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Review article

The selection of mosaic (MSC) phenotype after passage of cucumber (*Cucumis sativus* L.) through cell culture – a method to obtain plant mitochondrial mutants

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Abstract. Mosaic (MSC) mutants of cucumber (*Cucumis sativus* L.) appear after passage through cell cultures. The MSC phenotype shows paternal transmission and is associated with mitochondrial DNA rearrangements. This review describes the origins and phenotypes of independently produced MSC mutants of cucumber, including current knowledge on their mitochondrial DNA rearrangements, and similarities of MSC with other plant mitochondrial mutants. Finally we propose that passage of cucumber through cell culture can be used as a unique and efficient method to generate mitochondrial mutants of a higher plant in a highly homozygous nuclear background.

Keywords: *Cucumis sativus*, mitochondrial mutants, mutagenesis, plant mitochondria, somaclonal variation, tissue culture.

Introduction

Plants possess three major genomes carried in the nucleus, mitochondrion, and plastids. Plant mitochondrial genomes are much larger than those in animals and fungi. For example, melon and cucumber have the largest known mitochondrial genomes at 2 400 and 1 500 kb, respectively (Ward et al. 1981), similar in size to the entire genome of many prokaryotes. These huge mitochondrial genomes in plants are due in part to the transfer of DNA from the chloroplast or nucleus, and the accumulation of short repetitive DNAs (Unseld et al. 1997; Kubo et al. 2000; Lilly and Havey 2001; Notsu et al. 2002; Clifton et al. 2004; Ogihara et al. 2005). Recombination among these repetitive sequences produces rearrangements in the mitochondrial DNAs, shifting the linear order of genes even among relatively closely related species (Palmer and Hebron 1988; Fauron et al. 1995; Bartoszewski et al. 2004a).

Plants regenerated from *in vitro* cultures may show new phenotypes; a phenomenon referred to as somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation may have been pre-existing or arose during cell culture and may be either genetic (Evans and Sharp 1983; Karp 1991) or epigenetic (Kaeppler and Phillips 1993; Kaeppler et al. 2000). Genetic variability triggered by tissue culture may result from changes in the nuclear and/or organellar (plastid and mitochondrial) genomes. Observed changes include polyploidy, aneuploidy, deletions, inversions and translocations, activation of transposable elements, and single base changes. The chloroplast genome does not appear to be significantly af-

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fected by *in vitro* culture with the exception of albino plants derived from anther cultures (Day and Ellis 1985; Harada et al. 1991). Somaclonal variation is recognized as an important source of mutations for different qualitative and quantitative traits and is used in plant breeding (Karp 1991; Skirvin et al. 1994). Not all cell cultures produce somaclonal variants, for example shoot primordia cultures are considered to be free of somaclonal variation (Karp 1991).

Mitochondrial mutations can produce visible phenotypes, such as cytoplasmic male sterility (CMS), non-chromosomal stripe (NCS), mosaicism, etc. (reviewed by Newton et al. 2004). Mutations in the mitochondrial DNA also result in no visible phenotypes, for example the U mitochondrial DNA organization of *Nicotiana sylvestris* regenerated from protoplasts (Vitart et al. 1992). Most of the reported mitochondrial mutations are rearrangements or deletions (reviewed by Newton et al. 2004); however, single point mutations have also been reported (Ducos et al. 2001).

Plant cell cultures have been proposed as a method of producing mitochondrial mutants (Hauschner et al. 1998; Hartmann et al. 2000). The latter authors showed that changes in the mitochondrial genome may be influenced by nuclear genes, and suggested an effect of complex properties of a culture, such as medium composition and culture conditions, on their production. An example of a tissue culture that produced mitochondrial mutants included CMS mutants of *Nicotiana sylvestris* obtained after protoplast regeneration (Li et al. 1988; Chétrit et al. 1992).

In cucumber, passage of the highly inbred line B through cell cultures produces regenerated plants with a strongly mosaic (MSC) phenotype typified by chlorotic spots on leaves (Malepszy et al. 1996). This phenotype first appears as mosaic sectors on individual leaves of some R_1 plants and very rarely on R_0 plants. After self-pollination and selection of plants with mosaic sectors, stable MSC lines were produced (Malepszy et al. 1996).

Species in genus *Cucumis* show a differential transmission of the three plant genomes, maternal for the chloroplast, paternal for the mitochondrial, and biparental for the nuclear DNA (Havey 1997; Havey et al. 1998). The MSC phenotype shows paternal inheritance (Malepszy et al. 1996), indicating that MSC is conditioned by the mitochondrial DNA. Detailed studies of MSC lines showed that they possess complex rearrangements in the mitochondrial genome (Lilly et al. 2001; Bartoszewski et al. 2004b).

