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Detection of DNA Rearrangement in Rice Using a Cosmid Library

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Previously we reported the migration and rearrangement of a chloroplast gene cluster into mitochondria. The exact genomic locations of the clusters, modes of the gene rearrangement and mechanisms of the interorganellar migration of the clusters have yet to be understood. The detailed analysis needs to include a larger region of DNA surrounding each cluster. To study DNA rearrangement and migration in more detail a cosmid library was constructed using the total rice genomic DNA including nuclear, chloroplast and mitochondrial DNA. From this cosmid library, a sub-library was obtained by selecting the clones hybridized to various regions of chloroplast DNA. According to the hybridization pattern 136 clones from the sub-library were classified into 29 groups. Detailed analysis of these clones revealed that in addition to authentic chloroplast DNA, the clones contain its homologs resulted from rearrangement and mutation. We analyzed two clones in detail, which contain different *rpl2* homologs resulted from rearrangement and/or migration, respectively.

There are three distinct genetic systems in each plant cell. The two cytoplasmic organelles, chloroplast and mitochondria, have their own genetic systems which are different from the nuclear counterpart. The organelle genomes are much smaller than the nuclear genome and are incapable of coding for all of the mitochondrial or chloroplast proteins (Anderson et al., 1981; Wallace, 1982; Fauron et al., 1991; Shimada and Sugiura, 1991; Cummings, 1992; Haberhausen et al., 1992; Hallick et al., 1993; Skadsen, 1995). Molecular techniques have been applied to study the organelle genetic systems. In the past ten years, some advances were made in our knowledge on the organization, gene content, gene structure, and expression of organelle genomes (Kao et al., 1984; Moon et al., 1985; Moon et al., 1988b; Hirasuka et al., 1989; Gillham, 1994; Myata, 1995).

Molecular biology techniques, especially detection of sequence homology using Southern blot analysis, revealed that the movement of DNA sequences among nucleus, mitochondria and chloroplast had occurred during evolution (Moon et al., 1988a; Kadowaki, 1996; Nakazono, 1996; Kubo, 1996). Moon et al. (1987a, 1987b, 1988a) reported chloroplast *rbcL* gene clusters which underwent rearrangement in the mitochondria. To analyze the multiple *rbcL* clusters in more detail, it is necessary to have the clones containing a larger region of DNA that flanks the *rbcL* gene cluster. Analysis of the surrounding sequences will give more information on the exact genomic location of the individual cluster and the characteristics on the junction sequences. This information may provide clues to the mechanisms on the generation of the different *rbcL* gene clusters.

In attempting to get the clones containing a large region of DNA, the cosmid vector, pWE15, was chosen in that it can contain 35-45 kb as an insert (Evans and Wahl, 1987). Furthermore, it can be handled easily as a plasmid, and it is specially designed for "chromosome walking" to obtain even a larger region of continuous DNA by combining overlapping cosmid clones.

In this paper, we describe construction of a cosmid library of the total rice genomic DNA, and selection of a sub-library that contains part of the rice chloroplast DNA or its homologs. The clones containing rearranged chloroplast sequences were also identified and analyzed. We discuss the application of cosmid library to studies of DNA migration.

Materials and Methods

Germination of rice seedlings and preparation of the genomic DNA

Rice (*Oryza sativa* L.) seeds were sterilized in 20% Clorox overnight and rinsed thoroughly with sterile water. The seeds were germinated on a sterile air conditioner filter, keeping them under dark at 30℃ for 2-3 weeks. Five grams of rice seedlings were used to isolate the total rice DNA using the method described

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in Moon et al. (1987a).

