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# Physical Mappings of Chloroplast DNA from Liverwort *Marchantia polymorpha* L. Cell Suspension Cultures

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**Summary.** Physical maps of liverwort *Marchantia polymorpha* chloroplast DNA were constructed with restriction endonucleases, *Bam*HI, *Sma*I, *Kpn*I, and *Xho*I. The molecular size, calculated from the sum of the restriction fragments, is 121.0±1.0 kilobase (kb) pairs. This value is in good agreement with that of 118.7±2.0 kb pairs obtained from contour length measurements of the chloroplast DNA electron micrographs. The physical map indicates that the chloroplast DNA contains two copies of inverted repeats. The length of the inverted repeat is at most 11.7 kb pairs. Southern hybridization analysis indicates that two sets of the chloroplast ribosomal RNA genes are located in the inverted repeat regions. The site of the ribulose bisphosphate (RuBP) carboxylase (large subunit) gene was also determined on a *Bam*HI fragment, Ba5, by using $^{32}$P-labeled DNA fragments containing the tobacco chloroplast RuBP carboxylase gene as a probe.

## Introduction

There have been many reports on the construction of physical maps and ribosomal RNA gene cloning of chloroplast DNA from algae, *Chlamydomonas reihardii* (Rochaix 1978; Rochaix and Malnoe 1978), *Euglena gracilis* (Gray and Hallick 1977, 1978; Rawson et al. 1978), to higher plants, *Zea mays* (Bedbrook and Bogorad 1976; Bedbrook et al. 1977), *Spinacia oleracea* (Whitfeld et al. 1978; Driesel et al. 1979; Herrmann et al. 1980a), *Vicia faba* (Koller and Delius 1980), *Spirodela oligorrbiza* (Van Ee et al. 1980), *Nicotiana tabacum* (Sugiura and Kusuda 1979; Jurgenson and Bourque 1980), *Petunia hybrida* (Bovenberg et al. 1981), *Pennisetum americanum* (Rawson et al. 1981), *Pisum sativum* (Chu et al. 1981; Palmer and Thompson 1981) and *Oenothera parviflora* (Gordon et al. 1981). Ribulose bisphosphate (RuBP) carboxylase genes of *Zea mays* (Coen et al. 1977; Link and Bogorad 1980), *Chlamydomonas reihardii* (Gelvin et al. 1977; Malnoe et al. 1979) and *Spinacia oleracea* (Whitfeld and Bottomley 1980) have been mapped. The gene site of a 34000 dalton membrane protein was determined on *Zea mays* chloroplast DNA (Bedbrook et al. 1978). The sites of several genes, such as coupling factor subunits (Westhoff et al. 1981), 32 kb photosystem II polypeptide (Driesel et al. 1980) and a few tRNA genes (Driesel et al. 1979), were also determined on the chloroplast DNA from *Spinacia oleracea*. Chloroplast DNA fragmentation analysis with restriction endonucleases has also been used for comparison of plant species (Atchison et al. 1976; Vedel et al. 1976; Vedel et al. 1978; Gordon et al. 1982) as well as for identification of parasexual hybrid plants (Belliard et al. 1978). However, there has been no investigation of physical mapping of chloroplast DNA from lower plants which are taxonomically different from both algae and higher plants. A comparison of chloroplast DNA gene organization from taxonomically different plant species will help to elucidate the origin of chloroplasts in the process of evolution. We have reported a simple method for isolation of chloroplast DNA from liverwort *Marchantia polymorpha* cell suspension cultures (Ohyama et al. 1982).

In this paper we describe physical mapping of the entire chloroplast DNA of *Marchantia polymorpha* and determination of the sites of ribosomal RNA genes and the RuBP carboxylase (large subunit) gene on the chloroplast DNA.

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## Materials and Methods

*Cells and Chloroplast Isolation. Marchantia polymorpha* L. cells were photomixotrophically grown in 1-M51C medium (Ono et al. 1979). The chloroplast DNA was isolated by the procedure described previously (Ohyama et al. 1982).

*Electron Microscopy.* Covalently closed circular molecules of chloroplast DNA were purified by CsCl-EtBr centrifugation and mounted for electron microscopy by the formamide technique as modified by Yamagishi et al. (1976). The samples were rotary-shadowed with electron-beam evaporation of platinum-carbon. Micrographs were taken in a JEM 200 CX electron microscope at an instrumental magnification of 7,200 and the DNA molecules were traced as 10-fold-enlarged images with the aid of a Yokogawa-Hewlett-Packard 9864A digitizer. The actual magnification was calibrated with a grating replica. Double-stranded fd RFII DNA (6408 base pairs) was mounted on the same grids as a length standard.

*Restriction Endonuclease Digestions.* Restriction endonucleases *Bam*HI, *Xho*I, *Kpn*I, *Sma*I, *Hin*dIII, and *Eco*RI were purchased from Takara Shuzo Co. LTD., Kyoto, Japan. Chloroplast DNA (1–5 μg) was completely digested in a 50 μl reaction mixture, as suggested by the supplier, with 1–5 units of each enzyme for 2 h at 37° C.

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Double enzyme digestions of chloroplast DNA were carried out by restricting a primary DNA digest with a second enzyme after adjustment of the reaction mixtures. Partial digestions of chloroplast DNA were performed with less enzyme (0.1 unit) at a lower incubation temperature (30° C) for a short period of time.

*Agarose Gel Electrophoresis.* Chloroplast DNA fragments digested with various restriction endonucleases were analysed by agarose electrophoresis on horizontal slab gels ($20 \times 15 \times 0.3$ cm$^3$) or vertical slab gels ($15 \times 13 \times 0.3$ cm$^3$) containing agarose ME (Nakarai Chemicals, Japan, 0.4%–2.5%) or low-gelling-temperature agarose (BRL, USA, 0.4%–1.0%) in 0.04 M Tris-acetate buffer, pH 7.8, 5 mM sodium acetate, 1 mM EDTA, and EtBr (1 μg/ml). Electrophoresis was carried out in the same buffer at 40–150 V (20–100 mA) for 3–24 h. DNA fragments were visualized by illumination with a UV lamp and photographed with a Mamiya RB67 camera (Polaroid film 665) using a red filter.

For determination of the sizes of chloroplast DNA fragments, *Hin*dIII digests of λDNA and *Hae*III digests of ϕX174 RF II DNA were used as size standards.

*Secondary Digestion of DNA Fragments Excised from Low-Gelling-Temperature Agarose Gels.* DNA fragments digested by a first restriction endonuclease were separated by electrophoresis on low-gelling-temperature agarose gels. Gels containing a DNA fragment were excised and transferred to a 1.5 ml Eppendorf tube. The gels were heated to 65° C and treated with an equal volume of TE (0.01 M Tris-HCl, pH 8, 1 mM EDTA) buffer-saturated phenol. DNA in the aqueous phase was precipitated by addition of two volumes of cold ethanol. DNA was dissolved in TE buffer and used for a second restriction endonuclease digestion.

*Nomenclature.* The names Ba1 to Ba13, Xh1 to Xh11, Kp1 to Kp6, Sm1 to Sm3, were given to the fragments produced by *Bam*HI, *Xho*I, *Kpn*I, and *Sma*I restriction endonucleases, respectively. In double digestions, BX1 to BX21, BK1 to BK17, BS1 to BS15, KX1 to KX14, KS1 to KS8, and SX1 to SX14, were assigned to the subfragments produced in double digestions with *Bam*HI/*Xho*I, *Bam*HI/*Kpn*I, *Bam*HI/*Sma*I, *Kpn*I/*Xho*I, *Kpn*I/*Sma*I, and *Sma*I/*Xho*I restriction endonucleases, respectively.

