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# Rice chloroplast DNA: a physical map and the location of the genes for the large subunit of ribulose 1,5-bisphosphate carboxylase and the 32 KD photosystem II reaction center protein

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**Summary.** By homogenizing rice leaves in liquid nitrogen, it was possible to isolate intact chloroplasts and, subsequently, pure rice chloroplast DNA from the purified chloroplasts. The DNA was digested by several restriction enzymes and fragments were fractionated by agarose gel electrophoresis. The sum of the fragment sizes generated by the restriction enzymes showed that the total length of the DNA is 130 kb. A circular physical map of fragments, generated by digestion with SalI, PstI, and PvuII, has been constructed. The circular DNA contains two inverted repeats of about 20 kb separated by a large, single copy region of about 75 kb and a short, single copy region of about 15 kb. The location of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase (Fraction I protein) and the 32 KD photosystem II reaction center gene were determined by using as probes tobacco chloroplast DNAs containing these genes. Rice chloroplast DNA differs from chloroplast DNAs of wheat and corn as well as from dicot chloroplast DNAs by having the 32 KD gene located 20 kb removed from the end of an inverted repeat instead of close to the end, as in other plants.

**Key words:** Rice – Chloroplast DNA – Physical map – Restriction pattern

## Introduction

In the past, most breeding programs have concentrated on nuclear genomes with relatively little attention being paid to chloroplast genomes, primarily because the appropriate technology to improve the genomes was lacking. However, recent progress in molecular biology had established that genes important for photosynthesis are encoded in chloroplast DNA (ctDNA). Furthermore, progress has also enabled the study of ctDNA in crop plants at the molecular level, opening the way towards the eventual practical genetic manipulation of chloroplasts.

Since the physical map of restriction enzyme fragments in corn ctDNA was first constructed (Bedbrook and Bogorad 1976), physical maps of ctDNA from spinach (Herrmann et al. 1980), tobacco (Fluhr and Edelman 1981), wheat (Bowman et al. 1981), and soybean (Spielmann et al. 1983) have been reported. Thus, physical maps of ctDNA are available for all of the major crops except rice. Because of the accumulation of hard, amorphous silica gel in epidermal cells, rice leaves have been difficult to homogenize while still maintaining chloroplasts in their intact state. We have now overcome this difficulty by homogenizing rice leaves in liquid nitrogen and have isolated rice ctDNA from intact chloroplasts.

In this report, we will show the restriction patterns, the physical map of rice ctDNA and the location of the genes for the large subunit of ribulose 1,5-bisphosphate carboxylase (Rubisco: Fraction I protein) and the 32 KD protein of the photosystem II reaction center.

## Materials and methods

*Preparation of ctDNA*

Rice plants (*Oryza sativa* L.) were grown in a growth chamber at 28 °C under fluorescent lamp illumination. Rice leaves were blended in liquid nitrogen, and ctDNA was isolated by essentially the same method as that of Saltz and Beckman (1981). Fifty grams of leaves from two-week-old plants were briefly blended in liquid nitrogen. After the liquid nitrogen was completely evaporated, the frozen leaf powder was suspended in 250 ml of Kool's buffer A (50 mM Tris-HCl, pH 8.0; 0.35 M sucrose; 7 mM EDTA; 5 mM 2-mercaptoethanol) containing 0.1% bovine serum albumin. The liquid suspension was filtered through two layers of cheese cloth, and then two layers of Miracloth. The filtrate was centrifuged for 10 min at 1,000 × g, and the green pellet was resuspended in 2.5 ml of

Kool's buffer A. The second resuspension was loaded on top of a stepwise 20–45–60% sucrose gradient made in 50 mM Tris-HCl, pH 8.0, 0.3 M sorbitol, 7 mM EDTA, and centrifuged for 30 min at 2,000×g. The green band at the interphase between 20 and 45% sucrose was collevted, diluted 1 : 3 with Kool's buffer B (50 mM Tris-HCl, pH 8.0; 20 mM EDTA), and centrifuged for 10 min at 3,000×g. The chloroplast pellet was resuspended in Kool's buffer B, and the chloroplasts lysed by adding sodium dodecylsarcosinate at a final concentration of 3%. One-twentieth volume of 10 mg/ml Pronase E (Kaken Kagaku Co. Ltd.) was added to the solution, and the mixture incubated overnight at 37 °C. DNA was extracted twice from the lysate with phenol and once with phenol-chloroform (1 : 1). The DNA was precipitated from the aqueous phase to which 0.1 volume of 3 M sodium acetate had been added with 2.5 volumes of ethanol. The precipitate was washed twice with 70% ethanol and dissolved in water.

