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Large-scale deletions of rice plastid DNA in anther culture

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Summary. Plastid DNA (ptDNA) in albino rice plants regenerated from pollen by anther culture was investigated by Southern blotting. Of the 20 albino plants investigated, 7 contained ptDNA that had suffered large-scale deletion. The size and location of the deletions differed among the plants. In all cases about 30 kbp of the region containing the PstI-2 fragment (15.7 kbp) had been retained. The deleted ptDNA molecules were retained in calluses derived from the roots of each albino plant.

Key words: Plastid DNA – Anther culture – Rice – Albino – Pollen

Introduction

Doubled haploids are of great value for providing a rapid means of obtaining pure homozygous lines. While anther culture is at present a routine procedure in a number of rice breeding programs (Oono 1981), various problems still need to be resolved in order to raise its efficiency. Albinism is a serious problem encountered in *Gramineae* anther culture due to the high percentage of albino plants generated from pollen. Therefore, in the case of rice, such breeding programs have been restricted by the low overall yield of green plants. Many factors have been found to affect the degree of albinism, such as the genotype and physiological state of the anther donor plants (Bullock et al. 1982), developmental stage of the microscope (Chen and Lin 1976), culture temperature (Huang 1984), cold pretreatment (Genovesi and Magill 1979) and sucrose concentration in combination with growth hormones (Clampham 1973). However, the primary cause of the albinism is not known.

While albino plants have been shown to possess proplastids, which are the precursors of chloroplasts (Vaughn et al. 1980), they apparently lack the 23S and 16S chloroplast ribosomal RNAs and ribulose bisphosphate carboxylase (RuBisCo) (Sun et al. 1979). The large subunit of RuBisCO and chloroplast ribosomal RNAs are known to be encoded by the chloroplast genome. As regulatory mechnisms exist for coordinating the synthesis of subunits in the nucleus and plastids (Rodermel et al. 1988), the absence of this enzyme may be due to a mutation in the nuclear genome.

On the other hand, Day and Ellis (1984, 1985) have reported deletions in the ptDNA of albino wheat plantlets regenerated from anther culture as well as changes in the nature of variant plastid genomes in albino plants derived from barley anther culture. We have investigated the nature of plastid DNA in albino rice plants from anther culture. In this paper we report that some albino rice plants generated from pollen contain large-scale deletion structures of the plastid genome similar to those of wheat and barley.

Materials and methods

Studied Plants and their culture

Rice plants (*Oryza sativa* L. cv 'Kitaake') were grown in a greenhouse. When the auricle of a flag leaf stood 4–6 cm above the auricle of the leaf beneath it, the stem, including a panicle, was sampled and stored at 10 °C for 10 days. After this cold treatment, the panicle was removed from the stem, and anthers excised from a spike were floated on 5 ml of liquid medium in a petri dish. The liquid medium was N6 basal medium (Chu 1975) supplemented with 30 g/l sucrose, 1 g/l yeast extract and 2 mg/l 2,4-dichlorophenoxyacetic acid. Some of the pollen re-

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leased into the medium grew to form calluses, which, after 25 days, were transferred to solid regeneration medium (0.8% agar) to induce the formation of shoots and roots. The regeneration medium was the same as that used for the floating culture except that it was supplement with 0.5 mg/l IAA and 2 mg/l kinetin as growth hormones. Callus induction from the regenerated plants roots was carried out by the method of Abe and Futsufara (1984). Floating culture was carried out in the dark at 25 °C, and the regeneration culture was performed under light of about 3,000 lux from a white fluorescent lamp using a day length of 16 h at 25 °C.

Preparation of total DNA

The method described by Murray and Thompson (1980) was used for DNA extraction. About 0.5 g (fresh weight) of leaves or calluses were ground with 1.0 ml extraction buffer [50 m*M* TRIS-HCl pH 8.0, 0.7 *M* NaCl, 10 m*M* EDTA, 1% (W/V) cetyltriethylammonium bromide (CTAB)] using a mortar and pestle. The mixture was incubated in a waterbath at 56 °C for 20 min and extracted with 2 ml chloroform-octanol (24:1 v/v). The aqueous phase (top layer) was recovered, a 1/10 volume of 10% (w/v) CTAB in 0.7 *M* NaCl was added, and then the chloroform-octanol extraction procedure was repeated. The recovered aqueous phase was mixed with an equal volume of precipitation buffer (50 m*M* TRIS-HCl pH 8.0, 10 m*M* EDTA, 1% CTAB) and left to stand at room temperature for 30 min. The precipitate obtained by centrifugation at 10,000 *g* for 10 min was dissolved in a CsCl/EtBr solution, and the DNA was purified by CsCl centrifugation.