Twenty ug of the rice total genomic DNA was partially digested with *Sau* 3A using the limited amount of enzyme (1 unit) in 30 µl. At 0, 5, 10, 20, 30, 60 min after the initiation of incubation, 5 µl of the reaction mixture was taken, and 1 µl of 0.1 M EDTA was added. Each DNA sample was heat-inactivated at 70℃ for 10 min and loaded on a 0.3% agarose gel. *Hind* III and *Eco* RI digested λ DNA, uncut λ DNA and T_4 DNA were loaded together as size markers. After determining the optimum incubation time to generate mainly 35-45 kb fragments, the sample was scaled up to digest 400 µg of DNA to obtain a large amount of DNA as the insert for the cosmid vector. To collect DNA fragments of 35-45 kb, partially digested DNA was fractionated as follows: two of the 11 ml continuous NaCl gradient of 5-20% were made by gradually mixing 5.5 ml of 20% NaCl solution with 5.5 ml of 5% NaCl solution. On top of the gradients the partial DNA digest was loaded and centrifuged at 35,000 rpm for 7 h at 20℃ in SW41 rotor. Then, 1 ml fractions were collected. To each fraction, 100 ul of 3 M NaOAc and 2 ml of ethanol were added. The tubes were chilled at -70℃ for 20 min and then centrifuged. The DNA pellet was resuspended in 100 ul of TE buffer. Three ul of each DNA was checked on 0.3 kb agarose gel to identify the fraction that contains the DNA fragments of 35-45 kb in size.

Cosmid vector preparation and construction of the recombinant cosmid

Five mg of the cosmid vector pWE15 (Stratagene) was digested with *Bam*H1 restriction enzyme, and extracted with phenol and then with phenol-chloroform. The DNA was precipitated with ethanol and resuspended in 20 µl of TE. 2.5 µl of 10 X CIAP (calf thymus alkaline phosphotase) buffer (500 mM Tris-HCl, pH 9.5, 10 mM spermidine, 1 mM EDTA) and 2 µl (1 unit/ml) of CIAP was added and then the mixture was incubated at 37℃ for 30 min. After dephosphorylation, the reaction mixture was extracted with phenol and precipitated with ethanol. The pWE15 cosmid vector and the genomic DNA (in fraction 11) were mixed and ligated. The reaction mixture was extracted with phenol, precipitated with ethanol, and then resuspended in TE.

In vitro packaging of the recombinant cosmids

The recombinant cosmids were packaged as lambda phages using the Gigapack Gold (Straragene) *in vitro* packaging system and the cosmid library was constructed in the form of phage particles. The efficiency of the cosmid library construction was checked by counting the number of colonies containing the cosmid after infection.

Screening of the cosmid library

E. coli AG1 host was grown in TB broth containing 0.2% maltose and 10 mM $MgSO_4$ overnight. After the cells were pelleted at 2,000 rpm for 10 min, gently resuspended in 10 mM $MgSO_4$. The library in the form of the packaging mix and the AG1 cells were incubated at 37℃ for 15 min. Molten top agar with each aliquot of infected bacteria was spread evenly onto a 2X YT plate containing ampicillin. GeneScreen filters were used to lift colonies from the plates. Probe was labeled with [α-32P]dATP by a random hexamer labeling method. Positive cosmid clones were selected in the form of colonies.

Preparation and analysis of the recombinant cosmid DNA

Cosmid clones were grown in 5 ml 2X YT broth containing ampicillin at 37℃ overnight. Cosmid DNA was isolated as regular plasmid DNA using the PEG mini-preparation method (Moon et al., 1991). Restriction mapping analysis and Southern blot hybridization analyses were done as described by Moon et al. (1991).

Results and Discussions

Construction of the cosmid library

The total rice genomic DNA was digested partially with *Sau* 3A, and then fractionated on a 5-20% NaCl gradient. Fig. 1. shows the result of the size fraction ation. DNA in fraction 11 was used to construct a cosmid library. The titer of the library was determined to be 10^6 transformants/mg of DNA, which is high enough to be an efficient library.

Selection and analysis of the sub-library containing the chloroplast sequences or their homologs using hybridization analysis

The cosmid library was screened with the mixed probe of six chloroplast genes, petA (Wu, unpublished), *rbcL* (Moon et al., 1987), *psbAB* (Hanson, unpublished), *rpl2* (Moon et al., 1988b), *atpI* (Moon, unpublished), *ndhD* (Hanson, unpublished). Fig. 2 shows the locations of the genes on the chloroplast genome (Hiratsuka et al., 1989). From the primary screening using the mixed probe 200 hybridizing clones were selected.

