The Model Plant *Medicago truncatula* Exhibits Biparental Plastid Inheritance

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The plastid, which originated from the endosymbiosis of a cyanobacterium, contains its own plastid DNA (ptDNA) that exhibits a unique mode of inheritance. Approximately 80% of angiosperms show maternal inheritance, whereas the remainder exhibit biparental inheritance of ptDNA. Here we studied ptDNA inheritance in the model legume, *Medicago truncatula*. Cytological analysis of mature pollen with DNA-specific fluorescent dyes suggested that *M. truncatula* is one of the few model plants potentially showing biparental inheritance of ptDNA. We further examined pollen by electron microscopy and revealed that the generative cell (a mother of sperm cells) indeed has many DNA-containing plastids. To confirm biparental inheritance genetically, we crossed two ecotypes (Jemalong A17 and A20), and the transmission mode of ptDNA was investigated by a PCR-assisted polymorphism. Consistent with the cytological observations, the majority of F1 plants possessed ptDNAs from both parents. Interestingly, cotyledons of F1 plants tended to retain a biparental ptDNA population, while later emergent leaves tended to be uniparental with either one of the parental plastid genotypes. Biparental transmission was obvious in the F2 population, in which all plants showed homoplasmy with either a paternal or a maternal plastid genotype. Collectively, these data demonstrated that *M. truncatula* is biparental for ptDNA transmission and thus can be an excellent model to study plastid genetics in angiosperms.

**Keywords:** Biparental inheritance — *Medicago truncatula* — Model plant — Plastid — Pollen grain

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; dCAPS, derived cleaved-amplified polymorphic sequence; DiOC₆; 3,3'-dihexyloxycarbocyanine iodide; EST, expressed sequence tag; PMI, pollen mitosis I; PMII, pollen mitosis II; ptDNA, plastid DNA; SNPs, single nucleotide polymorphisms.

Nucleotide sequence data from this article have been deposited in the EMBL/GenBank Data Libraries under accession Nos. NC_003119, EF471904, NC_000932, EU220212, EU220213, EU220214, EU220215, EU220216, EU220217, EU220218, EU220219, EU220220, EU220221, EU220222, EU220223 and EU220224.

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**Introduction**

Mature angiosperm pollen grains are composed of two or three male reproductive cells that are derived from a sporogenous pollen mother cell (McCormick 2004). As the first step of pollen development, a tetrad formed by meiosis of the pollen mother cell becomes separated and gives rise to four microspores. Subsequently, each microspore undergoes pollen mitosis I (PMI) to divide into a larger vegetative cell and a smaller generative cell. Finally, the generative cell undergoes pollen mitosis II (PMII) to form two sperm cells. The timing of PMII varies among species, sometimes occurring within the anther, although more commonly it occurs during pollen tube growth.

Plastids and mitochondria, which are DNA-containing organelles that originated from endosymbiosis, have been studied by electron microscopy during pollen development (Clauhs and Grun 1977, Mogensen and Rusche 1985, Schröder 1985, Schröder 1986, Kuroiwa et al. 1993). They have been studied in particular because the behavior of these organelles in pollen grains is important in considering the transmission of organelle DNAs. In general, organelle DNAs are transmitted from either parent. Since the discovery of organelle DNAs in plastids and mitochondria in the 1960’s (Ris and Plaut 1962, Nass and Nass 1963a, Nass and Nass 1963b), non-Mendelian transmission of organelle DNAs has been genetically studied in many organisms: higher plants, mosses, ferns, algae (Sears 1980, Whatley 1982, Hagemann and Schröder 1989, Kuroiwa 1991), fungi (Mitchell and Mitchell 1952, Kawano et al. 1987), animals (Hutchison et al. 1974, Sutovsky and Schatten 2000) and humans (Giles et al. 1980).

