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Genetic and physical maps and a clone bank of mitochondrial DNA from rice

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Summary. Mitochondrial DNA (mtDNA) was isolated from young green leaves of rice plants. DNA fragments were cloned into lambda DNA, and clones that hybridized to mitochondrial genes from other plants were selected. Distal restriction fragments of these clones were used as probes for the selection of overlapping clones. A genetic map was finally created from the library by "walking" along the genome. The mitochondrial genome consists of five basic circles, with each circle sharing homologous sequences with one or two other circles. A master circle was constructed from the results of recombination across repeated sequences, and its size was estimated to be 492 kb. A physical map and a bank of overlapping clones were also constructed.

Key words: Rice (*Oryza sativa*) – Mitochondrial DNA – Genetic map – Physical map – Clone bank

Introduction

The mitochondrial genomes of higher plants are much larger and more complex than those of other eukaryotic organisms (Lonsdale et al. 1984). They are organized as multicircular molecules, and these subgenomic molecules are considered to be generated from a master circle by reciprocal recombination across recombination repeats (Palmer and Shields 1984; Lonsdale et al. 1983). If recombination occurs between direct repeats, two circular molecules are newly formed from the master circle. Therefore, the number of recombination repeats in a master circle determines the complexity of a mitochondrial genome. The sizes of the master circles also vary greatly among plant species (Pring and Lonsdale 1985; Newton 1988). Such complexity and variation in size contrast markedly with the physical characteristics of the chloroplast genomes of higher plants, which consist of a single kind of molecule and are relatively constant in size (Palmer 1985).

Lonsdale et al. (1984) constructed a physical map of maize mitochondrial DNA (mtDNA) by genome mapping and reported the existence of a 570-kb master circle with six pairs of recombination repeats. In a similar study on wheat mtDNA (Quetier et al. 1985) found a master circle of 430 kb, with ten recombination repeats. To date, the organization of mtDNA from rice, and important cereal crops, has not been analyzed. In this paper, we report the physical and genetic maps of rice mtDNA, as constructed by "walking" along the genome with cloned fragments.

Materials and methods

Isolation of the mitochondrial DNA

Rice plants (*Oryza sativa* cv 'Nipponbare') were grown in a growth chamber at 28 °C under continuous light. Mitochondrial DNA was isolated from 2-week-old green leaves by the method of Kadowaki et al. (1986). The leaves were harvested, minced with scissors and briefly homogenized in buffer (0.4 *M* sucrose, 50 m*M* TRIS-HCl, pH 7.5, 5 m*M* Na_2EDTA, 0.1% bovine serum albumin, 5 m*M* 2-mercaptoethanol) in a Waring blender. The homogenate was filtered through four layers of cheesecloth and two layers of Miracloth and centrifuged for 10 min at 17,000 *g*. Subsequent procedures were the same as those described by Umbeck and Gengenback (1983).

Cloning into lambda phage

Isolated mtDNA was partially digested with *Sau*3AI, and fragments were fractionated by density gradient centrifugation in 10–50% sucrose. Fragments of 13–20 kb were selected and lig-

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ated with lambda DASH that had been digested with *Bam*HI (STRATAGENE, USA). The ligated lambda DNA was packaged in vitro by GIGAPACK GOLD (STRATAGENE, USA), and used to transfect *E. coli* P2392. Since only inserts of 9–23 kb can be packaged in vitro, every recombinant lambda DNA should contain only a single insert.

Probes

Plasmids containing mitochondrial genes for subunit II of cytochrome c oxidase (*cox II*), for subunits of alpha and 9 of F_1-F_0 ATPase (*atpA* and *atp9*, respectively) and for 26S and 18S ribosomal RNA (*rrn26* and *rrn18*, respectively) were provided by K. Nakamura, Nagoya University, Japan. Wheat clones containing genes coding for subunit 3 of NADH dehydrogenase (*nad3*) and ribosomal protein S12 (*rps12*), and a wheat clone for apocytochrome b (*cob*) were a gift from J. M. Grienenberger, University Louis Pasteur, France and M. W. Gray, Dalhousie University, Canada, respectively. The plasmid pBM 2.5, containing the gene for subunit I of cytochrome c oxidase (*coxI*) from sugar beet, was provided by T. Mikami, Hokkaido University, Japan. A clone covering the gene for subunit 6 of F_1-F_0 ATPase (*atp6*) from *Oenothera* was a gift from W. Schuster, Institute of Gene Biology, Federal Republic of Germany.