To examine the chloroplast sequences or their homologs contained in the 200 cosmid clones in detail, the six genes used as the mixed probes in the first screening were used again individually. The hybridization results of 136 cosmid clones were summarized in Table 1. The clones were divided into 29 groups according to the hybridization pattern; each clone in a group hybridized to the same probes. Based on the locations of the probe genes on the genome (see Fig. 2), the chloroplast DNA contained in the 136 clones can be assumed to be one of the following three types. The first type is the authentic chloroplast sequences derived from the functional chloroplast

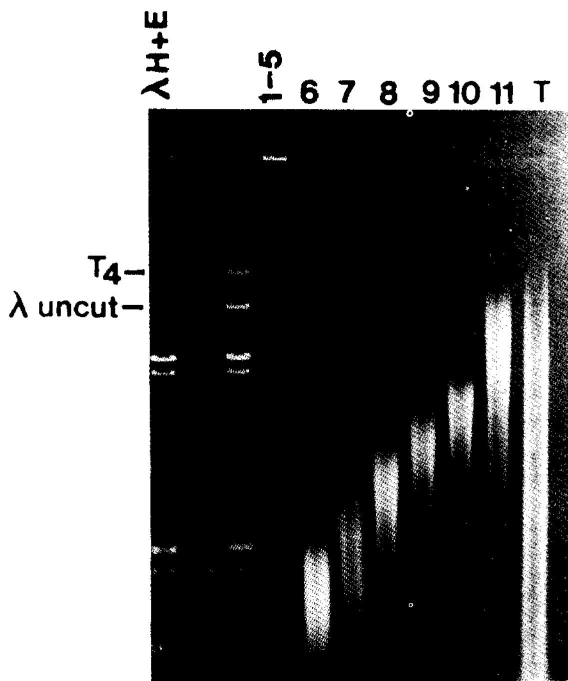

Fig. 1. Size fractionation of the partially digested DNA. 400 mg of DNA was digested with 20 unit of *Sau* 3A for 5 min. From this partially digested DNA, only 35-45 kb sized DNA fragments were collected by size fractionation. On the top of two 11 ml continous NaCl gradients of 5-20%, the partial DNA digest was loaded, and centrifuged at 35 K rpm for 7 h at 20℃ in SW41. One ml fractions were collected from the top of the tube. The DNA in each gradient fraction was pelleted and then resuspended in 100 ml of TE buffer. 3 ml of each DNA was checked on 0.3% agarose gel to identify the fraction that contains the DNA fragments of 35-45 kb in size. Eleven fractions were collected. Fraction 11 was collected near the bottom of the gradient tubes. The size markers are in lanes 1, 2 and 3: lane 1, *Hind* III-*Eco* RI digested lambda; lane 2, 4.5 kb DNA frgment, lane 3, mixture of T4 DNA, uncut lambda and *Hind* III-*Eco* RI digested lambda.

genome. Thus, the sequence maintains the proper gene order and gene structure (authentic type, Type I). The second type is a variant chloroplast sequence rearranged in the way where the genes are not in the proper order and thus reorganized (reorganized type, Type II). For example, the clones in Group 6 (clones 6, 28, 63, 92 and 99, see Table I) has this type of insert DNA in that they were hybridized to both *rbcL* and *rpl2* but not to *psaAB* and *atpI*. Another example of this type is the clone 15 of the group 13 which hybridized to *petA, psaAB, atpI* and *rpl2* but not to *rbcL*. Since the order of the authentic genes is *petA-rbcL-psaAB-atpI-rpl2*, it is clear that these clones contain the rearranged chloroplast gene cluster defined here as the reorganized type. The genetic event responsible for these sequences are rearrangement through recombination, deletion or insertion. The last type is isolated variants of any one of the chloroplast sequence (isolated type, Type III) such as the chloroplast homologs flanked by unknown sequences. For example, the clones in the group 5 (see Table 1) hybridized to *petA* only but not to any other genes. Based on the size of the insert and the distance from the ajacent genes

