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Mitochondrial plasmids in related cultivars of crookneck and stable and unstable butternut squash

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Summary. Two series of two to four plasmids are contained in mitochondria of butternut squash (*Cucurbita moschata*). The plasmids are composed of DNA, and are consistent in number within a cultivar but vary in number among cultivars. Plasmid patterns and homology suggest that the faster migrating series of plasmids are the supercoiled form of the slower running plasmids. Plasmid 1 was present in all cultivars except New Hampshire Butternut. Plasmid 2 was only observed in the original crookneck cultivar, Canada Crookneck. Plasmids 3 and 4 were present in all accessions tested. The presence of plasmid 1 in a cultivar does not follow a maternal pattern of inheritance; this may be due to the ability of the plasmid to insert into and excise from the main mitochondrial DNA, or the recombination of some of the smaller plasmids to create the larger plasmid 1. There is some homology between plasmid 1 and plasmids 3 and 4, and between plasmid 1 and main band mitochondrial DNA. The equal presence of plasmid 1 in mitochondria of F_1 seedlings from the pollination of New Hampshire Butternut by Ponca Butternut to that in F_1 seedlings of the reciprocal cross indicates that there is probably a dominant nuclear effect on the ability to produce plasmid 1 either by release from the mitochondrial genome or by recombination of the smaller plasmids. There is no obvious relationship between the presence or number of the mitochondrial plasmids and the butternut fruit shape or stability of the butternut trait of the cultivars.

Key words: Morphogenesis – Fruit shape – *Cucurbita moschata* – DNA

Introduction

Butternut squash is a class of winter squash (*Cucurbita moschata*) with a characteristic fruit shape. The origin and characteristics of this trait have been reviewed by Mutschler and Pearson (1987). The butternut fruit shape trait arose as a spontaneous mutant in a field of crookneck winter squash in the late 1920's. The seed-bearing bulb of the butternut fruit is attached to a short neck which is slightly narrower than the bulb and approximately as long as its diameter. A crookneck fruit has a bulb similar to that of the butternut fruit, but the neck of the fruit is usually half the diameter and twice the length of the bulb and is frequently curved or 'crooked'. The basis for the difference between the crookneck and butternut fruit shapes is the presence and absence, respectively, of some mechanism controlling the orientation of the planes of cell division in the ovary neck tissue (Owen 1954; Sinnott 1932, 1944). In crookneck fruit the planes of cell division are parallel and are perpendicular to the ovary wall, creating a long neck which bends (crooks) due to physical stresses during its rapid growth (Ibrahim et al. 1973). The planes of cell division are random in butternut fruit.

The original butternut stocks were highly unstable, with reversion rates in selfed progeny as high as 30% (Mutschler and Pearson 1987). The majority of the plants grown from self-pollinated seed produced from unstable butternut cultivars reflect the phenotype of the fruit from which the seed was obtained (Mutschler and Pearson 1987). A stable butternut cultivar, New Hampshire Butternut (NHBN), was developed in the 1950's. Circumstantial evidence suggests that the cytoplasm may be involved in the stable butternut trait. Although the butternut trait is inherited as a single dominant gene in crosses involving a stable butternut parent and a

crookneck cultivar, the stability of the butternut trait has not been successfully transferred through the pollen parent (Mutschler and Pearson 1987), and pedigrees show that all stable butternut cultivars available share the same cytoplasm (Coyne 1976; Coyne and Hill 1976; Mutschler and Pearson 1987; Yeager and Meader 1957). Results of a grafting experiment also suggest that the butternut trait may be graft transmissible (Pearson, personal communication). Crosses involving unstable butternut cultivars resulted in an inheritance pattern which was non-Mendelian and distinctly nonreciprocal (Mutschler and Pearson 1987).

