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Biparental inheritance of chloroplast DNA and the existence of heteroplasmic cells in alfalfa

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Summary. Mapping of chloroplast DNA (ctDNA) restriction fragment patterns from a chlorophyll deficient mutant and two phenotypically normal alfalfa genotypes (*Medicago sativa* L.) has demonstrated the existence of a distinct ctDNA genotype from each source. These unique restriction fragment patterns were utilized to identify maternal or paternal origin of ctDNA in hybrid plants from crosses involving the normal alfalfa genotypes as females and the yellow-green chlorophyll deficient sectors as males. Progeny from these crosses expressing the yellow-green sectored phenotypes contained paternal ctDNA in the chlorophyll deficient sectors and maternal ctDNA in the normal sectors, confirming biparental plastid inheritance. The existence of mixed cells containing both mutant and normal plastids at various stages of sorting-out was observed by transmission electron microscopy of mesophyll cells in mosaic tissue from hybrid plants. This observation verified the biparental transmission of plastids in alfalfa.

Key words: Alfalfa – Biparental plastid inheritance – Chlorophyll deficient mutants – ctDNA

Introduction

Plastid inheritance in higher plants has been demonstrated to follow maternal, paternal and biparental modes. Chlorophyll deficiency has traditionally been the chloroplast encoded trait utilized in studies of plastid inheritance (Kirk and Tilney-Basset 1978). Recently, however, chloroplast encoded antibiotic resistance has been used to select among large numbers of cultured cells for low frequencies of plastid transmission (Medgyesy et al. 1986).

Patterns produced following cleavage of chloroplast DNAs (ctDNA) with restriction endonucleases have provided another way to differentiate plastids with maternal or paternal origin in genetic studies. Variation in restriction patterns of ctDNA is limited, but detectable, among closely related species and among genotypes within a species (Palmer 1986). Analysis of ctDNA restriction fragment banding patterns of parents and progeny has been used to demonstrate the pattern of plastid transmission in interspecific crosses of wheat (Vedel et al. 1981), corn (Conde et al. 1979), sorghum (Pring et al. 1982), tobacco (Medgyesy et al. 1986), soybeans (Hatfield et al. 1985), *Oenothera* (Hatchel 1980), *Larix* (Szmidt et al. 1987) and *Pelargonium* (Metzlaff et al. 1981).

Two chlorophyll deficient mutants of alfalfa (*Medicago sativa* L.) that arise through somatic sectoring were studied by reciprocal cross analysis and found to sexually transmit their phenotypes through both parents (Smith et al. 1986). Developmental and genetic evidence suggested that the chlorophyll deficiencies represent plastid mutations and that plastids are biparentally inherited in alfalfa. Subsequent mapping of ctDNA from pure chlorophyll deficient and normal sectors from these phenotypes demonstrated the existence of a unique plastid genotype in the mutant tissue. Electron microscopy evidence also verified that development of mutant plastids is dramatically arrested in the chlorophyll deficient sectors. Differences in restriction fragment patterns between chlorophyll deficient and normal ctDNA could provide a definitive description of the mode of inheritance of plastids in alfalfa. Therefore, in this study ctDNA from normal and mutant tissue of parents and sexual progeny were compared by restriction fragment analysis. Tissue from sectoral (normal plus chlorophyll deficient) proge-

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ny undergoing the sorting out process were also examined by transmission electron microscopy for evidence of individual mesophyll cells containing both mutant and normal plastids.

Materials and methods

Plant Sources

The tetraploid *Medicago sativa* plants used in this study were a phenotypically normal male sterile tester line (6-4), a multifoliate fully fertile tester line that had no chlorophyll deficiency (MAG) and progeny from a cross between MAG and a sectored chlorophyll deficient (yellow-green) variant (YGS) described by Smith et al. (1986). Pure yellow-green sectors on progeny from the cross MAG × YGS (lines 33B-2 and 33B-4) produced fertile flowers that were utilized in the cross 6-4 × 33B-4 (female parents are listed first throughout). Progeny from this cross 12-1, 13-2, 13-4) were then selected for the yellow-green sectored phenotype. All plants were grown in the greenhouse under 16 h daylength.

DNA extractions and southern blotting

Tissue from pure yellow-green or normal sectors were removed from plants and either freeze dried in a VirTis freezedryer for 2–4 days for total DNA extraction (Saghai-Maroof et al. 1984) or homogenized fresh for ctDNA extraction (Kemble 1987). DNA samples were quantified by fluorometry in the presence of DNA specific dye (Hoescht 33258) and digested with Hind III or Bam HI restriction endonucleases according to suppliers' instructions (IBI or BRL).