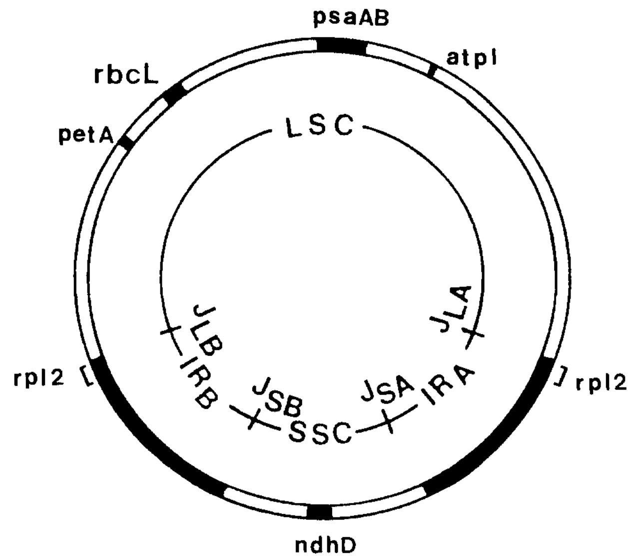

Fig. 2. Locations of the probe genes on the rice chloroplast genome. The outermost circle represents the rice chloroplast genome. Two inverted repeats, IR_A and IR_B, and the genes used as the probes are indicated by filling-in. J_{LA} and J_{LB} are junctions between the IR_A and IR_B and the LSCR (LSCR in the Figure) respectively. J_{SA} and J_{SB} are junctions between the IR_A and IR_B and the SSCR (SSC in Figure) respectively. The genome consists of 134,525 bp, and the size of the two IRs is 33,134 bp, respectively.

next to petA, it should hybridize to either *rbcL* or *rpl2* genes if the clone contains the authentic chloroplast sequences. Thus, it is reasonable to presume that some of the clones hybridized to only one gene among the six genes used as the probes possibly contain an insert of the isolated type. The possible genetic events giving rise to those variants are homologous recombination, DNA rearrangement, interorganeller migration followed by incorporation and mutations of various kinds.

According to the hybridization pattern shown in Table 1, there are many candidate clones containing an insert of Type II or Type III. The result strongly suggests that the above-mentioned genetic events seem to have occurred frequently, and they have contributed significantly to the organization, structure and thus evolution of the rice genomes.

Identification and analysis of the cosmid clones containing the authentic rpl2 gene and its homologs

Previously we have shown the two *rpl2* genes are separately located at each end of the two IRs near LSCR in the rice chloroplast genome (see Fig. 3, Moon and Wu, 1988b). To detect possible *rpl2* homologs elsewhere in the rice genome 608 bp Xba I fragment containing a part of *rpl2* gene (shown in the map of the clone 1 in Fig. 5) was used as the probe to hybridize the total rice genomic blot. As shown in Fig. 4A, all three digests show multiple bands with one or two major bands. According to our previous studies, the two copies of the authentic chloroplast *rpl2* gene correspond to the major bands in each lane (Moon et

Table 1. Summary of the hybridization analysis of 136 cosmid clones. The probe genes were listed accordiny to the relative order on the rice chloroplast genome. Hybridization was indicated by '+'

Group #	*petA*	*rbcL*	*psaAB*	*atpI*	*rp12*	*ndhD*	probe / clone #
1	+	+			+		1, 36, 38, 67, 73, 97, 113
2						+	2, 29, 41, 51, 77, 80, 88
3				+	+		3, 9, 42, 49, 52, 70, 84, 96, 133
4						+	4, 21, 65, 127
5	+						5, 22, 95, 98, 114
6		+			+		6, 28, 63, 92, 99
7		+	+	+			7, 104, 109, 110
8			+				8, 40, 112
9				+	+		9, 93
10					+	+	10, 14, 20, 25, 50, 57, 61, 66, 69, 85, 94, 101, 105, 111, 115, 119, 122, 123, 131
11					+		11, 13, 30, 62, 71, 86, 100, 108, 125, 132
12			+	+	+		12, 17, 23, 32, 72, 82
13	+		+	+	+		15
14			+		+		16, 55, 75, 103
15	+	+		+			18, 35, 89, 118
16	+	+	+		+		19
17		+		+			24, 26, 43, 54, 74, 129
18		+					27, 53, 68, 78, 90, 124, 135
19	+				+		31, 33, 130
20		+	+				34
21		+		+			37
22	+	+	+				39, 81, 134
23			+	+			45, 48, 59, 79, 91, 107, 120, 126, 128
24	+	+	+	+			46, 64, 116
25	+	+					47, 60, 76, 83, 102
26				+			56, 58, 136
27	+	+			+	+	87
28	+		+				106
29	+				+	+	121