*Isolation and $^{32}$P-Labeling of Chloroplast RNAs.* Chloroplast rRNAs were prepared from purified chloroplasts and labeled with $\gamma^{32}$P-ATP using T4 polynucleotide kinase (Sugiura and Kusuda 1979).

*Southern Image Hybridization.* DNA fragments on gels were denatured, neutralized and transferred to nitrocellulose filters (Schleicher and Schull, BA85) (Southern 1975). For hybridization with $^{32}$P-labeled rRNAs the filters were wetted with minimum volumes of $^{32}$P-rRNA (23S and 16S) solution in $5 \times$ SSC (0.15 M NaCl–0.015 M trisodium citrate, pH 8) buffer and 50% formamide, wrapped in Saran wrap and incubated overnight at 37° C. The filters were washed 4 times with $5 \times$ SSC buffer-50% formamide, and twice with $2 \times$ SSC buffer to remove free $^{32}$P-rRNA. For hybridization with $^{32}$P-DNA fragments, labeled with $\alpha^{32}$P-dATP by the nick translation reaction (Rigby et al. 1977; Seyer et al. 1981), the filters were incubated overnight with $^{32}$P-DNA fragments in Denhardt solution (Denhardt 1966) at 65° C. The filters were then washed with $2 \times$ SSC buffer containing 0.5% SDS.

Autoradiography was performed on Fuji X-ray film with Dupon Cronex® Lightning-plus UD.

## Results

### *Primary and Double Digestions*

Figures 1 and 2 show agarose electrophoreic patterns of DNA fragments produced by primary digeston with *Bam*HI, *Xho*I, *Kpn*I, and *Sma*I restriction endonucleases, and double digestions with combinations of two enzymes, respectively. Restriction endonucleases *Bam*HI, *Xho*I, *Kpn*I, and *Sma*I cleaved *Marchantia polymorpha* chloroplast DNA into 13, 11, 6, and 3 bands on agarose gel electrophoresis, respectively. The Xh7 band, and the Kp5 and Kp6 bands appeared to be present in two moles because the intensity of their UV-irradiation is twice as high as the other bands. Double digestions of the chloroplast DNA with the combinations *Bam*HI/*Xho*I, *Bam*HI/*Kpn*I, *Bam*HI/*Sma*I, *Kpn*I/*Xho*I, *Kpn*I/*Sma*I, and *Sma*I/*Xho*I produced 21 BX subbands, 17 BK subbands, 15 BS subbands, 14 KX subbands, 8 KS subbands, and 14 SX subbands. Tables 1 and 2 summarize the fragment sizes in primary and double digests. The sum of the molecular sizes of the fragments in each case is $121.0 \pm 1.0$ kb pairs (mean).

### *Electron Microscopy of Circular Molecules of Chloroplast DNA*

Fractions of the covalently closed circular molecules of chloroplast DNA (lower band in CsCl-EtBr gradients) were spread with fd RFII DNA (Fig. 3). The contour length of the circular molecules of chloroplast DNA (14 molecules) was measured as $32.32 \pm 0.65$ μm, whereas that of fd RFII DNA (55 molecules, 6408 bp) was $2.07 \pm 0.07$ μm. These

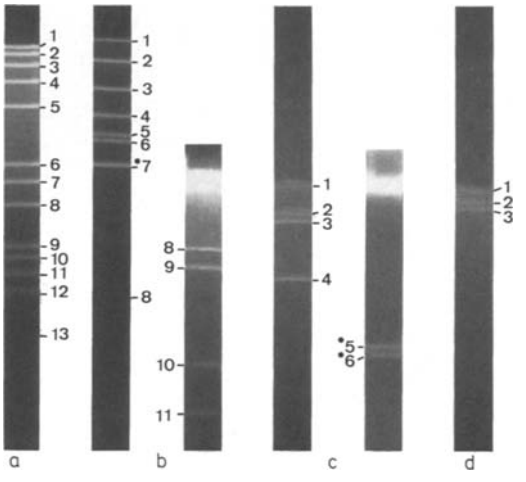


**Fig. 1.** Agarose gel electrophoresis of *Marchantia polymorpha* chloroplast DNA digests with: a, *Bam*HI; b, *Xho*I; c, *Kpn*I; d, *Sma*I restriction endonucleases. Samples were run on 1% agarose gels (a and left lane of b) and on 0.4% agarose gels (left lane of c and d). Samples were run on 2.5% agarose gels (right lanes of b and c) to get clear bandings. Asterisks (*) indicate 2 moles of DNA fragments

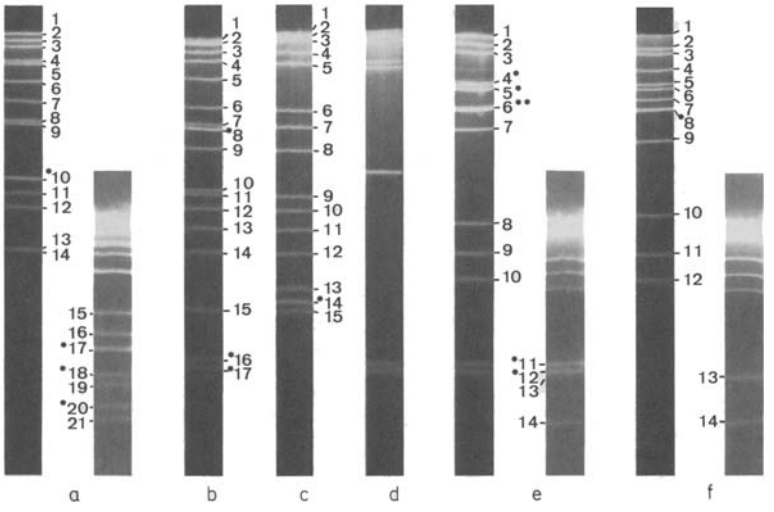


**Fig. 2.** Agarose gel electrophoresis of *Marchantia polymorpha* chloroplast DNA double digests with: a, *Bam*HI/*Xho*I; b, *Bam*HI/*Kpn*I; c, *Bam*HI/*Sma*I; d, *Kpn*I/*Sma*I; e, *Kpn*I/*Xho*I; f, *Xho*I/*Sma*I restriction endonucleases. Samples were run on 1% agarose gels except right lanes of a, e and f which were run on 2.5% agarose gels to give clear bands of small fragments. Single (*) and double asterisks (**) indicate 2 moles and 3 moles of DNA fragments, respectively