Southern hybridization

Restriction fragments of total DNA were separated on 0.8% agarose gel. Southern blotting was carried out as described by Sugiura and Kusuda (1979). The probes used were pTBa13, pTBa30 and pTX6 of tobacco ctDNA (Sugiura et al. 1986) and pH10R of rice chloroplast DNA (ctDNA) (9.5-kbp HindIII fragment containing the *rbc*L gene; Moon et al. 1988). DNA labeling with digoxigenin-dUTP was carried out by means of the random priming method (Heiles et al. 1988).

Results

Lack of rbcL gene in albino plants

The mitochondrial genomes of several plant species have been shown to contain DNA sequences showing extensive homology with their ctDNA sequence (Stern and Palmer 1984). The rice mitochondrial genome (mtDNA) contains an almost complete DNA sequence homologous with the chloroplast *rbc*L-*atp*B-*atp*E-*trn*M-*trn*V gene cluster (Moon et al. 1988). For this reason the probe pH10R (9.5-kbp HindIII fragment containing the *rbc*L gene) hybridizes to the mtDNA fragments that contain this homologous sequence. In the Southern blot of HindIII-digested total DNA from green rice plants, the pH10R probe indeed hybridized to the 6.9-kbp fragment of mtDNA as well as to a 9.5 kbp ctDNA fragment. However, with regard to the total DNA from some albino plants, this probe hybridized only to 6.9-kbp mtDNA fragment (Fig. 1). These results indicate that some albino plants lack the region coding the *rbc*L gene in the plastid genome. Digestion with other restriction enzymes also confirmed the lack of the region containing the *rbc*L gene in ptDNA of albino plants (Fig. 1).

Deleted forms of ptDNA

Tobacco and rice ptDNAs have been completely sequenced (Shinozaki et al. 1986; Hiratsuka et al. 1989), and from the sequence information, gene maps of the tobacco and rise plastid genomes have been constructed. Because there is a conservation of DNA sequence homology between the two ptDNA genomes, it is possible to use tobacco ptDNA clones as hybridization probes for rice ptDNA. ptDNA from albino plants was therefore analyzed by DNA-DNA hybridization using ptDNA clones of tobacco and rice (Fig. 2). Total DNA from 12 albino plants was digested with PstI, PvuII and SalI, and

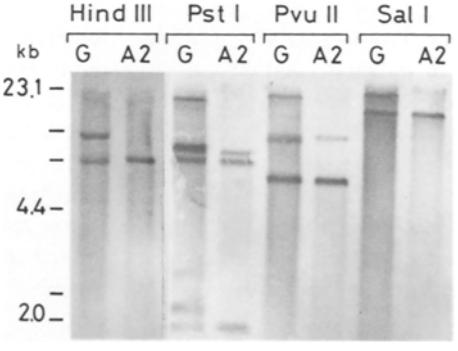

Fig. 1. Hybridization analysis of total DNA from rice albino clone 2 (A2). Total DNAs were digested with HindIII, PstI, PvuII and SalI, fractionated on agarose gel, and then transferred to nitrocellulose filters. Each filter was hybridized with the labeled 9.5-kbp fragment containing *rbc*L (pH10R). *Lane G* green plant; *lane A2* albino clone 2

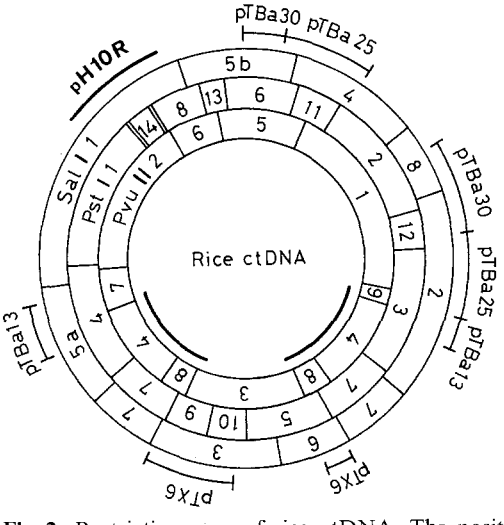

Fig. 2. Restriction map of rice ptDNA. The positions of the probes pH10R, pTBa13, pTBa25, pTBa30 and pTX6 are indicated