In this review we describe a method for the production of mitochondrial mutants in cucumber using *in vitro* cultures and assess their phenotypic similarities and differences.

MSC phenotype

All MSC lines described in literature were derived from cell cultures of one, highly inbred ($>S_{10}$) and homogeneous line of cucumber, obtained after self-pollination of single plants from the Polish cultivar 'Borszczagowski', referred to as line B (Kubicki, data unpublished). The MSC phenotype was not described for other cucumber cultivars. Somatic tissue of line B was passed through *in vitro* cultures and plants were regenerated from the leaf callus (Malepszy and Nadolska-Orczyk 1989; Pląder et al. 1998), the leaf callus with salt-tolerance selection (Baszczyk-Guzek and Szwacka 1994), leaf microexplants (Burza and Malepszy 1995a), cell suspension protoplasts (Burza and Malepszy 1995b), cytokinin dependent cell suspension (Burza and Malepszy 1998), or shoot primordia culture (Ladyżyński et al. 2002). Each independently regenerated plant (R_0) was self-pollinated in a greenhouse. The MSC mutants were first identified on the basis of mosaic sectors on the R_1 plants. For example, Malepszy and Nadolska-Orczyk (1989) found that three S_1 families from 60 R_0 plants (5%) possessed plants with the mosaic phenotype. The mosaic phenotype most often appeared on several leaves and 2–3 such plants usually occurred among 30 to 60 R_1 progenies. These mosaic plants were self-pollinated and progenies with the strongest mosaic phenotypes were selected in the next generation (Figure 1). This procedure was repeated until a phenotypically stable MSC line was obtained, usually in the R_1, R_2 or R_3 generation. In case of line MSC3, the regenerated R_0 plant possessed leaves with mosaic sectors and all R_1 plants were homogeneously mosaic (Ladyżyński et al. 2002). The homogeneity of each line was considered as stable after at least two self pollinations and the evaluation of at least 90 progenies. Homogenous lines were test-crossed as the male to wild-type plants to establish the transmission of the mosaic phenotypes. All lines listed in Table 1 showed paternal inheritance of the mosaic phenotype (Table 2; Malepszy et al. 1996). Detailed genetic studies performed for MSC16 and MSC19 eliminated paternal imprinting or nuclear recessive allele as the genetic basis for the MSC phenotype (Malepszy et al. 1996; Lilly et al. 2001).

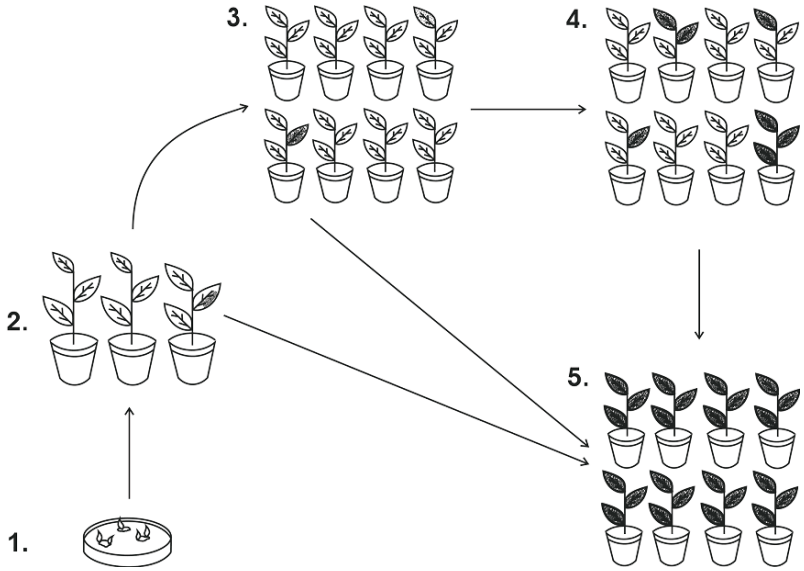

Figure 1. Scheme showing production and selection of cucumber lines from inbred line B possessing the mosaic (MSC) phenotype. 1. Identification of independent cell culture regenerants (R_0) from inbred line B. 2. Growth of R_0 plants in the greenhouse. All R_0 plants are self-pollinated. Some R_0 plants may display mosaic sectors. 3. R_1 families are grown in the field. Identification and selection of plants with mosaic sectors and self-pollination. 4. Selected R_2 families are grown in the field. Plants with the mosaic sectors are selected and self-pollinated. 5. Stable and homogenous mosaic lines are identified (R_3 generation). Sometimes R_1 or R_2 progenies are stable and homogenous.

