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Inheritance of large mitochondrial RNA's in alfalfa

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Summary. Several large RNA molecules that migrated to electrophoretic positions ranging from 1.7–10 kb were observed in preparation of alfalfa (*Medicago sativa*) mitochondria. F_1 progenies inherited the RNA's from both maternal and paternal parents (Fig. 1). Treatment of intact mitochondria with RNase A failed to remove the RNA's, indicating that they were contained within an RNase impermeable compartment. Further purification of mitochondria in linear sucrose gradients failed to separate the RNA's from mitochondria. Transmission electron microscopic examination of sucrose gradient purified mitochondria revealed that mitochondria were free of contamination by virus-like particles, indicating that the RNA's were contained within the mitochondrion. Biparental inheritance of large mitochondrial RNA's in alfalfa provides evidence that mitochondria are inherited biparentally in this species.

Key words: Alfalfa – *Medicago sativa* – Mitochondrial RNA – Organelle inheritance

Introduction

Uniparental-maternal inheritance of extranuclear organelles is a common, but not universal, phenomenon in higher plants. Twenty to thirty percent of plant species studied exhibit regular biparental inheritance of plastids (Kirk and Tilney-Bassett 1978; Smith 1988). In other species, uniparental-maternal inheritance of plastids appears to predominate, but a small proportion of hybrid offspring appear to contain paternal plastids (Kirk and Tilney-Bassett 1978; Medgyesy et al. 1986; Schmitz and Kowallik 1986). Analysis of restriction fragment length polymorphisms (RFLP's) in parental and hybrid genotypes has demonstrated uniparental-maternal inheritance of mitochondria in a limited number of plant species (Levings and Pring 1976; Quetier and Vedel 1977; Sisson et al. 1978; Pring et al. 1982; Medgyesy et al. 1986; Samoilov et al. 1986). However, relatively little research has been conducted on mitochondrial inheritance patterns in those species that are known to inherit plastids biparentally (Smith 1988).

Mitochondria of several plant species contain small circular or linear plasmid-like DNA molecules in addition to the main mitochondrial genome (Pring et al. 1977; Prowling 1981; Boutry and Briquet 1982; Palmer et al. 1983; Brown et al. 1986; Mignouna et al. 1987). These molecules have frequently, though not exclusively, been associated with cytoplasmic male sterility. Relatively large mitochondrial RNA molecules have also been reported in maize (*Zea mays*) (Sisco et al. 1984; Finnegan and Brown 1986), sugarbeet (*Beta vulgaris*) (Prowling 1981) and *Brassica* (Kemble et al. 1986). In all studies where inheritance was reported, both plasmid-like mitochondrial DNA's and large mitochondrial RNA's were inherited in a uniparental-maternal fashion.

Smith et al. (1986) demonstrated that alfalfa (*Medicago sativa*) exhibits regular biparental inheritance of plastids based on the sexual transmission of mutant chloroplasts that produce chlorophyll deficient phenotypes. Mitochondrial nucleic acid molecules of a size range similar to the plasmid-like mitochondrial DNA's and large mitochondrial RNA's of maize and other species have been observed in alfalfa (Nikiforova and Negruk 1983). Such molecules could be useful markers for studying mitochondrial inheritance in alfalfa. The objectives of this study were to document the existence of large mitochondrial RNA's in alfalfa and determine the pattern of their inheritance.

Materials and methods

Alfalfa genotypes used in this study were: (1) 6–4, a male-sterile plant selected from the cv Saranac; (2) CUF A, a male fertile plant selected from the cv CUF 101; (3) three sexual F_1 progeny plants from the cross 6–4 × CUF A, all male sterile; (4) SAMS 1, a male-sterile, sexual F_1 progeny plant from the cross 6–4 × CUF A that was used as a female parent in additional crosses; and (5) a male-sterile, sexual F_1 progeny plant from the cross SAMS 1 × Lew 30. Lew 30 was a male-fertile plant selected from the cv Lew and has since been lost. All plants were maintained in a greenhouse under natural light supplemented with artificial light to maintain an 18 h photoperiod.

