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RFLP analysis of nuclear DNAs homologous with mitochondrial plasmid-like DNAs in cultivated rice

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Summary. B1 and B2 are small, circular, mitochondrial plasmid-like DNAs found in male-sterile cytoplasm (cms-Bo) of rice. In this study, nuclear sequences homologous to these DNAs were investigated among a number of rice cultivars. Several copies of nuclear B1- and B2-homologous sequences were detected in all examined cultivars, regardless of the presence or absence of the B1 and B2 DNAs in mitochondria, indicating that the existence of the B1- and B2-homologous sequences in the rice nuclear genome was widespread. A restriction fragment length polymorphism (RFLP) was detected for both sequences, and we propose that these DNAs could be useful RFLP markers for the rice nuclear genome. To analyze these nuclear homologues genetically, segregation analysis of the RFLP was carried out in the F_2 progenies of an Indica-Japonica rice hybrid. Of the B1 homologues, there were two nonallelic fragments, one specific to the Indica parent and the other to the Japonica. These results indicate that the B1 and B2 homologues were dispersed in the nuclear genome. The integration of B1-homologous DNA into the nuclear DNA may have occurred independently after sexual isolation of the Indica and Japonica rice varietal groups, or a intranuclear transposition of these sequences took place during the process of rice differentiation into the varietal groups.

Key words: Plasmid-like DNA – Rice – Mitochondrial DNA – RFLP – Nucleo-mitochondrial DNA transmission

Introduction

In many higher plants small, linear, and circular DNAs, called plasmid-like DNAs, have been observed in mitochondria (for reviews, Pring and Lonsdale 1985; Newton 1988). Two well-characterized examples of these are present in the maize cms-S cytoplasms, S1 and S2, which share homologies with the main mitochondrial genome (Pring et al. 1977). These two DNAs have identical, 208-bp terminal inverted repeats (Kemble and Thompson 1982), by which homologous recombination can occur in the main mitochondrial genome (Schardl et al. 1984). However, unlike the examples from maize, most of the plasmid-like DNAs in other plant species lack homology with the main mitochondrial genome (Palmer et al. 1983; Powling and Ellis 1983; Chase and Pring 1985, 1986; Shikanai et al. 1987; Smith and Pring 1987), and their origin and function are unknown.

Small, circular, plasmid-like DNAs have also been observed in mitochondria of cytoplasmic male-sterile rice (Yamaguchi and Kakiuchi 1983; Mignouna et al. 1987; Nawa et al. 1987; Shikanai et al. 1987). Four plasmid-like DNAs – B1, B2, B3, and B4 – from cms-Bo cytoplasm (one of the male-sterile cytoplasms of rice) were cloned and characterized (Shikanai et al. 1987, 1989; Shikanai and Yamada 1988). Surprisingly, these four DNAs shared homologies with the nuclear genome but not with the main mitochondrial genome (Shikanai et al. 1987; Sakamoto et al. 1990). These plasmid-like DNAs were also present in mitochondria from normal cytoplasm (Sakamoto et al. 1989; Saleh et al. 1989). In many indigenous rice varieties, Kadowaki et al. (1988) found the presence of one or more of the plasmid-like DNAs in the mitochondria, which could be correlated to the geographical distribution of the rice varietal groups.

In order to better understand their origin and function, the nuclear sequences homologous to these plasmid-like DNAs were analyzed. We examined the distribution of the B1 and B2 homologous nuclear sequences of cultivated rice varieties and detected a restriction frag-

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ment length polymorphism (RFLP) among them. It was thus proposed that B1 and B2 DNAs are useful RFLP markers of the rice nuclear genome. Segregation analysis in the F_2 population of an Indica and Japonica hybrid indicated that the homologous sequences to B1 and B2 are dispersed throughout the nuclear genome. Their origin and transmission between organelles are discussed.

Materials and methods

Plant materials

Rice seeds (*O. sativa*) of cultivars 'Ginbozu' (Japonica), 'Nipponbare' (Japonica), 'IR24' (Indica), 'Jamuna' (Indica), and 'OK21' (*O. glabberima*) were germinated and grown at 28 °C for 2 weeks. Green seedlings were used for the isolation of chloroplast DNA (ctDNA), and etiolated seedlings were used for that of mitochondrial DNA (mtDNA).

