Nuclear-encoded chloroplast ribosomal protein L12 of *Nicotiana tabacum*: characterization of mature protein and isolation and sequence analysis of cDNA clones encoding its cytoplasmic precursor

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ABSTRACT

Poly(A)⁺ mRNA isolated from *Nicotiana tabacum* (cv. *Petite Havana*) leaves was used to prepare a cDNA library in the expression vector λ gt11. Recombinant phage containing cDNAs coding for chloroplast ribosomal protein L12 were identified and sequenced. Mature tobacco L12 protein has 44% amino acid identity with ribosomal protein L7/L12 of *Escherichia coli*. The longest L12 cDNA (733 nucleotides) codes for a 13,823 molecular weight polypeptide with a transit peptide of 53 amino acids and a mature protein of 133 amino acids. The transit peptide and mature protein share 43% and 79% amino acid identity, respectively, with corresponding regions of spinach chloroplast ribosomal protein L12. The predicted amino terminus of the mature protein was confirmed by partial sequence analysis of HPLC-purified tobacco chloroplast ribosomal protein L12. A single L12 mRNA of about 0.8 kb was detected by hybridization of L12 cDNA to poly(A)⁺ and total leaf RNA. Hybridization patterns of restriction fragments of tobacco genomic DNA probed with the L12 cDNA suggested the existence of more than one gene for ribosomal protein L12. Characterization of a second cDNA with an identical L12 coding sequence but a different 3'-non-coding sequence provided evidence that at least two L12 genes are expressed in tobacco.

INTRODUCTION

In higher plants, different classes of ribosomes exist in the cytoplasm, the chloroplast and the mitochondrion. Although chloroplasts code for and synthesize ribosomal RNA and many chloroplast ribosomal proteins (1,2,3), about two-thirds of these ribosomal proteins are synthesized in the cytoplasm (by translation of nuclear gene-derived mRNA) and are subsequently transported

into the chloroplast (4). Chloroplast ribosomal protein precursors in the cytoplasm have amino terminal transit peptides which are removed after protein transport across the membrane (5).

In *E. coli*, the L7/L12 protein (L7 is an amino-acetylated form of L12) constitutes the morphologically distinct stalk on the 50S ribosomal subunit (6,7). Demonstrated functions of L12 ribosomal protein in prokaryotes include involvement in binding to rRNA during ribosome assembly (8), maintenance of ribosome structure and function (8) and possible involvement in binding (9) and movement of peptidyl-tRNA during the translocation phase of the elongation cycle (10). *E. coli* ribosomal protein L12 plays an important role in EF-G-dependent GTP hydrolysis (11,12) and also binds to translation, elongation and initiation factors (9,13). L12 protein of *E. coli* possesses N-terminal and C-terminal domains which are connected by a relatively short, proline-alanine rich region which functions as a flexible hinge (14). In solution, the N-terminal domain forms a spontaneous dimer (15) and is essential for binding to ribosomal protein L10 (16). The C-terminal domain has been crystallized as a dimer which has a highly organized and stable secondary structure of alternating alpha helices and beta sheets (17). This region also contains a potential GTP binding site, a dimerization site and a site involved in interaction with protein synthesis elongation factors (17).

The L12 gene of *E. coli*, located in the L10 operon, is cotranscribed with genes for ribosomal protein L10 and the β and β' subunits of RNA polymerase. Ribosomal protein L12 synthesis is four to five-fold higher than that of L10 protein (18), while the distal β and β' subunits are synthesized at only 10-20% of the rate of L10. The mechanism whereby L12 is overproduced, relative to the downstream L10 gene is not known, but it results *in vivo* in the necessary stoichiometry (4:1) of L12:L10 in the 50S ribosome subunit (19). In higher plant chloroplasts (20) as in *E. coli* (19), L12 is present in the 50S subunit in multiple copies, relative to other ribosomal proteins.

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Since a ribosomal protein L12 gene is lacking in the published plant chloroplast genome sequences [i.e. tobacco (2), rice (21) and liverwort (22)], it must be a nuclear gene. Here we report characterization of cDNAs coding for a tobacco chloroplast ribosomal protein with homology to the large subunit L12 proteins of *E. coli* (23), spinach chloroplast (24) and eucaryotic ribosomes (25–28). In addition, we demonstrate that the amino-terminal sequence of mature tobacco L12 protein agrees with that predicted from the cDNA sequence. Determination of the size of L12 mRNA and evidence for the existence of more than one expressed gene coding for tobacco L12 is presented.

MATERIALS AND METHODS

Materials

Chemicals were from Sigma, Bethesda Research Laboratories (BRL), or Mallinckrodt. Agarose (Seakem) was from FMC. Nitrocellulose filters (HATF, 0.45 μ) were from Millipore. Restriction endonucleases, T4 DNA ligase and other DNA modifying enzymes were from either BRL, Boehringer Mannheim, Amersham, New England Biolabs, Promega Biotech or Stratagene. *Eco*RI linkers were from BRL. *Eco*RI adaptors and *Sma*I hexamers were from New England Biolabs. AMV reverse transcriptase was from Life Sciences. Predigested β gt11 arms, packaging extracts, bacterial strains, pBS(+) and pBluescript II KS(+) vectors were from Stratagene. [α - 32 P]dCTP was from ICN. [α - 35 S]dATP was from New England Nuclear. Sequenase was obtained from United States Biochemicals.

