Sex Plant Reprod (1995) 8:308–312

ORIGINAL PAPER

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Variation in generative cell plastid nucleoids and male fertility in *Medicago sativa*

Received: 6 March 1995 / Revision accepted: 9 March 1995

Abstract Biparental inheritance of plastids has been documented in numerous angiosperm species. The adaptive significance of the mode of plastid inheritance (uni- or biparental) is poorly understood. In plants exhibiting paternal inheritance of plastids, DNA-containing plastids in the microgametophyte may affect survival or growth of the gametophyte or the embryo. In this study the number of plastids containing DNA (nucleoids) in generative cells and generative cell and pollen volumes were evaluated in a range of genotypes of *Medicago sativa* (alfalfa). *M. sativa* exhibits biparental inheritance of plastids with strong paternal bias. The *M. sativa* genotypes used were crossed as male parents to a common genotype and the relationships between the gametophytic traits measured and male reproductive success were assessed. Generative cell plastid number and pollen grain size exhibited opposing associations with male fertility. Path analysis showed that generative cell plastid number was negatively associated with male fertility. This study provides evidence that there may be a competitive advantage at fertilization afforded sperm that have minimized their organelle content. The apparent lack of strong selection for reduced plastid number in generative cells of *M. sativa* may be a reflection of the diminished importance of reproductive success due to its perenniality or its long use in cultivation.

Key words Alfalfa · Chloroplast · Organelle inheritance · Pollen

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Introduction

Biparental inheritance of plastid DNA (cpDNA) has been documented in at least 15 angiosperm genera (Smith 1989b; Harris and Ingram 1991). Cytological analysis of generative or sperm cells with the DNA fluorochrome DAPI (4′-6-diamidino-2-phenylindole) indicates that biparental inheritance of plastids is also likely in many additional taxa (Corriveau and Coleman 1988). Little is known, however, regarding the possible adaptive significance of the various modes of plastid inheritance. Keys et al. (1995) demonstrated that the mean number of DNA-containing plastids (nucleoids) in generative cells from individual genotypes of 18 species from *Medicago*, *Melilotus*, *Trigonella* and *Trifolium* was unrelated to qualitative variation in mating system and life history characteristics of the species. The number of plastid nucleoids in these species was proportional to the volume of the pollen grain and generative cell nucleus in comparisons among *Medicago* species. A similar association was found between the number of plastids per generative cell and cytoplasmic and nuclear volumes of cells at the intraspecific level in *M. sativa* (Zhu et al. 1991).

There have been no studies that directly address relationships that may exist between variation in the number of microgametophytic plastids and male fertility. We have attempted to do so using *Medicago sativa* (alfalfa) as a model of an insect-pollinated herbaceous perennial. *M. sativa* exhibits biparental inheritance of plastids with a strong paternal bias (Smith et al. 1986; Schumann and Hancock 1989). Genetic differences have been observed in both paternal and maternal plastid transmission in this species (Smith 1989a). Significant variation has also been observed among different *M. sativa* genotypes in the number of plastids and DAPI-staining plastid nucleoids per generative cell (Shi et al. 1991), although this variation is not necessarily associated with extent of paternal plastid transmission (Shi et al. 1991; Zhu et al. 1991).

The objectives of this study were to: (1) quantify the numbers of plastid nucleoids in the microgametophytes

and the volumes of generative cell nuclei and pollen grains of a diverse range of alfalfa genotypes and (2) evaluate relationships between these variables and male reproductive success based on hand pollinations made onto a common female parent. Our results demonstrated opposing associations of generative cell plastid content and pollen grain size with male fertility. Because of the interrelated nature of the different variables contributing to male fertility and the random nature of the variables involved, path analysis was employed to clarify interpretation of the results. This analysis revealed that microgametophytic plastid content was negatively associated with male fertility.

