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RNA editing in tobacco chloroplasts leads to the formation of a translatable *psbL* mRNA by a C to U substitution within the initiation codon

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The *psbL* gene which codes for a 38 amino acid peptide of photosystem II, together with the photosynthetic genes *psbE* and *psbF*, is contained in a conserved position of many species of higher plant plastomes. The alignment of the *psbL* nucleotide sequences from ten species shows strong conservation, which is indicative of a functional gene. The tobacco and spinach *psbL* genes have, however, an ACG codon instead of the initiator ATG codon observed in the homologous position of the other eight species. Evidence is presented that in tobacco chloroplasts a translatable *psbL* mRNA containing an AUG initiator codon is formed by a C to U editing of the ACG codon. This observation, following the previously reported editing of an *rpl2* gene in maize chloroplasts, underlines a more widespread occurrence of this type of post-transcriptional mRNA modification and demonstrates its presence in a dicotyledon plant.

Key words: initiation codon/photosynthetic gene/*psbL*/RNA editing/tobacco chloroplasts

Introduction

The editing of mRNA as a post-transcriptional process was discovered in the kinetoplast genetic system of trypanosomes (Benne *et al.*, 1986) and later in the nuclear encoded mRNA of human apolipoprotein B (Powell *et al.*, 1987) and in a number of genes encoded by plant mitochondrial DNA (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989). Different types of editing, such as base substitutions and insertions or deletions of U residues, are observed in the various systems. In plant mitochondria, mainly a C to U editing but to some extent also the reverse substitution from U to C residues is found. As a result of these base substitutions, changes of amino acid sequences are observed which in many cases lead to conservation of functionally important amino acid positions. However, neutral base substitutions of third codon position, not altering the corresponding amino acids, have also been observed. In certain cases an initiation codon, as in the *nad1* gene of wheat mitochondria (Chapdelaine and Bonen, 1991), a termination codon as in the *atp9* gene of *Petunia* mitochondria (Wintz and Hanson, 1991) or an improvement of the intron secondary structure necessary for splicing as in the *Oenothera nad1* gene (Wissinger *et al.*, 1991) is created by editing. In contrast to the editing in trypanosomes, where guide RNA has been shown to act as a template as well as a substrate for transesterification reactions of U residues (Blum *et al.*, 1991), nothing is known about the mechanisms underlying the C to U editing reactions in plant mitochondria.

While mRNA editing appears to be a common processing step in the plant mitochondrial system, editing of a chloroplast mRNA was observed for the first time only recently (Hoch *et al.*, 1991). The *rpl2* gene of the maize and rice plastome contains an ACG codon at the position where *rpl2* genes of other chloroplast species have an AUG initiator codon. Instead of the ACG codon predicted from the gene sequence, an ATG codon was found in the amplified cDNA obtained from *rpl2* mRNA, showing that a C to U editing creates an AUG initiator codon for the *rpl2* mRNA. This observation has led us to search for other examples of RNA editing in plastids.

The situation similar to the *rpl2* gene of maize appears to exist for the *psbL* genes from tobacco and spinach chloroplasts. This gene, originally described as ORF38 in liverwort (Ohyama *et al.*, 1986; Fukuzawa *et al.*, 1988) and tobacco (Shinozaki *et al.*, 1986), was only later identified as the *psbL* gene coding for a hydrophobic 3.2 kDa polypeptide of photosystem II (Webber *et al.*, 1989). Subsequently, *psbL* was identified at homologous positions of several plastomes from higher plants. The adjacent genes, *psbE* and *psbF*, are cotranscribed together with the *psbL* gene to give a 1.1 kb polycistronic mRNA. A comparison of ten *psbL* genes shows that eight encode a conventional ATG initiator codon, while two of them, the tobacco (Shinozaki *et al.*, 1986) and the spinach (Herrmann *et al.*, 1984) *psbL* genes, contain ACG codons at the homologous position. Wolfe and Sharp (1988) and Hiratsuka *et al.* (1989) have postulated that these ACG codons function as initiation codons—as is suggestive from an ACG initiation codon occurring in Sendai virus RNA (Gupta and Patwardhan, 1988). In view of the editing of *rp12* mRNA observed in maize chloroplasts (Hoch *et al.*, 1991), we tested the possibility of a C to U editing in the *psbL* gene of tobacco by which the ACG codon would be converted to an AUG initiator codon. Here we present experimental evidence for this type of editing by which a translatable *psbL* mRNA containing an AUG initiator codon is created.

