Curr Genet (2004) 45: 45–53
DOI 10.1007/s00294-003-0456-6

RESEARCH ARTICLE

Grzegorz Bartoszewski · Stefan Malepszy
Michael J. Havey

Mosaic (MSC) cucumbers regenerated from independent cell cultures possess different mitochondrial rearrangements

Received: 22 July 2003 / Revised: 23 September 2003 / Accepted: 29 September 2003 / Published online: 29 October 2003

Abstract Passage of the highly inbred cucumber (*Cucumis sativus* L.) line B through cell culture produces progenies with paternally transmitted, mosaic (MSC) phenotypes. Because the mitochondrial genome of cucumber shows paternal transmission, we evaluated for structural polymorphisms by hybridizing cosmids spanning the entire mitochondrial genome of *Arabidopsis thaliana* L. to DNA-gel blots of four independently generated MSC and four wild-type cucumbers. Polymorphisms were identified by cosmids carrying *rrn18*, *nad5-exon2*, *rpl5*, and the previously described JLV5 deletion. Polymorphisms revealed by *rrn18* and *nad5-exon2* were due to one rearrangement bringing together these two coding regions. The polymorphism revealed by *rpl5* was unique to MSC16 and was due to rearrangement(s) placing the *rpl5* region next to the forward junction of the JLV5 deletion. The rearrangement near *rpl5* existed as a sublimon in wild-type inbred B, but was not detected in the cultivar Calypso. Although RNA-gel blots revealed reduced transcription of *rpl5* in MSC16 relative to wild-type cucumber, Western analyses revealed no differences for the RPL5 protein and the genetic basis of the MSC16 phenotype remains enigmatic. We evaluated 17 MSC and wild-type lines regenerated from independent cell-culture experiments for these structural polymorphisms and identified eight different patterns, indicating that the passage of cucumber through cell culture may be a unique mechanism to induce or select for novel rearrangements affecting mitochondrial gene expression.

Keywords Paternal transmission · Mitochondrial genetics · Sublimon

Communicated by A. Brennicke

G. Bartoszewski · M. J. Havey (✉)
Vegetable Crops Unit, Department of Horticulture,
Agricultural Research Service,
U.S. Department of Agriculture,
University of Wisconsin,
1575 Linden Drive,
Madison, WI 53706, USA
E-mail: mjhavey@wisc.edu
Fax: +1-608-2624743

G. Bartoszewski · S. Malepszy
Department of Plant Genetics,
Breeding and Biotechnology,
Faculty of Horticulture and Landscape Architecture,
Warsaw Agricultural University,
ul. Nowoursynowska 166,
02-787 Warsaw, Poland

Introduction

Cucumber (*Cucumis sativus* L.) and melon (*C. melo* L.) are unique among all plants in that the three genomes show differential transmission: maternal for chloroplast, paternal for mitochondrial, and biparental for the nuclear DNA (Havey 1997; Havey et al. 1998); and they possess the largest mitochondrial genomes among all eukaryotes (Ward et al. 1981). Biochemical and molecular analyses established that the cucumber mitochondrial genome has accumulated a plethora of short repetitive DNAs spread throughout the genome (Ward et al. 1981; Lilly and Havey 2001). Plants of the genus *Cucumis* represent a unique genetic system among herbaceous dicotyledonous plants because mitochondrially encoded traits can be easily separated from traits conditioned by the chloroplast genome, by taking advantage of differential transmission of the organellar genomes (Havey et al. 1998). Examples of plant phenotypes conditioned by the mitochondrial genome include cytoplasmic male sterility in many species (Kaul 1988), the nonchromosome striped mutants of maize (Coe 1983; Newton and Coe 1986; Newton 1995), the mosaic (MSC) mutants of cucumber (Malepszy et al. 1996; Lilly et al. 2001), and *chm*-conditioned mitochondrial rearrangements of *Arabidopsis thaliana* (Martinez-Zapater et al. 1992; Sakamoto et al. 1996).

The highly inbred ($>S_{18}$) cucumber line B originated from the Polish cultivar Borszczagowski and was selected during inbreeding for uniform regeneration from various

cell-culture systems (Burza and Malepszy 1995a). After plants of the wild-type inbred line B are passed through cell cultures, regenerated (R0) plants predominately have a normal phenotype (Burza and Malepszy 1995b; Plader et al. 1998). However, after self-pollination of R0 plants, occasional first (R1) or second (R2) generation selfed progenies from wild-type R0 plants show paternally transmitted, strongly MSC phenotypes expressed in seedlings and adult plants (Malepszy et al. 1996; Ladyzynski et al. 2002). All crosses, backcrosses, and self-pollinations of the wild type by MSC plants showed paternal transmission (Malepszy et al. 1996) and imprinting of paternal nuclear alleles was eliminated (Lilly et al. 2001). We demonstrated that MSC plants possess a deletion in the mitochondrial genome, relative to the parental inbred line B (Lilly et al. 2001), and that testcrosses with MSC as the male parent to wild-type plants produce wild-type seedlings at or below 1% (Malepszy et al. 1996; Lilly et al. 2001). MSC plants are heteroplasmic for both mutant and wild-type mitochondrial genomes, with the former predominating; and wild-type progenies result from sorting of the mitochondrial genome in MSC pollen (Lilly et al. 2001). The passage of cucumber through cell culture may allow mitochondria carrying the deleterious MSC lesion(s) to sort by their reducing negative effects, as previously observed in maize (Gu et al. 1994). Another explanation is that passage through cell culture may induce mutations or be conducive to recombination among direct repeats in the mitochondrial DNA to produce structural rearrangements or deletions affecting the expression of mitochondrial genes. If this latter scenario were true, the passage of cucumber through cell culture may offer a unique opportunity to affect mitochondrial gene expression. In this study, we evaluated for mitochondrial-DNA rearrangements affecting gene expression in MSC and wild-type lines and demonstrated that independently derived MSC lines possess different combinations of mitochondrial polymorphisms.

