Characterization of chloroplast DNA from sugar beet with normal and male sterile cytoplasms

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ABSTRACT

*Hind*III digestion of chloroplast DNA (ctDNA) from normal (N) and male sterile (S) lines of sugar beet generated 26 (N ctDNA) and 25 (S ctDNA) discrete bands ranging in size from 0.45×10^6 to 14.5×10^6 daltons (d). Densitometric tracings and a Southern hybridization experiment revealed one band (H7) missing and replacement of this band by two fragments (H12b and H14b) in S ctDNA. A slight intraspecific heterogeneity is likely to be due to the creation of a new *Hind*III site in the left part of the large single-copy region of S ctDNA. The summation of *Hind*III fragments indicated a molecular weight of 97.38 (N ctDNA) or 97.53 (S ctDNA) $\times 10^6$ d. Southern hybridization analyses demonstrated that the chloroplast ribosomal RNA genes are located on three *Hind*III fragments of H4, H20 and H22, the large subunit (LS) gene of ribulose-1,5-bisphosphate carboxylase/oxygenase on H6 and H17 fragments, the ribosomal protein CS-19 gene on H2 and H5a fragments and the 32,000 d thylakoid protein gene on a H5b fragment. Twelve (derived from N ctDNA) and 18 (derived from S ctDNA) *Hind*III fragments have been cloned into pBR 322.

1. INTRODUCTION

Chloroplast genomes of higher plants consist of circular double-stranded DNA molecules of approximately 10^8 daltons (d) in size (Bedbrook and Kolodner 1979; Sugiura *et al.* 1980). Since restriction endonuclease mapping is essential to analyze physical properties of chloroplast DNA (ctDNA), their detailed maps have been constructed in several plant species, e.g. maize (Bedbrook and Bogorad 1976), spinach (Whitfield *et al.* 1978; Steinmetz *et al.* 1979), pea (Chu *et al.* 1981), tobacco (Fluhr and Edelman 1981; Seyer *et al.* 1981) and wheat (Bowman *et al.* 1981; Ogihara and Tsunewaki 1982). Furthermore, recent biochemical studies (reviewed by Bedbrook and Kolodner 1979; Herrmann and Possingham 1980) have demonstrated that chloroplasts contain their own transcription-translation machineries, and ctDNA codes for structural genes for ribosomal RNAs, transfer RNAs, the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase/Oase), the sub-

nits of coupling factors, the 32,000 d thylakoid protein (P-32), ribosomal proteins and so forth.

However, our knowledge of sugar beet ctDNA is at present limited to an electron microscopic work (Herrmann *et al.* 1975) and restriction enzyme analyses (Powling and Ellis 1983; Mikami *et al.* 1984). In the previous report (Mikami *et al.* 1984), we found a marked heterogeneity in mitochondrial DNA between normal (N) and male sterile (S) cytoplasms of sugar beet, while less variation existed in ctDNA from the comparable cytoplasms. This suggests that mitochondrial genes are mainly responsible for the expression of sterility trait, though a definite conclusion has not been drawn yet.

In this study, we provided more detailed molecular characterization of ctDNA isolated from sugar beet with normal and male sterile cytoplasms. A preliminary results on molecular cloning of ctDNA were also presented.

2. MATERIALS AND METHODS

Plant materials and ctDNA preparation. A cytoplasmic male sterile line, NK169-MS and its maintainer, NK169-O were used in this work. These two lines are nearly isogenic except the constitution of cytoplasmic gene(s) conditioning male sterility; NK169-MS and NK169-O carry S and N cytoplasms, respectively. Chloroplast DNA was isolated essentially by the procedure of Saltz and Beckman (1981).

Probes and Southern hybridization. Tobacco rRNA or ctDNA probes used for hybridization experiments were the following; a mixture of chloroplast 23S and 16S rRNAs (Sugiura and Kusuda 1979); a 0.8×10^6 d *Bam*HI DNA fragment containing the LS gene; a 1×10^6 d *Pst*I DNA fragment carrying the CS-19 gene; a 2×10^6 d *Bam*HI-*Pvu*II fragment bearing the P-32 gene; a 12.5×10^6 d *Bam*HI fragment which contains the left part of the large single-copy region; a $5.8 \times 10^6 + 2.3 \times 10^6$ d *Bam*HI fragment which contains the right part of the large single-copy region. Chloroplast DNA from N and S cytoplasms of sugar beet was individually digested with *Hind*III and the resultant fragments were fractionated by electrophoresis in 0.7% horizontal agarose slab gels. After denaturation, DNA fragments in gels were neutralized and transferred to nitrocellulose filters. Hybridizations by using 32 P-labeled rRNA or nick-translated 32 P-DNA probes were carried out as described (Sugiura and Kusuda 1979; Shinozaki and Sugiura 1982).

