Mol Gen Genet (1992) 233:145–150

MGG

Pollen-derived rice calli that have large deletions in plastid DNA do not require protein synthesis in plastids for growth

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Received October 21, 1991

Summary. Albino rice plants derived from pollen contain plastid genomes that have suffered large-scale deletions. From the roots of albino plants, we obtained several calli containing homogeneous plastid DNA differing in the size and position of the deletion. Southern blotting and pulsed field gel electrophoresis experiments revealed that the DNAs were linear molecules having a hairpin structure at both termini, existing as monomers (19 kb) or dimers, trimers and tetramers linked to form head-to-head and tail-to-tail multimers. This characteristic form is similar to that of the vaccinia virus, in which the replication origin is thought to lie at or near the hairpin termini. Furthermore, polymerase chain reaction experiments revealed complete loss of the ribosomal RNA genes of the plastid DNA. The results suggest that plant cells can grow without translation occurring in plastids. All of the deleted plastid DNAs commonly retained the region containing the $tRNA^{Glu}$ gene (*trnE*), which is essential for biosynthesis of porphyrin. As porphyrin is the precursor of heme for mitochondria and other organelles, it is considered that *trnE* on the remnant plastid genome may be transcribed by an RNA polymerase encoded on nuclear DNA.

Key words: Rice – Albino – Plastid DNA – Linear DNA – $tRNA^{Glu}$

Introduction

The main function of plastids in higher plant cells is considered to be photosynthesis in leaves. The plastids are unique organelles in that they are able to undergo drastic changes in morphology and function according to the direction of cell differentiation (Kirk and Tilney-Bassett 1978). As most higher plant cells are totipotent, plastids unrelated to photosynthesis (i.e. amyloplasts, chlomoplasts, leucoplasts and so on) must maintain the capability to differentiate into chloroplasts (Possingam 1980). Plastids have their own DNA, which encodes all of their own tRNA and rRNA molecules and some of the proteins required by their genetic apparatus or for photosynthesis (Sugiura 1989). Therefore, all types of plastid must contain a complete plastid genome, which regulates gene expression by methylation or translational mechanisms (Ngernprasirtsiri et al. 1988; Deng and Gruissem 1988). Recently, dePamphilis and Palmer (1990) have reported that the plastid DNA of a parasitic flowering plant (*Epifagus virginiana*) has lost numerous photosynthetic genes, while retaining rRNA, tRNA and ribosomal protein genes and some open reading frames. Thus, they suggest that the remnant genome may express one or more gene products for plastid function unrelated to photosynthesis. In plants, algae and some photosynthetic bacteria, 5-aminolevulinic acid, the precursor of porphyrin, is formed from the intact carbon skeleton of glutamine, in a process requiring three enzymatic reactions and the $tRNA^{Glu}$ of plastids (Schön et al. 1986). Howe and Smith (1991) suggest that the remnant genome of the achlorophyllous plant produces the RNA polymerase necessary for the transcription of $tRNA^{Glu}$ to produce heme for mitochondria and other organelles.

The plastid DNA in albino plants regenerated from pollen of wheat and barley has undergone deletion (Day and Ellis 1984, 1985). The deleted plastid DNA can be a linear molecule containing hairpin termini and occur in monomer or dimer conformation (Ellis and Day 1986). However, more detailed information about these molecules is not yet available. Albino rice plants obtained from pollen also have deleted plastid DNA (Harada et al. 1991). As the rice plastid genome has been completely sequenced (Hiratsuka et al. 1989), more information about these deleted forms can be readily obtained. If an intact albino plant is used as the source material for DNA preparation, the elucidation of the structure of the deleted DNAs may be confused by the heterogeneity of deleted forms (Day and Ellis 1985). A relatively rapid sorting out of heterogeneous plastid DNA occurs by vegetative segregation during the culture of callus

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tissue (Pelletier et al. 1983). Therefore, we have induced calli from the roots of albino rice plants. After several subcultures of these calli, we were able to obtain a sample which, on the basis of Southern blotting, appeared to contain a homogeneous deleted plastid DNA. In this report, we focus on characterization in the rice callus of plastid DNA with large-scale deletions and discuss its putative replication system and residual function.

