Ultrastructural and molecular characterization of altered plastids in nuclear gene controlled yellow stripe mutant of *Pennisetum americanum*

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The ultrastructural and molecular biological studies were conducted to elucidate the changes in the nuclear gene controlled plastid alterations in yellow stripe mutant of *Pennisetum americanum*. The plastids in yellow tissue were bound by a double membrane envelope and no internal thylakoid membrane differentiation, whereas plastid development was normal in the green tissue. The nuclear gene apparently influences the individual plastids as evidenced by the presence of heteroplastidic cells. Ribosomal RNAs were extracted from green and yellow seedlings with homozygous recessive and heterozygous genotypes. Plastid specific 23S and 16S rRNAs were absent in the yellow seedlings irrespective of the genotype. The presence of disorganized lamellar membranes in the rRNA/ribosome deficient plastids indicate that they are synthesized outside the plastid compartment. Restriction endonuclease analysis revealed no detectable differences in fragmentation pattern of the plastid DNAs from green and reverted green seedlings. This is consistent with the suggestion that the nuclear gene is not inducing any change in the plastid genome size and in the restriction enzyme recognition sites.

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The widespread recognition of the physiological function of the plastids as the site of photosynthesis has far too long overshadowed the importance of the plastid as an organelle worthy of study for itself. With the advent of modern techniques and biochemists' growing appreciation for structure/function relationships, the emphasis has shifted towards studies designed to provide information on the plastid itself. Both nuclear and plastome genes are known to influence plastid development. But the identification of specific role(s) of cytoplasmic and nuclear genes in the biogenesis of plastids is a complex puzzle. A direct approach to this problem would be to identify the transcriptional and translational products of organelle and nuclear DNAs. A beginning has been made in this direction with the hybridization of RNAs (plastid specific rRNAs, tRNAs, and mRNAs) to the plastid and/or to nuclear DNAs (see SAGER 1972). The experimental approach to identify plastid specific proteins has not been notably successful (PARTHIER 1982). Studies with isolated organelles (ELLIS et al. 1973) revealed that the incorporation of labelled amino acids into polypeptides was slow and did not give meaningful results. Studies of intact cells by means of antibiotics (BOULTER et al. 1972; ELLIS et al. 1973) have provided some indirect evidence of protein synthesis either on cytoplasmic or on plastidic ribosomes. Such studies, however, did not provide conclusive information for which they were designed.

The use of mutants is potentially the most powerful method to identify the origin of specific proteins and to dissect the process of plastid biogenesis. Plastid DNA contains a selected set of genes, each of which presumably carries the essential information for an organelle development. Thus, the investigation of a mutant in plastid DNA should provide direct information on it as a source of altered protein(s) and the role of cytoplasmic genes in organelle biogenesis. The nuclear gene mutations affecting plastid function have shown complex pleiotropic phenotypes obscuring the primary effect of the mutation (HAGEMANN and BÖRNER 1978). Identification of the proteins coded by nuclear genes has thus far been a frustrating problem since a large number of nuclear genes seem to be involved in the

regulation of organelle development (PARTHIER 1982) and function. In this article, we present an ultrastructural and molecular study of changes associated with nuclear gene induced altered plastids and their relationship with the genetic basis of plastid alteration and their mode of transmission in the overall development.

Materials and methods

The stripe mutant (700430) was selfed and crossed with normal inbred lines of IP 7939 and IP 9382, taking the advantage of protogyny (BURTON 1980). The selfed and crossed progeny were grown in plastic trays inside a glass house.

Electron microscopy

Fully expanded green, stripe and white leaves were cut into 1–3 mm² pieces and immediately fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer pH 7.2 with 0.5 % sucrose, and kept overnight. The tissue was washed with cold phosphate buffer for 30 min and post-fixed in 2 % osmium tetroxide in phosphate buffer for 4 h. Samples were washed with water, and dehydration was carried out with a graded acetone series (30 %, 50 %, 70 %, 90 %, and 100 %). Later, the leaf material was transferred and infiltrated with 1:1 mixture of acetone and Spurr for 1 h and kept overnight in fresh Spurr. The samples were then embedded in Spurr's low viscosity epoxy resin. Ultrathin sections were cut on a Reichert-Jung ultramicrotome with a diamond knife and mounted on 200 mesh copper grids. Sections were stained with 2 % aqueous uranyl acetate for 15 min, and the excess stain was washed off with distilled water. The sections were restained with lead citrate for 6 min. Excess stain was removed from the sections and examined under Philips 201 C transmission electron microscope.

