A Mitochondrial Mutator System in Maize\[w]\n
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The P2 line of maize (Zea mays) is characterized by mitochondrial genome destabilization, initiated by recessive nuclear mutations. These alleles alter copy number control of mitochondrial subgenomes and disrupt normal transfer of mitochondrial genomic components to progeny, resulting in differences in mitochondrial DNA profiles among sibling plants and between parents and progeny. The mitochondrial DNA changes are often associated with variably defective phenotypes, reflecting depletion of essential mitochondrial genes. The P2 nuclear genotype can be considered a natural mutagenesis system for maize parents and progeny. The mitochondrial DNA changes are often associated with variably defective phenotypes, reflecting mutations. These alleles alter copy number control of mitochondrial subgenomes and disrupt normal transfer of mitochondrial genome stability and subgenome partitioning in plant mitochondria.

Spontaneous plant mitochondrial deletion mutants generated by irreversible recombination have been described in several cases. The nonchromosomal stripe (NCS) mutants of maize (Zea mays; Newton and Coe, 1986) contain deletions in essential genes, including nad4, cox2, and rps3/p116 (Newton et al., 1990; Hunt and Newton, 1991; Marienfeld and Newton, 1994; Newton et al., 2004). Each defect severely reduces plant growth, and plants are usually heteroplasmic, i.e. defective plants carry variable ratios of normal versus mutant mtDNAs. Large mtDNA deletions that remove the nad7 gene have been found in Nicotiana sylvestris (Li et al., 1988; Pla et al., 1995). Another example of plant mitochondrial deletions is cytoplasmic reversion to fertility from cytoplasmic male sterility (CMS; Schardl et al., 1985; Newton et al., 1996; Bellaoui et al., 1998; Newton et al., 2004), where usually only CMS-specific mtDNA regions are disrupted. Mitochondrial DNA deletion events are quite stable, despite the active reciprocal recombination that is constantly active during the plant life cycle, a second type of recombination occurs in higher plant mitochondria: rare, irreversible recombination across short (6- to less than 100-bp) repeats that can generate new mtDNA arrangements. On a generational timescale, plant mitochondrial genomic patterns appear to be quite stable, despite the active reciprocal recombination that maintains a multipartite structure of the mitochondrial genome. Currently, little is known about the mechanisms involved in nuclear control of mitochondrial genome stability and subgenome partitioning in plant mitochondria.

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There are a growing number of examples of specific nuclear effects on particular components of plant mitochondrial genomes. The most well studied are the Phaseolus vulgaris Fr gene, whose dominant allele drastically reduces the copy number of a single mitochondrial subgenome that confers male sterility (Janska and Mackenzie, 1993; He et al., 1995; Janska et al., 1998), and the chm mutation in Arabidopsis (Arabidopsis thaliana), which causes accumulation of characteristic rearrangements in mtDNA leading to maternally conferred leaf variegation and distortion (Martinez-Zapater et al., 1992; Sakamoto et al., 1996).

Here, we report a novel genetic system in maize characterized by highly destabilized mitochondrial genomes. The P2 line was derived from a South American strain of popcorn and was first described as having maternally transmitted abnormalities, such as poor plant growth and pale streaks on leaves (Brown and Duvick, 1958). We have found that mtDNA from plants in the P2 pedigree accumulate numerous rearrangements with surprising differences...
between sibling plants. We show that introducing P2-line genes through the male can induce dramatic changes in mtDNA structure and create novel genotypes; thus, the P2 line is a natural mitochondrial mutagenic system in maize.

RESULTS
Mitochondrial DNA Patterns of P2 Plants Show High Variability within a Family

mtDNAs from individual plants, representing two closely related P2 families, were analyzed by filter hybridization with a number of maize probes. A set of 20 cosmid clones (each containing 25 to 35 kb of mtDNA) covering the entire mitochondrial genome of B37N maize (normal, NB cytoplasm; Fauron and Havlik, 1988) was used for hybridization probes to screen for mtDNA polymorphisms in P2 siblings. Mitochondrial cosmid walking showed that the overall type of the P2 mitochondrial genome is very similar to NB. Two of the hybridization results are shown in Figure 1. The seven related P2 mtDNA samples analyzed represent four siblings from family 4712 (lanes 1–4) and three progeny from one of them, plant 4712-4 (lanes 5–7). There are a large number of differences between sibling mtDNAs, both qualitative (novel mtDNA arrangements) and quantitative (different abundance of the same arrangement). Some mtDNA regions are lost in the progeny (marked by “o”), whereas novel arrangements appear or increase in abundance (marked by an asterisk).

