Theor Appl Genet (1990) 79:49–55

High transmission of paternal plastid DNA in alfalfa plants demonstrated by restriction fragment polymorphic analysis *

S. A. Masoud [1], L. B. Johnson [1,] and E. L. Sorensen [2]**

Departments of Plant Pathology [1], Agronomy [2], and USDA-ARS [2], Throckmorton Hall, Kansas State University, Manhattan, KS 66506, USA

Received July 1, 1989; Accepted August 30, 1989
Communicated by R. Hagemann

Summary. A high frequency of paternal plastid transmission occurred in progeny from crosses among normal green alfalfa plants. Plastid transmission was analyzed by hybridization of radiolabeled alfalfa plastid DNA (cpDNA) probes to Southern blots of restriction digests of the progeny DNA. Each probe revealed a specific polymorphism differentiating the parental plastid genomes. Of 212 progeny, 34 were heteroplastidic, with their cpDNAs ranging from predominantly paternal to predominantly maternal. Regrowth of shoots from heteroplasmic plants following removal of top growth revealed the persistence of mixed plastids in a given plant. However, different shoots within a green heteroplasmic plant exhibited paternal, maternal, or mixed cpDNAs. Evidence of maternal nuclear genomic influence on the frequency of paternal plastid transmission was observed in some reciprocal crosses. A few tetraploid F_1 progeny were obtained from tetraploid ($2n=4x=32$) *Medicago sativa* ssp. *sativa* × diploid ($2n=2x=16$) *M. sativa* ssp. *falcata* crosses, and resulted from unreduced gametes. Here more than the maternal genome alone apparently functioned in controlling plastid transmission. Considering all crosses, only 5 of 212 progeny cpDNAs lacked evidence of a definitive paternal plastid fragment.

Key words: Chloroplast DNA – *Medicago sativa* – Maternal plastid transmission – Biparental plastid inheritance – Heteroplasmy

Introduction

Lilienfeld (1962) first reported biparental chloroplast inheritance in the genus *Medicago* in a study of nuclear-cytoplasmic incompatibility. Pronounced reciprocal differences in plastid behavior were noted in crosses between two races of *Medicago truncatula* Gaertn. Heteroplasmy occurred in some hybrid embryos. Somatic segregation of the plastids during development frequently occurred.

Biparental inheritance was reported in alfalfa (*Medicago sativa* ssp. *sativa*) following studies with plastid-encoded mutants for chlorophyll deficiency (Smith et al. 1986) and plastid DNA (cpDNA) polymorphisms (Lee et al. 1988). Higher transmission rates of yellow-green and albino plastids via the pollen parent, as opposed to the seed parent, strongly suggested biparental chloroplast inheritance in alfalfa (Smith et al. 1986). Lee et al. (1988) confirmed this by demonstrating both heteroplasmicity and biparental plastid transmission in alfalfa, using cpDNA restriction fragment analysis and electron microscopy. Evidence came from three F_1 plants of crosses between a paternal parent possessing a plastid-encoded chlorophyll deficiency mutant and a normal green maternal parent.

The potential of alfalfa for biparental plastid inheritance was confirmed by Corriveau and Coleman (1988), who detected plastid nucleoids in the pollen generative cells. Disappearance of plastid nucleoids from the generative cells may be responsible for maternal plastid inheritance in several higher plant species (Miyamura et al. 1987).

Heteroplasmy has been reported also in alfalfa with normal green chloroplasts, and presumably resulted from pollen plastid transmission. Rose et al. (1986) concluded that plastid sorting-out during vegetative propagation must have converted two protoplast donor plants

* Contribution No. 89-524-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan
** To whom offprint requests should be addressed

to homoplasmy, presumably before protoplast isolation. Johnson and Palmer (1989) clearly demonstrated heteroplasmy in several *Medicago* species by restriction fragment analysis. Five of 10 randomly selected plants, analyzed from populations of three subspecies of *M. sativa* exhibiting cpDNA heterogeneity, were heteroplasmic.

It is easy to find cpDNA polymorphisms in alfalfa, even within a single accession, and restriction maps of mutants are available (L. B. Johnson and J. D. Palmer, unpublished results). Thus, plastid transmission frequencies can be determined in reciprocal crosses, without concern for possible differences in competitiveness of plastids encoding their own chlorophyll deficiency. We report here high paternal and low maternal plastid transmission in several alfalfa genotypes. The maternal nuclear genome, and possibly the paternal nuclear and/or plastid genome(s) as well, apparently affected plastid transmission frequencies in these crosses.