Cytoplasmic RNA and DNA plasmids have been found in association with mitochondria of a number of higher plant species. Two circular DNA plasmids (Negruk et al. 1982) and a double-stranded RNA (Grill and Garber 1981) were observed in mitochondria of *Vicia faba.* Mitochondria of fertile sugarbeet (*Beta vulgaris* L) cultivars contained three DNA plasmids, of 1.3, 1.4, and either 1.45 or 1.5 kbp, while mitochondria of cms cultivars contained only a 1.5 kbp plasmid. The mitochondria of some sugarbeet cultivars also contained one or more high molecular weight RNA plasmids (Prowling 1981). Mitochondrial DNA plasmids have been found in normal, T, C and S cytoplasms of maize (*Zea mays* L.). The linear DNA plasmids of the S cytoplasm, S1 and S2, are approximately 6.2 and 5.2 kbp in length, respectively. The plasmids have similar 200 bp regions of homology in inverted orientation at their terminae, and have similar 1,200 bp regions near one of their ends adjacent to this 200 bp region (Kim et al. 1982). The loss of the S1 and S2 plasmids from S cytoplasm was thought to be involved in the reversion of S sterile plants to fertility (Levings et al. 1980), but more recent studies indicate that these plasmids do not always disappear in fertile cytoplasmic revertants (Laughnan et al. 1984). Two double stranded RNA plasmids of 2900 and 760 bp were also found in maize cultivars carrying L cytoplasm, which is a cytoplasm belonging to the S cytoplasm group (Sisco et al. 1984). Although the presence of these RNA plasmids is maternally inherited, the nuclear genotype affects the amount of these RNA plasmids which is detectable. An 11 kb linear DNA plasmid was found in mitochondria of several accessions of *Brassica campestris* and 2 lines of *B. oleracea* carrying the ogu cms cytoplasm derived from *Raphanus sativus* (Palmer et al. 1983). The plasmid does not have any homology with either the chloroplast or mitochondrial genomes, and like the maize S1 and S2 plasmids mentioned above, plasmid copy number may be affected by the nuclear genotype (Erikson et al. 1986).

Due to the slow replacement rate in butternut squash cultivars, it is still possible to obtain seed of most of the lines used in the development of stable butternut cultivars. As a part of a survey of related stable and unstable butternut cultivars, crude mitochondrial DNA preparations were assayed for the presence of plasmids to determine whether such plasmids existed, and if so whether the presence of the plasmid(s) had any association with the presence or instability of the butternut trait.

Materials and methods

Plant material

Seed of the cultivars used were obtained from several sources. Seed of Ponca, Ponca-BN, Ponca-CR, Patriot, Waltham, New Hampshire Butternut, Derived Crookneck and Canada Crookneck were produced by hand sib pollination at Cornell. Ponca-BN and Ponca-CR are a pair of isogenic lines derived by self pollinating butternut and crookneck scions rooted from a dimorphic Ponca plant (Mutschler, unpublished). Seeds of Waltham and Ponca were obtained from the Harris Seed Company. Additional seed of Patriot was obtained from Northrup King, and also produced at Cornell by hand sibbing plants grown from breeder seed provided by Dr. Dermot Coyne, University of Nebraska. Seed of Waltham and Eastern Butternut were obtained from Agway.

Chemicals and supplies

DNAse (type I) and low melting agarose (type VII) were obtained from Sigma. Agarose type LE was purchased from FMC Marine Colloids. DNAse free RNAse was provided by Dr. M. Goldberg, Dept. of Genetics, Cornell University.

Preparation of mitochondria

Squash seed were grown for 5 days in Cornell mix (Sheldrake and Boodley 1973) in the dark at 30 °C. The etiolated shoots were then placed in the light for 8 h followed by 16 h in the dark. The shoots were harvested with scissors, cotyledons were removed, and the hypocotyls were weighed (yield is approximately 150 g of tissue for 250–300 seeds) then washed in 10% chlorox solution and spun dry in a salad spinner. Isolation of mitochondria was similar to that of the procedure of Kemble and Bedbrook (1980) except that: (1) the hypocotyls were ground in 2 volumes of a modified grinding buffer (50 mM Tris-Cl, pH 7.8, 0.4 M sucrose, 20 mM EDTA, 1 mM B-mercaptoethanol, 0.1% BSA, 5 mM dithiothreitol); (2) the crude mitochondrial pellets were resuspended in organelle buffer (50 mM Tris-Cl, pH 7.8, 0.4 M sucrose, 20 mM EDTA), and (3) the low and high speed centrifugation steps were repeated once.

