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Nicotiana chloroplast genome

8. Localization of genes for subunits of ATP synthase, the cytochrome *b–f* complex and the 32 kD protein

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Summary. Using the existing restriction map and probes from wheat and pea ct-DNA, seven protein genes have been localized in the chloroplast genome of *N. tabacum*. On the clock-like map, the location of each gene is indicated by its time zone: the 15.2 kD polypeptide of the cytochrome b/f complex at 3:15, cytochrome f at 4:30, LS of RuBPCase at 4:50, both β and ε subunits of ATP synthase at or near 5:00, proton-translocating subunit of ATP synthase at 8:20, α subunit of ATP synthase at 8:40 and the 32 kD protein at 9:30. The genome organization of *Nicotiana* chloroplast DNA is similar to spinach.

Key words: Southern hybridization – Restriction map – Chloroplast relatedness

Introduction

The study of the structure and function of chloroplast genomes from many plant species occupies a very important position in the field of plant molecular biology. The chloroplast genome of *Nicotiana* (Fluhr and Edelman 1981; Jurgenson and Bourque 1980; Kung et al. 1982; Seyer et al. 1981; Shen et al. 1982; Zhu et al. 1982), together with chloroplast genomes from maize (Bogorad et al. 1983), spinach (Whitfeld and Bottomley 1983), wheat (Howe et al. 1983; Willey et al. 1983), and many other plant species (Chu et al. 1981; Gordon et al. 1981; Palmer et al. 1983; Poulsen 1983) has been well characterized. In the case of the *Nicotiana* chloroplast genome, many genes for ribosomal RNA (Takaiwa and Sugiura 1980, 1982; Tohdoh and Sugiura 1982) transfer RNA (Deno et al. 1982; Takaiwa and Sugiura 1982) and several proteins have been sequenced (Shinozaki and Sugiura 1982; Shinozaki and Sugiura 1982). However, the location of some of those genes has not been mapped.

The use of probes from one plant chloroplast DNA (ct-DNA) containing specific genes or fragment of a gene allows accurate mapping of the corresponding genes on the chloroplast genomes of other plant. By applying this technique, the genes for the subunits alpha, beta, epsilon and the proton-translocating subunit of ATP synthase, cytochrome f and the 15.2 kD polypeptide of the cytochrome b/f complex and the 32 kD protein have been mapped on the *N. tabacum* chloroplast genome. The relative locations of these genes are very similar to those mapped on the chloroplast genome of spinach (Alt et al. 1983 a, b; Westhoff et al. 1981; Whitfeld and Bottomley 1983; Zurawski et al. 1982 a, b). It is hoped that the information provided in this paper will be of value in the study of evolution as well as in the cloning and sequencing of chloroplast genes from other species of *Nicotiana*.

Materials and methods

Preparation of chloroplast DNA

ct-DNA was isolated from chloroplasts as described by Rhodes and Kung (1981). Freshly harvested leaves were homogenized in liquid nitrogen, filtered through Miracloth, and the chloroplast purified through discontinuous silica sol gradients according to the method of Kolodner and Tewari (1975).

Restriction endonuclease digestion; agarose gel electrophoresis, and DNA transfer

All restriction endonucleases were used according to the procedure as recommended by the supplier, BRL (Bethesda, MD). ct-DNA was digested with different restriction endonucleases and electrophoresed in TEA buffer (50 mM Tris-Acetate pH 8.05; 20 mM NaOAC; 2 mM EDTA; 18 mM NaCl)

for 20 h at 50 volts on 0.8% agarose gels. ct-DNA was transferred onto nitrocellulose paper as described by Smith and Summers (1980).