*Restriction endonuclease analysis*

Restriction enzymes were purchased from the Takara Shuzo Co. Ltd. and ctDNAs were digested by the enzymes according to the supplier's instructions. The sizes of the fragments were determined by electrophoresis on 0.9% and 0.6% agarose (Dotite: Dojindo Lab.) gels by the method of Sugiura and Kasuda (1979).

*Southern hybridization*

Nick translation of cloned plasmids, Southern blotting, and DNA-DNA hybridization were carried out as described by Shinozaki and Sugiura (1982).

*Cloning of PstI fragments*

PstI fragments of rice ctDNA were ligated to PstI site of pUC8 (Vieira and Messing 1982), and recombinant plasmids were selected by using McConky plates.

## Results

*1 Restriction endonuclease analysis of rice ctDNA*

Rice ctDNA was analyzed by several restriction enzymes to examine the purity and to determine the size of the DNA. The first group of restriction enzymes consisted of EcoRI, BamHI and HindIII. Although the pattern shown in Fig. 1 indicates that the ctDNA was pure, these enzymes generated a large number of fragments ranging from 20 to 0.5 kb. They were not suitable for determining the total size of fragments.

A second group of enzymes, consisting of SalI, PstI and PvuII, generated eight, fourteen and nine fragments, respectively (Fig. 2). Since the number and size of the fragments digested by the second group of three enzymes were suitable for constructing a physical map, we also analyzed the sizes of fragments by double-digestion between the three enzymes – the results also being shown in Fig. 2.

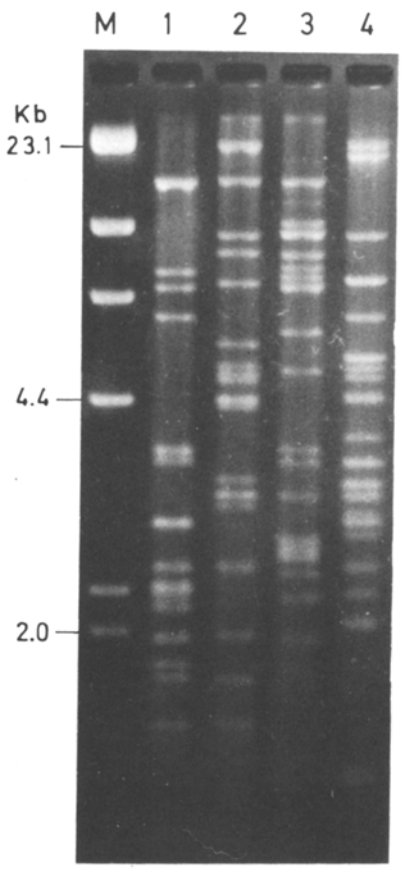


**Fig 1.** Restriction fragment patterns of rice ctDNA (*1–3*) and *Nicotiana gossei* ctDNA (*4*) generated by digestion with EcoRI (*1*), BamHI (*2, 4*), and HindIII (*3*). Fragments were separated by electrophoresis for 18 h in 0.9% agarose gels. *M*: Lambda DNA digested by HindIII

