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RESEARCH ARTICLE

Mutations at the Arabidopsis *CHM* Locus Promote Rearrangements of the Mitochondrial Genome

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Nuclear recessive mutations at the chloroplast mutator (*CHM*) locus of Arabidopsis produce a variegated phenotype that is inherited in a non-Mendelian fashion. Molecular analysis of the cytoplasmic genomes of variegated plants from two independent *chm* mutant lines, using specific chloroplast and mitochondrial probes, showed that the *chm* mutations reproducibly induce the appearance of specific new restriction fragments in the mitochondrial genome. The presence of these restriction fragments cosegregated with the variegated phenotype in the progeny of crosses between mutant and wild-type plants. Sequence analysis of one of the new restriction fragments found in the variegated plants suggested that it was the product of a rearrangement event involving regions of the mitochondrial genome. Thus, it appears that the *CHM* locus may encode a protein involved in the control of specific mitochondrial DNA reorganization events.

INTRODUCTION

Plant cells contain two extranuclear genetic systems, the chloroplast and the mitochondrion, whose functions are coordinately regulated by the nuclear genome. The nuclear genome encodes most if not all of the functions required for replication and expression of the cytoplasmic genomes (for reviews, see Umesono and Ozeki, 1987; Levings and Brown, 1989). However, most of the nuclear genes encoding those functions are poorly characterized. In this respect, nuclear mutations that cause cytoplasmically inherited alterations could be useful tools to identify nuclear genes required for chloroplast or mitochondrial replication and expression. Nuclear mutations that promote elevated rates of plastid mutations have been reported in petunia (Potrykus, 1970), Oenothera (Epp, 1973; Chiu et al., 1990), and maize (Thompson et al., 1983). Nuclear genes that control mitochondrial rearrangements are known in humans (Zeviani et al., 1989) and fungi (Seidel-Rogol et al., 1989; Belcour et al., 1991). In plants, there is evidence that the nuclear genetic background affects the frequency of mitochondrial genome rearrangements (Laughnan and Gabay-Laughnan, 1983; Newton and Coe, 1986), and at least one nuclear gene has been identified that is responsible for the loss of specific DNA sequences from the mitochondrial genome (Mackenzie and Bassett, 1987; Mackenzie and Chase, 1990).

Rearrangements involving both intramolecular and intermolecular recombination and amplification of specific recombinant molecules have been implicated in the generation of mutants with altered mitochondrial function (Dewey et al., 1986; Makaroff et al., 1989; Newton et al., 1990, Hunt and Newton, 1991) and have played an important role in the evolution of plant mitochondrial genomes (Small et al., 1989). In the nonchromosomal stripe (NCS) mutants of maize, the mitochondrial mutations result in a variegated (striped) phenotype (Newton and Coe, 1986; Newton et al., 1990; Hunt and Newton, 1991). It appears that the mitochondrial mutation exerts a deleterious pleiotropic effect on chloroplast structure and function (Roussell et al., 1991).

The chloroplast mutator (*CHM*) locus of Arabidopsis (Redei, 1973) was identified by the isolation of two ethyl methanesulfonate–induced recessive nuclear mutations, *chm-1* and *chm-2*, that produced a conspicuous variegated phenotype. Plants homozygous for the *chm* mutations expressed a variegated phenotype that was inherited in a non-Mendelian fashion. The phenotype of the variegated plants was thought to be produced by the induction of hereditary alterations in the plastids because their color and shape were affected in the mutant sectors of the variegated plants (Redei, 1973; Redei and Plurad, 1973).

We present here the characterization of a new variegated mutant of Arabidopsis whose phenotype is the result of a single nuclear recessive mutation, allelic to the *CHM* locus (Redei, 1973). Plants homozygous for this mutation develop variegation in all green organs, and the chlorosis is subsequently inherited as a cytoplasmic trait. Using specific chloroplast and mitochondrial probes, we have identified characteristic

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rearrangements of mitochondrial DNA (mtDNA) that are only detected in plants homozygous for mutations at the *CHM* locus and that appear to be responsible for the variegated phenotype.

RESULTS

Origin and Phenotype of the Variegated Mutant

The variegated mutant appeared spontaneously in the progeny of one transgenic Arabidopsis (ecotype Columbia) plant regenerated from tissue culture (Zhang and Somerville, 1987). This transgenic plant carried a single T-DNA insertion that segregated independently from the variegated phenotype (data not shown). Variegation in this mutant affected all chlorophyll-containing organs of the plant (cotyledons, leaves, shoots, and siliques) and was not affected by the irradiance conditions used during growth of the plants. Different mutant plants showed variegated phenotypes varying in the size and color of the sectors, as shown in Figures 1A and 1B. The size of chlorotic sectors ranged from single cell spots to sectors covering most of the plant. Sector color varied from white or yellow to various shades of green. Large sectors of one color frequently contained smaller sectors of other colors (Figure 1B). Generally, variegated plants did not show morphological abnormalities, indicating that yellow or white cells divided and grew at the same rate and to the same final size as green cells (Figures 1A and 1B). However, completely chlorotic sectors lacking green subsectors grew much more slowly than variegated sectors and produced flowers that were male sterile.

Seedlings derived from self-fertilized variegated plants showed a variety of variegated phenotypes that ranged from completely albino seedlings, unable to grow further, to yellow, variegated, and green seedlings that developed variegation later in their development. The abundance of chlorotic tissue in the seedlings was generally similar to the level of chlorosis of the maternal plant sectors from which they were derived (data not shown).

Genetic Analysis

The mode of inheritance of the chlorotic phenotype was determined by reciprocal crosses between variegated and wild-type plants. The plants used as females were marked with morphological mutations to verify the nuclear hybrid nature of the F_1 plants. When variegated plants were used as pollen donors, all F_1 plants were phenotypically normal. By contrast, when variegated plants were crossed as females, there was always a variable number of plants in the F_1 that had chlorotic sectors, as shown in Table 1. These results indicated cytoplasmic inheritance of the variegation. However, the presence of a large proportion of variegated individuals in the F_2 progenies derived from crosses between wild-type females and variegated males, as shown in Table 2, indicated that the origin of the chlorosis was associated with a single nuclear recessive mutation. All the individuals homozygous for the mutation were variegated, indicating that the penetrance of the mutation was 100%. The results obtained in the F_2 analysis support this complete penetrance.

