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Structural changes in the plastid DNA of rice (*Oryza sativa* L.) during tissue culture

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Abstract To investigate the rearrangement of the plastid genome during tissue culture, DNA from rice callus lines, which had been derived individually from single protoplasts isolated from seed or pollen callus (protoclones), was analyzed by Southern hybridization with rice chloroplast DNA (ctDNA) clones as probes. Among 44 long-term cultured protoclones, maintained for 4, 8 or 11 years, 28 contained plastid DNA (ptDNA) from which portions had been deleted. The ptDNA of all protoclones that had been maintained for 11 years had a deletion that covered a large region of the plastid genome. The deletions could be classified into 15 types from their respective sizes and positions. By contrast, no deletions were found in the ptDNA of 38 protoclones that had been maintained for only 1 month. These results indicate that long-term culture causes deletions in the plastid genome. Detailed hybridization experiments revealed that plastid genomes with deletions in several protoclones were organized as head-to-head or tail-to-tail structures. Furthermore, ptDNAs retained during long-term culture all had a common terminus at one end, where extensive rearrangement is known to have occurred during the speciation of rice and tobacco. Morphological analysis revealed the accumulation of starch granules in plastids and amyloplasts in protoclones in which the plastid genome had undergone deletion. Our observations indicated that novel structural changes in the plastid genome and morphological changes in the plastid had occurred in rice cells during long-term tissue culture. Moreover, the morphological changes in plastids were associated with deletions in the plastid genome.

Key words *Oryza sativa* L. · Plastid DNA (ptDNA) Deletion · Plastid morphology

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Introduction

The culture of plant cells generates genetic variability in the cultured tissues as well as in the regenerated plants (for a review see Karp 1991). The passage of plant tissues through a culture system in vitro can induce changes in the number of chromosomes (Blackely and Stuward 1964; Ogihara and Tsunewaki 1979; Wang et al. 1992), in the extent of methylation of the genome (Ngerprasirtsiri et al. 1988; Arnholdt-Schmitt 1993) and in the copy number of the rice genome (Kikuchi et al. 1987), as well as resulting in the activation of transposons (Peschke and Phillips 1991) and the molecular reorganization of genomes (Brown et al. 1991; Paven et al. 1992).

Plastids contain their own genomes, which are distinct from those of the nucleus and the mitochondria. To-date, the entire sequence of the chloroplast genome has been determined in the liverwort (*Marchantia polymorpha*; Ohyama et al. 1986), as well as in tobacco (*Nicotiana tabacum*; Shinozaki et al. 1986), rice (*Oryza sativa*; Hiratsuka et al. 1989) and beechdrops (*Epifagus virginiana*; Kenneth et al. 1992). Comparisons among the sequences have revealed that transition-biased substitutions and additions or deletions in coding, as well as non-coding, regions have been dominant during plastid evolution but that, in general, the plastid genome has changed only slowly (Zurawski and Clegg 1987). In rice, the evolutionary variability of the plastid genome was found to be less than that of the nuclear and mitochondrial genomes (Ishii et al. 1993). Deletion of ptDNA was

reported in cytoplasmic male-sterile (CMS) lines of sorghum (Chen et al. 1993). Large deletions of ptDNA in callus or albino plants that had been regenerated from pollen cultures were reported in wheat, barley (Day and Ellis 1984), and rice (Harada et al. 1991).

Changes in genetic organization have been monitored by digestion with restriction endonucleases and hybridization analysis. In many higher plants, structural changes in the nuclear and mitochondrial genomes have been shown to be induced by cell culture in vitro (for a review see Lee and Phillips 1988). However, there are few reports on alterations in the plastid genome during tissue culture. No changes in ptDNA were found in carrot cells that had been cultured for 10 years (Matthews and DeBonte 1985). Although large deletions were found in the ptDNA of some albino variants of barley (Day and Ellis 1984), it is unclear whether or not structural changes in ptDNA are induced by tissue culture.

