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Paternal plastid inheritance in alfalfa: plastid nucleoid number within generative cells correlates poorly with plastid number and male plastid transmission strength

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Summary. A previous study on alfalfa determined that the number of plastids/generative cell does not necessarily correlate with male plastid transmission strength in a given genotype. The objectives of the present study were to learn (1) whether plastid nucleoid number/generative cell is comparable to the number of plastids/generative cell, and (2) whether plastid nucleoid number/generative cell correlates with known male plastid transmission behavior in three alfalfa genotypes. Our results, which were based upon 150 generative cells examined by DAPI/epifluorescence microscopy, indicate that the mean plastid nucleoid number/generative cell is much less than the mean number of plastids/generative cell in genotype 7W (60 nucleoids/264 plastids) and genotype 301 (54 nucleoids/165 plastids). In genotype MS-5, mean plastid nucleoid number/generative cell (45) is similar to the mean number of plastids/generative cell (65). The significantly fewer plastid nucleoids/generative cell in MS-5, compared to that of 7W and 301, correlates positively with the relatively poor male plastid transmission strength of this genotype. However, the difference between the mean number of plastid nucleoids/generative cell in 7W and 301 is not significant, yet 301 is a much stronger transmitter of male plastids than is 7W.

Key words: Cytoplasmic inheritance – DAPI fluorochrome – *Medicago sativa* – Plastid DNA

Introduction

A recent study on mature alfalfa (*Medicago sativa*) pollen showed that the number of plastids/generative cell does not necessarily correlate with known patterns of male plastid transmission in certain genotypes (Zhu et al. 1991). Of the three genotypes investigated, MS-5, which is a relatively poor transmitter of male plastids (Smith, unpublished), has the fewest plastids/generative cell (mean = 65). However, another relatively weak male plastid transmitter, genotype 7W, has the most plastids/generative cell (mean = 264) of the three genotypes. The strongest male plastid transmitter, genotype 301, has an intermediate number of generative cell plastids; mean = 165 (Zhu et al. 1991). A possible explanation for these results is that not all the plastids present within generative cells of mature alfalfa pollen contain DNA. Since the plastid counts (Zhu et al. 1991) were made from serial ultrathin sections, no information was available regarding plastid DNA content.

In the present study, we used the DNA-fluorochrome/epifluorescence microscopic technique (Miyamura et al. 1987; Corriveau and Coleman 1988) to estimate the number of plastid DNA aggregates (plastid nucleoids) within generative cells of alfalfa. The purpose of this study was to determine: (1) whether the number of plastid nucleoids approximates the number of plastids within alfalfa generative cells, and (2) whether the number of plastid nucleoids/generative cell correlates with relative male plastid transmission strength in specific genotypes.

Materials and methods

Genotypes of *Medicago sativa* L. used in this study were the same as those used in a previous study on quantitative aspects of generative cells within mature pollen (Zhu et al. 1991). Briefly, genotype 301 is considered the "strongest" male of the three genotypes because, when used as the male parent in crosses with a weak female, over 90% of the progeny contained only paternal plastids. Genotype 7W is designated as a relatively weak male since, when crossed with the same female parent, 62% of the progeny contained only paternal plastids (Smith 1989b). The third genotype, MS-5, is considered to be an even weaker male plastid transmitter. In preliminary studies using a moderately strong female and MS-5 as the male parent 9.5% of the progeny contained only paternal plastids, whereas in crosses with the same female parent and 301 as the male parent 53.8% of the progeny contained only paternal plastids (Smith unpublished). Details of plant growing conditions, genotype-specific male plastid transmission strength, and genotype ancestry have been published previously (Smith et al. 1986; Smith 1989b; Zhu et al. 1991). Since plastid nucleoids are much more visible when the generative cells have entered the pollen tubes,