For isolation of mitochondrial nucleic acids, 10–15 g fresh weight of leaf tissue were obtained from an individual plant or from clones vegetatively propagated from a single plant. DNase I treated and washed mitochondrial pellets were isolated following the procedures of Kemble (1987). Mitochondria were lysed for 1 h at 37 °C in 50 m*M* Tris, 3% SDS, pH 7.5. Lysates were deproteinated with a single phenol-chloroform extraction and the nucleic acids ethanol precipated. Undigested nucleic acid molecules were separated using 1% agarose submarine gel electrophoresis for 16 h at 2.4 V cm^{-1} and stained with 0.5 μg ml^{-1} ethidium bromide. Gels were visualized and photographed under 302 nm transmitted UV light.

Three separate experiments were conducted with mitochondrial pellets from the genotype SAMS 1 in order to determine if large RNA's in progenies were the result of contamination of mitochondrial pellets by extraorganellar virus-like particles.

(1) RNase A (10 μg/g tissue) was added with DNase to the mitochondrial pellet and incubated for 1 h at 25 °C. Pellets were centrifuged 3 times through 1 ml wash buffer (0.6 *M* sucrose, 10 m*M* TES, 20 m*M* EDTA, pH 7.2) and subjected to lysis, phenol-chloroform extraction, ethanol precipitation and electrophoresis as described above.

(2) A DNase I treated and washed mitochondrial pellet isolated from approximately 200 g of tissue was suspended in 0.5 ml of wash buffer and layered onto a linear (20%–50%) sucrose gradient prepared using nuclease-free sucrose and 10 m*M* phosphate buffer, pH 7.5. Tubes were centrifuged for 1.5 h at 100,000 Xg. Light scattering bands were individually collected and diluted slowly 4 times with 10 m*M* phosphate buffer, pH 7.5. All samples were made to 3% SDS ands subjected to lysis, phenol-chloroform extraction, ethanol precipitation and electrophoresis as described above.

(3) Individual fractions collected from a sucrose gradient prepared as described above were adsorbed to carbon coated grids for 10 min, washed with 30 drops of sterile distilled water, and stained with 4% phosphotungstic acid, pH 7.0. At least 10 apertures per grid on each of 4 grids were examined using an Hitachi H-500 transmission electron microscope at an accelerating voltage of 100 kV and a range of magnification (10K–60K) suitable for detection of mitochondria and virus-like particles.

Results and discussion

Several secondary nucleic acid molecules in addition to the primary mitochondrial genome were present in preparations of undigested mitochondrial nucleic acids from the genotypes studied (Fig. 1). The molecules were composed of RNA (susceptible to RNase A and resistant to DNase I treatments) and migrated to positions ranging from 10–1.7 kb, although precise molecular weights of

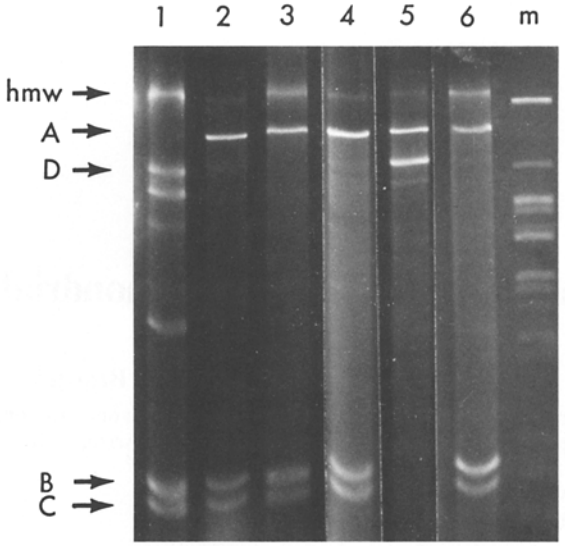

Fig. 1. Undigested alfalfa mitochondrial nucleic acids analyzed on 1% agarose gels stained with ethidium bromide. *Lane1*: 6–4, a male-sterile plant selected from the cv Saranac; *lanes 2–4*: individual sexual F_1 progeny plants from the cross 6–4 × CUF A, all male-sterile; *lane 4*: SAMS 1, an F_1 progeny plant from the cross 6–4 × CUF A selected as a female parent in additional crosses; *lane 5*: CUF A, a male fertile clone selected from the cv CUF 101; *lane 6*: sexual F_1 progeny plant from the cross SAMS 1 × Lew 30, male-sterile. *Lane m* contains molecular weight marker fragments from independent digestions of lambda DNA with *Eco* RI and *Eco* RV. The uppermost band (*hmw*) in *lanes 1–6* represents high molecular weight mitochondrial DNA. All other bands in *lanes 1–6* are RNA's. *Bands A, B, C,* and *D* are described in the text

RNA's cannot be assumed since native Tris-acetate gels and DNA markers were used. The RNA's were uniformly present or absent in all vegetative propagules of a given genotype, although the intensities of the RNA bands varied somewhat relative to the mitochondrial DNA bands. No secondary mitochondrial plasmid-like DNA molecules were observed in the plants used in this study.