Restriction enzymes

Restriction enzymes were purchased from Boehringer Mannheim, Federal Republic of Germany, and used according to the supplier's instruction.

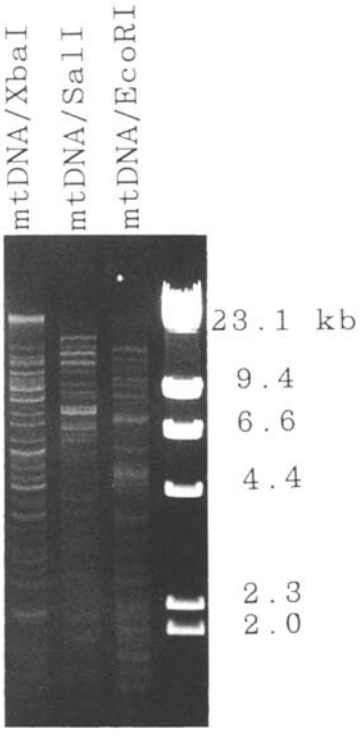

Fig. 1. Restriction patterns after agarose gel electrophoresis generated by rice mtDNA after digestion with restriction endonucleases *Xba*I, *Sal*I and *Eco*RI

Results

Mitochondrial DNA was isolated from green leaves as described in the Materials and methods. Restriction patterns of the isolated DNA are shown in Fig. 1. Chloroplast DNA (cpDNA) was co-electrophoresed with mtDNA, and the results indicated that contamination of the mtDNA by cpDNA was negligible (data not shown).

A library of mtDNA clones was constructed in order to study the organization of the mtDNA by "walking" along the genome. Mitochondrial clones were selected from the library by plaque hybridization with labeled fragments of DNA that included mitochondrial genes from other plants. Positive mitochondrial clones were digested with *Xba*I and *Sal*I, and their restriction maps were determined from patterns of bands after agarose gel electrophoresis and Southern hybridization.

As shown in Fig. 2, clone #20 was selected with a probe specific to *coxII*. Digestion with *Sal*I cleaved clone #20 into three fragments of 11.0 kb, 2.0 kb and 2.0 kb, respectively. The two 2.0-kb fragments hybridized with the probe. Mitochondrial DNA isolated from leaves was also analyzed, and fragments of 21 kb, 14 kb, 11 kb and 2.0 kb, generated by *Sal*I, hybridized with the same probe. One of the 2.0-kb fragments of clone #20 corresponds to the 2.0-kb fragment of total mtDNA; the other 2.0-kb fragment is the terminal fragment of clone #20 and corresponds to a portion of the longer fragments of

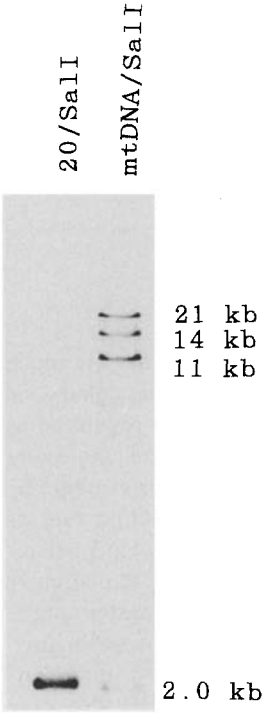

Fig. 2. DNA gel-blot analysis of *Sal*I-digested total mtDNA and lambda clone #20. Hybridization was with a 1.9-kb *Eco*RI fragment that contained the *coxII* gene from pea