Materials and methods

Plant materials. The callus lines were derived from the roots of albino rice plants regenerated by anther culture (Harada et al. 1991). The lines were maintained for 28 days on 0.8% agar medium, N6 basal medium supplemented with 30 g/l sucrose, 1 g/l yeast extract and 2 mg/l 2,4-dichlorophenoxyacetic acid.

Southern blotting. Total DNA was isolated from callus using the method described previously (Harada et al. 1991). To determine the position and extent of the deleted region, total DNA was digested with restriction enzymes, and fractionated on a 0.8% agarose gel. After partial depurination of the DNA in the gel and transfer to a nylon membrane, Southern hybridization analysis was carried out using an Amersham ECL gene detection system (Pollard-Knight et al. 1990). Probes used were 13 clones of rice chloroplast DNA kindly provided by Dr. M. Sugiura.

Pulsed field gel electrophoresis (PFGE). About 0.1 g of callus was ground to a fine powder in liquid nitrogen and the powder mixed with 1 ml 0.7% low melting agarose (in 10 mM TRIS-HCl, pH 7.8, 15 mM NaCl, 200 mM EDTA). The mixture was poured into a mold and the plugs incubated at 50° C in 10 mM TRIS-HCl, pH 8.0, 50 mM EDTA, 1% Sarkosyl, 1 mg/ml proteinase K for 16 h (Guidet et al. 1990). The low melting agarose with the embedded callus DNA was excised and analyzed by PFGE with a CHEF-DRII apparatus (Biorad). The gel (1% agarose in 0.5 × TBE; 1 × TBE is 0.9 M TRIS-borate, 2 mM EDTA pH 8) was subjected to electrophoresis in 0.5 × TBE at a constant temperature of 9° C, with 45 s pulse time, at 200 V for 12 h.

Alkaline electrophoresis. Total DNAs digested with restriction enzymes were dissolved in 50 mM NaOH and 1 mM EDTA; the DNA was then fractionated on a 0.8% alkaline agarose gel as described by Sambrook et al. (1989).

Polymerase chain reaction (PCR) experiments. DNAs (0.4 μg) were used for the PCR reaction with 1 μmol primers, for 25 cycles (94° C, 1.5 min; 55° C, 2.5 min; 72° C, 4 min), with Cetus *Taq* polymerase and buffer. About 10% of the total PCR reaction was loaded on 3% NuSieve agarose. Primers are listed 5′–3′ with the arrow designating orientation of the sequence: A,→(CTTAAATGTGTTTAGTATTTAGTAGCCCGA); B,←(CAGCTTTTAAAGGGAAGGGAGATAGACTG);C,→(CATAGTGGCAACTAAACACGAGGGT);D,←(TCATAGTTGCATTACTTATAGCTTC). The primer positions used are followed by their coordinates from the rice chloroplast genome sequence in the EMBL database (no. X15901): A, 12121–121150; B, 12602–12630; C, 122736–122760; D, 123821–123845.

Results

Linear conformation of deleted plastid DNA

Figure 1 shows the regions retained in the deleted form of plastid DNAs in each callus. Though some initial calli had heterogeneous plastid DNA, after several subcultures of these calli the DNA showed homogeneity. This result was considered to be due to the sorting of heterogeneous plastid DNA during callus tissue culture (Pelletier et al. 1983). The restriction enzyme sites of these retained regions were the same as those of a seed-derived callus, and agreed with a restriction map constructed from sequence data (Hiratsuka et al. 1989).

We performed a more detailed analysis of the deleted plastid DNA from callus line 5 using Southern blotting (Fig. 2). The non-digested plastid DNA separated by standard agarose gel electrophoresis was detected as two fragments, 19 and 38 kb in size. Furthermore, DNA fractionation by PFGE demonstrated monomeric to trameric lengths of the 19 kb unit (Fig. 2B). *Pst*I-digested DNA was also found to consist of two fragments, which differed in size from those of the controls (Fig. 2A). Two such fragments were detected after digestion with other restriction enzymes, as long as the probe used contained region A shown in Fig. 1. The differences in size between the control and the smaller, deleted fragments that hybridized to the 0.8 kb *Hin*cII fragment