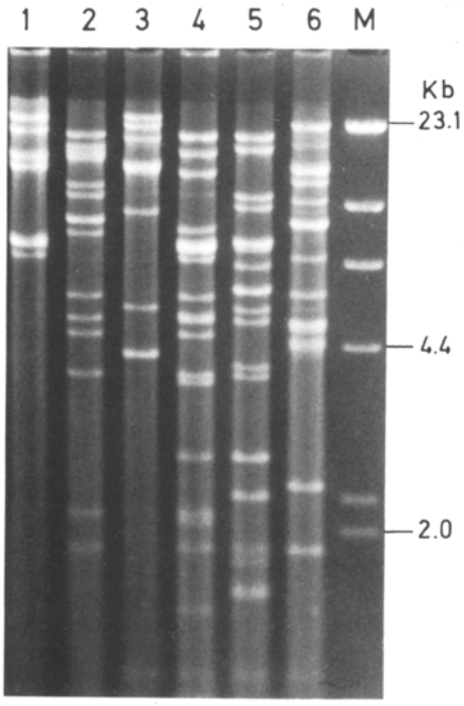


**Fig 2.** Restriction fragment patterns of rice ctDNA generated by digestion with SalI (*1*), PstI (*2*), PvuII (*3*), SalI + PstI (*4*), PstI + PvuII (*5*) and PvuII + SalI (*6*). Electrophoresis condition and the marker were same as Fig. 1

The sizes of each of the fragments are given in Table 1. The total size of fragments generated by each of the three enzymes was 130 kb; therefore the size of rice ctDNA was determined to be 130 kb.

**Table 1.** Size in kb of restriction fragments from rice chloroplast DNA

| | SalI | PstI | PvuII | Sal–Pst | Pst–Pvu | Pvu–Sal |
|---|---|---|---|---|---|---|
| 1 | 27.9 | 19.1 | 28.0 | 19.1 | 19.1 | 22.6 |
| 2 | 20.8 | 15.7 | 22.6 | 14.6 | 15.7 | 12.5 |
| 3 | 14.3 | 14.6 | 18.1 | 11.8 | 10.0 | 11.6 |
| 4 | 13.8 | 13.8 | 13.0×2 | 7.8 | 9.1 | 9.4 |
| 5 | 12.6×2 | 10.6 | 12.7 | 7.2×2 | 7.4×2 | 8.3 |
| 6 | 7.4 | 10.0 | 8.8 | 7.1×2 | 7.0 | 8.2×2 |
| 7 | 7.2×2 | 8.3×2 | 5.2 | 6.8 | 6.2 | 6.8 |
| 8 | 6.8 | 7.6 | 4.2×2 | 5.5 | 5.6×2 | 5.6 |
| 9 | | 5.5 | 1.0 | 5.0 | 5.2 | 4.8×2 |
| 10 | | 5.0 | | 4.8 | 5.0 | 4.6 |
| 11 | | 4.6 | | 4.6 | 4.0 | 4.4×2 |
| 12 | | 3.8 | | 3.8 | 3.9 | 4.3 |
| 13 | | 2.1 | | 3.5 | 2.7×2 | 2.4×2 |
| 14 | | 1.7 | | 2.8 | 2.3×2 | 1.8×2 |
| 15 | | | | 2.1×2 | 1.7×2 | 1.0 |
| 16 | | | | 2.0 | 1.5×2 | 0.8 |
| 17 | | | | 1.7×2 | 1.0×2 | |
| 18 | | | | 0.8×2 | 0.6 | |
| 19 | | | | 0.3×2 | 0.4 | |
| Sum | 130.6 | 130.7 | 130.8 | 130.5 | 130.6 | 130.7 |

*2 Construction of a physical map of rice ctDNA*

Since a physical map could not be constructed with confidence from the double digestion data, nick-translated Bam-2 fragments from tobacco ctDNA were hybridized with rice ctDNA fragments. In tobacco ctDNA, the Bam-2 fragments were known to occupy most of the short single copy region between two inverted repeats. The hybridization results are shown in Fig. 3. The rice fragments which hybridized to tobacco Bam-2 fragments were tentatively assumed to be located in a short single copy region.

Then we cloned the Pst-8, -11, -13, -14 fragments to pUC8, and the recombinant plasmids were digested by PstI, PstI + SalI, PstI + PvuII or PstI + SalI + PvuII and the fragments resolved by agarose gel electrophoresis with the results shown in Fig. 4A. These experiments revealed the restriction sites of each cloned fragment as illustrated in Fig. 4B. Finally, we labeled the cloned DNA and hybridized it to restriction fragments from rice ctDNA. The results are shown in Fig. 5. These data were analyzed together with double digestion data in order to construct the physical map shown in Fig. 6.