Table 1. Characteristics of morphological features among independently generated mosaic mutant lines

Line	Means ± SD for					
	Main shoot length [cm]		Number of lateral shoots		Third leaf length [cm]	Third leaf width [cm]
	2 w	4 w	2 w	4 w	4 w	4 w
B	49.4 ± 6.0	109.6 ± 9.6	9.8 ± 1.0	12.0 ± 1.1	11.3 ± 0.9	12.0 ± 1.2
BTC	42.9 ± 4.9	98.3 ± 6.0	8.7 ± 1.0	11.0 ± 1.2	9.6 ± 0.7	9.4 ± 1.3
MSC3	44.6 ± 6.0	89.4 ± 13.0	7.8 ± 2.1	10.6 ± 2.6	9.2 ± 0.9	10.5 ± 1.2
MSC12	28.0 ± 2.3	n.d.	5.6 ± 0.9	7.8 ± 1.6	6.5 ± 0.6	7.3 ± 1.0
MSC16	22.1 ± 2.4	51.9 ± 5.6	4.9 ± 0.9	6.8 ± 0.4	7.7 ± 1.0	9.0 ± 0.8
MSC19	24.1 ± 1.9	50.4 ± 4.2	6.2 ± 0.7	7.2 ± 0.6	8.3 ± 0.8	8.1 ± 0.9
MSC22	27.2 ± 5.9	56.3 ± 5.7	5.8 ± 1.3	7.5 ± 0.8	7.4 ± 1.3	7.8 ± 1.2
MSC23	61.7 ± 5.0	122.6 ± 8.1	10.0 ± 1.0	11.5 ± 1.5	9.8 ± 0.7	11.5 ± 0.8
MSC28	63.5 ± 6.7	124.6 ± 8.7	10.7 ± 0.5	11.7 ± 1.1	10.2 ± 0.5	11.2 ± 1.4

B – wild-type line B, BTC – wild-type line without mosaic regenerated from tissue culture of line B,
w – weeks after field planting, nd – not determined, SD – standard deviation

Table 2. Inheritance of the MSC phenotype of different mutants in test cross with the wild type line. Lines B and 2gg are wild type inbred lines and were used as a female parent in all test crosses. Mutant lines were a male parent in all test crosses.

Test cross	Number of plants with phenotype:	
	WT	MSC
2gg × MSC3	0	60
2gg × MSC12	0	66
2gg × MSC19	3	130
B × MSC22	2	76
2gg × MSC23	0	60
2gg × MSC27	0	60
B × MSC28	1	59

Morphological characteristics of MSC lines

Cotyledons of MSC plants are smaller, misshapen, and exhibit a sectoring of green and chlorotic tissues. True leaves are misshapen and chlorotic, and the flowers are reduced in size and misshapen as compared to the wild type (Malepszy et al. 1996). MSC plants possess reduced numbers of thylakoid membranes in chloroplasts, similar to the non-chromosomal stripe (NCS) mutants of maize (Roussell et al. 1991). MSC lines generated from independent cell culture experiments are not identical and differ in growth rates and mosaic intensities (Table 1, Figure 2 and 3). We measured

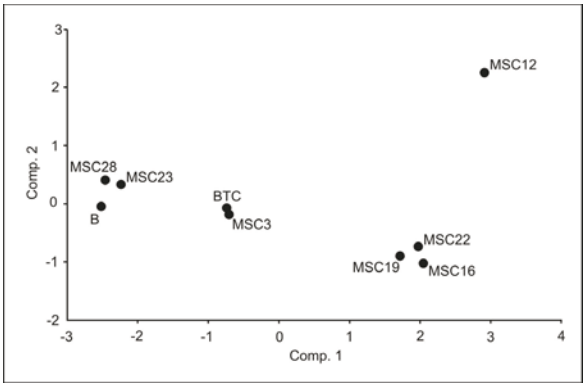

Figure 2. Comparison of the MSC lines based on morphological traits. The analysis divided MSC lines into four groups. Composite variables (Comp.1 and Comp. 2) were created using principal component analysis (PCA). Variables used to build principal components and % of explained variability are listed in table 3. B – wild-type line B, BTC – wild-type line without mosaic regenerated from tissue culture of line B.