**Table 1.** Size of *Marchantia polymorpha* chloroplast DNA fragments digested with *Bam*HI, *Xho*I, *Kpn*I, and *Sma*I restriction endonucleases

| Fragment number | Fragment size (kilobase pairs) | | | | Size standards[a] |
|---|---|---|---|---|---|
| | *Bam*HI | *Xho*I | *Kpn*I | *Sma*I | |
| 1 | 29.7 ±0.6 | 43.7 ±0.6[b] | 53.1 ±3.2[b] | 53.8±1.5[b] | 49 |
| 2 | 24.3 ±1.5 | 23.0 ±1.0 | 25.3 ±0.6 | 36.9±1.5[b] | 28.0 |
| 3 | 19.0 ±1.0 | 13.8 ±0.3 | 23.7 ±0.3 | 31.0±0.5[b] | 23.7 |
| 4 | 14.8 ±0.3 | 9.6 ±0.1 | 14.0 ±0.5 | – | 9.46 |
| 5 | 10.3 ±0.3 | 7.9 ±0.1 | 0.84±0.01(×2)[c] | – | 6.66 |
| 6 | 5.7 ±0.1 | 7.4 ±0.2 | 0.75±0.01(×2)[c] | – | 4.26 |
| 7 | 4.8 ±0.2 | 5.8 ±0.1(×2)[c] | – | – | 2.25 |
| 8 | 3.85±0.05 | 1.78±0.08 | – | – | 1.96 |
| 9 | 2.72±0.06 | 1.53±0.03 | – | – | 1.35 |
| 10 | 2.45±0.05 | 0.71±0.01 | – | – | 1.08 |
| 11 | 2.08±0.03 | 0.49±0.01 | – | – | 0.87 |
| 12 | 1.78±0.03 | – | – | – | 0.60 |
| 13 | 1.18±0.03 | – | – | – | 0.31 |
| Total | 122.66 | 121.51 | 119.28 | 121.70 | |

[a] Size standards of fragments are λDNA, λDNA fragments digested with *Hin*dIII, and ϕX174 RF II DNA fragments digested with *Hae*III

[b] Size was estimated by summing up sizes of subfragments

[c] Number in parentheses indicates moles of DNA fragment

values give 118.7±2.0 kb pairs for the chloroplast DNA. This value is in excellent agreement with the molecular size (121.0±1.0 kb pairs) estimated by the sum of restriction fragments of the chloroplast DNA. Two molecules containing an internal loop were found among 14 circular molecules. The internal loop may represent the replicative intermediate at the replication origin. Linear molecules were most common in fractions pooled from the upper band in CsCl-EtBr gradients.

### *Stragety of Determination of the Chloroplast DNA Physical Map*

The strageties used in the this physical mapping were (1) determination of primary fragments restricted with another restriction endonuclease in double digestions, (2) determination of subfragment composition of primary fragments by product analysis in secondary digestions, and (3) determination of the order of nonrestricted *Bam*HI fragments by *Bam*HI partial digestions. These results are summarized in Figs. 4–7.

### *Determination of Physical Maps of BamHI and XhoI Fragments*

As shown in Figs. 4 and 5, the fragment Ba1 has two end-subfragments, BX8 and BX2. Subfragment BX8 can also be seen in Xh7a. Therefore, Ba1 and Xh7a overlap via a common subfragment, BX8. The other end-subfragment of Xh7a is BX16, which is also an end-subfragment of Ba11. Ba11 contains another end-subfragment, BX15, which is found in Xh1. Thus, the order of *Bam*H1 fragments is Ba1-Ba11, and the order of *Xho*I fragments is Xh7a-Xh1. Next, the another end-subfragment of Xh1 is BX10. However, this subfragment can be seen in both Ba6 and Ba8. On the other hand, the Xh1 fragment contains a subfragment, KX4, in common with Kp4, which is from double digestion of the chloroplast DNA with *Kpn*I and *Xho*I restriction

**Table 2.** Fragment sizes of *Marhantia polymorpha* chloroplast DNA in double digestions with *Bam*HI/*Xho*I, *Bam*HI/*Kpn*I, *Bam*HI/*Sma*I, *Kpn*I/*Xho*I, *Kpn*I/*Sma*I, and *Xho*I/*Sma*I restriction endonucleases[a]

| Fragment number | Fragment size (kilobase pairs) | | | | | |
|---|---|---|---|---|---|---|
| | *Bam*HI/*Xho*I | *Bam*HI/*Kpn*I | *Bam*HI/*Sma*I | *Kpn*I/*Xho*I | *Kpn*I/*Sma*I | *Xho*I/*Sma*I |
| 1 | 23.7 | 17.0 | 28.0 | 27.0 | 42 | 37 |
| 2 | 18.0 | 16.0(×2)[b] | 22.4 | 16.0 | 27.0 | 16.5 |
| 3 | 14.0 | 13.0 | 19.0 | 13.0 | 20.0 | 13.0 |
| 4 | 10.5 | 10.5 | 12.6 | 8.1(×2)[b] | 11.0 | 9.8 |
| 5 | 9.8 | 8.0 | 10.5 | 7.5(×2)[b] | 10.0 | 8.1 |
| 6 | 7.5 | 5.9 | 5.9 | 6.1(×3)[b] | 3.5(×2)[b] | 7.5 |
| 7 | 6.1 | 4.8 | 4.8 | 4.7 | 0.80(×2)[b] | 7.2 |
| 8 | 5.0 | 4.5(×2)[b] | 3.9 | 2.15 | 0.75(×2)[b] | 6.1(×2)[b] |
| 9 | 4.8 | 3.9 | 2.8 | 1.82 | – | 4.6 |
| 10 | 3.0(×2)[b] | 2.9 | 2.45 | 1.52 | – | 2.40 |
| 11 | 2.8 | 2.8 | 2.15 | 0.80(×2)[b] | – | 1.82 |
| 12 | 2.45 | 2.45 | 1.80 | 0.75(×2)[b] | – | 1.52 |
| 13 | 1.82 | 2.10 | 1.48 | 0.71 | – | 0.71 |
| 14 | 1.80 | 1.80 | 1.32(×2)[b] | 0.47 | – | 0.47 |
| 15 | 1.15 | 1.15 | 1.15 | – | – | – |
| 16 | 0.98 | 0.80(×2)[b] | – | – | – | – |
| 17 | 0.82(×2)[b] | 0.75(×2)[b] | – | – | – | – |
| 18 | 0.72(×2)[b] | – | – | – | – | – |
| 19 | 0.64 | – | – | – | – | – |
| 20 | 0.53(×2)[b] | – | – | – | – | – |
| 21 | 0.47 | – | – | – | – | – |
| Total | 121.65 | 120.40 | 121.57 | 119.97 | 120.10 | 120.82 |

[a] Standard deviations from means were deleted from this Table
[b] Number in parentheses indicates moles of DNA fragments