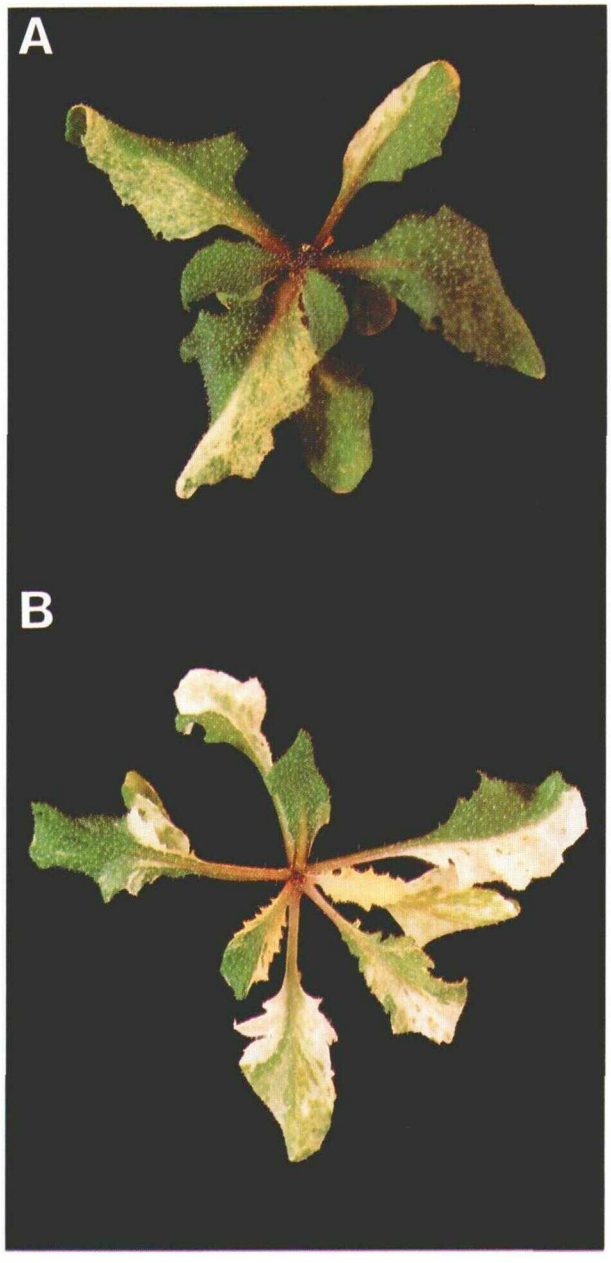


Figure 1. Phenotype of the Variegated Mutant.

(A) Variegated plant showing typical variegated and green leaf sectors.
(B) Highly variegated plant showing the presence of albino sectors and the appearance of an albino leaf.

Linkage analysis performed on the F_2 progeny derived from the cross between the marker line W100 (female parent) and the variegated mutant (male parent) indicated that the nuclear mutation responsible for the variegation was located on chromosome 3, between loci *glabra1* (*gl1*) and *long hypocotyl2*. The percentage of recombination between the *long hypocotyl2* locus and the mutation was estimated as 21.7 ± 5.6 from the analysis of 284 F_2 plants derived from a cross in repulsion phase. The percentage of recombination between the mutation responsible for the variegation and the *gl1* locus was estimated as 5.5 ± 1.6 after scoring 216 F_2 plants derived from a cross in coupling phase. The *CHM* locus, which has a similar phenotype and mode of inheritance, had previously been found to be linked to *gl1* (Redei, 1973). A complementation test between a line carrying the *chm-1* allele of this locus and our variegated mutant was performed by crossing phenotypically wild-type plants heterozygous for the mutation with plants heterozygous for the *chm-1* allele, also phenotypically normal. Nineteen of the 80 individuals derived from five independent crosses were variegated. This indicates that the mutation in the variegated line is an allele of the *CHM* locus that we have designated as *chm-3*.

Variegated Plants Show Additional Mitochondrial Restriction Fragments

The observation that a nuclear mutation is responsible for the induction of a maternally inherited phenotype suggested that the *chm-3* mutation promotes heritable changes in one or both of the cytoplasmic genomes. Therefore, several chloroplast and mitochondrial specific probes were used to perform a preliminary analysis of the integrity of the cytoplasmic genomes in the variegated plants. The tobacco chloroplast genes encoding the 70-kD chlorophyll apoprotein of photosystem I, the 50-kD chlorophyll apoprotein of photosystem II, and the β subunit of CF_1-ATPase (*atpB*) were used as chloroplast probes. The *Brassica campestris* genes encoding the α subunit of F_1-ATPase (*atpA*), the rRNA subunits 18s and 5s (rrn18s+5s), and the subunit 9 of F_0-ATPase (*atp9*) were used as mitochondrial probes. In addition, we employed a 2.3-kb BamHI clone designated pAr5 that was isolated from a total DNA library of Arabidopsis and initially identified as a middle repetitive sequence (Pruitt and Meyerowitz, 1986). Sequence analysis of this clone (GenBank accession number M88258) showed that it contains regions highly similar to open reading frame (ORF) 25 and ORF143 of maize and wheat mitochondrial genomes, respectively (Dewey et al., 1986; Pereira de Souza et al., 1991). This 2.3-kb BamHI restriction fragment has recently been mapped on the Arabidopsis mitochondrial genome at a position adjacent to exon c of the gene encoding subunit 5 of NADH ubiquinone reductase (*nad5*) (Knoop et al., 1991).

Variegated and wild-type plants showed identical restriction patterns when total DNA digested with EcoRI, BamHI, or HindIII was hybridized with the chloroplast probes or with the *atpA* and rrn18s+5s mitochondrial probes (data not shown). However, the two other mitochondrial probes (*atp9* and pAr5) hybridized with restriction fragments of DNA from the mutant that were not present in wild-type DNA. New restriction fragments were consistently found in total DNA from variegated plants digested with XhoI, PstI, EcoRI, BamHI, HindIII, HpaII, and MspI. The results found for the BamHI and EcoRI digests are shown in Figure 2. The *atp9* probe hybridized intensely with five EcoRI restriction fragments in the wild type and with three additional EcoRI fragments in the variegated mutant (10.7, 3.9, and 3.5 kb) (Figure 2A, filled arrows). Wild-type plants showed at least nine BamHI restriction fragments that hybridized to the *atp9* probe (four strong and five weak signals), whereas the *chm-3* mutant showed four additional BamHI fragments (6.3, 2.3, 1.6, and 1.4 kb) (Figure 2A, filled arrows).