In a parasitic flowering plant, *E. virginiana*, the plastid genome of which lacks chlororespiratory genes (de-Pamphillis and Palmer 1990), starch accumulates in the plastids (Walsh et al. 1980). Similarly, Kobayashi (1990) reported the accumulation of starch in cultured tissues of sycamore (*Acer pseudoplatanus*). However, the relationship between the accumulation of starch and changes in ptDNA is unclear.

In the present study, we have examined the changes in ptDNA that occurred during long-term and short-term tissue culture using rice protoclones derived from single cells. The results demonstrated that the rice plastid genome underwent deletions at high frequency during long-term culture, providing the first evidence of structural changes in ptDNA during tissue culture. The deleted plastid genome of some protoclones was organized as a head-to-head or tail-to-tail structure. The site of the deletion, at least at one end of each genome, was located within a region that is known to have been involved in extensive rearrangement during the evolution of angiosperms. Electron microscopy revealed that the accumulation of starch granules in the plastids and amyloplasts was associated with deletions in the ptDNA.

Material and methods

Culture of protoclones

Rice callus protoclones derived individually from single protoplasts that had been isolated from seed or pollen callus were established. All calli and protoplasts were cultured as described by Oono (1975) and Kawata et al. (1992). Each protoclone was maintained separately by subculture at 2-week intervals on the solid medium of Murashige and Skoog (1962), supplemented with 2 mg/ml 2, 4-D. The origin and culture age of the protoclones is given in Table 1.

Southern blotting and hybridization

Total DNA was extracted from calli or seedlings by the CTAB method (Murray and Thompson 1980), and was purified by CsCl/ethidium bromide density gradient centrifugation as described by Sambrook et al. (1989). Twenty-three clones of rice chloroplast (ct) DNA, generously provided by Dr. M. Sugiura, Center for Gene Research, University of Nagoya, Japan, were used as hybridization probes. The samples of total DNA were digested with restriction enzymes in accordance with the manufacturer's specifications and fragements were separated by electrophoresis on 0.6% or 0.8% agarose gels in TAE buffer, with subsequent blotting of the DNA fragments onto a nylon membrane by the alkaline method (Reed and Mann 1985). Hybridization was carried out using an ECL gene detection system from Amersham (UK), as described by Pollard-Knight et al. (1990).

Electron microscopy

Samples were immersed in a mixture of 2% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.2) for 24 h at 4 °C. They were then post-fixed in 2% (w/v) OsO_4 in 0.1 M cacodylate-HCl buffer (pH 7.2) for 6 h at 4 °C, dehydrated in a graded ethanol series and embedded in EPON 812 (TAAB Laboratory Equipment Ltd., Reading, UK). Ultrathin sections were cut with a diamond knife on an Ultratome NOVA (LKB, Bromma, Sweden). Sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEM-1200 EX; JEOL, Tokyo, Japan).

Results

Deletion of regions of ptDNA during tissue culture

No restriction fragment length polymorphysms could be identified from the Southern hybridization patterns of ptDNA from 38 protoclones that had been cultured for 1 month (Fig. 1 A) when 23 probes, which covered the entire rice ctDNA, were used. Different culture conditions, such as culture in darkness or under fluorescent light or with different basal media, solid or liquid, did not cause any alterations in ptDNA (data not shown). Apparently the plastid genome was retained intact during culture for a short period regardless of the culture conditions. However, the ptDNA of 1 of 12 protoclones that had been maintained for about 4 years underwent deletion (Fig. 1 B), retaining only about one-third of the entire normal ptDNA. The plastid genome of all of the protoclones that had been maintained for 8–11 years had also undergone deletion (Fig. 1 C). No hybrid bands were produced by any of the 11-year-old protoclones (Fig. 1 C; lanes 22–34), clearly demonstrating that deletion of part of the plastid genome had taken place during long-term tissue culture.