In higher plants, fluorescence microscopy, combined with 4',6-diamidino-2-phenylindole (DAPI) staining of organelle DNA, suggested that there is a positive correlation between the appearance of organelle DNA fluorescence in the mature sperm or generative cells and a biparental mode of organelle inheritance (Miyamura et al. 1987). Thus, the observation of DAPI-stained pollen grains with fluorescence microscopy allows us to estimate the mode of organelle inheritance in higher plants. Combination of
cytological and genetic data revealed that the absence of DAPI signals in sperm cells is highly correlated with maternal inheritance. In contrast to this, the presence of DAPI signals does not always support biparental inheritance, since there are several exceptions (Corriveau et al. 1989, Polans et al. 1990). Nevertheless, this DAPI staining method has been used for large-scale screening of angiosperm species to assess the frequency of the potential biparental or uniparental inheritance (Corriveau and Coleman 1988, Zhang et al. 2003).

In the case of plastid genomes, approximately 80% of angiosperm species display maternal inheritance, and the remaining species exhibit the potential for biparental inheritance (Corriveau and Coleman 1988, Zhang et al. 2003). In maternal inheritance systems, paternal transmission of plastids is impeded either during the PMI via unequal plastid distribution (Lycopersicon type), or during generative or sperm cell development via plastid degeneration (Solanum type) (Hagemann and Schroeder 1989, Mogensen 1996). Therefore, the generative and sperm cells in mature pollen grains tend to be free of plastids. Conversely, the generative and sperm cells of plant species that display biparental plastid inheritance reserve DNA-containing plastids and transmit plastids biparentally (Pelargonium type) (Sodmergen et al. 1992, Kuroiwa et al. 1993). The biparental types are known to amplify the plastid DNA (ptDNA) in the generative cells after PMI (Nagata et al. 1999). It should be noted, however, that plastids and/or ptDNAs in sperm cells are not excluded in a strict manner. A low-frequency biparental transmission has been reported in several plant species that were originally defined as exhibiting strict maternal plastid inheritance (Diers 1967, Simmonds 1969, Schmitz and Kowallik 1986, Avni and Edelman 1991) in different genetic lines.

Despite the knowledge gained from the aforementioned studies, little is known about the molecular mechanism underlying biparental inheritance. The mode of organelle inheritance raises the question of how this type of diversity emerged during evolution and which mode of transmission is an advanced form. To understand this, a study in a model plant such as Arabidopsis is a feasible approach. However, the majority of the current model plants inherit plastid DNA maternally (Martinez et al. 1997). In this study, we focus on Medicago truncatula, a model legume that is widely used for the analysis of root symbioses and legume biology (Stacey et al. 2006). In contrast to other model systems such as Arabidopsis, we show that M. truncatula inherits the plastid genome biparentally. We therefore propose that M. truncatula has potential to serve as a suitable model species to investigate the mechanisms of the biparental inheritance of the plastid genome.

Results

Cytological analysis of pollens in model plants

We examined pollen grains from seven model plant species (M. truncatula, Lotus japonicus, N. tabacum, Lycopersicon esculentum, Arabidopsis thaliana, Oryza sativa and Zea mays) by the conventional squash method and DAPI staining (Fig. 1A–G). Among these, we were particularly interested in testing model legumes since biparental inheritance has been implicated in M. truncatula and its related species. The mature pollen grains of M. truncatula (Fig. 1A, H), L. japonicus (Fig. 1B, I), N. tabacum (Fig. 1C, J) and L. esculentum (Fig. 1D, K) (matured before PMII) contain a generative and a vegetative cell, whereas those of A. thaliana (Fig. 1E, L), O. sativa (Fig. 1F, M) and Z. mays (Fig. 1G, N) (PMI completed before maturation) contain two sperm cells and a vegetative cell. Fig. 1A and H demonstrate that fluorescent spots were observed around the generative nucleus of M. truncatula, whereas no fluorescent spots were detected around the generative and sperm nucleus of the other plants (Fig. 1B–G, I–N). The fluorescent spots around the M. truncatula generative nucleus were hypothesized to represent organelle DNAs, suggesting that M. truncatula exhibits potential biparental inheritance of organelle genomes.