A similar situation was found when the pAr5 sequence was used as a probe, as shown in Figure 2B. According to the restriction pattern of the λbAt006 clone, which contains the mitochondrial genomic region surrounding pAr5 (Figure 2C), only two EcoRI restriction fragments (7.0 and 1.1 kb) and one BamHI restriction fragment (2.3 kb) were expected in the wild-type total DNA digests. However, five additional EcoRI (21.5, 11.5, 4.6, 2.3, and 1.7 kb) and seven additional BamHI restriction fragments (20, 8.5, 7.3, 3.7, 3.3, 1.7, and 1.3 kb) were found. We interpreted these additional restriction fragments, which

Table 1. Phenotype of F_1 Plants Derived from Reciprocal Crosses[a]

Cross[b]	*n*[c]	Phenotype		
		Green	Variegated	Albino
W1 × VAR	2	16	0	0
WT × VAR	8	29	0	0
W100 × VAR	3	34	0	0
VAR × WT	8	105	65	2

[a] The female parent is listed first in each cross.
[b] W1 and W100 are marker lines in Landsberg *erecta* genetic background; WT, wild-type Columbia; VAR, variegated plants homozygous for the *chm-3* mutation.
[c] *n*, number of independent crosses analyzed.

Table 2. Segregation of the Variegation in F_2 Progenies from Different Crosses

Cross[a]	Phenotypes		χ^2	P
	Green	Variegated		
WT × VAR	134	38	0.77	0.3–0.4
WT × VAR	76	21	0.58	0.4–0.5
W100 × VAR	220	64	0.92	0.3–0.4

Chi-square values were calculated to test the fit to the 3:1 hypothesis.
[a] W100 is a marker line in Landsberg *erecta* genetic background; WT, wild-type Columbia; VAR, variegated plants homozygous for the *chm-3* mutation.

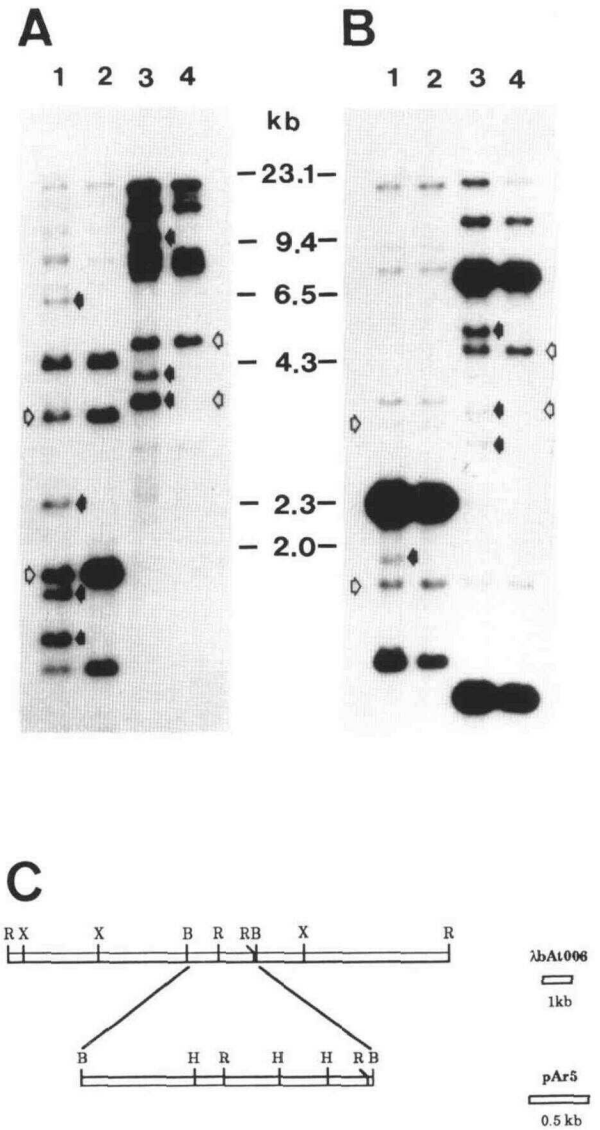


Figure 2. DNA Gel Blot Hybridization of Total DNA from *chm-3* and Columbia Wild-Type Plants with Two Mitochondrial Probes.

Total DNA (2 μg) was digested with BamHI (lanes 1 and 2) and EcoRI (lanes 3 and 4). Lanes 1 and 3 contain DNA from variegated plants. Lanes 2 and 4 contain DNA from wild-type plants. Filled arrows indicate the new restriction fragments present in variegated plants. Open arrows indicate fragments that appear to be recognized by both probes.
(A) Hybridization with the *atp9* sequence from *Brassica campestris*.
(B) Hybridization with the pAr5 sequence from Arabidopsis.
(C) Restriction map of the Arabidopsis mtDNA clone λbAt006 and the subclone pAr5. R, EcoRI; X, XhoI; B, BamHI; H, HindIII.

showed a lower hybridization signal, as DNA sequences that share limited similarity with the pAr5 probe or restriction fragments from subgenomic molecules that are present at lower copy number than the master mitochondrial chromosome. The major EcoRI restriction fragments detected by the pAr5 probe were conserved in nine different ecotypes of Arabidopsis (Bologna-1, Coimbra-1, Copenhagen-2, Landsberg *erecta*, Lund-1, Oystese-0, Rschew-0, Stockholm-0, and Turk Lake-0) (J. M. Martínez-Zapater and C. R. Somerville, unpublished results). However, as noted below, some variation between ecotypes was found for the presence of the 11.5- and 1.7-kb EcoRI fragments. Similar to the results found with the *atp9* probe, use of the pAr5 sequence as a probe also resolved new restriction fragments in the variegated plants. At least three additional EcoRI fragments (5.0, 3.5, and 3.0 kb) and one additional BamHI fragment (1.9 kb) were found in the mutant plants (Figure 2B, filled arrows). Comparison of the lengths of the restriction fragments on blots of wild-type and *chm-3* mutant DNA that had been probed with both the *atp9* and pAr5 clones indicated that some of the restriction fragments hybridized to both probes. This was the case for the 4.6-kb EcoRI and the 3.3- and 1.7-kb BamHI fragments of the wild type and the 3.5-kb EcoRI fragment of the *chm-3* mutant (Figures 2A and 2B, open arrows).

Because the chromosomal arrangement of the pAr5-homologous sequences was known, this probe was used for further analysis. Due to the use of two different ecotypes as parents during the genetic analyses, the variegated lines that were constructed had cytoplasms originating either from Columbia or from Landsberg *erecta*. As shown in Figure 3B, these two ecotypes showed slight differences in their restriction pattern when pAr5 was used as a probe. The Landsberg *erecta* ecotype completely lacks the 1.7-kb EcoRI fragment present in Columbia, and its 11.5-kb EcoRI fragment is underrepresented (Figure 3B).