Identification of chloroplast ribosomal protein L12

Tobacco chloroplast ribosomal subunits were isolated as previously described (29), and proteins from the 50S subunits were extracted, dissolved in 40 μ l of 10mM Tris-HCl, pH 7.6, 150mM LiCl, 6M urea and 0.4M mercaptoethanol and separated by HPLC using a 30nm pore C3 silica column (Ultrapore RPSC, Beckman). Unfractionated 50S ribosomal proteins and HPLC-purified protein L12 were separated by the 2-D PAGE system I of Madjar, et al (30). The amino-terminal sequence of HPLC-purified L12 protein was determined by automatic basic Edman degradation (31) using a Beckman AB1 477A liquid pulse/gas phase protein sequencer.

Preparation of antibodies to 50S chloroplast ribosomal subunit proteins

Purified proteins from 50S chloroplast ribosomal subunits were used to immunize rabbits by surgical injection in the popliteal lymph nodes (32) of 1 mg ribosomal proteins in 100 μ l phosphate-buffered saline emulsified with 100 μ l of Freund's adjuvant. Intraperitoneal booster injections of 1mg antigen were given after one month. Serum was collected at the end of the first month, or after an additional month if a second injection is required.

mRNA isolation

Young, expanding tobacco leaves (1 to 6cm long) were collected from greenhouse-grown plants, washed with tap water and used immediately for total leaf RNA preparation (33). Poly(A)⁺ mRNA was separated from the total leaf RNA on oligo(dT)-cellulose columns (34) and its size distribution was <0.5 kb to >3 kb, as determined by formaldehyde-agarose gel electrophoresis (35). When wheat germ extract *in vitro* translation products of this poly(A)⁺ mRNA were analyzed by SDS and

acid-urea gel electrophoresis (36), the diversity and molecular weight range of basic polypeptides (including chloroplast ribosomal proteins) observed among the translation products (data not shown), was as would be predicted for intact total leaf poly (A)⁺ mRNA. Tobacco genomic DNA was prepared from the young expanding tobacco leaves following the method of Bedbrook (37).

cDNA synthesis and expression library construction

cDNA was synthesized from poly(A)⁺ mRNA templates (38). The size of the synthesized first and second cDNA strands was monitored on alkaline gels (35). The double stranded cDNA was blunt-ended with T4 DNA polymerase, methylated with *Eco*RI methylase, ligated to *Eco*RI linkers, and digested with *Eco*RI to remove excess linkers. To obtain full-length L12 cDNAs, it was necessary to develop a method to avoid *Eco*RI methylation and subsequent *Eco*RI digestion of the cDNA library. This was accomplished by constructing the cDNA library after ligating blunt-ended, non methylated cDNA to preannealed *Sma*I-*Eco*RI adaptors (39). Separate libraries of both cDNA types were prepared by ligation to *Eco*RI-digested bacteriophage λ gt11 arms (40). The resulting recombinant cDNA was packaged to produce a library (1.6 $\times$ 10⁶pfu) of infectious phage stocks which was plated and amplified on *E. coli* strain Y1088 and screened using *E. coli* strain Y1090 (40).

Screening of the cDNA expression library

Coliform-reactive antibodies were removed from antiserum to tobacco chloroplast 50S ribosomal subunit proteins essentially as described by Kemp and Cowman (41). Five ml of serum were mixed with 5ml of cyanogen bromide-activated Sepharose 4B to which approximately 3mg of a lysate of *E. coli* strains BNN97 (C600- λ gt11 lysogen) and Y1090 had been bound. After stirring gently overnight in an end-over-end shaker at 4°C, the mixture was poured in a 5ml syringe plugged with glasswool, and the antiserum was eluted with an equal volume of Tris-buffered saline (25mM Tris, pH 7.4, 0.14M sodium chloride and 5mM

Figure 1. Maps and sequencing strategy for cDNA clones of tobacco chloroplast ribosomal protein L12. (A). Size and coding regions of three different length cDNA clones encoding the L12 protein are shown. The scale at the top designates the nucleotide position in bases from the 5'-terminus of the L12-1 cDNA. Recognition sites for *Eco*RI and *Hind*III are indicated. (B). Horizontal arrows indicate polarity of DNA fragments sequenced. Some regions were sequenced using synthetic oligonucleotides (open boxes) L12-1 (complementary to nucleotides 241–258, Figure 2) and L12-2 (nucleotides 699–719, Figure 2).

potassium chloride). Antibody-containing fractions which absorbed light at 280 nm were pooled.

