Organellar Genomes of the Cucurbits

Michael J. Havey

Abstract The cucurbit organellar DNAs possess distinctive characteristics of practical and theoretical significance. Whereas the cucurbit chloroplast DNAs are similar in size, structure, and transmission to most Angiosperms, their mitochondrial DNAs show enormous size differences. Plants in the genus *Cucumis* have some of the largest mitochondrial DNAs among all plants, due in part to accumulation of repetitive DNAs and inter-genomic transfers. Recombination among these repetitive motifs produces structurally diverse mitochondrial DNAs associated with paternally transmitted mosaic phenotypes and altered gene expression. The mitochondrial DNAs of *Cucumis* species are paternally transmitted, which is relatively rare among Angiosperms. The unique characteristics of the *Cucumis* organelles are interesting not only from an evolutionary point-of-view, but also may allow for characterization of beneficial organellar-nuclear interactions, generation of mitochondrial mutants, transformation of the mitochondrial DNA, and knock-downs of mitochondrial-gene expression.

Keywords DNA • Mitochondrion • Mutation • Plastid • Transformation • Transmission genetics

The Organellar Genomes

The plant cytoplasm carries two organelles, the plastid and mitochondrion, each with their own DNA. The organellar DNAs encode for proteins important for photosynthesis and respiration, as well as a subset of the transfer and ribosomal RNAs necessary for protein synthesis (Unseld et al. 1997; Mackenzie and McIntosh 1999; Notsu et al. 2002; Handa 2003; Ogihara et al. 2005; Sugiyama et al. 2005; Allen et al. 2007). In addition to organellar-encoded products, hundreds of nuclear-encoded proteins are required for functional organelles (Emanuelsson et al. 2000; Giegé et al. 2005). The intimate interaction between the organelles and nucleus is

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imperative for overall plant performance (Kihira 1982), stress tolerances (Millar et al. 2003; Atkin and Macherel 2009; Vanlerberghe et al. 2009; Gill and Tuteja 2010), and programmed cell death (Gottlieb 2000; Gechev et al. 2006; Reape and McCabe 2008).

Sizes and Structures of the Cucurbit Organellar Genomes

Plastids

Restriction-enzyme analyses of the plastid DNAs of the major cucurbits demonstrated that their sizes and structures are similar to most other angiosperms with large and small single-copy regions separated by two inverted repeats (Palmer 1982; Perl-Treves and Galun 1985; Lim et al. 1990). The sequences and annotations of the plastid DNAs from cucumber (Kim et al. 2006; Chung et al. 2007; Pląder et al. 2007) and melon (Rodriguez-Moreno et al. 2011) have been published. The reported sizes of the cucumber plastid DNA vary from 155,293 (Pląder et al. 2007) to 155,527 basepairs (bp) (Kim et al. 2006), likely due to small differences in intergenic regions. The melon plastid DNA is slightly larger than cucumber at 156,017 bp (Rodriguez-Moreno et al. 2011). GC contents were similar for cucumber (40 %) and melon (45 %). Although different numbers of coding regions were reported for the plastid DNAs of cucumber versus melon, sequence differences were only about 5 % primarily due to short indels and single nucleotide polymorphisms (Kim et al. 2006; Rodriguez-Moreno et al. 2011).

Variations in plastid sequences have been used to estimate maternal phylogenies within the Cucurbitaceae, which correlated well with floral and pollen morphologies (Kocyan et al. 2007). Schaefer et al. (2009) extracted DNA from herbarium specimens of cucurbit species, successfully amplified and sequenced regions from the plastid DNAs, and constructed a maternal phylogeny supporting long-distance transoceanic dispersal of cucurbits probably by floating fruit. Plastid-sequence variation, together with mitochondrial and nuclear sequences, revealed phylogenies indicating that monoecy may have evolved from a progenitor dioecious state numerous times in the Cucurbitaceae. Variation for fragment lengths and sequences in the genus *Cucumis* revealed wild species more closely related to cultivated species (Chung et al. 2006; Sebastian et al. 2010), information important for use of these species for genetic improvement of cucumber and melon.

