protochlorophyllide reductase as a screen. From a cDNA library of approx. 10^5 recombinant phages, several were initially selected as expressing the reductase reproducibly. One, p127, was selected on the basis of the insert size (approx. 1150 bp). The insert excised by *Eco*RI after purification was recloned into the *Eco*RI site of the plasmid pEMBL12(+). Fig. 8 shows that p127 cross-hybridizes during Southern-blot analysis with one of the clones (252) identified previously as a putative reductase clone. Confirmation of the sequence similarity between the two clones was obtained from base sequence analysis (see below).

Partial characterization of p127 was achieved by single and multiple restriction-enzyme mapping (Fig. 9), from which the sequencing strategy outlined in the Figure was devised. One six cutter (*Pst*I) and five four cutters (*Sau*3A, *Hae*III, *Alu*I, *Taq*I, and *Rsa*I) were used to cut the isolated purified *Eco*RI insert, and the resulting mixture of fragments was ligated into the appropriate phage M13 vector for sequencing. The resulting base sequence was translated into an amino acid sequence by selecting the reading frame showing minimum interruption by stop codons. This translated into a 315-

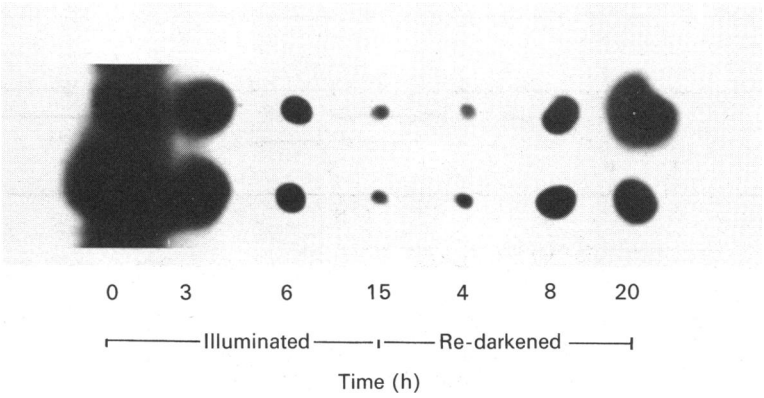

Fig. 7. 'Dot-blot' analysis of protochlorophyllide reductase transcript levels under various illumination conditions

Etiolated oat plants were illuminated for up to 15 h, followed by re-darkening for up to 20 h, during which time samples were taken as indicated and poly(A)$^+$ mRNA was isolated. Aliquots of the RNA (100 ng) were loaded in duplicate on to a nitrocellulose filter and hybridized with ^{32}P-labelled 113 (5×10^6 c.p.m.) as described in Fig. 6. Finally the washed filter was dried and autoradiographed.

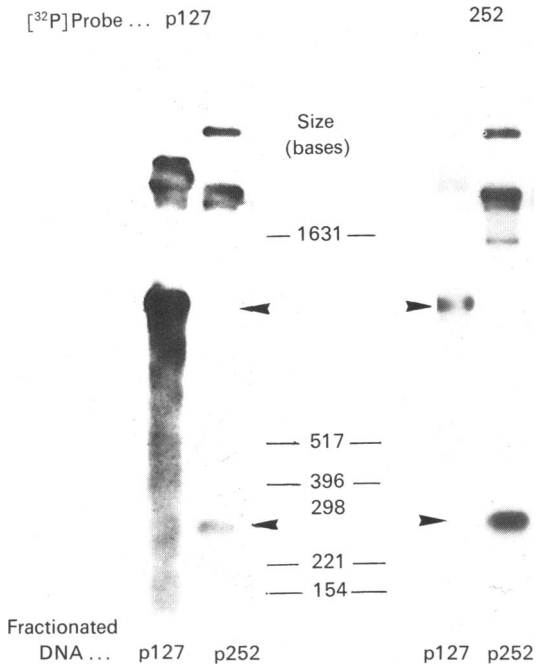

Fig. 8. Reciprocal hybridization of the two putative protochlorophyllide reductase clones 252 and p127