The mtDNA restriction fragment length polymorphism (RFLP) identified by the pAr5 probe in *chm-3* plants was cytoplasmically inherited, as was the variegation. Total DNA samples prepared from pooled F_1 plants derived from reciprocal crosses between variegated and wild-type plants were digested with EcoRI and hybridized with the pAr5 probe. Hybrid F_1 plants from the cross Columbia wild type × *chm-3* mutant were not variegated and did not show the mtDNA RFLP (Figure 3A, lane 1). Hybrid F_1 plants from the reciprocal cross, *chm-3* mutant (Landsberg *erecta* cytoplasm) × Columbia wild type, were frequently variegated and showed the mtDNA RFLP (Figure 3A, lane 2).

The New Mitochondrial Restriction Fragments Are Caused by the *chm* Mutations

Two criteria were used to assess whether the mtDNA RFLP in the mitochondrial genome of the mutant was caused by the *chm* mutations. First, we analyzed the restriction pattern of pAr5-homologous DNA in variegated plants homozygous for the independently derived *chm-1* allele. Second, we performed a cosegregation analysis in an F_2 population derived from a cross of Columbia wild type × *chm-3* mutant.

When total DNA from variegated plants homozygous for the *chm-1* allele was digested with EcoRI and hybridized with the pAr5 probe, additional restriction fragments of similar lengths to the mtDNA RFLP in the *chm-3* mutant were identified (Figure 3C). A similar result was obtained when *atp9* was used as a probe (data not shown).

For the cosegregation analysis, a cross was made between Columbia wild type (female) and the *chm-3* mutant. A total of 20 F_3 families derived from self-fertilization of F_2 plants of known phenotype were analyzed. Twelve phenotypically wild-type F_2 plants produced F_3 families that were entirely wild type in five cases or segregated variegated plants in seven cases. Whenever the variegated phenotype segregated in the F_3, the analysis of DNA from the pooled F_3 plants indicated the presence of the mtDNA RFLP, examples of which are shown in Figure 4. Because variegation appears late in development when originating de novo and the DNA was isolated from pooled F_3 plants, the hybridization signal of the new bands is of variable intensity. The mtDNA RFLP was never observed in the progeny of F_2 plants with the wild-type phenotype that bred true in the F_3, but was always present in the progeny of the eight variegated F_2 plants analyzed (Figure 4). Complete cosegregation was always found between the presence of the variegation and the mtDNA RFLP. Similar results were obtained for the RFLP identified by the *atp9* probe (data not shown).

Circumstantial evidence linking the variegated phenotype to the mtDNA RFLP is that the extent of the variegation was correlated with the intensity of the hybridization signal shown by the additional EcoRI restriction fragments on blots probed with pAr5, as shown in Figure 5. When total DNA from yellow

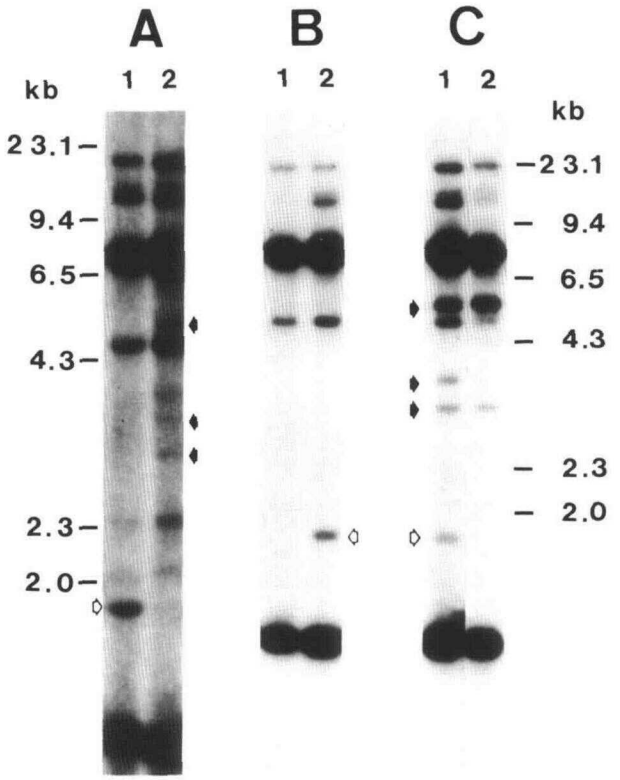


Figure 3. DNA Gel Blots of EcoRI Digests of Total DNA from Different Genotypes Hybridized with pAr5.

The filled arrows indicate the presence of the restriction fragments typical of the *chm* mutants. The open arrows indicate a 1.7-kb restriction fragment that is present in the Columbia cytoplasm but not in the Landsberg *erecta* cytoplasm.

(A) EcoRI digests of total DNA from F_1 hybrids. Lane 1, F_1 plants from the cross Columbia wild type (female) × *chm-3* mutant. Lane 2, *chm-3* mutant (female; Landsberg *erecta* cytoplasm) × Columbia wild type.

(B) EcoRI digests of total DNA from Landsberg *erecta* wild type (lane 1) and Columbia wild type (lane 2).

(C) EcoRI digests of total DNA from variegated plants homozygous for the *chm-3* mutation (lane 1) and the *chm-1* mutation (lane 2).

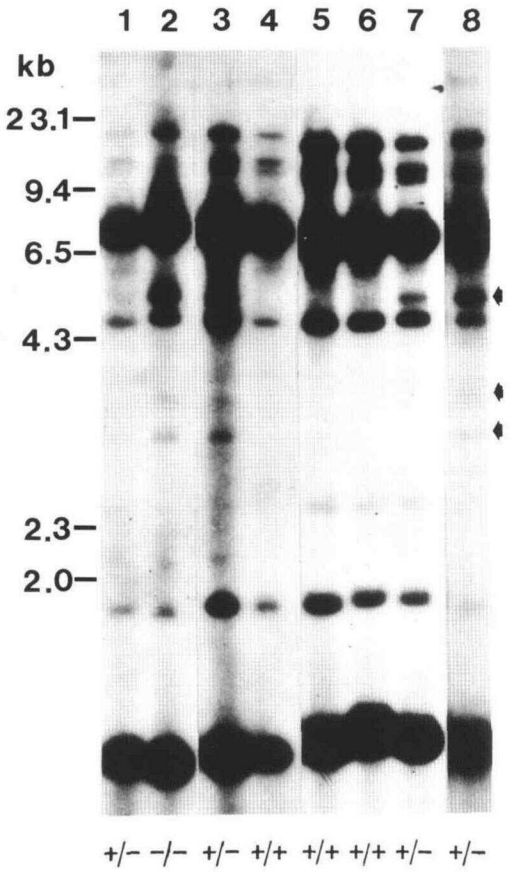


Figure 4. Cosegregation of the Variegated Phenotype with the mtDNA RFLP.