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pollen tubes were grown by sprinkling fresh pollen onto thin layers of 1% agar (containing 15% sucrose, 0.001 g/l boric acid, and 0.001 g/l $CaCl_2$ in distilled water) on microscope slides. After 2 h of incubation under moist conditions at room temperature, the young pollen tubes were flooded with a fixing solution of 95% ethanol and glacial acetic acid (3:1). Following an overnight fixation at 4 °C, the slides were transferred into 70% ethanol and stored at 4 °C. Slides were allowed to air dry before staining with 0.05 μg/l 4′,6-diamidino-2-phenylindole (DAPI) in McIlvaine's pH 4.1 buffer (four parts 0.2 M Na_2HPO_4 mixed with six parts 0.1 M citric acid). The number of cytoplasmic nucleoids was estimated in 150 generative cells (50 from each of the three genotypes) using a Zeiss Axioplan epifluorescence microscope. A combination of Zeiss 49-77-02 excitation and emission filters was used to produce an excitation peak between 340 and 370 nm. Photomicrographic negatives were taken with Kodak Tri-X Pan 400 film. Controls included (1) observations of generative cells without DAPI staining, and (2) observations of generative cells treated with deoxyribonuclease I (0.2 mg/ml in 1 mM Tris pH 7.5, 5 mM $MgCl_2$) for 2 h at 37 °C before DAPI staining. Because the generative cell nucleus also fluoresces after DAPI staining and would, therefore, mask any nucleoids near its periphery, and because overlying nucleoids may mask those below, the counts of this study must be considered as a conservative estimate. A one way analysis of variance and a mean separation using Duncan's multiple range test were conducted for comparisons of differences among genotypes.

Results and discussion

All generative cells, except those unstained or treated with deoxyribonuclease, gave a positive reaction for plastid nucleoids (Fig. 1). Results of plastid nucleoid counts are summarized in Table 1. Genotype MS-5 contained the lowest mean number of nucleoids/generative cell (45.4). Genotype 7W contained the largest mean number of nucleoids/generative cell (59.9); and genotype 301 contained an intermediate number of nucleoid/generative cell (54.4).

Mean plastid nucleoid number/generative cell is significantly less in genotype MS-5 than in either 7W or 301 ($P<0.05$). The difference between mean plastid nucleoid number/generative cell in genotypes 7W and 301 is not statistically significant ($P>0.05$).

Table 1 also summarizes the results of plastid counts/generative cell in the three genotypes as reported by Zhu et al. (1991). The ratio of mean plastid nucleoid number/mean plastid number is 0.73 in genotype MS-5 (weakest male), 0.23 in genotype 7W (intermediate male), and 0.31 in genotype 301 (strongest male).

Several considerations indicate that the technique used in this study is valid for estimating plastid nucleoid number within generative cells: (1) We used essentially the same methods as other workers who have studied putative plastid nucleoids within generative and/or sperm cells of flowering plants (Miyamura et al. 1987; Corriveau and Coleman 1988, 1990; Corriveau et al. 1989, 1990). (2) Control experiments using deoxyribonuclease demonstrate that plastid nucleoids do not stain following enzyme treatment (Corriveau et al. 1989; present study). (3) No nucleoids were observed in unstained generative cells of this study. (4) Only the 340–370 nm excitation range allowed detection of nucleoids. (5) Nucleoids were only seen within generative cells, not in pollen tube cytoplasm. (6) The fixative used in this study is believed to cause dispersion of mitochondrial DNA, thus mitochondrial nucleoids do not stain with DAPI after this treatment (Coleman 1984). (7) The presence of plastid DNA in alfalfa pollen has recently been detected using molecular techniques (Corriveau et al. 1990). (8) The general distribution of nucleoids is the same as that of plastids seen in three-dimensional reconstructions of alfalfa generative cells from serial ultrathin sections (Zhu et al. 1990a). (9) The relative differences in mean nucleoid number/generative cell agree with those of mean plastid number/generative cell as determined from serial ultrathin sections; i.e., genotype MS-5 has the fewest, followed by 301, and 7W (Table 1; Zhu et al. 1991).

Although it is possible that changes in plastid and/or plastid nucleoid number may occur after pollen germination, counts of plastid number within sperm pairs of genotype 301 indicate that plastid number does not change appreciably up to 17 h after pollen germination in this genotype (Zhu et al. 1990b). Therefore, we believe that the counts made from generative cells within the 2 h pollen tubes employed in the present study provide a good estimate of the number of nucleoids present within generative cells of mature pollen.