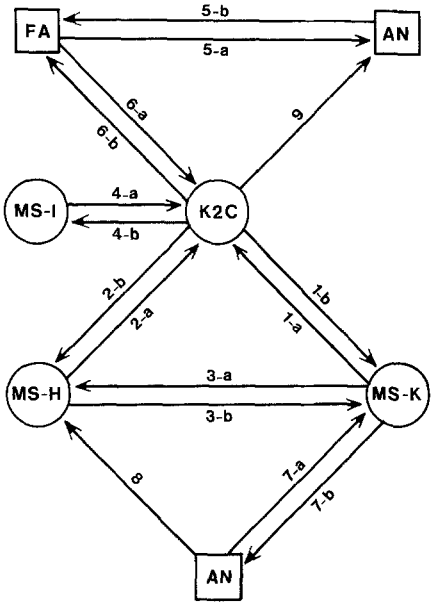

Fig. 1. A schematic representation of all sexual crosses made for evaluating paternal plastid transmission. *Circles* represent tetraploid parents (*Medicago sativa* ssp. *sativa*), *squares* represent diploid parents (*M. sativa* ssp. *falcata*). The *arrows* represent crosses. *Tails* start from the pollen parents. *Heads* point to the maternal parents. The crosses are numbered on or under the *arrows*. See 'Materials and methods' for an explanation of the plant genotypes

Materials and methods

Parental plants and sexual crosses

Crosses were made using two subspecies (ssp.) of *Medicago sativa* L. The four plants of *M. sativa* ssp. *sativa* (2n = 4x = 32) used were: RS-K2C, a ramet of a plant (Johnson and Palmer 1989) selected from Regen S (Bingham et al. 1975); and MS-H, MS-I, and MS-K, plants selected from P.I. 26590 obtained from the USDA Regional Plant Introduction Station, Pullman/WA. Two diploid (2n = 2x = 16) *M. sativa* ssp. *falcata* plants used were: FA selected from P.I. 234815 and AN selected from 'Anik' (Pankiw and Siemens 1976). All plants were cloned by ramets before crossing, in order to increase chances of plastid homozygosity. Parental plants were grown at 25 °C either in growth chambers (16-h photoperiod) or in a greenhouse with supplemental lighting at night, in order to induce flowering in the winter. Most reciprocal crosses were made between plants at the same ploidy level. A schematic representation of these crosses is shown in Fig. 1. Since all of the parents were self-fertile, most were emasculated by suction (Viands et al. 1988). Part of reciprocal cross no. 6 was made by alternately tripping flowers of paternal and maternal parents with a toothpick, counting on the competitive advantage of heterologous pollen in alfalfa.

Planting and identification of hybrid plants

Seeds were harvested, scarified with sandpaper, and germinated in darkness on wet filter paper in petri plates at 21 °C. Seedlings were planted individually in either 5 × 5 or 7 × 7 cm plastic pots. Plants from reciprocal crosses 2, 3, 4, and most plants of 1 were grown in growth chambers (16-h photoperiod and 130 μmol m^{-2} s^{-1} light intensity at 25 °C) for 4 weeks before extraction. The slow-growing diploid hybrids of reciprocal cross 5 were similarly maintained for 8 weeks. Plants from reciprocal crosses 6, 7, 8, and 9 and the remaining plants of reciprocal cross 1 were grown in the greenhouse with supplemental lighting, and were cut back several times before DNA extraction.

Four of five plants that showed only maternal cpDNA and about half of the plants in reciprocal crosses 1–3 were tested in order to determine, by isozyme analysis, whether they were hybrids. Hybrid origins could be confirmed by either isozyme or cpDNA restriction patterns. Hybrid plants from diploid-tetraploid crosses 6 through 9 exhibited variegated flowers (hybrid phenotype) and faster seedling growth than did diploid seedlings produced by selfing. Tetraploidy was established for six of these diploid × tetraploid progeny using procedures of Snow (1963). High fertility following selfing makes it unlikely that any of the others were triploid, and necessitates the involvement of unreduced gametes (Pfeiffer and Bingham 1983) in their production.