Results

The psbL genes of tobacco and spinach chloroplasts have lost the initiator codon

The position of the *psbL* gene on the tobacco plastome and within the *psbE/psbF/psbL*/ORF40 gene cluster is shown in Figure 1. This figure shows also the mRNA region around the initiation codon, which is encoded at the DNA level by an ACG. In Figure 2 an alignment of 10 *psbL* genes is presented which, on the one hand shows strong conservation at the nucleotide level throughout the gene, but on the

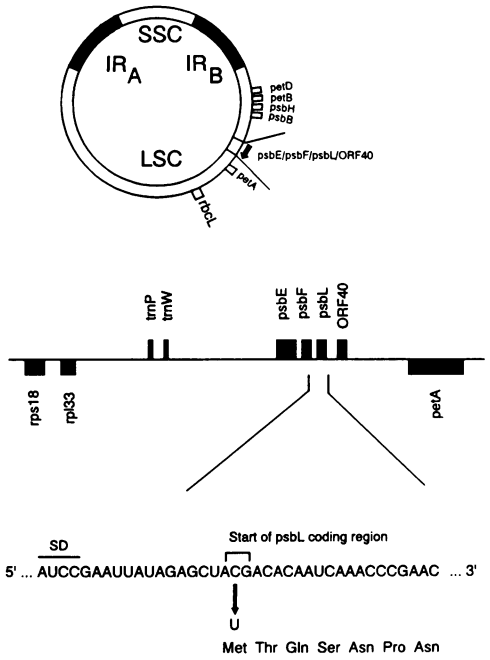

Fig. 1. Position of the *psbL* gene on the tobacco plastome. In the upper part, the tobacco plastome with the two inverted repeat regions IR_A and IR_B which separate the small (SSC) and large (LSC) single copy region is depicted together with several representative genes (*rbcL*, *petA*, *psbB*, *psbH*, *petB* and *petD*) surrounding the *psbE/psbF/psbL* gene cluster (Shinozaki *et al.*, 1986). In the middle, an enlargement of the region encoding this gene cluster is shown. It should be noted that the orientation with respect to the upper part has been reversed in order to obtain 5′ → 3′ polarity of the *psbL* transcript from left to right. At the bottom, the 5′-terminal region of the *psbL* coding region of the *psbE/psbF/psbL*/ORF40 transcript together with part of the *psbF–psbL* intergenic region and a putative ribosomal binding site marked by SD (Shine and Dalgarno, 1975) is depicted. The editing position leading to the AUG start codon is marked by a vertical arrow.

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                MetThrGlnSerAsnProAsnGluGlnAsnValGluLeuAsnArgThrSerLeuTyrTrp
                .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Tobacco         ACGACACAATCAAACCCGAACGAACAAAATGTTGAATTGAATCGTACCAGTCTCTACTGG  60
Snapdragon      ATGACACAATCAAACCCGAACGAACAAAGTGTTGAATTGAATCGTACCAGTCTCTACTGG  60
Barley          ATGACACAATCAAACCCGAATGAACAAAATGTTGAATTGAATCGTACCAGTTTATACTGG  60
Rice            ATGACACAATCAAACCCGAATGAACAAAATGTTGAATTGAATCGTACCAGTCTATACTGG  60
Maize           ATGACACAATCAAACCCGAATGAACAAAATGTTGAATTGAATCGTACCAGTCTATACTGG  60
Wheat           ATGACACAATCAAACCCGAATGAACAAAATGTTGAATTGAATCGTACCAGTCTATACTGG  60
Pea             ATGACACAATCAAACCCAAATGAACAAAATGTCGAATTGAATCGTACCAGTCTCTACTGG  60
Liverwort       ATGACACAACCAAATCCAAACAAACAAAGTGTAGAATTAAATCGTACTAGCCTCTATTGG  60
Euglena         ATGCCACAAACAAATTCAAATAAAAAAACAGTCGAATTAAATAGGACAAGTTTATACTGG  60
Spinach         ACGACACAATCAAACCCGAACGAACAAAATGTTGAATTG--------------------  39
                * *-***** **** -* **  **-*** -** ***** ***-*-** **- * **-***