Cloning of ctDNA. About $10 \mu\text{g}$ of ctDNA from each cytoplasm and $2 \mu\text{g}$ of pBR322 DNA were digested with *Hind*III. The cleaved pBR322 DNA was dephosphorylated with bacterial alkaline phosphatase (Worthington, BAPF). Ligation of the DNAs and transformation of *E. coli* HB101 were performed as described (Sugiura and Kusuda 1979). T4 DNA ligase was prepared as described (Sugiura 1980). Recombinant plasmid DNA was prepared by centri-

Fig. 1. Autoradiographs of *Hind*III digests of N (a) and S (b) ctDNAs hybridized to 32 P-labeled tobacco RNA or DNA probes and H14b fragment of sugar beet ctDNA. The *Hind*III digests were fractionated by electrophoresis in 0.7% agarose gels and transferred to a nitrocellulose filter for Southern hybridization.

fugation in CsCl-ethidium bromide equilibrium gradients (Sugiura and Kusuda 1979).

3. RESULTS AND DISCUSSION

Analysis of HindIII digests of ctDNA

Chloroplast DNA from N and S cytoplasms of sugar beet was subjected to complete digestion with *Hind*III followed by gel electrophoresis. Inspection of the cleavage patterns by UV photography or densitometric tracing revealed at least 26 (N ctDNA) and 25 (S ctDNA) bands which ranged in size from 0.45×10^6 to 14.5×10^6 d (Fig. 1). A mixture of λ DNA *Hind*III digest and ϕ x 174RF DNA *Hae*III digest was used as size markers to estimate molecular weights of the *Hind*III fragments. Table 1 presents molecular weights and stoichiometries of the visible bands. Densitometric scannings (Mikami *et al.*

Table 1. *Constitution of the N and S ctDNA fragments generated by HindIII digestion*

Fragment number	Size ($\times 10^6$)		Hybridized with
	N ctDNA	S ctDNA	
H1	14.5	14.5*	
H2	8.0	8.0*	12.5×10^6 BamHI fragment ¹⁾ , CS-19
H3	6.8	6.8*	$5.8 + 2.3 \times 10^6$ BamHI fragment ¹⁾
H4	6.2+++	6.2+++	$5.8 + 2.3 \times 10^6$ BamHI fragment ¹⁾ , rRNA (23S+16S)
H5a	5.2*	5.2*	CS-19
H5b	5.2	5.2	P-32
H6	3.65*	3.65*	LS
H7	3.5	—	H14b fragment, 12.5×10^6 BamHI fragment ¹⁾
H8	3.4*	3.4*	
H9	2.9	2.9	
H10	2.8	2.8	
H11	2.05*	2.05*	
H12a	1.95*	1.95*	
H12b	—	1.95	12.5×10^6 BamHI fragment ¹⁾
H13	1.8*	1.8*	12.5×10^6 BamHI fragment ¹⁾
H14a	1.7+++	1.7+++	
H14b	—	1.7*	12.5×10^6 BamHI fragment ¹⁾
H15	1.55*	1.55*	$5.8 + 2.3 \times 10^6$ BamHI fragment ¹⁾
H16	1.45	1.45	
H17	1.2+++	1.2+++	LS
H18	0.99	0.99*	
H19	0.95	0.95*	12.5×10^6 BamHI fragment ¹⁾
H20	0.87+++	0.87++	rRNA (23S+16S)
H21	0.82*	0.82*	
H22	0.74++	0.74+++	rRNA (23S+16S)
H23	0.67*	0.67*	
H24	0.61	0.61	
H25	0.52	0.52	
H26	0.45	0.45	
Sum	97.38	97.53	

Plus signs indicate stoichiometries of fragments. Cloned bands are marked with asterisks. 1) Tobacco ctDNA fragment.

1984) and a Southern hybridization experiment (as mentioned in a later section) revealed that a 3.5×10^6 d fragment (H7) detected in the N ctDNA was missing in the S ctDNA and was replaced by two fragments (H12b and H14b) with molecular weights of 1.95 and 1.7×10^6 d.

Molecular weights of the sugar beet ctDNAs were estimated by summing the molecular sizes of all the *Hind*III fragments present on given digestion

profiles. When the band multiplicity was taken into consideration, the sizes of N and S ctDNAs were 97.38 and 97.53×10^6 d, respectively (Table 1). These sums are not significantly different from the values, $99.9 \pm 2.0 \times 10^6$ d based on *SalGI* restriction digests (Powling and Ellis 1983) or 99.6×10^6 d estimated by the measurement of the contour length (Bedbrook and Kolodner 1979).