Screening for λ gt11 recombinants which expressed chloroplast ribosomal proteins was done after infecting *E. coli* strain Y1090 with 10^5 to 10^6 pfu of phage from the *Eco*RI-linker library and plating on 150mm plates. Plaques were transferred to nitrocellulose filters, incubated with antibody, washed, reacted with 125 I-protein A, dried and exposed to X-ray film to identify immunoreactive positive plaques. A partial length L12 cDNA was obtained which was then used to screen the *Sma*I-*Eco*RI adaptor-constructed cDNA library to obtain a full-length cDNA. Phage plating and plaque transfer to filters was as above for antibody screening. The nucleic acid probe was labelled with [α - 32 P] dCTP (3000 Ci/mmol) using the random primer method (42) and then purified on a Sephadex G-50 (fine) column (35). Prehybridization and hybridization were at 42°C in 50% formamide, 5×Denhardt's solution, 6×SSC, 50 mM sodium pyrophosphate (pH 6.5), 100 μ g/ml denatured salmon sperm DNA, 0.1% SDS and 10 mg/ml poly (A)⁺ RNA. Dextran sulfate (10%) was sometimes used in the hybridization mix. After hybridization for 12–18 hr, the filters were washed twice for 30 min in 6×SSC/0.1% SDS at room temperature and exposed with an intensifying screen to X-ray film at –80°C. Plaques which gave positive signals were further purified by two additional rounds of screening (40).

RNA and genomic DNA blot analysis

Northern blot analysis was done after electrophoresis of RNA in a 1.2% agarose gel in the presence of 2.2 M formaldehyde

		CCCCACCCCCCCCCCCCCCTTATCCGGTTTCTCTCATTT	41
		TGCTGTATCACACTCTCTTCTCTCTCTCTCATTTCTTCAACTCTCTGCTACA	96
-53		ATG GCT TCC ACT TTA TCC ACA ATT ACT CTT CGC TCT CCT TCT MET Ala Ser Thr Leu Ser Thr Ile Thr Leu Pro Ser	138
-39		CCT TCT ACC GCT TCC TCA ACA CAC GCC TCA ATT CCA TTC CCC Pro Ser Thr Ala Ser Ser Thr His Ala Ser Ile Pro Phe Pro	180
-25		AAA AAA GCC CTA <u>GAA TTT</u> CCC ATT CGC ACT CCC AAA CTC CAC Lys Lys Ala Leu Glu Phe Pro Ile Arg Thr Pro Lys Leu His	222
-11		CAC CGC CGA GCC ACT TTG CTC CGC CCT CTC GCC <u>GCC GTT GAA</u> His Arg Arg Ala Thr Phe Leu Arg Pro Leu Ala <u>Ala Val Glu</u>	264
4		<u>GCA CCT GAG AAG GTA GTC CAG CTC GGA</u> GAT GAA ATT TCC AAT Ala Pro Glu Lys Val Gln Leu Glu Gly Asp Glu Ile Ser Asn	306
18		TTA ACT CTC GCT GAC GCT CAG AAA CTC GTC GAG TAC CTT CAG Leu Thr Leu Ala Asp Ala Gln Lys Leu Val Glu Tyr Leu Gln	348
32		GAT AAG CTT GGT GTT ACC GCC GCA TCC TTC GCT CCG GCC GCC Asp Lys Leu Gly Val Thr Ala Ala Ser Phe Ala Pro Ala Ala	390
46		GTC GCC GCC CCA CTT GGC GCC GCC GCC GAA GCT CCT GCT GTG Val Ala Ala Ala Pro Gly Ala Ala Ala Glu Ala Pro Ala Val	432
60		GTG GAG GAG AAG ACT GAA TTT GAC GTT GTT ATT GAC GAA GTT Val Glu Glu Lys Thr Glu Phe Asp Val Ile Asp Glu Val	474
74		CCG AGT AAT GCG AGA ATT GCT ACG ATT AAG GCT GTT AGG GCT Pro Ser Asn Ala Arg Ile Ala Thr Ile Lys Ala Val Arg Ala	516
88		TTA ACT AGT TTG GCT TTG AAA GAG GCA AAG GAA TTG ATT GAA Leu Thr Ser Leu Ala Leu Lys Glu Ala Lys Glu Leu Ile Glu	558
102		GGA TTG CCT AAG AAA TTT AAG GAA GGT GTG TCT AAG GAT GAG Gly Leu Pro Lys Lys Phe Lys Glu Gly Val Ser Lys Asp Glu	600
116		GCT GAA GAT GCT AAG AAA CAG CTT GAA GAA GCT GGT GCT AAA Ala Glu Asp Ala Lys Lys Gln Leu Glu Ala Glu Ala Lys	642
130		GTT TCT ATT GCT TAACITGAAATTTGTGGTAAATGTTTAAATCTTATTTTGT Val Ser Ile Ala <u>TER</u>	692
		TTTGTGATATCTGGGAAAATGTTGTACTTTTTGTGTTTTGTAAAGAGAATC	747
		AGTTGTGATTGATAAAAAAAAAAAAAA	773

Figure 2. Nucleotide sequence of cDNA and deduced amino acid sequence of tobacco chloroplast ribosomal protein L12. The first 12 amino acids of the mature protein, confirmed by protein sequencing, are boxed. The initiation codon, the termination codon as well as the two internal *Eco*RI sites are underlined.