                GlyLeuLeuLeuIlePheValLeuAlaValLeuPheSerAsnTyrPhePheAsn---
                .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Tobacco         GGGTTATTACTCATTTTTGTACTTGCTGTTTTATTTTCCAATTATTTCTTCAATTAA   117
Snapdragon      GGGTTATTACTCATTTTTGTACTTGCTGTTTTATTTTCCAATTATTTTTTCAATTAG   117
Barley          GGTTTATTACTCATTTTTGTACTTGCTGTTTTATTTTCCAATTACTTCTTCAATTGA   117
Rice            GGTTTATTACTCATTTTTGTACTTGCTGTTTTATTTTCCAATTACTTCTTCAATTGA   117
Maize           GGTTTATTACTCATTTTTGTACTTGCTGTTTTATTTTCCAATTACTTCTTCAATTGA   117
Wheat           GGTTTATTACTCATTTTTGTACTTGCTGTTTTATTTTCCAATTACTTCTTCAATTGA   117
Pea             GGTTTATTACTCATTTTTGTACTTGCAGTTTTATTCTCAAATTATTTCTTCAATTAA   117
Liverwort       GGTTTATTACTAATATTTGTACTTGCTGTTTTATTTTCTAATTATTTTTTTAATTAA   117
Euglena         GGACTATTATTAATTTTTGTTTTGGCTGTTCTATTTTCTAGTTATATCTTTAATTAG   117
Spinach         ------------------------------------------------------------
                ** -*****-* **-******--*-**-***-****-** *-*** -* ** ****
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Fig. 2. Alignment of *psbL* nucleotide sequences. Except for the *Antirrhinum* (snapdragon) sequence (J.Kudla, unpublished) published sequences were taken for the alignment as follows: tobacco (Shinozaki *et al.*, 1986), barley (Krupinska and Berry-Lowe, 1988), rice (Hiratsuka *et al.*, 1989), maize (Haley and Bogorad, 1990), wheat (Webber *et al.*, 1989), pea (Willey and Gray, 1989), liverwort (Ohyama *et al.*, 1986), *Englena gracilis* (Cushmann *et al.*, 1988) and spinach (Herrmann *et al.*, 1984; partial sequence). The initiation codon including the non-edited ACG codon in tobacco and spinach are framed. The amino acid sequence derived from the tobacco *psbL* gene is given in the top line. Positions identical in all species are indicated in the bottom line by a star. Positions deviating only in one species are marked by −. Positions deviating in two or more species are unmarked.