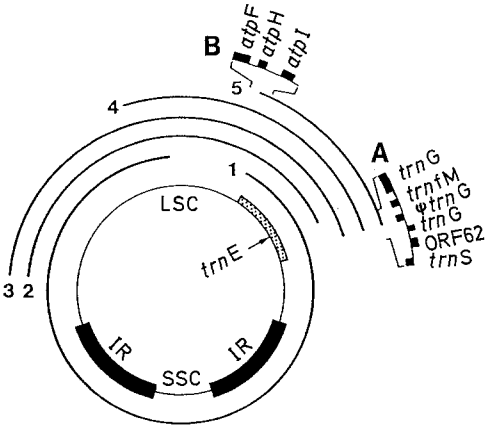

Fig. 1. Retained regions of the deleted form of plastid DNA in each callus (*curved lines 1–5*). Line 5 indicates the region for the callus line described here. The *inner circle* represents the rice plastid genome. The inverted repeats are indicated by *bold boxes*. LSC and SSC indicate the large and small single copy regions, respectively. The *dotted area* shows the region of the pRP2 clone, next to *trnE* (tRNA^{glu} gene). A and B denote regions described in the text

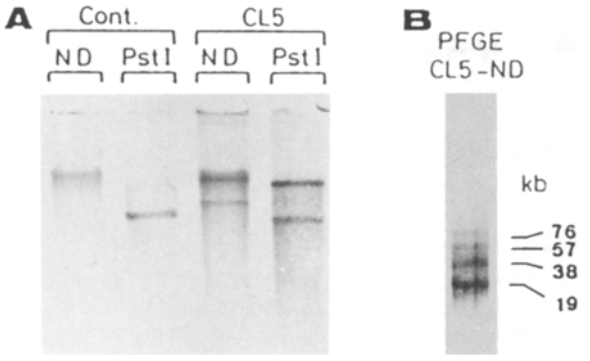

Fig. 2. **A** Southern blot of pRP2-homologous DNA in CL5 and control callus. Blotting and hybridization were carried out as described in Materials and methods. ND, not digested. The probe used was the pRP2 clone (Fig. 1). **B** The sizes of the two fragments in lane CL5-ND were determined to be 19 and 38 kb in another experiment using 0.3% high gel-strength agarose (Nippon-Gene AgaroseH) and pulsed field gel electrophoresis (PFGE)

probe (Fig. 3B) could all be accounted for by assuming that the 5′ region of *trnG* (dotted area in Fig. 3) had been lost. Therefore, we concluded that the molecules of deleted plastid DNA in callus line 5 were linear, and that one of the telomere sites was located in *trnG*. A search was made for the other telomere site using Southern blotting. The probes containing region B shown in Fig. 1 also hybridized with two fragments in the DNA after digestion with each of several restriction enzymes. The change in size of these fragments could be attributed to loss of the region including *atpH*, on physical map, the 3′ side of *atpI* (Hiratsuka et al. 1989), and hence we concluded that the other telomere site was on the 3′ flank of *atpI* (data not shown).

Multimeric configuration and hairpin terminal structure

As shown in Fig. 3B, the larger hybridizing fragments of the deleted forms produced by each enzyme were exactly twice the length of each corresponding terminal fragment. These fragments did not hybridize with any DNA clones from other regions. Furthermore, the same results were obtained from experiments using the probe for the other telomere. These data suggest that the larger fragments comprise multimers of the basic structure in inverted orientation. As the lengths of the intact larger molecules were also exactly 2, 3, and 4 times the length of 19 kb (Fig. 2), we concluded that callus line 5 contained head-to-head and tail-to-tail multimeric molecules in addition to the 19 kb monomer molecule.

Figure 4 shows the results of Southern blot experiment performed on DNA digested with three restriction enzymes and fractionated under alkaline conditions.