To determine the frequency and extent of deletions that had occurred in the ptDNA during tissue culture, we analyzed the ptDNA of 33 long-term cultured protoclones by Southern hybridization using rice ctDNA clones that covered the entire plastid genome as probes. Quantitation of deletions in the ptDNA from various protoclones (Table 1) revealed a relationship between the frequency of deletion and increasing duration of culture. Figure 2 and Table 2 summarize the structural changes in the plastid genome that occurred during the long-term culture of protoclones. It should be noted that one end point of the deletions detected in all seed-derived protoclones (S-1, S-2, S-3 and S-4) was the same.

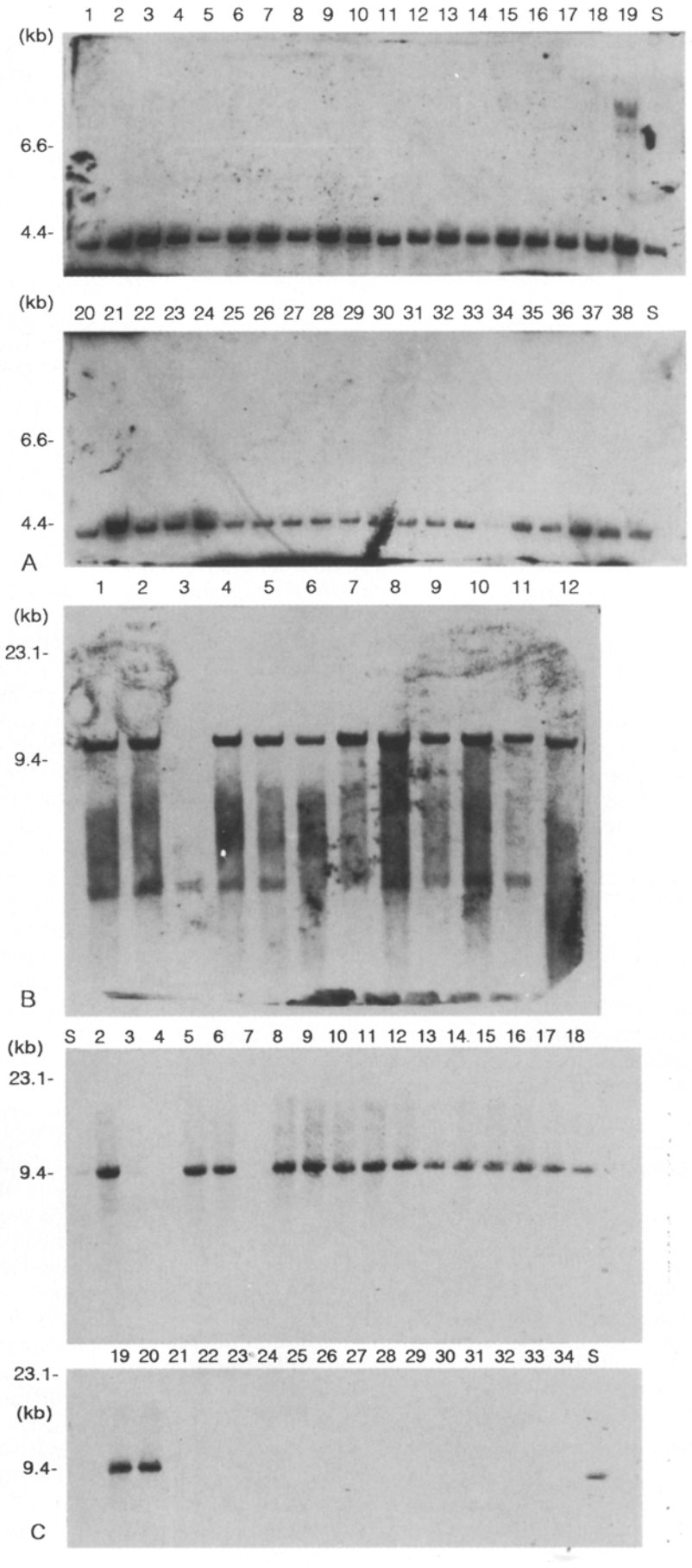

Fig. 1A–C Comparative analysis of changes in DNA in independent cell lines. **A** Thirty-eight cell lines that had been maintained for 1 month. DNA was digested with *Pst*I and probed with a 4.7-kb fragment of rice ctDNA that encoded ribosomal protein subunit 2. **B** Twelve cell lines that had been maintained for 4 years. DNA was digested with *Pst*I and was probed with a 0.8-kb fragment of rice ctDNA that included the $tRNA^{Gly}$-coding region. **C** Thirty-three cell lines that had been maintained for 8 years (*lanes* 2–21) or for 11 years (*lanes 22–34*). DNA was digested with *Bam*HI and was probed with the 2.2-kb fragment of ctDNA that encoded the 32-kDa protein of photosystem II. *Lane S* was loaded with genomic DNA from seed-grown seedlings as a control

Table 1 Frequency of deletions in ptDNA in different rice protoclones

Origin	Culture age	No. of protoclones examined	No. of protoclones with deletion	Frequency of deletion (%)
Seed	1 month	38	0	0
Seed	4 years	12	1	8
Seed	11 years	13	13	100
Pollen	8 years	20	14	70

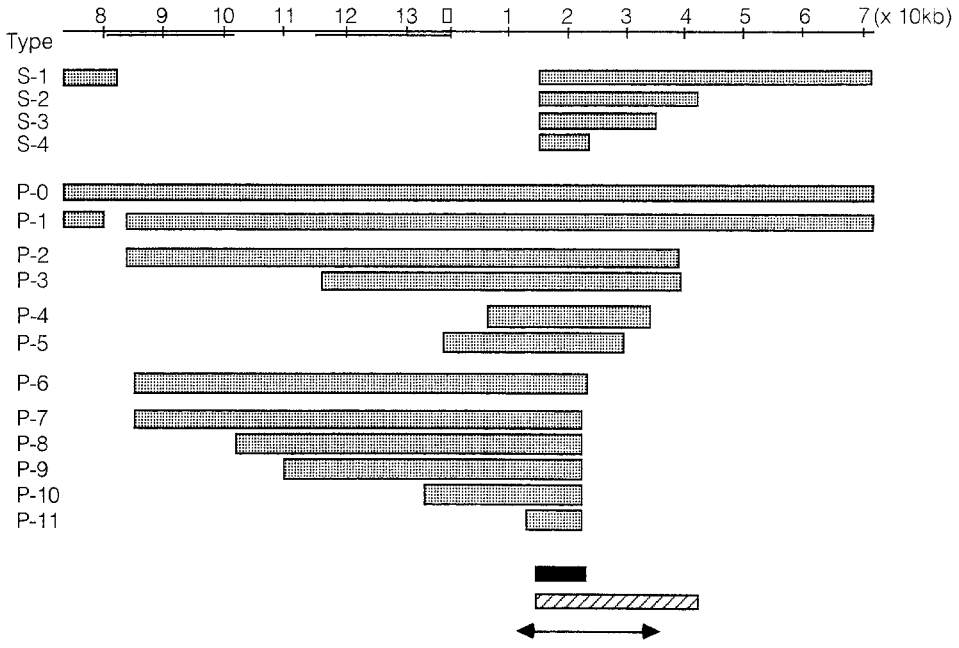

Fig. 2 Schematic diagram of ptDNAs retained in different protoclones. The unit scale represents 10 kb (after Hiratsuka et al. 1989). A *double line* shows inverted repeats. A *solid box* represents the region that was retained in all the protoclones examined. A *solid box* represents a region with in which one deletion end was frequently observed. The *double-headed arrow* represents a region in which extensive rearrangements are found when the chloroplast genomes of rice and tobacco are compared (Hiratsuka et al. 1989)