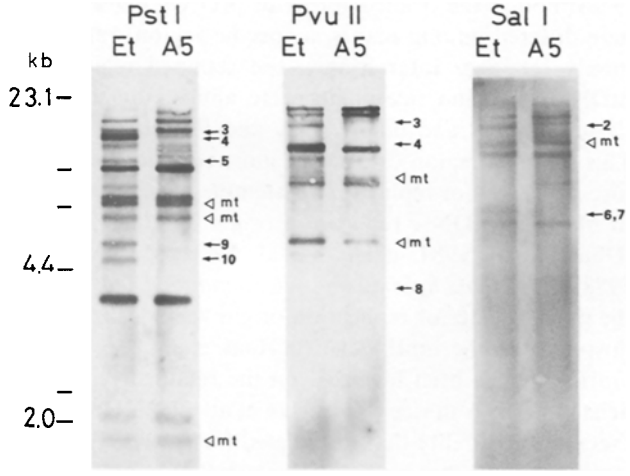

Fig. 3. Hybridization analysis of total DNA from rice albino clone 5 (A5). Total DNAs were digested with each enzyme, fractionated on agarose gel, then transferred to a nitrocellulose filter and hybridized with labeled probe pH10R, pTBa13, pTX6 and pTBa30. Fragment numbers not detected in the albino plant are shown by *arrows*. *mt* Fragment(s) of mtDNA (Fig. 1). *Lane Et* Etiolated plant; *lane A5* albino clone 5

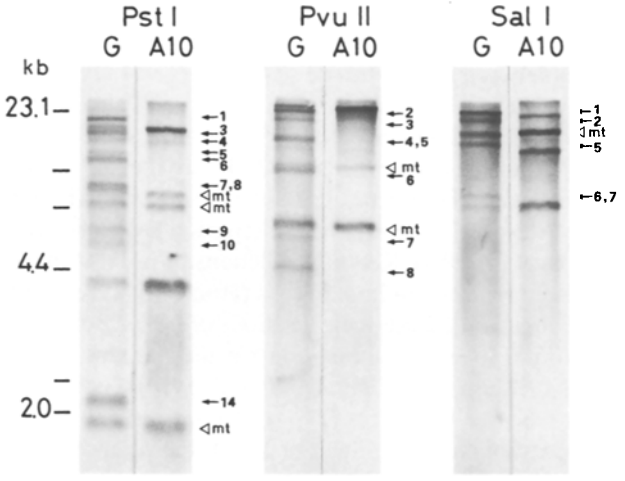

Fig. 4. Hybridization analysis of total rice DNA from albino clone 10 (*lane A10*). Electrophoresis, hybridization and probes used were identical to those in Fig. 3.

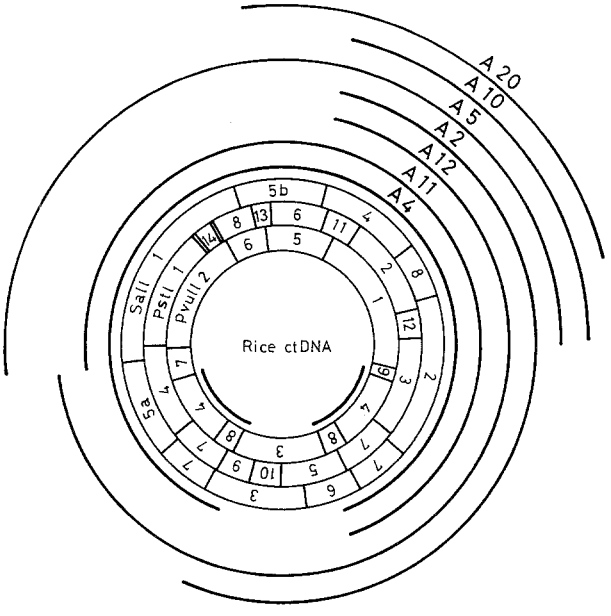

Fig. 5. Diagram summarizing retained ptDNA regions in rice albino plants. The retained regions in each of the albino plants are shown *outside* the ptDNA restriction map

then the Southern-blotted filters were hybridized with the probes. The absence of some ptDNA restriction fragments was detected in 7 albino plants. In the case of albino clone 4 (A4), the fragments located at the small single copy (SSC) region were not detected (data not shown). Albino clone 5 (A5) lacked the fragments corresponding to the inverted repeats and SSC regions (Fig. 3). The most marked deletion of ptDNA was observed in albino clones 10 (A10) and 20 (A20), in which fragments corresponding to a region of about 100 kbp of ptDNA were not detected (Fig. 4). Some fragments which were not present in the control were observed in the albino clones. These fragments appeared to be new fragments generated by the deletion. The results of these experiments are summarized in Fig. 5. The deleted regions of the plastid genome were different in each albino plant. All of the altered ptDNA in the albino plants examined retained the region containing the PstI-2 fragment (15.7 kbp).