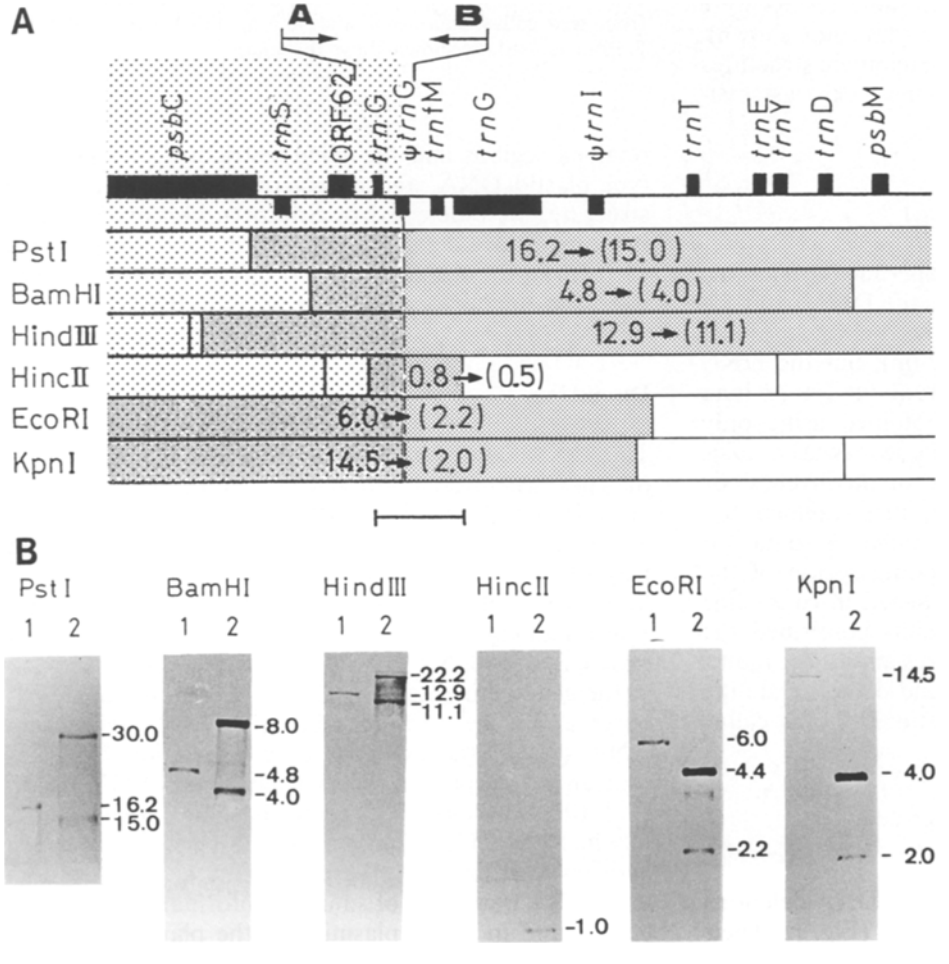

Fig. 3A, B. A restriction map of the terminus of the deleted plastid DNA in callus line 5 and results of Southern hybridization using the *Hin*cII 0.8 kb fragment as a probe. **A** A restriction map constructed from sequence data of rice chloroplast DNA. Locations of genes and the positions of primers A and B (Fig. 6) are shown above the map. The *vertical dashed line* indicates the terminus and the junction point of monomers in multimeric forms. The region of the probe used is shown below the map. The sizes of fragments that should hybridize with the probe are indicated in kb, and the sizes of terminal fragments are in *parentheses*. **B** Lanes 1 and 2 for each restriction enzyme show the hybridization pattern of total DNA from seed callus, and that from callus line 5, respectively. The sizes of the fragments detected in each lane are shown in kb

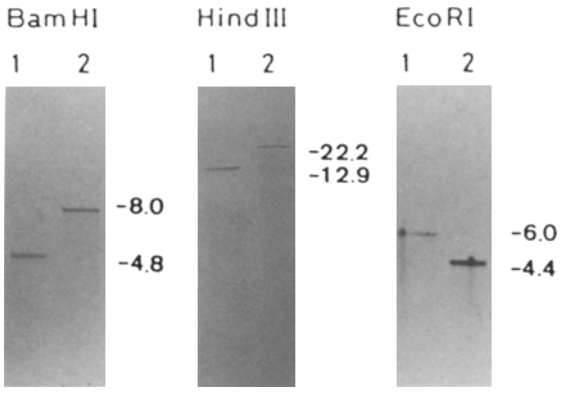

Fig. 4. Electrophoretic analysis under alkaline conditions of the terminal structure of the deleted plastid genome in callus line 5. Restriction enzyme digested DNAs were subjected to alkaline electrophoresis, Southern blotting and hybridization with the 0.8 kb *Hin*cII fragment. Lanes 1, seed callus; lanes 2, callus line 5

