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ORIGINAL PAPER

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Chloroplast mutations induced by 9-aminoacridine hydrochloride are independent of the *plastome mutator* in *Oenothera*

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Abstract *Oenothera* plants homozygous for the recessive *plastome mutator* allele (*pm*) show chloroplast DNA (cpDNA) mutation frequencies that are about 1,000-fold higher than spontaneous levels. The *pm*-encoded gene product has been hypothesized to have a function in cpDNA replication, repair and/or mutation avoidance. Previous chemical mutagenesis experiments with the alkylating agent nitroso-methyl urea (NMU) showed a synergistic effect of NMU on the induction of mutations in the *pm* line, suggesting an interaction between the *pm*-encoded gene product and one of the repair systems that corrects alkylation damage. The goal of the experiments described here was to examine whether the *pm* activity extends to the repair of damage caused by non-alkylating mutagens. To this end, the intercalating mutagen, 9-aminoacridine hydrochloride (9AA) was tested for synergism with the *plastome mutator*. A statistical analysis of the data reported here indicates that the *pm*-encoded gene product is not involved in the repair of the 9AA-induced mutations. However, the recovery of chlorotic sectors in plants derived from the mutagenized seeds shows that 9AA can act as a mutagen of the chloroplast genome.

Introduction

Mutators serve as genetic tools for the study of mutation avoidance and repair pathways (Miller 1998). Bacterial mutator genes encode proteins that contribute to the fidelity of DNA replication (e.g., *mut D* and *pol A* in *Escherichia coli*) as well as those that are involved in DNA repair (such as genes of the mismatch repair system: *mut H*, *L*, and *S* in *E. coli*). Studies with chloroplast mutator systems have sought similar characterizations of loci involved in chloroplast DNA (cpDNA) replication and/or repair pathways. One such mutator locus is defined by the nuclear-encoded *plastome mutator* (*pm*) allele in *Oenothera*.

The *plastome mutator* induces a high frequency of non-Mendelian chloroplast mutations when the *pm* allele is homozygous (Epp 1973; Sears and Sokalski 1991). Due to the high frequency of cpDNA mutations by the *plastome mutator*, the *pm*-encoded gene product has been hypothesized to have a function in cpDNA replication and/or repair. Studies by Blasko et al. (1988) and Chiu et al. (1990) showed restriction fragment length polymorphisms (RFLPs) in the chloroplast DNA (cpDNA) specific to the *pm* line. Subsequently, mutations caused by the *plastome mutator* were shown to occur at cpDNA microsatellite regions, resulting in the deletion or duplication of repeat(s) probably through replication slippage (Chang et al. 1996; Stoike and Sears 1998).

In order to test whether a general defect in chloroplast DNA repair or mutation avoidance is responsible for the *plastome mutator* activity, Sears and Sokalski (1991) looked for synergistic effects of ultraviolet light (UV light) and the chemical mutagen, nitroso-methyl urea (NMU), in several *pm* lines. No synergism in mutation induction was noted when the *pm* lines were irradiated with UV light, allowing the conclusion that the *pm*-encoded gene product is not involved in the repair of UV damage. In contrast, when the potent plastome mutagen NMU is applied to the *pm* lines, extremely high frequencies of mutation result, indicating a synergistic effect. Thus, the alkylation damage caused by NMU is probably repaired by a pathway that has the *pm*-encoded gene product as a component (Sears and Sokalski 1991).