Table 2 Protoclones carrying different types of plastid genome

Origin	Type	No. of protoclones	Protoclone
Seed	S-1	1	11-3
	S-2	10	11-1, 11-2, 12-3, 12-5, 13-1, 13-3, 14-1, 14-2, 14-3, 14-4
	S-3	1	12-4
	S-4	1	12-1
Pollen	P-O	6	2-1, 3-3, 4-5, 5-5, 6-4, 8-3
	P-1	1	7-4
	P-2	2	3-2, 5-4
	P-3	1	1-4
	P-4	1	8-5
	P-5	1	1-6
	P-6	3	4-3, 7-2, 8-4
	P-7	1	2-3
	P-8	1	3-5
	P-9	1	7-1
	P-10	1	2-4
	P-11	1	1-5

Thirty percent of the pollen-derived protoclones retained the entire plastid genome (P-O), whereas the ptDNA of the remainder was subject to deletions (Table 2). The region between position 15 000 and position 22 000 was retained in all the deleted ptDNAs (Fig. 2). With the exception of P-1, one end of all deletions was located in a defined area: between positions 15 000 and 43 000 (Fig. 2). This corresponds to the region in which molecular rearrangements are known to have occurred during the evolution of angiosperms (Hiratsuka et al. 1989). All but three of the seed-derived protoclones had the same deletion (Table 2; S-2). In the pollen-derived protoclones, the deletions could be classified into ten types by reference to their respective sizes and positions.

Head-to-head organization of ptDNA in which deletions had occurred

The border structure of the S-2 type of ptDNA (see Fig. 2), which represented the major type of deletion in the seed-derived protoclones, was investigated (Fig. 3 A). When DNA of this type was digested separately with *Bam*HI, *Eco*RI, *Hin*dIII and *Xba*I, hybrid bands of fragments of 2.8, 6.6, 17.0 and 9.2 kb, respectively, were generated when the 2.0-kb fragment of ctDNA that encodes several tRNAs was used as a probe. The sizes of hybrid fragments were quite different from those expected from the known sequence of rice ctDNA, and the actual hybrid fragments were exactly twice the expected length. Therefore, the fragments that hybridized with the probe must have been organized in a head-to-head conformation. As shown in Fig. 3 B, the end