Radioactive labeling of DNA probes carrying defined genes

The following probes carrying defined chloroplast genes were used in this study:
1) α subunit of ATP synthase: a 200 bp EcoRI-SalG1 fragment of wheat ct-DNA from within the gene cloned into M13mp8 (Howe et al. 1983); 2) β subunit of ATP synthase: a 150 bp Sau3A fragment of wheat ct-DNA from within the gene cloned into M13mp7; 3) ε subunit of ATP synthase: a Sau3A fragment of wheat ct-DNA containing the 3′ end of the gene and some 3′ non-coding sequence cloned into M13mp7; 4) Proton-translocating subunit of ATP synthase: a 140 bp HaeIII-HindIII fragment of wheat ct-DNA, corresponding to nucleotides 45–185 in sequence of Howe et al. (1983), cloned into M13mp7; 5) Cytochrome f: a 350 bp Xho-BamI fragment of pea ct-DNA from within the 5′ end of the gene cloned into M13mp8 (Willey et al. 1983); 6) 15.2 kD polypeptide of the cytochrome b/f complex: a 950 bp MspI fragment of pea ct-DNA containing the complete gene and some 5′ and 3′ flanking sequence was inserted into the AccI site of pUC8 plasmid (Phillips and Gray 1984). 7) P32 kD gene: the PstI-10 fragment of *N. tabacum* ct-DNA which carries the P32 kD gene of ct-DNA (Fluhr et al. 1984) was used as probe.

Single-strand DNA templates were prepared by the modification of Schreier and Cortese (1980). One ml of the supernatant of *E. coli* JM103 cultures (Messing et al. 1981) infected by various recombinant M13mp7 and M13mp8 clones was collected, precipitated by PEG solution (2.5 M NaCl; 20% PEG 6,000), and phenol extracted. The purified template DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Plasmid DNA was prepared as described by Holmes and Quigley (1981).

The radioactive labeling of M13 DNAs were prepared as described by Hu and Messing (1982) with the following modifications

Hybridization probe-primer was purchased from New England Biolab. One µl of primer was added into an annealing mixture which contained 1 µl of previously prepared template DNA, 1 µl of 10x Hin buffer (66 mM Tris-HCl pH 8.0; 66 mM NaCl; 66 mM $MgCl_2$), 1 µl of 0.1 M DTT, and 5 µl of H_2O. The mixture was boiled for 2 min, then incubated at 37 °C for 30 min. α-^{32}P dATP was taken up and mixed with the annealing mixture. One µl of a mixture of dGTP, dCTP, TTP, at 125 µM each, and 1 µl of DNA polymerase (Klenow fragment, 0.25 units) were added. The DNA synthesis reaction was allowed to proceed at room temperature for 90 min, and then stopped by the addition of 1 µl of 250 mM EDTA (pH 8.0). The probes were kept on ice before use in hybridization. The label of the 15.2 kD polypeptide of the cytochrome b/f complex gene and 32 kD protein gene was prepared according to the method of Maniatis et al. (1975).

Hybridization, washing and autoradiography

The nitrocellulose paper with ct-DNA to be tested was prehybridized at 37 °C for 4 h in a solution containing 10x Denhardt solution (Denhardt 1966), 3x SSC, 50% formamide, 1 mM phosphate buffer (pH 7.9), 0.1% SDS, 10 mM HEPES (pH 7.4), 1 mM EDTA (pH 7.9), and 100 µg/ml sonicated denatured salmon sperm DNA. The prehybridization solution was then replaced by the same solution to which 10% dextran sulfate and radioactive probes were added. Hybridization was allowed to proceed at 37 °C overnight. The nitrocellulose papers were then washed three times (20 min each) at room temperature with 2x SSC, 0.1% SDS; four times (30 min each) at 45 °C in 0.1x SSC, 0.1% SDS solution; two times (10 min each) at room temperature in 0.1X SSC. After filter was blot-dried, it was exposed at −70 °C to X-ray film in the presence of an intensifying screen, either overnight or as specified.

Results and discussion

To localize the seven genes on the *N. tabacum* chloroplast genome, we took advantage of the availability of the probes for those genes and the physical map of *Nicotiana* chloroplast genome which was constructed with six restriction enzymes (Tassopulu and Kung 1983). *N. tabacum* ct-DNA was digested with each of the seven enzymes: BamHI, EcoRI, HindIII, PvuII, SalI, SmaI and XhoI. With the exception of EcoRI and HindIII, the physical maps constructed from other five enzymes are available (Tassopulu and Kung 1983). The resulting fragments generated by each enzyme were separated on agarose gels. Each probe containing a defined gene or a segment of the gene was labelled with ^{32}P and hybridized with the fragments produced by a given enzyme. A typical result of such hybridization is presented in Fig. 1. The correlation of an autoradiographic signal with its corresponding fragment is excellent. In most instance, one probe hybridized with a specific restriction enzyme fragment and no cross hybridization among fragments was observed. The results obtained from such hybridization studies are listed in Table 1. It is evident from this Table that some genes may be contained in a single fragment. This close arrangement of these genes may indicate a high degree of coding capacity of chloroplast genome. Based on the information of Table 1, the positions of the seven genes on the *N. tabacum* chloroplast genome were determined and are illustrated in Fig. 2.