*3 The location of Rubisco LS and photosystem II 32 KD genes*

The cloned fragments containing the tobacco large subunit (LS) gene of Rubisco and the 32 KD of photosystem II reaction center gene were labeled by nick

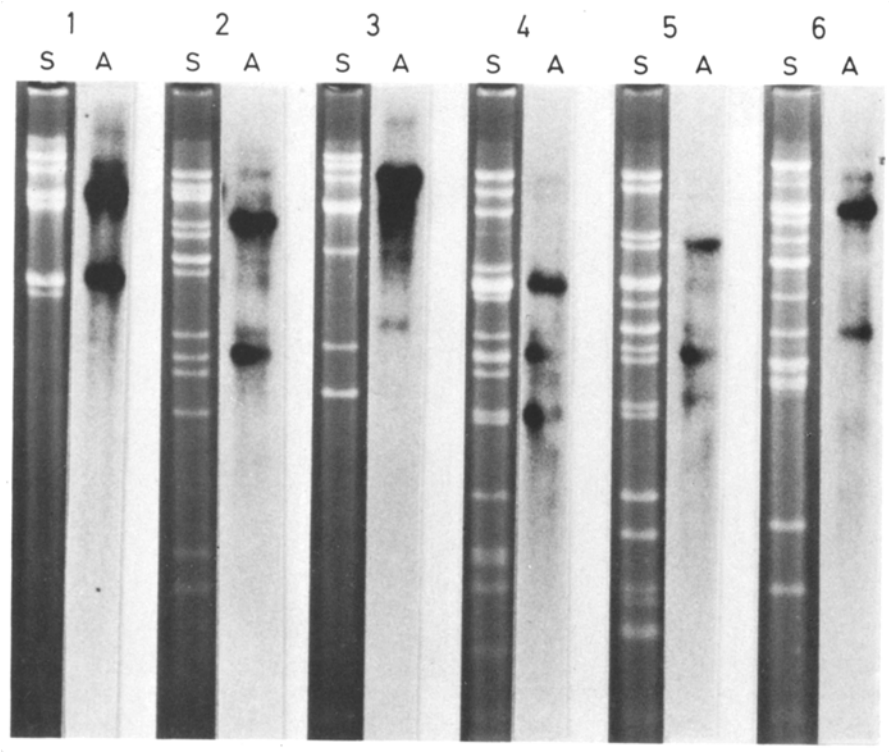


**Fig. 3.** Hybridization of cloned tobacco Bam-2 fragments to restriction fragments of rice ctDNA generated by digestion with SalI (*1*), PstI (*2*), PvuII (*3*), SalI + PstI (*4*), PstI + PvuII (*5*) and PvuII + SalI (*6*). *S*: Stained by EtBr; *A*: Autoradiograph