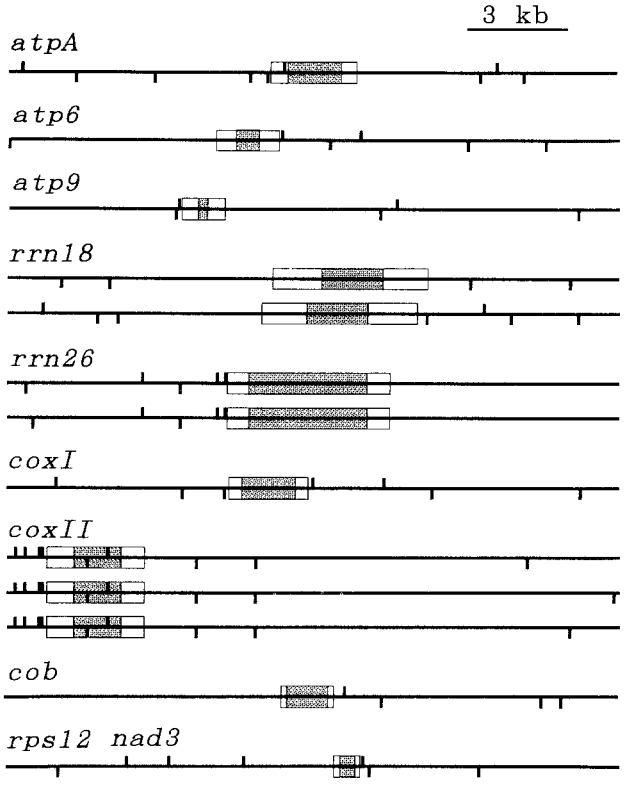

Fig. 3. Restriction maps of adjacent regions of mitochondrial genes constructed from cloned fragments. *Vertical bars above* and *below* horizontal lines indicate *Sal*I and *Xba*I sites, respectively. The *shaded boxes* indicate the lengths of the mitochondrial genes used as probes. The *open boxes* indicate the maximum extent of several overlapping clones as indicated by Southern hybridization

21 kb, 14 kb or 11 kb. This result revealed that there are three different regions adjacent to the *coxII* gene that have been formed by crossing at a recombination sequence, as shown in Figs. 3 and 4. The 21-kb fragment is found along the route from *coxII* to *atpA* through *rrn18*; the 14-kb fragment is found on the route from *coxII* to *cob* through *rrn18*; and the 11-kb fragment is found on the route from *coxII* to *atp6*.

We used eight other probes and constructed partial physical maps in the vicinity of the respective genes (Fig. 3). The results were subsequently extended by "walking" along the genome. Distal restriction fragments of the various clones were used as probes for the selection of overlapping clones.

As shown in Fig. 4, a complete genetic map of the mitochondrial genome was constructed. The locations of *coxI*, *coxII*, *atpA*, *atp6*, *atp9*, *cob*, *rps12*, *rrn18* and *rrn26* were determined by Southern hybridization, and those of *trnfM* and *trnN* were determined from sequencing data (data not shown). The mitochondrial genome consists of five basic circles, and each circle shares homologous sequences with one or two of the other circles. The sizes of the basic circles are 136 kb, 126 kb, 100 kb, 70 kb and 60 kb. The lengths of the repeated sequences are 47 kb, 44 kb, 18 kb, 6 kb and 3 kb. The accuracy of this map was confirmed by hybridization of each cloned fragment to total mtDNA.

A master circle was constructed from the results of recombination across repeated sequences and is illustrated in Fig. 5. Repeated sequences are shown by boxes inside the circle. The size of the master circle was calcu-