Total DNA from pooled F_3 plants was digested with EcoRI and hybridized with the pAr5 probe. Arrows indicate the positions of the new EcoRI restriction fragments that comprise the mtDNA RFLP. The genotypes of the corresponding F_2 plants, given below each lane, are based on the segregation of the variegated phenotype in the F_3 progenies.

seedlings without green sectors was digested with EcoRI and hybridized with pAr5, an increase in the ratio of mutant to wild-type restriction fragments was observed (Figure 5). Restriction fragments present in the wild-type plants were still found to some extent in these yellow seedlings. In fact, when these yellow seedlings were cultured in sucrose-containing medium, they developed green spots and sectors that permitted their growth to adult plants.

Origin of the mtDNA RFLP Found in Variegated Plants

To understand the origin of the additional EcoRI restriction fragments found in the variegated plants, two subclones of pAr5, the 1.2-kb BamHI-EcoRI (1.2BR) and the 1.1-kb EcoRI (1.1R)

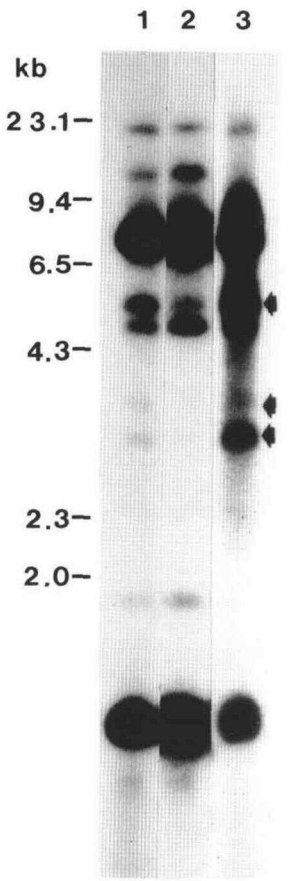


Figure 5. DNA Gel Blot Hybridizations of EcoRI Digests of Total DNA from Different Variegated Plants Probed with pAr5.

Lane 1 contains total DNA from a typical variegated plant; lane 2, total DNA from a slightly variegated plant; lane 3, total DNA from yellow seedlings without green sectors. Plants used for lanes 1 and 2 had a Columbia cytoplasm, whereas plants used for lane 3 had a Landsberg *erecta* cytoplasm. Arrows indicate the positions of the new EcoRI restriction fragments that comprise the mtDNA RFLP.

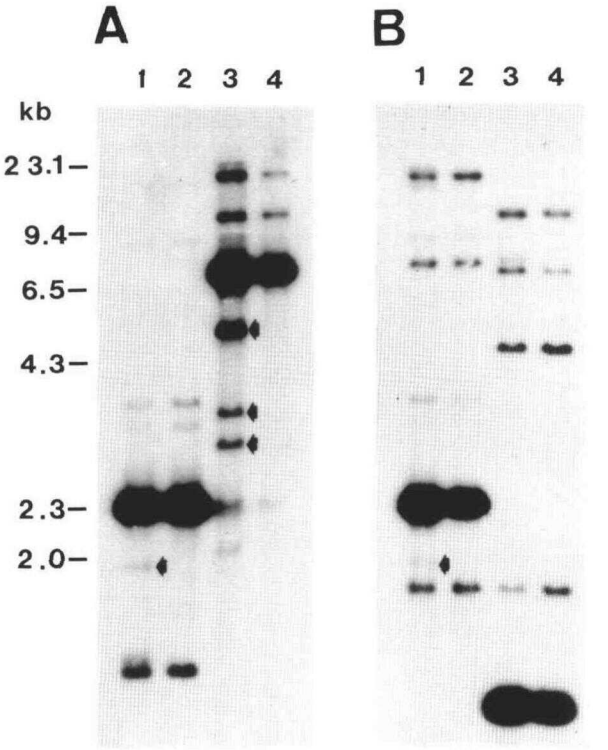


Figure 6. DNA Gel Blot Hybridizations of Wild-Type and *chm-3* Total DNA Digests Probed with Two pAr5 Subclones.

Total DNA of wild-type Columbia (lanes 2 and 4) or *chm-3* (lanes 1 and 3) was digested with BamHI (lanes 1 and 2) or EcoRI (lanes 3 and 4). The arrows indicate the EcoRI and BamHI restriction fragments present only in variegated plants.

(A) Hybridization with the 1.2BR subclone from pAr5.

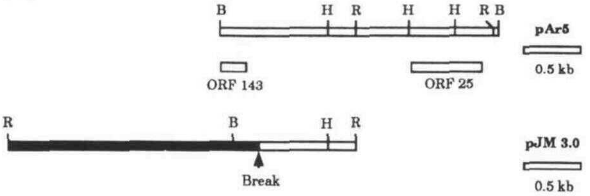
(B) Hybridization with the 1.1R subclone from pAr5.

restriction fragments, were used to probe DNA gel blots. The results are shown in Figure 6. The 1.2BR probe hybridized, as expected, with the 7.0-kb EcoRI fragment of λbAt006 (Figure 2C). This probe also hybridized with longer fragments and with all the new restriction fragments shown by the variegated plants (Figure 6A, arrows). The 1.1R probe hybridized with the 1.1R fragment, as expected, and less strongly with other fragments. However, this probe did not hybridize with any of the EcoRI restriction fragments comprising the mtDNA RFLP (Figure 6B). Thus, all the mtDNA RFLPs are due to rearrangements taking place in the 7.0-kb EcoRI fragment of λbAt006 (Figure 2C). Rearrangements taking place within the 1.2BR fragment of pAr5 would give rise to new BamHI fragments hybridizing with pAr5 and with both 1.2BR and 1.1R subclones. This is the case for the 1.9-kb BamHI fragment associated with the mtDNA RFLP (Figure 6, arrow).

To further confirm these results, we cloned the 3.0-kb EcoRI fragment found only in the variegated plants into plasmid pJM3.0. Comparison of the nucleotide sequences of pJM3.0

and pAr5 indicated that the 3.0-kb EcoRI fragment is the result of recombination between pAr5 and a foreign DNA sequence in the region predicted by the restriction analysis, as shown in Figure 7. This region is located downstream of exon c of *nad5* (Knoop et al., 1991) and also downstream of sequences that share 85% similarity with ORF143 of wheat mitochondrial DNA (Pereira de Souza et al., 1991).