Isozyme analysis

Alfalfa leaves (0.1–0.2 gm) were collected separately in microfuge tubes and ground in liquid nitrogen. An equal volume of protein isolation buffer (100 m*M* TRIS, pH 8.5, 5 m*M* $MgCl_2 \cdot 6\,H_2O$, 2 m*M* EDTA, 1% v/v 2-mercaptoethanol) was added to each microfuge tube before centrifuging at 15,000 × *g* at 4 °C for 10 min. Supernatants were transferred into new microfuge tubes containing 20 μl of loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol in isolation buffer). Protein extracts (50 μl) were loaded in high pH discontinuous vertical polyacrylamide gels (Hames 1981). Electrophoresis was in either 10% acrylamide gels for leucine aminopeptidases, or 7.5% acrylamide gels for esterases. Separations were terminated, respectively, when the xylene cyanol dye or the bromophenol blue dye reached the end of the gel. Gel-staining techniques were modified from Quiros (1981). Leucine aminopeptidase gels were equilibrated in 200 ml of cold 0.2 *M* TRIS-HCl (pH 5.8) for 30 min and transferred to 100 ml of the same buffer, containing freshly combined fast blue B salt (50 mg) and L-leucine β-naphthylamide (50 mg, dissolved in 1 ml methanol). Staining was in the dark at 37 °C for 1 h. Esterase staining was in 50 ml of 0.2 *M* TRIS-HCl (pH 7.1) containing freshly combined fast blue BB (75 mg) and α-naphthyl acetate (50 mg, dissolved in 5 ml acetone). Staining was at 21 °C for 30 min.

DNA extraction and Southern hybridization

Chloroplast DNAs of parents and a few hybrid plants were isolated from 10–30 gm of leaf tissue with the sucrose density gradient technique of Palmer (1986), with one modification: ethanol precipitation replaced dialysis. Total DNAs of single hybrid plants or their F_1 progeny were isolated from 200–300 mg leaf or root tissues (Rogers and Bendich 1985) after a 48-h dark treatment. Random leaf samples were collected from large plants, but all trifoliolate leaves were harvested from seedlings. When required, DNA was quantified either spectrophotometrically or by comparison on test gels containing known standards. DNA samples were digested by the restriction endonucleases BamHI, HindIII, PstI, or XbaI (Promega, Madison/WI), following the manufacturer's instructions. Fragments were separated in 0.7% horizontal agarose gels (HindIII, PstI, XbaI) or 1.1% gels (BamHI) after Maniatis et al. (1982). Gels were stained in ethidium bromide and examined under ultraviolet light. Alkaline transfer of depurinated DNA to membranes (Zeta-Probe, Bio-Rad, Richmond/CA; or Zetabind, AMF CUNO, Meriden/CT) was performed as published (Reed and Mann 1985). When a strong signal was needed, 1 *M* NaCl was used in the transfer buffer. Cloned alfalfa cpDNA fragments (Palmer et al. 1987) were used as probes to identify maternal and paternal cpDNA origin (Rose et al. 1986; Johnson and Palmer 1989; Lee et al. 1989), and were labeled with [α-^{32}P] dCTP, either by nick translation (Maniatis et al. 1982) or with an oligolabeling kit (Pharmacia, Piscataway/NJ). Unincorporated nucleotides were removed with a Sephadex G-50 spun column (Maniatis et al. 1982). Prehybridization and hybridization followed Zeta-Probe manufacturer's recommendations, but with 10% dextran sulphate added, for at least 6 h. Filters were washed in solutions of increasing stringency up to 0.1 × SSC (SSC is 150 m*M* NaCl and 15 m*M* sodium citrate) and 1% SDS (sodium dodecyl sulfate) at 65 °C for 30 min. Filters were exposed to X-ray film at −80 °C for 4–96 h. DuPont Lightning Plus intensifying screens were used when needed.

Results

Fragment length polymorphisms unique to each parental cpDNA were looked for in individual plants (Table 1). The BamHI polymorphisms shown in Table 1 are due to insertion/deletion events, while the polymorphisms with the other enzymes shown result from restriction site mutations (L. B. Johnson and J. D. Palmer, unpublished results). BamHI restriction analysis was generally used where possible, in order to eliminate possible confusion of mixed cpDNAs with incomplete digestion. Exceptions were crosses 2 and 5, which were tested by XbaI and PstI, respectively, the inter-subspecific crosses in which either BamHI, HindIII, or both, were used, and 12 plants from cross no. 1 in which both BamHI and XbaI were used.

A total of 212 hybrid progeny were analyzed for the parental source of their cpDNA. Plastid transmission was classified as paternal (P), biparental (BP), or maternal (M). Considering all crosses, only five hybrid plants showed no evidence of paternal cpDNA, while 82% of the plants had no detectable maternal cpDNA (Table 2). Isozyme analysis of four of the five hybrids (the fifth did not survive after DNA extraction) with only maternally derived plastids likewise showed that all were hybrids and not the result of self-fertilization (data not shown). The extent of plastid mixing in the 34 hybrid heteroplatidic (BP) plants averaged 59% paternal based on estimates of banding intensity, and ranged from predominantly paternal to predominantly maternal. The sensitivity for detection of a minor parental cpDNA population varied among autoradiograms. However, less than 1% of a minor cpDNA was detected in an in vitro mixture of two different cpDNAs (data not shown), a limit similar to the 0.1% value of Scowcroft and Larkin (1981).