DNAse treatment, nucleic acid extraction, and electrophoresis

Mitochondria of early preparations were DNAse treated to demonstrate that the plasmids were contained in this organelle, but later preparations omitted this step to increase yield. If the mitochondria were to be DNAse I treated, the pellets were resuspended in a minimal amount of DNAse I buffer (50 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 0.4 M sucrose) and DNAse treated by the method of Kolodner and Tewari (1975), then mitochondrial DNA was isolated as from the untreated mitochondria.

For DNA isolation, mitochondrial pellets were resuspended and lysed at 37 °C for at least 2 h in 4 ml of lysis buffer (50 mM Tris-Cl, pH 8.0, 25 mM EDTA, 0.036% Pronase which was

autodigested for 1 h at 37 °C then brought to 2% with lauryl sarcosine). The lysate was extracted twice with 1:1 phenol:chloroform, once with 24:1 chloroform:isoamyl alcohol, and once with water saturated ether before the DNA was ethanol precipitated, collected, washed, dried, and resuspended in minimal 1× TE buffer (typically 250 µl depending on pellet size). Then 15 µl of this resuspension was treated with DNAse I or, more routinely, RNAse A prior to agarose gel electrophoresis. For DNAse treatment (1 h, 37 °C), 2.0 µl of 10× DNAse I salts (0.5 M Tris-Cl (pH 7.8), 0.1 M $MgCl_2$) was added to 15 µl of crude nucleic acid resuspension, followed by 2 µl of ddH_2O and 1 µl of a 1 mg/ml DNAse I stock solution. For RNAse A reactions (1 h, 37 °C), 4 µl of ddH_2O was added to 15 µl of nucleic acid resuspension followed by 1 µl of 0.1 mg/ml RNAse A stock solution. Southern blots were performed essentially as in Maniatis et al. (1982) using a single-transfer dry-blotting apparatus.

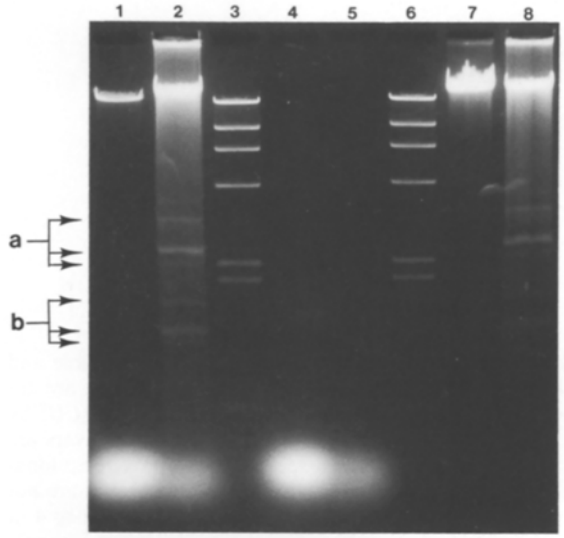

Fig. 1. Nuclease sensitivity of butternut mitochondrial plasmids. *Lanes 1, 4, and 7* contain 4 µg of tRNA and 0.25 µg of lambda DNA and *lanes 2, 5, and 8* contain a Derived Crookneck mitochondrial DNA preparation. The samples in lanes 1 and 2 were untreated; those in lanes 4 and 5 were pretreated with DNAse and those in lanes 7 and 8 were pretreated with RNAse. *Lanes 3 and 6* contain 0.13 µg of Hind III digested lambda DNA. pa1, pa3 and pa4 are indicated in descending crookneck order by the *arrows* labelled *a* and pb1, pb3 and pb4 are similarly indicated by the *arrows* labelled *b*