Of particular interest is the largest of these RNA's that migrated to a position of approximately 10 kb (Fig. 1, labeled *A*). This molecule was not detected in mitochondrial isolations using up to 200 g of tissue from 6–4. In the cross 6–4 × CUF A, this molecule appears to have been transmitted through the pollen to the F_1 progeny (Fig. 1, *lanes 2–4*). One of these progeny plants, designated SAMS 1 (Fig. 1, *lane 4*), was used as a female parent in the cross SAMS 1 × Lew 30. In this cross the same molecule appears to have been inherited maternally in F_1 progeny (Fig. 1, *lane 6*), although possible paternal inheritance cannot be ruled out since the male parent has been lost. Two other RNA's (Fig. 1, labeled *B* and *C*, 1.7 and 1.8 kb positions, respectively) were inherited maternally in the crosses 6–4 × CUF A and SAMS 1 × Lew 30. To date, every plant known to carry these two molecules is male-sterile.

An additional RNA that migrated to a position of approximately 8 kb was detected in sucrose gradient purified mitochondria isolated from a relatively large quantity of tissue (200 g) from the genotype SAMS 1 (data not shown). This RNA band is faint, but is visible in *lanes 2* and *4* (Fig. 1, labeled *D*). The parental origin of this molecule cannot be determined since both parents carry RNA's at this same position. The remainder of the RNA's present in the mitochondrial preparations of both 6–4 and CUF A were not detected in their progenies. The reason for this remains unexplained. Possibly the parent plants were heteroplasmic for mitochondria carrying different RNA's. If this is true, then germ cells may not contain all species of mitochondria present in the parent due to vegetative sorting-out of organelles. This would result in progeny with some, but not all, of the mitochondrial species present in the parent plants. A similar phenomenon occurs with plastid inheritance in alfalfa and other species that inherit plastids biparentally (Smith 1988; Smith et al. 1986).

Several plant viruses contain RNA molecules of the same general size range as those reported here, and the possibility exists that some viruses may copurify with mitochondria. No obvious viral symptoms were present in any of the plants studied and the same RNA's do not appear in unrelated plants grown in close proximity in the greenhouse for periods of over 2 years. In addition, the RNA's in the genotype SAMS 1 were unaffected by RNase A added to the unlysed mitochondrial pellet, indicating that they were within an RNase impermeable compartment.

Four distinct light scattering bands were visible in sucrose gradients of mitochondrial pellets isolated from the genotype SAMS 1. Three bands were located between the 22% and 32% sucrose concentrations, while the fourth was observed in approximately the 42%–44% zone, which is the typical location of the mitochondrial fraction (Douce 1985; Finnegan and Brown 1986). Nucleic acid molecules were detected only in the fourth band and the electrophoretic pattern of these molecules was similar to that of SAMS 1 (Fig. 1, *lane 4*), confirming that sucrose gradient purification was unable to separate large RNA's from the mitochondria. Fractions collected from the first, second and fourth gradient bands were examined by transmission electron microscopy. The third band, which consisted of a dark green pellicle, was not examined. Condensed mitochondria [typical of mitochondria prepared from sucrose gradients (Douce 1985)] were observed in preparations of the fourth band, which appeared to be free of contamination by other organelles. No mitochondria were detected in either of the other two bands. No virus-like particles were detected in any of the bands examined.

Biparental inheritance of large mitochondrial RNA's in alfalfa provides strong, though not conclusive, evidence that mitochondria are inherited biparentally in this species. The possibility exists that large mitochondrial RNA's have an inheritance mechanism that differs from the mitochondrion. However, the apparent localization of these molecules within the mitochondrion renders this possibility unlikely. It is also possible that the expression of large mitochondrial RNA's may be under the influence of nuclear genes, although there is currently no evidence for this. If this is the case, then the presence or absence of large mitochondrial RNA's may be a function of nuclear as well as organellar inheritance.

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