

Cucumber: A model angiosperm for mitochondrial transformation?

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Abstract. Plants possess three major genomes, carried in the chloroplast, mitochondrion, and nucleus. The chloroplast genomes of higher plants tend to be of similar sizes and structure. In contrast both the nuclear and mitochondrial genomes show great size differences, even among closely related species. The largest plant mitochondrial genomes exist in the genus *Cucumis* at 1500 to 2300 kilobases, over 100 times the sizes of the yeast or human mitochondrial genomes. Biochemical and molecular analyses have established that the huge *Cucumis* mitochondrial genomes are due to extensive duplication of short repetitive DNA motifs. The organellar genomes of almost all organisms are maternally transmitted and few methods exist to manipulate these important genomes. Although chloroplast transformation has been achieved, no routine method exists to transform the mitochondrial genome of higher plants. A mitochondrial-transformation system for a higher plant would allow geneticists to use reverse genetics to study mitochondrial gene expression and to establish the efficacy of engineered mitochondrial genes for the genetic improvement of the mitochondrial genome. Cucumber possesses three unique attributes that make it a potential model system for mitochondrial transformation of a higher plant. Firstly, its mitochondria show paternal transmission. Secondly, microspores possess relatively few, huge mitochondria. Finally, there exists in cucumber unique mitochondrial mutations conditioning strongly mosaic (*msc*) phenotypes. The *msc* phenotypes appear after regeneration of plants from cell culture and sort with specific rearranged and deleted regions in the mitochondrial genome. These mitochondrial deletions may be a useful genetic tool to develop selectable markers for mitochondrial transformation of higher plants.

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The structures of the higher plant organellar genomes

Plants possess three major genomes, carried in the chloroplast, mitochondrion, and nucleus. The organellar genomes are circular double-stranded DNA molecules present in many copies per organelle. The chloroplast DNA of higher plants is relatively conserved in structure and function. Tobacco, as a representative angiosperm, has a chloroplast genome of approximately 156 kb (SHINOZAKI et al. 1986) that carries approximately 160 genes (WAKASUGI et al. 1998). The molecule possesses two inverted repeats of approximately 25 kb encoding for the 16S and 23S ribosomal RNAs and ribosomal proteins (PALMER et al. 1988). The structure and linear arrangement of chloroplast coding regions is conserved among most higher plants, due in part to the inverted repeats (PALMER, STEIN 1986). When pairing and crossing-over occurs between inverted repeats in the same molecule, the circular structure and general order of genes remains the same. This structural stability of the chloroplast DNA has made this molecule useful in phylogenetic comparisons among relatively distantly related genera and families (PALMER et al. 1988).

The plant mitochondrial genome is also a circular double-stranded DNA molecule that encodes rRNAs, tRNAs, ribosomal proteins, and a portion of the enzymes used in respiration (UNSELD et al. 1997). Many mitochondrial enzymatic subunits are nuclear encoded, cytoplasmically translated, and imported into the mitochondria (NEWTON 1988). The important interaction between mitochondrial and nuclear-encoded products is a possible explanation for reduced performance associated with alien cytoplasms (ALLEN et al. 1989). Mitochondrial coding regions accumulate sequence changes very slowly; however the linear arrangement of genes changes relatively quickly (PALMER, HERBON 1988). In contrast to the chloroplast genome, the mitochondrial DNA possesses direct repeats spread throughout the genome. Pairing and recombination among these direct repeats produces smaller circular DNA molecules. Continued pairing and recombination among other direct repeats can shift the relative arrangements among coding regions, quickly producing polymorphic molecules among relatively closely related plants. As a result, structural analyses of the plant mitochondrial DNA has not been useful to estimate phylogenetic relationships.

