# Nucleotide Sequence and Linkage Map Position of the Gene for Maize Chloroplast Ribosomal Protein S14†

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ABSTRACT: A cloned fragment of maize chloroplast DNA is shown to contain the nucleotide sequence that encodes the protein homologous to *Escherichia coli* ribosomal protein S14. The nucleotide sequence was determined by the dideoxy chain termination method using the M13mp phage system. The gene is located between coordinates 71 759 and 72 068 on the physical map of maize chloroplast DNA [Larrinua, I. M., Muskavitch, K. M. T., Gubbins, E. J., & Bogorad, L. (1983) *Plant Mol. Biol. 2*, 129–140]. The 5′ upstream of the coding region has "−10" and "−35" promoter-like sequences in addition to the ribosome binding site. The deduced protein sequence is 103 amino acids long and has 39% identity with the ribosomal protein S14 of *Escherichia coli* [Yaguchi, M., Reithmeier, R. A. F., Wittmann-Liebold, B., & Wittmann, H. G. (1983) *FEBS Lett. 154*, 21–30] and 76% identity with the S14 of *Marchantia polymorpha* chloroplast [Umesono, K., Inokuchi, H., Ohyama, K., & Ozeki, H. (1984) *Nucleic Acids Res. 12*, 9551–9565]. The three homologous proteins exhibit a much closer identity at the level of predicted secondary structure.

Organelle-specific protein synthesis of the chloroplasts accounts for about half the protein mass in green leaves (Ellis, 1977). This function in the chloroplast is carried out by a 70S class of ribosome, assembled from 4 rRNAs and approximately 58 ribosomal proteins (r-proteins)[1] (Subramanian, 1985). The primary structure and gene organization of chloroplast rRNA genes in maize (Bedbrook et al., 1977; Schwarz & Kössel, 1980) and in several other plants have been determined, and the data show a close kinship of these genes with the corresponding genes of eubacteria. Similar structural data on chloroplast r-protein genes would be necessary to understand the evolutionary origins of the chloroplast, but such complete data are presently not available for any plant.

The r-protein genes of the chloroplast are distributed between the nuclear DNA and the chloroplast DNA (Bogorad et al., 1977). Therefore, the assembly of chloroplast ribosomes entails a coregulation of expression of genes in two cellular compartments. Experimental analysis of this problem can be facilitated if several of the genes (from both compartments) are isolated for use as constructs in transformation and other studies.

Our laboratory has been studying the chloroplast r-proteins of spinach and maize, which belong to the two large, distinct families of flowering plants (Bartsch et al., 1982; Subramanian et al., 1983; Leijonmarck et al., 1984; Bartsch, 1985). In the present paper, we report the identification, mapping, and nucleotide sequencing of a segment of maize chloroplast DNA that encodes a protein homologous to *Escherichia coli* ribosomal protein S14.

## Materials and Methods

*Plasmid and λ DNA Preparation.* Plasmid DNA was prepared by procedures described by Maniatis et al. (1982). Phage DNA was prepared as described by Yamamoto et al. (1970) and purified by equilibrium density gradient centrifugation in 0.75 g/mL CsCl (38 000 rpm, 24 h, 4 °C).

*Subcloning.* DNA fragments to be subcloned were isolated by electroelution from a 1.2% agarose gel. The fragments were ligated with the appropriate linearized and dephosphorylated vector by using 2 units of T4 DNA ligase/μg of DNA. When necessary, the DNA fragments were blunt-ended with the Klenow fragment of DNA polymerase I plus dNTPs and cloned into the *Hin*cII site of the vector. The plasmid DNAs were then allowed to transform *E. coli* strains HB101 or JM109. Procedures described by Maniatis et al. (1982) were used at all these steps.

*Plaque and Blot Hybridization.* Charon 4A phages containing maize chloroplast DNA fragments were allowed to infect *E. coli* C600 cells and were spread in soft agar on LB plates (200–300 pfu/plate). The plaques were transferred onto Biodyne filters (1.2-μm pore size). The filters were treated with 0.5 M NaOH/1.5 M NaCl for 5 min and neutralized with 3 M potassium acetate, pH 4.5, for 5 min. The air-dried filters were baked at 80 °C in a vacuum oven. The filters were prehybridized with 6 × SSC, 2 × Denhardt, and 100 μg/mL denatured calf thymus DNA for 2 h at 60 °C and hybridized with a $^{32}$P-labeled RNA transcript of *Marchantia rps*14 cloned in pSP64 (2 × $10^6$ cpm/mL) in 40% formamide, 6 × SSC, and 2 × Denhardt at 37 °C for 24 h. The filters were washed twice with 2 × SSC/0.1% SDS for 30 min at room temperature and in 0.1 × SSC/0.1% SDS for 5 min at 50 °C. The filters were autoradiographed on Fuji X-ray film overnight.