DNA for the segregation analysis was isolated from leaves of F_2 individuals of the Indica-Japonica hybrid, which was generated by a cross between 'Kasalath' (Indica) and 'Fl134' (a mutant line of Japonica).

Isolation of DNA

Total DNA was isolated from green leaves, according to the method of Murray and Thompson (1980), with the following modifications. Approximately 10 g of seedlings frozen with liquid nitrogen was minced in a Waring blender. The broken pieces were further powdered in liquid nitrogen by a Polytron treatment for 5 min. In a new tube, the powder was suspended in 10 ml of just-boiled 2 × E buffer [2% (w/v) CTAB, 0.1 *M* TRIS-HCl (pH 8.0), 20 m*M* EDTA, and 1.4 *M* NaCl] and incubated at 55 °C for 15 min while being shaken. An equal volume of chloroform-isoamylalcohol (24:1, v/v) was added to the suspension. After gentle extraction at room temperature for 30 min, the emulsion was centrifuged at $1,500 \times g$ for 15 min and the aqueous phase was collected. The chloroform-isoamylalcohol extraction was repeated, and 1/10 vol. of 10% CTAB (w/v), containing 0.7 *M* NaCl, was added to the final aqueous phase. An equal volume of ppt buffer [1% (w/v) CTAB, 50 m*M* TRIS-HCl (pH 8.0), 10 m*M* EDTA] was added and the solutions were mixed gently. After a 30 min incubation at room temperature, the DNA was recovered by centrifugation at $1,500 \times g$ for 15 min. The DNA precipitate was redissolved in 3 ml of TE buffer [10 m*M* TRIS-HCl (pH 8.0), 1 m*M* EDTA] containing 1 *M* NaCl, and an equal volume of isopropanol was added and mixed. After a 30 min incubation at room temperature. DNA was again recovered by centrifugation and redissolved in TE buffer. To the DNA solution, RNase A (Sigma) was added to a final concentration of 20 μg/ml and incubated at 55 °C for 1 h. The final DNA solution was stored at 4 °C.

mtDNA and ctDNA were isolated from seedlings as described previously by Kadowaki et al. (1986).

Southern blot analysis

Electrophoresis and blotting of DNA to nitrocellulose papers were carried out according to the protocol of Maniatis et al. (1982). Labeled DNA probes were prepared from the cloned, plasmid-like DNAs of B1 (2.1 kb) and B2 (1.5 kb) (Sakamoto et al. 1989). B1 and B2 clones were labeled with [α-^{32}P]dCTP (Amersham, >3,000 Ci/mmol) by using the multiprime DNA labelling system (Amersham) to a specific activity of $>10^8$ cpm/μg DNA.

Prehybridization of the filters was carried out in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution, 50 m*M* sodium phosphate (pH 6.5), 0.25 mg/ml denatured salmon sperm DNA, and 1% (w/v) glycine, at 42 °C overnight. Hybridization was performed in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 20 m*M* sodium phosphate, 0.1 mg/ml denatured salmon sperm DNA, and denatured probe, at 42 °C for 20 h. The filters were washed twice in 2 × SSC, 0.1% SDS at 42 °C for 15 min, twice in 1% SSC at 42 °C for 15 min, and finally in 0.1% SSC at 65 °C for 20 min. Autoradiographs were obtained by exposure of X-ray film (Fuji) to the filters at −80 °C for 3–4 days with the aid of an intensifying screen.

Results

Presence of the plasmid-like DNAs in mitochondria of Indica cultivars

To examine the degree of homology of B1 and B2 to the mitochondrial genome, mtDNAs were isolated from two Indica cultivars, 'IR24' and 'Jamuna', and *O. glaberrima* cultivar, 'OK21'. It had been previously proven that Japonica cultivars 'Ginbozu' and 'Nipponbare' share no homology with the mitochondrial genome (Sakamoto et al. 1989). Figure 1 shows the results of a Southern hybridization, wherein mtDNAs were probed with B1. Homologous sequences were detected only in 'Jamuna' mtDNA (Fig. 1 B, lanes 2 and 5). In uncut mtDNA, these homologous sequences were detected as multimeric bands, and B1 did not hybridize to the main mitochondrial genome at the high-molecular-weight region of the blot. When the mtDNA was digested with EcoRI, which cuts B1 once, these multimeric bands were all reduced to a single band of 2.1 kb, the linear length of B1, indicating that the homologous sequence to B1 was present in mitochondria of 'Jamuna' as a plasmid-like form.

