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A novel chlorophyll a/b binding (*Cab*) protein gene from petunia which encodes the lower molecular weight *Cab* precursor protein

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ABSTRACT

The 16 petunia Cab genes which have been characterized are all closely related at the nucleotide sequence level and they encode Cab precursor polypeptides which are similar in sequence and length. Here we describe a novel petunia Cab gene which encodes a unique Cab precursor protein. This protein is a member of the smallest class of Cab precursor proteins for which no gene has previously been assigned in petunia or any other species. The features of this Cab precursor protein are that it is shorter by 2-3 amino acids than the formerly characterized Cab precursors, its transit peptide sequence is unrelated, and the mature polypeptide is significantly diverged at the functionally important N terminus from other petunia Cab proteins. Gene structure also discriminates this gene which is the only intron containing Cab gene in petunia genomic DNA.

INTRODUCTION

The initial capture of light energy in the chloroplasts of higher plants is accomplished by an abundant, integral membrane complex - the light harvesting complex (LHC II), which is composed of chlorophylls a and b and nuclear-encoded binding proteins. These chlorophyll a/b binding (Cab) proteins are translated in the cytoplasm into precursor proteins, which are transported into the chloroplast, proteolytically processed to their mature size, and inserted into the thylakoid membranes. The light harvesting complex, LHC II, adapts to changes in light intensity and wavelength to optimize energy capture. This adaptation is mediated in part by the reversible phosphorylation of the Cab proteins (1). Changes in the pattern of Cab protein phosphorylation are also associated with light-induced stress (photoinhibition) and herbicide poisoning (2). The Cab precursor proteins, in most of the plant species which have been analyzed,

are separable into two major size classes by one-dimensional SDS gel electrophoresis (3,4,5). All of the Cab genes which have been characterized to date appear to specifically encode the larger precursors - as judged from hybrid selection experiments (4,6): thus at present the functional and structural relationships between the two major size classes of Cab precursor proteins are unclear.

In petunia, there are at least 16 Cab genes, which can be classfied into 5 subfamilies. Within a subfamily, the individual genes are closely related; they encode identical Cab proteins and have similar 3' and 5' flanking regions. However, the genes of different subfamilies can be distinguished by sequence divergence both 5' and 3' to the protein coding region, and they encode slightly different polypeptides (7). Ten petunia Cab genes have been isolated and sequenced. These genes are all structurally similar - they are without intervening sequences and they are closely related at the nucleotide sequence level (greater than 90% homology). In addition, these genes all specify Cab proteins of the larger precursor size (4). Most, if not all, of these genes are expressed, based upon the isolation of eight distinct Cab cDNA clones, and also from the reintroduction of individual petunia Cab genes into tobacco where their transcription can be measured without interference from other Cab genes (Dunsmuir, Gidoni and Bond-Nutter, in preparation).

We report the isolation and analysis of a different petunia Cab gene (Cab 37), which encodes a Cab precursor protein of the formerly uncharacterized shorter size class. This gene is divergent in sequence and structure from the petunia Cab genes already characterized. The protein encoded by Cab 37 differs significantly at the amino terminal region of the mature protein and its transit peptide is completely divergent from other Cab proteins of petunia.

MATERIALS AND METHODS

Plants:

Petunia (Mitchell) was grown under greenhouse conditions as described by Dunsmuir et al.(4). Petunia were dark-treated by placing greenhouse-grown plants in total darkness for 7 days.

Leaves were harvested which had emerged during the period of growth in the dark.

PolyA RNA Isolation and Library Construction:

Petunia RNA and DNA was prepared according to Dunsmuir et al.(4). Petunia leaf polyA RNA was isolated by chromatography on oligo dT cellulose columns (8). A lambda gt 10 cDNA library was constructed from petunia leaf polyA RNA (9), and consisted of 5000 independent recombinants. The genomic library was constructed in the lambda cloning vector EMBL3, from a Sau3A-partial digest of petunia genomic DNA (10).