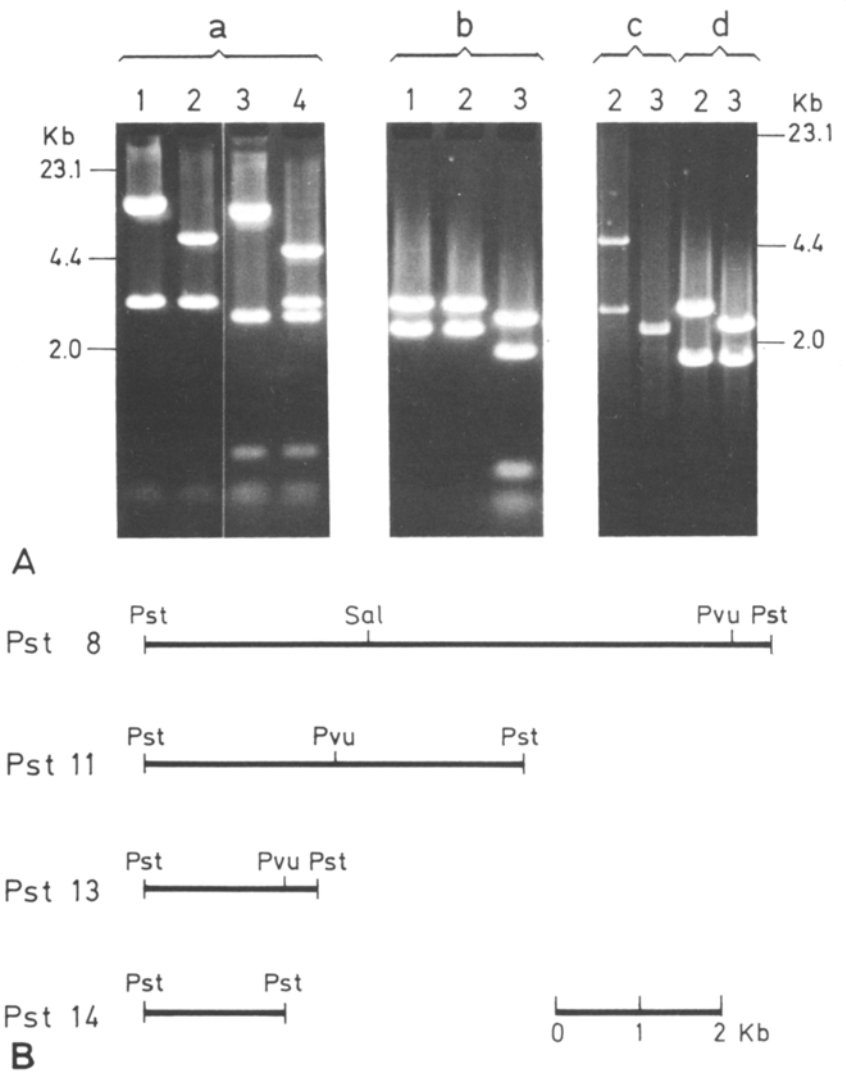


**Fig. 4.** **A** Restriction fragment patterns of recombinant plasmids containing Pst-8 (*a*), Pst-13 (*b*), Pst-11 (*c*) and Pst-14 (*d*). DNAs were electrophoresed in 0.9% agarose gels after being digested by PstI (*1*), PstI + SalI (*2*), PstI + PvuII (*3*) or PstI + SalI + PvuII (*4*). PstI + SalI digestion of the vector pUC8 generates a 2.8 kb fragment and PstI + PvuII digestion of pUC8 generates 2.3 kb and 0.5 kb fragments. **B** Restriction sites of cloned fragments derived from results of above experiments