The cDNA inserts were excised from the purified plasmids with either *Pst*I (252) or *Eco*RI (p127) and 2 μg of each separated in duplicate on a 1.5%-agarose/TBE(Tris/borate/EDTA) gel, together with DNA molecular-mass markers (pAT153 cut with *Hin*FI). After transfer of the DNA to nylon membrane (Hybond-N), both samples were hybridized separately to either the ^{32}P-labelled nick-translated inserts of either 252 or p127 as indicated and finally autoradiographed. Hybridizing bands are indicated by ►.

amino-acid peptide of M_r 33758, which, although it does not represent a full-length copy of the gene, must nevertheless represent more than 95% of the mature reductase sequence. Prior to this we had been successful in partially sequencing the 252 clone from its 5′ end, but had experienced considerable problems in obtaining any 3′ sequence data, owing to difficulties in cloning such fragments into M13. Attempts at sequencing the clone by the Maxam & Gilbert (1980) procedure, using the (two times) CsCl-density-gradient-purified plasmid also proved unsuccessful, owing to problems in reading through the poly(G/C) tail of the clone. What sequence we did have for the 252 was found to overlap at its 5′ end with the 3′ region of the 127, where complete sequence similarity in the open-reading-frame overlap of the two clones is seen (Fig. 10). As no further useful information was to be gained from sequence data on the non-coding part of the 252 clone, no attempts at further sequencing of this or the 113 clone were made. In the p127 sequence (Fig. 10) downstream of the translation termination codon there is a 218-base 3′ intranslated sequence which terminates in a poly(A)$^+$ tail 15 residues long. In the absence of any structural data on the protein, it is impossible to conclude exactly how much of the mature reductase our sequence represents. To confirm the identity of our derived sequence as originating from the reductase, some structural information on the reductase was sought.

The amino acid composition of the enzyme isolated from wheat etioplasts by two successive cycles of preparative SDS/PAGE was chemically estimated. The resulting percentage composition is plotted in Fig. 11, together with the corresponding data deduced from the p127 sequence. Despite the fact that the predicted composition may be based on an incomplete DNA sequence, a remarkable similarity between the two compositions is apparent, enhancing our confidence in identification of p127 as a protochlorophyllide reductase clone. Sequence data from the direct analysis of the protein proved very difficult to obtain, and all our attempts at *N*-terminal sequencing of the purified protein were unsuccessful, presumably owing to the protein being *N*-terminally blocked. As an alternative strategy, CNBr fragments of the purified protein were prepared and the six major fragments purified by SDS/PAGE. One of these (17.5 kDa) on analysis produced the *N*-terminal sequence:

Met-Xaa-Asp-Gly-Asp-Glu-Ser

which, as can be seen, corresponds to the segment of the derived sequence underlined in Fig. 10.