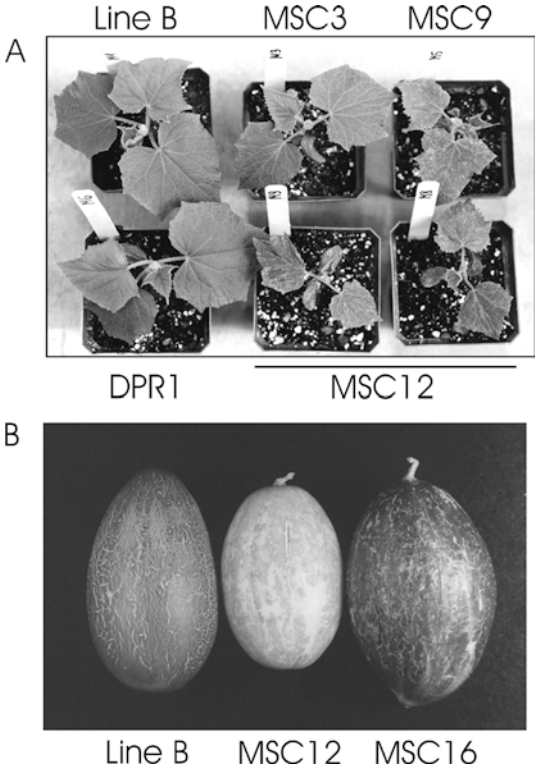

Fig. 1 Examples of phenotypic differences for leaves (**A**) and fruit (**B**) among mosaic (*MSC*) and wild-type (*DPR1*) cucumber lines regenerated from cell cultures started from the highly inbred line B. The origin of each line is listed in Table 1

Experimental procedures

Origin of MSC lines

The origins of wild-type inbred line B, the cultivar Calypso, and MSC lines 11, 16, and 19 have been described by Lilly et al. (2001). Additional MSC and wild-type lines (Fig. 1, Table 1) were produced after self-pollination of plants regenerated from independent cell-culture experiments (Malepszy et al. 1996; Ladyzynski et al. 2002). Wild-type sorters from MSC16 or MSC sorters from wild-type lines were identified at the seedling stage and DNA was isolated using a minipreparation (Lilly et al. 2001).

Hybridization of mitochondrial *Arabidopsis* cosmids and cucumber clones

Total DNA was isolated from cucumber populations using standard CTAB extraction (Havey 1997). The DNAs of MSC3, MSC11, MSC16, MSC19, and wild-type lines B, LCST1, LCST2, and Calypso were digested with 12 different restriction endonucleases (*Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Sac*I, *Xba*I, *Alu*I, *Hae*III, *Cfo*I, *Msp*I, *Mbo*I, *Mse*I), subjected to electrophoresis though 0.8% (six-base cutters) or 2% (four-base cutters) agarose gels, and transferred to Nylon membranes (Zetaprobe GT; Bio-Rad, Hercules, Calif.). Fifteen cosmids covering the entire *A. thaliana* mitochondrial genome (Klein et al. 1994) were individually radiolabeled by nick translation (Invitrogen, Carlsbad, Calif.) and hybridized overnight to blots as described by Havey et al. (1998). Blots were washed once for 5 min in 2× SSC with 0.1% SDS at room temperature and twice for 10 min in 0.1× SSC with 0.1% SDS at 65 °C. Specific coding regions (*nad5-exon2*, *rpl5*, *rrn18*) on cosmids that revealed polymorphisms were amplified by PCR, using primers designed based on *A. thaliana* (Unseld et al. 1997) and used as probes for hybridizations. Cucumber primers amplifying from the P1 and JLV5 mitochondrial regions (Table 2; Lilly et al. 2001) were P1 (5′-AGG CTC GAC CCC ATT CTA CT-3′) and P2 (5′-TTA GCT GGA CCG ATT TAC GG-3′) and MF1 (5′-GCA GAA GCA ACC TTC ACC TCC GAG AGT TAG-3′), JLV5R (5′-TAA GAC TGC CCA CTA CGC TCC GTG CAA-3′), and rpl5R2 (5′-GCC CTC GGA GAG TGC TTT CTC GT-3′). Primers specific for mitochondrial *rrn18* were designed from the mitochondrial region V7 (Neefs et al. 1993). PCR products were gel-purified, cloned by TA-tailing (Invitrogen), sequenced to verify similarities, and used for hybridizations.

Synthesis of mitochondrial DNA libraries

We synthesized mitochondrial genomic libraries of wild-type inbred B and MSC16 from DNA extracted using DNAseI treatments and Percoll-gradient centrifugation (Klein et al. 1994; Lilly and Havey 2001). Cucumber mitochondrial DNA was partially digested with *Sau*3A, subjected to electrophoresis, and fragments of approximately 10–20 kb were excised from the gel. DNA was purified

Table 1 Origins of wild-type and mosaic (MSC) cucumber lines and polymorphisms detected in their mitochondrial genomes. Cell cultures were described by Ladyzynski et al. (2002): *LC* direct regeneration from leaf callus, *Salt* regeneration from leaf callus after selection for salt tolerance, *Cytokinin* cytokinin-dependent embryogenic suspension cultures, *Meristem* liquid meristematic culture (shoot primordia culture), *Protoplast* direct protoplast regeneration. Polymorphisms are shown in Figs. 1, 2. Fragments were wild type (*WT*), MSC16 (*MSC*), or both (*WT*/*MSC*). *New* Unique fragments not observed in either wild type or MSC16

