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The consensus land plant chloroplast gene order is present, with two alterations, in the moss *Physcomitrella patens*

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Summary. A restriction endonuclease cleavage site map for the enzymes *Cla*I and *Bgl*II, and a partial map for *Sac*I, has been constructed for the chloroplast genome of the moss *Physcomitrella patens* (Hedw.) BSG. The plastid chromosome contains approximately 122 kb organized into small (21 kb) and large (82 kb) single-copy regions separated by two copies of a repeat sequence (9.4 kb) oriented in an inverted arrangement. Genes for 17 proteins and 2 ribosomal RNAs have been mapped using heterologous probes from corn, spinach, pea, and petunia. The general order and arrangement of the moss chloroplast genes are similar to the consensus land plant genome typified by that of spinach, with two major exceptions. First, there is an inversion of approximately 20 kb, bordered internally by *psbA* and *atpH*, and also containing the genes *atpF* and *atpA*. Second, *rpl*2 and *rps*19 have been relocated to a different position within the large single-copy region, adjacent to the 20 kb inversion.

Key words: Moss chloroplast DNA – Restriction/gene map

Introduction

Various structural aspects of the chloroplast genome have been elucidated in over 200 angiosperm taxa (reviewed in Bedbrook and Kolodner 1979; Wallace 1982; Whitfeld and Bottomley 1983; Crouse et al. 1985; Palmer 1985). Angiosperm chloroplast DNAs are circular, range in size from 120 kb to 217 kb, and with two exceptions are organized into two single copy regions of approximately 20 kb and 80 kb, separated by two inverted repeat regions (each containing a complete set of ribosomal RNA genes) ranging in size from 10 kb to 76 kb (Palmer 1985). The chloroplast genomes from the legumes broad bean (*Vicia faba*) and pea (*Pisum sativum*) lack the inverted repeats (Chu et al. 1981; Palmer and Thompson 1981; Kolodner and Tewari 1975; Köller and Delius 1980). Those chloroplast genomes that contain the inverted repeats exhibit a highly conserved linear order of common sequence elements (Fluhr and Edelman 1981a; Palmer and Thomson 1982; De Heij et al. 1983; reviewed in Palmer 1985). This observation has led to the emergence of a consensus angiosperm chloroplast gene order, typified by that of spinach (*Spinacia oleracea*) (Palmer 1985). Exceptions to this consensus are of two types. The most drastic deviation is found in the chloroplast genomes of mung bean and pea, which exhibit numerous and extensive sequence rearrangements scattered through-

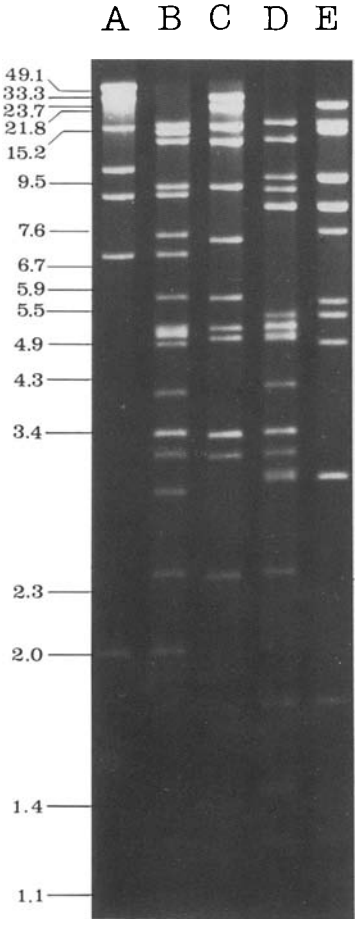

Fig. 1. Single and double digestion products of *Physcomitrella patens* chloroplast DNA with the following restriction endonucleases: Lane A *Sac*I; lane B, *Sac*I/*Cla*I; lane C, *Cla*I; lane D, *Cla*I/*Bgl*II; lane E, *Bgl*II. Fragment sizes were determined by co-electrophoresis with a range of phage λ DNA fragments produced by separate digestions with *Hin*dIII, *Eco*RI and *Sal*I, as well as undigested phage λ DNA. Molecular weight sizes are listed in kilobases along the *left* ordinate