Mitochondria

The cucurbit mitochondrial DNAs show several-fold size differences (Ward et al. 1981). Watermelon possesses a relatively small mitochondrial genome of 379 kilobases (kb), and squash has a mitochondrial genome 2.5 times bigger at 983 kb (Alverson et al. 2010). Cucumber and melon possess huge mitochondrial genomes of

1,685 kb (Alverson et al. 2011) and approximately 2,460 kb [calculation based on estimate by Ward et al. (1981) of 1,600 megadaltons divided by 650 daltons per base-pair], respectively. Sequencing revealed a larger mitochondrial DNA of melon than estimated by Ward et al. (1981), and assembled into five scaffolds covering 2,740 kb and corresponding to approximately 95 % of the genome (Rodriguez-Moreno et al. 2011). The significant size differences among cucurbit mitochondrial DNAs cannot be attributed to increased mitochondrial volume (Bendich and Gauriloff 1984); accumulation of conserved repetitive DNAs, microsatellites, or transposable elements (Ward et al. 1981; Rodriguez-Moreno et al. 2011); or increased numbers of coding regions (Alverson et al. 2010, 2011; Rodriguez-Moreno et al. 2011). In fact, only about 119 kb of highly conserved sequences were shared among the mitochondrial DNAs of melon, squash, and watermelon (Rodriguez-Moreno et al. 2011). Sequencing has revealed that accumulation of short repetitive motifs and DNA transfers from the chloroplast contributed to expansion of mitochondrial-DNA sizes in cucumber and melon (Lilly and Havey 2001; Bartoszewski et al. 2004a; Alverson et al. 2011; Rodriguez-Moreno et al. 2011). For melon, DNA transfer from the nucleus to the mitochondrion accounted for 1.14 megabases of sequence, or 42 % of its mitochondrial DNA. This large-scale transfer of nuclear DNA into the melon mitochondrion is the largest so far reported and significantly contributed to genome-size expansion.

Organellar Transmission

The vast majority of plants show maternal transmission of their organelles (Gillham 1978; Harris and Ingram 1991); however examples of paternal (Boynton et al. 1987; Neale and Sederoff 1989; Havey et al. 1998) or biparental (Medgyesy et al. 1986; Smith 1989; Mason et al. 1994; Erickson et al. 1989; Erickson and Kemble 1990) transmission of the organelles have been documented. A unique characteristic of organellar transmission among the cucurbits is paternal transmission of the *Cucumis* mitochondrial DNAs (Matsuura 1995; Havey 1997; Havey et al. 1998; Shen et al. 2013), versus maternal transmission of the squash and watermelon mitochondrial DNAs (Havey et al. 1998). The cucurbit plastids appear to be maternally transmitted, as evidenced by maternal transmission of chlorophyll deficiencies, presumably due to mutations in the chloroplast genome, in squash (*Cucurbita maxima* Duch.; Hutchins and Youngner 1952) and melon (Ray and McCreight 1996). Corriveau and Coleman (1988) used a DNA fluorochrome and epifluorescence microscopy to demonstrate physical exclusion of chloroplast DNA from the male gametophyte of cucumber, indirect evidence for maternal transmission.

Paternal transmission of the mitochondrial DNA is likely a derived state and possibly restricted to the genus *Cucumis*, although this exceptional transmission mode has been conclusively demonstrated only for cucumber, melon, and *C. hystrix* (Havey et al. 1998; Shen et al. 2013). Shen et al. (2015a) studied the prevalence of organellar DNAs in the developing male gametophyte of cucumber and presented evidence that the mitochondrial DNA is protected from nuclease(s) during microsporogenesis, resulting in retention and delivery via the sperm cells to progenies. One question commonly asked