Hybridization Probes and Genomic Blots:

Nick-translations were carried out by standard procedures (8). First strand, ^{32}P-labelled cDNA probes were prepared in a 50ul reaction mixture containing: 50 mM Tris-HCl pH 8.5, 40 mM KCl, 10 mM $MgCl_2$, 0.4 mM dithiothrietol, 50 uM each dATP and dCTP, 1 mM each dGTP and dTTP, 10 ug/ml oligo dT $^{12-18}$, 20 U of AMV reverse transcriptase, 10 U of RNasin, 100 uCi each of ^{32}P-dATP and ^{32}P-dCTP and 2 ug petunia leaf polyA RNA. The reaction was carried out by diluting the RNA in sterile, distilled water, heating at 70°C for 3 minutes, chilling 5 minutes on ice, and then adding the other reagents in the order indicated. The reaction was incubated at 42°C for 60 minutes. The cDNA:RNA duplexes were denatured and the RNA hydrolyzed by addition of 2 ul of 0.5 M EDTA and 25 ul of 175 mM NaOH. The mixture was heated at 65°C for 1 hour and neutralized with 25 ul of 1 M Tris-HCl pH7.5 and 25 ul of 1 N HCl. Denatured salmon sperm DNA (10 ug) was added as a carrier and the labelled cDNA isolated by chromatography over a 5 ml, G-50 column equilibrated in 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1% SDS.

Genomic blots were carried out according to Dean, et al. (10). RNA blots were as described by Maniatis, et al. (8).

In vitro Translation:

Raw wheat germ was purchased from Goodson's Health Foods and Vitamins, Berkeley, California. Wheat germ extract was prepared (11) and assays for protein synthetic activity (12) demonstrated a 20-30 fold dependence on added total RNA.

Hybrid Selection:

Specific Cab mRNAs were purified from total leaf mRNA by

hybridization to cloned DNA that was denatured and immobilized on nitrocellulose filters (13). DNA (20 ug) was diluted in 0.67 ml, 0.1X SSC and denatured with 67 ul of 1 N NaOH. The mixture was incubated at room temperature for 5 minutes, placed in a boiling water bath for 10 minutes, then diluted to 10 ml with 2 N NaCl. Nitrocellulose filters (25 mm circles) were wet with 2X SSC, placed in the filter apparatus and rinsed with 10 ml, 6X SSC. The DNA was loaded by gravity filtration, the filter was rinsed with 10 ml of 6X SSC and then dried and baked under vacuum at 80°C for 2 hours. The filters were placed in the bottom of siliconized glass scintillation vials and shaken overnight at 37°C in 1 ml, prehybridizaion buffer (20 mM PIPES, pH 7.5, 1 mM EDTA, 0.8 M NaCl, 0.2% SDS, 5X Denhardts, 50% formamide and 100 ug/ml poly A). Filters were rinsed in 2 ml of hybridzation buffer (prehybridzation buffer minus Denhardts and the poly A). The buffer was discarded and the filters shaken with 0.1 ml hybridzation buffer plus 100 ug/ml, petunia poly A RNA for 24-48 hours at 37°C.

Unbound RNA was removed and the filters washed twice with 3 ml of 2X SSC, 0.1% SDS at room temperature (15 minutes per wash). This wash regime was repeated with 0.1X SSC, 0.1% SDS at 50°C. The filters were chopped and transferred to 1.5 ml microfuge tubes. Bound RNA was eluted with 0.4 ml, 5 mM EDTA pH 7.2 by boiling for 2 minutes, then placing tubes on ice. The eluted RNA was removed to a new tube, NaCl added to 0.2 M, tRNA added to 20 ug/ml and ethanol to 70%. The mixture was chilled, the RNA recovered by centrifugation and the dried pellet was dissolved in water.