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Table 1. Molecular sizes of *Physcomitrella patens* chloroplast DNA fragments produced by digestion with restriction endonucleases[a]

Fragment designations	Fragment sizes (kb)				
	*Sac*I	*Sac*I/*Cla*I	*Cla*I	*Cla*I/*Bgl*II	*Bgl*II
1	53[b]	15.5	26.5	15.7	23.7
2	26	14.8	21.7	14.3	15.7
3	15.0	14.3	15.0	9.6	15.1
4	10.5	9.4	14.3	9.2	9.6 (×2)
5	8.8	8.8	9.4	8.4 (×2)	8.4 (×2)
6	6.9	7.8	7.4	5.4	7.6
7	2.0	6.9	5.8	5.2 (×2)	5.7
8		5.8	5.2	5.0 (×2)	5.4
9		5.2	5.0	4.2	4.9
10		5.1	3.4 (×2)	3.4 (×2)	2.9 (×2)
11		4.9	3.2	3.2	1.8
12		3.8	2.4	3.0	1.3
13		3.4 (×2)		2.9	
14		3.2		2.4	
15		2.9		1.8	
16		2.4		1.5	
17		2.0		1.3	
Total	122.2	119.6	122.7	121.7	123.0

[a] Fragments smaller than 1 kb were not measured
[b] Determined by summation of sizes of sub-fragments produced by digestion with a second restriction endonuclease

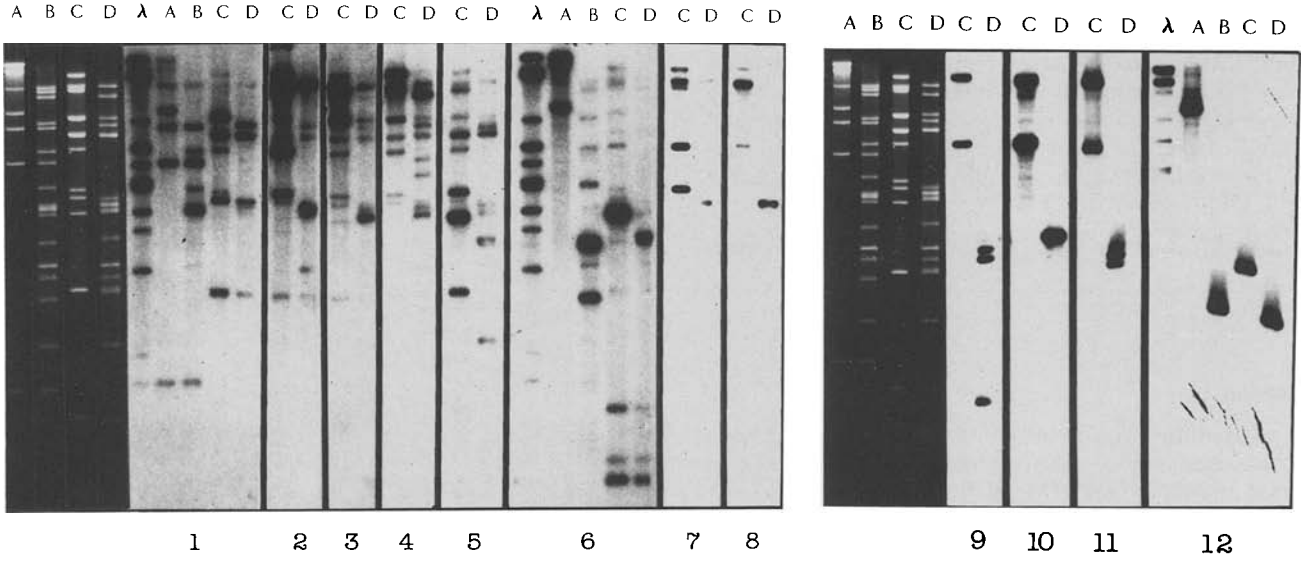

Fig. 2. Results of hybridizations with *Physcomitrella patens* radiolabeled *Cla*I chloroplast DNA fragments to chloroplast DNA digested by A, *Sac*I; B, *Sac*I/*Cla*I; C, *Bgl*II; D, *Bgl*II/*Cla*I. Numbers *below* the lanes refer to the specific *Cla*I fragments (Table 1) used as hybridization probes. Reference markers of phage λ DNA digested with *Eco*RI and *Hin*dIII, as well as intact phage λ DNA, are in lanes marked λ

out the chloroplast genome (Palmer and Thompson 1982). Several chloroplast genomes with the inverted repeat exhibit more restricted rearrangements, the result of one or two inversion events (Palmer and Thompson 1982; Palmer et al. 1983; Courtice et al. 1985; Hirai et al. 1985; Tyagi and Herrmann 1986; Jansen and Palmer 1987).