Probe preparation

The squash DNA plasmids designated pb1 and pb3 were isolated from Waltham Butternut crude mitochondrial DNA as follows: 530 µl of Waltham Butternut crude mitochondrial DNA suspension was precipitated with ethanol and resuspended in 150 µl of TE buffer. Then 10 µl of RNAse A (0.1 mg/ml) was added along with 40 µl of ddH_2O and the solution was incubated for 1 h at 37 °C. Then 20 µl of this mixture was loaded into each of 10 lanes of a 1.2% low melt agarose gel and electrophoresed at 4.3 volts/cm for 2 h. Bands corresponding to species pb1 and pb3 were located unter UV light and cut out of the gel. For each species, slices were pooled and DNA was recovered. Nick translations and hybridizations were performed essentially as in Maniatis et al. (1982) using high stingency wash conditions (0.1× SSC at 68 °C).

Results and discussion

A number of plasmids were observed in all the mitochondrial extracts. The plasmids were shown to be composed of DNA rather than RNA by their nuclease susceptibility (Fig. 1). The plasmids were observed when the mitochondria are DNAse I treated before lysis (Fig. 2a, lanes 2–4), as well as when they were not DNAse treated (Fig. 2a, lanes 5, 6; Fig. 2b, lanes 1–3), demonstrating that the plasmid DNAs were contained

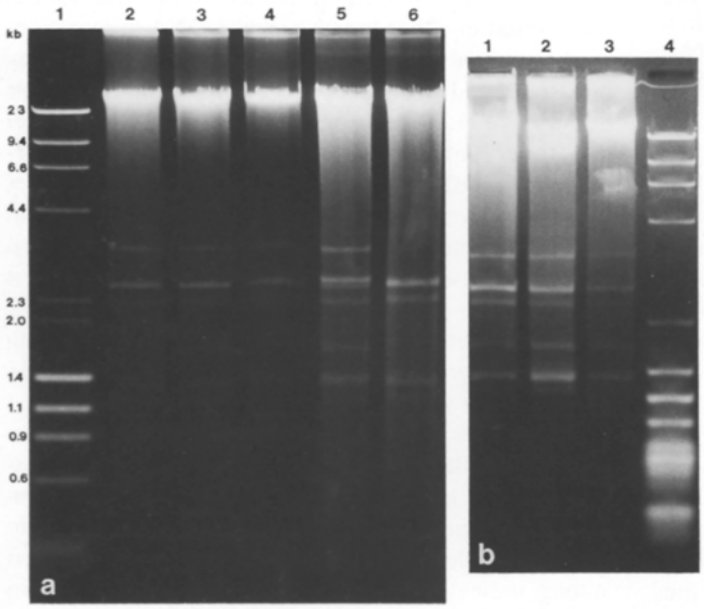

Fig. 2. a Mitochondrial plasmids in cultivars of winter squash. *Lanes 2–6* contain RNAse-treated mitochondrial DNA of Derived Crook, Ponca-CR, Ponca-BN, Canada Crookneck, and NHBN, respectively. *Lanes 2 to 4* contain DNA samples obtained from DNAse-treated mitochondria. *Lanes 5, 6* contain DNA from untreated mitochondria. *Lane 1* contains a mixture of 0.15 µg lambda DNA cut with Hind III and 0.6 µg of 0X174 DNA cut with Hae III. **b** *Lanes 1–3* contain RNAse-treated mitochondrial DNA preparations of Patriot, Waltham, and Butternut, respectively. *Lane 4* contains a mixture of 0.15 µg lambda DNA cut with Hind III and 0.85 µg 0X174 DNA cut with Hae III