Whereas the chloroplast genomes of most higher plants are similar in size and structure, both the nuclear and mitochondrial genomes show great size differences even among relatively closely related species. Amounts of nuclear DNA in plants vary widely (Figure 1), due to differences in basic DNA amounts and polyploidy. As opposed to the smaller mitochondrial genomes of humans and yeast (approximately 17 kb), the mitochondrial DNAs also show great size differences among higher plants (Figure 2). The smallest mitochondrial genome known among

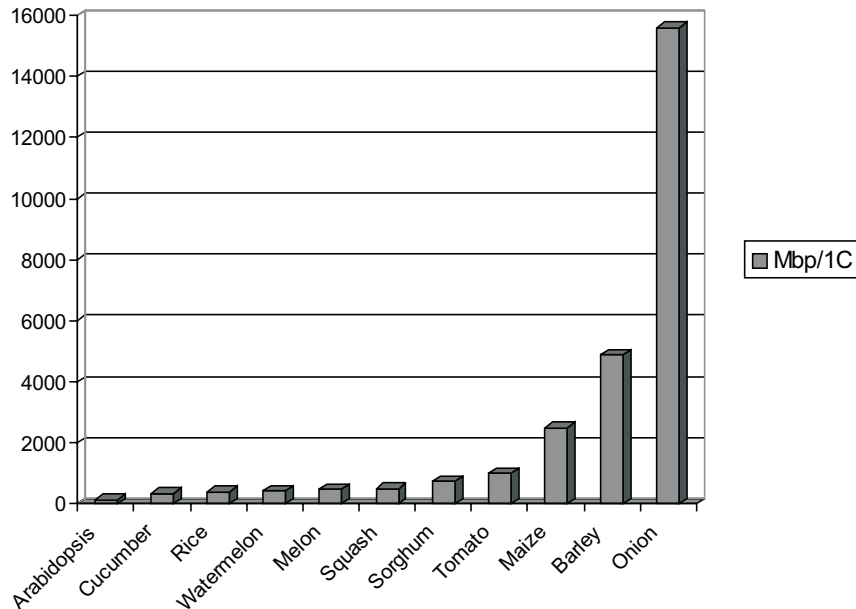


Figure 1. Relative amounts of nuclear DNA (megabase-pairs per 1C nucleus) in major diploid plant species (ARUMUGANATHAN, EARLE 1991)

higher plants is that of *Brassica hirta* at 218 kb (PALMER, HERBON 1987) and the largest known is that of *Cucumis melo* at 2300 kb (WARD et al. 1981). Great size variations in mitochondrial DNAs exist among closely related species. For example, species in the Cucurbitaceae possess chloroplast and nuclear genomes of similar sizes, but their mitochondrial genomes range from one of the smallest (watermelon at 230 kb) and to the largest known mitochondrial (melon at 2300 kb) genome among angiosperms (WARD et al. 1981). BENDICH (1985) and LILLY and HAVEY (2001) demonstrated that the huge *Cucumis* mitochondrial genomes are associated with the accumulation of short degenerate repetitive DNA sequences.

Organellar DNA transmission

For the majority of higher plants, the organellar genomes are maternally transmitted (MOGENSEN 1996). The intimate interaction between the nuclear and organellar genomes may be the reason for maternal transmission of organelles (HARRIS, INGRAM 1991, GILLHAM 1994). Strict maternal transmission would allow the organellar genomes to remain static, while the nuclear genome undergoes