(35). The resolved RNA species were transferred to nitrocellulose membranes and hybridized to ^{32}P -labelled L12-1 cDNA (cf., Figure 1). The transfer was in $10\times\text{SSC}$ and hybridization and washing were as for cDNA library screening.

Tobacco genomic DNA was digested to completion with restriction endonucleases (*EcoRI* and *BamHI*) and subjected to electrophoresis in 0.8% agarose gels. The DNA was transferred to nitrocellulose filters and hybridized to L12-1 cDNA (cf., Figure 1) as described for cDNA library screening.

Recloning of cDNA inserts

The positive phage identified by immunological screening was plated on *E. coli* strain Y1090 and allowed to produce confluent lysis. Phage DNA isolated (35) from the *EcoRI* linker cDNA library was digested with *EcoRI*, resolved on agarose gels, and electroeluted. cDNA inserts were recloned in the unique *EcoRI* site of a pBS(+) vector. The recombinant plasmids were amplified in transformed cells of *E. coli* strain XL1 Blue. For recloning cDNA inserts isolated from the *SmaI-EcoRI* adaptor-constructed library (39), the phage DNA was isolated and

Figure 3. Purification and identification of tobacco chloroplast ribosomal protein L12. (A) Purification of L12 by HPLC of total proteins from 50S ribosome subunits. Proteins were eluted at 1ml/min. from a reverse phase column (Ultrapore RPS-C3) in 0.1% trifluoroacetic acid using a gradient of acetonitrile as follows: 10–22% in 7 min.; 25–34% in 47 min.; 34–52% in 30 min.; and 52–70% in 6 min. (B) Identification of tobacco chloroplast ribosomal protein L12 by 2D-PAGE gel analysis. (a). 2D-PAGE of tobacco 50S ribosomal proteins. (b). 2D-PAGE of HPLC-purified ribosomal protein L12. Included in wells on the left side of the second dimension SDS gel slab in (a) are HPLC-purified tobacco L12 and molecular weight standards as indicated. The molecular weight of L12 (21kd) as estimated from electrophoretic mobility is an overestimate of that predicted (13,823) by the nucleotide sequence.

digested with *Sp*II (Amersham), and the inserts cloned into the unique *Asp*718 (Boehringer) site in a pBluescript II KS(+) vector.

DNA sequencing strategy

To facilitate DNA sequencing, partial clones (Figure 1) were generated by digesting the L12 DNA with *Eco*RI and *Hind*III. Single-stranded template DNA was prepared (43), and sequenced by the [α - 35 S]dATP dideoxy chain termination method (44) using Sequenase (45). Sequences were obtained using the T3, T7, M13 and reverse oligonucleotide primers as well as two synthetic oligonucleotide primers [L12-1 (complementary to nucleotides 241–258, Figure 2) and L12-2 (nucleotides 699–719, Figure 2)]. The sequence data was analyzed on an IBM PS2 Model 80 computer using programs of Mount and Conrad (46). Related L12 proteins were identified and aligned by searching the NBRF database (National Biomedical Research Foundation, Georgetown University) protein sequence bank using the computer programs Word Search and Gap of the Wisconsin GCG package. Identity refers to number of identical residues divided

by the number of residues of tobacco chloroplast ribosomal protein L12. Similarity refers to number of identical as well as conservative amino acid replacement residues divided by the number of residues of tobacco chloroplast ribosomal protein L12. Different L12 proteins were compared using the Kyte and Doolittle hydropathy measure (47). RNA secondary structure was predicted using the computer programs 'Fold' and 'Squiggles' of the Wisconsin GCG package.

RESULTS

Isolation and analysis of cDNAs coding for tobacco chloroplast ribosomal protein L12

Using antibody probes to screen the *Eco*RI linker-constructed cDNA library, only a partial cDNA (referred to as L12-2 in Figure 1, nucleotides 198–744, Figure 2) was repeatedly obtained. This cDNA codes for the mature L12 protein, part of the transit peptide and most of the 3'-non-translated sequence. In order to isolate a cDNA coding for the complete L12 precursor