Determination of whether the *plastome mutator* activity extends to the repair of damage caused by non-alkylating mutagens required a mutagen different from the well-characterized plastome mutagens such as NMU and *N*-nitroso-*N*-ethylurea (NEU) (Hagemann 1982). We chose to test 9-aminoacridine hydrochloride (9AA) for synergism with the *plastome mutator*. 9AA intercalates

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between the stacked nitrogen bases at the core of the DNA double helix (Nasim and Brychcy 1979). The intercalated 9AA mimics base pairs and thus causes deletions and additions of bases upon replication. In *Salmonella* (Hoffman et al. 1989; Kopsidas and MacPhee 1994) and *E. coli* (Gordon et al. 1991; Thomas and MacPhee 1985), 9AA causes single base-pair deletions or duplications that result in frameshift mutations, with preference for runs of mononucleotides (Skopek and Hutchinson 1984). Since an oligo-A stretch has been found to be a *plastome mutator* target in *Oenothera* (Chang et al. 1996), 9AA seemed to be a good choice to test for potential synergism with the *plastome mutator*.

In plants such as barley and onion, the application of 9AA has been reported to cause chlorotic mutations and growth retardation (D'Amato 1950, 1952). Therefore, as a first step in this study, our goal was to determine whether 9AA could cause similar mutations in *Oenothera* and/or could act specifically as a cpDNA mutagen. Once it was established that 9AA could cause mutations in the cpDNA, the next goal was to determine whether it could act synergistically with the *plastome mutator*.

Materials and methods

Plant material

Homozygous plants of *Oenothera hookeri* strain *Johansen* containing plastomes I, II, and IV were constructed originally by Professor W. Stubbe (University of Düsseldorf), and the lines were maintained by self-pollination. The *Johansen* strain containing either plastome I or II was used as the green parent in crosses with the variegated mutant A-E. Self-pollinations of the *pm*/*pm* line result in the accumulation of mutations (Chiu et al. 1990). Hence, efforts were taken to obtain a *pm*/*pm* line with a low background mutation rate. These seeds were produced by crossing a +/*pm* line as the female parent with a *pm*/*pm* line as the pollen donor which should result in an equal mixture of *pm*/+ and *pm*/*pm* seeds, with most plastids transmitted from the female parent.

9-Aminoacridine hydrochloride mutagenesis

After a 4-h imbibition period, seeds were surface-sterilized by incubating them in a solution of 50% bleach and 0.1% SDS for 30 m, followed by one rinse with 0.01 N HCl and several rinses with sterile water. To establish a dose curve, 9AA (Sigma) was added at specified final concentrations (see "Results") for 0.5 h, 8 h, 16 h, and 32 h. The seeds were swirled gently throughout the incubation period and rinsed several times before placing them in a beaker of sterile water where they were allowed to germinate. Control seeds were swirled for corresponding time periods in water.

Maintenance and scoring of plants

All the lines were maintained by leaf-tip cultures and by self-pollinations as described by Stubbe and Herrmann (1982). Seed germination medium was made according to Chiu et al. (1990). For the mutagenesis experiments, seedlings were planted in multipots (Hummert Seed Co.); the trays were placed under aquarium lights (GE) and were checked periodically for chlorotic sectors and other developmental abnormalities.

Transmission electron microscopy

Preparation, sectioning, and microscopy of leaf samples was performed by the MSU Center for Electron Optics.

Statistical analyses

To discern whether the 9AA treatment significantly altered the mutation frequency in the mixed *pm*/*pm, pm*/+ line, the data contained in Table 3 were analyzed by applying the chi-square goodness-of-fit test to the numbers of observed and expected mutations. If the chemical and the genetic condition independently cause mutations, then the following formula should predict the expected number of mutations: ([*CN*]+[*NP*])−(*CP*[½*N*])=Expected, where *N* is the population size of the *pm*/*pm*, *pm*/+ seeds, *C* is the frequency of mutations in the chemically treated wild-type plants, and *P* is the observed frequency of mutations of *pm*/*pm*, *pm*/+ alone without any chemical treatment. *CP* would give the expected value of both causing a mutation in the same plant, but is applicable to only half of the population, as half of the seeds are *pm*/*pm*.