Heteroplasmy was observed in hybrid plants from 13 of the 16 crosses (Table 2). Examples are shown in Fig. 2. The top growth of several heteroplastidic plants was removed and allowed to regrow from the crown after the first cpDNA analysis. The new shoots were analyzed in groups of two or one. Results of analysis of one heteroplastidic plant are shown in Fig. 2A and B. Homoplastidic maternal, homoplastidic paternal, and heteroplastidic shoots arose from the same crown. Analysis of regrowth from a few heteroplastidic plants showed slight changes in cpDNA ratios, presumably as a result of sorting-out (Fig. 2D). However, progeny from cross no. 1 showed no change in the number of paternal, biparental, and maternal plants, when extracts from a later cutting were compared with initial extracts. Bulk cpDNA analysis of progeny from five selfed hybrid plants, three

Table 1. Restriction fragment length polymorphisms (in kb) used to distinguish parental cpDNAs following restriction enzyme digestion and hybridization with alfalfa cpDNA fragments

Parents	Restriction enzyme/fragment[a]			
	BamHI[b]/ 12.5	HindIII[c]/ 12.5	PstI[c]/18	XbaI[d]/6.2
MS-K	2.00	10.0	18.0	7.8, 0.5
MS-I	2.00	10.0	18.0	7.8, 0.5
MS-H	1.90	10.0	18.0	7.8, 0.5
RS-K2C	1.85	10.0	18.0	8.3
AN	1.75	6.0, 4.0	10.0, 8.0	8.3
FA	1.70	6.0, 4.0	18.0	8.3

[a] Cloned alfalfa cpDNA probes (6.2-, 12.5-, and 18-kb PstI fragments) provided by J. D.. Palmer (Indiana University, Bloomington). A restriction map is published (Palmer et al. 1987). Note that the 12.5-kb PstI fragment was initially estimated at 12.7 kb (Johnson and Palmer 1989)

[b] These polymorphisms occur within the hypervariable region reported and mapped by Johnson and Palmer (1989). Sizes estimated to nearest 0.05 kb. Apparently correspond to polymorphisms seen by Lee et al. (1989)

[c] L. B. Johnson and J. D. Palmer (unpublished results) observed *Medicago sativa* ssp. *sativa* and *M. sativa* ssp. *falcata* cpDNA differences here

[d] Polymorphisms first observed here by Rose et al. (1986). See Johnson and Palmer (1989) for a restriction map

Table 2. Single and reciprocal crosses used for evaluating cpDNA transmission of different alfalfa genotypes

Cross no.	♀ parent	♂ parent	Plants/class			Total analyzed	Transmission (%/class)		
			P[a]	BP[b]	M[c]		P	BP	M
1-a	RS-K2C	MS-K	28	7	2	37	76	19	5
1-b	MS-K	RS-K2C	45	4	1	50	90	8	2
2-a	RS-K2C	MS-H	10	3	0	13	77	23	0
2-b	MS-H	RS-K2C	10	2	1	13	77	15	8
3-a	MS-H	MS-K	16	4	0	20	80	20	0
3-b	MS-K	MS-H	27	1	1	29	93	3	3
4-a	RS-K2C	MS-I	5	0	0	5	100	0	0
4-b	MS-I	RS-K2C	4	1	0	5	80	20	0
5-a	AN	FA	12	2	0	14	86	14	0
5-b	FA	AN	7	1	0	8	87	13	0
6-a	RS-K2C	FA	3	5	0	8	37	63	0
6-b	FA	RS-K2C	2	2	0	4	50	50	0
7-a	MS-K	AN	1	1	0	2	50	50	0
7-b	AN	MS-K	1	0	0	1	100	0	0
8	MS-H	AN	1	1	0	2	50	50	0
9	AN	RS-K2C	1	0	0	1	100	0	0
Total			173	34	5	212	82	16	2

[a] Paternal
[b] Biparental
[c] Maternal

of them heteroplastidic and two homoplastidic paternal, all exhibited expected restriction patterns (not shown). Also, roots from eight homoplastidic plants from different reciprocal crosses all contained the same plastid type as did their shoots (not shown).

Table 2 breaks down cpDNA transmission into paternal (P), biparental (BP), and maternal (M) classes for all crosses, as summarized by frequency of plants/class and percentage of plants. Frequencies of M were very low, making comparisons difficult. Because the percentage of M is small, the percentages of P and BP complement each other. The frequency of progeny exhibiting only P ranged from 37% to 100% with different crosses, and the 100% values occurred only where few plants were analyzed.