The blot shown in Figure 1 was rehybridized with two shorter probes (Fig. 2, A and B). A 7.8-kb XhoI fragment containing nad5 exons D and E was found at similar levels in all 4712 P2 siblings. In addition, all plants contained novel XhoI fragments homologous to this probe. This result shows the accumulation of novel sequence arrangements that do not replace the original one and lead to an increase in overall copy number. Another probe, a 3.7-kb BamHI fragment encoding ribosomal proteins RPS3 and RPL16 (Hunt and Newton, 1991), showed a decrease of the corresponding 16-kb XhoI fragment in two of three progeny from 4712-4 (i.e. H55 in Fig. 2B). Since no other XhoI fragments were found to hybridize with this probe, the plants appear to have reduced amounts of the rps3/rpl16 genes.

NB maize mitochondria contain a 2.3-kb linear plasmid (n-plasmid) that carries the only Trp tRNA gene of mitochondrial genome (Leon et al., 1989). Most P2 lines analyzed contained levels of n-plasmid comparable to NB. However, significant reductions in the n-plasmid copy number have been found occasionally in individual P2 plants, as in one of the four P2 siblings represented in Figure 2C. In this plant, no other TRNA-Trp-homologous sequences could be detected (Fig. 2C, left). A probe used for a loading control also detects novel arrangements of the target sequence in addition to the normal 12-kb XhoI fragment (Fig. 2C, right).

Collectively, the results shown in Figures 1 and 2 demonstrate that, in individual P2 siblings, the copy number of a particular mtDNA sequence may increase or decrease differentially, shifting in both directions. Moreover, individual P2 siblings can inherit different mtDNA rearrangements from the maternal parent.

Figure 1. Individual mtDNAs from P2-line families display multiple RFLPs that differ between sibling plants. mtDNA samples from four siblings (family 4712, lanes 1–4), and from three progeny of the self-pollinated 4712-4 individual (family 96H55, lanes 5–7) were digested with XhoI, and DNA gel blots were hybridized to the cosmid probes N8B1 (left) and N7C9 (right). B37N mtDNA is used for a normal (NB) control.
P2-Like Phenotypes Can Be Induced by P2 Nuclear Genes

Plants within the self-pollinated P2 pedigrees are extremely variable in appearance (Supplemental Fig. 1). Many types of leaf sectoring can be seen, ranging from yellow (A and E), pale-green, and cream stripes (B) to streaking (D) and necrotic striations (C). Sometimes, a combination of different striping patterns can be seen on the same plant (B). Leaf-stripping patterns often vary among sibling plants (plants A and B are siblings as well as plants D and E) and also between maternal P2 parents and their progeny. This feature distinguishes P2 mutants from the NCS mitochondrial mutants, in which striping patterns are conserved and sibling plants differ only in the extent, but not the type, of striping. Some maternal phenotypes in the P2 lineages were consistently inherited in all subsequent progeny generations. However, many defective leaf striping patterns were not following strict inheritance within a given lineage.

To test if specific nuclear alleles in the P2 lines are responsible for the mitochondrial genome instability and thereby induce defective phenotypes, plants with NB or CMS-T mitochondrial genotypes were crossed by pollen from P2 plants. The resulting F1 hybrids were backcrossed by P2 pollen twice before some of the progeny clearly showed P2-like sectors. Subsequent backcrossing or self-pollination of plants manifesting mutant leaf sectors usually gave high percentages of P2-like phenotypes in progeny. As in P2-inbred families, the P2-converted lines showed variable leaf striping patterns and a variable percentage of normal-looking plants within a single family (Supplemental Fig. 1, F–H).