The restriction fragments of DNA on agarose gels were transferred (Southern, 1975) to nylon membrane filters (0.2-μm pore size). The filters were probed with a $^{32}$P-labeled RNA transcript under the same conditions as described for plaque hybridization.

The $^{32}$P-labeled RNA transcript of the insert DNA cloned in pSP64 was prepared as described by Melton et al. (1984).

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[1] Abbreviations: bp, base pair; kbp, kilobase pair; r-protein, ribosomal protein; Pfu, plaque-forming unit(s); SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride. The gene for chloroplast ribosomal protein S14 is designated *rps*14 (instead of *rps*N as in *E. coli*) in accordance with the recommended nomenclature for chloroplast protein genes [see Hallick and Bottomley (1983)].

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*DNA Sequencing Strategy.* On the basis of the results of hybridization experiments, the S14 gene of maize was localized in a 1.4-kbp *Bam*HI fragment. This fragment and smaller subfragments of it produced by "4-cutter" restriction enzymes (*Alu*I, *Sau*3AI, and *Taq*I) were cloned into M13mp18 DNA (Yanish-Perron et al., 1985) and were sequenced by the dideoxy chain termination method (Sanger et al., 1977). DNA fragments with blunt ends were shotgun cloned into the *Hinc*II site of the M13mp18 DNA; others were blunt-ended with the Klenow fragment of DNA polymerase I before cloning. Microgram quantities of the 1.4-kbp DNA used for this purpose were purified from the restriction digest of a recombinant plasmid on a 10–30% sucrose gradient (in 0.5 M NaCl, 10 mM $Na_2EDTA$, and 10 mM Tris-HCl, pH 8.0) by centrifugation at 38 000 rpm for 18 h at 15 °C. The sequenced fragments were aligned and compared by using version 3 of the UWGCG program on a Vax computer (Devereux et al., 1984).

The coordinates of S14 gene on the physical map of maize chloroplast DNA (Larrinua et al., 1983) were determined by restriction analysis of the clone carrying the 1.4-kbp fragment using enzymes *Sal*I and *Nsi*I.

*Materials.* Restriction enzymes and other DNA-modifying enzymes used were purchased from Bethesda Research Laboratory (BRL), Boehringer Mannheim, New England Biolabs, or Amersham International (AI) and used under the conditions suggested by the respective manufacturer, M13 cloning and sequencing reagents were from BRL, and $^{32}P$-labeled nucleotide triphosphates were from AI. Nylon membrane filters (Biodyne) were from Pall Corp., Glen Cove, NY. Charon 4A clones of maize chloroplast DNA (Larrinua et al., 1983) and a clone of *Marchantia Polymorpha* chloroplast DNA fragment that contains *rps*14 (Umesono et al., 1984) were kindly provided by Prof. L. Bogorad (Harvard University) and Dr. K. Ohyama (Kyoto University), respectively.

## Results

*Subcloning of M. polymorpha S14 Gene.* Recombinant plasmid pMP227 is a clone of the *Bam* 7 fragment of *M. polymorpha* chloroplast DNA in pBR322 (Umesono et al., 1984). When digested with restriction enzymes *Xba*I and *Eco*RV, the pMP227 DNA released four fragments of size 5.89, 1.85, 0.96, and 0.539 kbp (Figure 1A). These fragments were separated on a 1.2% agarose gel. The fragment of 539 bp was isolated from the gel by electroelution. It contains 227 bp of the coding region of S14 gene, 102 bp of a spacer, and 210 bp of the coding region of a then unidentified protein (Umesono et al., 1984). It was blunt-ended with Klenow fragment and ligated into the *Hinc*II site of pSP64 (Figure 1A). The resulting plasmid, designated pBS182, has at the upstream of the insert a promoter specific for SP6 RNA polymerase and at the downstream a *Bam*HI restriction site. Thus, linearization of the plasmid DNA with *Bam*HI limits the RNA transcription to only the insert.