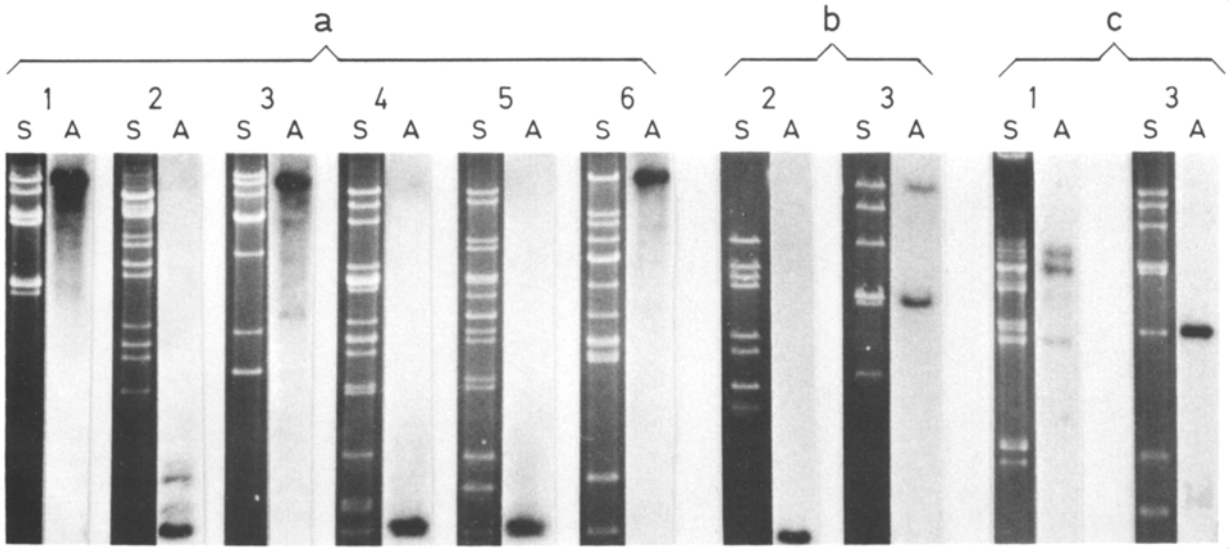


**Fig. 5.** Hybridization of Pst-14 (*a*), Pst-11 (*b*) and Pst-8 (*c*) fragments to restriction fragments generated by digestion with SalI (*1*), PstI (*2*), PvuII (*3*), SalI + PstI (*4*), PstI + PvuII (*5*) und PvuII + SalI (*6*). C-1 was partially digested. *S;A*, as in Fig. 3

translation and hybridized to rice ctDNA fragments. The results are shown in Fig. 7. The tobacco DNA containing the LS gene hybridized mainly with the Pst-14 fragment of 1.7 kb, and only very slightly with Pst-1 and Pst-8 fragments. We conclude, therefore, that the Pst-14 fragment contains most of the Rubisco LS gene. The tobacco DNA fragment containing the 32 KD gene hybridized to Pst-11 and Pst-6 and Pvu-5 and Pvu-1 fragments. Thus, both the 32 KD and LS genes are located far from the inverted repeats.

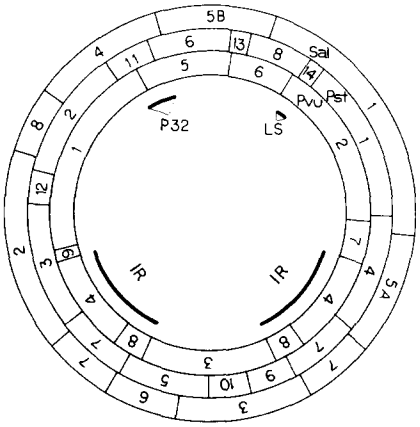


**Fig. 6.** Circular SalI + PstI + PvuII physical map of rice ctDNA, showing the two inverted repeats (*IR*) and the location of the rubisco large subunit (*LS*) and the 32 KD photosystem II gene (*P32*)

## Discussion

Rice ctDNA used in this report was completely digested by restriction enzymes without any further purification (such as CsCl-EtBr density gradient centrifugation). The DNA fragments were also successfully attached to plasmid pUC8, and recombinant DNAs were obtained. Therefore, rice ctDNA prepared by this method is pure enough to use for molecular biological experiments.

The size of monocot rice ctDNA is 130 kb, which is the same as other monocot ctDNAs from corn (Larrinua et al. 1983) and wheat (Bowman et al. 1981). However, monocot ctDNA is consistently smaller than the dicot ctDNA from tobacco (160 kb; Fluhr and Edelman 1981), spinach (150 kb; Herrmann et al. 1980) and soybean (150 kb; Spielmann et al. 1983). It would be of interest, therefore, to determine what parts of dicot ctDNA are missing in monocot ctDNA. The length of inverted repeats in rice ctDNA was estimated to be not less than 17.4 kb (Pvu-4 + Pvu-8; Fig. 6). However, it could be extended 1 kb to the Pvu-1 and Pvu-9 site, the large single copy region, and 4 kb to the Pst-9 and Pst-10 site, the short single copy region, so that the inverted repeat might be 22 kb long. This size would correspond to the 21.0 kb inverted repeat of wheat ctDNA (Bowman et al. 1981) and the 22.5 kb inverted repeat of corn ctDNA (Larrinua et al. 1983).