Our results demonstrate that P2 nuclear genes are responsible for the defective phenotypes of P2-converted plants. However, the genetic experiments to determine the number of genes involved in the onset of mitochondrial genome destabilization proved to be complicated by the lack of immediate phenotypic markers for the P2-specific nuclear alleles. The phenotypic defects were not apparent in the F2 or first backcross generation (Supplemental Table I), suggesting either one or a combination of the following: (1) recessive alleles of more than one nuclear gene are needed for destabilization of the mitochondrial genome; and (2) the penetrance of the P2 mutation(s) is markedly less than 100%. Low phenotype penetrance of the nuclear mutations destabilizing the mitochondrial genome is not unexpected. Judging from our mtDNA RFLP analysis of P2-inbred lines, not every change in the mtDNA profile correlates with a mutant phenotype. Many such changes may occur in non-functional regions of the large and redundant maize mitochondrial genome, and, even if functional regions are involved, novel arrangements may be amplified independently and may not replace the original ones. Also, it may take more than one generation to develop and sort mtDNA rearrangements in plants that already have a destabilizing allelic combination in the nucleus. The effect of heteroplasmy (as in the case of NCS) should act to lower the percentage of defective phenotypes in the progeny of a mutant plant.

P2-Converted Plants Show the Same Types of mtDNA Changes as the Original P2-Inbred Lines

A family of P2-converted plants with originally NB cytoplasm is shown on Figure 3, A to C (lanes 1–5). The Mo17N inbred was crossed by P2 males three consecutive times to give family 5535. Then one of the 5535 siblings was self-pollinated to give family 5714. A probe specific to the mitochondrial 28S rRNA (Fig. 3A) highlights a novel 22-kb XhoI fragment in mtDNA from two 5714 siblings (Fig. 3A, lanes 3 and 4). In one of them, the original 25-kb fragment is severely decreased. The same blot used for Figure 3A was
rehybridized with a 10.5-kb XhoI fragment containing genes for 18S rRNA and 5S rRNA and flanking regions (Fig. 3B). A novel 9.7-kb XhoI fragment is seen in all the P2-converted samples. In the 5714-3 individual, the original 10.5-kb fragment is significantly reduced. Figure 3C shows the hybridization with a 2.9-kb XhoI fragment from cosmid N7E8 (Fauron and Havlik, 1988). The 2.9-kb fragment of normal mtDNA is decreased in all 5714 plants tested but not in the parental family 5535. Another hybridizing region is a 9.7-kb fragment apparently identical to the one highlighted in Figure 3B. These results show that, similar to the original P2 lines, P2-converted families are characterized by (1) an accumulation of novel mtDNA arrangements and decreases in original arrangements, and (2) different mtDNA profiles among sibling plants and between maternal parents and their progeny.

The same set of three probes used to analyze the P2-converted family 5714 (Fig. 3, A–C) was hybridized also to XhoI-digested mtDNAs from P2-inbred families 4712 and H55 (Fig. 3, D–F). The 28S-rRNA-specific probe detected a similar 25-kb/22-kb XhoI polymorphism. A novel 9.7-kb fragment was detected as well in P2-inbred plants but at comparable levels with the normal 10.5-kb band. Probing with the 2.9-kb XhoI probe (Fig. 3F) showed that, although the 9.7-kb fragment was abundant in all the 4712 plants, there was no reduction of the normal 2.9-kb band. Interestingly, with this probe, low amounts of a 9.7-kb XhoI fragment could be detected (marked by an asterisk) in the NB mtDNA controls.

These results suggested that the novel 9.7-kb fragments were identical in P2-inbred and in P2-converted plants. This rearrangement has been cloned from the mtDNA of plant 5714-3 and partially sequenced. It contains the coding sequence for the ribosomal protein RPS2, reported previously to exist in wheat (Triticum aestivum), rice (Oryza sativa), and maize but not in dicots (Vaitilingom et al., 1998). Two copies of RPS2 coding sequence (rps2A and rps2B) have been found in maize (Perrotta et al., 2002). Analysis of the novel rps2 sequence isolated from the P2-converted plant showed that it was created by recombination between rps2A and rps2B, which share 560 bp of homology (Fig. 3G). PCR with primers for the 5′ region of rps2A and the 3′ region of rps2B detected the same 1,600-bp fragment not only in 5714-3 and 4712-1 mtDNAs, but also in the NB mtDNA from Mo17N (Fig. 3H). Partial sequencing showed that this recombin rps2 version was identical in normal, P2, and P2-converted plants and thus was an amplified sequence preexisting in normal mtDNA at the low level.
Comparison of mtDNA profiles among the P2-converted (families 5714 and 5535) and P2-inbred plants analyzed (Fig. 3) shows that there is no direct substitution of either rps2A or rps2B with rec-rps2. We can infer that stoichiometric differences in rps2 versions A and B in P2-converted plants developed independently.