Isolation of Ribosomal RNA

Ten days old seedlings grown in plastic trays were cut to the base, washed with distilled water, adherent water being removed by blotting onto a filter paper, and immediately frozen in liquid nitrogen and ground with chilled mortar and pestle. The nucleic acids were extracted following the method of ROSEN and MONAHAN (1984) except for the additional use of diethyl pyrocarbonate. The ground tissue was mixed with 3 ml/g of isolation buffer (100

mM Tris-HCl pH 8.0, 100 mM EDTA, 1 M NaCl, 2.4 % SDS and 2 % of diethyl pyrocarbonate) and kept in an ice-bath with occasional shaking. After 10 min, an equal volume of buffer saturated phenol was added. The emulsion was further incubated in ice-bath for 30 min with gentle shaking. The emulsion was centrifuged for 20 min at 10,000xg. The aqueous phase was removed and again mixed with an equal volume of buffer saturated phenol and incubated in ice-bath for 15 min with occasional shaking. The aqueous phase was extracted by centrifuging at 10,000xg for 20 min. The aqueous phase was repeatedly extracted with equal volumes of buffer saturated phenol and chloroform (1:1) until no protein layer at the interface is seen. Nucleic acids were precipitated from the aqueous phase with 2 volumes of 95 % ethanol containing 0.2M sodium acetate and kept overnight at −20°C. The precipitate was pelleted by spinning for 5 min in an eppendorf centrifuge. The pellet was dissolved in minimum volume of RNase free distilled water.

Electrophoresis of different rRNA species

The total nucleic acids were run on 3 % polyacrylamide gel. The slab gels (10 cm × 6 cm) were prepared according to BISHOP et al. (1967). The gels were submerged in the electrophoretic buffer (0.04M Tris pH 7.8, 0.02M sodium acetate, and 2 mM EDTA) and was prerun for 1 h at 10 V 5°C to remove any unpolymerised toxic chemicals in the gel. 20 μl samples (nucleic acids + Glycerol + Bromophenol blue) were loaded in each slot and electrophoresed under the same conditions for 150 min according to LOENING (1967). Subsequently, the gels were rinsed in 1 M acetic acid and stained with 0.2 % methylene blue in 0.4M sodium acetate pH 4.7 for 1 h according to PEACOCK and DINGMAN (1967). The gels were destained with distilled water. The nucleic acid bands stained blue. The gels were scanned at 578 nm using a gel scanner attached to Beckman 5260 spectrophotometer.

Extraction of plastid DNA

Ten days old seedlings were kept in dark for 24 h to exhaust the stored carbohydrates from the chloroplasts. Leaves from such plants were cut, frozen in liquid nitrogen, and ground in precooled mortar (RHODES and KUNG 1981). The ground tissue was mixed with extraction buffer (0.3M mannitol, 0.05M Tris pH 8, 0.003M EDTA, 0.1 % BSA and 0.001M beta-mercapto ethanol) passed through cheese cloth once and spun at 1000xg for 15 min.

The pellet was resuspended in a minimum quantity of the extraction buffer (KOLODNER and TIWARI 1975). The plastid suspension was layered on a two step sucrose discontinuous gradient consisting of 30 % and 60 % (w/v) in the isolation buffer and spun at 10,000xg for 45 min. The chloroplast band was collected from the interface of 30 %–60 % gradient and washed in isolation buffer and repelleted by spinning at 1000xg for 5 min.

The chloroplast pellet was resuspended in a minimum amount of buffer (0.4M NaCl, 0.02M Tris-HCl pH 7.8). The solution was adjusted to 2 % SDS using 20 % SDS in water and incubated at 37°C for 1h followed by RNase treatment (100 μg/ml) for 30 min at 37°C (SCOWCROFT and LARKIN 1981). Equal volume of phenol saturated with the buffer was added, shaken gently on ice bath for 10 min, and was spun at 1000xg for 10 min. This step was repeated twice. Equal volume of chloroform was added to the aqueous phase, gently shaken for 5 min on ice bath, and again spun at 1000xg for 10 min. The step was repeated until the interface was clear. The supernatant was extensively dialysed against a suitable buffer and the DNA was precipitated with 2 volumes of cold (−20°C) ethanol by keeping the mixture at −70°C for 2 h. The mixture was centrifuged in an eppendorf centrifuge for 5 min. The precipitate was washed with 70 % ethanol and dissolved in TE buffer (0.01M Tris pH 8, 0.001M EDTA).