Line	Culture	Phenotype	Polymorphism				
			rrn18	*nad5-exon2*	*rpl5*	Pseudo-*rpl5*	P1
B	None	WT	WT	WT	WT	WT	WT
Calypso	None	WT	WT	WT	WT	WT	WT
MSC16	LC	MSC	MSC	MSC	MSC	MSC	MSC
MSC19	LC	MSC	MSC	MSC	MSC	MSC	MSC
LC1	LC	WT	MSC	WT	WT	WT	WT
MSC11	Salt	MSC	MSC	MSC	WT	MSC	New
MSC12	Salt	MSC	MSC	MSC	WT	MSC	New
LCST1	Salt	WT	MSC	MSC	WT	MSC	New
LCST2	Salt	WT	MSC	MSC	WT	MSC	New
CDS1	Cytokinin	WT	MSC	WT	WT	WT	WT
CDS2	Cytokinin	WT	WT	WT	WT	MSC	WT
CDS3	Cytokinin	WT	WT/MSC	WT	WT	MSC	WT
CDS4	Cytokinin	WT	WT	WT	WT	MSC	WT
MSC3	Meristem	MSC	WT	WT	WT	MSC	WT
LMC1	Meristem	WT	WT	WT	WT	WT	WT
LMC2	Meristem	WT	WT	WT	WT	WT	WT
MSC9	Protoplast	MSC	MSC	MSC	MSC	MSC	MSC
DPR1	Protoplast	WT	WT	WT	WT	WT	WT
DPR2	Protoplast	WT	WT	WT	WT	MSC	WT

using the QiaEX gel extraction kit (Qiagen, Valencia, Calif.) and ligated into a dephosphorylated *Bam*HI-digested pUC18 vector, according to the manufacturer's directions (Amersham, Piscataway, N.J.). Standard electroporation into bacteria and blue-white selection were performed (Sambrook et al. 1989). The average insert size was 15 kb, requiring 1,375 clones for 3× coverage at 99% probability. Two wild-type libraries from Calypso (1,152 clones) and line B (3,072 clones) and one MSC16 library (3,072 clones) were created. Colonies from plates were spotted onto nylon membranes, allowed to grow overnight, and lyzed (Nizetic et al. 1991; Lilly and Havey 2001). Hybridization of the mitochondrial genomic or cDNA clones to these filters revealed genomic clones carrying homologous regions. These clones were aligned by end-sequencing and restriction-enzyme digests. Polymorphic regions were subcloned, sequenced, and compared with the wild-type region in inbred B to establish the genetic basis of rearrangements or deletions in MSC16. Sequencing was completed using random transposon insertions (EZ::Kn2 system; Epicentre, Madison, Wis.). Cycle sequencing reactions were performed (Lilly and Havey 2001) and analyzed on an automated DNA sequencer at the University of Wisconsin Biotechnology Center. Sequence data were edited and trimmed and contigs aligned with Sequencer 3.1.2 (Genecodes, Ann Arbor, Mich.). GeneBank accessions are listed in Table 2.

Mitochondrial *rpl5* transcription

Total RNA was isolated from cucumber leaf tissue (Lilly et al. 2001), 20 μg were subjected to electrophoresis on 1.2% formaldehyde-agarose gels (Sambrook et al. 1989), and transferred to Zetaprobe membranes. The membranes were then hybridized overnight at 65 °C in 1 mM EDTA, 0.5 M $NaHPO_4$, and 7% SDS, after which randomly primed, ^{32}P-labeled probes were added. The *rpl5* coding sequence and the untranslated 5′ region of *rpl5* (corresponding to the bases −3,280 to −2,482 of the wild-type *rpl5* genomic region; GenBank accession AY258275) were subcloned as described earlier and hybridized to the RNA-gel blots. After hybridization, membranes were washed twice at 65 °C for 20 min in 1 mM EDTA, 40 mM $NaHPO_4$, 5% SDS, and twice in 1 mM EDTA, 40 mM $NaHPO_4$, 1% SDS, and then subjected to autoradiography.

Mitochondrial *rpl5* antibody production and immunoblotting

RT-PCR with primers carrying *Xho*I restriction sites was used to isolate the cucumber *rpl5* cDNA from line B. Primer sequences were 5′-CAT ATG GGT CCG AGC CTA CAA CT-3′ (rpl5-*Nde*I) and 5′-CTC GAG TTA TTA CAA ATT CAA ATT CCA T-3′ (rpl5-*Xho*I). RT-PCR products were cloned by TA-tailing and 15 clones were sequenced in both directions. Six editing sites (all C-to-U) were shared among the *rpl5* cDNAs from MSC16 and line B. One of these edited clones from line B was chosen and directionally placed, using the *Xho*I and *Nde*I sites, into the expression vector pET28 (Novagen, Madison, Wis.). The correct construct was verified by sequencing and was introduced into *Escherichia coli* expression strain BL21 Codon Plus (Stratagene, La Jolla, Calif.) by heat-shock transformation. Protein expression was induced using isopropyl-*β*-D-thiogalactopyranoside and, after 4 h induction, the recombinant protein was collected from the insoluble fraction by centrifugation. The pellet was subjected to electrophoresis on SDS-PAGE gels, visualized after staining of adjacent lanes with Coomassie blue, and an adjacent lane of the gel containing the unstained RPL5 was excised, emulsified, and injected into rabbit at

Table 2 Genbank accessions for sequenced mitochondrial genomic regions of cucumber

Region	Clone	Source	Accession	Length (bp)
rrn18/*nad5-exon2*	WT-*rrn18*	Calypso	AY258278	5,219
	WT-*nad5-exon2*	Calypso	AY258277	8,511
	MSC-*rrn18-nad5-exon2*	MSC16	AY258271	11,003
JLV5/*rpl5*	WT-*rpl5*	Calypso	AY258275	8,626
	WT-JLV5 (extended)	Calypso	AF288044	42,769
	MSC-*rpl5*	MSC16	AY258273	13,495
	MSC-JLV5-DEL-front junction (with *rpl5*)	MSC16	AY258270	3,148
	MSC-JLV5-DEL-rear junction	MSC16	AY258272	9,303
Pseudo-*rpl5*	WT-pseudo-*rpl5*	Calypso	AY258274	11,291

the Polyclonal Antibody Service, University of Wisconsin, Madison, Wis. The second bleeding was affinity-purified (Sambrook et al. 1989) and used for Western blots. Mouse monoclonal antibody Anti-PORIN was kindly provided by Dr. Tom Elthon, University of Nebraska, USA. To prepare mitochondrial protein extracts, mitochondria were purified as described above and resuspended in sample buffer (McCabe et al. 1998). For immunoblotting analysis, 30–40 μg of total plant proteins or mitochondrial proteins were loaded on 15% SDS-PAGE gels, subjected to electrophoresis, and blotted onto Hybond C Extra nitrocellulose membrane (Amersham). Membranes were blocked using 5% milk powder and probed with antibodies, according to the membrane manufacturer's instructions. RPL5 antibody was diluted 1:1,000 and PORIN 1:500. Alkaline phosphatase conjugated anti-rabbit or anti-mouse antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) were used as secondary antibodies. Both primary and secondary antibodies were diluted in PBS containing 0.1% Tween. Immunoreactive proteins were visualized using the 1-Step NBT/BCIP system (Pierce, Rockford, Ill.).