PtDNA is present in generative cells of M. truncatula

To identify which organelle DNA yields DAPI-stained fluorescent spots in the generative cell of M. truncatula, we prepared Technovit sections of pollen grains and performed double staining with DAPI and a mitochondria-prefering dye, 3,3’-dihexyloxacarbocyanine iodide (DiOC₃). This procedure made it possible to distinguish plastids and mitochondria in pollen grains (Nagata et al. 1999). Consistent with the result from our squash method (Fig. 1A), DAPI signals in the Technovit sections were observed as small spots in the generative cell cytoplasm in addition to a large signal corresponding to the generative nucleus (Fig. 2A, C). No DAPI signals were observed in the cytosol of vegetative cells. In contrast to DAPI staining, DiOC₃ stain detected many fluorescent spots in the cytosol of both vegetative and generative cells (Fig. 2B, D). Since earlier studies showed that DiOC₃ selectively stains mitochondria in pollen grains but not in plastids (Nagata et al. 1999), this differential staining preference enabled us to distinguish plastids and mitochondria effectively. To clarify whether the DAPI and DiOC₃ signals in the generative cell overlap, we followed the outlines of DAPI
and DiOC₆ signals (Fig. 2E, F) and reconstructed two signals into one merged picture (Fig. 2G). The majority of DAPI signals did not merge with DiOC₆ signals. Therefore, these results suggested that the observed DAPI signals most probably resulted from ptDNA.

Electron microscopic observation of plastid and ptDNAs in generative cells of M. truncatula

Our results from DAPI and DiOC₆ stains imply that a substantial number of plastids containing ptDNA should be retained in the generative cells of M. truncatula. To confirm this, the generative cell of M. truncatula pollen grain was examined by transmission electron microscopy. The result shown in Fig. 3 indicates that the generative cell actually contains many plastids. These plastids were easily distinguished by their characteristic morphology; a round shape of about 1 μm, double membranes of envelopes and a few thylakoids. We also observed mitochondria in generative cells, whose sizes are smaller than those of mitochondria in vegetative cells. In addition to these analyses, we also performed immunoelectron microscopic analysis of generative cells using anti-DNA antibodies. Immunogold labeling was detected within plastids and generative nuclei, whereas gold particles were never detected in mitochondria (Fig. 3D). The absence of the gold particles in mitochondria was consistent even by our serial sectional study (data not shown). Taken together, these results confirmed our findings by DAPI and DiOC₆ staining methods and allowed us to conclude that the generative cell of M. truncatula has plastids that contain ptDNA.

Biparental transmission of ptDNA in M. truncatula

Although our cytological analysis described above strongly suggests the mode of biparental inheritance of ptDNA in M. truncatula, this mode needed to be verified genetically. We first attempted to detect a ptDNA polymorphism among ecotypes, which is necessary to distinguish one of the ptDNA genotype from another in a crossed population. Several chloroplast genes were sequenced among 15 ecotypes (unpublished results), and
consequently we found two polymorphisms (Fig. 4A). Both polymorphisms are single nucleotide polymorphisms (SNPs) within *ndhE* (encoding NADH dehydrogenase subunit 4L). Neither SNP causes amino acid substitutions since they occur at a degenerate third nucleotide position of the corresponding codon (data not shown).

The result showed that Jamelong A17, one of the common ecotypes used for genetic analysis, is distinguishable from all other ecotypes (Fig. 4A). We therefore selected Jemalong A17 and the other ecotype A20 to generate F1 progeny from a reciprocal cross. We subsequently designed derived cleaved-amplified polymorphic sequence (dCAPS) primers to detect one of the SNPs (see Materials and Methods). PtDNAs from the two ecotypes were successfully distinguished by PCR using this dCAPS (Fig. 4B). Under our PCR conditions, the dCAPS primers appeared to anneal to both parental ptDNAs with equal efficiency. Reflecting this fact, we found that the ratio of the band intensities in each PCR correlated well with the ratio of mixed A17 and A20 ptDNAs (Fig. 4C).