Restriction enzyme analysis

Plastid DNA samples 1 or 2 μg each were digested at 37°C for 100 min with six different restriction enzymes, viz. Bam HI, Bgl I, Cla I, Eco RI, Pvu II, and Sma I at the rate of 5 units/μg DNA. The reaction was terminated with the addition of 1/10 volume of a solution containing 15 % Ficoll, 0.2 % bromophenol blue, and 0.5M EDTA, and the assay mixture was further incubated at 60°C for 10 min followed by chilling on ice-bath for a minimum of 10 min. Each assay mixture consisted of DNA sample in 0.1×TE buffer, specific incubation buffer supplied with each enzyme, restriction enzyme, and made up to a final volume of 15 μl using sterile distilled water. The controls consisted of the mixture used above except the enzyme. The reference was a Hind III digest of φX174 DNA. The restricted DNA samples were loaded on 1 % agarose horizontal gels (15 cm × 15 cm × 0.5 cm) submerged in 1.5 litres of TAE (0.04M Trisacetate, 0.002M EDTA pH 8) buffer and were run for 15h at 60V. After the electrophoresis, the gels were stained with 0.05 % aqueous solution of ethidium bromide for 45 min and photographed under U.V. using a photodyne transilluminator.

Results

Stripe, green, and yellow seedlings were obtained in the selfed progeny of stripe plant, whereas only green and yellow seedlings were obtained in the crossed progeny of stripe plant with normal inbreds. Electron microscopic studies were conducted on green and yellow areas of stripe plants and revealed ultrastructural differences between the plastids of green and yellow areas. The differences were apparent at all stages. The plastids from green tissue of fully expanded stripe leaves were normal (Fig. 1) with extensive grana fretwork system. Ribosomes, osmophilic granules, and occasional starch grains were observed. The normal chloroplasts of vascular bundle sheath cells in fully expanded leaves were similar to that of normal plastids of mesophyll cells but contained fewer small grana thylakoids. Sometimes only stroma thylakoids were present.

The plastids from yellow tissue of fully expanded leaves were aberrant (Fig. 2). They were bound by a typical double membrane envelope. They were irregular in shape and relatively smaller than the normal plastids. However, they were present in the same number as normal plastids. The thylakoid membrane differentiation was lacking. The disorganized lamellar membranes were found as vesicles in lightly stainable stroma. Plastid ribosomes were absent in aberrant plastids while cytoplasmic ribosomes were present in the surrounding cytoplasm. There were no detectable differences in mesophyll and bundle sheath plastids in yellow tissues.

Majority of the cells in pure yellow stripes contain no normal plastids. However, in the overlapping regions of yellow and green stripes, occasionally cells with normal and aberrant plastids were found (Fig. 2). In heteroplastidic cells, the presence of normal plastids did not affect the appearance of aberrant plastids.