Changes in mtDNA Profiles Lead to Changes in Profiles of Mitochondrial Transcripts

It was shown earlier (Perrotta et al., 2002) that the rps2B copy generates a low-abundance transcript that, in contrast to the abundant rps2A transcript, is unedited in two highly conserved positions of the rps2 coding sequence. This suggests that the rps2A gene is the only functional one. It is located within the 2.9-kb XhoI fragment seen in Figure 3. All plants of the P2-converted family 5714 had low amounts of rps2A and variable ratios of rps2B and rec-rps2 (Fig. 3, B and C). Northern analyses (Fig. 4A) showed that the major RPS2 RNA in Mo17 normal plants was represented by a 3-kb rps2A transcript (in agreement with Perrotta et al., 2002). The rec-rps2 transcript, which also is approximately 3 kb, was detectable in P2-converted plants. The 2.4-kb rps2B transcript, which is normally a minor transcript, is further decreased in mutant plants. The transcript levels of the three rps2 versions roughly correlated with their copy numbers within mtDNA (Fig. 3, B and C). Necrotic leaf striations, characteristic for the 5714-3 individual, were similar to the phenotypes of NCS3 and NCS4 maize mutants that are depleted for mitochondrial ribosomal protein RPS3 (Hunt and Newton, 1991). Thus, the phenotype of plant 5714-3 probably results also from the decrease in mitochondrial ribosomal protein, in this case, RPS2. Our results suggest that a P2-induced mutant phenotype is likely to be caused by a decrease in copy number of a functional mitochondrial gene, rather than by an accumulation of alternative mtDNA arrangements.

As we have shown here, independent changes in the copy numbers of several mtDNA regions occur among sibling P2 plants. Thus, it could be expected that P2 siblings will differ within the same family in their mitochondrial transcript profiles. Indeed, as seen in Figure 4C, siblings from the P2-converted family 5714 have different steady-state levels of several mitochondrial mRNAs. This fact may explain the occurrence of phenotypically different mutants among the progeny of a single P2-inbred or P2-converted plant (Supplemental Fig. 1).

A P2-Specific Nuclear Allele Directly Controls the Amplification of the rps2 Rearrangement

A significant copy number increase of the rec-rps2 gene was detected for almost all of the P2 families tested. Therefore, we asked whether a single recessive gene in the P2 genome can directly control this effect. F1 hybrids, obtained by pollinating Mo17N-inbred plants with P2 pollen, were backcrossed with a second P2 male parent, and the ratio of rec-rps2 versus rps2A was scored on Southern blots of total DNA isolated from random sets of progeny seedlings. One of them is shown in Figure 5A. The controls for low (normal) and high stoichiometry of the rec-rps2 are represented by the Mo17N and 5535-22 samples, respectively (Fig. 5A). A significant increase in the rec-rps2 (4.36-kb HindIII fragment) relative to the rps2A (2.91-kb HindIII fragment) is seen in 7 out of 14 tested progeny, suggesting a 1:1 Mendelian segregation. The progeny of the independent cross between Mo17NxP2 female and P2 male showed a segregation ratio 11:13 (Fig. 5D). These results indicate that a single P2-specific allele may be responsible for the copy number increase of the rec-rps2-containing mitochondrial subgenome.

The rps2A and rps2B coding sequences form 560-bp-long direct repeats. This suggests that rec-rps2, which is always present at a very low level in maize NB-type mitochondria, might be continuously generated by homologous recombination. Thus, an increase in the rec-rps2 copy number could be due to an increase in the replication efficiency or to an increase in the recombination efficiency between rps2A and rps2B. In the latter case, the higher level of rec-rps2 should cosegregate with...
an increased amount of the reciprocal recombination product, rec-rps2* (formed by the 5’ flank of rps2B and the 3’ flank of the rps2A; Fig. 5E). The DNA blot shown in Figure 5A was rehybridized with the rps2B 5’ probe. In addition to the 3.1-kb HindIII fragment (rps2B), a 1.88-kb HindIII fragment, corresponding to reciprocal recombination product rec-rps2*, was detected at similarly low levels in all of the backcross progeny (Fig. 5B), with no correlation to elevated rec-rps2. Thus, a P2-specific allele appears to affect the replication rate of a subgenome carrying rec-rps2 and not the recombination rate between the rps2 open reading frame (ORF) repeats.