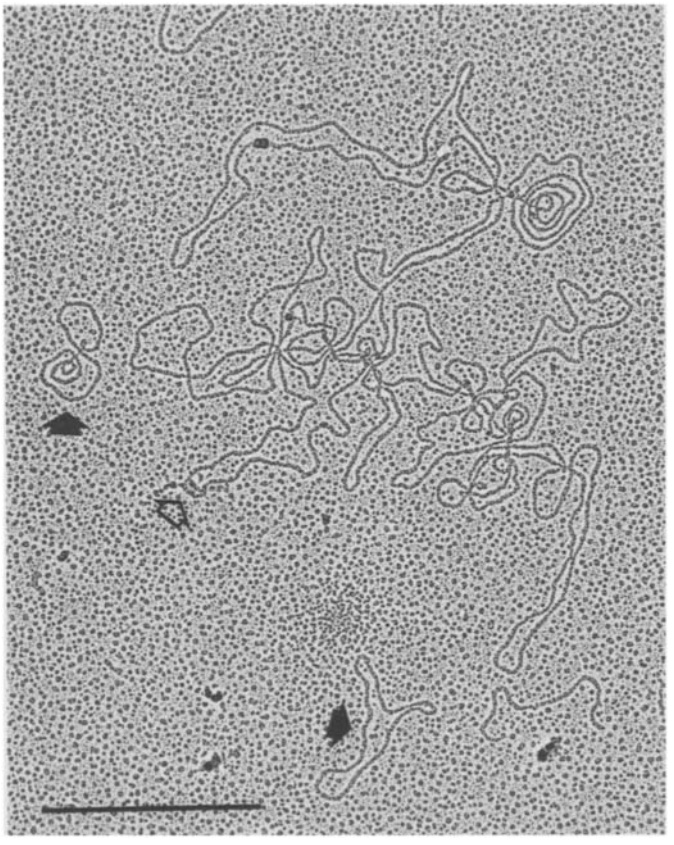

**Fig. 3.** Electron microgram of a circular *Marchantia polymorpha* chloroplast DNA. The open arrow indicates internal loop. Bacteriophage fd RFII DNA (solid arrows) is included as length reference. The bar indicates 1.0 μm

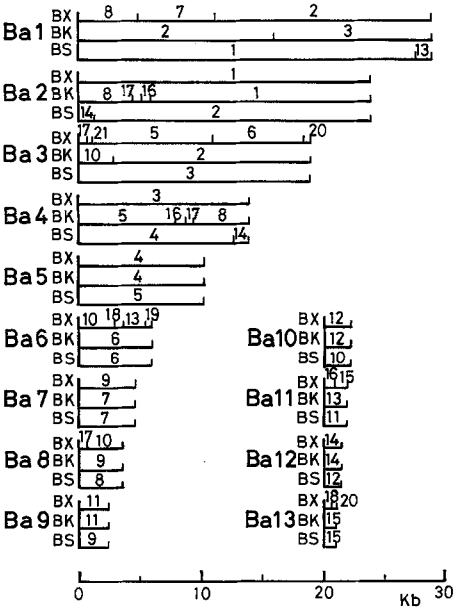


**Fig. 4.** Summary of subfragment composition in primary *Bam*HI fragments. *Bam*HI fragments were individually excised from gels and redigested with *Xho*I, *Kpn*I, and *Sma*I restriction endonucleases, respectively. Numbers indicate subfragment number produced (see Table 2). Order of subfragments (BK16 and BK17) in Ba2 and Ba4 fragments were determined by redigesting with *Hin*dIII, *Eco*RI endonucleases (see Fig. 10). BX subfragment order in Ba6 fragment was determined by partial digestion with *Xho*I restriction endonuclease (data not shown). Subfragment order (BX5, BX6, BX21) of Ba3 was determined by BK subfragment analysis

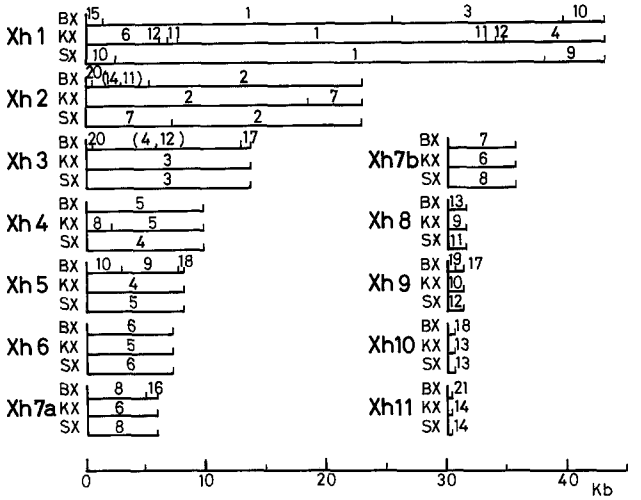


**Fig. 5.** Summary of subfragment composition in primary *Xho*I fragments. *Xho*I fragments were individually excised from gels and redigested with *Bam*HI, *Kpn*I, and *Sma*I restriction endonucleases, respectively. Numbers indicate subfragment number (see Table 2). Numbers in parentheses indicate nonoverlapping fragments which were not ordered in this analysis

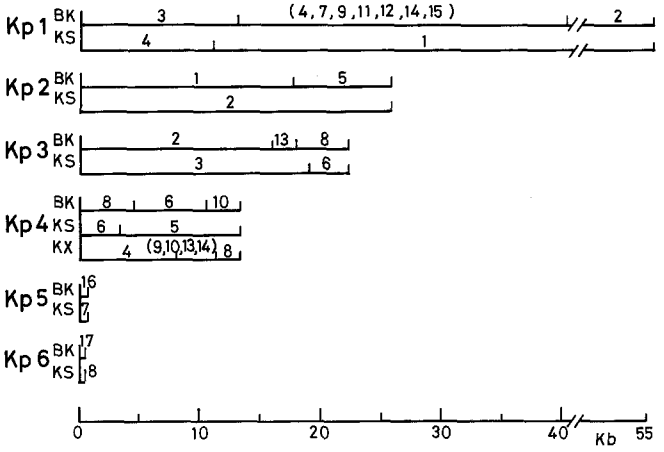


**Fig. 6.** Summary of subfragment composition in primary *Kpn*I fragments. *Kpn*I fragments were separately excised from gels and redigested with *Bam*HI and *Sma*I restriction endonucleases, respectively. *Kpn*I fragment, Kp4, was also redigested with *Xho*I restriction endonuclease. Numbers in parentheses indicate nonoverlapping fragments which were not ordered in this analysis

endonucleases. Kp4 also contains the nonrestricted Ba6 fragment (Fig. 6). These results indicate that Xh1 overlaps with Ba6 via a common subfragment, BX10, but does not overlap with Ba8. Ba6 then carries the other end-subfragment, BX19, which is found in Xh9. Thus far, the orders Ba1-Ba11-Ba6 for the *Bam*HI fragments and Xh7a-Xh1-Xh9 for the *Xho*I fragments have been determined. The other end-subfragment, BX17, of Xh9 fragment is found in both the Ba8 and Ba3 fragments. Since the Kp4 fragment, which carries the nonoverlapping Ba6 fragment, contains an end-subfragment, BK10, of Ba3 fragment (Fig. 6), Ba6 must be linked to Ba3 through BX17 as a common subfragment. The other end-subfragment of Ba3, BX20, also occurs among the products of *Bam*HI-*Xho*I double digests from Xh2 and Xh3. If subfragment BX20 of Ba3 occurs in Xh2, Xh2 must overlap with Ba1 via BX2 as