Total DNA isolation

Total DNA was isolated from *Oenothera* leaves using a protocol adapted from Rether et al. (1993) and Stoike and Sears (1998). CTAB extraction of whole plant DNA was done according to the protocol adapted from Doyle and Doyle (1987) and Stoike and Sears (1998). After the DNA was precipitated with isopropanol, the pellet was resuspended in sterile water and a Caylase treatment was then performed to counteract the high polysaccharide content of the plant DNA according to Rether et al. (1993).

Isolation of DNA from a mitochondria-enriched fraction

For Southern analysis, DNA was isolated from a mitochondria-enriched fraction using a protocol adapted from Sears et al. (1989). The leaf tissue from the plants was first ground using a ten-fold vol/wt ratio of homogenization medium (50 mM TRIS, 6% sorbitol, 6 mM EDTA, 0.1% BSA, 0.3% polyvinyl polypyrrolidone, 1 mM ascorbic acid and 3 mM cysteine) to leaf tissue. The mixture was filtered through two layers of cheese cloth and one layer of miracloth, and the filtrate was centrifuged in a Sorvall centrifuge using a GSA rotor at 5,900 g for 6 m to remove chloroplasts and starch. The supernatant was centrifuged at 25,000 *g* to pellet the mitochondria. The mitochondrial pellet was then resuspended in 3 ml CTAB buffer and DNA was extracted using the protocol described above.

PCR amplification

The *oriB* primers and conditions for amplifying the *16S* rRNA-*trnI* region of the cpDNA of *Oenothera* are described by Stoike and Sears (1998), except for the annealing temperature, which was set at 48°C. The PCR products were run on a 1% agarose gel along with a 123-bp DNA ladder (GIBCO BRL) and photographed using a Polaroid camera. The gel picture was digitally scanned using a Hewlett Packard ScanJet 4C and displayed using the Adobe Photoshop software (San Jose, Calif.).

Southern analysis

The DNA from the mitochondria-enriched fraction was digested with *Bam*HI (GIBCO BRL) and then electrophoretically separated on a 0.8% agarose gel at 60 V for approximately 8 h. The DNA was transferred overnight to a nylon membrane according to the method of Sambrook et al. (1989). The membrane was probed with a radioactively labeled CMS-Sprite cosmid clone 651-12C2 (containing 12 random mitochondrial DNA fragments from *Phaseolus vulgaris*) using the standard nick translation method described by Sambrook et al. (1989); the ^{32}P-dATP used for the labeling was obtained from Andotek Sciences Co. The filter was washed according to the method described by Sambrook et al. (1989), and then exposed to film (Kodak X-O-Mat) at −80°C for 17 h.

Results

9-Aminoacridine hydrochloride dosage trials on wild-type line

In *Oenothera*, different plastid DNA (plastome) types (I, II, III, and IV) are transmitted differentially in crosses and also have specific RFLPs associated with them (Chiu et al. 1988, 1990). Previous studies have shown that the different plastome types are equivalent targets for the *plastome mutator* (Chang et al. 1996; Sears and Sokalski 1991). The different plastome types also responded similarly to preliminary 9AA dosage trials to determine the concentration and the time interval that would result in the highest frequency of mutations. Complete data from the dosage trials from only plastome II are reported here (Table 1) since the mutant plant (A-E) used for further characterizations was isolated from one of these trials.

In two sets of experiments, 500 seeds were treated with 0-, 2-, or 10-μg/ml concentrations of 9AA for 0.5–32 h (Table 1). Mutant sectors were observed at both the 2- and 10-μg/ml concentrations when the seeds were exposed for 16.5 h. In the preliminary trials, mutations were observed in seeds incubated for other periods of time, but their most frequent occurrence was always after a 16-h treatment.

Mutant phenotypes from acridine treatment

The 9AA treatment produced a spectrum of pigment mutations in *Oenothera*, with white, yellow, and light green mutations being observed as both solid and mottled sectors in leaves. Developmental anomalies such as abnormal leaves with fused midribs and plants without trichomes were also observed in the 9AA treated seedlings, but these were transient and never seen beyond the second set of leaves. Because so few mutants were recovered, only one segregated into the germline and could be analyzed by crossing. This mutant was labeled "A-E" and had pale yellow sectors.