In contrast to all other ctDNAs mapped so far, the gene for 32 KD protein in rice is located 20 kb from the

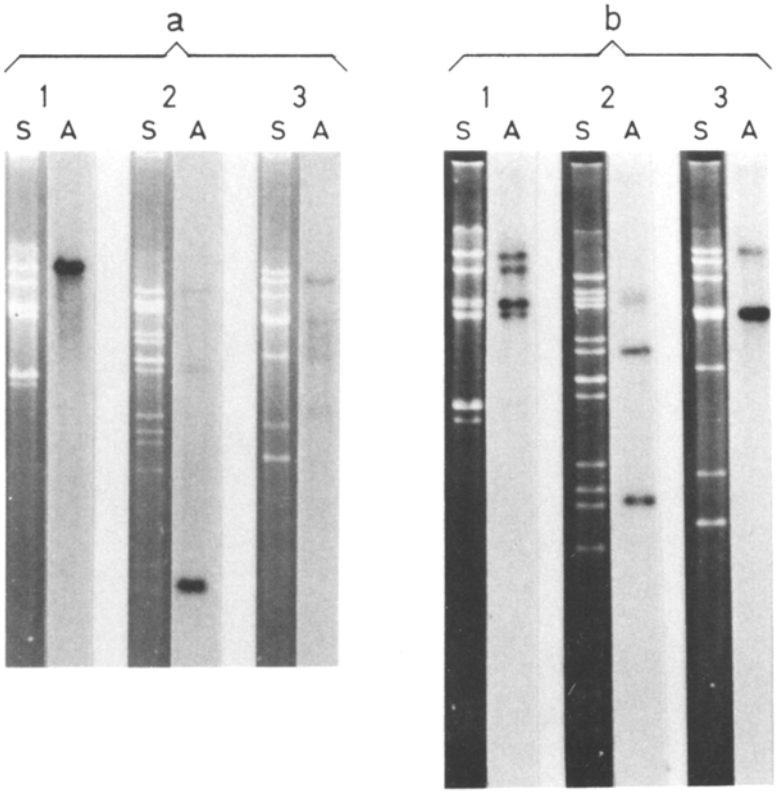


**Fig. 7.** Hybridization of tobacco rubisco LS gene (*a*) and 32 KD gene (*b*) to restriction fragments of rice ctDNA generated by digestion with SalI (*1*), PstI (*2*) and PvuII (*3*). *S;A*, as in Fig. 3

end of an inverted repeat. It is possible (cf. Palmer and Thompson 1982) that this divergence could have resulted from a large, unique inversion.

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## References

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

Bowman CM, Koller B, Delius H, Dyer TA (1981) A physical map of wheat chloroplast DNA showing the location of the structural genes for the ribosomal RNAs and the large subunit of ribulose 1,5-bisphosphate carboxylase. Mol Gen Genet 183:93–101

Fluhr R, Edelman M (1981) Physical mapping of *Nicotiana tabacum* chloroplast DNA. Mol Gen Genet 181:484–490

Herrmann RG, Whitfeld PR, Bottomley W (1980) Construction of a Sal/PstI restriction map of spinach chloroplast DNA using low-gelling-temperature-agarose electrophoresis. Gene 8:179–191

Larrinua IM, Muskavitch KMT, Gubbins EJ, Bogorad L (1983) A detailed restriction endonuclease site map of the *Zea mays* plastid genome. Plant Mol Biol 2:129–140

Palmer JD, Thompson WF (1982) Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. Cell 29:537–550

Saltz Y, Beckman J (1981) Chloroplast DNA preparation from *Petunia* and *Nicotiana.* Plant Mol Biol Newslett 2:73–74

Shinozaki K, Sugiura M (1982) The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Gene 20:91–102

Spielmann A, Ortiz W, Stutz E (1983) The soybean chloroplast genome: construction of a circular restriction site map and location of DNA regions encoding the genes for rRNAs, the large subunit of the ribulose-1,5-bisphosphate carboxylase and the 32 KD protein of the photosystem II reaction center. Mol Gen Genet 190:5–12

Sugiura M, Kusuda J (1979) Molecular cloning of tobacco chloroplast ribosomal RNA genes. Mol Gen Genet 172: 137–141

Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268