To detect any influence of the P2-specific allele on the general rate of reciprocal recombination in the mitochondrial genome, the recombination patterns mediated by another repeated sequence in the NB mtDNA were tested in the segregating population shown in Figure 5, A and B. Several pairs of repeats, ranging in size from 0.7 to 14 kb, have been reported to be recombinationally active in the maize NB mitochondrial genome (Lonsdale et al., 1984; Fauron et al., 1995; Lupold et al., 1999). The direct 0.7-kb repeat is close in size to the rps2 repeat and was reported to possess low but readily detectable recombination activity (Lonsdale et al., 1984; Lupold et al., 1999). Both copies of 0.7-kb repeat and their flanking regions were sequenced from cosmids N6A6 and N5G8 (see “Materials and Methods”) for copies A and B (as in Lupold et al., 1999), respectively. The flanking sequence of the copy A was amplified with primers P13 and P14 and used
to reprobe the DNA blot. Figure 5C shows the major hybridizing 6-kb HindIII fragment, corresponding to copy A of the repeat, and a 5-kb fragment of lower stoichiometry, generated by recombination. The ratio between these fragments is comparable to the earlier reported data (Lupold et al., 1999). Similar to the result in Figure 5B, there are no stoichiometric shifts, cosegregating with the amount of the rec-rps2.

The data presented in Figure 5 strongly suggest that one of the P2-specific genes increases the abundance of the previously rare mtDNA arrangement without affecting the efficiency of reciprocal recombination.

A P2-Specific Allele Controls Amplification of Several mtDNA Rearrangements

Several P2-converted lines have been derived from plants carrying CMS-T cytoplasm. After CMS-T plants were serially pollinated four times by P2 pollen parents, individuals from three families were then crossed with pollen from a Mo17N/P2 hybrid. The progeny from each last cross were expected to segregate the mutation responsible for the amplification of the rec-rps2 sequence (described above). mtDNA profiles of three sets of segregating siblings derived from each of the three independent crosses are shown in Figure 6. Because both the rps2A and rps2B genes in CMS-T maize mtDNA lie within large regions of identity with NB-type mtDNA (Fauron et al., 1990), the same sizes of XhoI fragments that carry rps2 isomers in CMS-T were expected. Indeed, Figure 6A shows that, in addition to the constant 2.9-kb XhoI fragment (rps2A), a 9.7-kb XhoI fragment (rec-rps2) is amplified in some individuals in each of the families. A probe for nad9 coding sequence (Fig. 6B) highlights a constant 24-kb XhoI fragment, which is the usual arrangement in CMS-T (Fauron et al., 1990), as well as a segregating 18-kb band. Figure 6C shows hybridization with an rps3/rpl16 probe. This probe detects partially homologous 16-kb and 13-kb XhoI fragments. Three different types of novel bands (3.8, 5, and 18 kb) are segregating within individuals.

Figure 6 demonstrates that in all three families, all novel mtDNA rearrangements cosegregate. Thus, a P2-derived recessive allele deregulates copy-number control of multiple mitochondrial subgenomes rather than of a single one.

The P2-Nuclear Background Alters Mitochondrial Genome Partitioning

Three independently maintained sublines of P2 (A, B, and C) were established from related individual plants and were propagated by self-pollination for many generations. The P2(B) lineage was the only one.
where most of the plants were of normal phenotype. P2(B) mtDNA contained rec-rps2 at the normal (very low) level (data not shown). P2(B) plants appeared to be homozygous dominant for the allele controlling copy number of mitochondrial subgenomes. One of the progeny from self-pollination of a P2(A) individual (4712-4; see Fig. 1) had been crossed as a female by a P2(B)-inbred plant. (A gametophytic factor found in popcorn lines prevents fertilization of P2 plants with pollen of nonpopcorn normal inbreds [Nelson, 1994].) The resultant offspring family (5677) would be expected to be heterozygous for the mutation disrupting copy number control. Indeed, all 5677 siblings tested contained very low (normal) levels of rec-rps2 (Fig. 7A). Nevertheless, variation among the mtDNAs of these siblings was observed, as well as differences from the mtDNA of the maternal grandparent (4712-4; Fig. 7B). Such variation could not be explained by deregulated mitochondrial replication. Thus, we suggest that another mutation, present in the P2-nuclear background, disrupts the proper partitioning of maternal mtDNA molecules into egg cells.