The percentage of P plants was compared among all combinations of the three-plant reciprocal crossing scheme (reciprocal crosses 1, 2, and 3), where more plants were analyzed (Table 2). Higher percentages of paternal transmission were noted in crosses 1-b and 3-b, where the MS-K plant was used as a female parent (90% and 93%), than when it was used as a male parent in crosses 1-a and 3-a (76% and 80%). With RS-K2C and MS-H, the percentage of paternal transmission was 77% in either direction.

Differences were apparent also when comparisons were made between intra-subspecific and inter-subspecific crosses (Table 3). Intra-subspecific reciprocal crosses were more alike in overall paternal transmission (84%–86% P) than were inter-subspecific reciprocal crosses (42%–67% P). Small sample sizes, in part due to the low success rate of interploidy crosses through unreduced gametes, made comparisons within an individual interploidy reciprocal cross meaningless. Thus, data were pooled. When total inter-subspecific crosses were compared, female (58% BP) and male (67% P) tetraploid parents transmitted their plastids at a higher rate than did female (33% BP) and male (42% P) diploid parents. Paternal plastid transmission followed two patterns. Intra-subspecific crosses showed a high percentage of P and low percentage of BP in the progeny. Inter-subspecific crosses exhibited a relatively low percentage of P and a high percentage of BP in the hybrids.

Discussion

Our cpDNA analyses clearly establish the role of pollen transmission of plastids in the initiation of heteroplasmy (Fig. 2A–D), a phenomenon noted in randomly selected green *Medicago* plants by Johnson and Palmer (1989). As might be expected, plastids in heteroplastidic plants can sort out, resulting in homoplastidic tissues (Fig. 2B). Eight homoplastidic plants possessing paternal plastids were analyzed. All showed the same plastid type in both root and shoot. Clearly, the high frequency of paternal homoplasmy is not the result of any preferential sorting-out in the embryo during early cell divisions that give rise to root:shoot differentiation.

Maternal nuclear control of plastid transmission frequency seems apparent in our three-plant reciprocal

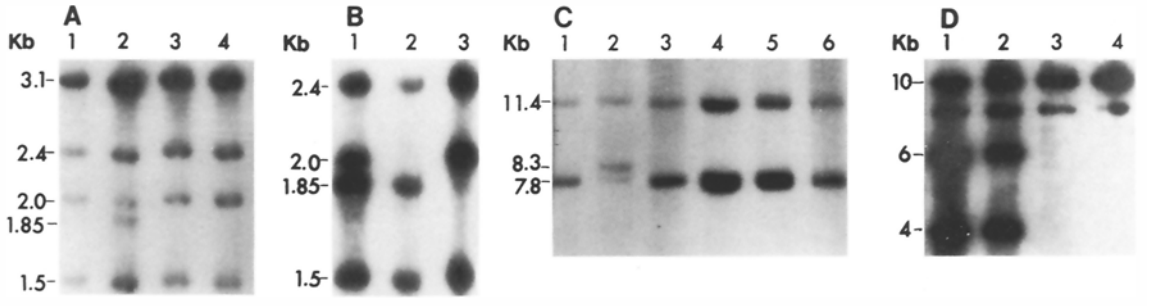

Fig. 2 A–D. Autoradiograms showing cpDNA transmission in alfalfa sexual crosses, and conversion of heteroplasmy to homoplasmy during vegetative propagation. **A** BamHI-digested DNAs from leaves of four hybrid seedlings of RS-K2C × MS-K, separated in a 1.1% agarose gel and hybridized to the 12.5-kb alfalfa cpDNA probe. Hybrids in *lanes 1, 3,* and *4* show a 2.00-kb fragment from the MS-K paternal plastids, while the heteroplastidic hybrid in *lane 2* has both paternal and maternal (1.85 kb) plastid fragments. **B** BamHI-digested DNA, of regrowth shoots from the hybrid in *lane 2* above, separated in a 1.1% agarose gel and hybridized to the 12.5-kb alfalfa cpDNA probe. Leaves from three different sectors, each containing one or two shoots regrown from the crown, were separately analyzed for their plastid source. Sectors in *lanes 2* and *3* show maternal and paternal plastids, respectively. The sectors in *lane 1* exhibit both plastids. **C** XbaI-digested DNA extracted from six RS-K2C × MS-H hybrids, separated in a 0.7% agarose gel and hybridized to the 6.2-kb alfalfa cpDNA probe. *Lane 2* contains a heteroplastidic plant with 7.8 and 8.3 kb fragments. All other *lanes* have plants with only paternal plastids, as shown by the presence of 7.8-kb fragments. **D** HindIII-digested DNA from two plants from a FA × RS-K2C cross at two consecutive harvests, separated in a 0.7% agarose gel and hybridized to the 12.5-kb alfalfa cpDNA probe. The maternal FA cpDNA contains an additional restriction site, producing 6.0- and 4.0-kb fragments instead of a 10-kb fragment. *Lanes 1* and *2* contain extracts from a heteroplastidic hybrid, and show decreased maternal cpDNA and increased paternal cpDNA in the later extract (*lane 2*). Only paternal cpDNA was detected in extracts of the hybrid plant represented in *lanes 3* and *4*