Results

MSC and wild-type cucumbers regenerated from cell cultures possess rearrangements in the mitochondrial genome

We revealed five structural polymorphisms (Fig. 2) in the mitochondrial genome among wild-type and MSC lines (Fig. 1, Table 1), all tracing back to the highly inbred line B. In addition to the previously described P1 and JLV5 regions (Lilly et al. 2001), new polymorphisms were revealed by *rrn18*, *nad5-exon2*, and *rpl5* (Fig. 2). All polymorphisms were paternally transmitted (data not shown). We assembled and sequenced mitochondrial contigs across the polymorphic regions. The polymorphisms revealed by *rrn18* and *nad5-exon2* were due to a rearrangement placing these two coding regions together (Fig. 3, Table 2). For the polymorphism revealed by *rpl5*, all accessions possessed a 5.2-kb *Sac*I fragment corresponding to the functional *rpl5* gene (Fig. 2, Table 2). Wild-type lines B and Calypso possessed a pseudo-*rpl5* gene (as defined by premature stop codons) carried on a 15.0-kb *Sac*I fragment (Fig. 2, Table 2). MSC16 and the wild-type lines LCST1 and LCST2 were regenerated from the same leaf-callus experiment (Table 1) and did not possess this pseudo-*rpl5* (Fig. 2). MSC16 possessed a unique 6.2-kb *Sac*I fragment (Fig. 2), due to a rearrangement placing a duplicated *rpl5* gene next to the forward junction of the JLV5 region (Fig. 3, Table 2). JLV5 is a 15.1-kb region missing in MSC16, present in wild-type line B, and sorting with the MSC phenotype (Lilly et al. 2001). Hybridization of the region (P1) adjacent to the front junction of the JLV5 region revealed polymorphisms between MSC and wild-type lines (Fig. 2). Sequencing an expanded contig of the wild-type JLV5 region (Lilly et al. 2001; Table 2) established that the forward and reverse junctions were located near regions of previously identified short repetitive DNA motifs in cucumber mitochondria (Lilly and Havey 2001). For example, repeat motifs 1, 3, 4, and 5 were located near the front junction of the JLV5

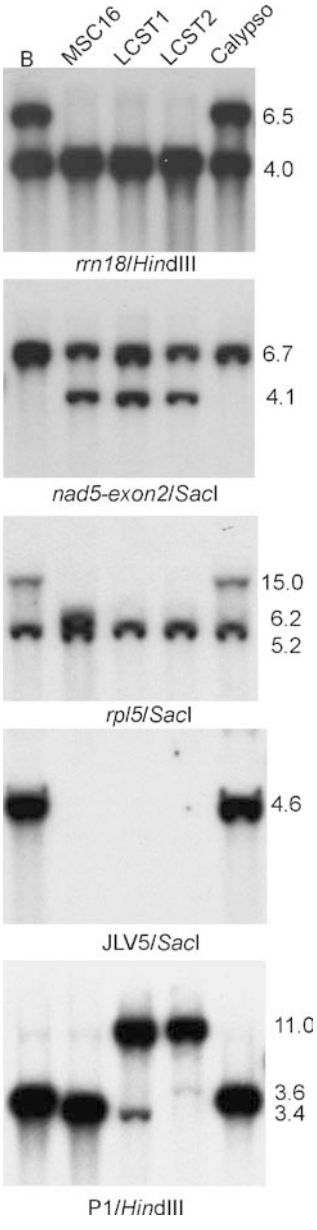

Fig. 2 Autoradiograms showing RFLPs in the mitochondrial DNA between wild type inbred line B (*lane 1*), MSC16 (*lane 2*), wild-type tissue culture lines 1 and 2 (LCST1 in *lane 3*, LCST2 in *lane 4*), and wild-type cultivar Calypso (*lane 5*). Restriction enzymes and probes revealing polymorphisms are *shown below the autoradiograms*. RFLPs were revealed by cosmids from the *Arabidopsis* mitochondrial genome carrying the *rrn18*, *rpl5*, and *nad5-exon2* coding regions (Unseld et al. 1997). The JLV5 deletion was described by Lilly et al. (2001), but was not characterized in lines LCST1 and LCST2. P1 is the genomic region adjacent to the JLV5-DEL rearrangement (Lilly et al. 2001). Approximate fragment sizes (in kilobases) are *shown at the right*

deletion at positions −150 to −110 and near the rear junction at positions −120 to −81. The exact junction points were not homologous to these previously described motifs (Lilly and Havey 2001). The rearrangement that fused the genomic region carrying the *rpl5* with the forward junction of the JLV5 deletion occurred at ATTTAGAAGCAA. The rear junction was at or

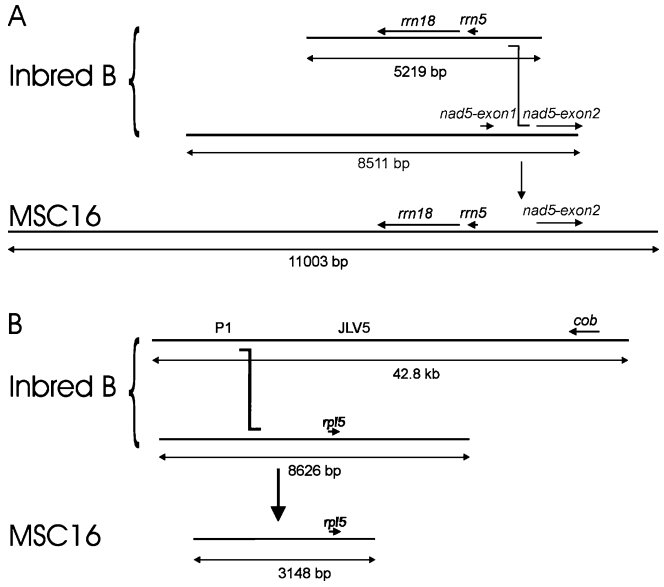

Fig. 3 Schematic diagrams of mitochondrial-DNA rearrangements near *rrn18-rrn5* with *nad5-exon2* (**A**) and *rpl5* with JLV5 (**B**) in MSC16 relative to the wild-type parental inbred line B. Regions or genes are *indicated above the lines*