al., 1988b). Thus, the existence of minor bands implies that there are several *rpl2* homologs in the rice genome responsible for the minor bands in each digests. It is also possible that some of the *rpl2* homologs contribute to the major bands in part. The Southern hybridization analysis clearly shows that there are *rpl2* homologs in addition to the two authentic *rpl2* genes in the rice genomes.

The cosmid clones containing the authentic *rpl2* gene or *rpl2* homologs were first identified by colony hybridization. Based on the results shown in Table 1, eight such cosmid clones (clones 1, 3, 6, 10, 11, 13, 28 and 30) were chosen and the *Eco* RI digest of the recombinant cosmid DNA from each clone was hybridized to the same *rpl* 2 probe. As shown in Fig. 3B, each clone has one of the three hybridizing bands, 2.4 kb, 3.4 kb and 3.7 kb, which were detected in the hybridization of the total rice genomic DNA. To characterize the nature of the *rpl* 2 sequence in detail, the DNA from each clone was digested with *Bgl* II and *Hind* III in addition to *Eco* RI restriction enzymes, respectively, and then they were subjected to Southern hybridization. Fig. 4 shows the hybridization results of the clones 1, 3, 6 and 13, and it reveals that the four clones are different from each other. Based on this and the similar hybridization analysis using *rpl23*, *rpl22* and *psbA*, the restriction map of the region containing the *rpl2* sequence was determined, and the genes were localized on the map (Fig. 5). It is clear that clones 1 and 3 contain one of the two authentic *rpl2* genes which are separately on the inverted repeats of the chloroplast genome (Type I). It is also clear that the clone 6 contains the reorganized rbcL-*rpl2* cluster (Type II). According to the map shown in Fig. 5, the clone 13 contains an isolated *rpl2* flanked by unidentified sequences. Thus, it contains an insert of Type III according to our definition. In summary, using a cosmid library we have identified authetic *rpl2* and rearranged *rpl2* corresponding to four different hybridizing bands in the total genomic Southern blot (see *Bgl* II digest in

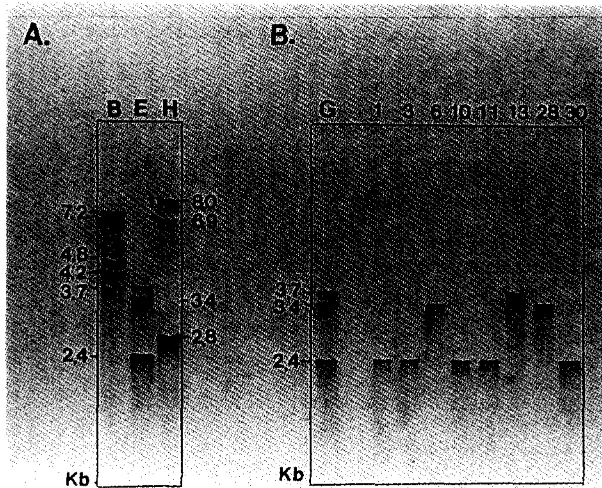

Fig. 3. Southern blot analysis of rice genomic DNA (A) and cosmid clones (B). (A) Total rice DNA (10 mg in each digestion) separately digested with indicated restriction enzymes (B, E and H), and (B) cosmid DNA (1 mg each) was digested with *Eco* RI. Digested DNA was fractionated on a 0.9% agarose gel in separate lanes, and then transferred to a Nytran filter. The filter was hybridized to the labeled *rpl2* gene (608 bp Xba I fragment, indicated on the map of clone 1 in Fig. 5). G on the first lane in (B) indicates total genomc DNA, and numbers on each lane in (B) indicate the cosmid clone number. B: Bam HI, E: *Eco* RI, H: *Hind* III.