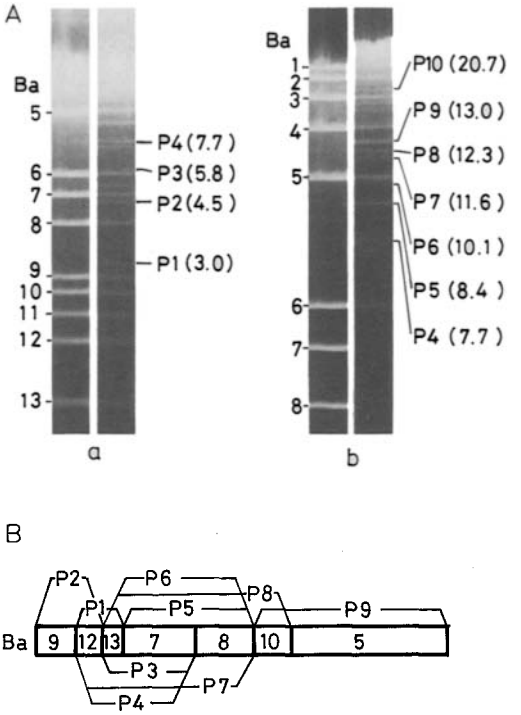


**Fig. 7A, B.** Analysis of partial *Bam*HI fragments. **A** Agarose gel electrophoresis of partial *Bam*HI fragments (p1 to p10). Samples were run on 1% agarose gels. Pictures were taken at 2 h (a) and 6 h (b). Numbers in parentheses indicate sizes (kb) of partial *Bam*HI fragments. Left lanes are complete *Bam*HI digestion (Ba1 to Ba13) of the chloroplast DNA. **B** Determination of *Bam*HI fragment order. Composition of the partial fragments was determined by summation of *Bam*HI fragment lengths and the orders were constructed by examining the overlapping fragments

a common subfragment, because this common subfragment can only be seen in Ba1. If this is the case, however there is no way for Xh3 to overlap with *Bam*HI fragments in the DNA map. Therefore, the BX20 subfragment of Ba3 must be in Xh3. The other end-subfragment, BX17, of Xh3 is now left in Ba8, since BX17 also occurred in Ba3. The other end-subfragment of Ba8, BX10, occurs in Xh5, because it was also found as an end-subfragment of Xh1. Xh5 contains BX18 as an end-subfragment, and BX18 is produced from *Xho*I digestion of Ba13. The other end-subfragment of Ba13, BX20, is in Xh2. This Xh2 fragment overlaps with Ba1 via a common end-subfragment, BX2. Thus, circular physical maps with overlapping fragments produced by *Bam*HI and *Xho*I double digestion are constructed as Ba1-Ba11-Ba6-Ba3-Ba8-Ba13 and Xh7a-Xh1-Xh9-Xh3-Xh5-Xh2, respectively.

Nonoverlapping fragments in *Bam*HI and *Xho*I digests were then determined by subjecting a primary *Bam*HI fragment to secondary digestion with *Xho*I restriction endonuclease and vice versa. For instance, Xh1 contains two nonoverlapping fragments Ba2 and Ba4. Xh3 has Ba10 and Ba5. Xh5 carries nonoverlapping Ba7. Xh2 produces two nonoverlapping fragments Ba9 and Ba12. Therefore, the order of *Bam*HI fragments is Ba1-Ba11-(Ba2, Ba4)-Ba6-Ba3-(Ba10, Ba5)-Ba8-Ba7-Ba13-(Ba12, Ba9)-Ba1, in circular form. The ciruclar map of *Xho*I fragments was determined to be Xh7a-Xh1-(Xh10, Xh8)-Xh9-(Xh11, Xh4, Xh6)-Xh3-Xh5-Xh2-Xh7b-Xh7a.

Finally, the orders of *Bam*HI and *Xho*I fragments in parentheses above were determined by analysis of *Bam*HI

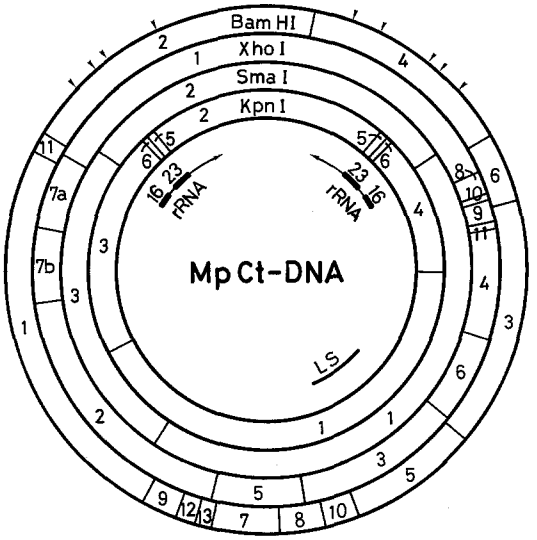


**Fig. 8.** Physical mapping of *Marchantia polymorpha* chloroplast DNA. Narrow lines with arrows inside the circular map indicate inverted repeat regions. Chloroplast rRNA genes were expressed as rRNA (16S and 23S). LS indicates the site of RuBP carboxylase (large subunit) gene. Arrow heads on *Bam*HI fragments Ba2 and Ba4 indicate *Eco*RI cleavage sites

and *Xho*I partial digests. Partial digestion of *Bam*HI fragments gave a variety of partial fragments. The composition of *Bam*HI fragments is shown in Fig. 7. These results gave the *Bam*HI fragment order shown in Fig. 7B. Also, *Bam*HI partial digestion of the chloroplast DNA produced the Ba4-Ba6 partial fragment (Fig. 7A, P10). On the other hand, the BX subfragment order of Ba6 was determined as BX10-Xh10-Xh8-BX18 because *Xho*I digestion of Ba6 gave partial fragments, BX10-Xh10, BX10-Xh10-Xh8 (data not shown). Also, Kp4 and Ba3 each produced nonoverlapping Xh11 when digested with *Xho*I restriction endonuclease. *Kpn*I restriction endonuclease cleaved both Xh4 and Ba3 but not Xh6 (Figs. 4 and 5). These results gave the order Xh11-Xh4-Xh6 in the region overlapping with Ba3. The entire physical maps of *Bam*HI and *Xho*I fragments are shown in Fig. 8.

### *Determination of Physical Maps of KpnI and SmaI Fragments*

As described above, the Kp4 fragment contains KX4 as an end-subfragment which can also be seen as an end-subfragment in *Kpn*I digests of Xh1. Xh1 also produced KX6 end-subfragment as well as nonoverlapping fragments KX1 (Kp2), KX11 (Kp5, 2×), and KX12 (Kp6, 2×). On the other hand, fragment Ba3, which overlaps with Kp4, produced end-subfragment BK2. This end-subfragment (BK2) must overlap with Kp1 because the Kp1 fragment contains the Ba5 fragment which is adjacent to Ba3, as shown in the *Bam*HI map. Thus, the order [Kp2, Kp5 (2×), Kp6 (2×)]-Kp4-Kp1 was determined. The other end-subfragment, BK3, of Kp1 is located in Ba1. Ba1 produced the other end-subfragment of Ba1, BK2, which also occurs in Kp3. Kp3 contains nonoverlapping Ba11 and subfragment BK8 (in common with Ba2), through which Kp3 is linked to the fragment [Kp2, Kp5(2×), Kp6(2×)]-Kp4-Kp1. The order of [Kp2, Kp5(2×), Kp6(2×)] was also determined by detailed physical mapping with *Hin*dIII and