The isolated RNA was translated in a wheat germ extract and the ^{35}S-methionine labelled proteins sized by SDS polyacrylamide gel electrophoresis. The gel was fixed for 1 hour in destaining solution, soaked 20 minutes in Amplify™ (Amersham, Inc., Chicago, Illinois, USA), dried on Whatman 3MM paper and exposed to Kodak XAR-5 X-ray film at -70°C.

DNA Sequencing:

Both strands of the Cab 1B cDNA clone were sequenced. One strand of the Cab 37 genomic clone coding region and 3' flanking region was sequenced. Both strands of the Cab 37 5' flanking

sequence were determined. The chain termination method of DNA sequencing (14) was used throughout.

Primer Extensions:

A Cab 37-specific oligonucleotide primer was 5' end-labelled, hybridized with 10 ug of petunia total leaf RNA and extended with reverse transcriptase to the 5' end of the mRNA template (15). The products were separated on a DNA sequencing gel and detected by autoradiography. The optimal hybridization temperature for this primer ($40^{o}C$) was determined empirically. In addition, the annealling reactions were carried out in a large excess of primer so that the amount of the extended primer reflected the concentration of Cab 37 mRNA in the sample. The 18-base primer is complementary to the Cab 37 mRNA sequence at a position which encodes the transit peptide (nucleotides 134 to 151, Figure 2).

RESULTS AND DISCUSSION

Isolation of a cDNA clone for the smaller Cab precursor

To identify genes encoding the small precursor proteins of the light harvesting complex, we initiated a general search for nuclear-encoded, light-inducible petunia cDNAs, which did not cross-hybridize at high stringency to characterized Cab and SSU (the small subunit of ribulose bisphosphate carboxylase) cDNA probes. A library of cDNA clones, prepared from petunia leaf polyA^{+} RNA, in the vector lambda gt10, were screened with ^{32}P-labelled cDNAs to a) poly A^{+} RNA from light grown leaves, b) poly A^{+} RNA from dark grown leaves, c) pCab 3 and d) pSSU 71. One of the resulting clones, cDNA 1B which hybridized strongly with the a, but not b, c, or d probes, specified a polyadenylated RNA of approximately 950 bases which was strongly light-induced (Figure 1).

Nucleotide Sequence Analysis of Clone 1B

The cDNA clone 1B has a length of 927 nucleotides. Both strands of this clone have been sequenced using the dideoxy sequencing method. Nucleotide sequence analysis confirms cDNA 1B as a Cab gene (Figure 2); it shows a low level of homology (75%) at the nucleotide level to the formerly characterized petunia Cab genes (7). The nucleotide sequence of the Cab 1B clone encom-

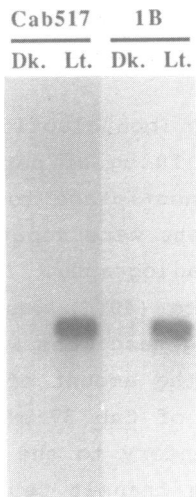

Figure 1 An autoradiograph of a northern blot hybridization carried out with petunia polyA RNAs isolated from dark-treated plants (Dk.) and light-grown plants (Lt.). Each track of the formaldehyde-agarose gel contained 1 ug of RNA. The posthybridization wash was at 55°C in 0.1% SSC, 0.1% SDS. (Cab 517 is a PstI-Hind3 subclone from the Cab 51 gene).

passes an open reading frame which corresponds to a complete Cab coding region of 265 codons, with 19 bases of 5' untranslated and 113 bases of 3' untranslated sequence. The lack of a poly A sequence at the 3' end most likely reflects an artifact of cDNA synthesis or cloning. Alignment of the amino acid sequence with the described Cab protein sequences predicts a transit peptide 36 or 37 amino acids in length, which is longer by two amino acids than any other Cab transit peptide in petunia (Figure 2 and Figure 3). Furthermore the Cab 1B transit peptide shows no homology with the other petunia Cab gene transit peptides (Figure 3, Table 1). By contrast, among the characterized petunia Cab gene subfamilies, 88-91% transit peptide sequence homology is observed (7). The Cab 1B transit peptide sequence is, however, closely related to the transit sequence of a Cab gene (AB19) from Lemna gibba, an aquatic monocotyledonous species (Figure 3) (16). Conservation of this transit peptide sequence over such a wide evolutionary distance suggests that there may be functional distinctions among the various transit peptides.