Approximately 5 μg of digested DNA per lane were loaded onto a 0.8%, 110 × 135 mm agarose gel and electrophoresis was carried out on a BRL horizontal apparatus at 1 V/cm for 16 h in TBE gel buffer (Maniatis et al. 1982). Gels were then stained with ethidium bromide and photographed under UV light. Mobilities of the Lamdba Hind III markers were measured from the photograph. Transfer of restriction fragments from agarose gels to Zeta-Probe nylon membranes was performed according to the methods of Reed and Mann (1985).

Hybridization probes

A library constructed by Dr. J. D. Palmer consisting of alfalfa and mung bean ctDNA fragments cloned into pBR322 or pUC18 plasmid vectors served as hybridization probes to the filter-bound DNA fragments. These 11 fragments cover the entire alfalfa chloroplast genome (J. D. Palmer, personal communication). Plasmids were isolated from *E. coli* hosts using the mini-prep procedure of Birnboim and Doly (1979). DNA from mini-preps was labeled with 32P dNTPs using nick-translation (Rigby et al. 1977). Unincorporated nucleotides were separated from labeled recombinant probes using centrifuged Sephadex G-50 1 cc columns. Prior to hybridization, the labeled probes were denatured in solution with 200 M/s of 0.2 *N* NaOH with heating to 100 °C for 10 min.

Hybridizations, washing, autoradiography

Zeta Probe filters were prehybridized in 25–30 mls of a 1.5 × SSPE, 1.0% SDS and 0.5% Blotto solution at 67 °C in a water incubator with gentle shaking for 4–24 h. Denatured probes were added to the bagged filters and prehybridization mix and the bags were resealed and incubated at 67 °C for 20–24 h. After hybridization, filters were washed at room temperature with 300–500 mls of 2 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS and 0.1 × SSC/0.1% SDS solutions successively, followed by one wash with 300–500 mls of 0.1 × SSC/1% SDS at 55°–60 °C. Washed filters were wrapped in Saran wrap and taped to a Kodak X-ray exposure cassette. Autoradiography was performed at room temperature for 2–72 h. Sizes of hybridized filter-bound fragments detected by autoradiography were determined by the regression analysis method described by Schaffer and Sederoff (1981).

Electron microscopy

Ultrastructural features of mesophyll cells from fine mosaic sectors of yellow-green sectored progeny were examined by transmission electron microscopy. Tissue was prepared for ultra-thin sectioning largely according to the procedure of Carroll and Mayhew (1976). Finely chopped leaves were fixed in 3.0% glutaraldehyde in 0.01 *M* potassium phosphate buffer, pH 7.2, for 24 h. Fixation in 2.0% osmium tetroxide in the same phosphate buffer followed. Tissue dehydration was in a graded concentration series of ethanol (50%–100%). Ethanol was replaced with propylene oxide before infiltration of the fixed tissue with Spurr's embedment. Polymerized tissue was then thick sectioned for cytological observation under the light microscope. Selected regions of embedded tissue were thin sectioned on an ultramicrotome, mounted on grids and stained with uranyl acetate and lead citrate. Grids were then observed under the Zeiss EM 10C transmission electron microscope.

Results and discussion

Restriction fragment polymorphisms

Hybridization of filter-bound ctDNA with the alfalfa and mung bean ctDNA probes revealed four distinct polymorphisms among the genotypes studied. In all cases, hybridization patterns of total and chloroplast filter-bound DNA in these autoradiographs were identical. Bam HI, Hind III and Xho I restriction fragments were physically mapped (Lee et al. 1988) and the polymorphisms were found to be due to two insertion/deletion events and two point mutations. These polymorphisms provided markers to distinguish the ctDNA genotypes used in this study (Table 1).

Table 1. Hybridization pattern differences detected with alfalfa ctDNA Pst I fragments

ctDNA	12.7 kb probe 6[a]	18.0 kb probe 7	21.0 kb probe 2
Bam HI fragment polymorphisms (kb)			
6-4	1.9	7.6+1.5	5.5
MAG	1.9	9.1	5.6
YGS	1.8	9.1	5.6
Hind III fragment polymorphisms (kb)			
6-4	11.5	4.4	2.6
MAG	11.5	2.8+1.6	2.7
YGS	11.4	2.8+1.6	2.7

[a] Probe designation from Lee et al. (1988)