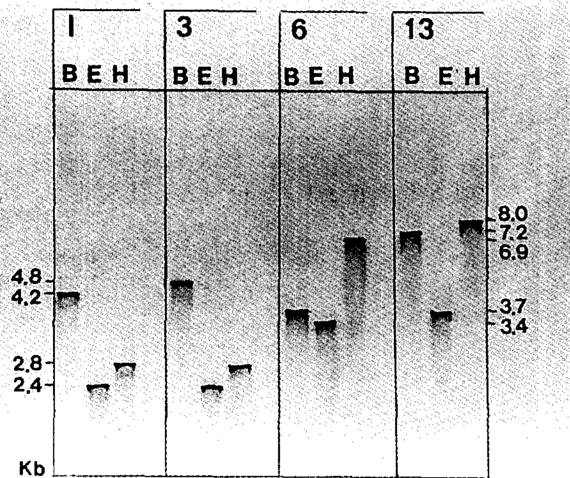

Fig. 4. Southern blot analysis of four cosmid clones (1, 3, 6 and 13) that contain *rpl2* sequence. 1 mg of cosmid DNA was used in each digestion. Each sample, separately digested with indicated restriction enzymes, was fractionated on a 0.9% agarose gel in separate lanes, and then transferred to a Nytran filter. The filter was hybridized to the labeled *rpl2* sequence (a 608 bp XbaI fragment, see the map of clone 1 in Fig. 5). Numbers on the top indicate the cosmid clone number. B: *Bam* HI, E: *Eco* RI, H: *Hind* III.

Fig. 4A and Fig. 5). Using the same approach, the cosmid library can be applied not only to identify any rearranged region of chloroplast DNA but also to identify any rearrangement of mitochondrial DNA.

Analysis of the cosmid clone containing the rearranged rbcL-rpl2 gene cluster

As shown in Table 1, the clone 6 hybridizes to both rbcL and *rpl2* genes but not petA nor psaAB, both of which are located between *rbcL* and *rpl2* on the chloroplast genome (Fig. 2). The hybridization result implies that the clone contains the chloroplast DNA rearranged in the way which led to the joining of *rbcL* and *rpl2* genes and deleting *psaAB* from the middle of the two genes (Fig. 5). Further analysis by restriction mapping and by gene localization using the Southern blot hybridization method revealed that the clone 6 contains MT-O. MT-O is a chloroplast DNA sequence migrated into mitochondria and rearranged through 151 bp repeat sequences as previously discussed (Moon et al., 1988a). Since these cosmid clones contain longer inserts than the previous plasmid clone, MT-0, the sequences beyond the rearranged *rbcL-rpl2* sequences can now be obtained from the cosmid clone 6. The restriction mapping and sequence analysis of this region revealed the junctions between chloroplast and mitochondrial DNAs and thus, integration site. The chloroplast sequence starts from the middle of the *trnV* gene of the *trnV-trnM-atpE-atpB* gene cluster which is located upstream of the *rbcL* gene. According to the map of the clone 6 in Fig. 5, it starts from the location which is 694 bp downstream of the *Hind* III site on the left end. The chloroplast sequence ends at a point which is 12 bp past the *Eco* RI site downstream of the *rpl2* gene.

The integration junction was sequenced, and it did not show homology to any sequences in the data bank. The result imlplies that the sequence beyond the integration site has not been reported so far. Thus it is clear that it is not chloroplast sequence. If the sequence information is available for the whole mitochondrial genome, the integration site can be exactly identified by homology search.

Based on the Southern hybridization analysis in many different plant systems, it is possible that any region of the chloroplast DNA can be migrated into the nucleus or the mitochondria (Baldauf et al., 1990; Thorsness and Fox, 1990; Ayliffe and Timmis, 1992a; Ayliffe and Timmis, 1992b; Kadowaki, 1996; Nakazono, 1996; Kubo, 1996). In a series of analyses on mung beans, spinach, corn, and peas, it was found that their mtDNA contain species-specific patterns of sequence homology to their chloroplast DNA (Stern and Palmer, 1984; Schuster and Brennicke, 1988; Lonsdale, 1989; Hanson and Folkers, 1992; Palmer, 1992). These studies, however, focused mainly on the comparison of restriction maps of chloroplast DNA fragments with their homologous sequences in the mitochondrial genome. At this level it is impossible to reveal precise differences of the sequences and exact locations of the transferred sequences.