Similar forms of DNA were observed when B2 was used as a probe, where homologous sequences to B2 existed as plasmid-like mtDNA and not within the main mitochondrial genome of both Indica cultivars 'IR24' and 'Jamuna' (Fig. 1 D). Identical hybridization experiments using isolated ctDNAs and probes B1 and B2 showed no sequence homology between the DNAs in all tested cultivars (data not shown). Summarizing the hybridization results, mitochondria of 'Jamuna' contained as plasmid-like DNA both B1 and B2, 'IR24' had B2, and *O. glaberrima* 'OK21' lacked both B1 and B2; in all cultivars the main mitochondrial genome had no homologous sequences to B1 and B2.

Homologies to B1 and B2 in the nuclear genome and their RFLPs among rice cultivars

Total DNA isolated from 'Ginbozu', 'Nipponbare', 'IR24', 'Jamuna', and 'OK21' was digested with restriction enzymes and subsequently hybridized to B1 and B2 DNA. We used restriction enzymes which cut B1 and B2

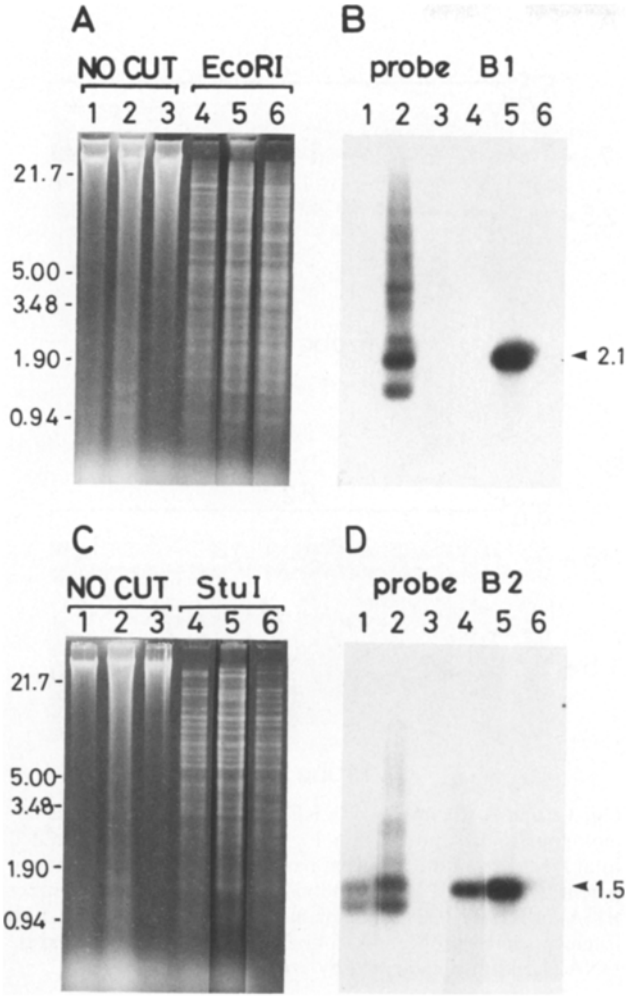

Fig. 1 A–D. Agarose gel electrophoresis and Southern blot analysis of rice mtDNAs. mtDNAs from cultivar 'IR24' (*lanes 1* and *4*), 'Jamuna' (*2* and *5*), and 'OK21' (*3* and *6*) were digested with either EcoRI (**A**) or StuI (**C**), and then electrophoresed in an 0.7% agarose gel together with non cut mtDNAs. Panels **B** and **D** are the autoradiograms of panels **A** and **C** probed with either B1 or B2, respectively