Structural analyses of chloroplast DNAs from non-angiosperm land plants have extended our understanding of the tempo and mode of structural evolution in the chloroplast genome. The chloroplast DNAs of three species in the pteridophyte genus *Osmunda* are highly conserved in sequence arrangement, and closely resemble the consensus angiosperm chloroplast genome (Stein et al. 1986). Furthermore, the structure and arrangement of DNA sequences are highly conserved among *Osmunda cinnamomea,* the gymnosperm *Ginkgo biloba,* and the angiosperms petunia (*Petunia hybrida*) and mung bean (*Vigna radiata*) (Palmer and Stein 1986). Even more remarkable is the demonstration that the chloroplast genomes of the liverwort *Marchantia polymorpha* (Ohyama et al. 1986) and the angiosperm tobacco (*Nicotiana tabacum*) (Shinozaki et al. 1986) are almost identical with respect to the organization of over 120 genes, despite their evolutionary distance and their genome size differences (121 kb versus 156 kb, respectively).

Table 2. Summary of restriction fragment mapping hybridization data

*Cla*I Probe fragment	Filter-bound fragments			
	*Bgl*II[a]	*Bgl*II/*Cla*I[a]	*Sac*I	*Sac*I/*Cla*I
1 (26.5)	4 (9.6) 5 (8.4) 8 (5.4) 10 (2.9)	4 (9.2) 5 (8.4) 6 (5.4) 13 (2.9)	4 (10.5) 5 (8.8) 6 (6.9) 7 (2.0)	5 (8.8) 7 (6.9) 11 (4.9) 17 (2.0)
2 (21.7)	1 (23.7) 2 (15.7) 3 (15.1) 6 (7.6) 7 (5.7)	1 (15.7) 7 (5.2) 8 (5.0)	–	–
3 (15.0)	3 (15.1) 4 (9.6)	3 (9.6 8 (5.0)	–	–
4 (14.3)	1 (23.7)	2 (14.3)	–	–
5 (9.4)	5 (8.4) 7 (5.7) 9 (4.9)	5 (8.4)	–	–
6 (7.4)	9 (4.9) 11 (1.8) 12 (1.3)	9 (4.2) 15 (1.8) 17 (1.3)	4 (10.5)	12 (3.8) 15 (2.9)
7 (5.8)	1 (23.7) 2 (15.7) 6 (7.6) 7 (5.7)	1 (15.7) 7 (5.2)	–	–
8 (5.2)	3 (15.1)	7 (5.2)	–	–
9 (5.0)	1 (23.7) 6 (7.6)	11 (3.2) 12 (3.0) 16 (1.5)	–	–
10 (3.4)	1 (23.7) 6 (7.6)	10 (3.4)	–	–
11 (3.2)	1 (23.7) 6 (7.6)	11 (3.2) 12 (3.0)	–	–
12 (2.4)	10 (2.9)	14 (2.4)	4 (10.5)	16 (2.4)

Hybridization signals due to artifact or contamination are excluded. Fragment designations are as in Table 1; molecular weights in kb pairs are listed in parenthesis

[a] Based on data from replicate experiments (Calie 1986)

Thus the gross structure of the chloroplast genome appears to be highly conserved in those characterized taxa representing diverse evolutionary lineages of land plants.

To expand further our understanding of non-angiosperm chloroplast genomes we undertook the characterization of the chloroplast genome of a moss. A map of *Physcomitrella patens* chloroplast DNA has been constructed, showing the locations of recognition sites for the restriction endonucleases *Bgl*II, *Cla*I, and *Sac*I, and the approximate positions of two ribosomal RNA (rRNA) and 17 protein-encoding genes have been determined.