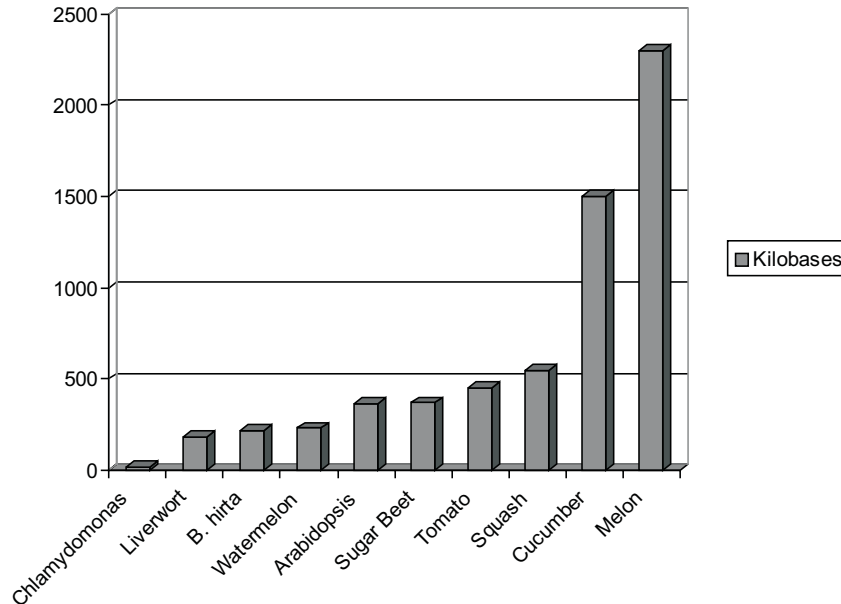


Figure 2. Relative amounts of mitochondrial DNA in (kilobases for a sample of plants (PALMER, HERBON 1987, WARD et al. 1981, ULSELD et al. 1997, KUBO et al. 2000, ODA et al. 1992, SHIKANAI et al. 1998).

selection to optimize their intergenomic relationships (GILLHAM 1994). Chloroplast DNA has been detected (MOGENSEN, RUSCHE 2000) and mitochondria (CONNETT 1987) are almost always present in the generative and sperm cells of the male gametophyte. Mitochondria have been observed entering the egg with the sperm nucleus (CONNETT 1987). Maternal transmission of the organelles may be due to specific degradation or debilitation of the paternal organellar DNA (VAUGHN et al. 1980, VAUGHN 1981, DAY, ELLIS 1984) or sloughing off of the pollen-tube cytoplasm during syngamy (CONNETT 1987, MOGENSEN 1988).

Exceptions to maternal transmission of the organelles are common in plants. The chloroplast genomes of some gymnosperms are paternally transmitted (NEALE, SEDEROFF 1989). Among angiosperms, both the chloroplast and mitochondrial genomes predominately show maternal transmission, although occasional biparental transmission is well established (MEDGYESY et al. 1986, SMITH 1989b, MASON et al. 1994, ERICKSON, KEMBLE 1990) and can be under nuclear control (CORNU, DULIEU 1988, SMITH 1989a, TILNEY-BASSETT et al. 1992). We established transmission of the organellar genomes for four species of the Cucurbitaceae, cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), squash (*Cucurbita pepo* L.), and watermelon (*Citrillus lanatus* L.). Polymorphisms in the chloroplast and mitochondrial genomes of squash were mater-

nally transmitted (HAVEY et al. 1998). This chloroplast result agrees with maternal transmission of a chlorophyll-deficient mutant of *C. maxima* (HUTCHINS, YOUNGNER 1952). Watermelon also showed maternal transmission of both the chloroplast and mitochondrial genomes. However, melon showed maternal transmission of the chloroplast and paternal transmission of the mitochondrial genomes (HAVEY et al. 1998). The chloroplast genome of melon was previously known to be maternally transmitted (RAY, MCCREIGHT 1996). The cucumber mitochondrial genome was paternally transmitted (HAVEY 1997). Although we were not able to identify polymorphisms to establish transmission of the cucumber chloroplast genome, epifluorescence microscopy demonstrated exclusion of the chloroplast DNA from the male gametophyte of cucumber, supporting maternal transmission (CORRIVEAU, COLEMAN 1988). Because maternal transmission of the mitochondrial genome predominates among angiosperms and occurs in the related genera *Citrullus* and *Cucurbita*, paternal transmission in the genus *Cucumis* is likely the derived state (HAVEY et al. 1998). Therefore, species in genus *Cucumis* are unique among the dicots in that they show differential transmission of the three plant genomes, maternal for chloroplast, paternal for mitochondrial, and biparental for the nuclear DNA (HAVEY 1997, HAVEY et al. 1998). A similar result has been reported for the monocot genus *Musa* (FAURE et al. 1994).