Figure 4. Alignment of tobacco and spinach (24) chloroplast L12 with prokaryotic L12 ribosomal proteins. Prokaryotic L12 sequences are those of *Escherichia coli* (23); *Desulfovibrio vulgaris* (70); *Micrococcus luteus* (71); *Streptomyces griseus* (72); *Bacillus stearothermophilus* (73); *Rhodospseudomonas sphaeroides* (74); Halophilic eubacterium NRC41227 (NBRF database) and *Bacillus subtilis* (75). Symbols indicate identical amino acids (+) and conservative amino acid substitutions (Δ) relative to the tobacco L12 sequence. Gaps (-) were introduced to optimize alignment. Unrelated amino acids are indicated by blank spaces. The terminal amino acids are indicated explicitly for each protein. Invariant and conservative amino acid residues in the chloroplasts and procaryotic proteins are boxed. The numerical scale above the amino acids refer to the tobacco L12 protein. Percent identities indicated were calculated for comparison of the mature tobacco L12 protein with the mature spinach L12 protein (exclusive of the transit peptides) and with the entire sequence of each of the procaryotic species. Initially, the amino acid sequence of each individual protein was aligned separately with the amino acid sequence of tobacco L12 ribosomal protein. Sequence similarities were then detected visually and edited using a text editor. The vertical arrow indicates the transit peptide-mature protein junction.

protein, this partial cDNA was used as a probe to screen a primary unamplified library cloned into λ gt11 using *EcoRI-SmaI* adaptors (39). Complementary DNA inserts in this library were excised with *SpII* and recloned in the unique *Asp718* site of the Pbluescript KS(+) vector. Development of this method permitted isolation of a cDNA (L12-1, Figure 1) coding for the complete tobacco chloroplast ribosomal protein L12 precursor polypeptide. This cDNA (773 nucleotides) has a 5'-leader sequence of 96 nucleotides, a coding sequence of 558 nucleotides and a 3'-non-coding sequence of 119 nucleotides. There are two internal *EcoRI* sites, one within the transit peptide coding sequence and another one only 25 nucleotides upstream from the 3'-end of the cDNA. Resistance to methylation at these internal *EcoRI* sites is likely to have prevented isolation of a cDNA containing a full-length L12 coding sequence from libraries constructed by the method of Huynh, et al (40).

The L12 coding sequence (nucleotides 97 to 654) translates as a 13,823 molecular weight polypeptide with a transit peptide of 53 amino acids (Ala⁻¹ to Met⁻⁵³, Figure 2) and a mature protein of 133 amino acids (Ala⁺¹ to Ala⁺¹³³). The amino-terminal sequence of HPLC-purified mature L12 protein was determined directly after identification and isolation of L12 from a mixture of chloroplast 50S ribosomal subunit proteins. The HPLC peak corresponding to L12 protein (Figure 3A) was identified as a relatively acidic protein on two-dimensional PAGE gels (Figure 3B). The amino-terminal 12 amino acids of this protein, determined by protein sequencing, were identical to those deduced from the cDNA sequence (Figure 2).

The transit peptide (53 amino acids) of L12 has an estimated molecular weight of 5,819. While lacking several amino acids (Table 1), serine and threonine account for 28.3%, with about half as much each of proline, alanine and leucine. The basic amino acid (arginine, lysine and histidine) content is high (20.8%), compared to acidic residues (glutamic acid, 1.9%), giving the presequence a strong net positive charge. These characteristics are typical of nuclear-encoded chloroplast protein transit peptides which, in general, have comparable amino acid composition but lack significant sequence similarity (48). The most abundant residues in the mature L12 protein are alanine, valine, and leucine (Table 1). Unlike the transit peptide, acidic

residues (18.1%) in the mature protein predominate the basic amino acids (12%), imparting the characteristic acidity of L12-type ribosomal proteins (6).

Similarity to other L12 ribosomal proteins

The amino acid identity between tobacco and spinach L12 ribosomal proteins is 79% when the mature polypeptide sequences are compared (Figure 4). The transit peptides (Figure 4) of tobacco and spinach (24) are 43% identical, with that of tobacco having about twice as many basic residues, and somewhat less serine and threonine. Sequence identity between mature higher plant chloroplast and procaryotic L12 proteins ranges from 36% to 48% (Figure 4). The distribution of acidic and basic amino acids (Figure 5B) indicates conservation of hydrophobic and charged regions of the L12 proteins of tobacco, spinach and *E. coli*. Regions of conserved amino acids and clusters of charged amino acids in these proteins occur centrally and towards the

Table 1. Amino acid composition (mole%) of tobacco L12 transit peptide and mature protein.

Amino acid	Transit peptide	Mature protein
Ala	11.3	21.1
Arg	9.4	1.5
Asp/Asn	0.0/0.0	5.3/1.5
Glu/Gln	1.9/0.0	12.8/3.0
Gly	0.0	4.5
His	5.7	0.0
Ile	5.7	4.5
Leu	11.3	9.0
Lys	5.7	10.5
Met	1.9	0.0
Phe	5.7	2.3
Pro	13.2	4.5
Ser	15.1	4.5
Thr	13.2	3.8
Cys	0.0	0.0
Trp	0.0	0.0
Tyr	0.0	0.8
Val	0.0	10.5

Figure 5. Hydropathy and location of charged amino acids of L12 ribosomal proteins from tobacco, spinach and *E. coli*. (A). Kyte-Doolittle hydropathy plots (47). The vertical arrow points to the putative transit peptide-mature protein junction. (B). Location of basic and acidic amino acids in L12 ribosomal proteins from tobacco, spinach and *E. coli*. The numerical scale refers to the tobacco ribosomal protein L12 amino acid sequence. Basic amino acids are indicated above and acidic amino acid below, relative to tobacco L12 amino acid sequence.