Materials and methods

Isolation of DNA and restriction endonuclease analysis. Chloroplast DNA was isolated and purified from moss gametophyte tissues as described (Calie and Hughes 1987). Plasmid DNAs were isolated by the alkaline lysis method of Birnboim and Doly (1979).

Chloroplast DNA was digested with restriction endonucleases under conditions prescribed by the supplier (New England BioLabs, Beverly, MA). Analytical digests were electrophoresed through 0.7% horizontal agarose gels (20 × 25 × 0.4 cm) in TBE buffer (Maniatis et al. 1982).

^{32}P Radiolabeling of DNA. Chloroplast DNA restriction fragments, generated by *Cla*I, were labeled with ^{32}P at the 3′ termini as described (Calie 1986). Plasmid DNA restriction fragments containing chloroplast rRNA gene probes were radiolabeled with ^{32}P by a nick-translation reaction without DNaseI (Maniatis et al. 1975). Plasmid DNAs containing the other chloroplast gene probes were also labeled with a nick-translation reaction with DNaseI (Davis et al. 1980).

Radiolabeled restriction fragments were fractionated on either 0.4% or 1% low melting temperature agarose gels and prepared for hybridization to filter-bound chloroplast DNA digests as described (Palmer 1986). Nick-translated plasmid DNAs were purified and prepared for hybridization as described (Davis et al. 1980).

Table 3. Descriptions of gene probes

Designation	Gene(s)	Plant source	Insert	References
1	*atpB*	*Spinacia oleracea*	*Eco*RI–*Eco*RI 1980 bp	Zurawski et al. (1982)
2	*atpE*	*Spinacia oleracea*	*Eco*RI–*Xba*I 420 bp	Zurawski et al. (1982)
3	3′ region of *atpH*	*Pisum sativum*	*Pst*I–*Bam*HI 800 bp	Huttley and Gray (1984)
4	*atpF*, 5′ region of *atpA*	*Spinacia oleracea*	*Sal*I–*Hin*dIII 1.5 kb	Westhoff et al. (1985)
5	*atpF, atpA*	*Spinacia oleracea*	*Sal*I–*Sal*I 2.4 kb	Westhoff et al. (1985)
6	*psaA*1, 5′ region of *psaA*2	*Spinacia oleracea*	*Bam*HI–*Bam*HI 2.4 kb	Alt et al. (1984)
7	3′ region of *psaA*2	*Spinacia oleracea*	*Bam*HI–*Bam*HI 1.6 kb	Alt et al. (1984)
8	5′ proximal 200 bp of *psbD*	*Pisum sativum*	*Bam*HI–PstI 720 bp	Rasmussen et al. (1984)
9	3′ proximal 830 bp of *psbD*	*Pisum sativum*	*Pst*I–*Pst*I 1.1 kb	Rasmussen et al. (1984)
10	3′ region of *psbC*	*Spinacia oleracea*	*Bam*HI–*Pst*I 350 bp	Alt et al. (1984)
11	5′ region of *petA*	*Pisum sativum*	*Bam*HI–*Bam*HI 3.2 kb	Willey et al. (1984)
12	3′ region of *petA*	*Pisum sativum*	*Bam*HI–*Bam*HI 1.1 kb	Willey et al. (1984)
13	5′ region of *psbE*	*Spinacia oleracea*	*Eco*RI–*Eco*RI 650 bp	Herrmann et al. (1984)
14	3′ region of *psbE*	*Spinacia oleracea*	*Eco*RI–*Eco*RI 500 bp	Herrmann et al. (1984)
15	5′ region of *psbB*	*Spinacia oleracea*	*Bam*HI–*Bam*HI 375 bp	Heinemeyer et al. (1984)
16	3′ region of *psbB*	*Spinacia oleracea*	*Bam*HI–*Sal*I 1.5 kb	Heinemeyer et al. (1984)
17	*petB*	*Spinacia oleracea*	*Sal*I–*Bam*HI 2.4 kb	Heinemeyer et al. (1984)
18	5′ region of *petD*	*Spinacia oleracea*	*Bam*HI–*Bam*HI 300 bp	Heinemeyer et al. (1984)
19	3′ region of *petD*	*Spinacia oleracea*	*Bam*HI–*Sal*I 2.0 kb	Heinemeyer et al. (1984)
20	*rpl2* intron + 3′ region, *rps19*	*Petunia hybrida*	*Pst*I–*Pst*I 1.5 kb	Sugita and Sugiura (1983); J.D. Palmer, personal communication
pZmc100	16S rRNA 23S rRNA	*Zea mays*	*Bam*HI–*Bam*HI 3.2 bp *Hin*dIII–*Hin*dIII 1.2 kb	Bedbrook et al. (1977)
pZmc461	*rbcL*	*Zea mays*	*Pst*I–*Pst*I 567 bp	McIntosh et al. (1980)
pZr12	*psaA1, psaA2*	*Zea mays*	*Eco*RI–*Eco*RI 6.3 kb	Fish et al. (1985)
pZr48	*atpB, atpE*	*Zea mays*	*Eco*RI–*Eco*RI 2.8 kb	Krebbers et al. (1982)
pZmc427	*psbA*	*Zea mays*	*Bam*HI–*Eco*RI 2.2 kb	Bogorad et al. (1980)