We next applied this plastidic dCAPS for F1 progeny. Successful crossing was confirmed by the detection of heterozygosity in a nuclear polymorphic marker (Fig. 4D). To perform dCAPS in these F1 plants, genomic DNA was prepared from four different parts of each F1 individual: two cotyledons (the arrangement of each cotyledon relative to other leaves was not taken into consideration), a primary leaf and a first trifoliate leaf (Fig. 5A). Our PCR analysis allowed us to estimate the ratio of one parental ptDNA to the other in these leaf tissues, although ptDNA amounts were different between the samples (due to the difference in leaf area subjected to DNA isolation). The result from 12 F1 plants is shown in Fig. 5B (band intensities were quantified and displayed in Supplementary Table S1). Both reciprocal crosses produced several F1 plants that showed evidence of biparental transmission of ptDNA. It is interesting to note that cotyledons showed a tendency to have a mixed ptDNA population (plant Nos. 1–5 and 7–10). However, in contrast to this mixed parental ptDNA population in cotyledons, a first trifoliate leaf that is an advanced leaf in development tends to contain only one type of ptDNA (plant Nos. 1–4 and 7–9). Even in the presence of both ptDNAs in trifoliate leaves, one parental type was dominant over the other: plant No. 5 showed the ratio of approximately 3 : 1 for A20 (male) and A17 (female) ptDNAs, and plant No. 10 showed the ratio of approximately 5 : 1 for A20 (female) and A17 (male) ptDNAs. Plant Nos. 6, 11 and 12 have only maternal ptDNA in all tissues examined.

The results described above suggest that a sorting out of either plastid genotype occurs during plant growth, at least in the F1 generation. We subsequently tested segregation of plastid genotypes in the corresponding F2 generation. DNAs were isolated from two cotyledons of each F2 plant and were subjected to dCAPS analysis (Fig. 6). We tested at least two F2 individuals that derived from an identical F1 parent. None of the F2 progeny exhibited heteroplasmy (examples shown in Fig. 6A), suggesting that the sorting out was completed in the F1 population. The appearance of the paternal genotype in F2 plants, although the preference is for the maternal genotype, clearly indicates that the paternal ptDNA can be transmitted through generations. In F2 plants, the maternal genotype was dominant irrespective of which one was used as a maternal parent (Fig. 6B). Among the plants described in Fig. 5B, we found that the female type corresponded to plant Nos. 1, 2, 3, 6, 8, 9, 10 and 12, the male type corresponded to plants Nos. 5 and 7, and the biparental type corresponded to plant No. 4 (no F2 were harvested in plant No. 11). Dominant parental ptDNA in the trifoliate leaves of F1 plants tends to represent the genotypes of their F2 progeny. The plastid genotypes of F2 progeny from F1 plant (No. 4) were different among individuals. In our experiment, we collected seeds from different siliques together, and it remains unclear whether biparental segregation took place in the identical siliques of plant No. 4.

![Fig. 2](https://academic.oup.com/pcp/article-abstract/49/1/81/1904132/473)
Discussion

It is well documented that the presence of DAPI-detected ptDNA in sperm cells and generative cells is a prerequisite for biparental inheritance. With collective evidence provided by DAPI and DiOC₆ staining (Fig. 2), immunoelectron microscopy (Fig. 3D), and dCAPS analysis of F₁ and F₂ progeny (Figs. 5B, 6), we confirmed that *M. truncatula* transmits ptDNA biparentally.

To transmit paternal ptDNA to progeny, several obstacles should be taken into account. First, the exclusion or destruction of plastids themselves in sperm cells needs to be overcome. Secondly, the plastids retained in sperm cells need to be able to replicate ptDNA. Thirdly, the plastid with ptDNA in sperm cells needs to be transmitted into a zygotic cell. Since the volume of the sperm cell is much smaller than that of the egg cell, plastids from sperm cells may not be incorporated at the onset of fertilization. Finally, paternal plastids need to survive the competition with maternal plastids. In this final step, the initial number of plastids from both parents in the zygote may be important for subsequent segregation, supposing that the division frequency is even. Our observation indicates that in *M. truncatula*, a substantial number of plastids should be transmitted after fertilization, since several F₁ progeny retained paternal ptDNA in cotyledons (Fig. 5B), and that the paternal ptDNA could survive even in some F₂ plants (Fig. 6).