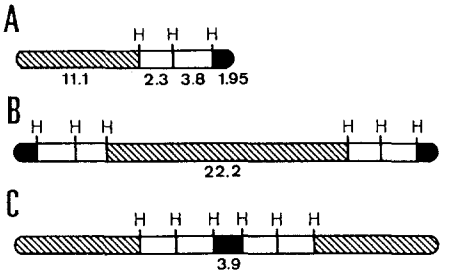

Fig. 5A–C. The structures of deleted plastid DNA in callus line 5. **A** Structure of monomer; **B** structure of head-to-head dimer; **C** structure of tail-to-tail dimer. Terminal and concatemeric fragment sizes are indicated in kb. H, *Hin*dIII restriction site

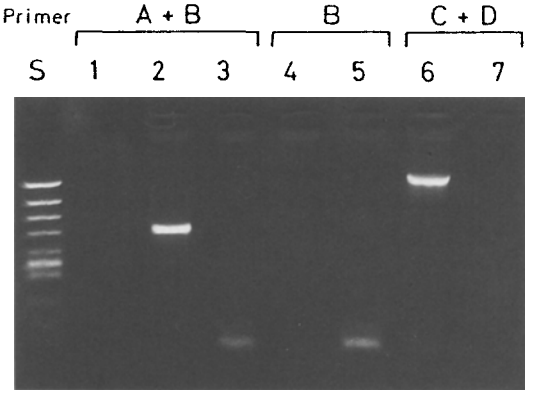

Fig. 6. Polymerase chain reaction (PCR) analyses of the deleted plastid DNA. Lane 1, H_2O control; lanes 2, 4 and 6, total DNA from seed callus; lanes 3, 5 and 7, total DNA from callus line 5. Primers used are shown above the lanes

Though the internal multimeric fragments (larger fragments in Fig. 3B) were clearly detected, the smaller fragments present under neutral fractionation conditions were not evident. However, if one terminus of such a fragment were a covalently closed hairpin, then the size of the fragment would double in the alkaline agarose gel (Sambrook et al. 1989). Thus, it was concluded that the smaller fragments have a hairpin structure and double in size under denaturing conditions, thus overlapping with the multimeric fragments. The same results were obtained for the other telomere site (data not shown). Thus, it was revealed that both of the telomere structures of the linear plastid DNA in callus line 5 exist as covalently closed hairpins (Fig. 5).

Complete loss of wild-type plastid DNA

We then carried out amplification experiments using the PCR. Using primer A+B (Fig. 3A), the DNA from the callus derived from control seed showed clear amplified fragments of the expected size (569 bp), but the DNA from callus line 5 showed a fragment about 130 bp long (Fig. 6). This fragment was also obtained using only primer B. These results reveal that the sequence containing primer B is located at the site of an inverted or hairpin repeat. Furthermore, the amplified fragment hybridized to the probe containing terminal A (data not shown). On the other hand, the coding region of the 16S rRNA gene (*rrs16*) was not detected in callus line 5 (Fig. 6, primer C+D). These results confirmed the presence of telomere hairpin structures and of the multimeric form in inverted orientation and demonstrate the complete absence of wild-type plastid DNA in callus line 5.

Other types of deleted plastid DNA

We obtained calli that contained plastid DNA deletions in regions differing from those in line 5 (Fig. 1). Their retained regions had the same restriction sites as wild-type plastid DNA, and their termini and concatemeric structures were detected by Southern blot experiments. Although more detailed experiments have not yet been performed, these deleted plastid DNAs were considered to have a similar form to those of callus line 5.