Transfer of DNA to nitrocellulose filters/hybridization to radiolabeled probes. Gel-fractionated analytical chloroplast DNA digests were transferred to nitrocellulose filters in a bidirectional manner (Smith and Summers 1980). Prehybridization and hybridization conditions were as described (Meinkoth and Wahl 1984). Nitrocullulose filters were washed and autoradiography of hybridized filters was performed under standard conditions (Maniatis et al. 1982).

Results and Discussion

Restriction fragment mapping

Physcomitrella patens chloroplast DNA was digested to completion with the restriction endonucleases *Cla*I, *Bgl*II, and *Sac*I. Summation of restriction fragment sizes, taking into account fragment stoichiometries, indicates a genome size of 122 kb (Fig. 1, Table 1).

Restriction endonuclease cleavage sites were mapped by the following principles. A set of chloroplast DNA restriction fragments (e.g. generated by *Cla*I) were radiolabeled and then fractionated by gel electrophoresis. The separated fragments were then used as hybridization probes against filter-bound chloroplast DNA digests generated by *Cla*I, *Bgl*II, *Sac*I, *Bgl*II/*Cla*I, and *Sac*I/*Cla*I. Hybridizations to single digests generated overlaps between probe and filter-bound fragments. Hybridizations to the double digests yielded the precise locations of cleavage sites within the probe fragments (Palmer 1982, 1986). These data are illustrated in Fig. 2 and summarized in Table 2. Self-hybridizations of probe fragments to chloroplast DNA digests generated by the same enzyme (i.e. *Cla*I) revealed the presence of reiterated sequences within the probe fragments (Fluhr and Edelman 1981b). Hybridizations with defined gene probes resolved mapping ambiguities due to either cross-contamination of restriction fragment probes, or difficulties in establishing the order of the smaller restriction fragments (Spielmann et al. 1983).

The inverted repeat

The inverted repeat has a minimum size of 3.4 kb (the doublet fragment *Cla* 10), and a maximum size of 12.4 kb [*Cla* 10 (3.4 kb) + *Cla* 7 (5.8 kb) + *Cla* 11 (3.2 kb)]. The actual size of the inverted repeat was estimated by comparing the autoradiographic intensities of cross- and self-hybridizing fragments located at the termini of the repeats (Palmer and Stein 1986). *Cla* 7 (5.8 kb), located at the upper right end