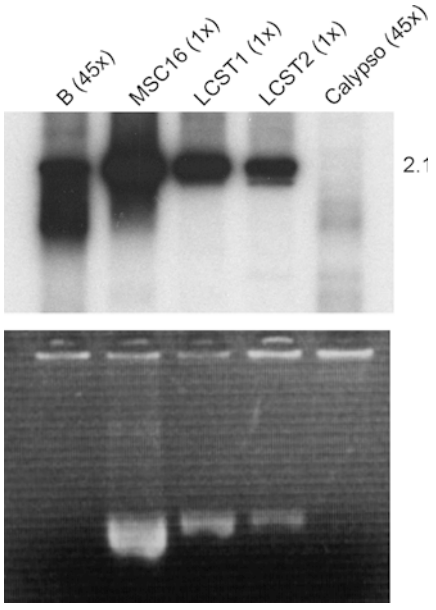

Fig. 4 Autoradiogram (*top*) from the hybridization of *rpl5* to PCR-amplified DNA, using primers flanking the *rpl5* rearrangement in the MSC16 mitochondrial genome. Lane contents are *shown at the top* and are listed in Table 1. The approximate size of the hybridizing fragment (in kilobases) is *shown at the right*. Each lane in the gel (*bottom*) carries 5 µl of the PCR reaction. The gel blotted to produce the autoradiogram (*top*) was loaded with 22.5 µl from the PCR reactions of B and Calypso and 0.5 µl from the PCR reactions of MSC16, LCST1, and LCST2

near CTCATTAACGAT. The exact sequence at the rear junction could not be established, because we could not isolate one of the wild-type regions involved in the rearrangement.

We evaluated for the presence of the rearranged *rpl5* region among wild-type sorters by intercrossing among MSC16 plants. The frequency of wild-type progenies from MSC16 was 3.3% (24 out of 720). In all cases, DNA-gel blots revealed that the wild-type *rpl5* mitochondrial region was recovered with the wild-type phenotype (data not shown). Hybridizations of the P1 region to *Hin*dIII-digested DNA of the wild-type line LCST1 revealed both unique (11.0 kb) and MSC16-specific (3.4 kb) fragments (Fig. 2); and intercrossing among LCST1 plants produced 504 wild-type and seven MSC progenies (1.4%). Although line LCST2 possessed no detectable levels of the 3.4-kb MSC-specific fragment in the P1 region (Fig. 2), intercrossing among plants of this line produced 469 wild-type progenies and one MSC progeny (0.2%).

We used primers MF1 and rpl5R2 to amplify a 2076-bp fragment across the front junction of the *rpl5*-JLV5 rearrangement from MSC16 and wild-type lines B, LCST1, LCST2, and Calypso. Amplicons were observed from MSC16, LCST1, and LCST2; and no fragments were amplified from B and Calypso (Fig. 4). Amplicons from LCST1 and LCST2 were less intense than MSC16, as expected because hybridization of the P1 region revealed weak RFLPs on autoradiograms (Fig. 2). To evaluate for the presence of substoichiometric levels of this rearranged fragment, we loaded onto an agarose gel 45 times the volume of the PCR reaction from B and Calypso (22.5 µl), as compared with MSC16, LCST1, and LCST2 (0.5 µl from each PCR reaction). The autoradiogram from hybridization of the internal *rpl5* probe to this gel blot clearly revealed the presence of the 2076-bp amplicon from B, but not Calypso (Fig. 4).

Gene expression and protein accumulation in MSC16

We used mitochondrial-RNA-gel blots to evaluate for *rpl5* expression in MSC16 and wild-type lines. Transcripts for *rpl5* were 4.4 kb and 2.0 kb, both of which were reduced in MSC16 relative to wild-type lines (Fig. 5). We cloned the region upstream from the functional *rpl5* from Calypso corresponding to positions −3,280 to −2,482 relative to the *rpl5* start codon. Because the recombination event that brought together the P1 and *rpl5* regions in MSC16 occurred between positions −1,379 and −1,390, this probe hybridized upstream from the wild-type *rpl5*, not the rearranged duplicated *rpl5* region in MSC16 (Fig. 2), and revealed only the 4.4-kb transcript (Fig. 5). The amount of the 2.0-kb *rpl5* transcript was less in MSC16 than in the wild-type lines, but showed a stronger signal intensity than the 4.4-kb transcript (Fig. 5). In spite of reduced transcripts, Western analyses of the RPL5 protein revealed no differences between MSC16 and B (Fig. 6). The nuclear-encoded, cytoplasmically translated, and mitochondrially imported PORIN protein also showed no differences (Fig. 6), indicating that the MSC phenotype does not significantly reduce the transcription or translation of this nuclear-encoded gene.

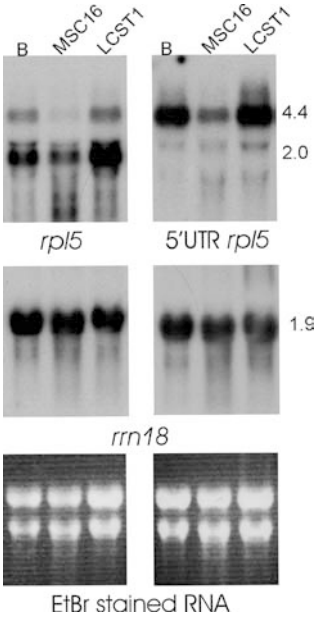

Fig. 5 RNA-gel blot analyses of *rpl5* and the untranslated region immediately upstream from *rpl5* (*5′UTR rpl5*). Lane contents are (*from the left*) wild-type inbred line B, MSC16, and wild-type line LCST1. The approximate sizes of transcripts (in kilobases) are *shown at the right*. The same blot was hybridized with a mitochondrial *rrn18* (V7 fragment) probe and the gel was stained with ethidium bromide to check equal RNA loading