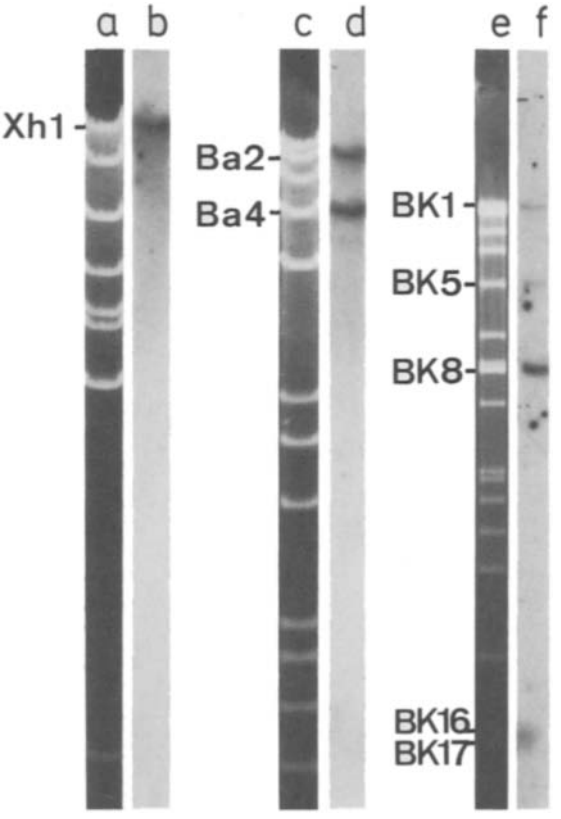


**Fig. 9.** Southern image hybridization of *Marchantia polymorpha* chloroplast $^{32}$P-labeled rRNAs. Agarose gel electrophoresis of *Marchantia polymorpha* chloroplast DNA digested with: a, *Xho*I; c, *Bam*HI; e, *Bam*HI/*Kpn*I restriction endonucleases. Autoradiograms (b, d, f) of *Marchantia polymorpha* chloroplast $^{32}$P-labeled rRNA (23S and 16S rRNA mixture) hybridization of fragments produced in a, c, e, respectively

*Eco*RI digestions of Ba4, which is carring fragments Kp5 and Kp6 (see K3 and K4 fragments in Fig. 10B).

An *Sma*I physical map was determined by fragment-size analysis of double digests with *Kpn*I and *Sma*I restriction endonucleases, together with the *Kpn*I physical map. The entire physical maps for *Kpn*I and *Sma*I restriction endonucleases on *Marchantia polymorpha* chloroplast DNA are shown in Fig. 8.

### *Inverted Repeats and their Size*

Both Ba2 and Ba4 fragments produced BK8, BK16 and BK17 subfragments in secondary digestion with *Kpn*I restriction endonuclease (Fig. 4. BK digests in Ba2 and Ba4). The Kp3 and Kp4 fragments which overlap with Ba2 and Ba4, respectively, also produced BK8 subfragment in secondary digestion with *Bam*HI, and BS6 subfragment with *Sma*I restriction endonuclease (Fig. 5. BK and BS digests in Kp3 and Kp4). These results indicate that Ba2 and Ba4 fragments as well as Kp3 and Kp4 fragments contain repeated fragments. Further analyses of secondary digestions of Ba2 and Ba4 fragments with *Sma*I restriction endonuclease showed that the order of repeated fragments (BK8, BK16 and BK17) was inverted on Ba2 and Ba4 fragments (Fig. 4. BS digests in Ba2 and Ba4). The size of the inverted repeat was determined by *Eco*RI digestions of Ba2 and Ba4 fragments (mapping data not shown). The Ba2 fragment

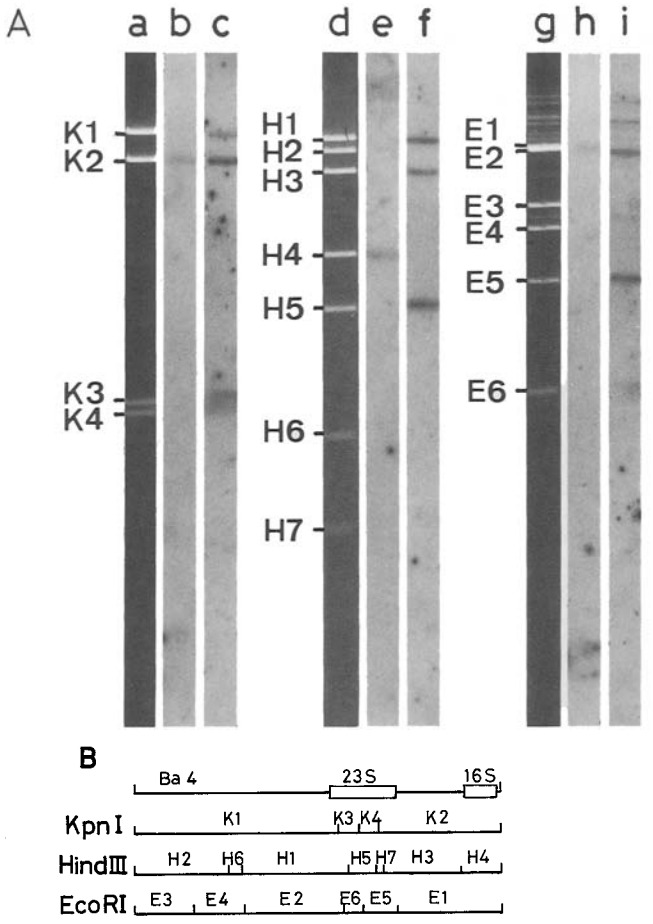


**Fig. 10A, B.** Orientation of 23S and 16S rRNA on *Marchantia polymorpha* chloroplast DNA. **A** Southern image hybridization of *Marchantia polymorpha* chloroplast $^{32}$P-labeled 23S rRNA and 16S rRNA. Agarose gel electrophoresis of Ba4 fragment redigested with: a, *Kpn*I; d, *Hin*dIII; g, *Eco*RI restriction endonucleases. Autoradiograms (b, e, and h) of 16S $^{32}$P-labeled rRNA hybridization to *Kpn*I subfragments, *Hin*dIII subfragments, and *Eco*RI subfragments, respectively. Autoradiograms (c, f, and i) of 23S $^{32}$P-labeled rRNA hybridization to *Kpn*I subfragments, *Hin*dIII subfragments, and *Eco*RI subfragments, respectively. **B** Physical mappings of Ba4 fragment and sites of 23S and 16S rRNA genes. Fragments, K3 and K4, represent exactly Kp5 and Kp6 fragments, respectively, in *Kpn*I digestion of the chloroplast DNA

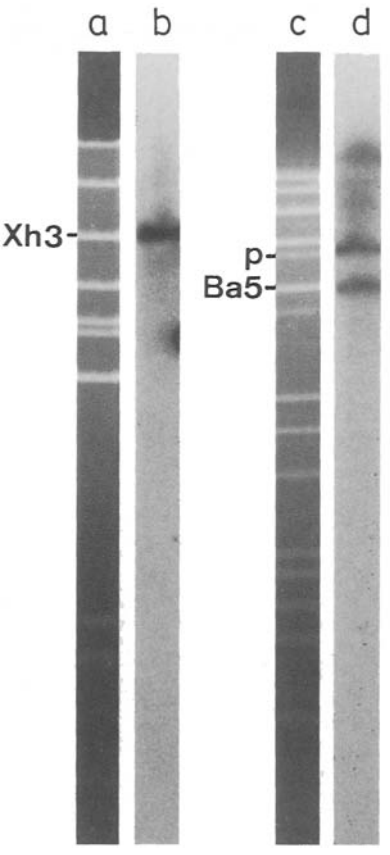


**Fig. 11.** Southern image hybridization of $^{32}$P-labeled DNA fragment containing the tobacco RuBP carboxylase gene. Agarose gel electrophoresis of *Xho*I fragments (a) and *Bam*HI fragments (c) and autoradiograms (b and d) of $^{32}$P-labeled DNA containing tobacco RuBP carboxylase (large subunit) gene hybridized to *Xho*I and *Bam*HI fragments, respectively. The letter (p) indicates partial Ba10-Ba5 fragment

produced subfragments E1, E2, E5, and E6, but neither E3 nor E4, all of which were produced from Ba4 by *Eco*RI digestion (Fig. 10B). Therefore, the size of the inverted repeats is in the range from 9.8 kb pairs (total length of E1, E2, E5 and E6) to 11.7 kb pairs at most (plus E4, see Fig. 10B).