In higher plants, plastids are not thought to fuse frequently, although they do divide (Arimura et al. 2004, Miyagishima 2005). Thus, it is unlikely that our results of F₁ progeny represent a recombination event of ptDNAs. Instead, our results demonstrate that plastids tend to become homogenous by excluding plastids from either parent (Figs. 5B, 6). This phenomenon is also observed in
yeast and mammalian mitochondria and is termed vegetative segregation (Birky 1994, Birky 2001). Vegetative segregation appears to be a general property of organelle inheritance, which is due to the stochastic partitioning of organelles during every cell division (Birky 2001). In the case of M. truncatula, vegetative segregation can be complete within the F1 generation, because all F2 plants were homoplasmic (Fig. 6A). In addition, our data demonstrate that vegetative segregation proceeds toward either parental type, although a maternal parent seems preferable (Fig. 6B). The reason for such maternal preference remains unclear and needs to be tested with other combinations of ecotypes.

It should be noted that one plant (No. 4) segregated both paternal and maternal homoplasmy in the F2 population, despite only a trace amount of paternal ptDNAs in F1 trifoliate leaves. Conversely, we observed no paternal homoplasmy in plant No. 10, although paternal ptDNAs were detected in F1 trifoliate leaves. Therefore, our observations suggest that vegetative segregation may not necessarily be consistent among different tissues, but it is completed up to the F2 generation. In contrast to the nuclear genome that is tightly regulated as a diploid in somatic cells, the mode of inheritance of the plastid genome is highly diverse and is most probably due to the various steps that are involved. It will be interesting to examine in

![Diagram](https://example.com/diagram.png)

**Fig. 4** Plastid DNA polymorphisms between 15 ecotypes of M. truncatula to distinguish plastid genomes. (A) Schematic representation of genomic context around the ndhE gene and partial nucleotide sequences of ndhE genes. Highlighted letters indicate the two single-nucleotide polymorphisms (SNPs). One of the polymorphisms (arrowed) is used for designing derived cleaved-amplified polymorphic sequence (dCAPS) primers. Numbers in parentheses correspond to the positions of NC_003119 (M. truncatula Jemalong A17 chloroplast complete genome sequence), EF471904 (Jemalong A20), EU220212 (Sephi), EU220213 (Salernes), EU220214 (Parabinga), EU220215 (Paraggio), EU220216 (Hannaford), EU220217 (SA32023), EU220218 (SA32026), EU220219 (SA32029), EU220220 (Borung), EU220221 (Caliph), EU220222 (Cyprus), EU220223 (SA32024) and EU220224 (R108-1). The sequences except NC_003119 are partial. (B) dCAPS analysis to distinguish the plastid genome of A17 and A20. Open and closed arrowheads indicated the band positions specific to A20 and A17, respectively. (C) PCR was performed with a variable amount of A17 genomic DNA and a constant amount of A20 genomic DNA. The numbers above the gel image indicate the ratio of A17 and A20 genomic DNA used for PCR. The numbers under the gel image indicate the ratio of band intensities of A17 (closed arrowhead) versus A20 (open arrowhead). (D) Confirmation of nuclear DNA heterozygosity. F1 progeny from reciprocal crosses between A17 and A20 (indicated on the top) were examined by a nuclear marker 19O4L. Open and filled arrowheads indicate the band positions specific to A20 and A17, respectively.
future works fundamental questions such as why organelle genomes tend to be homogenous and inherited uniparentally, or whether biparental and maternal inheritance is advantageous for plant adaptation.