when I speak or write about the paternal transmission of *Cucumis* mitochondria is what evolutionary advantage would be provided by paternal transmission of an organelle, since its occurrence is so rare? One hypothesis that I can offer is that as the size of the mitochondrial DNA increases due to accumulation of repetitive DNAs in direct and reverse orientations, the probability of pairing among these repeats increases allowing for intra- and inter-molecular recombination to shift the order of genes and/or produce sub-genomic molecules (sublimons). These recombination events can affect the transcription of mitochondrial genes by removing or altering the positions of up-stream promoter region(s) (Bartoszewski et al. 2004b), as well as affecting relative copy numbers because sublimons without origins of replication could become rarer [a process known as substoichometric shifting (Woloszynska 2010)] and potentially lost. These events would negatively affect mitochondrial function. With strict maternal transmission of the mitochondrial DNA, it may be more difficult to select against or eliminate deleterious sublimons from the population of mitochondrial DNAs transferred to progenies via the egg cell. In the case of paternal transmission, presumably relatively few mitochondria are delivered via the male gametophyte to the zygote and subsequently to progenies. This may provide a bottleneck restricting the number of mitochondria present in the sperm cells, representing a method to select for superior-performing, or against poor-performing, mitochondria. However if this hypothesis were true, one would expect that other plants with enormous mitochondrial DNAs would tend to show paternal transmission. At least in the case of *Silene conica*, the size of its mitochondrial DNA is estimated at 11,318 kb and transmission is maternal (Sloan et al. 2012); however there is evidence for occasional paternal or bi-parental transmission of the mitochondrial DNAs in the genus *Silene* (McCauley et al. 2005; Pearl et al. 2009; Bentley et al. 2010). Banana (*Musa acuminata*) also shows paternal transmission of its mitochondria (Fauré et al. 1994); however I could find no estimates of the mitochondrial DNA sizes for *Musa*. It will be interesting to determine the transmission modes and sizes of mitochondrial DNAs throughout the genus *Cucumis*, and establish if any relationship exists between size and transmission. An approach would be to first estimate the sizes of the organellar DNAs across *Cucumis* species, for example by differential centrifugation (Ward et al. 1981) or building contigs among large genomic clones (Bartoszewski et al. 2009), followed by next-generation sequencing and assembly to the cucumber and melon organellar DNAs to identify polymorphisms between plants of the same species. Then reciprocal hybrids can be genotyped for these polymorphic markers to establish transmission of the organellar DNAs. Another important line of research would be to determine if any cucurbits outside of the genus *Cucumis* show paternal transmission of the mitochondrial DNA.

Organellar Phenotypes

Maternal transmission of chlorophyll deficiencies, presumably due to mutations in the plastid DNA, has been demonstrated for *Cucurbita maxima* (Hutchins and Youngner 1952) and melon (Ray and McCreight 1996). A maternally transmitted tolerance to

cold temperature has been reported from the pickling cucumber 'Chipper' (Chung et al. 2003). Sequencing of the plastid DNAs from cold-tolerant and susceptible cucumbers revealed three single nucleotide polymorphisms, one of which resulted in a single amino-acid change in the atpB protein (Chung et al. 2007). It remains unclear how this single amino-acid change would condition cold tolerance; however because sequencing was completed using amplicons from the polymerase chain reaction, it is possible that rarer sublimons exist among the plastid DNAs from cold-tolerant cucumber that could condition or elicit this abiotic-stress tolerance. To the knowledge of the author, no other maternally transmitted phenotypes have been documented in the cucurbits.

A paternal effect on chilling tolerance has been reported in cucumber (Ali et al. 2014). Although reciprocal crosses between chilling-tolerant (CH1) and susceptible (CH4) cucumbers resulted in identical phenotypes in F_1 families, the F_2 family from CH4×CH1 was more tolerant to chilling than the F_2 family from CH1 x CH4 indicating a parent-of-origin effect on this trait which could be due to a mitochondrial effect or preferential expression of paternal nuclear allele(s).