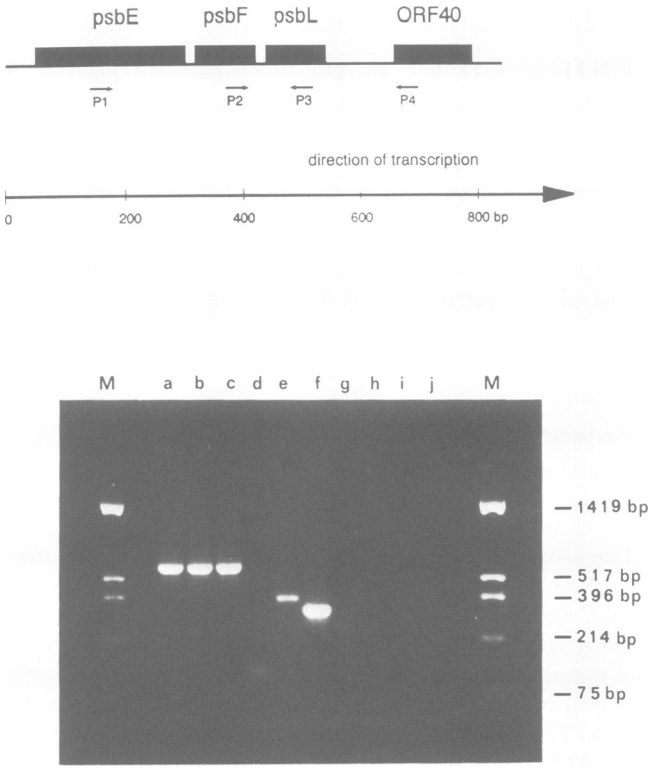

Fig. 3. Amplification of *psbL* sequences from tobacco chloroplasts by polymerase chain reactions. In the upper part, the positions and orientations of primers P1, P2, P3 and P4 within the *psbE/psbF/psbL*/ORF40 gene cluster are indicated. Primers P1 and P2 have the mRNA polarity whereas primers P3 and P4 are complementary to the transcript. In the lower part characterization of symmetrical amplification products by electrophoresis on 2% agarose gels is shown. The individual lanes show products derived from the following reaction components: Lane a, chloroplast DNA and primer pair P1+P4; lane b, total DNA and the same primer pair; lane c, chloroplast RNA reverse transcribed following by amplification with primer pair P1+P4; lane d, same as c but using primer pair P2+P3; lane e, same as c but using primer pair P1+P3; lane f, same as c but using primer pair P2+P4; lane g, chloroplast RNA without reverse transcription and using primer pair P1+P4 for the amplification reaction; lane h, chloroplast DNA treated with DNase I followed by amplification reaction with primer pair P1+P4; lane i, chloroplast RNA treated with RNase A followed by reverse transcription and amplification reaction with primer pair P1+P4; lane j, buffer control without RNA or DNA using primer pair P1+P4. DNA size markers of the marker lanes M were obtained by digestion of plasmid pUC18 with restriction enzyme *Hin*fI.

other hand shows a T to C transition in the first codon of the tobacco and spinach genes. No in-frame ATG and GTG codons can be found in the vicinity of the ACG codons of these species (see also lower part in Figure 1). Therefore, functional compensation of the mutated initiator codons by other initiator codons can be excluded.

Amplification of psbL sequences from tobacco chloroplasts

In Figure 3, amplification products containing *psbL* sequences are presented. The primer combination P1+P4 produces single amplification products of the expected size of 589 bp with chloroplast DNA (lane a), with total leaf DNA (lane b) and with cDNA obtained from reverse transcription of chloroplast RNA (lane c). This product is absent in the controls presented in lane g (chloroplast RNA without reverse transcription), h (chloroplast DNA treated with