*Identification of Maize S14 Gene.* Approximately 92% of the maize chloroplast genome is contained in 10 recombinant clones in λ Charon 4A vector described by Larrinua et al. (1983). These 10 clones (1, 2, 5, 8, 9, 11, 13, 14, 16, and 17) were screened with the in vitro synthesized, [$^{32}P$-UTP]RNA transcript of pBS182 by plaque hybridization. Only one of the clones, namely, 11, gave a strong signal (Figure 1B).

The Charon 11 recombinant phage has an 18.7-kbp maize chloroplast DNA insert from the large single-copy region between coordinates 64 420 and 83 140 (Larrinua et al., 1983). In order to localize the S14 gene of maize on a smaller DNA fragment, the Charon 11 DNA was cut with restriction enzymes *Bam*HI, *Eco*RI, *Pst*I, and *Sal*I, and the restriction digests were electrophoresed on an agarose gel. The DNA fragments on the gel were Southern transferred onto a membrane filter and probed with the RNA transcript of pBS182. The result of this experiment is shown in Figure 1C,D. Three *Bam*HI fragments (2.7, 1.6, and 1.4 kbp) and one each of *Eco*RI, *Pst*I, and *Sal*I fragments gave hybridization signals. The *Eco* fragment that gave the signal corresponds to *Eco* b, and, therefore, the three *Bam* fragments that gave signals are all derived from *Eco* b. These three fragments correspond to a part of *Bam* 14 (2.7 kbp), entire *Bam* 21′ (1.6 kbp), and *Bam* 24 (1.4 kbp; shown by an arrow) of maize chloroplast DNA. A previous paper by Fish et al. (1985) has described the nucleotide sequence of all *Bam* fragments of *Eco* b except *Bam* 24. The S14 gene was not present in the sequenced region. It was apparent, therefore, that S14 gene must be present on *Bam* 24.

The reason *Bam* 21′ gave a stronger signal than *Bam* 24 is that the 210-bp coding region of an unidentified protein in our hybridization probe matches almost perfectly to a segment of the published nucleotide sequence of *Bam* 21′ (Fish et al., 1985). This sequence in maize encodes the C-terminal region of a highly conserved protein component of chloroplast photosystem I.

*Nucleotide Sequence and Coordinates of Maize S14 Gene.* The *Bam* 24 fragment was subcloned into pUC19 (clone designated pBS183), and the insert DNA was isolated in microgram quantities and sequenced as described under Materials and Methods. Of the more than 20 shotgun clones that were sequenced, 5 contained the coding region and 5′-upstream/3′-downstream regions of *rps*14 (Figure 2).

The *Bam* 24 fragment of maize chloroplast DNA has the coordinates 71 470 and 72 870 on the physical map of this genome, and it contains a single *Sal*I site at 72 770 (Larrinua et al., 1983). The digestion of pBS183 with *Sal*I released a fragment of 1.2 kbp, suggesting that *Bam* 24 is oriented in pBS183 with its coordinate 71 470 near the *Sal*I site on the vector. From the sequencing data (Figure 2), it was possible to locate a *Nsi*I site on the S14 gene. pUC19 has no *Nsi*I site. Restriction of pBS183 with *Eco*RI and *Nsi*I released a fragment of 1 kbp, suggesting that the *NsiI* restriction site is located 1000 bp away from the *Eco*RI site on *Bam* 24. These data allowed the assignment of the coordinates for the S14 gene (Figure 2).

*Some Features of Maize S14 Gene.* The coding region of maize S14 gene is 309 nucleotides long and is rather rich in AT content (64%). The 5′ upstream of the coding region has "–10" and "–35" promoter-like sequences. In addition, 10 bp upstream of the initiating ATG it has a Shine–Dalgarno sequence (ribosome binding site) of four bases complementary to the phylogenetically conserved 3′-end sequence of maize chloroplast 16S RNA.

The codon usage of maize S14 gene is given in Table I. As is observed in the case of other chloroplast genes (Fish et al., 1985), there is a preference for codons with A or T at the third position. In all, only 44 of the 61 amino acid codons are used in *rps*14; the corresponding number for maize chloroplast *rps*4 is 54 (Subramanian et al., 1983).

The 3′ downstream of the coding region of maize and *Marchantia rps*14 each has an identical palindrome of eight nucleotides. The 3′ downstream of maize *rps*4 has a similar palindrome, but it is twice as long as that of *rps*14.