Figure 6. Alignment of tobacco chloroplast ribosomal protein L12 with those of spinach chloroplast, *E. coli* and eucaryotic ribosomes. Comparison is with L12 ribosomal proteins of *E. coli* (23), spinach chloroplast (24), rat liver protein P2 (25), *Artemia salina* protein eL12/eL12-P (26), yeast (*Saccharomyces cerevisiae*) protein YP A1 (27) and *Homo sapiens* L12e (28). Sequence similarities in the overlapping region between tobacco L12 C-terminal region (residues 61–133) and the eucaryotic proteins are 42% (residues 16–77) for rat liver P2, 32% (residues 16–75) for *Artemia salina* eL12/eL12-P, 34% (residues 16–77) for *Saccharomyces cerevisiae* YP A1 and 42% (residues 16–77) for *Homo sapiens* L12e. Symbols indicate identical amino acids (+) and conservative amino acid substitutions (·) relative to the tobacco L12 sequence. Gaps (–) were introduced for optimum alignment. Unrelated amino acids are indicated by blank spaces. Symbols above the C-terminal region refer to amino acids thought to be involved in structural features of the L12 protein (17,49). Residues involved in the interaction with extrinsic translation factors (♦); residues involved in the dimerization site (◆); and residues involved in the anion (potential GTP) binding site (★). Invariant and conservative amino acid residues in the chloroplast and *E. coli* proteins are shaded. Identical residues and conservative amino acid replacements between the C-terminal region of tobacco and eucaryotic L12 proteins are boxed. The numerical scale (indicating positions 1–206) is arbitrary. Numbers which are in parentheses (right ends of sequences) correspond to the location of that amino acid residue in each protein. The alignments were generated as for Figure 4.

carboxyl termini (Figure 5B). Hydropathy plots (Figure 5A) of tobacco, spinach and *E. coli* L12 type proteins are very similar, being more hydrophobic towards their amino and carboxyl termini and more hydrophilic in the central portions. The transit peptides of both tobacco and spinach L12 ribosomal proteins have hydrophobic amino and hydrophilic carboxyl termini (Figure 5A).

Chloroplast, *E. coli*, and eucaryotic L12 proteins were aligned (Figure 6) to reveal similarities between modular elements using the strategy of Shimmin, et al. (49). We propose (Figure 6), for the chloroplast L12 proteins, that N-terminal (residues 1–60, Figure 6) and C-terminal domains (residues 61–133) are connected by an alanine-proline rich hinge region (residues 42–60). The N-terminal domain of mature tobacco L12 protein has 65% amino acid identity with the corresponding portion of spinach L12 (residues 1–60) but only 27% identity with the related region (residues 1–49) of *E. coli* L12 protein. This N-terminal domain of tobacco L12 is more similar (40%) to the carboxyl region (residues 92–133, positions 117–162) than to any other portion of the eucaryotic L12 proteins. The C-terminal region (residues 61–133) of tobacco L12 protein has 88% and 58% amino acid identity with the comparable region of spinach and *E. coli* L12 proteins, respectively. The C-terminal region of tobacco L12 is related to corresponding portions (Figure 6) of several eucaryotic L12 proteins. In *E. coli* L12, This C-terminal region contains a putative dimerization site, anion (potential GTP) binding site and a conserved face for interaction with extrinsic translation factors (17). This domain of *E. coli* and spinach chloroplast L12 ribosomal proteins was predicted to have very similar folding patterns (50). The hinge regions (aligned

Table 2. Codon usage in the nuclear-located gene for tobacco chloroplast ribosomal protein L12.

Phe	TTT	2	Ser	TCT	5	Tyr	TAT	0	Cys	TGT	0
	TTC	4		TCC	5		TAC	1		TGC	0
Leu	TTA	3		TCA	2	TER	TAA	1	TER	TGA	0
	TTG	4		TCG	0	TER	TAG	0	Trp	TGG	0
	CTT	4	Pro	CCT	7	His	CAT	0	Arg	CGT	0
	CTC	6		CCC	3		CAC	3		CGC	4
	CTA	1		CCA	1	Gln	CAA	0		CGA	1
	CTG	0		CCG	2		CAG	4		CGG	0
Ile	ATT	8	Thr	ACT	7	Asn	AAT	2	Ser	AGT	2
	ATC	1		ACC	2		AAC	0		AGC	0
	ATA	0		ACA	2	Lys	AAA	8	Arg	AGA	1
Met	ATG	1		ACG	1		AAG	9		AGG	1
Val	GTT	7	Ala	GCT	16	Asp	GAT	4	Gly	GGT	3
	GTC	3		GCC	13		GAC	3		GGC	1
	GTA	1		GCA	4	Glu	GAA	12		GGA	2
	GTG	3		GCG	1		GAG	6		GGG	0

with residues 42–60 in tobacco L12 protein) are well conserved among the classes of proteins examined. This region of L12 proteins is thought to function as a flexible hinge (14), allowing for high mobility of the C-terminal domain of *E. coli* L12 (51). The N-terminal end of the L12 protein in *E. coli* (residues 1–15) appears to be a duplication of part of its dimerization site (53% similarity, Figure 2 in reference 49). The corresponding region of tobacco L12 (residues 1–18) has only 28% amino acid similarity to its dimerization site.