Novel mtDNA Rearrangements Are Amplified in P2 Plants

An almost-complete copy of a linear R1 plasmid, originally found in maize RU cytoplasms (Weissinger et al., 1982), is integrated into the mitochondrial chromosome of NB mtDNA, next to a 5.2-kb repeated region (Fauron and Havlik, 1988). A probe corresponding to the R1-derived region was hybridized to two sets of P2 sibling mtDNAs (Fig. 8A). In addition to the expected 7-kb PstI fragment, novel 12-kb, 6-kb, and 2.1-kb bands could be detected in individual progeny of two self-pollinated sibling P2 plants (3999-7 and 3999-10). None of these fragments were detectable in the B37N control mtDNA, and only the 12-kb fragment was seen in a plant from the parental generation (3999-4). The 2.1-kb PstI fragment was cloned, sequenced, and found to contain a rearrangement between the integrated R1 sequence and the gene for ribosomal protein S13 (Fig. 8C). A short repeat apparently mediated this nonreciprocal recombination, which is
similar to the processes generating NCS mutants of maize (Newton et al., 2004). PCR was used to test for preexisting copies of the R1/rps13 rearrangement. Among mtDNA samples used for Southern analysis (Fig. 8A), the specific 1.1-kb PCR fragment was only detected in 3999-7 progeny (Fig. 8B). It was not detected in NB mtDNA, or in the cousins (3999-10 progeny), or in the 3999-4 individual, a sibling of the 3999-7 plant. Thus, the 2.1-kb PsI fragment is unlikely to represent a stoichiometric shift in a preexisting sublimon but rather represents an amplified product of a de novo nonreciprocal recombination event.

DISCUSSION

We have described a novel type of maize mutant with destabilized mitochondrial genomes. The destabilization is caused by recessive nuclear alleles in the P2 popcorn-derived lines and leads to large-scale changes in mitochondrial genomes, some of which then become maternally inherited. Readily detectable changes in P2 mtDNA include: accumulation of numerous arrangements not seen previously at high levels; a significant decrease in the copy number of some normal regions; and multiple differences in the mtDNA profiles among sibling plants and between parents and their progeny, which constitutes a unique feature of the P2 genetic system.

Three types of events could be involved in the P2-specific destabilization of mitochondrial genome: (1) loss of nuclear control over the relative copy numbers of different mitochondrial subgenomes; (2) failure to transmit all of the subgenomes to progeny; and (3) increase in the level of nonreciprocal recombination, which is normally very low. We show here that the P2-nuclear background confers the first two effects.

Our results suggest that a recessive nuclear mutation could be responsible for the amplification of regions in mtDNA that are not normally abundant. This allele does not affect the reciprocal recombination and apparently disrupts the negative regulation of replication for a number of mitochondrial subgenomes. We speculate that such regulation normally prevents the accumulation of aberrant and spurious mtDNA arrangements generated both by the continuous, reciprocal and by the rare, nonreciprocal types of recombination in plant mitochondria.

Our analysis of the maize P2 lineages suggests the involvement of another mutation(s), altering the stable inheritance of mitochondrial genomes. It is genetically distinct from the copy number controlling allele(s) and causes a differential transmission of mitochondrial subgenomes to individual progeny. Further evidence for an altered control of the mtDNA inheritance is provided by the random reductions in some chromosomal (Fig. 2, A and B) and extrachromosomal (Fig. 2C) regions of mtDNA among P2 siblings.

The combined effect of deregulated replication of numerous mitochondrial subgenomes and of inconsistent transfer of subgenomes to progeny results in the differences in mtDNA profiles, mitochondrial transcript patterns, and even phenotypes among direct progeny of a single P2 plant. The abnormal phenotypes of P2 plants can be explained by the reduction in copies of functional mitochondrial genes and, hence, by depletion of important mitochondrially encoded proteins.

We do not know if the nonreciprocal recombination rate is significantly higher in the P2 nuclear background. However, the products of such rare recombination events, even if they are generated with wild-type (i.e. extremely low) frequencies, have a much higher probability of being amplified and becoming readily detectable in P2 lines, where they escape negative copy number control. This effect, clearly seen in Figure 8, also contributes to mtDNA polymorphisms in P2 plants.