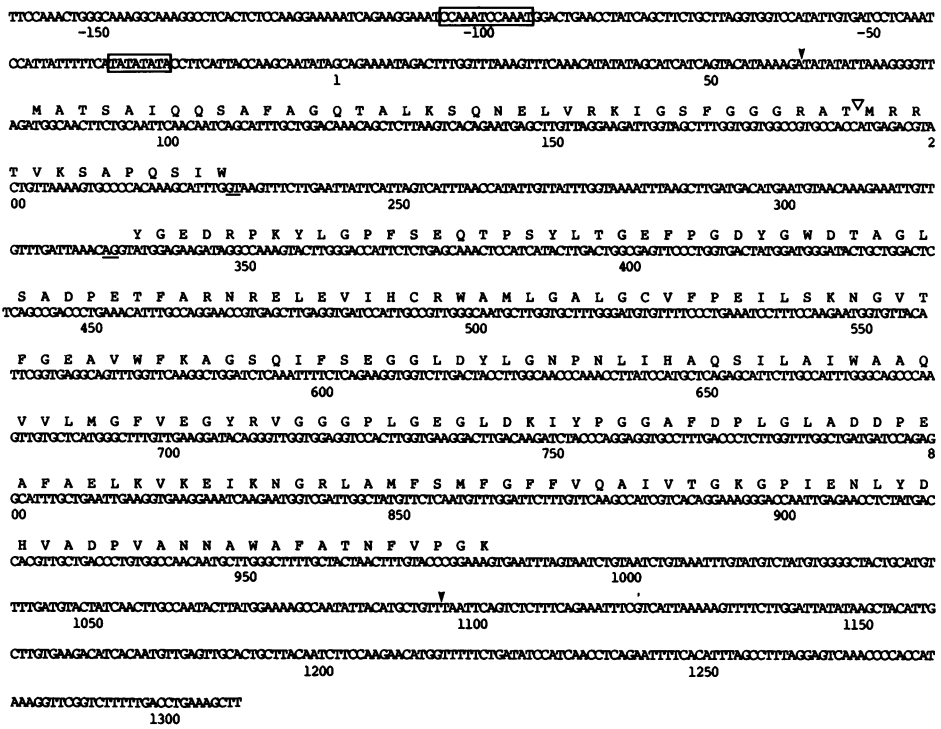

Figure 2 Nucleotide sequence and translated amino acid sequence of Cab 37 and Cab 1B. Nucleotides are numbered from the CAP site. The extent of the Cab 1B cDNA sequence is indicated with arrows (▼). (The Cab 1B sequence does not contain the intron sequence). The CAAT and TATA sequences are boxed. The terminal GT and AG dinucleotides of the single intron are underlined. A triangle (▽) marks the putative processing site within the Cab precursor protein.

Within the mature protein coding region, the amino acid sequence homology between Cab 1B and the characterized petunia Cab proteins is only 86% (Table 1), compared to 96-98% among the described petunia Cab gene subfamilies (7). Alignment of the amino acid sequences demonstrates that differences are concentrated in the N-terminal region of the mature protein (Figure 3). Within the N-terminal thirteen amino acids of Cab 1B there are six amino acid substitutions and a deletion of four amino acids relative to Cab 91R (a formerly characterized Cab gene). However, compared to the Lemna AB19 gene, this region of Cab 1B shows only two amino acid substitutions (Figure 3).