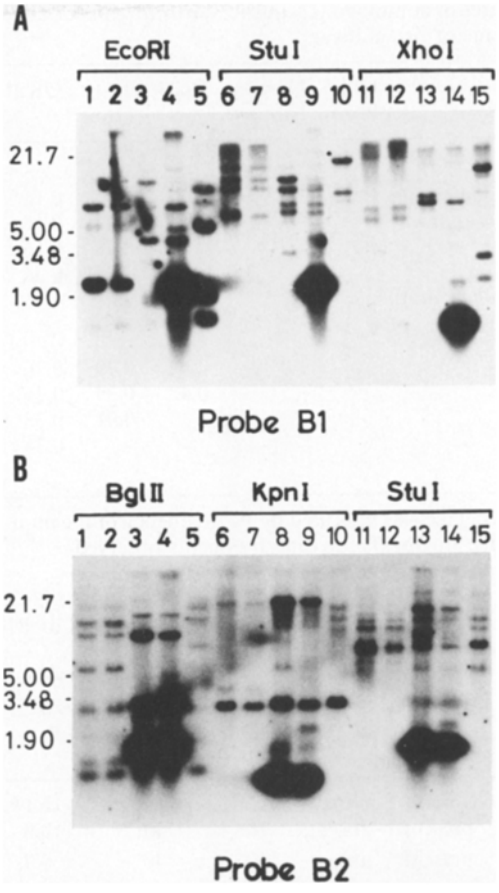

Fig. 2 A and B. Southern blot analysis of total DNAs from rice cultivars 'Ginbozu' (*lanes 1, 6,* and *11*), 'Nipponbare' (*lanes 2, 7,* and *12*), 'IR24' (*lanes 3, 8,* and *13*), 'Jamuna' (*lanes 4, 9,* and *14*), and 'OK21' (*lanes 5, 10,* and *15*) probed with either B1 (**A**) or B2 (**B**). Total DNAs were digested with the restriction enzymes indicated above each panel

at one or two sites. Digestion of B1 DNA with either EcoRI or StuI generate a single, 2.1-kb fragment, while XhoI generates two fragments of 1.1 and 1.0 kb. Digestion of B2 DNA with either BglII or StuI yields a single, 1.5-kb fragment, while KpnI results in 0.8 and 0.7 kb bands. Figure 2 A and B shows hybridizing bands in all cultivars. Five to nine homologous fragments were observed when B1 was used as a probe, and four to nine were observed when B2 was used, indicating the presence of several B1 and B2 homologous copies in the nuclear DNA. All cultivars had homologous sequences to both B1 and B2 in their nuclear genome, regardless of the presence or absence of the two DNAs in the mitochondria. At present, we have detected the homologous sequences to B1 and B2 in the nuclear genome of all 130 rice cultivars collected from various places around the world (data not shown).

As presented in Fig. 2, the nuclear sequences homologous to B1 and B2 appeared to show a RFLP among the rice cultivars. To describe the genetic aspect of the homologous sequence, segregation analysis of the RFLP was done (Table 1). While a RFLP could not be detected between Japonica varieties 'Ginbozu' and 'Nipponbare' when either B1 or B2 were used as probes, a slight polymorphism was observed between Indica varieties 'IR24' and 'Jamuna'. The polymorphic degree of B2-homologous sequences among the cultivars was almost the same. On the other hand, the polymorphic degree of B1-homologous sequences was higher, and remarkable polymorphisms were observed between Indica and *O. glaberrima,* and between Japonica and *O. glaberrima.* These results suggested not only the presence or absence of B1 and B2 in mitochondria, but also that the restriction fragments having nuclear sequences ho-

Table 1. Degree of homologous sequences with B1 and B2 in the nuclear genome of rice cultivar

Varieties	Ginbozu	Nipponbare	IR24	Jamuna	OK21
B1 probe					
Ginbozu (Japonica)	–	1.0	0.35	0.4	0.16
Nipponbare (Japonica)		–	0.35	0.4	0.16
IR24 (Indica)			–	0.78	0.16
Jamuna (Indica)				–	0.26
OK21 (*O. glaberrima*)					–
B2 probe					
Ginbozu	–	1.0	0.42	0.39	0.36
Nibbonbare		–	0.42	0.39	0.36
IR24			–	0.91	0.38
Jamuna				–	0.34
OK21					–

Homologous degree was indicated by the frequency of the number of the same fragments for total fragments observed

Table 2. Segregation analysis of homologous fragments to B1 and B2 in the F_2 population

Fragment	P1[a]	P2[a]	F_2 population		$\chi^2_{(3:1)}$
			Present	Absent	
B1 7.1 kb	absent	present	128	15	16.1*
2.5 kb	present	absent	108	35	0.0
B2 15 kb	present	absent	103	40	0.7