### *Southern Image Hybridization of $^{32}$P-Labeled rRNAs and $^{32}$P-Labeled DNA Containing RuBP Carboxylase (LS) Gene*

Southern hybridization of $^{32}$P-labeled rRNAs (23S and 16S rRNA mixture) of *Marchantia polymorpha* chloroplasts with the *Bam*HI fragments, *Xho*I fragments, and *Bam*HI/*Kpn*I double-digested fragments was performed. The chloroplasts rRNAs (23S and 16S) hybridized to the *Xho*I fragment Xh1, *Bam*HI fragments Ba2 and Ba4, and *Bam*HI/*Kpn*I double-digested fragment BK1, BK5, BK8(2×), BK16(2×), and BK17(2×) (Fig. 9). This result clearly shows that chloroplast rRNA genes were located at two different sites in inverted repeats because $^{32}$P-labeled rRNAs hybridized to the subfragments BK8, BK16 and BK17, which were located in inverted repeat regions of Ba2 and Ba4 fragments, respectively (Fig. 8). To determine the orientation of 23S and 16S RNA genes on the chloroplast DNA map Southern image hybridization of individual $^{32}$P-labeled 23S rRNA and 16S rRNA with *Kpn*I, *Hin*dIII, and *Eco*RI subfragments of Ba4 fragment, which contains one of the rRNA gene clusters, was performed (Fig. 10A). The result shows clockwise 23S-16S rRNA gene orientation on Ba4 in the physical map (Fig. 8) of the chloroplast DNA.

Southern hybridization of $^{32}$P-labeled DNA (Seyer et al. 1981) containing the gene of tobacco RuBP carboxylase (Large subunit, LS) with *Bam*HI fragments and *Xho*I fragments was carried out. An autoradiogram showed that *Bam*HI fragment Ba5, and *Xho*I fragment Xh3, have homology with the DNA sequence of tobacco RuBP carboxylase (LS) gene (Fig. 11).

## Discussion

This is the first report on physical mapping of chloroplast DNA isolated from lower plant (liverwort) cells especially grown photomixotrophically in cell suspension culture. Under our cell culture conditions, biologically active chlo-

roplasts multiply in the cells at a high rate (the cell doubling time is approximately 30 h). This may be reflected by the frequent appearance of replicative intermediates of DNA, as shown in Fig. 3. Physical mapping of the replicative origin is under way.

The molecular size of *Marchantia polymorpha* chloroplast DNA is approximately 121 kb pairs, determined by contour length measurement as well as by restriction fragment analysis. Herrmann et al. (1980b) also reported that the chloroplast DNA from *Sphaerocarpos donnellii* (liverwort) is unusally low in contour length. This value is considerably smaller than those (140–180 kb) reported in the higher plants (Bedbrook and Bogorad 1976; Herrmann et al. 1980a; Bovenberg et al. 1981; Chu et al. 1981; Jurgenson and Borque 1980 and algae so far examined (Manning and Richards 1972; Rochaix 1978), except in *Vicia faba* (Koller and Delius 1980) and *Pennisetum americanum* (Rawson et al. 1981).

The fragment map of the chloroplast DNA shown in Fig. 8 contains two copies of an inverted repeat in opposite orientation and two single copy regions. The size of the small single copy region was calculated to be at least 15.7 kb pairs by substracting the size ($11.7 \times 2$ kb) of two inverted repeats from Ba2 plus Ba4 fragment size (39.1 kb). The large single copy region is, therefore, approximately 81.9 kb pairs (121.0 kb in total size minus a single copy region and two inverted repeat regions). An inverted repeat containing chloroplast rRNA genes has been found in chloroplast DNA from higher plants with the exception of pea (Palmer and Thompson 1981) and *Vicia faba* (Koller and Delius 1980). The maximum length of the inverted repeat in *Marchantia polymorpha* chloroplast DNA is 11.7 kb pairs. This size is also smaller than those (20–26 kb) reported in higher plants. As described above, *Marchantia polymorpha* chloroplast DNA contains two copies in inverted repeat. Nevertheless, the molecular size of the chloroplast DNA is closer to that of *Vicia faba* which does not have inverted repeats (Koller and Delius 1980). This suggests that *Marchantia polymorpha* chloroplast DNA may have been exposed to deletion of a nonfunctional part of the DNA during chloroplast evolution. This is also likely to have happened to part of the small single copy region (15.7 kb) because its size is smaller than those seen in higher plants. It will be confirmed by DNA-DNA hybridization of the small single copy region between liverwort and higher plants.

Our finding that the inverted repeat in liverwort chloroplast DNA also contains chloroplast ribosomal RNA genes suggests a highly conserved form of inverted repeat in higher and lower plant species. Southern hybridization of $^{32}$P-labeled *E. coli* rRNA with liverwort chloroplast DNA fragment showed also obvious homology of rRNA genes between liverwort chloroplasts and *E. coli* (unpublished results). Further DNA sequence analysis of rRNA genes will elucidate the chloroplast DNA origin. Detailed physical mappings of liverwort chloroplast rRNA genes (23S, 16S, 5S, and 4.5S) will be published elsewhere.

Although the DNA sequence of the RuBP carboxylase (LS) gene has homology with that in different sources of chloroplasts (Zurawski et al. 1981), our results also indicate the DNA sequence homology in the RuBP carboxylase (LS) gene between tobacco and liverwort chloroplast DNA. Surprisingly, the location of the RuBP carboxylase (LS) gene relative to the inverted repeat is very similar to that found in maize (Bogorad 1979), *Chlamydomonas reihardii* (Malnoe et al. 1979), and spinach (Whitfeld and Bottomley 1980). These results strongly suggest that chloroplast gene organization may be highly conserved among green-plant cells in chloroplast evolution.

Palmer and Thompson (1981) suggested that pea and broad bean have lost an inverted repeat during divergence of the mung-bean line (contains two copies of inverted repeat) from the pea-broad bean line. Therefore, it would be of interest to study physical mappings of taxonomically related moss chloroplast DNA in comparison with that of liverwort chloroplast DNA.