Independently generated MSC plants possess different mitochondrial rearrangements

We scored all mitochondrial polymorphisms among MSC and wild-type lines generated from independent cell-culture experiments, all started from the highly inbred line B, and revealed eight unique patterns. MSC16 and MSC19 were both regenerated from the same leaf-callus experiment and shared all polymorphisms (Table 1; Lilly et al. 2001). MSC9 was regenerated after protoplast culture and shared all polymorphisms with MSC16 and MSC19 (Table 1, Fig. 7). MSC12 did not possess the same 6.2-kb *rpl5* fragment observed in MSC9, MSC16, and MSC19 (Table 1). However, we were able to amplify this rearranged *rpl5* fragment from MSC12, although the

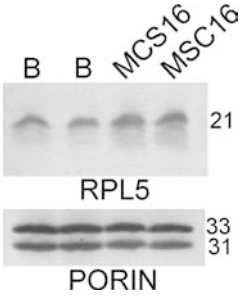

Fig. 6 Western analyses revealing relative amounts of mitochondrially encoded RPL5 and nuclear-encoded PORIN in mitochondrial protein extracts from inbred B and MSC16. The approximate sizes of polypeptides (in kiloDaltons) are *shown at the right*

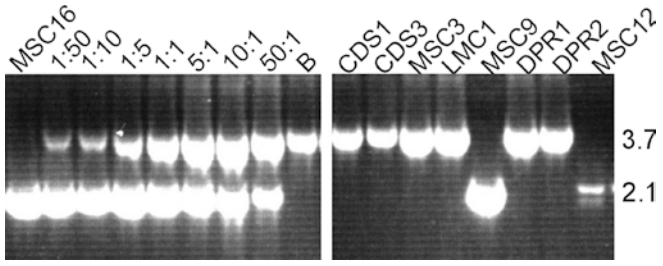

Fig. 7 Agarose gel showing PCR-amplified DNA fragments using primers MF1, JLV5R, and rpl5R2 (sequences given in the Materials and methods) and (*from the left*) genomic DNA of MSC16, wild-type line B, mixtures of DNA from B and MSC16, and wild type cucumber cultivars and independently arising MSC lines. The approximate sizes of fragments (in kilobases) are *shown at the right*

intensity of the amplicon was reduced (Fig. 7). MSC3 and four groups of wild-type lines: (1) LC1and CDS1, (2) CDS2, CDS4, and DPR2, (3) CDS3, and (4) LCST1 and LCST2 possessed unique combinations of mitochondrial polymorphisms (Table 1).

Discussion

We revealed structural polymorphisms (Fig. 2) in the mitochondrial genome among wild-type and MSC lines (Table 1) after hybridizing DNA-gel blots with ordered cosmids covering the entire *Arabidopsis* mitochondrial genome (Klein et al. 1994). Only the polymorphism revealed by *rpl5* was unique to MSC16 and not present in the wild-type lines (Fig. 2). This polymorphism resulted from duplication and fusion of the *rpl5* genomic region with the previously described JLV5 region (Fig. 3; Lilly et al. 2001). We sequenced across the rearrangement junctions to reveal pockets of repetitive DNAs, consistent with our hypothesis that the MSC phenotype is conditioned by mitochondrial DNA rearrangements due to recombination among short direct repetitive DNA motifs (Lilly et al. 2001). We demonstrated that the rearrangement near *rpl5* existed as a sublimon in B, but was not detected in Calypso (Fig. 4). We evaluated for the presence versus absence of the rearranged *rpl5* region among wild-type sorters from self-pollination of MSC16, taking advantage of the highly homozygous nuclear genotype to avoid the segregation of nuclear genes affecting the prevalence of sublimons (Hartmann et al. 1992; He et al. 1995; Janska et al. 1998) or conditioning rearrangements (Goff et al. 2002). All 24 wild-type progenies out of 720 selfed progenies possessed the wild-type *rpl5* mitochondrial region. The simplest explanation is that wild-type progenies resulted from sorting among wild-type and MSC mitochondria, with the wild type predominating.

We scored the mitochondrial polymorphisms among the wild type and MSC independently regenerated from different cell culture experiments (Table 1) and revealed eight different patterns (Fig. 7, Table 1). These results indicate that the MSC phenotype does not trace back to a single sublimon existing in the B

mitochondrial genome and there may be numerous rearranged mitochondrial genomes existing substoichiometrically in inbred B, as reported in *Phaseolus* (Janska and Mackenzie 1993; Janska et al. 1998; Arrieta-Montiel et al. 2001; Woloszynska et al. 2001). The prevalence of these sublimons may increase during tissue culture (Gu et al. 1994, Kanazawa et al. 1994). This conclusion is supported by the fact that MSC9 and MSC16 shared all mitochondrial polymorphisms, even though these lines were regenerated from independent cell-culture experiments. Another explanation is that passage through cell culture generates de novo rearrangements in the cucumber mitochondrial genome.

RNA-gel blots hybridized with *rpl5* revealed two fragments at approximately 2.0 kb and 4.4 kb, both of which showed reduced transcription in MSC16 relative to the wild-type lines (Fig. 5). The longer transcript possessed homology with the genomic region upstream from *rpl5*. Longer mitochondrial transcripts have been reported in cucumber (Stern and Newton 1985) and other plants (Hoffmann et al. 1999) and may undergo post-transcriptional modifications to reduce their size (de Souza et al. 1991). In spite of the reduced amounts of *rpl5* transcripts in MSC16 (Fig. 5), we observed no differences between MSC16 and wild-type lines for the RPL5 protein (Fig. 6). The reduced transcription of *rpl5* could be offset by longer protein longevities (Giegé et al. 2000), allowing for the accumulation of similar amounts of protein. Regardless, we were not able to establish that the rearrangement near *rpl5* is responsible for the MSC16 phenotype; and its genetic basis remains an enigma. Potential causes of the MSC phenotype not revealed by our analyses could include rearrangements affecting genes present in cucumber but missing from *Arabidopsis* mitochondrial DNA, such as *rps1*, *rps10*, *rps13*, and *sdh3* (Adams et al. 2002), relatively short deletions, insertions, or rearrangements not revealed by DNA-gel blot analyses, or post-transcriptional (Sutton et al. 1993; Maier et al. 1996) or post-translational (Sarria et al. 1998) modifications.