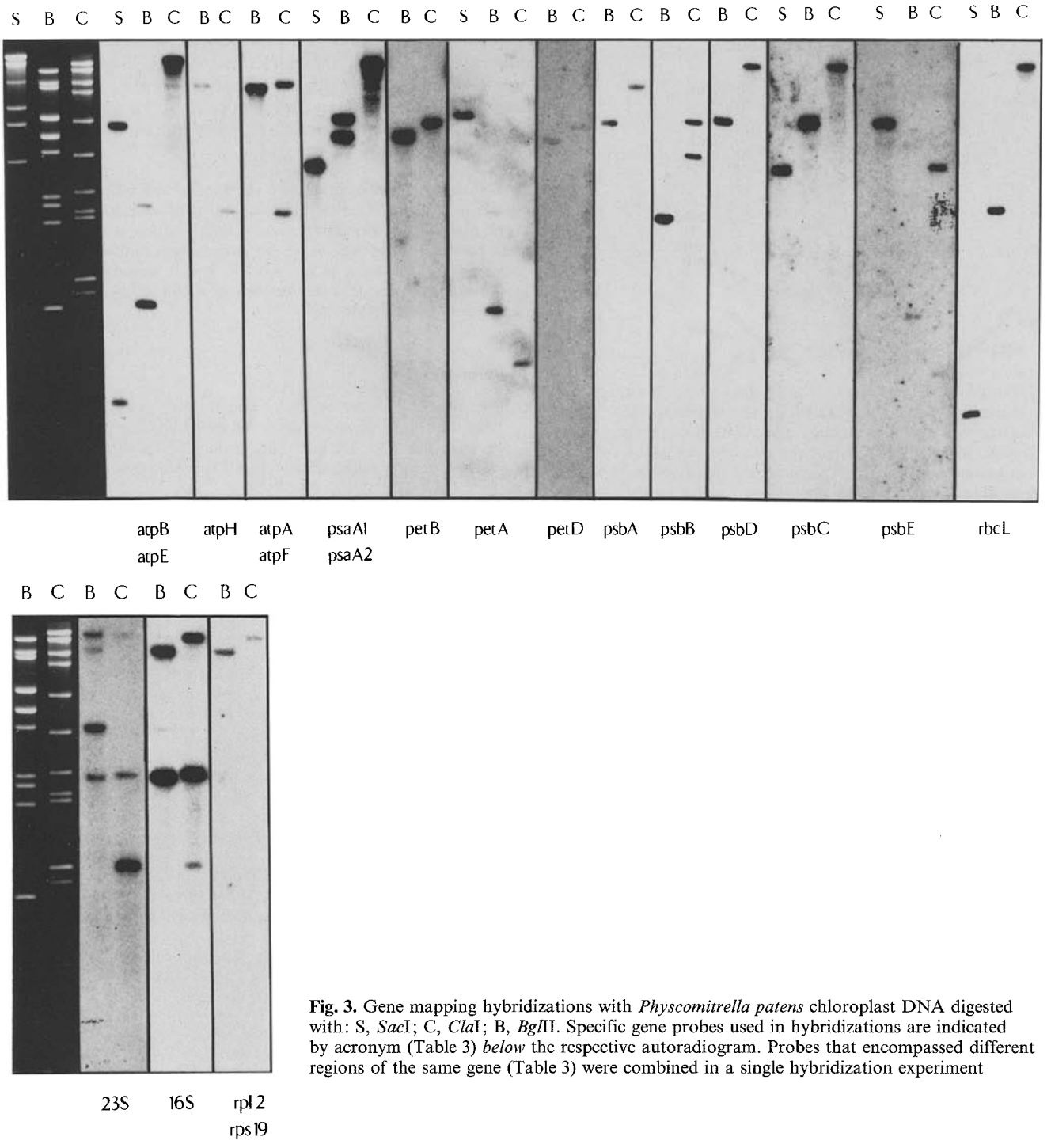

Fig. 3. Gene mapping hybridizations with *Physcomitrella patens* chloroplast DNA digested with: S, *Sac*I; C, *Cla*I; B, *Bgl*II. Specific gene probes used in hybridizations are indicated by acronym (Table 3) *below* the respective autoradiogram. Probes that encompassed different regions of the same gene (Table 3) were combined in a single hybridization experiment

of the repeat, hybridized approximately half as strongly to *Cla* 2 (21.7 kb) as to itself. Similarly, *Cla* 11 (3.2 kb), located at the lower left end of the repeat, produced a hybridization signal of almost equal intensity to *Cla* 9 (5.0 kb) as to itself (Calie 1986). We estimate that the repeats extend approximately 3 kb into *Cla* 7 and *Cla* 2, and 3 kb into *Cla* 11 and *Cla* 9. The actual size of each copy of the inverted repeat is estimated to be 9.4 kb.