Important mitochondrial traits

Although cytoplasmic effects on overall plant performance are well documented (KIHIRA 1982), phenotypes conditioned by mutations in the plant mitochondrial DNA are relatively rare. Examples of mitochondrially encoded plant phenotypes include cytoplasmic-genic male sterility (CMS) (LASER, LERSTEN 1972), the non-chromosomal stripe (*ncs*) mutations of maize (COE 1983, NEWTON, COE 1986), the mosaic (*msc*) mutations of cucumber (MALEPSZY et al. 1996, LILLY et al. 2001), the *chm*-induced mutations of *Arabidopsis* (MARTINEZ-ZAPATER et al. 1992, SAKAMOTO et al. 1996), and the plastome mutator phenotypes of *Oenothera* (EPP 1973, REDEI 1973, CHANG et al. 1996). With the exception of CMS, most of these mutant phenotypes are due to deletions or chimeric rearrangements involving mitochondrial coding regions and are maintained in plant populations by heteroplasmy (NEWTON, COE 1986). A pleiotropic effect of mutations in the mitochondrial genome is poor development of chloroplasts appearing as chlorotic sectors on leaves (ROUSSELL et al. 1991). A nuclear effect on the expression or predominance of polymorphic mitochondrial DNAs has been well documented (HE et al. 1995a, b, JANSKA et al. 1998).

CMS is an economically important mitochondrial trait that conditions no pollen production; plants can reproduce only as females allowing for the production of hybrid seed. CMS has been identified and exploited for hybrid-seed production

in many crops, including beet, cabbage, carrot, canola, maize, onion, sorghum, among others (LASER, LERSTEN 1972). To develop a male-sterile line for hybrid-seed production, a CMS source is usually backcrossed for at least six generations to a superior male-fertile inbred line to combine the male-sterile cytoplasm with the nuclear genome of the elite male-fertile parent. This superior male-sterile line is then used as the female in hybrid seed production.

Organellar DNA transformation

Transformation of the plant organellar genomes has been demonstrated. The algae *Chlamydomonas reinhardtii* is a model organism for chloroplast transformation because each cell possesses one relatively large chloroplast (GILLHAM 1994). Microprojectile bombardment and polyethylene glycol (PEG) mediated protoplast transformation has been successfully used to transform the chloroplast genomes of *Chlamydomonas* (BOYNTON et al. 1988, KINDLE et al. 1991), tobacco (SVAB, MALIGA 1993, O'NEILL et al. 1993, KOOP et al. 1996), *Arabidopsis* (SIKDAR et al. 1998), and tomato (RUF et al. 2001). For *Chlamydomonas*, the selectable markers were the *atpB* gene complementing photosynthetic-deficient mutants (BOYNTON et al. 1988) or 5-fluorodeoxyuridine treatments to reduce chloroplast DNA amount (KINDLE et al. 1991) and the bacterial *aadA* gene conditioning resistance to streptomycin or spectinomycin (GOLDSCHMIDT-CLERMONT 1991). The transformation cassettes for tobacco consisted of the desired transgene coupled with a chloroplast intergenic or coding region and resistance to the antibiotics kanamycin (CARRER et al. 1993) or spectinomycin (SVAB, MALIGA 1993). These constructs were precipitated onto relatively small (0.6 μm) gold particles and introduced into the chloroplast by particle bombardment (CARRER et al. 1993, SVAB, MALIGA 1993, ZOUBENKO et al. 1994, SKIDAR et al. 1998, RUF et al. 2001). After the gold particle enters the chloroplast, the transgene enters into the chloroplast DNA by homologous recombination. This site-specific recombination differs from transformation of the plant nuclear genome by *Agrobacterium* or particle bombardment, where the transgene enters randomly into the nuclear DNA. After bombardment, cells must be repeatedly plated on the selective agent or minimal medium to select for cells carrying transformed chloroplast genomes and to reduce heteroplasmy. Because of the relatively large number of chloroplasts per cell and chloroplast genomes per chloroplast, transgenes in the chloroplast DNA show extremely high levels of expression (MCBRIDE et al. 1995, STAUB et al. 2000, DECOSA et al. 2001, RUF et al. 2001).