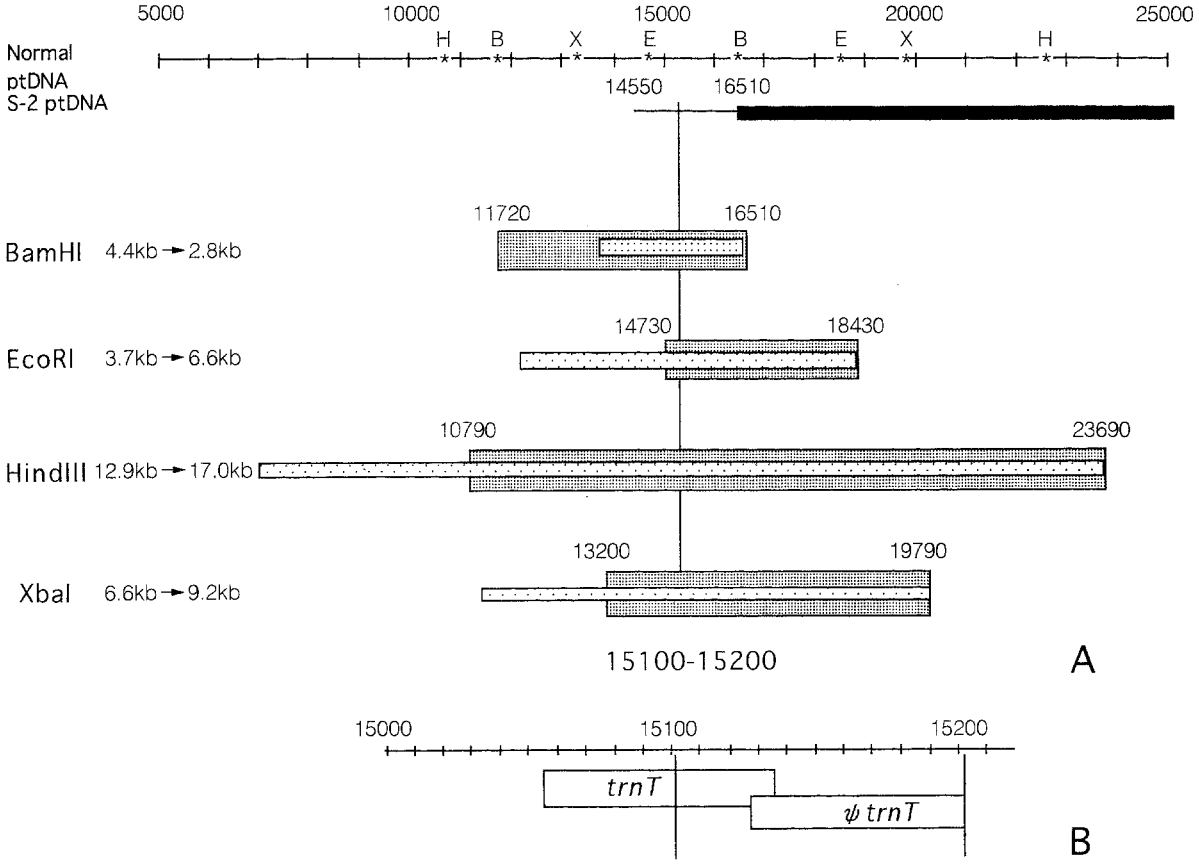

Fig. 3 **A** Restriction map of the S-2 type of ptDNA from a long-term cultured rice protoclone (see Table 2). A *thick solid bar* represents a region identical to the corresponding region of normal ptDNA. A *heavily dotted box* represents the hybrid fragment expected from the sequencing data, whereas a *thinly dotted box* represents the hybrid fragment that was actually observed. Restriction sites are indicated by the initials of the respective enzymes. The predicted and observed sizes of each restriction fragement are shown on the left. The center of all four hybrid fragments was located within a narrow region, between postions 15 100 and 15 200. **B** Detailed physical map of rice ctDNA between positions 15 000 and 15 200 (Hiratsuka et al. 1989)

point of the deletion was located between positions 15 100 and 15 200, where part of the coding region of both the *trnT* gene and its pseudogene, *ψtrnT*, are located (Hiratsuka et al. 1989). In two types of deletion, namely, S-2 and S-3, a head-to-head organization was detected at both ends, forming a head-to-head and tail-to-tail structure. Similarly, the P-3 type of ptDNA from a pollen-derived protoclone also had a head-to-head organization (Fig. 2). The remaining protoclones had a complex set of end points.

Electron microscopy

The most striking feature of the long-term cultured protoclone cells with deletions in their ptDNA was the accumulation of starch granules in the plastids (Fig. 4 B, C). By contrast, protoclone cells with a normal plastid genome had few starch granules in their plastids (Fig. 4 A). These results suggest the possible association of morphological changes in plastids with the deletion of parts of their genomes.

Discussion

Although ptDNA is a simple circular molecule and appears to be more stable than either mitochondrial or nuclear DNA during tissue culture (Rode et al. 1985; Brown et al. 1991; Paven et al. 1992), the occurrence of recombination has been documented in *Chlamydomonas* (Boynton et al. 1988) and has also been suggested in higher plants (Palmer 1983; Medgyesy et al. 1985). Although changes in ptDNA, including deletions, have been found in calli, as well as in callus-derived albino plants of wheat, barley (Day and Ellis 1984) and rice (Harada et al. 1991), their occurrence has not previously been unequivocally related to culture in vitro. The present study, using the protoclones derived individually from single protoplasts prepared from pollen or seed callus, has demonstrated for the first time that deletions in the ptDNA do occur during tissue culture.