A



B

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      730                                              780
AAAAAAAAAGGAATAATTTACACCGACTAAGATCAAAGAAACTAGCAGACTAATCATTAA
AAAAAAAAAGGAATAATTTACACCGACTAAGATCAAAGAAACTAGCAGACTAATCATTAA
      730                                              780

      790                                              840
AAAGGCCTTTGGTTCAAATTGTTACATCTATCTTAACTTACTCAATTATCTCGTGAACTA
AAAGGCCTTTGGTTCAAATTGTTACATCTATCTTAACTTACTCAATTATCTCGTGAACTA
      790                                              840

      850                                              900
TCTGCTTCAAAAAGAGAGTTGGTTGAATGAGACTGAAACCTTCTTTCTTCGAGGCGGG-A
TCTGCTTCAAAAAGAGAGTTGGTTGAATGAGACTGAAACCTTCTTTCTTCGAGGAGGGCA
      850                                              900

      909                                              958
CTCATTCTCCTGTTGC-CTTACTAATTCCTGATCAAGCCCCTGCCTCCATATGTCGAGTT
CCACTTCGACTGATGCGGGCGCGGTTCCCTCGTCGTGAAATAAGTAATGAAAGGGAAGAC
      910                                              960

      968                                             1015
GAGCATCTCGT--ACCATATCCCAGGCGCGATTGCCTACTTTTCCCTCCAGCTCGC-GCA
CCGCCCCTGGTCAACTTTATTGTTGCCTGTATAGCTTTTTAGAACAGAGATCTAGCAACG
      970                                             1020

     1025                                             1075
CCTCCCAATCGATGCGGGCGGCTAGTTCCCGCCCACGGTAGGGAGACCTCTCGTTCTCCA
CATTCTAGGCACAGATCATTGGTA-TTGGATGTATCGTTAGGTTGTGC-CGAGTCAAATC
     1030                                             1078

     1085
GGTTACGTTCCATTTCCTGGATCC
TGCTCCGTTTTTTTTTTTCGTTGC
     1088
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Figure 7. Origin of the 3.0-kb EcoRI Restriction Fragment.

(A) Organization of the pAr5 probe and the 3.0-kb EcoRI fragment. The filled-in region of pJM3.0 represents the sequence that is not homologous to λbAt006. The open boxes show the location of the regions similar to ORF143 of wheat and ORF25 of maize. Abbreviations are as given for Figure 2.

(B) Comparison of the nucleotide sequences of pAr5 (bottom) and pJM3.0 (top) in the region of the rearrangement. The sequences are numbered from the right EcoRI site of pJM3.0. The sequences of the 1102-bp BamHI-EcoRI fragment in pJM3.0 and the 2250-bp BamHI fragment in pAr5 were submitted to GenBank as accession numbers M88259 and M88258, respectively.

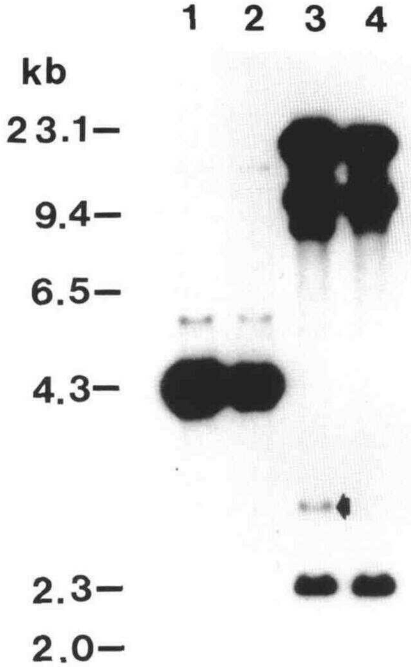

Figure 8. DNA Gel Blot of Wild-Type and *chm-3* Total DNA Digests Hybridized with the 1.9RB Subclone from pJM3.0.

The 1.9RB subclone has no homology to λbAt006. Lanes were loaded as given for Figure 6. The arrow indicates the 3.0-kb EcoRI restriction fragment.

The 1.9-kb EcoRI-BamHI (1.9RB) and the 1.1-kb BamHI-EcoRI (1.1BR) restriction fragments of pJM3.0 were subcloned into plasmids and used as probes. The 1.9RB subclone, containing a sequence nonhomologous to pAr5, was used as a probe in DNA gel blots of wild-type and variegated total DNA digests, as shown in Figure 8. As expected, it hybridized with the new 3.0-kb EcoRI fragment (Figure 8) and, with stronger signal, to 21.5-, 11.5-, and 2.3-kb EcoRI fragments. Those fragments are approximately the same length as some of the minor fragments detected by the 1.2BR subclone of pAr5 (Figure 6A) and could correspond to the same restriction fragments. The 1.9RB subclone did not hybridize to any of the other restriction fragments only found in the variegated plants. A sequence similarity search performed between the regions of the Arabidopsis mitochondrial genome already sequenced in A. Brennicke's laboratory and the 205 bp of 1.1BR that are not homologous to pAr5 (Figure 7B) located this fragment within

the Arabidopsis mitochondrial genome in a cosmid overlapping with the cosmid containing pAr5 (V. Knoop and A. Brennicke, personal communication). Therefore, the distance between the sequences involved in the rearrangement appears to be less than 40 kb.

DISCUSSION

Nuclear Mutations at the *CHM* Locus Cause the Cytoplasmically Inherited Variegation

We have characterized a new allele of the *CHM* locus that appeared spontaneously in the progeny of a plant regenerated from tissue culture. The new allele, *chm-3*, produced a variegated phenotype that was similar, in terms of inheritance (Tables 1 and 2) and size and color of the chlorotic sectors (Figure 1), to the two previously described alleles (Redei, 1973). Completely albino sectors in variegated plants also grew poorly and showed male sterility as described by Redei (1973) for the *chm-1* and *chm-2* alleles. We have not observed other cytoplasmically inherited phenotypes.

There are several previous reports of nuclear mutations giving rise to cytoplasmically inherited variegation (Potrykus, 1970; Epp, 1973; Walbot and Coe, 1979; Thompson et al., 1983). In the best characterized examples, the nuclear mutations seem to affect the function and genetic stability of the chloroplasts in an irreversible way (Walbot and Coe, 1979; Chiu et al., 1990). Nuclear mutations that produce a cytoplasmically inherited variegation because of their effect on mitochondrial stability and function have not been reported. However, as noted later, the presence of specific alterations of mtDNA has been correlated with variegation of green tissue in maize (Newton and Coe, 1986; Newton et al., 1990; Hunt and Newton, 1991) and has been shown to affect chloroplast structure and function (Roussell et al., 1991). Moreover, although a specific locus has not been identified, there is genetic evidence suggesting an effect of certain nuclear genetic backgrounds on the origin of the mtDNA alterations (Newton and Coe, 1986; Newton et al., 1990). The possibility that mutations at the *CHM* locus could produce mitochondrial alterations was not considered in the original report on this locus, although the phenotypic and genetic data presented are compatible with that hypothesis (Redei, 1973).