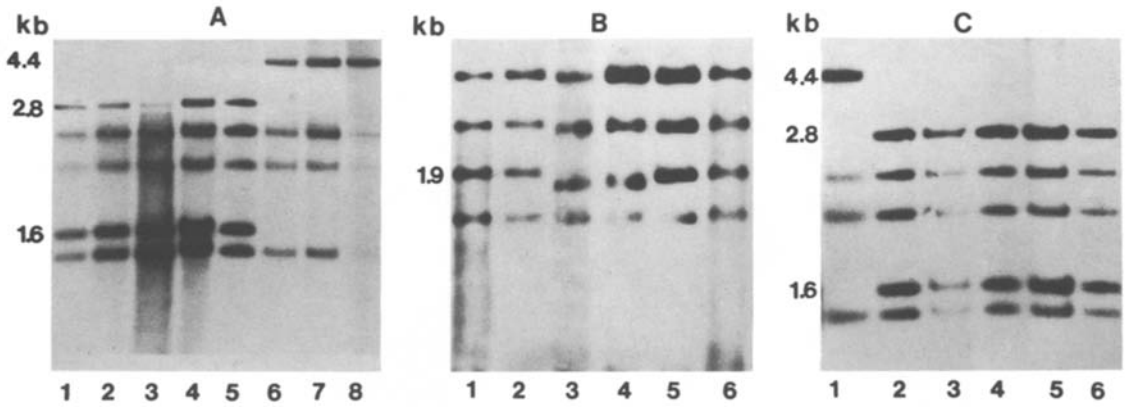

Fig. 1 A–C. Autoradiographs demonstrating ctDNA restriction fragment banding patterns of parents and progeny. **A**: Hind III digested DNA hybridized with an 18 kb alfalfa ctDNA probe. *Lane 1,* MAG ctDNA; *lane 2,* MAG total DNA; *lane 3,* 33B-4 normal sector total DNA; *lane 4,* 33B-4 mutant sector total DNA; *lane 5,* 13-4 mutant sector total DNA; *lane 6,* 13-4 normal sector total DNA; *lane 7,* 6-4 total DNA; *lane 8,* 6-4 ctDNA. **B**: Bam HI digested DNA hybridized with a 12 kb alfalfa ctDNA probe. *Lane 1,* MAG ctDNA; *lane 2,* 33B-4 normal sector ctDNA; *lane 3,* 33B-4 mutant sector total DNA; *lane 4,* 13-4 mutant sector total DNA; *lane 5,* 13-4 normal sector ctDNA; *lane 6,* 6-4 ctDNA. **C**: Hind II digested total DNA hybridized with the 18 kb alfalfa ctDNA probe. *Lane 1,* 13-2 normal sectors; *lane 2,* 33B-2 mutant seoctors; *lane 3,* 12-1 mutant sectors; *lane 4,* 13-4 mutant sectors; *lane 5,* 13-2 mutant sectors; *lane 6,* 33B-4 mutant sectors

Autoradiographs of ctDNA fragments of parents and progeny in Fig. 1 demonstrate that three distinct plastid genotypes exist in the plants studied and that plastid transmission occurs biparentally in alfalfa. Figure 1 A shows that MAG (*lanes 1* and *2*) and 6-4 (*lanes 7* and *8*) have distinct restriction fragment patterns with Hind III digested fragments hybridized with an 18 kilobase pair (kb) alfalfa ctDNA probe. This polymorphism is due to an extra Hind III recognition site in MAG that results in a 4.4 kb fragment being cleaved into a 2.8 kb and a 1.6 kb fragment. *Lanes 3* and *4* have the same fragment pattern, indicating that Hind III ctDNA fragments from 33B-4 normal sectors (*lane 3*) and yellow-green mutant sectors on the same plant (*lane 4*) were indistinguishable with this probe. Normal and mutant sectors on 13-4, however, are distinguishable. Hind III ctDNA fragments from mutant tissue (*lane 5*) have the same pattern as the paternal parent (33B-4 mutant sectors, *lane 4*), while the normal tissue fragment pattern (*lane 6*) is the same as 6-4, the female parent (*lane 7*).

Figure 1 B demonstrates unique Bam HI restriction patterns of mutant ctDNA hybridized with a 12.7 kb alfalfa ctDNA probe. Yellow-green mutant tissue from both 33B-4 and 13-4 (*lanes 3* and *4*) contains ctDNA with a fragment that is slightly smaller than the 1.9 kb Bam HI fragment in the normal tissue ctDNAs (*lanes 2* and *5*). The mobility of this fragment is the same in MAG, 6-4, and normal tissue from 33B-4 and 13-4.