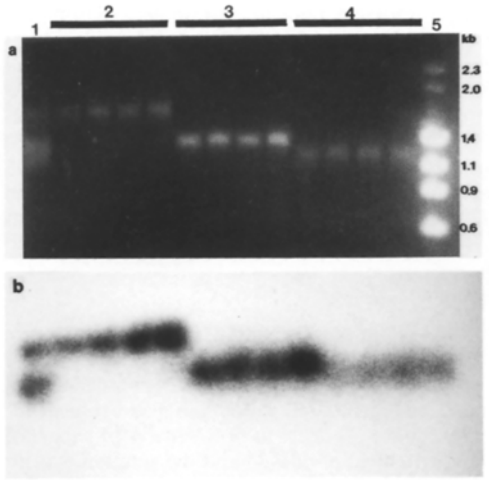

Fig. 3. **a** Preparation and purity test of plasmid 1 and 3 for use as probes. Plasmids pb1 (*lanes 2–5*), pb3 (*lanes 6–9*), and pb4 (*lanes 10–13*) isolated from segments cut from low-melt agarose gels loaded with RNAse treated mitochondrial DNA of Waltham butternut. *Lane 14* contains an equal mix of the three plasmids. *Lane 1* contains a mix of 0.15 μg of lambda DNA cut with Hind III and 0X174 DNA cut with Hae III. **b** Southern blot of the gel in Fig. 4a probed with nick-translated pb1

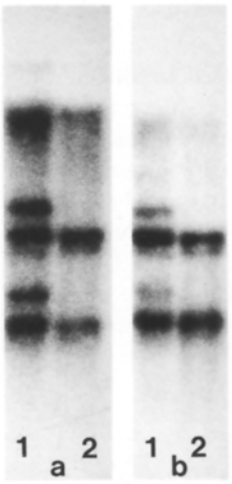

Fig. 4a, b. Southern blot of RNased treated mitochondrial DNA of Canada Crookneck (*lane 1*) and NHBN (*lane 2*) probed with **a** nick-translated pb1 and **b** pb3. This blot was made from the gel in Fig. 2a, lanes 5 and 6

within the mitochondria. Plasmids were observed in two region of the gels. Two to 4 plasmids (pa1 to pa4) were observed migrating to the extent expected of linear DNAs of 3.28, 2.59, 2.50, and 2.22 kb (Fig. 2). Additionally, 2 to 4 faster migrating plasmids (pb1 to pb4) were also observed if the DNA samples were treated with RNAse before loading the gels (as in Fig. 2) or if the gels were run longer (as in Fig. 1). These plasmids migrated to the extent expected of linear DNAs 1.56, 1.32, 1.30, and 1.3 kb. However, the pattern (relative distance between plasmids 1 and 3 versus 3 and 4), number (2 to 4), and relative intensity of plasmids within the faster and slower migrating groups were always the same. This suggests that the plasmids may be circular, with the

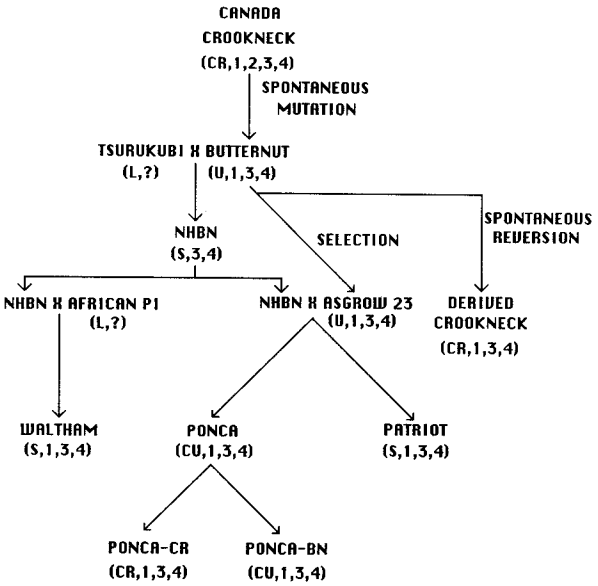

Fig. 5. Relationship among the crookneck and the stable and unstable butternut cultivars. The butternut cultivars are indicated as stable (*S*), unstable (*U*) or cross unstable (*CU*) by the letter codes beneath their names. Crookneck cultivars are designated *CR;* the two foreign accessions are considered long-necked, since they have fruit with long thin necks but are not of crookneck ancestry. The presence of the plasmids 1 to 4 in the mitochondria of any cultivar is indicated by the numbers in parenthesis below the cultivar names