The universal arrangement of rRNA genes within the inverted repeat, i.e. the 23S rRNA gene proximal to and the 16S gene distal to the small single copy region, is present in the moss chloroplast genome. These were the only genes found within the inverted repeat region using the probes listed in Table 2.

Gene mapping

Results of hybridizations with defined chloroplast gene probes (Table 3) from three dicots (spinach, petunia, and pea) and one monocot (maize) to moss chloroplast DNA digests are illustrated in Fig. 3. The composite restriction site and gene map is shown in Fig. 4.

As gene probes 2, 5, 10, pZmc461, and the 1.2 kb *Bam*HI subfragment from pZmc100 contain only specific

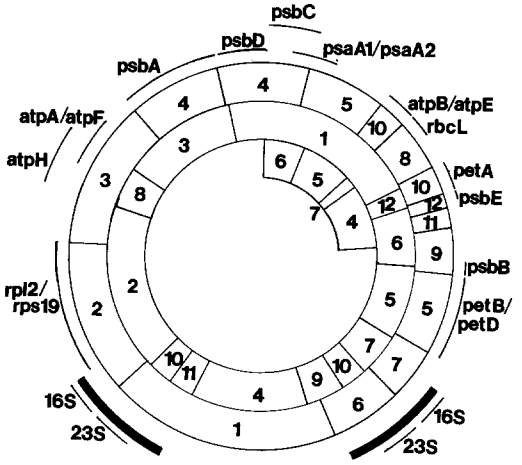

Fig. 4. Physical and gene map of the *Physcomitrella patens* chloroplast genome. *Bgl*II sites are shown on the *outermost circle*, *Cla*I sites on the *complete inner circle*, and *Sac*I sites on the *partial inner circle*. Restriction fragment designations are as in Table 1, gene acronyms as in Table 3. The estimated extent of the inverted repeat is indicated by the two *filled lines* parallel to the circular map

gene sequences, hybridization between these chloroplast gene probes and moss chloroplast DNA restriction fragments must be due to actual homology between the angiosperm and the moss genes. Probes 1, 3, 4, 6–9, 11–20, pZr48, pZr12, and pZmc427 and the 3.2 kb *Hin*dIII subfragment from pZmc100 contain flanking sequences in addition to specific chloroplast genes. We cannot exclude the possibility that the hybridization with these probes is due to homologies that lie outside the regions containing the gene-specific sequences.

The most significant finding of this investigation is that the moss chloroplast genome contains a gene order similar to the consensus land plant chloroplast genome. Thus the majority of the chloroplast genes have been conserved in order and arrangement since the divergence of the moss lineage from the other land plant lineages approximately 345–375 MYA (Scagel et al. 1982; Schofield 1985).

The deviations in the moss chloroplast gene order from the consensus land plant gene order are quite notable. The first involves an exchange of the positions of *atpH* and *psbA*. The second is the relocation of *rpl2* and *rps19* from a position adjacent to the *petB*/*petD* gene cluster to a locus flanking *atpH*. The first situation is clearly due to an inversion of a 20 kb region, resulting in a reorientation of *psbA* and *atpH* (*atpA* and *atpF* are also present within this inversion, but our mapping data do not allow for a precise evaluation of the repositioning of these two genes). The second situation is also probably due to an inversion involving the region encompassing both inverted repeats, flanking portions of the large single-copy region, and the small single-copy region. Inversion events within chloroplast genomes are not without precedent, and are found in a number of land plant taxa (reviewed in Palmer 1985). Significantly, the liverwort *Marchantia polymorpha*, a representative of the land plant lineage that has the nearest phylogenetic affinity to the mosses (Mishler and Churchill 1984) contains an inversion similar to the *atpH*/*psbA* inversion found in the moss chloroplast genome.

In summary, the chloroplast genome of the moss *Physcomitrella patens* contains a gene order which reflects the consensus land plant chloroplast gene arrangement with the exception of two rearrangement events. Thus, although rearrangements are evident in some chloroplast DNAs, the land plant chloroplast genome appears to be a highly conserved system, evolving at a very slow rate on a gross structural level.

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