Total nucleic acids were extracted and purified from yellow seedlings with homozygous (*vi/vi*) and heterozygous (*vi/+*) nuclear constitution and the corresponding green segregants. On electrophoretic separation of the nucleic acids of green leaves, 4 bands corresponding to the high molecular weight rRNAs, viz. 25S and 18S RNAs of cytoplasmic ribosomes and 23S and 16S RNAs of plastid ribosomes
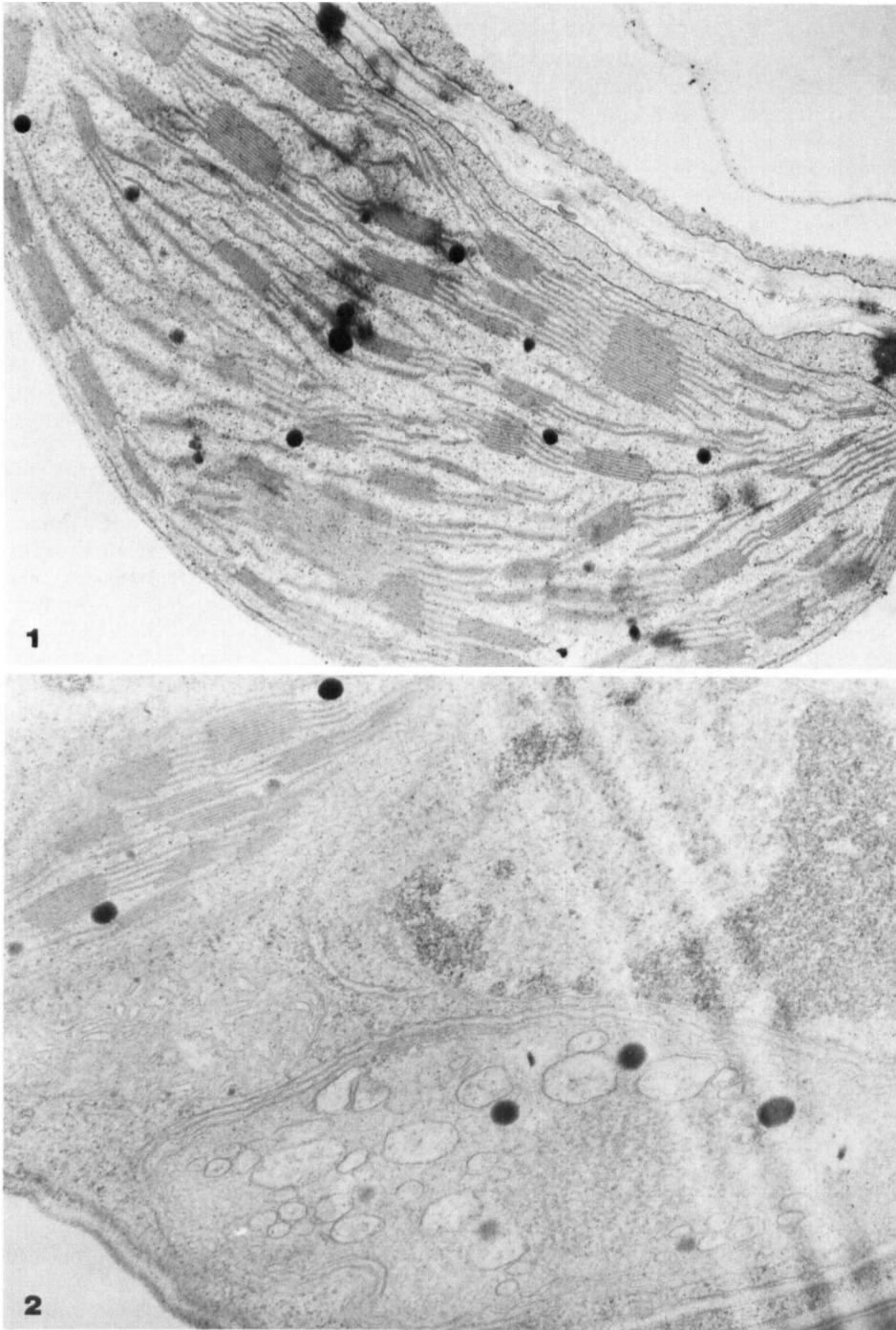
Fig. 1 and 2. Fig. 1. Ultrastructure of normal mesophyll plastid (23,000× of stripe plant. **Fig. 2.** Heteroplastidic cell with aberrant and normal plastids (31,900×).

were observed (Fig. 3A). In contrast, the electrophoretic pattern of the nucleic acids of yellow leaves showed only 2 bands corresponding to 25S and 18S rRNAs of cytoplasmic ribosomes while plastid specific 23S and 16S rRNAs were absent in homozygous and heterozygous yellow seedlings (Fig. 3 B, C).

Intact plastids were isolated from 700430 green sib and also from green F_1 plants arising from the crosses of stripe florets with wild type. Under similar conditions of extraction, it was not possible to isolate altered plastids from yellow tissue because of their poor structural development. DNAs extracted from the plastids of normal plants and from the reverted plastids were digested with six different restriction enzymes, viz. Eco RI, Cla I, Bam HI, Bgl I, Sma I, and Pvu II, and the fragmentation patterns were compared (Fig. 4). No detectable differences were observed in the fragmentation patterns of the plastid DNAs from green sibs (700430) and those from the reverted plastids in the F_1 plants.