The mosaic (MSC) mutants of cucumber are paternally transmitted phenotypes showing chlorotic to necrotic regions on leaves, reduced vigor and fertility, and enhanced expression of stress-response genes (Malepszy et al. 1996; Bartoszewski et al. 2007; Juszczuk et al. 2007; Szal et al. 2009). The MSC mutants were first observed among progenies from plants regenerated from cell cultures established using the highly inbred line 'B' selected from the Polish pickling cultivar 'Borszczagowski' (Fig. 1; Malepszy et al. 1996; Ładyżyński et al. 2002; Bartoszewski et al. 2004b, 2007). Paternal transmission of the MSC phenotypes (Malepszy et al. 1996) immediately focused attention on their mitochondrial DNAs. The MSC mutants possess mitochondrial DNAs with structural polymorphisms and deleted regions relative to wild-type inbred B (Lilly et al. 2001; Del Valle-Echevarria et al. 2015), and independently derived MSC mutants possess different mitochondrial polymorphisms indicating that they do not trace back to a single sublimon in B (Lilly et al. 2001; Bartoszewski et al. 2004b; Del Valle-Echevarria et al. 2015). Passage of cucumber through cell cultures may allow mitochondria carrying deleterious lesion(s) to sort by their reducing negative effects. Another possibility is that passage through cell culture may enhance recombination among repetitive DNAs to produce structural rearrangements or deletions affecting the expression of mitochondrial genes. Sequencing of the mitochondrial DNAs from three independently derived MSC lines (3, 12, and 16) revealed that all lines differed for regions that were missing or of significantly lower copy number relative to inbred B. MSC3 possessed significantly fewer copies of the polycistronic region coding for exons 4 and 5 of NADH dehydrogenase subunit 5 and ATPase subunit 4, while MSC 12 and 16 possessed an under-representation of the ribosomal protein S7 (rps7) coding region (Del Valle-Echevarria et al. 2015). Although sharing an under-representation of the same rps7 coding region, the mitochondrial DNAs of MSC12 and 16 differ both structurally (Bartoszewski et al. 2004b) and for sizes of overall missing or under-represented regions (Del Valle-Echevarria et al. 2015).

MSC plants are heteroplasmic for both mutant and wild-type mitochondrial genomes, with the former predominating, and relatively rare wild-type progenies result from sorting of the mitochondrial genome in MSC pollen (Lilly et al. 2001).
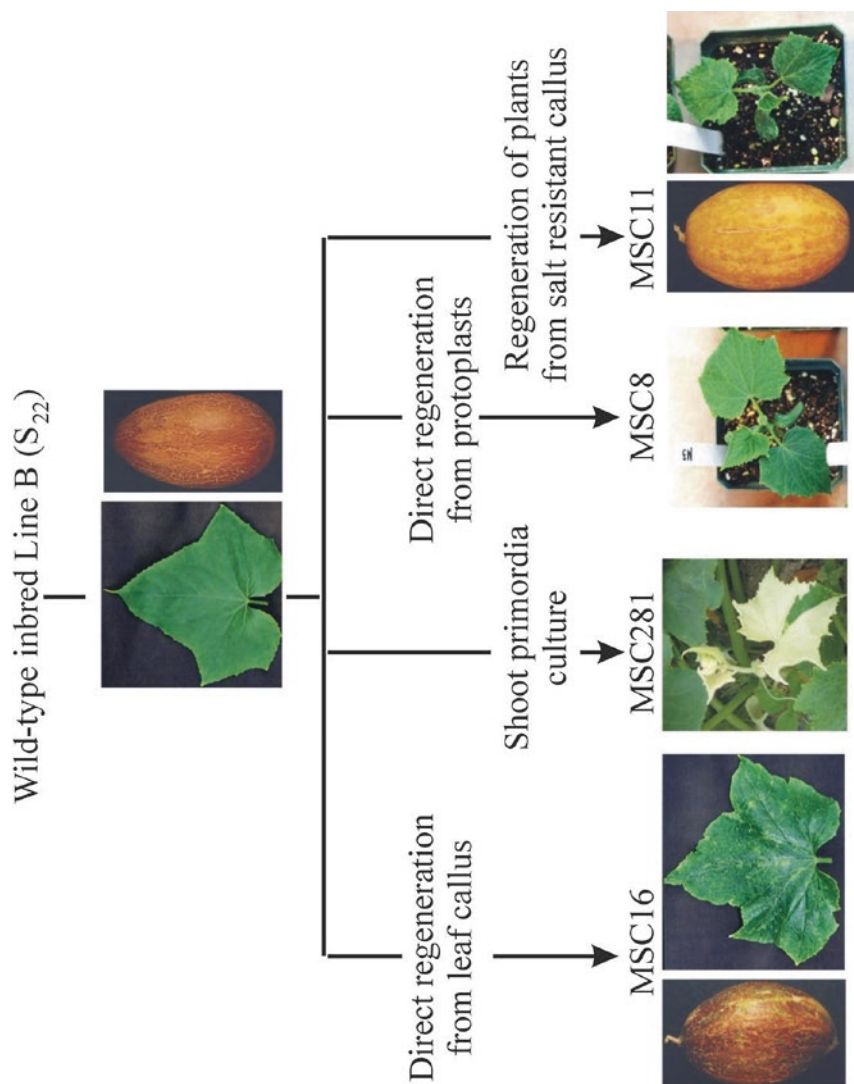
Fig. 1 Phenotypes of mosaic (MSC) lines identified among regenerated plants after passage of the wild-type inbred line B through various cell cultures