Since the sizes of the restriction fragments are usually larger than the genes mapped, the location of each gene within any given fragment was assigned and confined in the region overlapped by the two nearest restriction sites. For example, the probe containing a segment of the gene for the α subunit of ATP synthase hybridized with fragments BamHI-4, PvuII-5, SalI-5, SmaI-5 and XhoI-5 (Table 1). Therefore, this gene is contained in the region overlapped by all these fragments (Fig. 2). Since the smallest area overlapped by two restriction sites is between the BamHI-4 and SmaI-5, the gene is thus map in this region as marked on Fig. 2. This area is estimated to be of 2 kb and is slightly larger than the actual size of the α gene which is determined as having 1,521 bp (Deno et al. 1983). The gene for the 15.2 kD polypeptide of the cytochrome b/f complex is contained in the fragments BamHI-I,

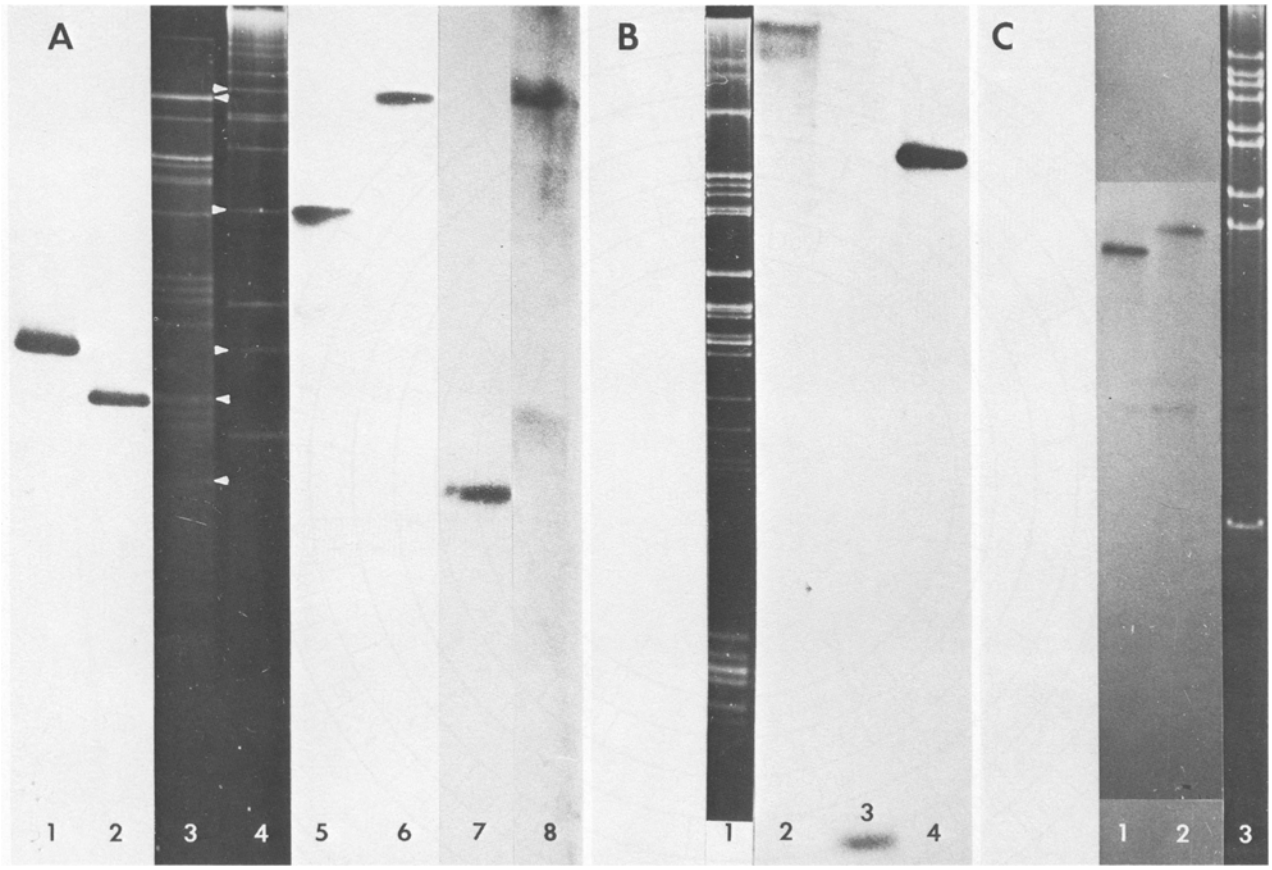

Fig. 1 A–C. Localization of various protein genes on *N. tabacum* chloroplast genome using probes from wheat and pea ct-DNA as described in the 'Materials and methods'. After nick-translation, the probes were hybridized to various restriction fragments. The autoradiograph showed that: In **A**, the probes for proton-translocating subunit (*1, 2*) β and ε subunits (*5, 6*) and α