Figure 7. Analysis of tobacco L12 mRNA and genes. (A). Hybridization of L12 mRNA with L12-1 cDNA. The left lane contains leaf poly(A)⁺ mRNA while the right lane contains total leaf RNA. Horizontal arrow indicates the site of the L12 message. (B). Hybridization of tobacco L12-1 cDNA to tobacco genomic DNA digested with either *Eco*RI or *Bam*HI and resolved on 0.8% agarose gels.

L12 MRNA and Genes

Total leaf RNA and poly(A)⁺ mRNA from tobacco leaves were electrophoresed in formaldehyde denaturing agarose gels and probed with the L12-1 cDNA. The size of the mRNA (about 0.75–0.8 kb) was similar for the poly(A)⁺ mRNA as well as the total leaf RNA (Figure 7A) and is in close agreement with the size of the longest cDNA (773 bp). The uniqueness of the mRNA band in both the poly(A)⁺ mRNA and total leaf RNA suggested that the poly(A)⁺ mRNA was intact.

Tobacco genomic DNA was digested to completion with either *Eco*RI or *Bam*HI, resolved on a 0.8% agarose gel, and transferred to nitrocellulose filters. DNA hybridization to a randomly-labelled L12-1 cDNA probe revealed a major *Eco*RI fragment (about 0.6 kbp) and two minor bands (about 2.5 kbp and 3.0 kbp, Figure 7B). The 0.6 kbp band probably corresponds to the L12-2 cDNA shown in Figure 1. *Bam*HI cleavage gave two major bands, one at about 4.0 kbp and one at about 3.0 kbp. There is no *Bam*HI recognition site in the L12 cDNAs. The presence of more than one hybridizing genomic DNA fragment indicates either the existence of introns in the L12 gene which harbors *Bam*HI sites, or that more than one L12 gene exists in the tobacco genome. Each of these assumptions is likely to be valid.

While searching for a full-length tobacco L12 cDNA, we identified another cDNA (clone L12-1a, Figure 8A) coding for the same protein. This cDNA is identical to the L12-1 cDNA (Figure 1) except, in the 3'-non-coding region, nucleotide 667 is T instead of G and the cDNA is 52 bp longer (Figure 8A). The 3'-noncoding sequences of these two L12 cDNAs could be folded into very similar stem-loop structures (Figure 8B). While

Figure 8. Predicted secondary structures of the 3'-non coding sequences of tobacco L12 ribosomal protein mRNA. (A) 3'-non-coding sequence of L12-1 and L12-1a cDNAs. The numbering of nucleotides in A is as in Figure 2. (B) predicted secondary structures of the two 3'-end cDNA sequences shown in A.

more than one loop is possible for L12-1 cDNA, the longer L12-1a cDNA is predicted to fold as a single stem-loop structure. These predicted hairpin loop elements provide clues regarding RNA folding patterns which may be important in regulation of mRNA stability and polyadenylation of mRNA transcripts *in vivo* (52).

DISCUSSION

Although the sequences of several nuclear-encoded chloroplast ribosomal proteins have been deduced from DNA sequences, the only complete protein sequence is that of spinach L12 (20). Comparing the spinach L12 amino acid sequence to that deduced for tobacco L12 revealed several differences. The published spinach L12 protein sequence (20) lacks three amino acids (Ala-Pro-Ala) corresponding to residues 56 to 58 of tobacco L12. Comparison of the nucleotide sequence of a cDNA coding for spinach L12 (24) with the earlier reported amino acid sequence revealed that Ala-Ala-Pro must occur at this position. The several different cDNA sequences now available for chloroplast L12 ribosomal proteins from tobacco and spinach provide evidence that three amino acids do occur in this location, although the sequence differs between plant species.

The cDNA for spinach L12 protein contains two AUG codons, both in the consensus initiator context [A at position -3 and G at position +4 (53)] at the beginning of its transit peptide (24). Spinach L12 protein synthesis *in vitro* occurs from both initiation codons, with 70–80% attributable to initiation from the first and 20–30% from the second (54). In tobacco, however, each of the two different L12 cDNA sequences has only one AUG codon in the consensus initiator context. The location of this codon corresponds to that of the first AUG of spinach L12. Whether the *in vitro* translation initiation observed from the second AUG of spinach L12 also occurs *in vivo* remains to be tested. Since L12 is required in multiple copies per 50S ribosome subunit, establishment of several functional genes (as in tobacco) may represent one evolutionary solution to insure high levels of expression of L12. Another possible solution could be the use

of multiple initiation codons on the same mRNA, as may be the case for spinach L12.