Identification of ctDNA fragments containing structural genes

Tobacco ctDNA fragments and a mixture of tobacco chloroplast 23S and 16S rRNAs were used as probes to hybridize individually to *HindIII* digests of ctDNA from N and S cytoplasms of sugar beet. As shown in Fig. 1, the 23S and 16S rRNAs hybridized exclusively to the three bands with molecular weights of 6.2 (H4), 0.87 (H20) and 0.74 (H22) $\times 10^6$ d. These three *HindIII* fragments were represented as doublet or triplet (Fig. 1, Table 1), suggesting that chloroplast rRNA genes of sugar beet are located within the inverted repeat regions of ctDNA molecules similar to those of maize (Bedbrook and Bogorad 1976), spinach (Whitfeld *et al.* 1978; Steinmetz *et al.* 1979), tobacco (Fluhr and Edelman 1981; Seyer *et al.* 1981) and so forth.

The 32 P-labeled tobacco ctDNA fragment containing the LS gene hybridized specifically to 3.65 (H6) and 1.2 (H17) $\times 10^6$ d fragments of sugar beet ctDNA. In a similar manner, tobacco CS-19 and P-32 probes hybridized to 8.0 (H2) and 5.2 (H5), and 5.2 (H5) $\times 10^6$ d bands respectively. The CS-19 gene is known to be located on the left margin of the large single-copy region, while the P-32 gene on the opposite margin of the region in tobacco chloroplast DNA (Sugita and Sugiura 1983, 1984). The linear order of common sequence elements is highly conserved among angiosperm chloroplast genomes which contain the inverted repeat (Palmer *et al.* 1983). Extensive sequence rearrangements are found only in the leguminous species, pea and broad bean, that have deleted one entire segment of the inverted repeat (Palmer and Thompson 1982). Thus, the H5 band probably consists of two kinds of fragments (H5a and H5b) which differ in sequence but are indistinguishable by mobility (Table 1).

The DNA fragments hybridized to the described tobacco probes are shared in common between the N and S ctDNAs of sugar beet. On the other hand, the H14b band which is unique to the S ctDNA hybridized strongly to the H7 band in the N cytoplasm (Fig. 1). The H7 band was also found to hybridize with a 12.5×10^6 d *BamHI* fragment of tobacco ctDNA which occupies the left margin of the large single-copy region (Table 1). It is therefore likely that the difference between N and S ctDNA sequences is due to the creation of a new *HindIII* site in the above-mentioned region of sugar beet S ctDNA. The results of hybridization tests are summarized in Table 1.

Fig. 2. The recombinant plasmids carrying various sizes of N (A) and S (B) ctDNA assigned electrophoretically in a 0.7% agarose slab gel using *Hind*III fragments of N per plasmid. Each cloned fragment is marked by an arrow.

Cloning of ctDNA

One goal of this research work is to construct DNA libraries of sugar beet ctDNAs which enable the detailed analysis of their chloroplast genes. We cloned sugar beet ctDNA digested with *Hind*III, because only *Hind*III digests of N and S ctDNAs were found to give distinct patterns so far examined (Mikami *et al.* 1984).

Initially, ctDNAs from N and S cytoplasms were digested individually with *Hind*III and the resultant fragments were ligated to *Hind*III-cleaved pBR 322 DNA. After the ligation mixture was introduced into competent *E. coli* HB 101 cells, the transformants were selected on ampicillin plates. We have screened about 300 transformants with ampicillin-resistance for the presence of ctDNA inserts after digestion of isolated plasmids with *Hind*III and agarose gel electrophoresis. We have so far cloned 12 (derived from N ctDNA) and 18 (derived from S ctDNA) distinct fragments into pBR 322

inserts. After *Hind*III digestion of recombinant plasmids, each cloned fragment was (a) and S (b) ctDNAs as standards. In several cases there are more than one insert

(Table 1). Fig. 2 shows the bands of the cloned fragments in parallel with the total ctDNA *Hind*III fragments, which can be assigned the inserted DNA fragments. Since recombinant DNA technique offers opportunities for investigating structure and function of specific gene(s), the construction of DNA library is one of the important operations in exploiting the potentials of genetic manipulation in higher plants. Further analysis on ctDNA organization of sugar beet is in progress in our laboratory by using the cloned ctDNA fragments as DNA sources.