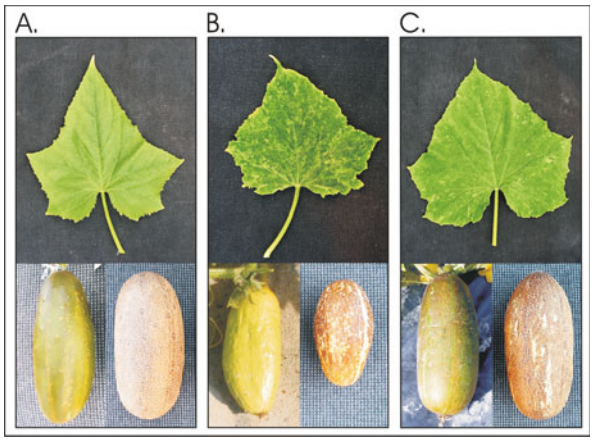

Figure 3. Phenotypes of independently generated MSC lines. A. inbred line B (wild type), B. MSC12 line shows mosaic on leaves, yellow immature fruit and mature fruit, C. MSC3 line shows less severe mosaic on leaves and fruit.

growth parameters, such as the main shoot length, third leaf width and length, and the number of lateral shoots, for seven independently generated MSC lines (Table 1) and were able to distinguish at least four groups of similarly performing lines (Table 3 and Figure 2). Two MSC lines (MSC23 and MSC28) had growth rates similar to that of control line B. The second group included line MSC3; the third group MSC16, MSC19 and MSC22; and the last group included only MSC12 (Figure 2). Differences in the degree of mosaic on leaves can be distinguished. For lines MSC9, MSC12, MSC16, MSC19, and MSC22, the combination of a large number of small yellow spots and large irregular silvery spots on leaves

Table 3. Results of principal component analysis (PCA). As two first principal components explained above 95 % of the total variability, they were used for line grouping. The analysis divided MSC lines into four groups (Figure 2).

Principal component designation	Principal component value	% of explained variability	Cumulated % of explained variability
1	4.733	78.880	78.880
2	0.970	16.172	95.052
3	0.154	2.561	97.613
4	0.108	1.792	99.405
5	0.040	0.594	99.999
6	0.00	0.001	100.000

was typical of the mosaic phenotype. Line MSC3 had a similar spotting pattern, but spots were less severe (Figure 3). Immature fruit of MSC12 showed no mosaic and were yellow in color (Figure 3B). Line MSC27 did not show any mosaic on the leaves, but had clearly visible mosaic on mature fruit. All these mosaic phenotypes were paternally transmitted (Table 2; Malepszy et al. 1996).

Mitochondrial DNA rearrangements associated with MSC phenotype

Passage of plants through cell cultures is known to reveal rearrangements in the mitochondrial DNA (Hanson and Conde 1985; Hartmann et al. 1987; Shirzadegan et al. 1989; Dörfel et al. 1989; Vitart et al. 1992; Kanazawa et al. 1994; Hauschner et al. 1998), which also appear as mutant phenotypes in regenerated plants (De Paepe et al. 1990; Martinez-Zapater 1992; Sakamoto et al. 1996; Pupilli et al. 2001). Occasionally, the polymorphic mitochondrial DNAs are the result of the amplification of substoichiometric molecules already present in the mitochondria before passage through cell cultures or are the result of *de novo* rearrangements during culture after recombination among homologous sequences. Mitochondrial DNA rearrangements in plants usually consist of deletions of coding regions (Lelandais et al. 1998) or complex duplications and/or deletions of non-coding mitochondrial DNA regions (Lilly et al. 2001; Bartoszewski et al. 2004b).

A large deletion (JLV5-DEL) was identified, spanning a putatively non-coding region in the mitochondrial genomes of MSC lines 11, 16, and 19 (Lilly et al. 2001). JLV5 deletion was paternally transmitted and sorted with the MSC phenotype.