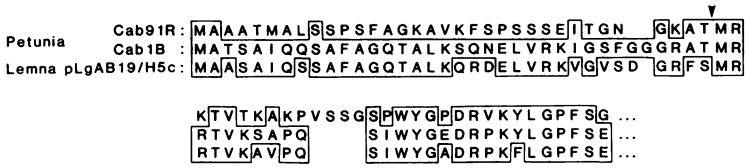

Figure 3 A comparison of the N-terminal amino acid sequence of the precursor protein of Cab 1B, Cab 91R (Dunsmuir, 1985) and the Lemna Cab gene AB 19 (Karlin-Neumann, et al., 1985). The arrow marks the putative processing point for the mature peptide. Gaps are introduced to improve sequence alignment.

Genomic hybridization pattern with Cab 1B cDNA and isolation of a genomic clone

Under conditions of low hybridization stringency (42°C, 50% formamide, 6x SSC), Cab 1B hybridizes to the same pattern of genomic EcoRI restriction fragments as formerly characterized Cab genes. However, the relative intensity of hybridization of specific bands differs dramatically between Cab 1B and Cab 517 which was used as an example of an already characterized Cab gene (Figure 4). By increasing the post hybridization wash temperature it is possible to demonstrate that Cab 1B is most closely related to sequences on a single 7 kb EcoRI fragment. A genomic library in the lambda vector EMBL3, constructed from a partial Sau3a digest of petunia nuclear DNA (10) was screened with the Cab 1B probe and two overlapping phage clones, phage 37 and phage

TABLE 1: Cab Gene Sequence Homology

Comparison	The Transit Peptide % Sequence Homology		The Mature Protein % Sequence Homology	
	Amino Acid	Nucleotide	Amino Acid	Nucleotide
Cab 1B vs. Cab 91R[1]	16	29	86	75
Cab 1B vs. Lemna Cab[2]	71	59	92	75
Petunia Cab[1] vs. Cab 91R	88-91	81-88	96-98	89-90

[1] Petunia Cab and Cab 91R sequences were from Dunsmuir (7).
[2] Lemna Cab sequence data were from Karlin-Neumann, et al.(16).

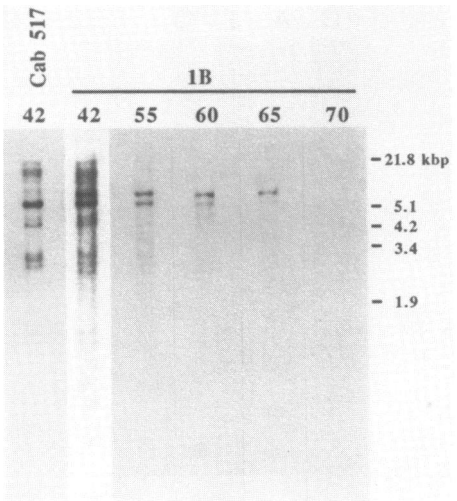

Figure 4 Autoradiograph of a genomic Southern blot hybridization. Each track of the agarose gel contained 10 ug of petunia nuclear DNA cut with EcoRI. After transfer to nitrocellulose, the filter was cut into strips and hybridized (4X SSC, 50% formamide, 42°C, 24 hours) with the specified probes. The posthybridization wash was in 0.1X SSC, 0.1% SDS at the indicated temperatures.