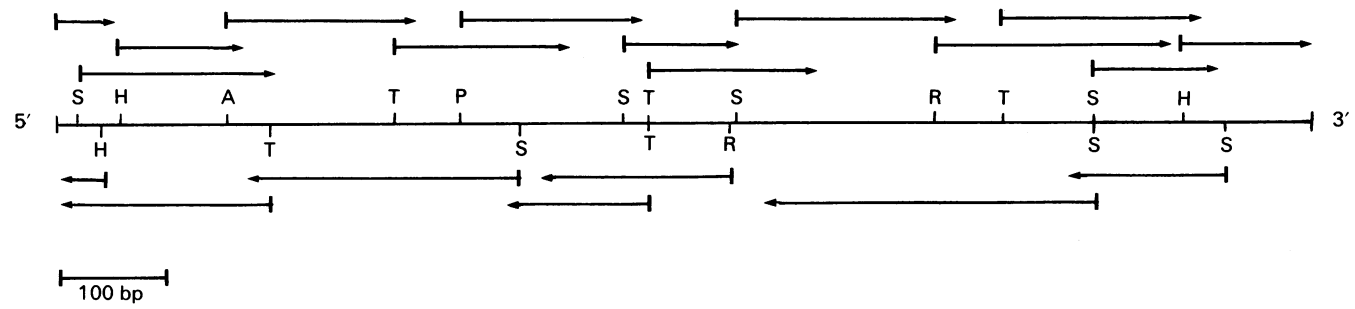

Fig. 9. Restriction-endonuclease map and sequencing strategy for cDNA clone p127

The *Eco*RI fragment of p127 was digested separately with the various restriction enzymes as indicated and the resulting fragments cloned into appropriately cut M13mp18 or M13mp19 for sequencing by the dideoxy method; these read in the directions indicated by the arrows. The restriction enzymes used were: S, *Sau*3A; H, *Hae*III; A, *Alu*I; T, *Taq*I; P, *Pst*I; and R, *Rsa*I.