When the highly inbred cucumber line B is passed through cell-culture systems, regenerated (R0) plants are predominately wild type with relatively few somaclonal variants (Burza and Malepszy 1995a; Plader et al. 1998; Ladyzynski et al. 2002). After self-pollination of these wild-type R0 plants, R1 plants are occasionally produced showing the strongly MSC phenotype. Selfed progenies from MSC plants almost exclusively possess the MSC phenotype, with wild-type progenies appearing at about 3% for MSC16. A salient question is why does the MSC phenotype appear after self-pollination of wild-type R0 plants? We demonstrated that the MSC phenotype is paternally transmitted, is not conditioned by paternal imprinting, and is associated with rearrangements in the mitochondrial genome (Malepszy et al. 1996; Lilly et al. 2001; Fig. 2). The passage of cucumber through cell culture may allow for the sorting of previously rearranged sublimons or induce new rearrangements in the cucumber mitochondrial genome. The wild-type R0 plants could possess a mixture of wild-type and rearranged mitochondrial genomes with enough wild-type genomes to condition the wild-type phenotype. The R0 plant producing LCST1 was regenerated from the same leaf-callus experiment that produced MSC11 (Table 1). LCST1 possessed a mixture of wild-type and rearranged mitochondrial genomes (Fig. 2) and produced 0.2% MSC progenies. Abreu et al. (1982) observed relatively few, large mitochondria in cucumber microspores. These few, large mitochondria may represent a bottleneck and contribute to significant random drift among mitochondria incorporated into the sperm cells of cucumber pollen and transferred by the male gametophyte to progenies. The appearance of the MSC phenotype among sexual progenies from wild-type R0 plants would occur when significant numbers of the randomly transferred mitochondria carry the MSC mitochondrial lesion(s).

The MSC16 phenotype is associated with reduced transcription of *rpl5* (Fig. 5). Similar DNA rearrangements affecting mitochondrial gene expression have been documented in maize (Feiler and Newton 1987; Lauer et al. 1990). Adjacent mitochondrial coding regions can be co-transcribed, producing relatively long transcripts (Hoffmann et al. 1999). DNA rearrangements upstream from coding regions could affect the *cis*-acting promoter region, significantly reducing transcription. Even if the rearrangement completely stopped transcription, some wild-type transcript would likely be produced from wild-type sublimons, enabling the plant to survive, depending on the frequency of wild-type mitochondrial genomes and the transcript and protein longevities (Giegé et al. 2000). Presently, there is no efficient way to mutate or alter the expression of specific mitochondrial genes (Jacobs 2001). If the passage of cucumber through cell-culture systems caused de novo rearrangements, or allowed for the sorting of a relatively large number of rearranged substoichiometric mitochondrial DNAs, it could provide a unique opportunity to affect the expression of mitochondrial genes. Relatively large numbers of independently generated MSC lines can be produced after cell culture (Ladyzynski et al. 2002). MSC lines can then be evaluated for rearrangements affecting specific mitochondrial genes to study interactions between mitochondrial and nuclear genes (Moneger et al. 1994; Karpova et al. 2002).

Acknowledgements We thank Drs. Joseph Walker (University of Wisconsin, USA) for help with protein analyses, Tom Elthon (University of Nebraska, USA) for providing the anti-PORIN mouse monoclonal antibody, Axel Brennicke (Universität Ulm, Germany) for the *Arabidopsis* mitochondrial cosmids, and Hanna Janska (Uniwersytet Wrocawski, Poland) for critical reading of this manuscript. G.B. was partially supported by the NATO Advanced Fellowships Programme for post-doctoral research at the University of Wisconsin. Product names are necessary to report factually on available data. However, the United States Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product; and the use of a name by the USDA implies no

approval of the product to the exclusion of others that may also be suitable.

References

Abreu I, Santos A, Salema R (1982) Atypical mitochondria during microsporogenesis in *Cucumis sativus* L. J Submicrosc Cytol 4:369–375

Adams K, Qiu Y, Stoutemyer M, Palmer J (2002) Punctuated evolution of mitochondrial gene content: high and variable rates of mitochondrial gene loss and transfer to the nucleus during angiosperm evolution. Proc Natl Acad Sci USA 99:9905–9912

Arrieta-Montiel M, Lyznik A, Woloszynska M, Janska H, Tohme J, Mackenzie S (2001) Tracing evolutionary and developmental implications of mitochondrial stoichiometric shifting in common bean. Genetics 158:851–864

Burza W, Malepszy S (1995a) Direct plant regeneration from leaf explants in cucumber (*Cucumis sativus* L.) is free of stable genetic variation. Plant Breed 114:341–345

Burza W, Malepszy S (1995b) In vitro culture of *Cucumis sativus* L. XVII. Plants from protoplasts through direct somatic embryogenesis. Plant Cell Tissue Organ Cult 41:259–266

Coe EH Jr (1983) Maternally inherited abnormal plants in maize. Maydica 28:151–167

Feiler HS, Newton KJ (1987) Altered mitochondrial gene expression in the nonchromosomal stripe 2 mutant of maize. EMBO J 6:1535–1539

Giegé P, Hoffmann M, Binder S, Brennicke A (2000) RNA degradation buffers asymmetries of transcription in *Arabidopsis* mitochondria. EMBO Rep 1:164–170

Goff S le, Lachaume P, Touraille S, Alziari S (2002) The nuclear genome of a *Drosophila* mutant strain increases the frequency of rearranged mitochondrial DNA molecules. Curr Genet 40:345–354

Gu J, Dempsey S, Newton KJ (1994) Rescue of a maize mitochondrial cytochrome oxidase mutant by tissue culture. Plant J 6:787–794

Hartmann C, De Buyser J, Henry Y, Morere-LePaven MC, Dyer TA, Rode A (1992) Nuclear genes control changes in the organization of the mitochondrial genome in tissue cultures derived from immature embryos of wheat. Curr Genet 21:515–520