When the calluses induced from roots of albino plants were transferred to the regeneration medium, all of the regenerated plants became albino. Hybridization analysis revealed that these calluses and albino plants possessed almost the same deleted form of ptDNA as their original plants. Thus, the molecules of deleted ptDNA can be maintained in callus tissues. Characterization of the deleted ptDNA structure is now in progress.

The deleted ptDNA molecules were also detected in some of the calluses derived from pollen by floating anther culture. The deleted fragments of ptDNA were not detected in 12 of the 20 albino plants. However, we could not exclude the possibility that small internal changes had taken place within restriction fragments of apparently unaltered sizes.

Discussion

The presence of deleted ptDNA has been detected in albino rice plants derived from anther culture; this agrees with results from wheat and barley anther cultures (Day and Ellis 1984, 1985). The presence of mtDNA molecules that have suffered large-scale deletions has also been

found in higher plants. These subgenomic molecules are known to arise through intra-molecular recombination events between large repeated sequences (1–14 kbp), giving the genome a multipartite structure (Palmer and Shields 1984; Lonsdale et al. 1984; Stern and Palmer 1986; Siculella and Palmer 1988; Fauron et al. 1989). However, such large repeated sequences are absent in rice ptDNA except for one inverted repeated sequence (Hiratsuka et al. 1989). Recently, deletions of mtDNA have also been reported in human cells (Holt et al. 1988), and a major cause of large-scale deletions of DNA molecules was found to be recombination via the flanking direct repeats (Schon et al. 1989; Mita et al. 1990). As several short repeated sequences (11–17 bp) are present in rice ptDNA (Shimada and Sugiura 1989), the ptDNA deletions may also arise from intra-molecular recombination(s) between these repeated sequences.

Our results showed that the ptDNA is highly conserved in the course of tissue culture; this agrees with results from numerous other studies (Chowdhury et al. 1988; Kemble et al. 1988; Rode et al. 1985). In fact, we have not yet detected any variation of ptDNA in calluses derived from seed. Therefore, the deletion of ptDNA can be considered to be a specific phenomenon of anther culture. Vaughn et al. (1980) reported organelle alteration in pollen-derived albino rice plants as observed by electron microscopy. Miyamura et al. (1987) demonstrated that plastid and/or mitochondrial genomes disappear through division of the male generative cell of *Triticum aestivum* during development of the mature pollen grain from a monocellular pollen grain. Day and Ellis (1984) suggested that ptDNA deletions in albino pollen-derived plants may result from a mechanism similar to the maternal inheritance of chloroplasts (Kuroiwa et al. 1982; Vaughn et al. 1980). Thus, there is a possibility that the high yield of albino plants in rice anther culture is correlated with a normal cytological phenomenon in pollen.

In plastids which have large-scale genome deletions, it is considered that protein synthesis activity no longer remains. Oelmüller and Mohr (1986) reported that the inhibition of chloroplast protein synthesis in mustard cotyledons with chloramphenicol also inhibited the transcription of nuclear genes coding for integral chloroplast proteins. Burgess and Taylor (1988) proposed that a signal of chloroplast origin is a necessary component for optimal transcription of nuclear genes encoding chloroplast proteins. From these report it is reasonable to conclude that plastids containing deleted DNA result from a deficiency in the ability of plastids to differentiate into chloroplasts. In addition, from our results and those of earlier experiments (Hagemann and Borner 1978; Scott et al. 1982; Sasaki and Kuroiwa 1988), we suggest that plastid genes are not important for the amplification of ptDNA and the division of plastids.

Although the deleted forms of ptDNA investigated here differed among plants, a specific region was commonly retained. Interestingly, the retained regions of ptDNA in albino rice plants were almost the same as those in wheat and barley (Day and Ellis 1984, 1985). This retained region of ptDNA must contain the structure necessary for replication. Attempts have been made to map the ptDNA replication origin in higher plants (Ohtani et al. 1984; deHaas et al. 1986; Meeker et al. 1988). In *Petunia hybrida* and *Nicotiana tabacum,* one of the putative sites of replication origin was localized just upstream of the *atp*H gene (deHaas et al. 1986). This *atp*H gene has been localized on the retained region of deleted ptDNA in rice (Hiratuska et al. 1988). However, there is a possibility that the deleted forms arise from an intact molecule that is present at a very low copy number and possess a replication system different from that of wild-type genomes (Ellis and Day 1986). By characterizing these deleted ptDNAs, it may be possible to elucidate the interaction between the nucleus and plastome. Characterization of the ptDNA structure in albino clone 20 (about 32 kbp) is currently in progress.

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