## Discussion

The deduced sequence of maize chloroplast r-protein S14 (including the initiating Met) is 103 amino acids long, com-

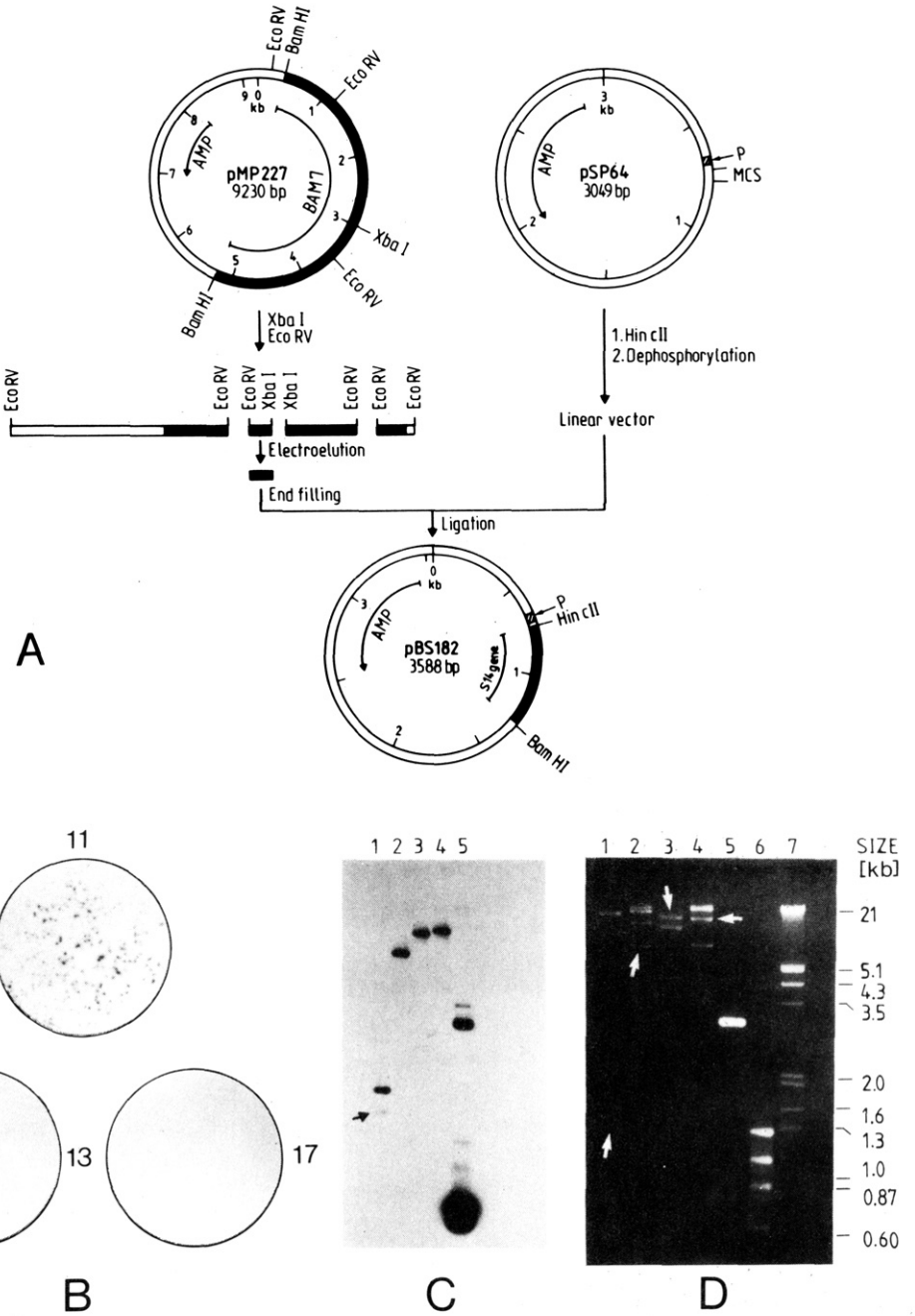
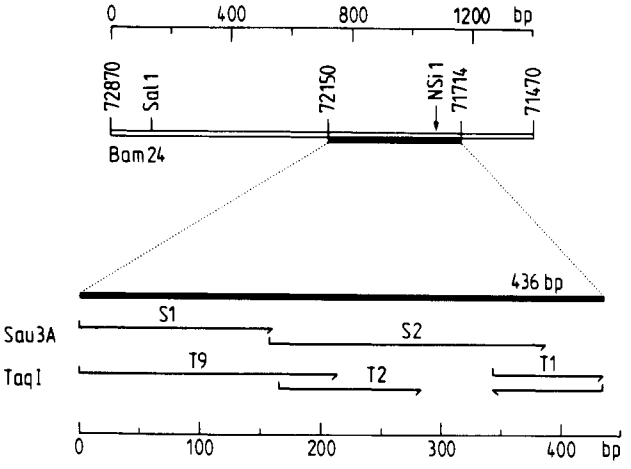