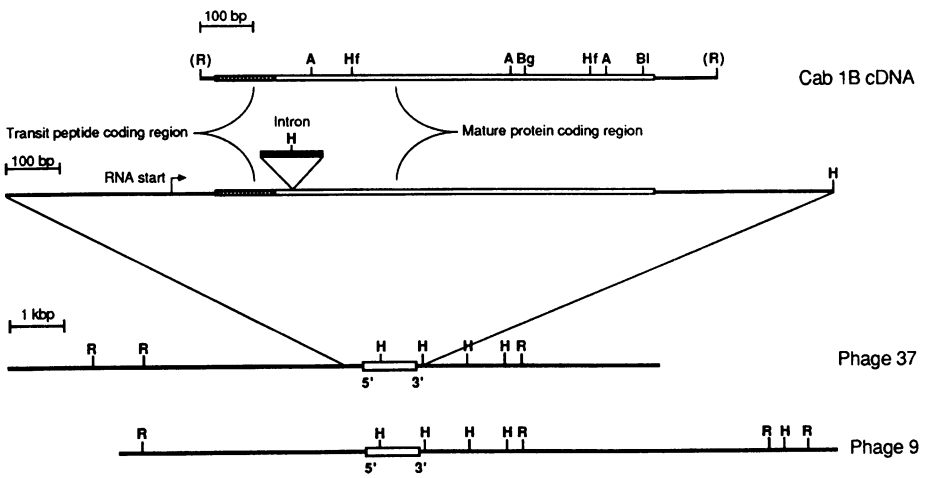

Figure 5 Restriction maps of the phage genomic clones, 37 and 9 and the Cab 1B cDNA clone. Phage 9 and phage 37 carry the 7kb, petunia genomic EcoRI fragment, which hybridizes specifically to the Cab 1B cDNA clone. The EcoRI sites in the cDNA originate from the cDNA cloning procedure. Abbreviations: A - AvaII; Bg - BglII; Bl - BalI; H - HindIII; Hf - HinfI; R - EcoRI.

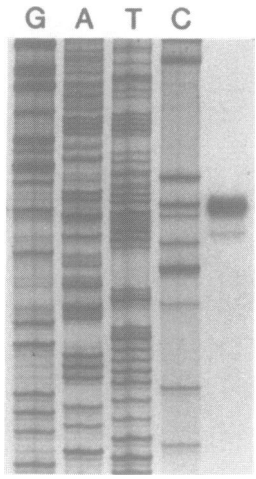

Figure 6 Primer extension analysis of leaf RNA. A Cab 1B specific oligonucleotide primer was 5' end labelled, hybridized with 10 ug leaf RNA, extended by reverse transcriptase to the 5' end of the mRNA template, then sized on a sequencing gel.

9, were isolated. These phage both contain a 7 kb EcoRI fragment which hybridizes with Cab 1B at high stringency and comigrates with the 7 kb genomic EcoRI fragment. The Cab gene carried on phage 37 and phage 9 was designated Cab 37 (Figure 5).

Nucleotide sequence analysis of Cab 37 genomic region

Nucleotide sequence analysis indicates that the Cab 37 genomic sequence is identical to the Cab 1B cDNA sequence, except for a 106 bp intron located 146 bp 3' from the translational start site (Figure 2). Cab 1B hybridizes only with the Cab 37 genomic sequence (7kb EcoRI fragment) under conditions of high stringency, and we estimate from reconstruction experiments (data not shown) that there is only a single copy of Cab 37 in the genome. Therefore, we presume that the cDNA clone 1B is derived from a transcript of Cab 37, an intron containing Cab gene.

The 5' untranslated sequence of Cab 37 is unrelated to the corresponding sequence of the described petunia Cab genes. Primer extension experiments establish a 5' untranslated RNA leader length of about 80 bases and confirm that Cab 37 is present at high steady state levels in light grown leaf tissue (Figure 6). Upstream from the transcription start site are two