Table 3. Summary of plastid transmission in crosses within and between two *Medicago sativa* subspecies

Parents	Plants/class			Total analyzed	Transmission (%/class)		
	P[a]	BP[b]	M[c]		P	BP	M
Total reciprocal crosses within ssp. *sativa*	145	22	5	172	84	13	3
Total reciprocal crosses within ssp. *falcata*	19	3	0	22	86	14	0
♀ *sativa* × ♂ *falcata*	5	7	0	12	42	58	0
♀ *falcata* × ♂ *sativa*	4	2	0	6	67	33	0
Total	173	34	5	212	82	16	2

[a, b, c] Same as in Table 2

crossing scheme (crosses 1, 2, and 3 in Fig. 1, and Table 1), where three parents are each reciprocally crossed with the other two. Crosses 1-b and 3-b involving MS-K as the maternal parent show a higher paternal plastid transmission than do the other four crosses, where MS-H or RS-K2C are the maternal parents. The percentage of plants showing biparental transmission is correspondingly reduced. Maternal nuclear regulation appears to explain these data. Regulation by paternal or maternal plastids is not involved in this difference, since the same plastids, when transmitted to other nuclear backgrounds, are all equally successful. A plastid which solely regulates its own high transmission rate should increase its transmission frequency in either direction in a reciprocal cross, which was not the case here. However, plastid influences on their own transmission are well documented in other species such as *Oenothera* (Chiu et al. 1988). For our material, the hybrid nuclear genotype also would not appear to be a major factor, again because of differences in plastid transmission frequency within a reciprocal cross. It appears that MS-K, when used as a female parent, transmitted its plastids at a lower rate than did either MS-H or MS-K as maternal parents.

The maternal parent contributes much toward controlling biparental chloroplast inheritance in *Pelargonium*. A *Pr* gene in the female nucleus predominantly controls plastid inheritance patterns, presumably through its effect on plastid replication (Kirk and Tilney-Bassett 1978; Tilney-Bassett and Birky 1981; Tilney-Bassett and Abdel-Wahab 1982). Two different distribution frequencies of maternal, biparental, or paternal plastid transmission result with different female parents of *Pelargonium* (Tilney-Bassett and Birky 1981). The first has a high maternal, intermediate biparental, and low

paternal transmission. The second has a high frequency of both maternal and paternal transmission, but biparental plastid transmission is generally low (Tilney-Bassett and Birky 1981). Both patterns are different than in alfalfa, where paternal transmission is high and maternal, low.

Two observations are of interest in the inter-subspecific crosses (Table 3). First, our tetraploid *M. sativa* ssp. *sativa* plants, used as either male or female parents, transmitted their plastids at a higher rate than did the diploid *M. sativa* ssp. *falcata* genotypes. Plastid load may contribute to the efficiency of plastid transmission (Kirk and Tilney-Bassett 1978; Birky 1983). Plastid number in diploid cells, which are typically smaller, should be lower than that in larger tetraploid cells (Molin et al. 1982; Pyke and Leech 1987). However, our hybrid plants produced by these crosses were all tetraploids, indicating fertilization by unreduced gametes, which are of similar size (Pfeiffer and Bingham 1983) and presumably equal proplastid number to tetraploid gametes. This suggests that more than plastid load is involved.

Second, in both inter-subspecific reciprocal crosses, a higher percentage BP and a lower percentage P occurred relative to the intra-subspecific crosses. Genetic differences between the two subspecies may influence plastid transmission frequencies in crosses between them. These subspecies, while sexually compatible, are considered by some to be distinct species (Lesins and Lesins 1979). The genetic control of plastid transmission in these crosses is difficult to evaluate, in part because of pooling of crossing data necessitated by the small sample size. However, it appears that factors other than the maternal nuclear genotype contribute to the control of plastid transmission. For example, plant RS-K2C was used as a female parent in four different crosses. Paternal plastid transmission ranged from 37% to 100%. Differences are apparent even though sample size for some of these crosses was small (Table 2). Presumably, paternal nuclear and/or plastid factor(s) also are involved. For whatever reason, a high frequency of heteroplastidic plants results.