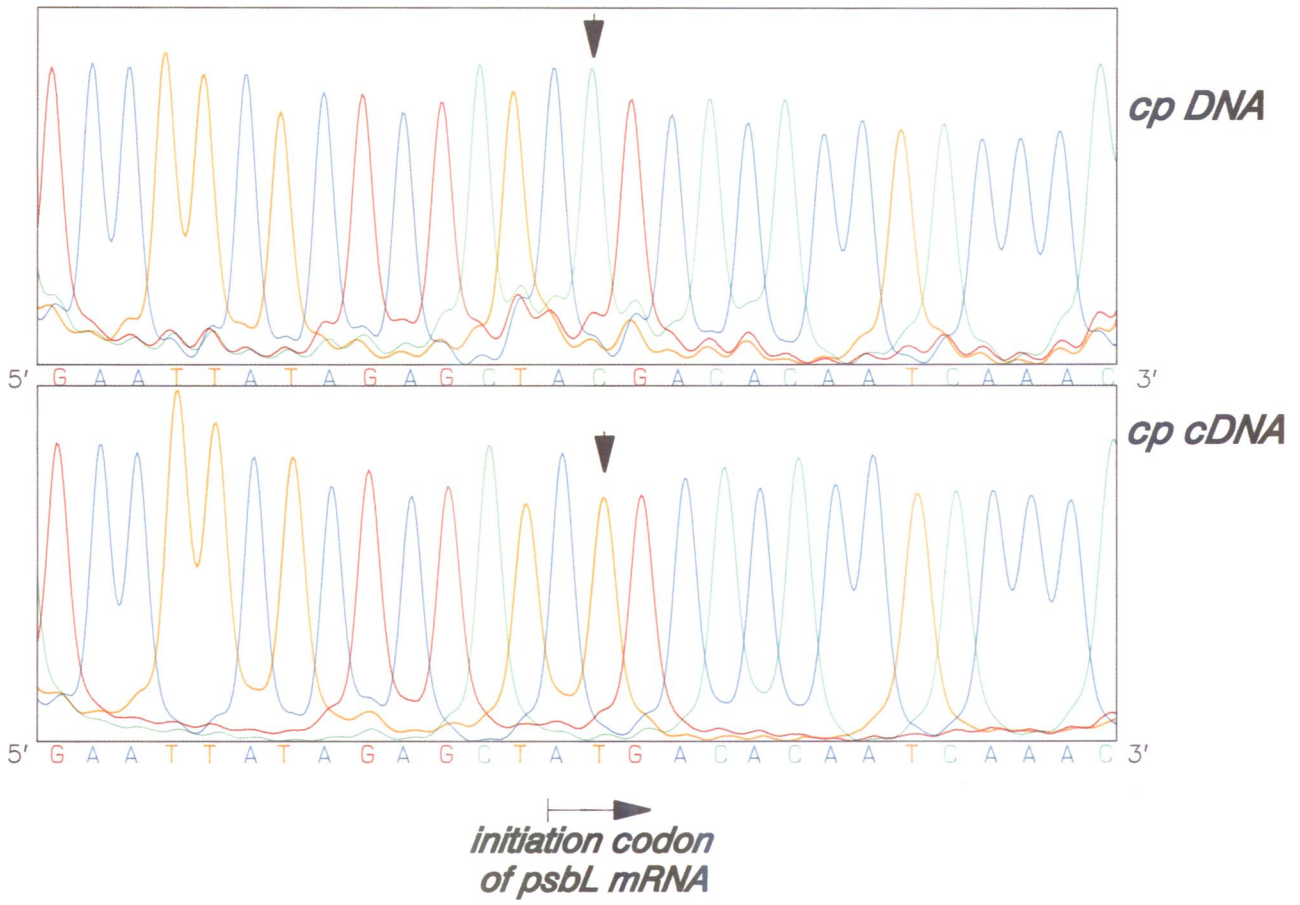

Fig. 4. Comparison of the *psbL* nucleotide sequences obtained from amplification products of tobacco chloroplast DNA (top) or cDNA (bottom). The chloroplast DNA sequence was obtained from the 589 bp product of lane a in Figure 3 after further asymmetrical amplification with primer P4 in excess. The single-stranded amplification product was then sequenced by using the fluorescently labelled primer P2. The same procedure was applied for sequencing the 589 bp cDNA amplification product of lane c of Figure 3. The positions where the sequences recorded by the automated laser fluorescence detection system differ between the chloroplast DNA and cDNA derived sequences by a C to T transition are marked with vertical arrows.

DNase I), i (chloroplast RNA treated with RNase before reverse transcription) and j (buffer control). The control with DNase I demonstrates that the amplification products of lanes a and b are derived from DNA. The controls without reverse transcription and with RNase, on the other hand, clearly show that the product of lane c is derived from an RNA (hence this product is termed cDNA). The expected sizes of 121 bp, 384 bp and 326 bp of the amplification products are obtained from reverse transcribed chloroplast RNA also with the primer combinations P2+P3, P1+P3 and P2+P4 (lanes d, e and f of Figure 3). Here too, unique products are observed, excluding size variability of the amplified regions.

Sequence analysis of psbL derived cDNA

The amplification product obtained with the primer combination P1+P4 and reverse transcribed tobacco chloroplast RNA (lane c of Figure 3) was used for sequence analysis with the primer P2 in a fluorescently labelled form. In a parallel experiment a chloroplast DNA derived amplification product from lane a of Figure 3 was used for sequencing with the same primer. As shown in Figure 4, the two sequences are identical with each other (and with the previously published sequences; compare with Figures 1 and 2) with the only exception of a C to U transition which converts the ACG codon encoded in the chloroplast DNA to the AUG codon of *psbL* mRNA. This C to U editing has been confirmed with several independent RNA preparations and also by sequencing of the complementary strands with the conventional technique using primer P3 (data not shown). A virtually complete absence of C residues in the edited position of cDNA is also observed when total RNA from tobacco leaves is used instead of chloroplast RNA as template for reverse transcription (data not shown).