The preservation of alternative subgenomic variants at very low levels in plant mitochondria (sublimons) was first described by Small et al. (1987), and a model for the generation of mitochondrial genome diversity has been suggested (Small et al., 1989; Fauron et al., 1995). Under normal nuclear control over mitochondrial genome replication and partitioning, any dramatic stoichiometric shifts for rare alternative mtDNA arrangements could require homologous recombination with major stoichiometric subgenomes. These amplified sequences could eventually replace an original subgenome, creating a novel mitochondrial genotype. Such changes are normally rare, and fixation of a new mtDNA organization may require many generations under nonselective conditions (see Small et al., 1987, 1989). The P2-type destabilization of mitochondrial genomes considerably facilitates and accelerates such microevolution. This genetic system can be used for the generation of novel mitochondrial mutants of maize after backcrossing of mutated plants by compatible normal pollen to remove the P2 mutator genes.

The P2-specific combination of mutations can be compared to the chm mutation in Arabidopsis (for review, see Sakamoto, 2003). Plants homozygous for the chm mutation have a variegated leaf phenotype (Redei, 1973; Martinez-Zapater et al., 1992), and progeny from chm/chm plants have novel mtDNA fragments that are maternally inherited (Martinez-Zapater et al., 1992; Sakamoto et al., 1996). In the case of the chm-derived MDL mutant, a defective mitochondrial ribosomal protein gene, rps3, replaces the normal copy (Sakamoto et al., 1996). The mechanism was shown to be an amplification of a rearranged subgenome that was already present in wild-type mtDNA at a very low level (Sakamoto et al., 1996). It has been suggested (Abdelnoor et al., 2003; Sakamoto, 2003) that the normal CHM allele acts to suppress a particular mitochondrial genomic component, similar to the Fr gene in Phaseolus, which reduces a specific mitochondrial subgenome but which does not destabilize mitochondrial genomes.
The CHM gene was recently identified as one of several Arabidopsis homologs to the bacterial mutS gene involved in DNA mismatch repair; it encodes a mitochondrially localized protein resembling the yeast MSH1 product (Abdelnoor et al., 2003). The molecular mechanism of the CHM/AtMSH1 action explaining its copy number effects on mtDNA is not known, nor is it known if chm affects recombination or replication of mtDNA (Abdelnoor et al., 2003; Sakamoto, 2003) and whether chm mutation makes mitochondrial genome continuously change in generations and between siblings (Abdelnoor et al., 2003; Sakamoto, 2003).

It is possible that a deficiency in an MSH-related function contributes to the P2 phenomenon in maize. However, an ortholog of CHM/AtMSH1 has not yet been described in maize. Comparative proteomics of mitochondrial DNA-binding proteins from P2 and normal plants may help to identify the lesions causing the extreme mtDNA instability in P2 lines of maize.

**MATERIALS AND METHODS**

**Plant Material**

All P2 plants derive from the original outcrossed popcorn families (Brown and Duvick, 1958). They had been propagated by crosses with a pollen-compatible line called P2ST. A large pedigree was established with the progeny grown from a single ear. Three plants within the family were self-pollinated and used to establish sublines designated A, B, and C, which were maintained by self or sib pollinations. Thus, all the plants in the P2 lineages were highly inbred. P2-converted plants described in this study originate from the Mo17 inbred line or CMS-T cytoplasm in a hybrid nuclear background, as the original female parents and the P2-inbred lines as males.