Materials and methods

Plant materials and crosses

Eight *M. sativa* plants from cultivated populations representing a range of the genetic variation in this species (Barnes et al. 1977) were used in this study (Table 1). Plants were identified by the name of the cultivar from which they originated or the nation from which they were collected. Plants were maintained in a greenhouse with continuous fluorescent illumination and hand pollinations were made to evaluate male fertility. All crosses were made using a male sterile genotype (6–4 ms, selected from the cultivar Saranac and provided by ET Bingham, University of Wisconsin) as a female parent. Pollen was collected by tripping flowers onto a spatula, which was then used to trip flowers of the female, thereby forcing the stigma into the pollen mass. Up to ten flowers were pollinated on a single raceme for each pollen parent on four different days. When the pods had matured, the number of pods and number of normal (filled) and aborted seeds per pod were recorded. Fertilized ovules that aborted early in seed development (in the first 7–10 days post-pollination) could not be identified as such in this research. These data were expressed as number of pods and numbers of filled and shrivelled seeds per flower pollinated for each raceme. All plants were rated for the amount of pollen produced using a 1–5 scale, with 1 being low and 5 being high pollen production. Scores were based on evaluation of a minimum of ten flowers on a single day.

Pollen germination and cytology for quantification of plastid nucleoids

Quantification of plastid nucleoids was done using in vitro germinated pollen because: (1) plastid nucleoid content can vary depending on stage of development of the microgametophyte (Corriveau and Coleman 1991), (2) plastid content of the developing microgametophyte would be more relevant to male fertility than that of ungerminated pollen, and (3) visualization of plastid nucleoids is facilitated in pollen tubes. Pollen tubes were grown using a modification of method iv described by Corriveau and Coleman (1988). Pollen from 5–8 flowers was placed in a depression well to which 100 µl of germination medium (15% sucrose, 0.16 mM H_3BO_3, 0.20 mM KNO_3, and 0.13 mM $Ca(NO_3)_2 \cdot 4H_2O$) was added. Germination was allowed to proceed for 2.5–3 h at 22–27°C in a petri plate containing moistened filter paper. One hundred microliters of 3:1 (v/v) 95% ethanol:glacial acetic acid was added to the well and the contents transferred to a microcentrifuge tube. This process was repeated with an additional 100 µl of the ethanol:glacial acetic acid solution. Using this fixative, mitochondrial DNA aggregates would not be preserved (Corriveau and Coleman 1988), thus assuring that any DNA aggregates that were visualized were of plastid origin. After 12–18 h storage at 4°C, the tubes were centrifuged at 100 rpm for 5 min and the supernatant was replaced with 230 µl of 70% ethanol. This suspension was stored at 4°C. For staining, 50 µl of the fixed pollen was spread on an acid-cleaned microscope slide and allowed to dry. Two drops of 0.05 µg/ml DAPI in Aqua-Poly/Mount (Polysciences, Warrington, Pa.) were placed on each slide and covered with an acid-cleaned coverslip.

Plastid nucleoids in the generative cell were visualized at ×1000 using a Zeiss Axiophot epifluorescence microscope and the Zeiss 487902 combination of excitation and emission filters. The number of nucleoids in each generative cell was recorded from 45 intact generative cells from developing microgametophytes of each male parent. Counts were taken only from cells in which nucleoids were clearly visible and there was no indication of mitotic activity. This procedure assured that microgametophytes from all male parents were evaluated at a similar stage of development,

Table 1 Description, mean nucleoid number, generative cell nuclear volume, pollen volume, pollen production and fertility attributes of *Medicago sativa* plastid parents (standard errors in parentheses)