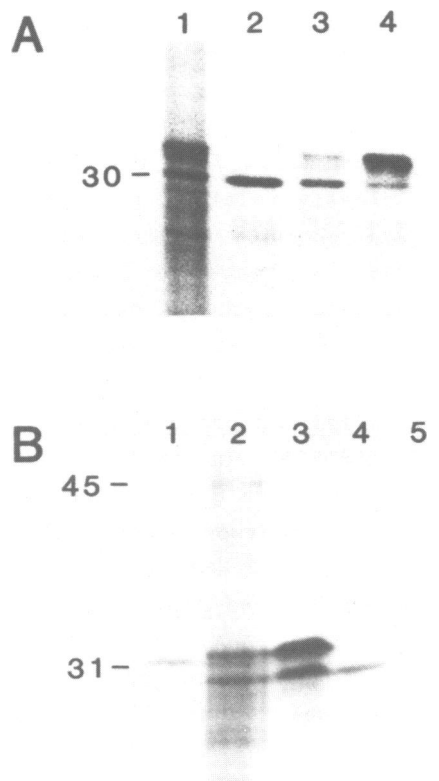

Figure 7 A) Fluorograph of ^{35}S methionine-labelled Cab precursor proteins from hybrid selection/translation, separated on an SDS polyacrylamide and separated on an SDS gel. 1 - in vitro translation of petunia leaf polyA RNA; 2 - protein translated from petunia polyA RNA, hybrid selected by Cab 1B at 50°C. 3 - Cab 1B hybrid selection at 45°C; 4 - Cab 517 hybrid selection at 45°C. B) Fluorograph of ^{35}S methionine-labelled Cab precursor proteins from in vitro transcription/translation separated on an SDS polyacrylamide gel. 1 - Cab 24 precursor protein obtained from in vitro transcription/translation of the Cab 24 gene. 2 - in vitro translation of petunia leaf polyadenylated RNA; 3 - proteins precipitated from a translation of petunia leaf polyA RNA by rabbit antibodies directed against petunia LHCII; 4 - Cab 1B precursor protein obtained from in vitro transcription/translation of the Cab 1B cDNA; 5 - proteins precipitated from a translation of petunia leaf polyA RNA by rabbit preimmune serum.

sequence elements common to most eukaryotic promoters (17), a TATA sequence (TATATATA) at -29 bp and a CAAT-like sequence (CCAAAT) at -100bp. Four of the five petunia Cab gene sub-

families share a region of homology centered at about -130 bp from the CAP site (7); this sequence is not conserved in Cab 37. The 3' untranslated sequence of the Cab 37 gene also differs from the formerly characterized Cab genes except for the sequence TTTGTAT located 25bp downstream from the stop codon, TGA. The sequence TTTGTTT, or closely related variants, occurs in most of the Cab and SSU genes in petunia (4,18). Since the Cab 1B cDNA does not have a 3' polyA sequence, we cannot precisely identify the extent of transcription to the 3' end. The polyadenylation signal sequence observed in animal genes, AATAAA (19), is not found.

Identification of the precursor protein specified by Cab 37.

In view of the striking nucleotide sequence differences between Cab 37 and all other Cab genes in petunia we have concentrated upon defining the precursor protein which corresponds to this gene. Initially a hybrid-select translation method was used; polyA RNA, complementary to Cab 1B, was isolated by hybridization to immobilized Cab 1B DNA. This RNA was translated in vitro and the labelled proteins were separated by electrophoresis on an SDS polyacrylamide gel (Figure 7A). Under normal hybrid selection/translation conditions (0.8 M NaCl, 50% formamide, 45°C) the 1B clone selects mRNAs which translate to give a set of proteins, some of which correspond in size to those which are hybrid selected by the formerly characterized Cab cDNAs (as indicated here by Cab 517). However, the relative stoichiometries of the proteins selected by Cab 1B and Cab 517, differ. After a higher stringency selection of RNA with Cab 1B (0.8 M NaCl, 50% formamide, 50°C) only a single protein is translated and this protein is smaller than any of the proteins which are hybrid selected at high stringency with other Cab genes. Furthermore, the protein, which is translated from mRNA hybrid selected against Cab 1B, is quantitatively precipitated by antibodies directed against maize or petunia LHC-II (data not shown). Thus Cab 1B must encode a precursor protein of unique size which is related antigenically to the already characterized Cab proteins of the LHC-II. The protein encoded by Cab 1B comigrates on an SDS gel with the smallest Cab precursor protein (30kdal) (Figure 7A).