Although spinach L12 cDNA has a plant polyadenylation signal (ATAAA) 217 nucleotides downstream from the stop codon (24), none occurs in the two sequenced cDNAs coding for tobacco chloroplast ribosomal protein L12. The absence of typical polyadenylation signals in tobacco L12 genes implies that plants can use other criteria (55) than the consensus sequence AATAAA (56) as a polyadenylation signal.

The codon usage of tobacco chloroplast ribosomal protein L12 (Table 2) does not show the preference for those codons ending in T or A displayed by ribosomal protein genes located in the chloroplast genome (57). The G+C content of higher plant chloroplast DNA (37%, ref. 58) is generally lower than that of nuclear DNA (34–50%; ref. 59). Thus, the codon usage pattern of the tobacco L12 gene probably reflects an adaptation to the tendency for maintenance of a high G+C content in nuclear protein-coding genes (60). The termination codon of chloroplast L12 is UAA, the most frequently used termination codon in nuclear-encoded plant genes (61).

Interspecific comparisons among species of the genus *Nicotiana* indicated that molecular weights and net charges of chloroplast ribosomal proteins have been highly conserved (62–64). Biochemical properties of chloroplast ribosomal proteins are more similar to prokaryotic ribosomal proteins than to cytoplasmic (eucaryotic type) proteins from the same plant species (65). The identical proportions and locations of basic and acidic amino acids of spinach and tobacco L12 proteins (Figure 5B) indicates significant evolutionary conservation between these unrelated plant species. Alignment and comparison of amino acid sequences of tobacco and spinach L12 with bacterial and eucaryotic type L12 proteins (Figure 6) illustrates evolutionary conservation of L12 amino acid sequence elements and functional motifs, in agreement with previous analyses (25–28). Difficulties in aligning eubacterial proteins with their eucaryotic counterparts (25,26,66,67) were overcome when Shimmin, et al. (49) developed an elegant analytical approach which revealed a logical alignment of these proteins. Our analysis indicates that the chloroplast L12 protein gene has probably undergone rearrangement of modular elements by gene fusion events, proposed by Shimmin et al. (49) to have occurred during evolution of L12 proteins. However, the lower similarity scores between the putative dimerization sites of tobacco L12 suggest that the chloroplast protein represents an intermediate evolutionary step between the eubacterial and eucaryotic L12 proteins (Figure 6). These observations suggest that eucaryotic and organellar (chloroplast) ribosomal proteins must be evolutionarily related. We have recently shown that the coding sequences are conserved among another nuclear-encoded tobacco chloroplast ribosomal protein (L27), a nuclear-encoded yeast mitochondrial ribosomal protein (MRP7), and procaryotic L27 proteins (68).

The deduced sequence identity with eubacterial L12 proteins suggests that tobacco chloroplast ribosomal protein L12 is likely to be localized in a 'stalk' structure and to have similar functions in chloroplast 50S ribosomal subunits to those of *E. coli* L7/L12. Using monospecific antibody to the tobacco L12 protein, these possibilities could be evaluated by immune electron microscopy and measurement of antibody interference with GTPase activities of 50S chloroplast ribosomal subunits which have been depleted of L12 by well-documented procedures (7,69). Preliminary comparisons suggest that each of the α -helical and β -pleated sheet

structural elements (deduced from the X-ray structure of the carboxyl-terminal fragment; refs. 17 and 23) of *E. coli* L12 also occur in the carboxyl-terminal half of tobacco L12 protein (data not shown). The three-dimensional structure of tobacco L12 is likely to be very similar to that of *E. coli* L12 (S. White, personal communication).

Our results indicate that tobacco chloroplast ribosomal protein L12 is coded by nuclear genes as a precursor polypeptide which is cleaved to the mature L12 protein after transport across the chloroplast membrane. The identities between chloroplast, bacterial and eucaryotic type L12 proteins (Figure 6) indicate that the structure and functions of this ribosomal protein have probably been conserved during the course of organelle evolution. The degree of identity (Figure 4) between chloroplast and bacterial L12 proteins supports a common evolutionary origin. Assuming a procaryotic evolutionary origin of chloroplasts, codon usage and sequence comparisons suggest that the tobacco nuclear-encoded L12 gene sequence may have diverged subsequent to its translocation to the nucleus. Further experiments are needed to show whether chloroplastic and eucaryotic L12 proteins have the same functions as L12 does in prokaryotic ribosome systems. Regulation of expression of the chloroplast L12 gene might occur by mechanisms which combine elements of regulation of both prokaryotic and eucaryotic genes. What mechanisms govern the regulation of L12 synthesis, transport into chloroplasts, and interaction with other chloroplast ribosomal proteins during 50S ribosome subunit assembly are unknown.

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