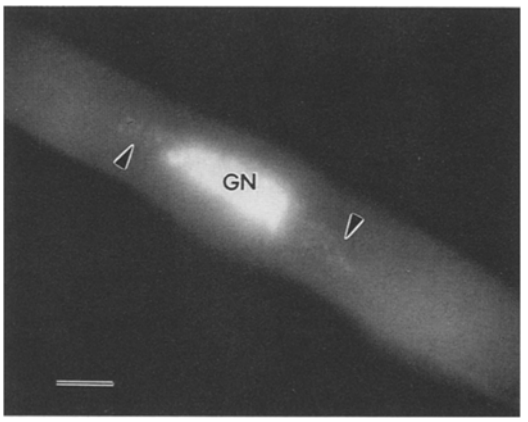

Fig. 1. Fluorescence micrograph of an alfalfa pollen tube after 2 h of in vitro growth. The generative nucleus (*GN*) and putative plastid DNA aggregates (nucleoids; *arrowheads*) fluoresce after staining with DAPI. Not all nucleoids are visible at a given plane of focus. Magnification bar = 10 μm

Table 1. Comparison of plastid nucleoid number with plastid number in generative cells from three genotypes exhibiting different levels of male plastid transmission, i.e., MS-5 weakest, 7W intermediate, and 301 strongest

Genotype	Nucleoids/generative cell[a]			Plastids/generative cell[b]		
	Mean	SD[c]	Range	Mean	SD[c]	Range
MS-5	45.4[d]	14.4	20–80	62.0[d]	10.4	50–69
7W	59.9[e]	24.5	16–155	264.7[e]	107.6	190–388
301	54.4	21.5	18–113	174.7	26.3	151–203

[a] Counts from 150 generative cells (50 from each genotype)
[b] Data from Zhu et al. 1991; based on counts from serial ultrathin sections of nine generative cells (three from each genotype)
[c] SD = Standard deviation of the mean
[d] Significantly less than genotype 301 or 7W ($P<0.05$)
[e] Not significantly different from 301 ($P>0.05$)

The finding that a considerable number of plastids within generative cells of genotypes 301 and 7W do not stain with the DNA-specific fluorochrome, DAPI, was unexpected. There is evidence that plastid DNA may be degraded throughout vegetative and generative cell cytoplasm during pollen development in species exhibiting maternal plastid inheritance (Miyamura et al. 1987; Corriveau and Coleman 1988), but this is the first case where the data suggest that a majority of the plastids within generative cells of a plant having biparental plastid inheritance may either lack DNA or have a plastome copy number too low to be detected with the techniques used in this study. While keeping in mind that the counts of this study are conservative, the amount of generative cell plastids lacking detectable DNA would still appear to be well over 50% in two of the three genotypes investigated. If plastids without stainable nucleoids do lack DNA, this condition could result from degradation of plastid DNA or from the formation of daughter plastids that do not receive DNA from parent plastids (Sodmergen et al. 1989). Since the generative cells of genotypes 301 and 7W contain numerous very small plastids (Zhu et al. 1990a, 1991), it seems likely that these may contain little or no DNA and were formed from dividing plastids in which little or no DNA synthesis occurred. No such small plastids were observed within generative cells of genotype MS-5 (Zhu et al. 1990a, 1991), whose plastid nucleoid number approximates the plastid number/generative cell. The apparent absence of DNA from a portion of the plastids within a given cell has been reported in other systems, such as the vegetative cells of the algae *Acetabularia* and *Batophora*, where approximately 75% of the plastids apparently lack DNA (Coleman 1979).

The fact that plastid nucleoid number/generative cell does not differ significantly between genotypes 301 and 7W, even though male plastid transmission strength is much greater in 301 (Smith 1989b), indicates that factors other than the mean number of DAPI-stainable plastid nucleoids/generative cell must influence male plastid inheritance in these genotypes. Another study has recently found that plastid nucleoid number/generative cell does not correlate well with genetic evidence for male plastid transmission in four plastome types of *Oenothera* (Corriveau and Coleman 1990). Genetic studies have demonstrated that both paternal and maternal nuclear genotypes affect male plastid inheritance in alfalfa (Smith 1989b). There are several factors that may predominate over male plastid input (Sears 1980; Connett 1987; Smith 1989a; Hagemann and Schroder 1989) including: (1) Differential reduction (according to genotype) in male plastid and/or plastid nucleoid number at a developmental stage, or stages, later than that of this study. (2) Differential exclusion of male plastids at the time of gametic fusion. (3) Preferential degeneration or replication of male plastids after zygote formation. (4) Sequestering of male or female plastids into embryonic cells that do not become part of the mature plant.

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