Tissue culture acts, by itself, as an environmental stress, inducing rearrangements in plastid, mitochondrial and nuclear genomes. We found that the number of

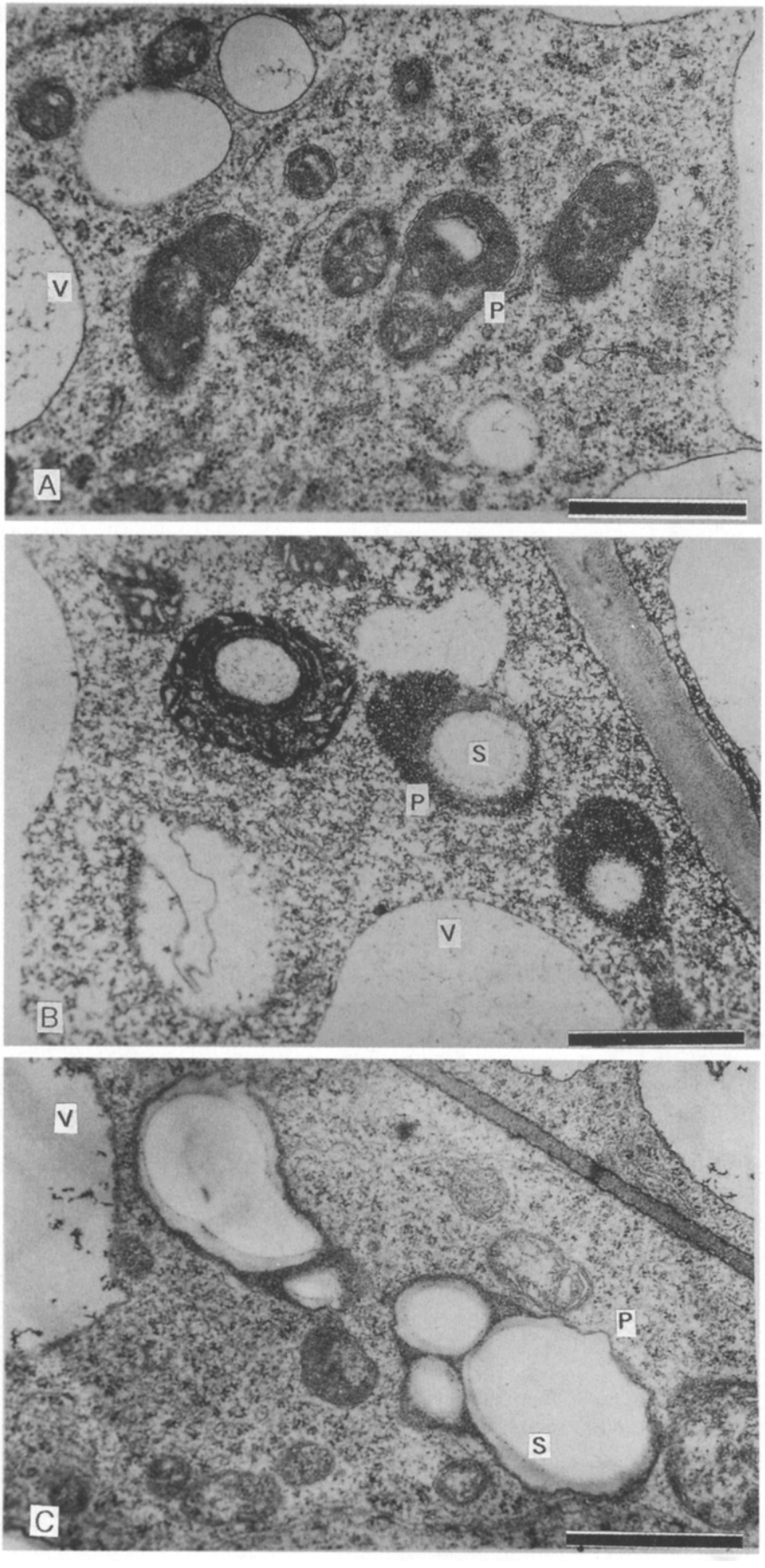

Fig. 4A–C Electron micrographs of parts of cells from long-term cultures of protoclones of rice. **A** Protoclone P-O, with a normal plastid genome. **B** and **C** Protoclones S-3 and P-5, with plastid genomes in which deletions had occurred. *P* Plastid; *V* Vacuole; *S* Starch. Bar = 1 μM