Lilienfeld (1962) first implicated the occurrence of biparental plasmid inheritance in *M. truncatula* through a genetic study of nuclear–cytoplasmic incompatibility, but this needed to be verified at the molecular level. He noted the pronounced reciprocal differences in plastid behavior in crosses between two races that have different chlorophyll contents. Consistent with this study, his cartoon of F1 plants showed that heteroplasmy and somatic segregation of the plastids occurred during development. Genetic analysis using a tetraploid alfalfa (*M. sativa*) also provided

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**Fig. 5** dCAPS analysis of plastid DNA inheritance using two ecotypes of *M. truncatula*. (A) Schematic representation of a young soil-grown seedling. Genomic DNA was prepared from two cotyledons (c), primary leaf (p) and trifoliate leaf (t). (B) dCAPS analysis of F1 progeny of reciprocal crosses between A17 and A20. Organs from which genomic DNA was prepared are indicated by c, p and t. Polymorphisms were detected as shown in Fig. 4B.
Plastid inheritance in *M. truncatula*

*A* dCAPS analysis of *F*₂ progeny. (A) Segregation pattern of ptDNA genotypes categorized parental *F*₁ plants into three types (female, male and biparental). The female type represents *F*₁ plants that contain only female ptDNA in the *F*₂ progeny (this pattern derives from plant No. 1 in Fig. 5B). The male type represents *F*₁ plants that contain only male ptDNA in the *F*₂ progeny (*F*₁ plant No. 7). The biparental type represents *F*₁ plants that contain both ptDNAs in the *F*₂ progeny (*F*₁ plant No. 4). The upper numbers in each panel indicate *F*₂ plant numbers. PtDNAs were prepared from the two cotyledons and each was subjected to the dCAPS analysis. (B) Classification of *F*₁ types according to the ptDNA segregation pattern. The female type corresponds to plant Nos. 1, 2, 3, 6, 8, 9, 10 and 12 in Fig. 5B. Similarly, the male type corresponds to plant Nos. 5 and 7, and the biparental type corresponds to plant No. 4.

![Image of ptDNA analysis](https://example.com/dCAPS_analysis.png)

Table: Number of *F*₁ plants whose *F*₂ progenies have female, male or biparental ptDNA.

<table>
<thead>
<tr>
<th>Parental cross</th>
<th>Female</th>
<th>Male</th>
<th>Biparental</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁7 x A₂₀</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A₂₀ x A₁7</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The degree of paternal transmission has been shown to be influenced by parental nuclear and plastid genotypes in *Oenothera* and *Pelargonium* (Tilney-Basset 1970, Chiu et al. 1988, Smith 1989, Zhu et al. 1993). Our sample size is too small to draw any conclusion for the variable frequencies between A₁7 and A₂₀. Identification of DNA polymorphisms within ecotypes in *M. truncatula* (Fig. 4A) can now allow us to study the genotype-dependent effect on transmission rate in future work.

*Meditago truncatula* is a model legume that is widely used for the analysis of root symbioses and legume biology. Recently a genome project has generated a large number of expressed sequence tag (EST) sequences from a wide range of libraries (Stacey et al. 2006). Transformation of nuclear genes is possible by *Agrobacterium tumefaciens* and *A. rhizogenes* (Cran et al. 2006), and molecular genetic approaches are also applicable to isolate the genes responsible (Penmetsa and Cook 2000). To dissect molecular mechanisms of organelle inheritance, our screening of *M. truncatula* mutants that have no organelle DAPI signals in generative cells is currently underway. The findings reported here suggest that *M. truncatula* is an excellent model system for the study of the biparental inheritance of the plastid genome.