Havey MJ (1997) Paternal transmission of the cucumber mitochondrial genome. J Hered 88:232–235

Havey MJ, McCreight J, Rhodes B, Taurick G (1998) Differential transmission of the *Cucumis* organellar genomes. Theor Appl Genet 97:122–128

He S, Lyznik A, Mackenzie SA (1995) Pollen fertility restoration by nuclear gene *Fr* in CMS bean, nuclear-directed alternation of a mitochondrial populations. Genetics 97:955–962

Hoffmann M, Dombrowski S, Guha C, Binder S (1999) Cotranscription of the *rpl5-rps14-cob* gene cluster in pea mitochondria. Mol Gen Genet 261:537–545

Jacobs HT (2001) Making mitochondrial mutants. Trends Genet 17:653–660

Janska H, Mackenzie SA (1993) Unusual mitochondrial genome organization in cytoplasmic male sterile common bean and the nature of cytoplasmic reversion to fertility. Genetics 135:869–879

Janska H, Sarria R, Woloszynska M, Arrieta-Montiel M, Mackenzie SA (1998) Stoichiometric shifts in the common bean mitochondrial genome leading to male sterility and spontaneous reversion to fertility. Plant Cell 10:1163–1180

Kanazawa A, Tsutsumi N, Hirai A (1994) Reversible changes in the composition of the population of mtDNAs during dedifferential and regeneration in tobacco. Genetics 138:865–870

Karpova OV, Kuzmin EV, Elthon TE, Newton KJ (2002) Differential expression of alternative oxidase genes in maize mitochondrial mutants. Plant Cell 14:3271–3284

Kaul M (1988) Male sterility in higher plants. Springer, Berlin Heidelberg New York

Klein M, Eckertossenkopp U, Schmiedeberg I, Brandt P, Unseld M, Brennicke A, Schuster W (1994) Physical mapping of the mitochondrial genome of *Arabidopsis thaliana* by cosmid and YAC clones. Plant J 6:447–455

Ladyzynski M, Burza W, Malepszy S (2002) Relationship between somaclonal variation and type of culture in cucumber. Euphytica 125:349–356

Lauer M, Knudsen C, Newton KJ, Gabay-Laughnan S, Laughnan JR (1990) A partially deleted mitochondrial cytochrome oxidase gene in the NCS6 abnormal growth mutant of maize. New Biol 2:179–186

Lilly JW, Havey MJ (2001) Short repetitive motifs contributed significantly to the huge mitochondrial genome of cucumber. Genetics 159:317–328

Lilly JW, Bartoszewski G, Malepszy S, Havey MJ (2001) A major deletion in the mitochondrial genome sorts with MSC phenotype of cucumber. Curr Genet 40:144–151

Maier RM, Zeltz P, Kossel H, Bonnard G, Gualberto JM, Grienenberger JM (1996) RNA editing in plant mitochondria and chloroplasts. Plant Mol Biol 32:343–365

Malepszy S, Burza W, Smiech M (1996) Characterization of a cucumber (*Cucumis sativus* L.) somaclonal variant with paternal inheritance. J Appl Genet 37:65–78

Martinez-Zapater JM, Gil P, Capel J, Somerville C (1992) Mutations at the *Arabidopsis chm* locus promote rearrangements of the mitochondrial genome. Plant Cell 4:889–899

McCabe TC, Finnegan PM, Millar AH, Day DA, Whelan J (1998) Differential expression of alternative oxidase genes in soybean cotyledons during post-germinative development. Plant Physiol 118:675–682

Moneger F, Smart CJ, Leaver CJ (1994) Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. EMBO J 13:8–17

Neefs JM, Van de Peer Y, De Rijk P, Chapelle S, De Wachter R (1993) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 21:3025–3049

Newton KJ (1995) Aberrant growth phenotypes associated with mitochondrial genome rearrangements in higher plants. In: Levings CS, Vasil IK (eds) The molecular biology of plant mitochondria. Kluwer, Amsterdam, pp 585–596

Newton KJ, Coe EH Jr (1986) Mitochondrial DNA changes in abnormal growth mutants of maize. Proc Natl Acad Sci USA 83:7363–7366

Nizetic D, Drmanac R, Lehrach H (1991) An improved bacterial colony lysis procedure enables direct DNA hybridization using short (10-bases, 11-bases) oligonucleotides to cosmids. Nucleic Acid Res 19:182

Plader W, Malepszy S, Burza W, Rusinowski Z (1998) The relationship between the regeneration system and genetic variability in the cucumber (*Cucumis sativus* L.). Euphytica 103:9–15

Sakamoto W, Kondo H, Murata M, Motoyoshi F (1996) Altered mitochondrial gene expression in a maternal distorted leaf mutant of *Arabidopsis* induced by chloroplast mutator. Plant Cell 8:1377–1390

Sambrook J, Fitsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sarria R, Lyznik A, Vallejos, CE, Mackenzie SA (1998) A cytoplasmic male sterility-associated mitochondrial peptide in common bean is post-translationally regulated. Plant Cell 10:1217–1228

Souza AP de, Jubier MF, Delcher E, Lancelin D, Lejeune B (1991) A *trans*-splicing model for the expression of the tripartite *nad5* gene in wheat and maize mitochondria. Plant Cell 3:1363–1378

Stern DB, Newton KJ (1985) Mitochondrial gene expression in Cucurbitaceae: conserved and variable features. Curr Genet 9:395–405

Sutton CA, Conklin PL, Pruitt KD, Calfee AJ, Cobb AG, Hanson MR (1993) Editing of *rps3*/*rpl16* transcripts creates a premature truncation of the *rpl16* open reading frame. Curr Genet 23:472–476

Unseld M, Marienfeld JR, Brandt P, Brennicke A (1997) The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nat Genet 15:57–61

Ward BL, Anderson RS, Bendich AJ (1981) The mitochondrial genome is large and variable in a family of plants (Cucurbitaceae). Cell 25:793–803

Woloszynska M, Kieleczawa J, Ornatowska M, Wozniak M, Janska H (2001) The origin and maintenance of the small repeat in the bean mitochondrial genome. Mol Gen Genomics 265:865–872