Although mitochondrial transformation has been reported for single-celled *Chlamydomonas* (RANDOLPH-ANDERSON et al. 1993) and yeast (BUTOW et al. 1996), there is no routine method to transform the higher-plant mitochondrial genome. The main hurdles to overcome are the introduction of foreign DNA into

the mitochondrion, incorporation of the transgene into the mitochondrial DNA, the absence of selectable mitochondrial markers, and the relatively large numbers of mitochondria per cell and mitochondrial genomes per mitochondrion. In addition, RNA editing (MAIER et al. 1996) may render ineffective foreign genes introduced into the mitochondrial genome.

Cucumber as a model system for mitochondrial transformation

Cucumber possesses three unique attributes that may allow the development of a mitochondrial transformation system for higher plants: the occurrence of huge mitochondria in microspores (ABREU et al. 1982), paternal transmission of mitochondria (HAVEY 1997), and the existence of rearrangements and/or deletions in the mitochondrial genome that condition severe mosaic (*msc*) phenotypes (MALEPSZY et al. 1996, LILLY et al. 2001). Cucumber microspores possess relatively few, huge mitochondria (ABREU et al. 1982). At the end of meiosis, the mitochondria in cucumber microspores are dumb-bell to 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 mononucleated microspores and may result from organelle elimination or fusion. After the first mitotic division that produces binucleated pollen grains, the mitochondria divide and resume normal shape, size, and numbers (ABREU et al. 1982). The reason for this unique mitochondrial change is not understood. The formation of relatively few mitochondria of huge size during microsporogenesis may create a bottleneck, reducing the diversity among mitochondrial genomes transferred to the progeny.

A second unique attribute of cucumber is paternal transmission of the mitochondrial genome (HAVEY 1997). This unique mode of mitochondria transmission, together with the formation of relatively few mitochondria of huge size during microsporogenesis, provide a unique opportunity to transform the plant mitochondrial genome. Introduction and incorporation of foreign DNA into the mitochondrial genome of cucumber microspores would allow for the delivery of transformed mitochondria via the male gametophyte to the zygote and progenies. Numerous researchers have reported successful pollen transformation using biolistics (HAY et al. 1994, JARDINAUD et al. 1995, LEEDE-PLEGT et al. 1995, NISHIHARA et al. 1995, STOGER et al. 1995, HORIKAWA et al. 1997), electroporation (MATTHEWS et al. 1990, JARDINAUD et al. 1993, SMITH et al. 1994, OBERMEYER, WEISENSEEL 1995), and co-cultivation with *Agrobacterium* (HESS, DRESSLER 1989). Many of these studies generated haploid plants from transformed pollen; few used the pollen for direct crossing. TOURAEV et al. (1995, 1997) pointed out that often these approaches are not repeatable. The group of Dr. E. Herbele-Bors, University of Vienna, Austria, demonstrated that in vitro maturation of microspores to pollen is possible for dicot tobacco (BENITO-MORENO et al. 1988) and monocot wheat (STAUFFER et al. 1991). This group also developed

a reproducible method for male-gametophyte transformation and demonstrated that biolistic transformation of the nuclear genome of microspores must occur at the single nucleus stage, before the first mitotic division (TOURAEV et al. 1995, 1997). This is precisely the stage when cucumber microspores possess relatively few, huge mitochondria (ABREU et al. 1982). Subsequently, the microspores are matured in vitro and pollen used for crosses (BENITO-MORENO et al. 1988, TUPY et al. 1991, STAUFFER et al. 1991).

MALEPSZY et al. (1996) identified unique mosaic (*msc*) phenotypes among cucumber plants regenerated from cell cultures. All crosses, backcrosses, and self pollinations of wild-type by *msc* plants showed paternal transmission; imprinting of paternal nuclear genes has been eliminated as a possibility (MALEPSZY et al. 1996, LILLY et al. 2001). Plants with *msc* phenotypes were recovered from independent cell-culture experiments using the same highly inbred parental line (MALEPSZY et al. 1996, LILLY et al. 2001); in all cases, cell cultures were started from independent plants from a highly inbred (>S₁₁) line "B" derived from the Polish cultivar 'Borszczagowski'. This inbred line was developed over many years as Polish researchers worked on cell-culture systems for cucumber and was chosen because it showed the best and most uniform regeneration (BURZA, MALEPSZY 1995). Plants showing the *msc* phenotype were recovered from independent plants from line B passed through different culture conditions (MALEPSZY et al. 1996, LILLY et al. 2001). This indicates that the mutations or lesions conditioning the *msc* phenotypes may exist heteroplasmically in inbred line B or that passage through cell culture may induce mutations or be conducive to recombination among direct repeats to produce deletions in the cucumber mitochondrial genome. Cell-culture systems may allow the *msc* phenotype to sort by reducing the negative effects of the *msc* mutation, as previously observed in maize (GU et al. 1994) and *Brassica* (SHIRZADEGAN et al. 1989).