Figure 1 C demonstrates that all yellow-green sectoring mutant progeny studied by this analysis had the same mutant ctDNA. *Lane 1* contains total DNA from normal sectors on 13-2 while the remaining lanes contain total DNA from mutant sectors from 5 different sectored plants from 2 different generations. Hind III digested ctDNA fragments from 33B-4 mutant sectors (*lane 6*) had the same pattern when hybridized with the 18 kb probe as its sibling, 33B-2 (*lane 2*), and progeny (*lanes 3, 4* and *5*). The stability of this genotype was confirmed by hybridization of these filter-bound Hind III fragments with other alfalfa ctDNA probes (data not shown).

Electron microscopy

Ultrastructural features of mesophyll cells from pure yellow-green mutant sectors were compared with normal tissue by transmission electron microscopy. The mutant phenotype was found to have distinct features indicative of arrested plastid development (Lee et al. 1987). These features included a reduction in thylakoid membrane development, an absence of thylakoid stacking to form grana, large numbers of osmiophyllic globuli in many of the plastids and a higher degree a vacuolization that was apparently due to thylakoid disassociation. Fine mosaic sectors examined in this study revealed mesophyll cells that contained both mutant plastids and normal chloroplasts.

Electron micrographs in Fig. 2 show sections of mesophyll cells from mosaic tissue in 13-4 plants. Figure 2 A and B ($\times$ 8,000) shows cells containing only mutant plastids (*p*) or only normal chloroplasts (*c*) adjacent to cells with with both types of plastids. Mutant plastids can be distinguished from normal chloroplasts in these mixed cells by their smaller size and absence of starch (*s*). Thylakoid membrane stacking is absent or extremely reduced in the mutant plastids (*r*), while large grana are present in the normal chloroplasts (*g*). Osmio-