Pollen parent	Region of origin	Nucleoid number/ generative cell	Generative cell nuclear volume (μm^3)	Pollen volume ($\mu m^3 \times 10^3$)	Pods/ flower[a]	Filled seeds/pod	Empty seeds/pod	Filled seeds flower
Oman A	Arabia	108.7 (1.8)	34.4 (6.0)	19.8 (1.4)	1.0 A (0.03)	3.2 ABC (0.2)	0.7 A (0.13)	3.0 AB (0.3)
Egypt III D	N. Africa	95.4 (1.6)	41.4 (4.8)	19.1 (2.0)	0.9 A (0.04)	2.9 BC (0.2)	0.8 A (0.12)	2.7 AB (0.2)
Lahonton E	C. Asia	98.4 (2.5)	33.3 (4.0)	21.1 (2.1)	0.9 A (0.03)	3.5 ABC (0.2)	0.8 A (0.13)	3.3 A (0.4)
Malone A	C. Asia	66.9 (1.9)	31.5 (3.8)	15.5 (1.4)	1.0 A (0.03)	4.0 A (0.3)	0.4 A (0.12)	3.9 A (0.3)
Ladak 25	S. Asia	105.3 (4.2)	42.8 (3.5)	14.0 (1.5)	0.7 C (0.03)	1.9 D (0.2)	0.6 A (0.15)	1.2 C (0.1)
Russia A	N. Europe	89.1 (2.5)	32.5 (3.7)	18.6 (2.5)	0.9 A (0.03)	3.3 ABC (0.3)	0.5 A (0.11)	3.1 AB (0.5)
DuPuits B	C. Europe	93.1 (2.5)	25.0 (3.6)	19.4 (1.6)	0.8 BC (0.08)	3.8 AB (0.3)	0.4 A (0.12)	2.9 AB (0.5)
1T, green	C. Europe	113.0 (2.0)	28.2 (4.6)	19.1 (1.5)	0.8 AB (0.05)	2.6 CD (0.2)	0.4 A (0.09)	2.1 BC (0.3)

[a] Means followed by the same upper-case letter are not significantly different by Duncan's Multiple Range Test, $P \leq 0.05$

thus avoiding possible confounding ontogenetic effects on plastid nucleoid content (Corriveau and Coleman 1991). Photographs of five DAPI-stained generative cells per male parent were taken using 400 ASA Tri-X Pan film. Dimensions of the nuclei of these cells were estimated by projecting the negative image at a fixed magnification, tracing their outlines on white paper, and measuring their maximum lengths and widths. Nuclear volumes were estimated by assuming that the nuclei were ellipsoids with circular cross-sections, and using the equation for the volume of an ellipsoid (Volume=$4/3\pi b^2 a$, where b=1/2 width and a=1/2 length). Using phase contrast microscopy at ×1000, diameters along the widest and narrowest axes were measured on ungerminated, but hydrated, pollen grains. An average diameter was calculated for each grain using these values. Volume of pollen grains was estimated as $4\pi r^3/3$, where r=1/2 diameter.

Statistical analyses

Data were analyzed using the general linear models procedure (SAS Institute, 1985), and mean separations were accomplished using Duncan's Multiple Range Test. Because all of the measured variables were random, they were subjected to path analysis (Sokal and Rohlf 1981) according to the model shown in Fig. 1. The ratio of the number of plastid nucleoids to pollen grain volume was also calculated, and partial correlation coefficients between these ratios and male fertility variables were calculated.

Results

Pollen production did not differ significantly among plants (data not shown). However, there was significant variation among plants for all other variables, with the exception of the number of aborted seeds per pod (Table 1). Region of origin of the genotypes had no apparent effect on any of the variables. Male reproductive success of the plants used in this study was in the range generally expected for alfalfa in which filled seed-to-ovule ratios are typically less than 0.5 (Sayers and Murphy 1966; Smith et al. 1990). Floral abortion (mean±SE=12.5± 10.1% of all flowers pollinated over all male parents) was somewhat lower in this experiment than has been observed with other male parents crossed onto 6–4 ms (25.9±2.0%; Smith et al. 1990). Conversely, mean seed production per pod appeared slightly lower with this sample of male parents (3.15±0.24%) than was observed in previous experiments with 6–4 ms (4.9±0.2%, Smith et al. 1990).