Discussion

An abundant 1.1 kb transcript of the *psbE/psbF/psbL*/ORF40 gene cluster has been shown to exist in tobacco chloroplasts (Carrillo *et al.*, 1986) as well as in other chloroplast species (Webber *et al.*, 1989) including spinach (Westhoff *et al.*, 1985). Mapping of the termini of this transcript in wheat (Webber *et al.*, 1989) and pea (Willey and Gray, 1989) shows a 5′ end preceded by a promoter structure proximal to the *psbE* gene and a 3′ end distal to the ORF40, indicating the inclusion of the *psbL* gene in the 1.1 kb transcript. In the absence of editing, amplification of the tobacco *psbL* cDNA would lead to a C residue in the second position of the first codon. However, as evident from Figure 4, a virtually complete substitution by a U residue is observed, which clearly shows that the C residue of the primary transcript is subject to C to U editing.

We have considered the possibility of a transfer of a translatable ATG-containing *psbL* gene to the nucleus or to the mitochondrial genome (for review see Palmer, 1985, 1991) followed by divergence of the chloroplast-encoded gene to a pseudogene without an initiation codon. Transfer to the nucleus would, however, necessitate the acquisition of a transit peptide coding sequence of the transferred *psbL* gene in order to allow import of the encoded polypeptide into chloroplasts. In addition to this, transfer to the mitochondrial genome would also necessitate export of the polypeptide from the mitochondria (prior to import into chloroplasts), which has so far not been observed for any polypeptide encoded by the mitochondrial genome. The absence of transit peptide sequences and the identity of the cDNA amplified sequences with the plastome-encoded *psbL* gene (except for the editing position), however, exclude the possibility that the edited *psbL* mRNA is encoded outside the chloroplast and that trace contamination of chloroplast RNA with nuclear or mitochondrial RNA were the sources for amplification of the edited *psbL* cDNAs. The observed amplification products of expected size using various primer combinations (Figure 3), is also an indication that splicing has not caused a size variation of the RNA which might be considered as evidence for the transfer of an initiator codon-bearing sequence to the primary transcript. Furthermore, the identity of the cDNA amplified sequences and the plastome encoded *psbL* gene (except for the editing position) also excludes the possibility that an AUG codon is created by a splicing event. We therefore conclude that the observed C to U transition reflects an RNA editing event which is necessary to produce a functional initiator codon for the *psbL* mRNA.

RNA editing of a chloroplast mRNA was described for the first time only recently (Hoch *et al.*, 1991). In that case, editing of a gene (*rp12*) coding for a component of the genetic apparatus of a monocot plant (maize) was shown. In contrast to this, the editing described in this paper is in a photosynthesis gene of a dicot plant. It appears therefore likely that RNA editing in chloroplasts is not restricted to particular gene classes or plant phyla. It is interesting in this connection that the *psbL* gene of the closely related species pea and snapdragon have retained the functional ATG initiator codon (see Figure 2), indicating that sequence divergence, creating editable positions, may occur at high frequency.

It is not possible to estimate the general frequency of chloroplast editing based on the two editing positions identified so far. The detection (by computer search as well as experimentally) of further editing sites is anticipated which may necessitate revisions of individual peptide sequences derived from many plastome encoded genes and open reading frames identified in the past. It is even conceivable that, as in the case of certain plant mitochondrial genes, the lengths of open reading frames or of identified genes will have to be modified due to editing events which create initiation or termination codons.

Finally, we would like to point out that the C to U editing—if it is the only or the predominant type of editing in organelles—may have contributed to the observed bias of AT pairs in chloroplast and mitochondrial genomes (Shimada and Sugiura, 1991; Murray *et al.*, 1989; Palmer, 1985). Although a high specificity of the editing process is necessary in order to guarantee high fidelity of gene expression, a certain low level of unspecific C to U editing, which would not interfere with the majority of proteins being translated correctly, is conceivable. Reverse transcription of the RNAs containing random C to U edited sites followed by recombination of the resulting cDNAs with organelle DNA could thus lead to a slow but steady increase of AT pairs.