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REFERENCES

- BEDBROOK, J. R. and BOGORAD, L. (1976) Endonuclease recognition sites mapped on *Zea mays* chloroplast DNA. *Proc. Natl. Acad. Sci. USA* **73**, 4309-4313.
- BEDBROOK, J. R. and KOLODNER, R. (1979) The structure of chloroplast DNA. *Ann. Rev. Plant Physiol.* **30**, 593-620.
- BOWMAN, C. M., KOLLER, B., DELIUS, H. and DYER, T. A. (1981) A physical map of wheat chloroplast DNA showing the location of the structural genes for the ribosomal RNAs and the large subunit of ribulose 1, 5-bisphosphate carboxylase. *Mol. Gen. Genet.* **183**, 93-101.
- CHU, N. M., OISHI, K. K. and TEWARI, K. K. (1981) Physical mapping of the pea chloroplast DNA and localization of the ribosomal RNA genes. *Plasmid* **6**, 279-292.
- FLUHR, R. and EDELMAN, M. (1981) Physical mapping of *Nicotiana tabacum* chloroplast DNA. *Mol. Gen. Genet.* **181**, 484-490.
- HERRMANN, R. G., BOHNERT, H. J., KOWALLICK, K. V. and SCHMITT, J. M. (1975) Size, conformation and purity of chloroplast DNA from some higher plants. *Biochim. Biophys. Acta* **378**, 305-317.
- HERRMANN, R. G. and POSSINGHAM, J. V. (1980) Plastid DNA-The plastome. In *Chloroplasts* (ed. REINERT, J.) pp. 45-96. Springer-Verlag, Berlin.
- MIKAMI, T., SUGIURA, M. and KINOSHITA, T. (1984) Molecular heterogeneity in mitochondrial and chloroplast DNAs from normal and male sterile cytoplasms in sugar beets. *Curr. Genet.* **8**, 319-322.
- OGIHARA, Y. and TSUNEWAKI, K. (1982) Molecular basis of the genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. I. Diversity of the chloroplast genome and its lineage revealed by the restriction pattern of ct-DNAs. *Jpn. J. Genet.* **57**, 371-396.
- PALMER, J. D. and THOMPSON, W. F. (1982) Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. *Cell* **29**, 537-550.
- PALMER, J. D., SINGH, G. P. and PILLAY, D. T. N. (1983) Structure and sequence evolution of three legume chloroplast DNAs. *Mol. Gen. Genet.* **190**, 13-19.
- POWLING, A. and ELLIS, T. H. N. (1983) Studies on the organelle genomes of sugarbeet with male-fertile and male-sterile cytoplasms. *Theor. Appl. Genet.* **65**, 323-328.
- SALTZ, Y. and BECKMAN, J. (1981) Chloroplast DNA preparation from *Petunia* and *Nicotiana*. *PMB Newsletter* **11**, 73-74.
- SEYER, P., KOWALLICK, K. V. and HERRMANN, R. G. (1981) A physical map of *Nicotiana tabacum* plastid DNA including the location of structural genes for ribosomal RNAs and the large subunit of ribulose bisphosphate carboxylase/oxygenase. *Curr. Genet.* **3**, 189-204.
- SHINOZAKI, K. and SUGIURA, M. (1982) The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase, *Gene* **20**, 91-102.
- STEINMETZ, A., MUBUMBILA, U., KELLER, M., BURKARD, G., WEIL, J. H., DRIESSEL, A. J., CROUSE, E. J., GORGON, K., BOHNERT, H. J. and HERRMANN, R. G. (1979) Mapping of the tRNA genes on the circular DNA molecule of *Spinacia oleracea*. In *Chloroplast Development* (ed. AKOYUNOGLU, G.) pp. 573-580. Elsevier, Amsterdam.
- SUGITA, M. and SUGIURA, M. (1983) A putative gene of tobacco chloroplast coding ribosomal protein similar to *E. coli* ribosomal protein S19. *Nucl. Acids Res.* **11**, 1913-1918.
- SUGITA, M. and SUGIURA, M. (1984) Nucleotide sequence and transcription of the gene for the 32,000 dalton thylakoid membrane protein from *Nicotiana tabacum*. *Mol. Gen. Genet.* **195**, 308-313.
- SUGIURA, M. (1980) Purification of the T4 DNA ligase by blue sepharose chromatography. *Anal. Biochem.* **108**, 227-229.
- SUGIURA, M. and KUSUDA, J. (1979) Molecular cloning of tobacco chloroplast ribosomal RNA genes. *Mol. Gen. Genet.* **172**, 137-141.
- SUGIURA, M., KUSUDA, J., SHINOZAKI, K. and TAKAIWA, F. (1980) Cloning and characterization of chloroplast ribosomal RNA genes. In *Genetics and Evolution of RNA polymerase, tRNA and ribosomes* (eds. OSAWA, S., OZEKI, H., UCHIDA, H. and YURA, T. pp. 437-449. Univ. Tokyo Press, Tokyo.
- WHITFIELD, P. R., HERRMANN, R. G. and BOTTOMLEY, W. (1978) Mapping of the ribosomal RNA genes on spinach chloroplast DNA. *Nucl. Acids Res.* **5**, 1741-1751.