[a] P1 and P2 represent maternal parent of Kasalath (Indica) and paternal parent of Fl134 (Japonica), respectively
* Significant deviation at the 1% level

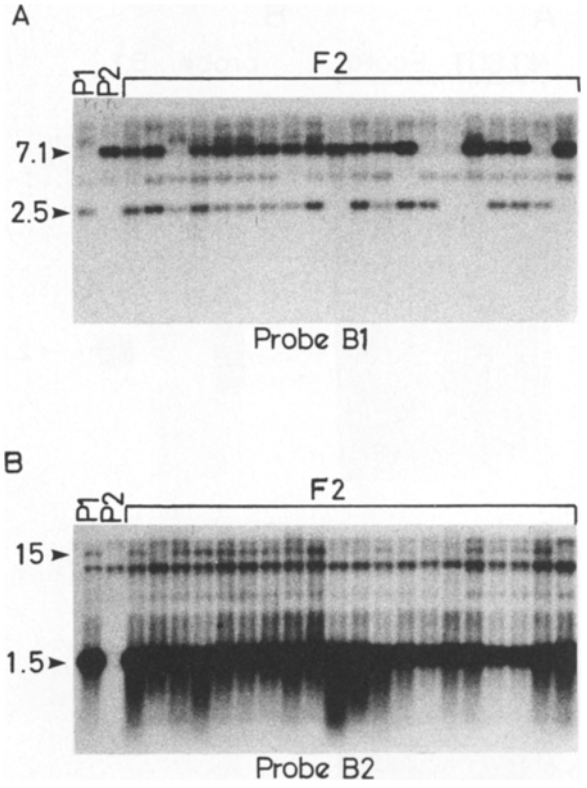

Fig. 3A and B. Examples of a RFLP analysis of sequences homologous to B1 and B2 in a F_2 population. EcoRV-digested total DNAs from the maternal parent 'Kasalath' (P1), paternal parent 'Fl134' (P2), and F_2 individuals were probed with either B1 (**A**) or B2 (**B**). The fragments showing RFLP (7.1- and 2.5-kb fragments in B1 probe, and 15-kb fragment in B2 probe) and B2 DNA (1.5 kb) are indicated by *arrowheads*

mologous to B1 and B2 were divergent and seemed to be correlated with rice varietal groups.

F_2 segregation analysis of the nuclear homologous sequences to B1 and B2

Since the nuclear homologues to B1 and B2 showed a RFLP among rice cultivars, a genetic analysis could trace these homologues in the F_2 population of an Indica-Japonica rice hybrid. In this study, we examined 143 F_2 progenies derived from the cross of 'Kasalath' (Indica, maternal) with 'Fl134' (Japonica, paternal), and we examined their inheritance of the B1 and B2 homologues in the nuclear genome.

Figure 3 shows two examples of a Southern blot analysis. Total cellular DNA was isolated and digested with EcoRV, which restricts B2 at one site, and the digested DNA was probed with B1 and B2. A strongly hybridizing band of 1.5 kb was detected in each F_2 individuals when B2 was used as a probe. Among the parents, this band was observed only in the maternal parent Kasalath (P1) and not in Fl134 (P2). Because of maternal inheritance and because it was the same size as the mitochondrial plasmid-like B2 DNA, this band was considered to be the B2 sequence present in mitochondria. The other band observed in approximately three-fourths of the F_2 had a length of 15 kb. Again, it was detected in P1 but not in P2. Based on its size and the segregation, the F_2 15-kb fragment corresponded to the B2 nuclear homologue from P1.

When the same total DNA was probed with B1, a 2.5-kb fragment corresponding to the RFLP of the P1 and a 7.1-kb fragment corresponding to that of P2 appeared. While the 2.5-kb fragment of P1 and the 7.1-kb fragment of P2 might be considered allelic, there were individuals in the F_2 population that lost both fragments at the same time, demonstrating that these two fragments were nonallelic.