subunit (*7, 8*) hybridized to: (*1*) PvuII-11; (*2*) HindIII-14, (*5*) PvuII-9, (*6*) HindIII-3, (*7*) HindIII-17, and (*8*) PvuII-5. (*3*) and (*4*) are HindIII and PvuII fragments, respectively. In **B**, the probe for cytochrome f hybridized to BamHI-1 (*2*), EcoRI-28 (*3*), and HindIII-5 (*4*). The BamHI fragment pattern is illustrated in (*1*). In **C**, the probe for the 15.2 kD polypeptide of the cytochrome b/f complex hybridized to HindIII-4 (*1*) and SalI-8 (*2*) and (*3*) is the SalI fragment pattern

Table 1. Hybridization of probes containing a defined gene (or partial gene) with various restriction fragments of *N. tabacum* ct-DNA

	ATP synthase		ATP synthase				ATP synthase		15.2 kD polypeptide			
Wheat Tobacco	α (1,512 bp) (1,521 bp)		β (1494 bp) Wheat and ε (411 bp)		Cytochrome f (960 bp)		Proton-translocating subunit (243 bp)		Cytochrome b/f complex (417 bp)		32 kD (1,059 bp)	
Restriction enzymes	No. fragment	Kb	No. fragment	Kb	No. fragment	Kb	No. fragment	Kb	No. fragment	Kb	No. fragment	Kb
BamHI	4	9.0			1	20.0	4	9.0	1	20.0		
EcoRI	3	7.2	7	4.6	28	0.6	3	7.2	4	6.5	7	2.9
HindIII	17	1.8	3	10.0	5	6.6	14	2.6	4	6.8	5	7.6
PvuII	5	10.0	9	6.35	1	22.0	11	3.5	1	22.0		
SalI	5	16.7	6	15.2	6	15.2	5	16.7	8	11.4	3	22.2
SmaI	5	10.5	1	28.0	1	28.0	6	9.5	1	28.0	12	3
XhoI	5	12.0	7	9.0	2	20.0	5	12.0	13	3.0	8	3.4