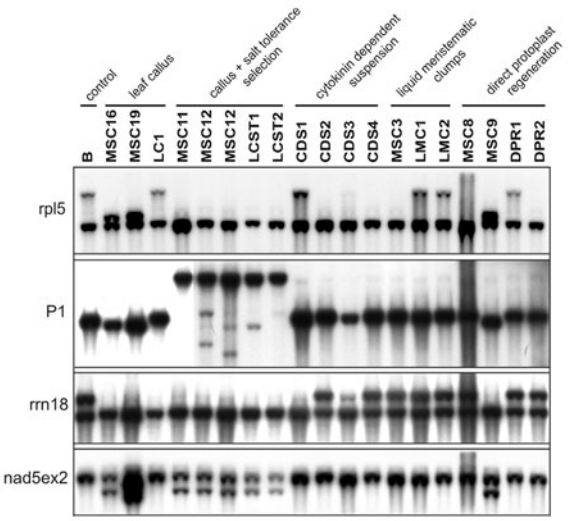

Figure 4. Autoradiogram showing RFLP polymorphisms in the mitochondrial genome of lines obtained in different cell culture regeneration experiments. Four probes were used (*rpl5*, P1, *rrn18*, and *nad5*ex2). Lines MSC9, 16, and 19 possess the same rearrangement pattern. All lines from callus with salt tolerance selection have the same rearrangement pattern (relatively few plants were regenerated from two callus lines obtained in this experiment and lanes on the gel are from closely related progenies). Other rearrangement patterns were detected that were not associated with the MSC phenotype, for example in cytokinin dependent cell suspension lines CDS2, 3, and 4, the pseudo-*rpl5* band is missing (Lilly et al. 2001; Bartoszewski et al. 2004b).

The mitochondrial genome of cucumber shows paternal transmission and there are no reports of variation for mitochondrial DNA transmission in cucumber, such as an occasional maternal transmission (Havey et al. 2004). The mitochondrial DNA of MSC lines was heterogeneous and approximately one wild-type genome was found for every 500 genomes carrying the JLV5 deletion as detected by PCR (Lilly et al. 2001) and wild-type progeny scoring (Bartoszewski et al. 2004b). Sequence analyses revealed that the JLV5 deletion was not a single event and was part of a complex rearrangement involving mitochondrial regions carrying *rpl5* and JLV5 (Bartoszewski et al. 2004b). Other rearrangements not associated with the MSC phenotype were found in cell-culture-derived lines. These rearrangements include deletion of a region carrying *rrn5-rrn18* genes, duplication of the region carrying exon 2 of the *nad5* gene, and deletion of a pseudo-*rpl5* gene (Bartoszewski et al. 2004b). Seventeen MSC and wild-type lines, all regenerated from independent cell-culture experiments, were evaluated for mitochondrial polymorphisms and different patterns were revealed. Lines MSC9, MSC16, and MSC19 showed the same rearrangements (Figure 4). Line MSC3 did not possess the pseudo-*rpl5* region and may carry additional rearrangements not found in MSC16.

Possible origins of the cell culture-induced mitochondrial rearrangements

Nuclear control over reorganization of the wheat mitochondrial genome in cell cultures has been demonstrated (Hartmann et al. 2000). For MSC lines there is no evidence that mitochondrial genome reorganization is under the control of nuclear gene(s). Analysis of MSC lines obtained from independent cell culture experiments indicated that the MSC phenotype does not trace back to a single mitochondrial sublimon existing in the parental B line and numerous rearranged mitochondrial genomes may exist at the substoichiometric level. Because similar rearrangements were recovered from independent cell cultures (e.g. MSC9, MSC16 and MSC19 in Figure 4), sorting from a relatively large number of sublimons, rather than *de novo* production of unique rearrangements, is consistent with the rearrangement patterns observed. The cucumber mitochondrial genome is rich in short repetitive DNAs (Lilly and Havey 2001) and recombination among these repeats may have produced a large number of sublimons in cucumber mitochondria. Stresses due to cell cultures and regeneration of plants may expose pre-existing variants in the cucumber mitochondrial genome, which can be fixed and stably maintained in sexual progenies. Passage of line MSC16 through repeated cell culture did not revert to the wild-type, indicating that the mosaic phenotype and mitochondrial rearrangements were not selected against under these culture conditions (Ziółkowska et al. 2005).

Epigenetic modification of nuclear genes which have been induced during *in vitro* culture cannot be excluded and could be involved in the MSC phenotype formation. The fact that a local phenotype (one or two leaves) could spread all over the plant suggests systemic information – for example small RNA that can manage DNA methylation could be involved in this mechanism. However, grafting experiments with MSC and wild-type line B did not provide any evidence that the MSC phenotype could be spread systemically .