Path coefficients showing the relationships among microgametophytic traits and male fertility are shown in Fig. 1. The overall coefficient of determination for the model was 0.906. Within a path diagram the correlation between two variables equals the sum of the products of the chain of path coefficients or correlations along all of the paths by which they are connected (Sokal and Rohlf 1981). This correlation coefficient, derived from values in the path diagram (Fig. 1), showed that plastid nucleoid number per generative cell was negatively and significantly ($P \leq 0.05$) correlated with the number of filled seeds per flower (r=–0.717). Correlations were nonsignificant between seeds per flower and both generative cell nuclear volume (r=–0.230) and pollen volume (r=0.265). Generative cell nuclear volume was negligibly

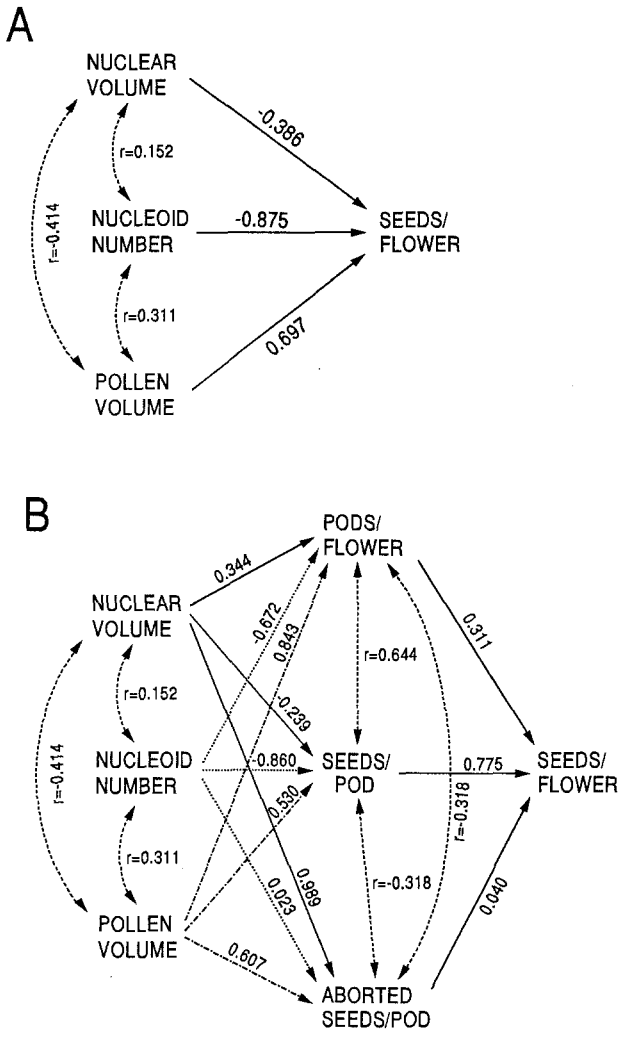

Fig. 1 Path analysis diagrams describing microgametophytic characters and components of male reproductive success for eight genotypes of *Medicago sativa* when crossed with the male-sterile genotype 6–4 ms. *Double-headed arrows* depict correlations and numerical values are path coefficients. *Single-headed arrows* depict cause-and-effect relationships (paths) and numerical values are standardized regression coefficients. **A** Microgametophytic characters and seeds per flower pollinated. **B** Microgametophytic characters, pod and seed retention, and seeds per flower pollinated

associated with filled seeds per flower, largely because of countervailing associations with pods per flower and filled seeds per pod. The number of aborted seeds per pod had a negligible effect on number of filled seeds per flower. Plastid nucleoid number showed strong negative association with both pods per flower and seeds per pod, and a slightly positive association with the number of aborted seeds per pod. Pollen volume, on the other hand, showed strong positive association with these fertility traits. Partial correlations of the ratio of plastid nucleoid number to pollen volume with the numbers of seeds/pod (r=–0.951), pods/flower (r=–0.752) and seeds per flower (r=–0.951) were all significant ($P \leq 0.01$), except for the number of aborted seeds per pod (r=0.206).