Discussion

The presence of aberrant plastids at all developmental stages of yellow seedlings indicates the developmental failure of plastids unlike photodestruction of plastids in *Helianthus* (WALLES 1972). The ultrastructural studies indicate the poor development of plastids in non-green regions of leaves and the absence of ribosomes in the plastid compartment. The absence of plastid specific 23S and 16S ribosomal RNAs in the yellow regions of the mutant is also evident from the comparisons of RNA profiles (Fig. 3) of the yellow and normal tissue of stripe mutant. These results indicate a direct relationship between the lack of plastid specific rRNAs and ribosomal assembly. This would also imply that the absence of plastid rRNAs and ribosomes leads to a block in protein synthesis within such plastids similar to the observations of BÖRNER et al. (1972) in *Pelargonium zonale*. These ribosome deficient plastid mutants would be useful in unravelling the precise contribution of the nuclear controlled proteins in the functional development of plastids. Previously, different methods (BOULTER et al. 1972; ELLIS et al. 1973) are used to determine which component of the plastid is of cytoplasmic origin and which component is synthesized inside the organelle. Firstly, antibiotics are used to selectively inhibit translation either on cytoplasmic ribosomes or on plastid ribosomes (BOULTER et al. 1972). But in many cases, controver-
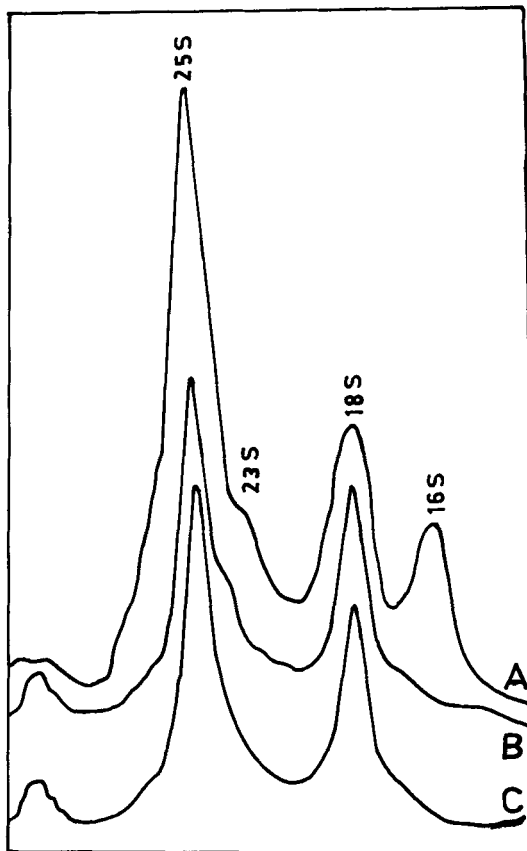
Fig. 3. Ribosomal RNA profiles of green and yellow seedlings of stripe mutant (A) homozygous recessive (*vi/vi*) green seedlings (B) heterozygous (*vi/+*) yellow seedlings (C) homozygous recessive (*vi/vi*) yellow seedlings.

sial results are obtained because of the lack of 100 % specificity or efficiency of these antibiotics and also because of some indirect side effects on in vivo systems. Secondly, the incorporation of labelled amino acids into discrete polypeptides by isolated plastids (ELLIS et al. 1973) may not give a complete picture in view of the physiological imbalance in such isolated plastids. An additional approach to this problem is the analysis of mutant with deficient plastid protein synthesis. All the protein components found in such mutant plastids originate from the protein synthesis outside the plastids.

The presence of double layered envelope in the altered plastids of stripe seedlings like normal plastids (Fig. 2) makes it conceivable that the major components of plastid envelope are synthesized outside the plastid and form an apparently normal double layered envelope for the ribosome deficient

Fig. 4. Restriction fragmentation pattern of plastid DNA from green (*vi/vi*) and reverted green (*vi/+*) seedlings of stripe mutant A- reverted green (B) green.

plastids. The presence of disorganized thylakoid membranes within the defective plastids may represent non-plastome coded proteins. This argument is in agreement with Börner et al. (1976) who found a similarity between the electrophoretic separation pattern of membrane proteins of ribosome-deficient plastids and the membrane proteins of etiolated normal plastids which are very close to the green plastids in the albostrain mutant of *H. vulgare.*

The yellow plastids are relatively smaller than green plastids but they are present in approximately same numbers per cell, indicating that these plastids multiply and assort during cell divisions and that these plastids have their own DNA. This implies that DNA also replicates in the yellow plastids along with plastid multiplication and that the plastid specific DNA polymerase and other enzymes which are necessary for the replication of plastid DNA are encoded in the nucleus, synthesized on the cytoplasmic ribosomes and then transported into the plastid compartment. These interpretations are in agreement with KNOTH et al. (1974), who observed the DNA replication autoradiographically in the albostrain mutant of the *H. vulgare.* HERMANN and FEIERABEND (1980) also observed the presence of

normal amounts of DNA in high temperature induced ribosome deficient plastids of rye. These results indicate that the double layered membrane envelope of defective plastids retain plastid specific proteins and enzymes inside the compartment similar to the normal plastid envelope. The selective transport of cytoplasmically synthesized plastid specific enzymes or proteins into plastid compartment may require special protein-transporting system.