FIGURE 1: (A) Scheme for subcloning a portion of the S14 gene of *Marchantia polymorpha* (p, SP6 promoter; MCS, multiple cloning site). (B) Autoradiogram of filters containing plaques of recombinant Charon phages 11, 13, and 17 hybridized with a 32p-labeled RNA transcript of *Marchantia* S14 gene. (C) Autoradiogram of Southern filters of the restriction fragments of Charon 11 DNA hybridized with the RNA probe used in (B): lanes 1–4, *Bam*HI, *Eco*RI, *Pst*I, and *Sal*I restriction fragments, respectively; lane 5, plasmid pBS182 DNA cut with *Bam*HI. (D) Restriction pattern of the Charon 11 DNA on a 1.2% agarose gel: lanes 1–5, same as in (C); lane 6 and 7, *Hae*III digest of $\phi$X174 RF DNA and *Eco*RI + *Hin*dIII digest of $\lambda$ DNA (size markers).

pared to 99 and 100 residues in S14's of *E. coli* and *Marchantia*, respectively. It has 39% identity to *E. coli* S14 and 76% identity to *Marchantia* (a lower plant of the bryophyte family) chloroplast S14. The amino acid compositions of the two chloroplast proteins are very similar, but they differ from that of *E. coli* in distinct ways (Table II). They are poorer in alanine and valine but richer in histidine and leucine than *E. coli* S14, a feature also observed for maize chloroplast S4 (Subramanian et al., 1983). The chloroplast r-proteins are also poorer in the sum of acidic amino acids (Asp + Glu) as compared to their *E. coli* counterpart, also previously reported in the case of maize S4. The chloroplast proteins are therefore more basic: the excess of basic (Arg + Lys + His) over Asp + Glu is 24, 21, and 16, respectively, for the S14 of maize, *Marchantia*, and *E. coli*. The proportion of total aliphatic hydrophobic amino acids to the hydrophilic amino acids in all three S14 proteins is comparable. Thus, the hydropathy plots (Kyte & Doolittle, 1982) of the three proteins are very similar (data not shown). All four prolines (important for the formation and stabilization of secondary structure) of *E. coli* S14 are conserved in *Zea mays* S14. Computer-predicted secondary structures of the three proteins (Figure 4) are very similar in spite of the up to 60% divergence in their amino acid sequence. Interestingly, a sequence segment of S14 from residues 30 to 50 (where gaps in alignment were introduced; Figure 3) shows great dissimilarity in both primary and predicted secondary structure, even between the two chloroplast proteins which are otherwise very similar.

In *E. coli*, S14 is one of the late assembly proteins. It is located by immune electron microscopy in the neighborhood




Nucleotide Sequence of rpS14:

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        10                  30                  50
GATCCGCCTTTTTAGATTTTTGCTATTTATTTTTCTATCTACCATTAGAACCATATACTT
        70                  90                 110
GGTAATAATAGGAACGGCACATTATGGCAAAAAAAAGTTTGATTCAGAGGGAGAAGAAGC
                          MetAlaLysLysSerLeuIleGlnArgGluLysLysA
       130                 150                 170
GGCAGAAATTAGAACAAAAATATCATTTGATTCGTCGATCCTCAAAAAAAAAGATAAGAA
rgGlnLysLeuGluGlnLysTyrHisLeuIleArgArgSerSerLysLysLysIleArgS
       190                 210                 230
GCAAAGTTTCCCCTTTGAGTTTCAGCGAAAAAACAAAAATGCAAGAAAAATTGCAATCCC
erLysValSerProLeuSerPheSerGluLysThrLysMetGlnGluLysLeuGlnSerL
       250                 270                 290
TACCACGTAATAGTGCACCTACACGCCTTCATCGACGTTGTTTTTTAACCGGAAGACCTA
euProArgAsnSerAlaProThrArgLeuHisArgArgCysPheLeuThrGlyArgProA
       310                 330                 350
GAGCTAACTATCGAGACTTTGGGCTATCCGGACACATACTTCGAGAAATGGTTTATGCAT
rgAlaAsnTyrArgAspPheGlyLeuSerGlyHisIleLeuArgGluMetValTyrAlaC
       370                 390                 410
GTTTGTTACCGGGTGCAACAAGATCCAGTTGGTAAGGATAAAATACCCTTTCTTTCATAT
ysLeuLeuProGlyAlaThrArgSerSerTrp***
       430
TTGTATGGTTTTCTCG
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FIGURE 2: Physical map of maize chloroplast *Bam* 24 fragment, the sequencing strategy, and the nucleotide sequence. The nucleotide sequence of the coding region along with the 5′-upstream and 3′-downstream flanking regions is shown. The "−10" and "−35"-like sequences are underlined, and the ribosome binding site is boxed. The deduced protein sequence is given in the three-letter code.

Table I: Codon Usage in Maize Chloroplast *rps*14

| | T | C | A | G | |
|---|---|---|---|---|---|
| T | Phe 2 | Ser 0 | Tyr 3 | Cys 2 | T |
| | Phe 1 | Ser 5 | Tyr 0 | Cys 0 | C |
| | Leu 3 | Ser 1 | Stop 1 | Stop 0 | A |
| | Leu 5 | Ser 0 | Stop 0 | Trp 1 | G |
| C | Leu 2 | Pro 3 | His 2 | Arg 3 | T |
| | Leu 0 | Pro 0 | His 1 | Arg 1 | C |
| | Leu 2 | Pro 1 | Gln 3 | Arg 4 | A |
| | Leu 0 | Pro 1 | Gln 2 | Arg 1 | G |
| A | Ile 2 | Thr 0 | Asn 1 | Ser 4 | T |
| | Ile 0 | Thr 1 | Asn 1 | Ser 2 | C |
| | Ile 2 | Thr 3 | Lys 10 | Arg 4 | A |
| | Met 3 | Thr 0 | Lys 3 | Arg 1 | G |
| G | Val 2 | Ala 1 | Asp 0 | Gly 1 | T |
| | Val 0 | Ala 0 | Asp 1 | Gly 0 | C |
| | Val 0 | Ala 4 | Glu 4 | Gly 2 | A |
| | Val 0 | Ala 0 | Glu 1 | Gly 1 | G |

of S3, S9, S13, and S19 on the head of the small ribosomal subunit [reviewed in Wittmann (1983)]. This region is known to be involved in binding tRNA to the ribosome [reviewed in Ofengand (1980)]. Although the function or the location of S14 on the maize chloroplast ribosome is not known, on the basis of the homology it has with *E. coli* S14 at the level of primary (and secondary) structure, it could be suggested that maize S14 has a role similar to that of *E. coli* S14. On the other hand, a 60% divergence seen between the primary

Table II: Amino Acid Composition[a] of S14 Proteins

| amino acid | mol % | | |
|---|---|---|---|
| | maize S14 | *Marchantia* S14 | *E. coli* S14 |
| Ala | 4.8 | 4.0 | 11.1 |
| Arg | 13.6 | 11.0 | 14.1 |
| Asn | 1.9 | 4.0 | 3.0 |
| Asp | 1.0 | 2.0 | 5.0 |
| Cys | 1.9 | 2.0 | 1.0 |
| Gln | 4.8 | 4.0 | 4.0 |
| Glu | 4.8 | 6.0 | 5.0 |
| Gly | 3.9 | 3.0 | 6.0 |
| His | 2.9 | 3.0 | 1.0 |
| Ile | 3.9 | 3.0 | 4.0 |
| Leu | 11.6 | 14.0 | 8.1 |
| Lys | 12.6 | 15.0 | 11.1 |
| Met | 2.9 | 2.0 | 3.0 |
| Phe | 2.9 | 3.0 | 3.0 |
| Pro | 4.8 | 5.0 | 4.0 |
| Ser | 11.6 | 9.0 | 7.1 |
| Thr | 3.9 | 5.0 | 2.0 |
| Trp | 1.0 | 2.0 | 1.0 |
| Tyr | 2.9 | 2.0 | 1.0 |
| Val | 1.9 | 1.0 | 5.0 |

[a] Derived from the nucleotide sequence (maize, this paper; *Marchantia*, Umesono et al., 1984; *E. coli*, Cerretti et al., 1983).