Discussion

Our results indicate that the presence of additional DNA-containing plastids in generative cells of *M. sativa* does not improve male fertility, at least under the conditions of this study. In fact, the association between the number of plastid nucleoids per generative cell and male fertility was negative, suggesting that greater plastid loads are actually associated with reduced male fertility. This reduction in male fertility was mediated through strong negative associations between plastid nucleoid number and both the proportion of flowers that form pods and the number of seeds produced per pod. Seed abortion after pollination was a relatively unimportant factor affecting seed production per flower in this research. Therefore, reduction in male fertility associated with plastid nucleoid number is probably the result of events occurring immediately before or during fertilization, or events occurring in the zygote shortly after fertilization. Keys et al. (1995) pointed out the importance of evaluating differences among genera in generative cell plastid content only after correction for pollen grain size. In this study, plastid content adjusted for pollen grain size still resulted in negative associations with male fertility variables. Therefore, generative cell plastid content was clearly negatively associated with male fertility at the intraspecific level in this study.

Pollen limitation was probably not responsible for variation in male fertility in this study since there were no measurable differences in pollen production among the male parents and crosses were made using an excess of pollen. Smith et al. (1990) found that applying excess pollen to the stigma, providing approximately 27 times more pollen tubes per ovary than light pollen application, merely doubled the seed-to-ovule ratio in *M. sativa*. Variation in pollen application in the present study did not approach this differential, yet the number of filled seeds per pod varied by a factor of two.

Our results showed that plants with inherently larger pollen grains were more fertile as males, but that this effect is confounded with plastid nucleoid content because larger grains also contain more nucleoids. Under conditions permitting pollen competition, larger pollen grains tend to have a competitive advantage in penetrating ovules (Willson and Burley 1983; Cruzan 1990). If this is true for *M. sativa* when pollen competition is a factor, then the more successful grains would also contain more plastid nucleoids that have a depressing effect on male fertility. Variability in male fertility would probably be reduced if compared to pollinations in which pollen was limited, because plastid nucleoid content would be more random without selection due to pollen competition. However, it is not possible to determine whether male fertility would increase or decrease when pollen competition is limited since the relative sizes of the effects of pollen grain size and generative cell plastid nucleoid number are not known.

The strong negative relationship between male reproductive success and plastid nucleoid number we observed provides some support for the hypothesis presented by Kirk and Tilney-Bassett 1978 that there may be a competitive advantage at fertilization afforded nonmotile sperm cells that have minimized their organelle content. If this is the case, selection would be expected to reduce plastid nucleoid number to optimize reproductive success. However, considerable variation was observed in both male fertility and plastid nucleoid number among the *M. sativa* genotypes studied. This suggests that if such selection occurs in *M. sativa*, it is not highly effective. This may be a reflection of the highly artificial selection these plants were exposed to in cultivation for herbage where reproductive traits are typically of secondary importance (Rumbaugh et al. 1988). It may also be due to the fact that *M. sativa* is a long-lived perennial with multiple reproductive opportunities. This would reduce the relative intensity of selection operating on all traits associated with sexual reproduction compared to what would be common in populations of annual plants (Wiens 1984).

These results do not demonstrate that the genotype of paternally derived plastids is responsible for reduced male fertility in alfalfa. Any effects of plastid nucleoid number were confounded with the different male nuclear and plastid genotypes involved in this study. If plastid nucleoid number is a heritable trait, then populations could be developed with isonuclear backgrounds that would permit separation of nuclear and plastid effects on male fertility. There could also be male nucleus/male plastid, male nucleus/female plastid, or female nucleus/male plastid interactions, or combinations of these, that are important in the developing zygote and endosperm. Zhu et al. (1993) have shown that sorting out of maternal plastids begins in egg cells prior to fertilization. It has also been demonstrated that sorting out of male and female plastids begins immediately after fertilization in *M. sativa* (H.L. Mogensen, unpublished data). Nuclear/plastid and maternal/paternal interactions at this early stage of zygote development could be quite important. Larger male plastid populations may present increased difficulties at this stage, leading to abortion. A single female was used for crossing in this study. Using a range of both maternal and paternal backgrounds could help to elucidate male/female, nuclear/plastid interactions in developing zygotes in this species.

Acknowledgements The authors acknowledge the assistance provided by L. Shi, J. Verbeke and T. Zhu. This research was supported by National Science Foundation grant IBN-9119740.

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