The *msc* phenotype is similar to other mitochondrially encoded mutations affecting leaf shape and chloroplast development, such as *ncs* (NEWTON, COE 1986), *chm* of *Arabidopsis* (MARTINEZ-ZAPATER et al. 1992, SAKAMOTO et al. 1996), and plastome mutator of *Oenothera* (EPP 1973, REDEI 1973, CHANG et al. 1996). For these mitochondrial mutations, rearrangements in the mitochondrial DNA produce deletions or chimeric genes that have been closely associated with the mosaic or striping phenotypes. We studied independently arising *msc* lines of cucumber and demonstrated that all share a major deletion in the mitochondrial genome (LILLY et al. 2001). Analyses of relatively rare wild-type sorters demonstrated that this deletion sorts with the *msc* phenotype. Although there were no open-reading frames in the deleted region, the genetic bases of the *msc* phenotype in *msc16* appears to be associated with rearranged mitochondrial coding regions associated with this deletion (BARTOSZEWSKI, HAVEY, unpublished).

There exist potential selectable markers for mitochondrial transformation in higher plants. Antimycin A (AA) and myxothiazol are inhibitors of electron transfer in the cytochrome pathway in mitochondria of animals, fungi, and plants

(SCHNAUFER et al. 2000). Susceptibilities to AA and myxothiazol are associated with highly conserved amino acids in the cytochrome B component of the mitochondrial respiratory complex III (JAGOW, LINK 1986). ORTEGA et al. (2000) demonstrated that tobacco protoplasts or suspension cultures were sensitive to AA and myxothiazol. Comparisons of the *cob* coding regions among tobacco, *Chlamydomonas*, yeast, and mouse revealed that tobacco possesses glycine (position 43) and phenylalanine (position 135) residues at positions consistent with susceptibility to AA and myxothiazol in other organisms (ORTEGA et al. 2000). Alternation by site-directed mutagenesis of glycine (43) to valine or phenylalanine (135) to leucine in tobacco should confer resistance to AA and myxothiazol, respectively (ORTEGA et al. 2000). Cucumber *cob* possesses amino acid sequences identical to tobacco across these regions (LILLY, HAVEY unpublished). Unfortunately, there is no technique to introduce into the mitochondrial genome and select for these engineered *cob* coding region(s). After bombarding the engineered *cob* region into cell cultures, microspores, or plants, it is presently not possible to distinguish between non-incorporation of the transgene and ineffectiveness of the incorporated gene.

The main challenge to mitochondrial transformation remains the identification of an acceptable selectable marker. If cucumber microspores were bombarded with a transformation cassette carrying antibiotic resistance and no antibiotic-resistant pollen or progenies were observed, one does not know if the selectable marker did not enter the mitochondria, did not incorporate into the mitochondrial DNA, was not expressed, or was rendered ineffective by post-transcriptional or post-translational events. Cucumber may be a potential model system to develop or identify a selectable marker for mitochondrial transformation of higher plants. Passage of line B through cell culture and regeneration of plants has produced *msc* plants with independent rearrangements and/or deletions in the mitochondrial genome (MALEPSZY et al. 1996, LILLY et al. 2001). If we could identify a *msc* line with a deletion or rearrangement affecting the expression of *cob* [referred to as *msc(cob)*], this line could be used to establish whether an engineered *cob* region confers resistance to AA or myxothiazol. To do this, cucumber wild type *cob* would be altered by site-directed mutagenesis to change glycine at position 43 to valine or phenylalanine at position 135 to leucine (ORTEGA et al. 2000). The cucumber nuclear genome would be transformed, using established techniques (TRULSON et al. 1986, NISHIBAYASHI et al. 1996, SZWACKA et al. 1996, Tabei et al. 1998), with this engineered *cob* fused to a mitochondrial targeting sequence. This engineered *cob* would be nuclearly transcribed, cytoplasmically translated, and the modified *cob* protein imported into the mitochondria, as previously demonstrated for the mitochondrial protein *atp9* (HERNOULD et al. 1993). When cucumber carrying the nuclear engineered *cob* is crossed as the female with an *msc(cob)* plant, wild-type plants would be expected from complementation of the *msc(cob)* with the nuclear-encoded, cytoplasmically translated, and imported engineered *cob* protein. These hybrid