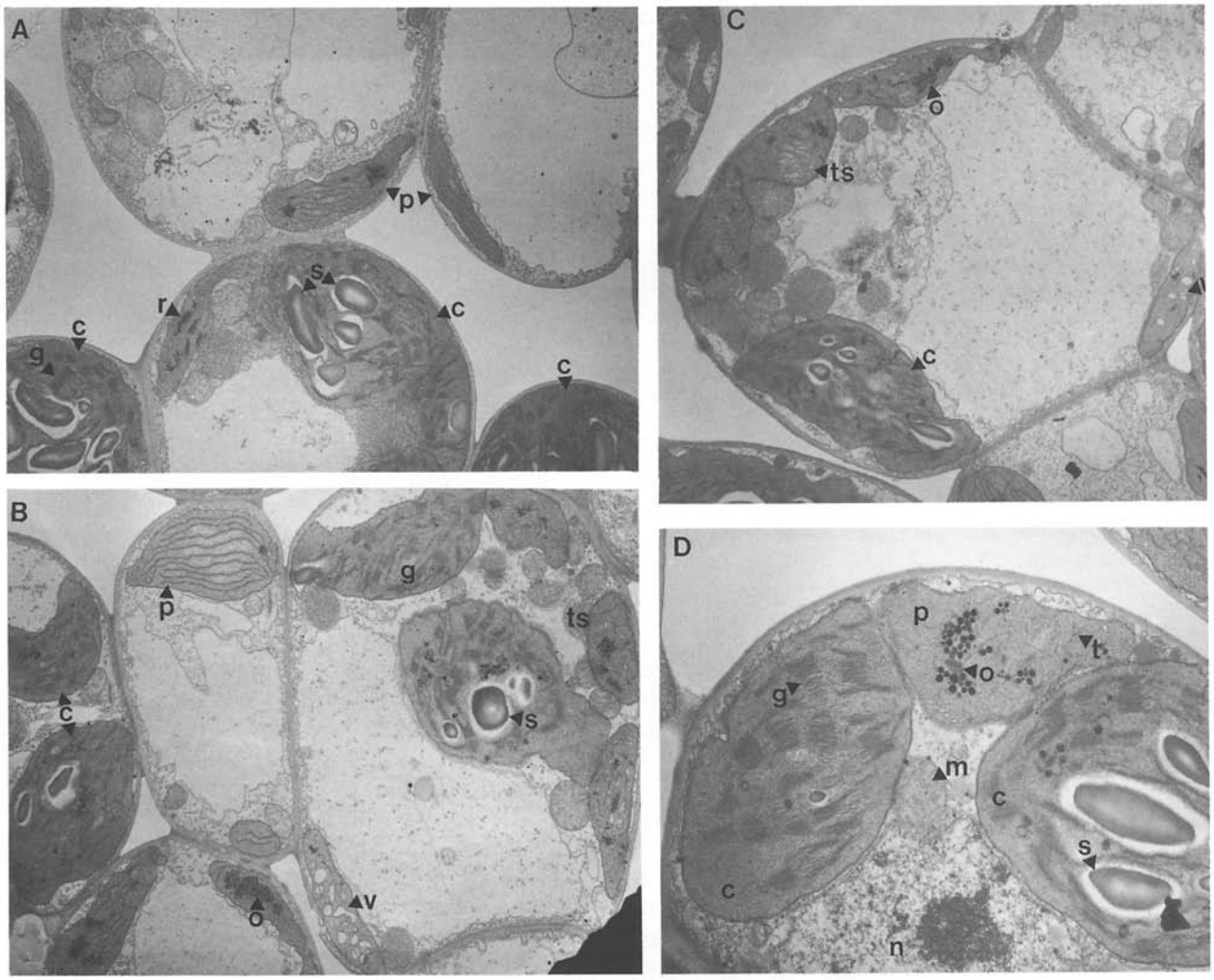

Fig. 2 A–D. Electron micrographs of mesophyll cells from 13-4 mosaic tissue. **A**: Micrograph (× 8,000) showing mesophyll cells containing normal chloroplasts (*c*), mutant plastids (*p*), of both. Starch grains (*s*) and grana (*g*) are present in the chloroplasts. Mutant plastids show only rudimentary thylakoid development (*r*). **B**: Micrograph (× 8,000) showing adjacent cells with normal (*far left*), mutant (*top center*) and mixed (*right*) plastid types. Mutant plastids contain dissociating thylakoid membranes (*ts*) that apparently lead to vacuolization. Osmiophyllic globuli are present in both plastid types but are much more predominant in the mutants (*o*). **C**: Slightly higher magnification (× 10,000) of a mixed cell showing mutant plastid features. **D**: Mixed cell (× 20,000) with normal nucleus (*n*), mitochondria (*m*), chloroplasts (*c*) and a mutant plastid (*p*). Large starch grains (*s*) and grana (*g*) are indicative of normal photosynthetic function while mutant plastids contain large numbers of osmiophyllic globuli (*o*) and rudimentary thylakoids (*t*)

phyllic globuli (*o*) are found in normal chloroplasts and mutant plastids, but to a much greater extent in the mutants. Vacuolization (*v*) is apparent in mutant plastids in Fig. 2 B and C. Mutant plastids also show the initation of vacuolization due to thylakoid membrane separation (*ts*). A mixed cell in Fig. 2 D (× 20,000) demonstrates the ultrastructural differences between the mutant plastids (*P*) and normal chloroplasts (*C*) with starch grains and large grana present in the normal organelle, while the mutant plastid (*p*) has only rudimentary thylakoids (*t*) and many osmiophyllic globuli (*o*). The nucleus (*n*) and a mitochondria (*m*) appear normal in this cell.

The observation of biparental transmission of chlorophyll deficiency in alfalfa by Smith et al. (1986) provided evidence for the biparental inheritance of plastids in this species. The mutant phenotypes appeared as yellow-green sectors on otherwise normal tissue and sorted out through a fine mosaic to coarse mosaic pattern eventually giving rise to pure mutant or normal sectors.

Mapping of ctDNA from normal and pure yellow-green sectors from chimeric plants has verified the existence of a mutant genotype as well as two distinct ctDNA genotypes in normal alfalfa. Utilization of the unique restriction fragment patterns of each genotype in crosses

involving these two normal tester lines and the yellow-green sectors on mutant plants has positively confirmed the biparental inheritance of plastids proposed by Smith et al. (1986). The ability to differentiate between the two normal plastid genotypes allowed for the determination of the maternal origin of ctDNA in normal tissue from 6-4 × 33B-4 progeny, while the chlorophyll deficient phenotype provided the marker with which to select for tissue with paternal plastids. Comparison of yellow-green tissue ctDNA from several plants indicates that the mutant genotype is stable and not likely to be due to de novo events.

Biparental inheritance of plastids necessitates the existence of "mixed" cells in the sexual progeny containing plastids from both parents. As such plants develop, random sorting-out of the two plastid types will eventually lead to pure sectors having only maternal or paternal plastids. Tissue in the fine mosaic state should consist of cells in the process of sorting to pure mutant or normal types. The electron micrographs of mosaic tissue from 13-4 show cells with both normal and mutant plastids at various stages of sorting-out, confirming the existence of biparental plastid inheritance.

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