faster migrating ones representing the supercoiled species. This was also supported by the hybridization data. Southern blots of mtDNA preparations were probed with nick-translated pb1 and pb3. First, purity of the probes was checked by electrophoresing a sample of the isolated pb1 and pb3 used to prepare the probes, blotting, and hybridizing the filter with nick-translated pb1. No detectable amounts of the other plasmid species were found in either the pb1 or pb3 DNA preparations by this method (Fig. 3). The Southerns performed using nick-translated plasmids pb1 and pb3 probes on blots from gels of mtDNA preparations showed that the faster and slower running series of plasmids hybridize similarly to pb1 and pb3, as would be expected if the two series of plasmids were the supercoiled and relaxed forms of the same plasmids (Fig. 4). The blots also demonstrated homology between plasmid pb1 and plasmids 3 and 4 (Figs. 3 and 4).

The number of plasmids observed varied among squash cultivars (Fig. 2), but remained constant within cultivar when seed lots from different sources are used (data not shown). Since the cultivars used are all related, it is possible to compare the cultivars, degree of relationship, fruit characteristics, and stability of fruit form with the absence or presence of each of the plasmids to determine whether any associations are indicated among these traits (Figs. 2 and 5). No clear pattern

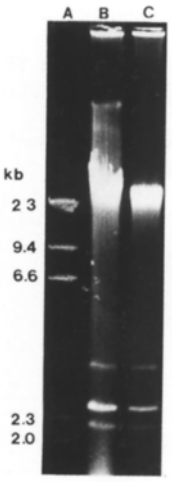

Fig. 6. Presence of plasmid 1 in DNA isolated from mitochondria of F_1 seedlings from the reciprocal crosses Ponca BN × NHHN (*lane B*) and NHBN × Ponca BN (*lane C*). *Lane A* contain lambda DNA cut with Hind III

emerged. All cultivars contained plasmids 3 and 4. The stable butternut cultivar New Hampshire Butternut was the only cultivar which lacked plasmid 1, and plasmid 2 were only found in the original crook cultivar, Canada Crookneck.

The presence or absence of plasmid 1 (pa1 and pb1) is not necessarily a function of which female parent was used in the development of new cultivars. Waltham, Ponca, and Patriot butternuts were all obtained from pedigree breeding programs from a cross with New Hampshire Butternut as the female parent, yet all three have the plasmid 1, which is absent in New Hampshire Butternut (Fig. 5). There are three possible explanations for this. First, the later butternut cultivars may have received plasmid 1 from the paternal parent through the pollen, rather than from the maternal parent, NHBN. There is no information in the literature that indicates whether this biparental inheritance exists in *Cucurbita moschata.* Secondly, plasmid 1 could be a large plasmid formed by recombination of the smaller plasmids and/or one of the smaller plasmid and the main band mtDNA. Thirdly, plasmid 1 may actually be present in New Hampshire Butternut carried as an insert in the mitochondrial genome instead of free plasmid, and the inserted plasmid may have been released during development of the later stable butternut cultivars from NHBN. The ability to create or maintain the plasmid 1 could be under nuclear control. The Southerns performed using nick-translated plasmids 1 and 3 as probes on blots from gels of mtDNA preparations showed that New Hampshire Butternut does not contain even a reduced level of free plasmid 1 (Fig. 4). Homology exists between plasmid 1 and 3, and between plasmid 1 and the main band mitochondrial genome in New Hampshire Butternut, but not between plasmid 3 and the main band mitochondrial genome. Furthermore, plasmid 1 is similarly present in mitochondrial DNA from F_1 seedlings produced from the reciprocal crosses Ponca BN × NHBN and NHBN × Ponca BN (Fig. 6). These data tend to support the second and third explanations above and suggest the action of a dominant nuclear factor affecting the production and/or maintenance of plasmid 1 in the mitochondria. This could be similar to the nuclear effect on the presence of the RNA plasmids in mitochondria of LBN cytoplasm maize (Sisco et al. 1984). It is less likely that pollen transmission could account for the strong plasmid 1 band obtained from the NHBN × Ponca BN F_1 seedlings, unless some factor favoring the increase and presence of the plasmid is operative.

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