The method for production of mitochondrial mutants in cucumber

The availability of mutants is imperative for studying gene functions. For traits encoded by the mitochondrial DNA, the production of mutants is difficult due to the importance of respiration for plant survival and uniparental transmission. Recently the P2 mutator system in maize was proposed as a method to generate variation in the mitochondrial DNA among siblings and between parents and progeny (Kuzmin et al. 2005). We propose to consider that cucumber cell culture is a more efficient and simpler method for the production of mitochondrial mutants in a highly inbred nuclear background. Highly inbred genotypes of cucumber, such as inbred line B or a doubled haploid population, can be passed through cell culture and plants regenerated. Visual screening of R_0 plants or their S_1 progenies will reveal plants with the MSC phenotype on the cotyledons or leaves. Phenotypically stable lines can be produced after two to three generations of self-pollinations, maintaining the highly homozygous nuclear background (Figure 1). Specific types of cell cultures, such as shoot primordia cultures, can be exploited to efficiently produce high frequencies of plants with the MSC phenotype (Ladyżyński et al. 2002).

Cucumber MSC mutants were produced in each of the evaluated *in vitro* culture types, indicating that the changes in the mitochondrial genome in cucumbers are common after regeneration. It is also possible that passage through cell cultures may be an efficient method to produce mitochondrial rearrangements in other species; mosaic mutants have been reported in both dicots (Buiatti et al. 1985; Barwale and Widholm 1987; Martinez-Zapater et al. 1992) and monocots (Oono 1978). These mutants were produced using various culture types, which supports our observations that the changes in the mitochondrial genome are common among *in vitro* regenerated plants. However, the association of the mosaic phenotypes and mitochondrial rearrangements is more difficult because of maternal transmission of both the chloroplast and mitochondrial genomes.

To date, we have produced 10 MSC lines characterised by unique phenotypes (Malepszy et al. 1996; Figure 3) or mitochondrial DNA profiles (Figure 4). Unique MSC phenotypes include the presence of mosaic on only one organ, for example the fruit (MSC27), or strong mosaic on the cotyledons and weak on leaves (MSC3). Presumably these different phenotypes may be conditioned by independent mutations or rearrangements affecting different mitochondrial genes.

The type of cell culture can affect the occurrence of plants with mosaic (R_0 or R_1) or the frequency of partially mosaic plants in the R_1 generation. The lowest frequency was recorded after regeneration from the leaf callus (Malepszy and Nadolska-Orczyk 1989), and the highest after leaf direct protoplast regeneration (Burza and Malepszy 1995b). Specific culture types may produce between 1 and 5% of plants with the mosaic phenotype as early as the R_0 generation. Therefore, the use of specific culture types may significantly accelerate the production of mitochondrial mutants.

It is presently difficult to characterize mitochondrial rearrangements or mutations associated with the MSC phenotype because of a lack of information about the structure of the cucumber mitochondrial genome. We have characterized some mitochondrial rearrangements (Lilly et al. 2001; Bartoszewski et al. 2004b), but other important rearrangements could remain undetected and the genetic basis of the MSC phenotype remains unknown. The development of a physical map of the cucumber mitochondrial genome and a mitochondrial gene chip would enable faster screening for mitochondrial polymorphisms among MSC lines. These resources would allow us to establish the genetic basis of the MSC phenotypes and assess the potential of the MSC system to produce a complete set of mitochondrial mutants.

Concluding remarks

The mitochondrial genome of cucumber is unique for its large size (Ward et al. 1981), paternal transmission (Havey 1997; Havey et al. 1998), and the production of mosaic phenotypes after *in vitro* culture (Malepszy et al. 1996) associated with mitochondrial DNA rearrangements (Lilly et al. 2001; Bartoszewski et al. 2004b). The paternal transmission of cucumber mtDNA makes it easy to distinguish phenotypes conditioned by the chloroplast or mitochondrial DNAs. A relatively large number of MSC lines can be produced after regeneration from cell cultures. Such lines can then be evaluated for mitochondrial DNA rearrangements, characterised in detail, and used to establish a collection of mitochondrial mutants. This collection would be extremely useful for genomic analyses of the interactions among the chloroplast, mito-

chondrial, and nuclear genomes. For example, mitochondrial mutants affect chloroplast organization and photosynthesis in *Arabidopsis* (Martinez-Zapater et al. 1992; Sakamoto et al. 1996), maize (Newton et al. 1990; Hunt and Newton 1991; Marienfeld and Newton 1994), and cucumber (Malepszy et al. 1996). Different MSC mutants could be used to reveal specific changes in nuclear gene expression in a highly homozygous nuclear background. Finally, cucumber mitochondrial mutants could also be used to study genetic and physical bases of organellar transmision. Recently the MSC16 mutant was used to describe the cucumber *Psm* locus, which uniquely controls sorting of paternally transmitted mitochondria (Havey et al. 2004).

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