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## References

Atchison BA, Whitfeld PR, Bottomley W (1976) Comparison of chloroplast DNAs by specific fragmentation with Eco RI endonuclease. Mol Gen Genet 148:263–269

Belliard G, Pelletier G, Vedel F, Quetier F (1978) Morphological characteristics and chloroplast DNA distribution in different cytoplasmic parasexual hybrids of *Nicotiana tabacum*. Mol Gen Genet 165:231–237

Bedbrook JR, Bogorad L (1976) Endonuclease recognition sites mapped on *Zea mays* chloroplast DNA. Proc Natl Acad Sci USA 73:4309–4313

Bedbrook JR, Kolodner R, Bogorad L (1977) *Zea mays* chloroplast ribosomal RNA genes are part of a 22,000 base pair inverted repeat. Cell 11:739–749

Bedbrook JR, Link G, Coen DM, Bogorad L, Rich A (1978) Maize plastid gene expressed during photoregulated development. Proc Natl Acad Sci USA 75:3060–3064

Bogorad L (1979) The chloroplast, its genome and possibilities for genetically manipulating plants. In: Setlow JK, Hollaender A (eds) Genetic engineering, vol I. Plenum Press, New York, p 181

Bovenberg WA, Kool AJ, Nijkamp HJJ (1981) Isolation, characterization and restriction endonuclease mapping of the *Petunia hybrida* chloroplast DNA. Nucl Acids Res 9:503–517

Chu NM, Oishi KK, Tewari KK (1981) Physical mapping of the pea chloroplast DNA and localization of the ribosomal RNA genes. Plasmid 6:279–292

Coen DM, Bedbrook JR, Bogorad L, Rich A (1977) Maize chloroplast DNA fragment encoding the large subunit of ribulosebisphosphate carboxylase. Proc Natl Acad Sci USA 74:5487–5491

Denhardt DT (1966) A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641–646

Driesel AJ, Crouse EJ, Gordon K, Bohnert HJ, Herrmann RG (1979) Fractionation and identification of spinach chloroplast transfer RNAs and mapping of their genes on the restriction map of chloroplast DNA. Gene 6:285–306

Driesel AJ, Speirs J, Bohnert HJ (1980) Spinach chloroplast mRNA for a 32,000 dalton polypeptide. Size and localization on the physical map of the chloroplast DNA. Biochim Biophys Acta 610:297–310

Van Ee JH, Vos YJ, Planta RJ (1980) Physical map of chloroplast DNA of Spirodela oligorrbiza; analysis by the restriction endonucleases *PstI, XhoI* and *SacI*. Gene 12:191–200

Gelvin S, Heizmann P, Howell SH (1977) Identification and cloning of the chloroplast gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase from *Chlamydomonas reinhardii*. Proc Natl Acad Sci USA 74:3193–3197

Gordon KHJ, Crouse EJ, Bohnert HJ, Herrmann RG (1981) Restriction endonuclease cleavage site map of chloroplast DNA

from *Oenothera parviflora* (*Euoenothera* plastome IV). Theor Appl Genet 59:281–296
Gordon KHJ, Crouse EJ, Bohnert HJ, Herrmann RG (1982) Physical mapping of differences in chloroplast DNA of the five wild-type plastomes in *Oenothera* subsection *Euoenothera*. Theor Appl Genet 61:373–384
Gray PW, Hallick RB (1977) Restriction endonuclease map of *Euglena gracilis* chloroplast DNA. Biochemistry 16:1665–1671
Gray PW, Hallick RB (1978) Physical mapping of the *Euglena gracilis* chloroplast DNA and ribosomal RNA gene region. Biochemistry 17:284–289
Herrmann RG, Whitfeld PR, Bottomley W (1980a) Construction of a *SalI/PstI* restriction map of spinach chloroplast DNA using low-gelling-temperature-agarose electrophoresis. Gene 8:179–191
Herrmann RG, Palta HK, Kowallik KV (1980b) Chloroplast DNA from three Archegoniates. Planta 148:319–327
Jurgenson JE, Bourque DP (1980) Mapping of rRNA genes in an inverted repeat in *Nicotiana tabacum* chloroplast DNA. Nucl Acids Res 8:3505–3516
Koller B, Delius H (1980) *Vicia faba* chloroplast DNA has only one set of ribosomal RNA genes as shown by partial denaturation mapping and R-loop analysis. Mol Gen Genet 178:261–269
Link G, Bogorad L (1980) Sizes, locations, and directions of transcription of two genes on a cloned maize chloroplast DNA sequence. Proc Natl Acad Sci USA 77:1832–1836
Malnoe P, Rochaix J-D, Chua NH, Spahr P-F (1979) Characterization of the gene and messenger RNA of the large subunit of ribulose 1,5-diphosphate carboxylase in *Chlamydomonas reinhardii*. J Mol Biol 133:417–434
Manning JE, Richards OC (1972) Isolation and molecular weight of circular chloroplast DNA from *Euglena gracilis*. Biochim Biophys Acta 259:285–296
Ohyama K, Wetter LR, Yamano Y, Fukuzawa H, Komano T (1982) A simple method for isolation of chloroplast DNA from *Marchantia polymorpha* L. cell suspension cultures. Agric Biol Chem 46:237–242
Ono K, Ohyama K, Gamborg OL (1979) Regeneration of the liverwort *Marchantia polymorpha* L. from protoplasts isolated from cell suspension culture. Plant Sci Lett 14:225–229
Palmer JD, Thompson WF (1981) Rearrangements in the chloroplast genomes of mung bean and pea. Proc Natl Acad Sci USA 78:5533–5537
Rawson JRY, Kushner SR, Vapnek D, Alton NK, Boerma CL (1978) Chloroplast ribosomal RNA genes in *Euglena gracilis* exist as three clustered tandem repeats. Gene 3:191–209
Rawson JRY, Clegg MT, Thomas K, Rinehart C, Wood B (1981) A restriction map of the ribosomal RNA genes and the short single-copy DNA sequence of the pearl millet chloroplast genome. Gene 16:11–19
Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113:237–251
Rochaix JD (1978) Restriction endonuclease map of the chloroplast DNA of *Chlamydomonas reinhardii*. J Mol Biol 126:597–617
Rochaix JD, Malnoe P (1978) Anatomy of the chloroplast ribosomal DNA of *Chlamydomonas reinhardii*. Cell 15:661–670
Seyer P, Kowallik KV, Herrmann RG (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. Current Genetics 3:189–204
Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517
Sugiura M, Kusuda J (1979) Molecular cloning of tobacco chloroplast ribosomal RNA genes. Mol Gen Genet 172:137–141
Vedel F, Quetier F, Bayen M (1976) Specific cleavage of chloroplast DNA from higher plants by Eco RI restriction nuclease. Nature 263:440–442
Vedel F, Quetier F, Dosba F, Doussinault G (1978) Study of wheat phylogeny by Eco RI analysis of chloroplastic and mitochondrial DNAs. Plant Sci Lett 13:97–102
Westhoff P, Nelson N, Bünemann H, Herrmann RG (1981) Localization of genes for coupling factor subunits on the spinach plastic chromosome. Current Genetics 4:109–120
Whitfeld PR, Herrmann RG, Bottomley W (1978) Mapping of the ribosomal RNA genes on spinach chloroplast DNA. Nucl Acids Res 5:1741–1751
Whitfeld PR, Bottomley W (1980) Mapping of the gene for the large subunit of ribulose bisphosphate carboxylase on spinach chloroplast DNA. Biochem International 1:172–178
Yamagishi H, Inokuchi H, Ozeki H (1976) Excision and duplication of $su3^+$-transducing fragments carried by bacteriophage $\phi 80$. I. Novel structure of $\phi 80sus2psu3^+$ DNA molecule. J Virol 18:1016–1023
Zurawski G, Perrot B, Bottomley W, Whitfeld PR (1981) The structure of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucl Acids Res 9:3251–3270

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