**DNA and RNA Isolation**

Mitochondrial DNA samples were isolated from unpollinated ear shoots of individual plants as described previously (Hunt and Newton, 1991; Newton, 1994). Total DNA was isolated from 4-d-old seedling roots according to Dellaporta (1994). DNA was analyzed by digestion with restriction endonucleases and electrophoresis in 0.7% to 1% agarose gels by standard techniques (Dellaporta et al., 1994). Total DNA was isolated from 4-d-old seedling roots according to Dellaporta and colleagues (1994). mtDNA was amplified using MasterTaq kit (Brinkmann Instruments, Westbury, NY) according to the manufacturer’s protocol. The standard PCR program used was 94°C 2 min; 5 cycles at 94°C 30 sec, 72°C 1 min; 5 cycles of 94°C 30 sec, 70°C 30 sec, 72°C 1 min; and 20 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 1 min. The following oligonucleotide primers were used: P1, 5′-CTCTCATCGGTCATTGCACGAC-3′; P2, 5′-CAAGACCGACAGTG-TTTCGTC3′; P3, 5′-ATGATCCGGCATCGTACG-3′; P4, 5′-ATACG-GCTCTAGATTTAAGACC-3′; P5, 5′-GCTCAGTTCATCCATGACATG3′; P6, 5′-TCTGTTTCCCCAGATACGGTGGC-3′; P7, 5′-AGAACAGAACACTGTCGCC-3′; P8, 5′-CAAGCTCATTGGAACAC-3′; P9, 5′-GCTC-CTTCAAAGCTCTGAC-3′; P10, 5′-GAAGGCCATATAGCATACAT-3′; P11, 5′-GAAGACCATCCGACACAAAAGATAC-3′; and P12, 5′-AATAAGGCCAGGC-3′; P13, 5′-CGTACATTTCATTTGTC-3′; P14, 5′-GAGCTCTGAGCATCATCAG-3′; P15, 5′-GAAGACACGAGGAAAAGG-3′; P16, 5′-GAAATCCTCACCACCTTTAG-3′; P17, 5′-GAGGAAGGCGTAGGACAC-3′; and P18, 5′-GCTCGCGCTGCTACTGCGC-3′. Amplification of the following fragments was performed with probe for rRNATrp region of plasmid, P1 and P2. For the 19 kb Xhol fragment between 14- and 11-kb repeats, P3 and P4; for the 3′ untranslated region (UTR) of rps24, P5 and P6; for the 3′ UTR of rps2B, P7 and P8; and for the rps2 ORF, P9 and P10; for the 5′ UTR of rps2B, P11 and P12; and for the wheat (Triticum aestivum) nad9, P13 and P14; for probes for the R1/rps13 rearrangement, P15 and P16; and for the 0.7-kb repeat flank, P17 and P18.

**PCR Experiments**

Mitochondrial DNA was amplified using MasterTaq kit (Brinkmann Instruments, Westbury, NY) according to the manufacturer’s protocol. The standard PCR program used was 94°C 2 min; 5 cycles at 94°C 30 sec, 72°C 1 min; 5 cycles of 94°C 30 sec, 70°C 30 sec, 72°C 1 min; and 20 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 1 min. The following oligonucleotide primers were used: P1, 5′-CCCTCAGCCTCTGTTCAAGCCAC-3′; P2, 5′-CAAGACCGACAGTG-TTTCGTC3′; P3, 5′-ATGATCCGGCATCGTACG-3′; P4, 5′-ATACG-GCTCTAGATTTAAGACC-3′; P5, 5′-GCTCAGTTCATCCATGACATG3′; P6, 5′-TCTGTTTCCCCAGATACGGTGGC-3′; P7, 5′-AGAACAGAACACTGTCGCC-3′; P8, 5′-CAAGCTCATTGGAACAC-3′; P9, 5′-GCTC-CTTCAAAGCTCTGAC-3′; P10, 5′-GAAGGCCATATAGCATACAT-3′; P11, 5′-GAAGACCATCCGACACAAAAGATAC-3′; and P12, 5′-AATAAGGCCAGGC-3′; P13, 5′-CGTACATTTCATTTGTC-3′; P14, 5′-GAGCTCTGAGCATCATCAG-3′; P15, 5′-GAAGACACGAGGAAAAGG-3′; P16, 5′-GAAATCCTCACCACCTTTAG-3′; P17, 5′-GAGGAAGGCGTAGGACAC-3′; and P18, 5′-GCTCGCGCTGCTACTGCGC-3′. Amplification of the following fragments was performed with probe for rRNA-Trp region of plasmid, P1 and P2; for the 19 kb Xhol fragment between 14- and 11-kb repeats, P3 and P4; for probe for the 3′ untranslated region (UTR) of rps24, P5 and P6; for the 3′ UTR of rps2B, P7 and P8; for the rps2 ORF, P9 and P10; for the 5′ UTR of rps2B, P11 and P12; and for the wheat (Triticum aestivum) nad9, P13 and P14; for probes for the R1/rps13 rearrangement, P15 and P16; and for the 0.7-kb repeat flank, P17 and P18.

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**LITERATURE CITED**


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