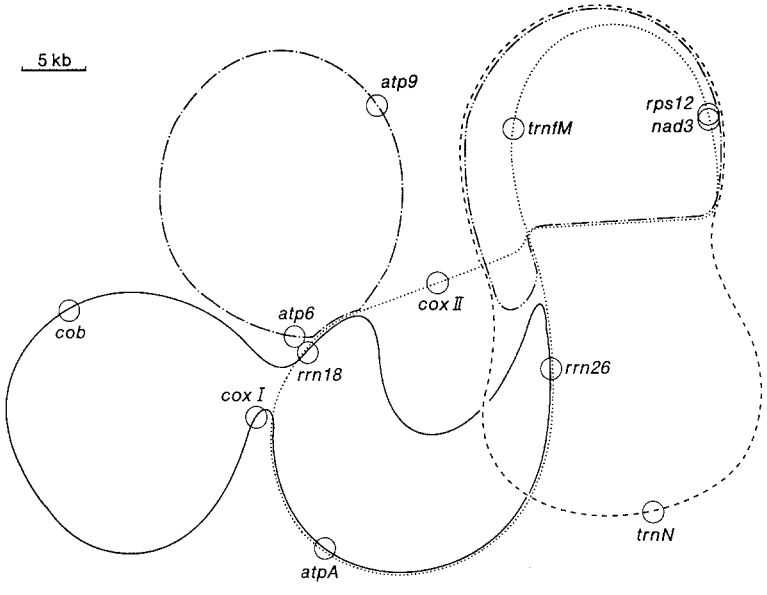

Fig. 4. A genetic map of the rice mitochondrial genome. Each type of line indicates a set of basic circles. Overlapping regions are regions repeated among circles

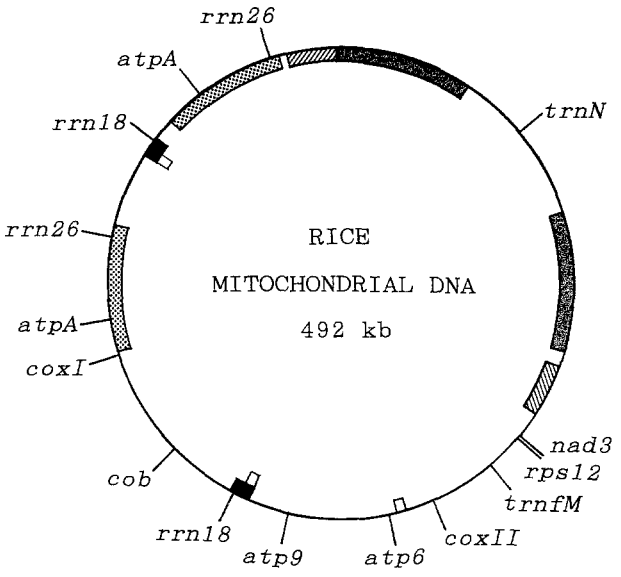

Fig. 5. A master circle of the mitochondrial genome with locations of genes and repeated sequences. Repeated sequences are indicated by *boxes inside* the master circle. Each set of repeats is indicated by *boxes* of a given pattern. The locations of mitochondrial genes are indicated outside the circle

lated to be 492 kb by summation of the sizes of the restriction fragments. A physical map of *Sal*I and *Xba*I fragments and overlapping clones are shown in Fig. 6. The circular map has been linearized at the terminus of the 47-kb repeat. The resolution of the map is approximately 0.3 kb; fragments of smaller size were ignored.

Our bank of overlapping clones of rice mtDNA contains 54 lambda phage clones. The entire rice mitochondrial genome is covered by these clones.

Discussion

A genetic map of rice mtDNA was created by "walking" along the genome in a lambda phage library constructed from mtDNA from green leaves. As shown in Fig. 4, rice mtDNA is organized as five basic circular DNAs, each of which shares homologous sequences with others, and the total length of a unique sequence is 374 kb. A larger population of subgenomic circles is presumably generated by recombination across homologous sequences.

A master circle can be constructed on the assumption that recombination occurs via these repeated sequences,