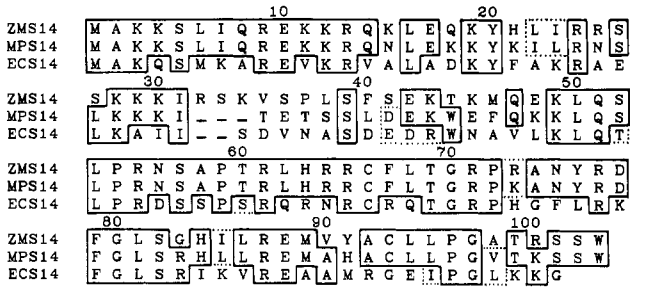


FIGURE 3: Comparison of the amino acid sequences of the S14 proteins of maize chloroplast (ZM), *Marchantia* chloroplast (MP), and *E. coli* (EC). Identical amino acids are boxed, and conservative replacements are indicated by dots. The derived sequences of ECS14 and MPS14 are from Cerretti et al. (1983) and Umesono et al. (1984), respectively.

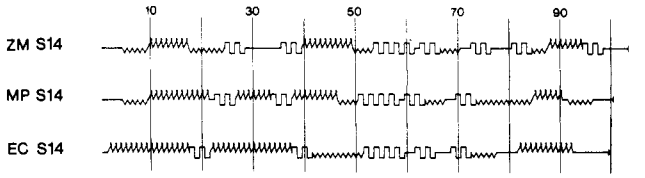


FIGURE 4: Plot of computer-predicted secondary structure of maize, *Marchantia*, and *E. coli* S14 according to the Chou and Fasman algorithm described by Rawlings et al. (1983). The symbols of zig-zags, loops, steps, and straight lines represent respectively $\beta$-sheet, $\alpha$-helix, $\beta$-turn, and random coil.

structure of S14's of maize and *E. coli* would mean that S14 has regions to accommodate structural variations necessary to cope with evolutionary pressure (e.g., endosymbiosis). The higher percentage of homology between maize and *Marchantia* S14 naturally reflects their more recent separation after chloroplast evolution.

In *E. coli*, *rps*14 is one of the genes of the *spc* operon in the large, 27-membered r-protein gene cluster (Nomura et al., 1984). Our data together with the data of Fish et al. (1985) argue against the presence of another r-protein gene near *rps*14 in maize. The data on the chloroplast genome sequence of tobacco and *Marchantia* also show that *rps14* is not clustered with other r-protein genes in these organisms (Sugiura et al., 1986; Ohyama et al., 1986). The gene for chloroplast S4 (Subramanian et al., 1983) shows the same picture; it is also a member of the *E. coli* gene cluster but is unclustered (with

Table III: Differing Codon Preferences of *E. coli* and Chloroplast Genomes with Respect to S14 Gene

| amino acid | codon | % of usage | | |
|---|---|---|---|---|
| | | | chloroplast | |
| | | *E. coli* | *Marchantia* | maize |
| Ala | GCA | 9 | 50 | 80 |
| Asn | AAT | 0 | 100 | 50 |
| Cys | TGT | 0 | 100 | 100 |
| Gly | GGA | 0 | 67 | 50 |
| Pro | CCT | 0 | 40 | 60 |
| Ser | AGT | 0 | 33 | 36 |
| Thr | ACC | 0 | 40 | 25 |
| Tyr | TAT | 0 | 50 | 100 |
| Leu | CTG | 50 | 0 | 0 |

other r-protein genes) in the chloroplast.

In the S14 genes of both maize and *Marchantia*, there is a preference for codons with A or T at the third position as is observed in the case of other chloroplast genes (Subramanian et al., 1983; Fish et al., 1985). Table III lists the more striking condon preferences in *E. coli* and chloroplast *rps*14. It is not known if these differing preferences reflect aspects of differing regulatory control or merely the different levels of the corresponding isoacceptor tRNAs in the two systems.

**Registry No.** DNA (corn chloroplast ribosomal protein S14 gene), 107710-99-6; protein S14 (corn chloroplast ribosomal), 107711-00-2.

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