Smith et al. (1986) evaluated transmission frequencies with chlorophyll-deficient plastid mutants in reciprocal crosses. Their transmission frequencies are difficult to compare with ours, because of differences in the parental material and the scoring procedure, although they also saw a high frequency of paternal transmission. Recovery of the chlorophyll deficiency used in either parent in the progeny was an indicator of chloroplast transmission by that parent. However, in our data this is equivalent to the summation of biparental transmission frequency, and paternal or maternal frequency, depending on the direction of the cross.

Extracts from several alfalfa plants were tested with two restriction enzymes for evidence of cpDNA recombination. As might be expected (Chiu and Sears 1985), none was found when 12 hybrid plants of cross 1 (RS-K2C × MSK) were compared following separate digestions with BamHI and XbaI. The two sites are located almost opposite each other on the circular alfalfa plastid genome (Johnson and Palmer 1989) and the parent cpDNAs differ at both sites. To our knowledge, recombination between two cpDNAs from higher plants has been demonstrated only in somatic hybrids derived from tissue culture (Medgyesy et al. 1985; Thanh and Medgyesy 1989). A much more extensive study would be required to detect recombination in our material, and should probably concentrate on progeny derived from selfing heteroplastidic plants.

Biparental plastid inheritance has been demonstrated by various DNA restriction techniques in conifers (Neale and Sederoff 1989; Szmidt et al. 1987; Wagner et al. 1987), the genus *Pelargonium* (Metzlaff et al. 1981), and alfalfa (Lee et al. 1988). The use of cpDNA as a marker for plastid inheritance, when combined with controlled crosses, allows a more meaningful estimation of the frequency of transmission than does the use of chlorophyll deficiency mutants, which may be transmitted at a competitive disadvantage. In *Pelargonium,* green plastids were transmitted at a higher rate than were mutant plastids (Tilney-Bassett and Birky 1981; Tilney-Bassett and Abdel-Wahab 1982). Two chloroplast mutants of *Oenothera* exhibited reduced transmission from the female parent relative to a third mutant, which was transmitted similarly to the wild type (Chiu et al. 1988).

There are other advantages as well for using cpDNA restriction fragment length polymorphisms for plastid transmission studies. A relatively large number of reciprocal crosses can be analyzed with high sensitivity for detection of a minor plastid component in a tissue. Crosses are not limited to genotypes that differ in plastid-encoded chlorophyll deficiency mutants. Both chloroplast and leucoplast analyses are possible. Aberrant transmission ratios due to seedling death resulting from chlorophyll deficiency mutants are eliminated. Induction and reversion of plastid mutations by nuclear mutator genes are common, at least in some species, and may interfere with plastid inheritance studies when chlorophyll deficiency markers are used (Gillham 1978; Cornu and Dulieu 1988).

Extraction of total plant DNA, followed by Southern hybridization, allows analysis of small seedlings and is much more sensitive than is ethidium bromide cpDNA staining. Comparative studies of plastid transmission involving both chlorophyll deficiency and cpDNA restriction pattern markers are not available.

We are unaware of other studies where plastid transmission frequency and vegetative segregation have been evaluated by cpDNA restriction analysis with green tissues rather than with plastid-encoded chlorophyll deficiency mutants. The high plastid paternal transmission

observed with alfalfa, similar in some ways to *Pelargonium,* suggests the potential of this species for studies on the regulation of plastid transmission through the pollen.

Acknowledgements. Joint contribution (No. 89-524-J) of the Department of Plant Pathology and USDA-ARS, Kansas Agricultural Experiment Station, Manhattan, KS 66506, USA. The provision by Dr. J. D. Palmer, Indiana University, of the cloned alfalfa chloroplast DNA fragments used as probes is gratefully acknowledged. We also thank Dr. M. Thomas for helpful comments.

References

Bingham ET, Hurley LV, Kaatz DM, Saunders JW (1975) Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci 15:719–721

Birky CW Jr (1983) Relaxed cellular controls and organelle heredity. Science 222:468–475

Chiu W-L, Sears BB (1985) Recombination between chloroplast DNAs does not occur in sexual crosses of *Oenothera.* Mol Gen Genet 198:525–528

Chiu W-L, Stubbe W, Sears BB (1988) Plastid inheritance in *Oenothera:* organelle genome modifies the extent of biparental plastid transmission. Curr Genet 13:181–189

Cornu A, Dulieu H (1988) Pollen transmission of plastid-DNA under genotypic control in *Petunia hybrida* Hort. J Hered 79:40–44

Corriveau JL, Coleman AW (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 Angiosperm species. Am J Bot 75:1443–1458

Gillham NW (1978) Organelle heredity. Raven Press, New York

Hames BD (1981) An introduction to polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D (eds) Gel electrophoresis of proteins, a practical approach. Practical Approaches Series. IRL Press, Oxford, pp 1–91