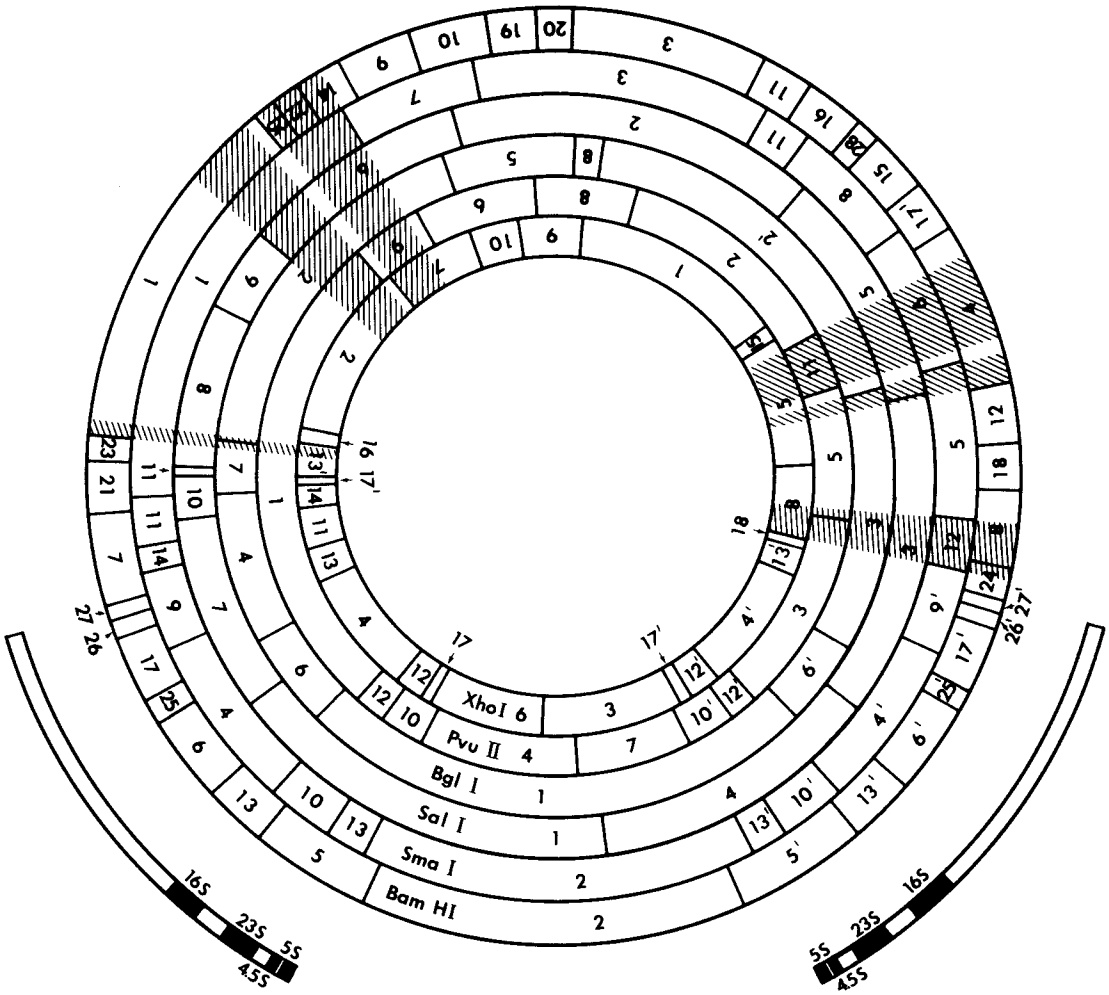

Fig. 2. Localization of seven protein genes on *N. tabacum* chloroplast genome. The physical map is from Tassopula and Kung (1983). The hatched areas indicate the location of protein gene or genes as determined from the data of Table 1 (see text).

PvuII-1, SmaI-1, SalI-8 and XhoI-13. A portion of XhoI-13 is overlapped with BamHI-1. The exact location of this gene is thus confined to the overlapped area between the border lines of BamHI-1 and XhoI-13 (Fig. 2). This area is estimated to be 1.2 kb in size which is easily large enough to host this gene of 417 bp (Phillips and Gray 1984). The mapping of those two genes is very precise. This is due to the use of multiple enzymes in constructing the physical map as well as in generating restriction fragments for hybridization. The locations of the genes for the proton-translocating subunit of ATP synthase and cytochrome f are less precise due to the large size of the overlapped area (Fig. 2). The gene for the proton-translocating subunit is confined in PvuII-11 which is contained in BamHI-4, SalI-5, SmaI-6 and XhoI-5. The size of PvuII-11 is 3.5 kb which is considerably larger than the gene (243 bp) (Howe et al. 1982). The gene for cytochrome f is contained in the largest fragments generated by BamHI, PvuII and SmaI and the second largest fragment produced by XhoI. Because the probe also hybridized with SalI-6, it is evident that this gene is located between the border lines of PvuII-1 and SalI-6 (Fig. 2). This region has a size of approximately 4 kb and is much larger than the gene which consists of 960 bp (Willey et al. 1984 b).