Mutations at the *CHM* Locus Promote Rearrangements in the Mitochondrial Genome

DNA gel blot analyses of total DNA from variegated and wild-type plants using both chloroplast and mitochondrial probes indicated the presence in the variegated plants of additional restriction fragments, collectively designated as the mtDNA RFLP, that hybridized with the *atp9* and pAr5 mitochondrial probes (Figure 2). These new restriction fragments were cytoplasmically inherited (Figure 3), excluding the possibility that they were the result of mtDNA transfer to the nuclear genome. Transfer of genetic information from the mitochondria to the plastid genome has so far not been reported (Schuster and Brennicke, 1988), and our limited DNA gel blot analysis has not detected any evidence indicating that the chloroplast genome is also affected in this mutant. More extensive analysis performed in the laboratory of G. Redei has not revealed any evidence of gross alterations of chloroplast DNA (G. Mourad, personal communication) in homoplastidic mutant lines (Mourad, 1987).

Two lines of evidence indicate that the mtDNA RFLP is specifically produced as a consequence of nuclear mutations at the *CHM* locus: (1) The new restriction fragments were always present in the F_3 progeny of F_2 plants homozygous or heterozygous for the *chm-3* allele, but were never observed in the progeny of F_2 plants homozygous for the wild-type allele (Figure 4). (2) Plants homozygous for the independently isolated *chm-1* allele showed restriction fragments of identical size when total DNA was hybridized with the pAr5 probe (Figure 3). The fact that two independently isolated alleles of the *CHM* locus induce the formation of three EcoRI restriction fragments of the same size strongly suggests that these restriction fragments are the result of highly specific rearrangements of the mitochondrial genome.

The results of the hybridizations, performed with two subclones of the pAr5 probe, indicated that all the EcoRI restriction fragments, typical of variegated plants, are the result of rearrangement events involving a common region of the λbAt006 clone (Figures 2 and 6). This extent was confirmed by cloning and sequencing the 3.0-kb EcoRI fragment (Figure 7). Every rearrangement event should theoretically give rise to one or two detectable restriction fragments, depending on the amplification of the two products of the rearrangement and the restriction enzymes and probes used. Therefore, the fact that three new EcoRI restriction fragments are observed suggests that at least two different rearrangements take place in the same region of the mitochondrial genome. These rearrangements could be the result of homologous recombination. This hypothesis is supported by the fact that the 1.9RB subclone from pJM3.0 hybridizes strongly with three EcoRI restriction fragments of 21.5, 11.5, and 2.3 kb (Figure 8) that correspond in length to the fragments showing low hybridization signal when probed with the 1.2BR subclone from pAr5 (Figure 6A). Their lower hybridization signal could be due to limited similarity with the probe. If the mtDNA RFLP is the result of homologous recombination between specific repeated sequences, the kind and number of possible recombinant molecules that can appear in the mutant genetic background must be limited, thus limiting the mutator capability of the nuclear locus.

The presence of repeated sequences in the mitochondrial genome has been frequently documented in several plant species (Lonsdale et al., 1984; Rottman et al., 1987; Makaroff et al., 1991). A few of these families are actively involved in high-frequency recombination generating a series of subgenomic molecules characteristic of the multipartite organization of the plant mitochondrial genomes (Levings and Brown, 1989). The other families of repeats do not recombine so frequently, and/or

their recombination products are not efficiently amplified (Small et al., 1989). However, infrequent recombination events between those repeats have been suggested to be the cause of some mitochondrial mutations (Newton et al., 1990) and could play an important role in the evolution of mitochondrial genomes (Small et al., 1989).

There are several reports that demonstrate the influence of the nuclear genetic composition in the production of heritable changes in the mitochondrial genome. The NCS mutations of maize, which involve alterations in mtDNA, are recovered at higher frequency in specific nuclear genetic backgrounds (Newton and Coe, 1986). The NCS5 mutation of maize has been shown to be associated with a recombination event that deletes part of the gene encoding subunit 2 of cytochrome oxidase (Newton et al., 1990), whereas the NCS3 mutation corresponds to the deletion of two mitochondrial ribosomal protein genes (Hunt and Newton, 1991). The nuclear genetic background has also been implicated in the frequency of reversion of the S male sterile cytoplasm (CMS-S) to fertile cytoplasm through mitochondrial rearrangements (Laughnan and Gabay-Laughnan, 1983; Escote-Carlson et al., 1990). However, in both examples, the responsible nuclear loci remain to be identified. The common bean fertility restorer gene (*Fr*) is a well-characterized example of a nuclear gene that produces inheritable alterations in the mitochondrial genome (Mackenzie et al., 1988; Mackenzie and Chase, 1990). When a dominant allele of this gene is crossed into a common bean CMS line, it results in a permanent restoration of fertility (Mackenzie and Bassett, 1987). This restoration has been correlated with the loss of a portion of mtDNA that could contain specific sequences responsible for the male sterility (Mackenzie and Chase, 1990; Johns et al., 1992). Because a new restriction fragment associating the regions flanking the excised DNA has not been observed (Mackenzie and Chase, 1990), these authors suggested that the *Fr* gene could promote the loss of an autonomous replicating subgenomic molecule. In a similar way, the *CHM* locus of Arabidopsis could prevent the appearance of the mtDNA RFLP by regulating the amplification of specific recombinant subgenomic molecules. Following this hypothesis, dominant alleles at both the *Fr* and *CHM* genes would prevent the amplification of specific subgenomic molecules that, when amplified, would result in male sterility or variegation. Alternatively, the product of the *CHM* locus could regulate the recombination frequency between specific regions of the mitochondrial genome. Nuclear genes with those features have been identified in humans (Zeviani et al., 1989) and fungi (Seidel-Rogol et al., 1989; Belcour et al., 1991), although the molecular mechanisms involved in the mitochondrial recombinations remain to be characterized.

Relationship between the Mitochondrial Rearrangements and the Variegation

Sequence analyses of clones pAr5 and pJM3.0 indicate that the rearrangement involves a region of the mitochondrial genome located between exon c of *nad5* and ORF25, downstream of sequences that are similar to the ORF143 of wheat mitochondrial DNA (Figure 7). Although this rearrangement does not seem to interrupt any identified ORF, it could disturb the transcription and/or mRNA processing of other ORFs in this chromosomal region. Recent reports indicate that *trans*-splicing events are involved in the processing of the *nad5* messenger RNA in different plant species, including Arabidopsis (Knoop et al., 1991; Pereira de Souza et al., 1991). The presence of a rearrangement downstream of exon c could affect its correct transcription and/or splicing. In addition, other rearrangements responsible for the origin of the other new restriction fragments could alter mitochondrial activity in different ways. More experiments are required to establish the molecular mechanisms that are responsible for the origin of mutant mitochondria.