Materials and methods

Oligonucleotides

The following four oligonucleotide primers were synthesized using a 380A automated DNA synthesizer from Applied Biosystems: P1, 5′-CCTTCC-CTATTCATTGCGGGTTGG-3′; P2, 5′-GGGATCAATATCAGCAA-TGCAG-3′; P3, 5′-CCCCAGTAGAGACTGGTACG-3′; and P4, 5′-GGAATCCTTCCAGTAGTATCGGCC-3′. The positions and orientations of these oligonucleotides used as primers for the polymerase chain reaction or for DNA sequence analysis are depicted in the upper part of Figure 3. Primer P2 was synthesized in a fluorescently labelled form and used for non-radioactive DNA sequencing (Ansorge *et al.*, 1986; Voss *et al.*, 1989). A random mixture of synthetic hexanucleotides was used as primer for cDNA synthesis (Kawasaki, 1990).

Isolation of nucleic acids from tobacco plants

Leaves from adult tobacco plants (*Nicotiana tabacum*, cv. Havanna) grown under greenhouse conditions were used for nucleic acid extraction and for chloroplast preparation. Prior to use, plants were kept in the dark for at least 24 h in order to reduce the starch content. Total plant RNA and DNA were isolated according to standard procedures (Kloppstech and Schweiger, 1976; Wienand and Feix, 1980). Chloroplasts, chloroplast DNA and RNA were isolated as described by Shaw (1988). Isolated RNA was treated with proteinase K in order to eliminate RNase contamination. Incubation of RNA with DNase I (RNase free) had to be carried out twice in order to remove trace amounts of DNA which gave rise to amplification products without reverse transcription.

Reverse transcription of RNA

5 μg of RNA (total RNA from tobacco leaves or from isolated tobacco chloroplasts) and 3 μg of random hexanucleotide primers dissolved in 20 μl of water were heated for 1 min to 95°C and then rapidly cooled on ice. The volume was then adjusted to 96 μl by addition of water, PCR buffer (final concentration: 10 mM Tris−HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine) and the four deoxynucleoside triphosphates at 0.25 mM. Reverse transcription was started by addition of 20 U of RNase inhibitor (Pharmacia) and 18 U of AMV reverse transcriptase. The reaction was carried out at room temperature for 10 min and continued at 42°C for 60 min. Finally the resulting product mixtures were heated to 95°C for two minutes and then stored at −20°C.

Polymerase chain reactions

Amplification of DNA (total DNA extracted from tobacco leaves or isolated from tobacco chloroplasts) and of cDNA obtained by reverse transcription was carried out according to standard protocols using 50−100 ng of DNA dissolved in PCR buffer containing the four dNTPs or using directly the cDNA product mixture described above. After addition of the respective primer pairs (1.5 μg of each primer) amplifications were started by addition of 3 U of *Taq* DNA polymerase (Amersham). After 30 cycles at 92°C for denaturing (1 min), 55°C for annealing (1.5−2 min) and 72°C for synthesis (1.5 min) the resulting product mixtures were extracted with chloroform for the removal of paraffin oil layers.

Asymmetrical amplification for generation of single-stranded DNA were carried out under identical conditions using 5 μl of the mixture resulting from a symmetrical amplification or 100 ng of chloroplast DNA as template but applying to 100:1 ratio of the primer concentration (100 pmoles of excess primer and 1 pmol of the limiting primer). The single-stranded amplification products after chloroform extraction were further purified by chromatography on Qiagen columns as recommended by the supplier (Diagen, Düsseldorf, FRG).

DNA sequence analysis

Sequencing reactions were performed on single-stranded templates obtained from asymmetrical amplification reactions (Wilson *et al.*, 1990). The chain termination method was applied but using the fluorescent primer P2 for labelling of the products. The products of the sequencing reactions were

separated by denaturing polyacrylamide gel electrophoresis and analysed by an automated laser fluorescence detection system obtained from EMBL (Ansorge *et al.*, 1986). Alignment of the *psbL* sequences was performed using the program PC/GENE (IntelliGenetics Inc., Mountain View, USA and Genofit GmbH, Heidelberg, FRG).

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