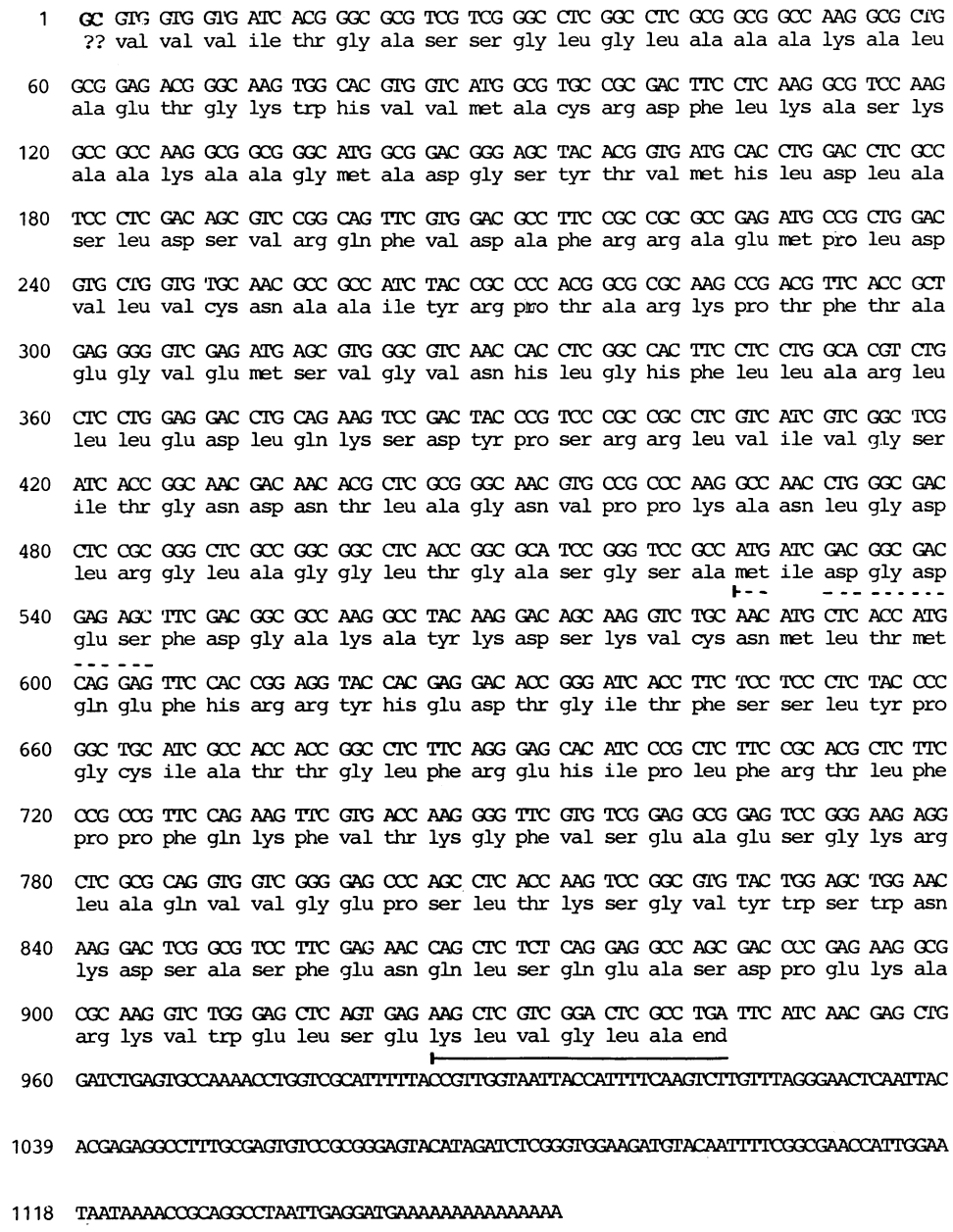

Fig. 10. Nucleotide and derived amino acid sequence of clone p127, a protochlorophyllide reductase clone

The sequence is shown in the 5′ → 3′ direction. The region of overlap in the open reading frame with the clone 252 is shown (continuously) underlined in the sequence. The position of overlap with the chemically derived amino acid sequence of the CNBr cleavage fragment is indicated by the broken underlining (-------).

DISCUSSION

The effect of light on plant development is nowhere more obvious than its effect on the development of chloroplasts. The illumination of etiolated plants results in the transition of etioplasts, with their characteristic paracrystalline prolamellar bodies, into mature chloroplasts containing grana and stroma lamellae. This transition offers a convenient system for studying the process of chloroplast formation and has, as such, contributed significantly to progress in this area.

Studies over the past few decades have shown that the etioplast-to-chloroplast transition is accompanied by an increased accumulation within the developing organelle of certain proteins, e.g. light-harvesting chlorophyll *a*/*b* protein (LHCP) and carboxylase, whereas some others, notably protochlorophyllide reductase, decrease in amounts. Further developments have indicated that the changes reflect light modulation of transcription of the nuclear genes coding for these chloroplast proteins (see review by Tobin & Silverthorne, 1985). Such conclusions have only been possible as a consequence of the application of the techniques of modern molecular biology, and cloned DNA sequences complementary to the

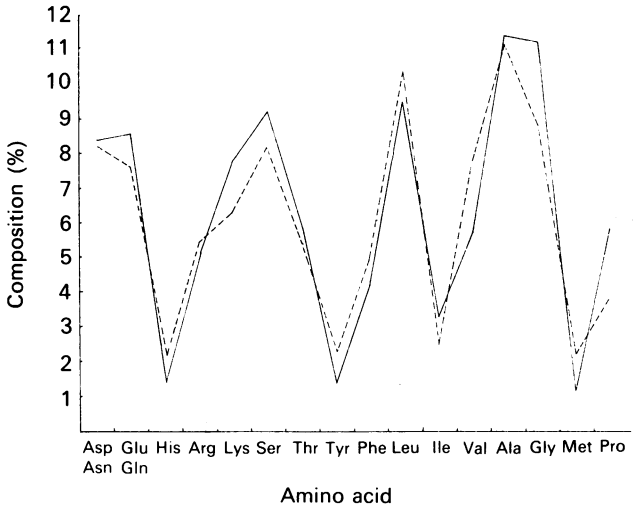

Fig. 11. Comparison of the amino acid composition of protochlorophyllide reductase as derived from the cDNA sequence or chemical analysis

The amino acid composition predicted from the cDNA sequence (Fig. 10) (----) or from direct chemical analysis of the purified mature reductase (——) are plotted. Aspartic acid + asparagine and glutamic acid + glutamine are considered together as they cannot be distinguished by chemical analysis.

mRNAs of several of the chloroplast proteins regulated by light have been isolated.