Johnson LB, Palmer JD (1989) Heteroplasmy of chloroplast DNA in *Medicago.* Plant Mol Biol 12:3–11

Kirk JTO, Tilney-Bassett RAE (1978) The plastids: Their chemistry, structure, growth, and inheritance, 2nd edn. Elsevier/North-Holland Biomedical Press, Amsterdam

Lee DJ, Blake TK, Smith SE (1988) Biparental inheritance of chloroplast DNA and the existence of heteroplasmic cells in alfalfa. Theor Appl Genet 76:545–549

Lee DJ, Blake TK, Smith SE, Bingham ET, Carroll TW (1989) Chloroplast genome mapping and plastid ultrastructure analysis of chlorophyll deficient mutants of alfalfa. Crop Sci 29:190–196

Lesins KA, Lesins I (1979) Genus *Medicago* (Leguminosae), a taxogenetic study. W. Junk, The Hague

Lilienfeld FA (1962) Plastid behavior in reciprocally different crosses between two races of *Medicago truncatula* Gaertn. Seiken Zihô 13:3–38

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6960–6964

Metzlaff M, Börner T, Hagemann R (1981) Variations of chloroplast DNAs in the genus *Pelargonium* and their biparental inheritance. Theor Appl Genet 60:37–41

Miyamura S, Kuroiwa T, Nagata T (1987) Disappearance of plastid and mitochondrial nucleoids during the formation of generative cells of higher plants revealed by fluorescence microscopy. Protoplasma 141:149–159

Molin WT, Meyers SP, Baer GR, Schrader LE (1982) Ploidy effects in isogenic populations of alfalfa. II. Photosynthesis, chloroplast number, ribulose-1,5-bisphosphate carboxylase, chlorophyll, and DNA in protoplasts. Plant Physiol 70:1710–1714

Neale DB, Sederoff RR (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor Appl Genet 77:212–216

Palmer JD (1986) Isolation and structural analysis of chloroplast DNA. Methods Enzymol 118:167–186

Palmer JD, Osorio B, Aldrich J, Thompson WF (1987) Chloroplast DNA evolution among legumes: Loss of a large inverted repeat occurred prior to other sequence rearrangements. Curr Genet 11:275–286

Pankiw P, Siemens B (1976) Anik alfalfa. Can J Plant Sci 56:203–205

Pfeiffer TW, Bingham ET (1983) Abnormal meiosis in alfalfa, *Medicago sativa:* cytology of 2 N egg and 4 N pollen formation. Can J Genet Cytol 25:107–112

Pyke KA, Leech RM (1987) The control of chloroplast number in wheat mesophyll cells. Planta 170:416–420

Quiros CF (1981) Starch gel electrophoresis technique used with alfalfa and other *Medicago* species. Can J Plant Sci 61: 745–749

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 13:7207–7221

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76

Rose RJ, Johnson LB, Kemble RJ (1986) Restriction endonuclease studies on the chloroplast and mitochondrial DNAs of alfalfa (*Medicago sativa* L.) protoclones. Plant Mol Biol 6:331–338

Scowcroft WR, Larkin PJ (1981) Chloroplast DNA assorts randomly in intraspecific somatic hybrids of *Nicotiana debneyi.* Theor Appl Genet 60:179–184

Smith SE, Bingham ET, Fulton RW (1986) Transmission of chlorophyll deficiencies in *Medicago sativa,* evidence for biparental inheritance of plastids. J Hered 77:35–38

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 38:9–13

Szmidt AE, Aldén T, Hällgren J-E (1987) Paternal inheritance of chloroplast DNA in *Larix.* Plant Mol Biol 9:59–64

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum.* Plant Mol Biol 12:87–93

Tilney-Bassett RAE, Abdel-Wahab OAL (1982) Irregular segregation at the *Pr* locus controlling plastid inheritance in *Pelargonium;* gametophytic lethal or incompatibility system? Theor Appl Genet 62:187–191

Tilney-Bassett RAE, Birky CW Jr (1981) The mechanism of the mixed inheritance of chloroplast genes in *Pelargonium.* Evidence from gene frequency distributions among the progeny of crosses. Theor Appl Genet 60:43–53

Viands DR, Sun P, Barnes DK (1988) Pollination control: mechanical and sterility. In: Hanson AA, Barnes DK, Hill RR Jr (eds) Alfalfa and alfalfa improvement. Agron. Monogr 29. Am Soc Agron, Crop Sci Soc Am, and Soil Sci Soc Am, Madison, pp 931–960

Wagner DB, Furnier GR, Saghai-Maroof MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097–2100