**Materials and Methods**

*Plant material and growth*

*Meditago truncatula* Gaertn. was used as the source of plant material for all studies. For germination, seeds were scarified and surface sterilized by immersion in concentrated sulfuric acid for 8 min, rinsed in deionized water five times and approximately 10 seeds were spread on 0.8% (w/v) Agar TC-6 (Funakoshi, Tokyo, Japan) in Petri plates (10 × 100 mm). The plates were wrapped with parafilm and aluminum foil, and seeds were vernalized by incubating them at 4°C. After 2 weeks of cold treatment, germinated seeds were transferred to soil-filled pots (8 cm diameter). Plants were grown in glasshouse conditions under 14 h day lengths with a light intensity of 30–40 μmol m⁻² s⁻¹ and a temperature range of 22–25°C. *L. japonicus* was grown under the same condition as *M. truncatula*. *N. tabacum* and *L. esculentum* were grown in a glasshouse with a temperature range of 25–30°C under natural light. *Z. mays* was grown in field conditions at Peking University during the summer of 2006. *A. thaliana* was grown in a growth room maintained at 22°C under a photoperiod of 12 h light, 12 h darkness with a light intensity of 30–40 μmol m⁻² s⁻¹.

*Genetic cross*

Artificial hybridization was performed according to a previously described method (Pathipanawat et al. 1994) with the minor exception that flowers were not placed in polystyrene tubes after artificial pollination. Briefly, immature buds were emasculated 1–2 d before the flower was fully open. Specifically, a cut was made in the middle of the standard petal between the sepals and tip of the bud on the concave side. Using fine tweezers, the standard petal was opened and folded back, starting from the middle.
portion up towards the tip. The wing petals were then prised apart and folded. This exposed the staminal bundle surrounding the style, with the anthers at the top of this bundle surrounding the stigma. Pollen grains were applied to the stigma just after removing the anthers. Success in the cross was confirmed by a nuclear CAPS marker 1904L (Cho et al. 2004).

Thin sections of Technovit 7100 resin and double stain with DAPI and DiOC₆. DNA staining of pollen grains with DAPI and DiOC₆ was performed according to a previous method (Hu et al. 2005). Pollen grains were fixed in 2.5% (v/v) glutaraldehyde and 1% (w/v) paraformaldehyde in cacodylate buffer (pH 7.4) for at least 24 h at room temperature. Samples were subsequently dehydrated through a graded ethanol series [20, 40, 60, 80 and 100% (v/v)] and then embedded in Technovit 7100 resin (Kulzer and Company, Wehrheim, Germany). The embedded samples were cut in 0.3 μm sections with an Ultracut N ultramicrotome (Reichert-Nissei, Tokyo, Japan) and diamond knives, and dried on coverslips. Thin sections were stained with 100 μg ml⁻¹ DAPI in 100 μM phenylmethylsulfonyl fluoride, washed with 50% ethanol and distilled water, and were further stained with 1 μg ml⁻¹ DAPI (Invitrogen, Tokyo, Japan) and diamond knives, and dried on coverslips. Thin sections were stained with 100 μg ml⁻¹ DAPI, 0.4 M phenylmethylsulfonyl fluoride, and 1.2 mM spermidine. In order to prevent fading, 1 μg ml⁻¹ n-propyl gallate in 50% (v/v) glycerol was added to the samples prior to fluorescence microscopic examination.

Fluorescence microscopy

Fluorescence microscopic examination of pollen cytoplasmic DNA was performed according to the method of Kurioka and Suzuki (1980). Briefly, mature pollen grains were placed on a glass slide and immersed in a drop of deionized water that was supplemented with 3% glutaraldehyde and 1–10 μg ml⁻¹ DAPI. Pollen grains were squashed by exerting gentle pressure on a coverslip that was placed on the glass slide. The samples were subsequently examined under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

In order to detect DAPI signals, we used a filter set (U-MWU2, Olympus) consisting of the following components: excitation filter (BP330-385), dichroic mirror (DM400) and a barrier filter (420 LongPass). To detect DiOC₆ signals, we used a filter set (U-MWU2, Olympus) consisting of the following components: excitation filter (BP330–385), dichroic mirror (DM400) and a barrier filter (420 LongPass). To detect DAPI signals, we used a filter set (U-MWU2, Olympus) consisting of the following components: excitation filter (BP330–385), dichroic mirror (DM400) and a barrier filter (420 LongPass). To detect DiOC₆ signals, we used a filter set (U-MWU2, Olympus) consisting of the following components: excitation filter (BP330–385), dichroic mirror (DM400) and a barrier filter (420 LongPass).