A putative cDNA clone for protochlorophyllide reductase was reported by Apel *et al.* (1983), and this has been used extensively to demonstrate that light turns off the transcription of the reductase gene (see Harpster & Apel, 1985). This work has gone a long way to explaining the interesting response of the enzyme to light. Protochlorophyllide reductase, however, besides showing this light-modulated accumulation, is also of interest in its own right as an unique light-dependent enzyme. Its presence as the major component of the etioplast's crystalline prolamellar body has inevitably led to suggestions of its importance as a structural component of the latter (Ryberg & Sundquist, 1988). Some sequence information on any reductase clone would inevitably provide valuable information regarding these aspects of the enzyme. Such information is provided for the first time in the present paper. Also, during isolation of the reductase clones, we have qualitatively confirmed most of the findings of Apel *et al.* (1983) with respect to the light regulation of the synthesis of protochlorophyllide reductase.

Using a protochlorophyllide reductase-specific polyclonal antibody and a wheat-germ '*in vitro*' translation system, we have shown (Fig. 1) that a poly(A)$^+$ mRNA fraction prepared from etiolated oats represents an active source of translatable reductase transcripts. Furthermore, this translatability decreased dramatically on illumination of the leaves prior to the RNA extraction, with 15 h-illuminated leaves retaining only 10% of the reductase translatability of the etiolated leaves (Fig. 4). Interestingly, return of the 15 h-illuminated leaves to darkness for 15 h restores the reductase-translatable mRNA level to 90% of the original 'dark' level (Fig. 4). With the availability of a cloned cDNA probe for the reductase (see below), confirmation of this light-regulated transcription was forthcoming by a procedure ('Northern dot-blot' analysis) free of the criticisms normally directed at translatability assays (Fig. 7). These data we take as supporting the suggestion of a natural ability of the plants to display diurnal variation in the extent of transcription of the protochlorophyllide reductase gene, confirming our previous work (Griffiths *et al.*, 1985) on the daily fluctuations in the reductase peptide levels in greening oat plants.

In contrast with the previous findings with barley (Apel, 1981), we find that the '*in vitro*' translation product which is immunoprecipitated by the reductase antibody (Fig. 1) and which shares common partial cleavage products with the isolated mature oat enzyme (Fig. 2) has an apparent M_r (41000), lower than the 44000 reported for the barley product.

Another feature of protochlorophyllide reductase formation we find rather unclear concerns processing of the primary precursor P41. This, in our hands, always migrates, in all gel systems tested, as a single polypeptide of M_r 41000. This compares with a doublet of M_r 35000 and 37000 as the mature peptides in 6-day-old etiolated oats. Both components of this doublet are enzymically active (Oliver & Griffiths, 1981), appear structurally related from peptide mapping and are immunologically cross-reactive (L. Jacobs & W. T. Griffiths, unpublished work). We have shown that the relative proportions of the 35 and 37 kDa peptides vary in plants in an age-dependent manner, with the 37 kDa component predominating in young plants, e.g. 5-day-old etiolated oats, whereas the 35 kDa component is the most abundant in older, say 10-day-old, etiolated oats (Griffiths & Walker, 1987). This suggests that the protochlorophyllide reductase mRNA is translated into a single 41 kDa precursor, which in young tissues is processed to a 37 kDa mature reductase but which in older tissues can be processed a stage further to the 35 kDa form, perhaps associated with membrane relocation of the enzyme. A multiplicity of peptide forms is not an uncommon feature among plant proteins, and a number of other cytoplasmically synthesized chloroplast proteins also appear as multiple, mostly doublet, forms. In both pea (*Pisum sativum*) (Cuming & Bennett, 1981) and barley (Bellemare *et al.*, 1982) mature LHCP is a doublet, each peptide produced from a specific precursor, each of which is the product of a specific mRNA. This conclusion is in keeping with identification of a number of genes encoding LHCP mRNAs in pea (Timko & Cashmore, 1983). Similarly, 'malic' enzymic in maize (*Zea mays*) bundle-sheath chloroplasts exists as a doublet centred at 63 kDa (Collins & Hague, 1983). These are produced from a precursor doublet of M_r approx. 75 kDa, again implying the presence of two mRNAs. In contrast, however, mature cytoplasmic phosphoenolpyruvate carboxylase of maize exists as a doublet, produced in a situation analogous to that of protochlorophyllide reductase, from a single precursor obviously the product of a single mRNA (Sims & Hague, 1981). Therefore although the occurrence of doublets of plant proteins is quite common, such doublets can originate from either two mRNA species, as in LHCP, or by limited proteolysis of the product of a single mRNA, as in protochlorophyllide reductase. Despite this, the physiological significance of multiple forms of proteins remains a mystery.