To further establish the Cab 1B precursor protein size class, Cab 1B (which we know to contain a complete coding region) and Cab 24 (a member of the Cab 102 gene subfamily which also contains a complete coding region) were each cloned downstream from the SP6 bacteriophage RNA polymerase promoter in pSP65 and the SP6 transcripts were synthesized in vitro as described by Krieg and Melton (20). The RNAs were capped and translated in a wheat germ extract (See Materials and Methods). The proteins specified by these two distinct Cab genes were electrophoresed adjacent to a translation of petunia leaf polyA RNA. Figure 7B demonstrates that the protein encoded by the Cab 1B is significantly smaller than that specified by Cab 24 (which is the smallest Cab precursor protein thus far identified in petunia). The protein encoded by the Cab 37 gene (Cab 1B cDNA) in fact specifies the precursor protein of the smallest size class - a group of proteins for which genes have not previously been isolated in petunia.

CONCLUSION

The Petunia Cab genes characterized to date were isolated initially by virtue of cross-homology to a pea Cab cDNA clone (4). In an effort to identify more distantly related genes which encode proteins of the LHC-II, we initiated a general search for light-inducible petunia cDNA clones. This screen produced a novel Cab cDNA clone, Cab 1B. Sequence analysis of Cab 1B and its corresponding genomic clone, Cab 37, showed this gene to be structurally distinct from the known Cab genes of petunia.

The already characterized class of petunia Cab genes, "Type 1 genes", consists of 16 closely related genes, ten of which have been cloned and sequenced. They share about 90% amino acid sequence homology within the transit peptide coding regions and greater than 96% homology within the mature protein sequence. None of these genes contain introns.

Cab 37 represents a second class of petunia Cab gene, "Type 2 genes". It is a highly-expressed, single-copy, intron-containing gene, encoding a protein which is significantly divergent from the other petunia Cab proteins and which represents the smallest size class of Cab presursor proteins. The shorter class

of Cab precursor protein is distinguishable from the longer size class in the following ways: 1) the Cab 1B precursor protein is 2-3 amino acids shorter, 2) the transit peptide sequences are unrelated, and 3) the mature protein coding sequences are significantly diverged, particularly at the N-terminus, the site of Cab protein phosphorylation.

Limited proteolysis of thylakoid membranes has established that the N-termini of the Cab poteins extend into the stroma and are responsible for grana stacking (21). Phosphorylation of this region of the proteins, which occurs in vivo at a threonine residue within the N-terminal seven amino acids, acts to reduce grana stacking and functions to shift light-harvesting capacity from photosystem II to photosystem I (2,22). These changes reflect the adaptation of the photosynthetic apparatus to the conditions of shade (1) and illustrate the functional importance of the N-terminal sequences. In view of the sequence homologies between Cab 1B and the Lemna AB19 gene and the regulatory significance of the N-terminal amino acid sequences, it is possible that the Cab 1B protein is functionally distinct from the other protein Cab proteins.

Uptake and processing of Lemna Cab precursor proteins in isolated chloroplasts has been demonstrated by Kohorn et al.(23). During transport into the cloroplast and thylakoid membrane insertion, the Lemna AB30 (Type 1) gene product was cleaved to form three proteins of slightly differing sizes. The smaller two species corresponded in size to mature light harvesting complex proteins. We have shown, in preliminary experiments, that the Cab 37 precursor protein can be transported into isolated pea chloroplasts, proteolitically processed and inserted into thylakoid membranes - although the uptake efficiency was low. The Cab 37 precursor protein was processed to a collection of three or four proteins which differed slightly in size. We have not yet determined, in our system, whether this results from a normal ambiguity in the proteolysis reaction or is an artifact of chloroplast fractionation. Control experiments carried out by Kohorn, et al.(23) suggest that the multiple cleavage sites are legitimate.

The Cab 37 gene, although very different from the 16 other

Cab genes which have been analyzed in petunia, is very similar to the Cab gene (AB19) which has been sequenced in the aquatic monocot Lemna (16). We have established for the first time that this "Type 2" class of Cab gene actually specifies a major component (by mass) of the light harvesting complex - the smaller precursor class. In view of the conservation of this "Type 2" Cab gene in evolution, and the functional importance of the regions of the protein which are unique to the Type 2 class, these proteins must be important components of the LHC II.

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