DNA isolation and PCRs

Cotyledons, primary and trifoliate leaves were harvested and ground in 500 μl of DNA extraction buffer [200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS] to isolate genomic DNA of M. truncatula. Particulates were subsequently removed from the sample by centrifugation. DNA was precipitated by adding an equal volume of isopropanol and centrifuging for 15 min at 16,000×g to pellet DNA. The resulting DNA pellet was washed in 70% ethanol prior to resuspension in 100 μl of distilled water. PCR was conducted on a thermal cycler using 2 μl of genomic DNA solution as the template with the following primer pair: 5'-GCTTACCGCTGCAAATTCTAT-3' and 5'-GCTTGTGAAGATATGTGGTTCGAG-3'. The primers were designed based on the Arabidopsis chloroplast genome (NC_000932, 117,889–117,911 and 119,546–119,567, respectively). The region between the primers contains the ndhE, ndhG and ndhI genes. The PCR conditions were as follows: 94°C for 2 min; 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 2 min; and a final elongation step of 7 min at 72°C. Sequences of the PCR products from A17 and A20 plants were compared for the detection of polymorphisms between them. To detect one of the polymorphisms by dCAPS, we conducted PCR using the following primers: 5'-ATTATCACA AAAATCAGAAAAGTCTC-3' and 5'-CATGATCTTTGTTT GAGTGGCC-3'. The PCR conditions were as follows: 94°C for 30 s; 33 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 1 min; and a final elongation step of 4 min at 72°C. The PCR product was digested with XhoI, and PCR products were subsequently separated by 15% PAGE and detected with ethidium bromide staining. In the case of A17, a PCR product (150 bp) was digested into 125 and 25 bp fragments. In the case of A20, the PCR product (150 bp) was not digested. To investigate whether the band intensities reflect the ratio of the amount of parental ptDNA, PCR was performed with a variable amount of A17 genomic DNA and a constant amount of A20 genomic DNA. Band intensities were subsequently analyzed with the NIH Image J 1.37v software (http://rsb.info.nih.gov/j/).

For a nuclear CAPS marker 1904L, a primer set (5'-GGAA TGATAATGTTGATATGAAAATG-3' and 5'-CTATGC CAGACTGCTCAATG-3') was used. The PCR conditions were as follows: 94°C for 30 s; 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 1 min; and a final elongation step of 4 min at 72°C. After HindIII digestion, PCR products were detected on a 3% agarose gel. In the case of A17, a PCR product (435 bp) was digested into 297 and 138 bp fragments. In the case of A20, the PCR product (435 bp) was not digested.

Electron microscopy

For transmission electron microscopy, pollen grains were fixed in 4% glutaraldehyde and 5% paraformaldehyde in cacodylate buffer (pH 7.4) for at least 24 h at room temperature. A second fixation was performed in 5% (w/v) potassium permanganate solution at room temperature for 20 min. After rinsing in distilled water, the fixed pollen grains were dehydrated through a graded alcohol series (20, 40, 60, 80 and 100%) and embedded in Spurr’s resin (Polysciences, Warrington, PA, USA). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a 2% glutaraldehyde solution to enhance the electron density of the DNA. The ultra-thin sections were then stained with 1% (w/v) uranyl acetate and 0.5% (w/v) lead citrate, and examined with an electron microscope (H-7100, Hitachi High-Technologies, Ibaraki, Japan) operating at 75 kV.

Immunoelectron microscopy for stable detection of DNA was based on a previously described method (Johnson and Rosenbaum 1990). Pollen grains were fixed with glutaraldehyde as described above, except that the post-fixation step was omitted and samples were embedded in LR White resin (Sigma-Aldrich Chemie, Steinheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). Ultrathin sections were placed onto nickel grids (150 mesh size). The grids were subsequently incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.
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References


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