Although our results do not preclude the existence in the *chm-3* mutants of chloroplast mutations responsible for the chlorosis, all the available evidence suggests that the rearrangements of mtDNA, produced by the *chm* mutations, are the cause of the morphological changes observed in the chloroplasts of these mutants (Redei, 1973; Redei and Plurad, 1973). Because the appearance of chlorotic tissue is always correlated with the presence of the mtDNA RFLP (Figures 3 to 5), putative mutant chloroplast would always have to segregate with the affected mitochondria, which seems highly unlikely. Moreover, both chloroplast and mitochondrial mutations would be produced by mutations in the same nuclear gene. This seems unlikely because there are no nuclear gene products known to enter both the chloroplast and the mitochondrion. The fact that yellow sectors, or even complete yellow seedlings, develop green sectors at high frequency (Figure 1) could be explained by sorting of mutant and wild-type mitochondria during cell division or by recombination between mitochondrial subgenomic molecules, which restores mitochondrial activity, followed by mitochondrial sorting. The appearance of green spots on pale yellow sectors (Figure 1B) suggests that mitochondrial genetic information is not completely lost, although rearrangements would impair mitochondrial gene function. The NCS2 mutation of maize produces an abnormal arrest of chloroplast development that alters its physiological function in different aspects (Roussell et al., 1991). It is likely that other NCS mutations also have pleiotropic effects on chloroplast structure and function because they produce different kinds of chlorotic tissue (Newton and Coe, 1986; Newton et al., 1990). These pleiotropic effects are probably not due to a single cause, but to the mitochondrial and chloroplast physiological interdependence within the plant cell (Roussell et al., 1991).

The molecular characterization and physical mapping of the mitochondrial genome of Arabidopsis is now in progress (W. Schuster, U. Eckert, and A. Brennicke, personal communication). This information will help in understanding the genomic rearrangements taking place in the *chm* mutants and to elucidate the mechanisms that regulate the complex structure of plant mitochondrial genomes. In addition, the molecular tools available to study this species should facilitate the eventual cloning and characterization of the corresponding *CHM* gene.

METHODS

Plant Material

The chloroplast mutator (*chm-3*) mutant was isolated as a variegated plant in the progeny of a transgenic Arabidopsis (ecotype Columbia) plant (Zhang and Somerville, 1987). The original mutant line was backcrossed twice as male parent with Landsberg *erecta* marker lines to incorporate morphological markers and once with Columbia wild type. The marker lines used were W1 (*an, py, er, gl1, cer2, ms1*) and W100 (*an, ap1, er, py, hy2, gl1, bp, cer2, ms1, tt3*). Both marker lines were derived from the Landsberg ecotype and were kindly provided by M. Koornneef. A mutant line homozygous for *chm-1* was kindly provided by G. Redei.

Growth Conditions

Plants were grown in pots containing a mixture of perlite, vermiculite, and sphagnum (1:1:1) and irrigated with mineral nutrient solution at 2-week intervals (Haughn and Somerville, 1986). Plants were grown at 22°C under continuous fluorescent illumination (100 μmol m^{-2} sec^{-1}). For the growth of albino plants, seeds were surface-sterilized and grown in sterile culture on Murashige and Skoog (1962) agar medium containing 1% (w/v) sucrose. Plates were incubated under continuous illumination (70 μmol m^{-2} sec^{-1}) at 23°C.

DNA Extraction, Cloning, and Sequencing

Total DNA from variegated and wild-type plants was purified as described by Leutwiler et al. (1984) from leaves of 3- to 4-week-old Arabidopsis plants. Total DNA from F_1 and F_3 progenies was prepared using 15 to 20 pooled plants from each progeny, including green and variegated ones, following the procedure of Dellaporta et al. (1983). To clone the 3.0-kb EcoRI restriction fragment from the variegated plants, 50 μg of total DNA from the *chm-3* plants was digested with EcoRI, and the restriction fragments were separated by preparative agarose electrophoresis. DNA eluted from the region of the gel corresponding to approximately 3.0-kb fragments was used to make a partial library in phage vector λGT10 (Huynh et al., 1985) using standard procedures. All phage and plasmid manipulations were performed using standard protocols (Sambrook et al., 1989). Sequencing was carried out following a modification of the dideoxy chain termination method of Sanger et al. (1977).

Restriction and Hybridization Analysis

Following digestion with restriction enzymes, the DNA restriction fragments were separated in 0.8% agarose gels and transferred to Hybond N^+ membranes following the conditions recommended by the manufacturer (Amersham Corp.). Membranes were prehybridized for 1 hr at 65°C in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.5% SDS, and 0.5 mg mL^{-1} denatured herring sperm DNA and hybridized for an additional 12 hr in the same solution with 10^7 cpm per mL of a ^{32}P-labeled probe. After hybridization, membranes were washed twice in 2 × SSPE, 0.1% (w/v) SDS at room temperature for 10 min, once in 1 × SSPE, 0.1% SDS at 65°C for 15 min, and two to three times in 0.1 × SSPE, 0.1% SDS at 65°C for 10 min. Chloroplast probes corresponding to the coding sequences of 70-kD chlorophyll apoprotein of photosystem I (2.618-kb SpeI-BamHI, coordinates 38302-40920), 50-kD chlorophyll apoprotein of photosystem II (0.723-kb NheI-KpnI, coordinates 75822-76545), and β subunit of CF_1-ATPase (*atpB*) (0.915-kb BamHI-NheI, coordinates 55921-56836) from *Nicotiana tabacum* (Shinozaki et al., 1986) and mitochondrial probes corresponding to α subunit of F_1-ATPase (*atpA*) (0.8-kb BamHI fragment), rRNA subunits 18s and 5s (rrn18s+5s) (3.0-kb PstI-EcoRI fragment), and subunit 9 of F_0-ATPase (*atp9*) (1.3-kb EcoRI-PstI fragment) from *Brassica campestris* (Palmer and Shields, 1984; Shirzadegan et al., 1989) were kindly provided by J. Palmer. The clone λbAt006 (Pruitt and Meyerowitz, 1986) and the pAr5 subclone, containing Arabidopsis mitochondrial sequences, were kindly provided by E. Meyerowitz. All the DNA sequences used as probes were gel purified from the vector sequences and labeled with α-^{32}P-dCTP by priming denatured DNA with random hexamers (Feinberg and Vogelstein, 1982).

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