The presence/absence ratios of the three fragments described above were estimated in 143 plants of the F_2 population (Table 2). Linkage analysis revealed that the three fragments were not linked to one another, suggesting that the sequences homologous with B1 and B2 DNAs were dispersed in the nuclear genome. The ob-

served ratios of two of the fragments, the B1-homologous 2.5-kb fragment and the B2-homologous 15-kb fragment, were consistent with a 3:1 inheritance ratio and they were considered to segregate in Mendelian fashion, confirming their nuclear location. However, segregation of the B1-homologous 7.1-kb fragment was unusual and deviated from the 3:1 ratio. This deviation might be explained by the linkage of the B1-homologous 7.1-kb fragment to the gametophyte gene (Nakagahra 1972), which affects pollen fertility, because other rice RFLP probes found to be linked to the gametophyte gene also showed a deviated segregation pattern (A. Saito, personal communication).

Discussion

In this paper we have shown that the nuclear sequences homologous to B1 and B2 exist in all rice cultivars examined, regardless of the presence or absence of the plasmid-like DNA in the mitochondria. Therefore, the presence of nuclear sequences homologous to B1 and B2 DNAs may be common in cultivated rice, including *O. glaberrima*. With respect to the homologous sequences, a RFLP was detected among rice cultivars. We propose that B1 and B2 can serve as RFLP markers to analyze the rice nuclear genome despite being isolated from mtDNA.

The nuclear B1 and B2 homologues are positioned at different loci in the varietal groups Indica and Japonica. If one considers the origin of B1 and B2 to be the mitochondria, then there are two possible explanations for the existence of the RFLP between the subspecies. The first is that B1 and B2 integrated into the nuclear genome independently in both Indica and Japonica. Once stably integrated, the mitochondrial forms might no longer have been needed and were subsequently lost from mitochondria. Therefore, most of the rice cultivars may share homology with B1 and B2 DNA in the nuclear genome, while the presence or absence of the B1 and B2 would show polymorphism. The second explanation is the possibility of transposition of these sequences in the nuclear genome. In rice, McCouch et al. (1988) constructed a rice RFLP map in which they pointed out that a significant portion of RFLPs in rice resulted from insertions or deletions of DNA sequences. As evidence for sequence transposition, they reported the RFLP probe, RG229, which had different loci in Indica and Javanica. The characteristics of B1 and B2 homologues in the nuclear genome are similar to that of RG229, although a sequence homology among them is unknown. If the homologous nuclear sequences to B1 and B2 DNAs are transposons, they may play a role in the rearrangement of the rice nuclear genome. However, the sequence data of B1 and B2 does not reveal any characteristic features of transposable elements (Shikanai et al. 1987, 1989).

Plasmid-like DNAs that share no homology with the mitochondrial main genome, but do share homology with the nuclear genome have been also reported in maize (Kemble et al. 1983; Abbott et al. 1985; Smith and Pring 1987). Shikanai et al. (1989) noted that B1 and B2 have highly conserved regions with the maize 1.9- and 1.4-kb plasmid-like DNAs, respectively, and suggested that they were closely related to one another. Accordingly, it may be considered that the nuclear sequences homologous with the 1.9- and the 1.4-kb DNAs in maize are also dispersed in the maize nuclear genome. However, in our experiments no sequence homology with B1 and B2 was detected in total DNAs from maize, barley, or wheat (Sakamoto et al. 1990). The sequences of B1 and B2 themselves seemed characteristic to rice, although plasmid-like DNA in various plants may have evolved from a common ancestral sequence.

Sequence homology between mitochondrial plasmid-like DNAs and ctDNA as well as nuclear DNA has been reported (Bedinger et al. 1986; Sederoff et al. 1986). The main mitochondrial genome also contains ctDNA sequences (Stern and Lonsdale 1982; Stern and Palmer 1984). These facts suggest that transmission of genetic information has happened between organelles. However, little is known about the mechanism allowing DNA sequences to be transferred between organelles. Schuster and Brennicke (1987) assumed that sequence transfer between organelles occurred via an RNA intermediate, because most of the sequences sharing homologies between organelles were transcribed in *Oenothera*. Most plasmid-like DNAs detected in plant species are transcriptionally active (Chase and Pring 1985, 1986; Traynor and Levings 1986; Smith and Pring 1987), and B1 and B2 are also transcribed (Y. Yamada, unpublished data). Therefore, it may be possible that plasmid-like DNAs are transferred between organelles via an RNA intermediate.

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