wild-type plants could be grown in the presence of AA or myxothiazol to establish whether the engineered cob is effective in conditioning resistance to these metabolic poisons. This is similar to the procedure used by SVAB et al. (1990a) to demonstrate that introduction of the bacterial *aadA* gene into the tobacco nuclear genome conditioned resistance to spectinomycin. Later, the *aadA* gene was successfully used as the selectable marker for chloroplast transformation in tobacco (SVAB et al. 1990b, SVAB, MALIGA 1993).

Once a selectable marker for mitochondrial transformation has been identified, one could employ biolistic transformation of the huge mitochondria of cucumber microspores (TOURAEV et al. 1997) with a transformation cassette carrying the selectable marker, the gene of interest, and flanking mitochondrial regions. The flanking mitochondrial regions would allow site-specific recombination in the mitochondrial genome of cucumber microspores, as demonstrated for plastid transformation (ZOUBENKO et al. 1994). These microspores would be matured to pollen (BENITO-MORENO et al. 1988, ALWEN et al. 1990) and used to pollinate wild-type cucumber. Progenies resistant to the selectable agent could be analysed by PCR and Southern hybridizations to establish that the recombinant DNA molecule has been introduced into the mitochondrial genome.

Once selectable markers for mitochondrial transformation become available, the technique could then be applied to higher plants showing maternal transmission of mitochondria. Methods of transgene incorporation into the mitochondrial genome could include particle bombardment of cell cultures (CARRER et al. 1993, SVAB, MALIGA 1993, ZOUBENKO et al. 1994, SKIDAR et al. 1998), PEG treatments of protoplasts (O'NEILL et al. 1993, KOOP et al. 1996), or microinjection of modified mitochondria to plant cells (VREHOEVEN, BLAAS 1992), followed by treatment with the selective agent.

Advantages of organellar DNA transformation

A mitochondrial-transformation system for a higher plant would allow geneticists to introduce and study genetic changes into this important genome, applicable both to basic research on the efficacy of engineered mitochondrial genes as well as to practical research on genetic improvement of the mitochondrial genome. One major advantage of organellar DNA transformation is transgene sequestering (DANIELL et al. 1998). Transgenes in the chloroplast and mitochondrial genome would greatly reduce the probability of transgene escape via pollen to non-transgenic populations. However, occasional biparental transmission of both organellar genomes has been well documented (MOGENSEN 1988) and extremely low levels of paternal organellar transfer must be expected. A second advantage of organellar transformation is the production of huge amounts of product (MCBRIDE et al. 1995, STAUB et al. 2000, DECOSA et al. 2001, RUF et al. 2001). Finally, transformation of the mitochondrial genome will allow for the efficient

production of CMS lines for hybrid seed production. Breeders of hybrid crops, especially those with longer generation times, would greatly benefit from a technique to routinely transform the mitochondrial genome. Once an elite male-fertile inbred line is identified, male-sterility-inducing factor(s) could be introduced into the mitochondrial genome of the male-fertile inbred, converting it to a male-sterile line of the same nuclear genotype. The male-fertile inbred would then become the maintainer line (JONES, DAVIS 1944) for seed propagation of the male-sterile inbred, allowing relatively rapid seed increases to production levels. This breeding scheme avoids the generations of laborious backcrossing presently required to develop male-sterile lines.

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