We demonstrated that testcrosses with MSC as the male parent to wild-type plants produce rare wild-type progenies at or below 1 % (Malepszy et al. 1996; Lilly et al. 2001). At the end of meiosis, the mitochondria in cucumber microspores are cup-shaped. By the time free microspores are produced, the mitochondria are few and gigantic (Abreu et al. 1982). These huge mitochondria are only observed in uninucleate microspores and may result from organelle elimination or fusion. After the mitotic division that produces bi-cellular microspores, the mitochondria divide and resume normal shape, size, and numbers (Abreu et al. 1982). The formation of relatively few, huge mitochondria during microsporogenesis may create a bottleneck and reduce the diversity among mitochondrial genomes transferred to progenies via the male gametophyte, contributing to the sorting of mitochondrial genomes revealed during transmission studies (Lilly et al. 2001; Bartoszewski et al. 2004b).

Uniparental Effects

Differential transmission of the *Cucumis* organellar DNAs allows for the separation of chloroplast and mitochondrial effects by reciprocal crossing. Because the vast majority of plants show maternal transmission of both the plastid and mitochondrial DNAs, one would expect that significant cross-talk between the organelles and nucleus would result in beneficial interactions. However the large size and frequent recombinations within and among the mitochondrial DNAs can produce structurally polymorphic molecules among plants with a population (Havey et al. 1998; Lilly et al. 2001; Bartoszewski et al. 2004b; Alverson et al. 2011; Rodriguez-Moreno et al. 2011). Plants in the genus *Cucumis* provide a unique system to identify and characterize chloroplast and mitochondrial effects on phenotypes by exploiting differential transmission of the organelles. Shen et al. (2015b) crossed among doubled haploid (DH) lines from divergent cucumber populations to produce a complete set of reciprocal hybrids. Significant differences were detected for plant growth between reciprocal hybrids possessing identical nuclear genotypes, revealing the potential of beneficial organellar effects on plant performance. The significantly better performance of a DH as the male parent could be due to beneficial mitochondrial interactions with the nucleus. Superior performance as the female parent may reveal better interacting chloroplasts. These results indicate that different organellar types may exist within *Cucumis* species, and inbreds possessing specific organelles may perform better as the male or female to produce more vigorous hybrids.