Discussion

The DNA conformations characterized in this study (hairpin structures at both termini and head-to-head and tail-to-tail multimeric forms) were basically the same as those of deleted plastid DNA from barley (Ellis and Day 1986; Collin and Ellis 1991). Quite a lot of information on rice plastid DNA is available (Hiratsuka et al. 1989), making the structure of deleted forms simple to reveal. Similar DNA conformations have been reported in the genome of vaccinia virus. Electron microscopical observation and in vivo labeling of early replicating DNA suggest the existence of a replication origin at or near the hairpin terminus (Esteban et al. 1977; Pongo et al. 1981). Baroudy et al. (1983) have proposed a model in which the terminal hairpin structure forms a free 3′-hydroxyl end and can serve as a primer for DNA replication. DNA molecules of similar conformation have also been found in linear plasmids of the plant pathogenic

fungus *Rhizoctonia solani* (Miyashita et al. 1990), and a model for DNA replication has been proposed based on that for vaccinia virus DNA. As initiation replication from the hairpin telomere accounts for the presence of head-to-head and tail-to-tail multimers, these replication systems are of immense interest. However, the replication mechanism of genomes having hairpin termini are still ill-defined, and the deleted plastid DNA contained the region upstream of the *atpH* gene, which is one of the putative replication origin sites of plastid DNA in higher plants (deHaas et al. 1986). Additional studies will be needed to determine whether the hairpin termini of the DNA are active replication origins in these deleted forms of plastid DNA.

Recently, it has been reported that wild-type plastid DNA is also linear and includes monomeric to oligomeric lengths of the unit genome (Deng et al. 1989; Bendich and Smith 1990). Interestingly, a similar oligomeric series of DNA molecules has been observed in the replication process in temperature-sensitive mutants of a vaccinia virus (Delange 1989; Merchlinsky and Moss 1989). Though a circular map has been constructed for the plastid genome of many plants, circular DNA molecules could be derived from linear molecules with circularly permuted sequences as in the T4 bacteriophage. Therefore, there is a possibility that the molecule investigated in this report is one of the natural linear molecules. The PCR amplified fragment of the deleted plastid DNA in the clone 5 callus was not detected in the callus containing wild-type plastid DNA. However, this result may not exclude the possibility that the deleted forms arise from an intact molecule that is present in very low copy number. More detailed analyses are required.

The quantity of deleted DNA was almost the same as in controls, suggesting that the retained region contains a physiologically functional gene. However, the plastid genome lacks nearly all the genes encoding its own translational apparatus (i.e. rRNA and tRNA genes). Even if some gene(s) on the remnant DNA were still functional, it is impossible that the gene product could fulfill its function as a translated product in the plastid. In plants, the formation of 5-aminolevulinic acid from glutamate during the biosynthesis of porphyrin requires activation of glutamate by a plastid-encoded tRNA (Schön et al. 1986; Smith 1988). A ligase and two other enzymes involved in this reaction are encoded by the nuclear genome (Kannangara et al. 1988; Grimm 1990). All the deleted forms of plastid DNA that have been characterized in rice indeed retain the *trnE* gene region (Hiratsuka et al. 1989; Harada et al. 1991). Furthermore, the deleted molecules of plastid DNAs in barley and wheat invariably retain the region corresponding to the *trnE* locus of rice (Day and Ellis 1984, 1985). We suggest that the function of the remnant plastid genome is to produce $tRNA^{Glu}$, which is essential for heme production in mitochondria and other organelles. Although the chloroplast *rpo* genes (*rpoA*, *rpoB* and *rpoC*) are functional and these polypeptides are functional components of the RNA polymerase (Hu and Bogorad 1990), it has been reported that another type of RNA polymerase is contained in plastids (Zaitlin et al. 1989). The molecules of $tRNA^{Glu}$ in plastids may be transcribed by an RNA polymerase encoded by the nuclear genome. In the present study, we used callus tissue as the experimental material, and it was not clear whether the callus still had totipotency. We conclude that plant cells can at least undergo growth without the translation system present in plastids. One or more proteins encoded by the plastid genome may be essential for differentiation into a mature plant.

Acknowledgements. We thank Dr. M. Sugiura for supplying rice chloroplast DNA clones. We are also grateful to Dr. Y. Kikuta, Dr. T. Mikami and Dr. T. Wakasugi for valuable suggestions. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

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Communicated by R. Hagemann