To reveal the mechanism of transfer and the rearrangement of DNA fragments in other organelles, a detailed structural and functional analysis is needed, and it requires cloning and determining a physical map for large regions of DNA containing these fragments. Cosmid vectors are useful for this analysis because of their ability to clone large fragment of genomic DNA.

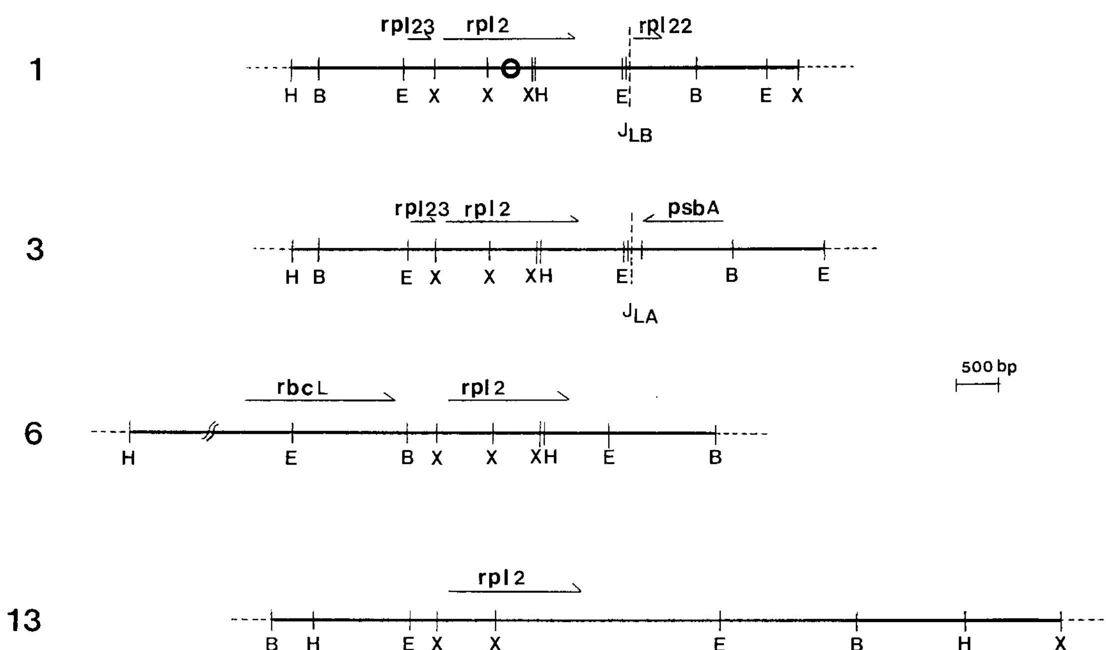

Fig. 5. The restriction maps and gene locations of the four *rpl2* containing cosmid clones, 1, 3, 6 and 13. Heavy lines indicate the region analyzed in detail, and the dotted lines at both ends of each straight lines indicate the rest of the insert DNA in each cosmid. The location and the direction of genes are indicated by arrows. The Xba I fragment marked by a circle (on the map of clone1) is the 608 bp fragment used as the *rpl2* probe. Wavy line on the map of clone 6 indicates reduction of length, and the actual *Hind* III fragment is 6.9 kb. J_{LA} and J_{LB}: indicate the location pf the two junctions between the inverted repeat and the large single copy region. B, *Bgl* II; E, *Eco* RI; H, *Hind* III; X, *Xba* I.

To expand studies to identify rearrangement and migration of other parts of the chloroplast genome, other clones in the chloroplast sub-library (Table 1) can be selected as candidates. We are constructing the cosmid sub-library representing the entire rice mitochondrial genome by probing the library with mitochondrial genes available from other species as we did in the case of chloroplast. Having sequence information of the authentic mitochondial genome, and by extensive analysis of genes in the DNA fragments, it is possible to reveal rearrangement pattern, detailed differences in their sequences, different times and possible mechanisms of gene rearrangement and transfer.

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