Future Research

Evolution of Organellar Transmission in the Cucurbits

Paternal transmission of the *Cucumis* mitochondrial DNA is likely a derived state because of the prevalence of maternal transmission in other Cucurbits and most other plants (Havey et al. 1998). A systematic evaluation of mitochondrial-DNA

transmission in other *Cucumis* species and related genera should reveal the commonness of this unique transmission mode, or if occasional or strict maternal transmission occurs in this genus. If the latter were true, interspecific crossing and segregation analyses may reveal the genetic basis of differential mitochondrial transmission and ultimately lead to identification of causal gene(s). This approach may be more fruitful than trying to alter organelle transmission by mutagenesis of plants showing strict maternal transmission of both organelles. Occasional biparental transmission of one or both organellar genomes may also exist, and would be useful to determine any nuclear role and its inheritance. A deeper understanding of any genetic control of organellar transmission would allow identification and introgression of superior plastids or mitochondria to enhance plant performance.

Mitochondrial "Knock-Downs"

The MSC mutants offer a unique opportunity to develop "knock-downs" of mitochondrial genes. Presently there is no efficient way to mutate mitochondrial genes (Jacobs 2001). Passage of cucumber (and presumably melon) through cell culture, regeneration of plants, and screening of progenies may provide a mechanism to identify and select plants with under-representation of mitochondrial genes. I recommend the development of doubled-haploid (DH) lines from diverse populations of cucumber and melon, in order to guarantee a uniform nuclear genotype and sample a wide range of putative mitochondrial DNA diversity. These DHs could be passed through various cell cultures, and regenerated plants evaluated for paternally transmitted phenotypes. Complete absence of a mitochondrial gene would likely be lethal; however substoichometric shifting could result in under-representation of specific coding regions (Del Valle-Echevarria et al. 2015) and produce distinct phenotypes, as observed in cucumber (Fig. 1). After confirming paternal transmission of the phenotype, sequencing of the mitochondrial DNAs can be completed to reveal genes carried on regions significantly under-represented relative to the wild-type parental DH line. These mitochondrial "knock-downs" would be a useful tool to study their effects on plant growth and development, mitochondrial function, and nuclear responses.

Mitochondrial Transformation

There is presently no method to efficiently introduce foreign DNA into the mitochondrion. Although mitochondrial transformation has been reported in *Chlamydomonas* (Randolph-Anderson et al. 1993) and yeast (Butow et al. 1996), it has never been successfully developed a higher plant. Microprojectile bombardment is used to transform the chloroplast genomes of *Chlamydomonas* (Boynton et al. 1987; Kindle et al. 1991), tobacco (Svab and Maliga 1993; Zoubenko et al.

1994), tomato (Ruf et al. 2001), and Brassica (Cheng et al. 2010). The lack of a robust technique to transform the mitochondrial DNA restricts assessment of mitochondrial effects on overall performance, stress tolerances, and programmed cell death, as well as retrograde (mitochondrion-to-nucleus) signaling and associated nuclear responses. Challenges for transformation of the mitochondrial DNA include efficient introduction of foreign DNA into a mitochondrion, recombination to incorporate the transgene into the mitochondrial DNA, the absence of selectable markers, and the relatively large numbers of mitochondria per cell and mitochondrial genomes per mitochondrion. Cucumber has potential as a model plant for mitochondrial transformation (Havey et al. 2002) because of two unique attributes: uninucleate microspores of cucumber possess relatively few, huge mitochondria (Abreu et al. 1982) and mitochondria show paternal transmission (Havey 1997). These two attributes may allow for delivery of transformed mitochondria to progenies via the male gametophyte. A potential approach would be to isolate cucumber microspores at the uninucleate stage, biolistically introduce a DNA construct conditioning antibiotic resistance into the huge mitochondria of uninucleate microspores, mature microspores to functional pollen (Alwen et al. 1990), and finally deliver transformed mitochondria to progenies via the male gametophyte.

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