The existence of both defective and normal plastids in the same cell as shown in Fig. 2 suggests that each plastid compartment differentiates independently. The presence of normal plastids does not affect the appearance of the defective plastids in the same cell. Thus the target of the nuclear gene is the individual plastid rather than the complete cell. Even within a cell, some plastids are altered while others are normal. How the same nuclear gene controls the development of individual plastids separately within a cell is not clear at this stage.

The persistence of altered plastids in homoplastidic egg cells in spite of acquiring a dominant allele (REDDY 1986) can be explained by the absence of protein synthesis in altered plastids. If exclusively altered plastids are present in the egg cell (as in the egg cells of yellow spikelets) they are unable to send

any signal to the nucleus to respond at (at least some nuclear genes) for the functional development of plastids. When they are associated with normal plastids in the egg cell (as in the heteroplastidic egg cells of stripe spikelets) an interaction between the normal plastids and the nuclear gene(s) leads to the normal development of the altered plastids into green plastids. This is consistent with the earlier proposal of plastid reversion (REDDY 1986). These results suggest that the nuclear gene in the stripe plants leads to a programmed loss of plastid ribosomes. When all the plastids in an egg cell are devoid of ribosomes, they can not develop their ribosomes even on acquiring a dominant allele. However, the yellow plastids develop their ribosomes when they are associated with normal plastids in heterozygous condition, similar to the inability of the rye plastids which completely lost their ribosomes under non-permissive temperature to regain plastid ribosomes under permissive temperatures (FEIERABEND and SCHRADER-REICHHARDT 1976). The nuclear gene induced plastid mutations were reported in maize (RHOADES 1943, 1946; STROUP 1970), barley (HAGEMANN and SCHOLZ 1962) and many other plants (see KIRK and TILNEY-BASSETT 1978), but change in plastid DNA was not demonstrated. However, the maternal transmission of altered plastids irrespective of its nuclear gene in subsequent generations was interpreted to indicate that nuclear gene(s) induced a heritable change in plastid DNA. The persistence of altered plastids even in the absence of mutant genotype may not exclusively suggest that nuclear gene induced a mutation in plastid DNA. The present results indicate a programmed loss of plastid ribosomes. In other words, the altered plastids lost their translating machinery to express their genetic material rather than loss/change in their genetic material.

Our studies led us to the more general question how the protein synthesizing systems of nucleo-cytoplasmic compartment and plastid compartment interact with each other. The transport of polypeptides from cytosol into the plastid is well established (PARTHIER 1982). Although experimental demonstration for the transport in the opposite direction is lacking so far, it can not be excluded a priori. The general lack of such evidence encouraged Ellis (1977) to postulate a "cytoplasmic control principle" which states that cytoplasmic products control protein synthesis in plastid compartment and rule out the converse. The present results, i.e., the essentiality of normal plastids in bringing about the reversion of ribosome deficient plastids to normal in the presence of a dominant nuclear genotype

suggest that the plastid coded signal(s) are essential to elicit nuclear gene products. Therefore, we assume that the "cytoplasmic control principle" formulated by ELLIS (1977) should be complemented by "plastidic control principle" to complete the complex network of co-operation among the two genetic (nuclear and plastome) and two translational systems (80S and 70S ribosomes) for the functional development of plastids. The absence of any detectable differences in the restriction fragmentation patterns of the DNA from normal and that of the reverted plastids indicates that there are no assignable differences in the DNAs of the two types (Fig. 4). From the ultrastructural and molecular characteristics, it is evident that the defective plastids are deficient in their ribosomal rRNAs, thus the protein synthesizing machinery. These evidences and the genetic data (REDDY 1986) suggest that the "plastidic control principle" involved in the reversion of defective plastids to normal is likely to be a protein.

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