As reported in spinach (Whitfeld and Bottomley 1983) and maize (Bogorad et al. 1983) and wheat (Howe et al. 1982), the *Nicotiana* genes for β and ε subunits of ATP synthase are also linked together and situated near the gene for the LS of RuBPCase. The probes for these two genes hybridized with the same fragments: PvuII-9, SmaI-1, SalI-6, and XhoI-7 (Fig. 2). It is thus clear that they are located in the same region which is between the border lines of SmaI-1 and XhoI-7. It is also known that the gene for the LS of RuBPCase is also contained in this fragment (Tassopulu and Kung 1983). Similarly, the 32 kD gene is contained in SalI-3, Sma-12 and Xho1-8. The results obtained from the hybridization studies (Table 1)

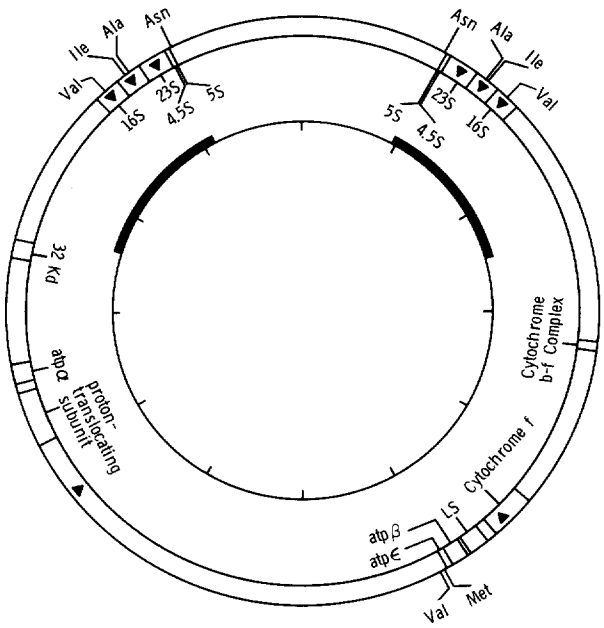

Fig. 3. Map of *N. tabacum* chloroplast genome showing the location of genes for ribosomal RNAs, transfer RNAs and eight proteins. The ▲ marks the sites of promoters. The thick lines on the inner circle represent the inverted repeat regions. This circle is divided into 12 time zones. The relative location of each gene is indicated by its time zones. (*atp* = ATPase)

are not only essential in localizing the seven genes but also confirm the validity of the restriction map of these regions. Thus, the locations mapped for all seven genes are reliable (Fluhr et al. 1984).

Many of the genes for chloroplast proteins exist in clusters as in the case of ribosomal RNA genes. The genes for the β and ε subunits of ATP synthase and for the LS of RuBPCase and cytochrome f are contained in a single SalI-6 fragment (Fig. 2). The fragment PvuII-9 has a size of 6.35 kb and contains three genes, the β and ε subunits of ATP synthase and the LS of RuBPCase. It is evident from Fig. 2 that all the protein-encoding genes studied so far are situated in the larger single-copy region of the *N. tabacum* chloroplast genome. This may represent a general organization of *Nicotiana* chloroplast genes which is similar to that of spinach (Whitfeld and Bottomley 1983) and different to that of some legume species (Palmer et al. 1983).

Figure 3 represents a general organization of the *Nicotiana* chloroplast genome constructed on the basis of the available information on the locations of many *Nicotiana* chloroplast genes, including all ribosomal RNA genes, several transfer RNA genes, eight protein genes and eight promoters (Kong et al. 1984). For the purpose of easy identification of various genes on the physical map of *N. tabacum* chloroplast genome, the map is divided, clock-like, into 12 regions. The location of each gene is indicated by pointing to the time zones; e.g., at nine o'clock or eight-thirty (Fig. 3). This map includes eight genes coding for chloroplast proteins. Their respective positions are: 15.2 kD polypeptide of the cytochrome b/f complex at 3 : 15, cytochrome f at 4 : 30, LS of RuBPCase at 4 : 50, both β and ε subunits at or near 5 : 00, proton-translocating subunit at 8 : 20, α subunit at 8 : 40 and 32 kD at 9 : 30. All the ribosomal RNA genes are situated in the inverted repeat region between 10 : 30–11 : 00 and 1 : 00–1 : 30. In the same regions, eight transfer RNA genes and six promoters are also mapped. Two more transfer RNA genes are localized at 5 : 10. Two promoters are also detected at 4 : 35 and 7 : 40, respectively.

It is clear that the function of the bulk of this genome is still unknown. It is fully expected that genes for all transfer RNAs will be mapped soon. This will be followed by the identification and localization of more genes coding for proteins. The utilization of the expression of the *E. coli galK* gene to localize promoters will certainly stimulate the study of promoters of the chloroplast genome from which the coding capacity of the genome may be evaluated.

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