From the initial plasmid cDNA library, two potential

reductase cDNA clones, labelled 113 and 252, were selected on the basis of various screening procedures. The clones hybridized to the expected size mRNA (1.6 kDa) during Northern-blot analysis of 'dark' mRNA (Fig. 6), selected an mRNA species translatable into a product of similar M_r to the reductase precursor (P41) during hybrid released translation (Fig. 5). This product recovered in similar experiments using the 113 plasmid bound to nylon membrane (Hybond-N; Amersham International) was also shown to be specifically immunoprecipitated by the reductase antibody (S. A. Kay, unpublished work), further supporting the identity of the hybrid-released translation product as the protochlorophyllide reductase precursor. Again the 252 plasmid, already shown to cross-hybridize with 113 (see above) also hybridized during Southern blots with sequences from a λgt11 clone (p. 127) already selected on the basis of expression of the reductase protein (Fig. 8). Confirmation of the identity of the 252 clone was eventually obtained from sequence analysis, indicating it as showing 100% similarity to a short section (18 bases) of the 5′ end of the reductase open reading frame (Fig. 10). In view of the complications of sequencing the 113 and 252 clones and the success with the p127 analysis, no further structural work on the two plasmid clones was undertaken. Selection of the p127 clone from the λ-phage library was based, after initial screening, on the length of the cDNA insert in the recombinant phage and its strong hybridization with 252 (Fig. 8). Sequencing of the 1461-bp clone revealed an open reading frame coding for a peptide of M_r 33758. Identity of this peptide as the reductase was confirmed by comparison of the amino acid composition, calculated from the derived sequence, with that experimentally determined for the purified protein (Fig. 11). Also, a comparison of the derived sequence with chemical sequence data obtained from analysis of CNBr fragments of the protein (Fig. 10) offered further confirmation of the identity. In the absence of any information on the *N*-terminal residue of the mature protein, it is impossible to say what proportion of the mature reductase is co-represented by our sequence. However, from a comparison of the M_r of the derived sequence and mature proteins, bearing in mind also the similar percentage compositions (Fig. 11), it is likely that the derived sequence represents at least 95% of the mature protein. This represents the first report of any structural information on protochlorophyllide reductase.

The amino acid composition of the reductase from oats was reported by Roper *et al.* (1987). This result is fairly similar to that given here, with the exception that chemically, they found a single cysteine residue per mole of the reductase, whereas our predicted sequence gives four cysteine residues (Fig. 10). Recalling the implication of cysteine at the active site of the reductase (Oliver & Griffiths, 1981) the position of this residue(s) could help to define the active site of the enzyme. We have used the computer program WORDSEARCH (Devereux *et al.*, 1984) to search the protein data banks NBRF and EMBL for sequence similarity with potential nucleotide-binding sites in our sequence and also looked for conserved sequences associated with the Rossmann nucleotide-binding fold (Rossmann *et al.*, 1975) in the structure. These studies, together with other considerations such as, e.g., the presence of a hydrophobic cysteine residue etc., highlight the *N*-terminal end of the structure, namely residues 2–31 (Fig. 10), as the most likely NADPH-binding site. However, much more work is required to make such an identification unambiguous.

Another feature of the reductase sequence which might be important in assessing its structural role is the relatively high proportion of hydrophobic amino acids (30%). However, from secondary-structure predictions on the sequence, there is no obvious membrane-spanning region. Again, the predicted reductase sequence (Fig. 10) with its relatively high basic amino acid content (Arg and Lys accounting for 12%) makes it possible to comprehend the high pI values (8.2–8.5) reported for the reductase (Ikeuchi & Marakami, 1982; Bergweiler *et al.*, 1983). This basic property might also be considered in assigning a role for the reductase in the etioplast membrane.

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