deletions in plastid genomes increased with increasing duration of the culture period. Similar observations have been reported for the nuclear and mitochondrial genomes of several higher plants (Peschke and Phillips 1992; Wang et al. 1992). Orton (1984) noted that a growing callus has metabolic requirements different from those of a mature plant, and that selection results in the loss of functionally dispensable genes. Such deletions in tissue culture may be reflected by our observations of large deletions in the ptDNA. Moreover, photosynthetic and chlororespiratory genes have been shown to have been lost from the plastid genome of a parasitic flowering plant (dePamphillis and Palmer 1990).

The present analysis of the plastid genome of rice revealed that a variety of deletions occurred during tissue culture. A common region was retained, however, in all protoclones, namely, the region located between positions 15 000 and 22 000 of the rice ctDNA, which includes the genes and pseudogenes for several tRNAs (*trnT*, *trnT*, *ψtrnE*, *trnE*, *trnY*, *trnD* and *trnC*). This region was also retained in the deleted ptDNAs of callus-derived albino rice plants (Harada et al. 1992). Clearly this region must contain sequences that are essential for DNA replication. Although putative origins of replication of ptDNA have been identified in higher plants (de Haas et al. 1986), it is unclear whether or not the region between positions 15 000 and 15 200 of rice ptDNA contains sequences necessary for its replication. It is worth mentioning, however, that this region includes the *trnT* gene for $tRNA^{thr}$ and the corresponding pseudogene, *ψtrnT*, and it corresponds to the region where rearrangements of ctDNA are known to have occurred during the speciation of rice and tobacco (Hiratsuka et al. 1989).

The deletions in ptDNA found in the pollen-derived protoclones were much more variable than those in seed-derived protoclones. It is not clear that this difference in variability is related to differences in the duration of culture or origin, although Paven et al. (1992) reported that tissue cultures derived from different explants of wheat exhibited organ- and/or tissue-specific genome changes in the mitochondrial genome.

The plastid genomes of several protoclones were organized as head-to-head and tail-to-tail structures. Ellis and Day (1986) and Harada et al. (1992) reported that the deleted ptDNA of albino plants exists as a linear molecule, which forms a hairpin or head-to-head and tail-to-tail structure. Recently, Maruta et al. (1994) obtained evidence that albinism of rice regenerants is associated with a splicing abnormality. We also found head-to-head structures in the case of some deleted ptDNAs from our rice protoclones. Taken together these results indicate that the plastid genome has an active recombination system, and that deletions of ptDNA, and/or a head-to-head conformation, are not strictly related to albinism. Further studies of the origin, function and significance of the head-to-head structure found in some deleted plastid genomes are needed.

Electron microscopy revealed that starch granules accumulated in the plastids of protoclones in which plastid genomes had undergone deletion. Although chloroplast DNA is present in amyloplasts (Scott et al. 1984) it is methylated and the genes involved in photosynthesis are transcriptionally repressed (Ngernprasirtsiri et al. 1988). Kobayashi (1990) observed the accumulation of starch in cultured tissues of *A. pseudoplatanus*, while a parasitic flowering plant, *E. virginiana*, lacks photosynthetic ability and contains amyloplastic plastids (Walsh et al. 1980). These reports suggest a possible relationship between the accumulation of starch and genetic changes in plastids. Using protoclones derived from single protoplasts, we have now for the first time demonstrated a direct relationship between the accumulation of starch granules in plastids and deletions in ptDNA, although the functional relationship between the two phenomena requires further study.

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