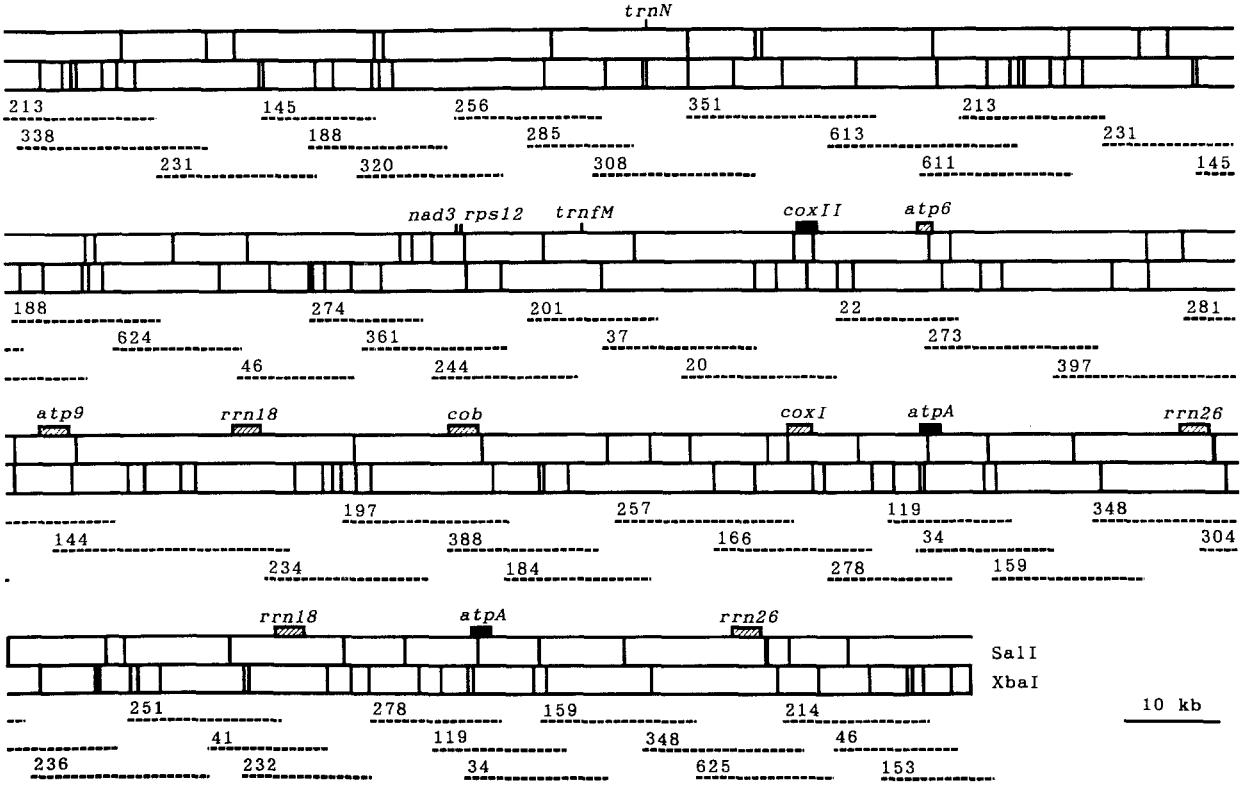

Fig. 6. A physical map and clone bank of the rice mitochondrial genome. The circular map has been linearized at the terminus of a 47-kb repeat. The *black boxes* indicate the locations of genes determined from sequence data. *Striped boxes* indicate the approximate locations of genes determined by hybridization. *Horizontal lines* and *numbers* indicate lambda clones and clone numbers, respectively

and its size is 492 kb. Thus, the organization of rice mtDNA is similar to that of maize and wheat mtDNA. Two of the repeated sequences, namely those of 47 kb and 44 kb, in the rice master circle are much longer than those reported in maize and wheat mtDNA. However, a 165-kb direct repeat was detected recently in a maize fertile cmsT revertant line (Fauron et al. 1990).

Three copies of a single recombination repeat have been found in *Petunia* (Folkerts and Hanson 1989) and in wheat (Quetier et al. 1985). We also found three copies of a 3-kb repeat in rice. However, in rice, the 3-kb repeat was located inside two copies of a 6-kb repeat. This repeat may be important in studies of the origin of the repeated sequence.

We used nine gene probes from other plant mitochondrial genomes to identify the mitochondrial clones in our library. All the genes were located in the rice mitochondrial genome, therefore, we can conclude that the gene content of rice mtDNA is similar to that of other plant mitochondrial genomes. However, the relative location of the genes appears to differ between reported mitochondrial genomes. An exception is *nad3-rps12*; these two genes are located less than 50 bases apart in the mtDNA of maize, wheat, *Petunia* and rice (Gualberto et al. 1988; Folkerts and Hanson 1989; Suzuki et al. 1991). Co-transcription of these two genes may be important for these plants.

Considering the number of genes identified, the genome size of plant mtDNA is extremely large. One explanation for this large size is that chloroplast sequences are found in mtDNA. It will be of interest to determine how these fragments were transferred from one genome to the other and to elucidate the function of these fragments. We are now sequencing these fragments so that we can compare them with chloroplast sequences, since the complete sequence of cpDNA is available from the same cultivar as our mtDNA clone bank.

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