Table 1 Sector frequencies for wild-type seedlings containing plastome II treated with 0-, 2-, and 10-μg/ml concentrations of 9AA for different exposure times. Each trial contained 500 seeds, except for one treatment, which contained 512 seeds

9AA (μg/ml)	Exposure time (h)	Percentage germination	Percentage viability	Mutation frequency[a]
Experiment 1				
0	32	41.8	40.2	0
10	1[b]	100	55.9	0
10	6.5	72.6	53.4	0
10	16.5	93.2	68.2	3/318 (0.9%)
10	32	80.6	59.3	0
Experiment 2				
0	32	92.0	63.9	0
2	1	90.4	83.8	0
2	6	90.2	83.3	0
2	16.5	75.2	93.6	3/352 (0.9%)
2	32	92.6	86.2	0

[a] $\text{Mutationfrequency} = \frac{\text{Numberofseedlingswithsectors}}{\text{Numberofviableseedlings}}$

[b] Trial contained 512 seeds

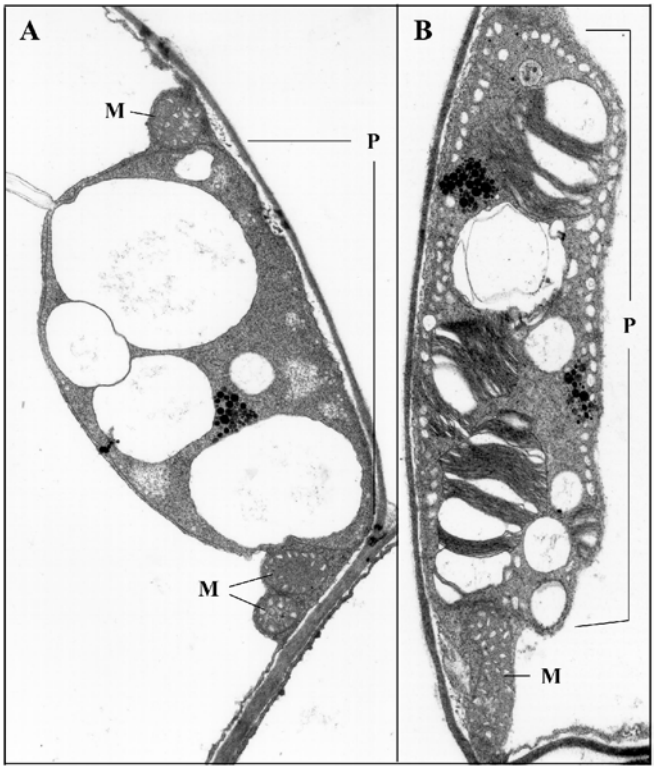

Fig. 1A, B Electron micrographs of plastids (*P*) with aberrant thylakoid structures and mitochondria (*M*) from the mutant yellow sectors of A-E

Transmission electron microscopy

The mutant A-E was examined by transmission electron microscopy (TEM) to characterize organelle ultrastructures. The plastids in the mutant sectors contained swollen vesicles along with either no (Fig. 1A) or few grana stacks

(Fig. 1B). In contrast, the mitochondria in the mutant sector appeared normal (Fig. 1A, B).

Inheritance of the acridine-induced mutation

Initial inheritance studies showed non-Mendelian inheritance and vegetative segregation of the A-E trait, indicating that the lesions caused by 9AA could reside either in the cpDNA or mitochondrial DNA (mtDNA). In *Oenothera*, the differential pattern of transmission of an organelle mutation through a cross can be used to determine whether the mutation lies in the cpDNA or mtDNA. The mtDNA is inherited only from the maternal parent (Brennicke and Schwemmle 1984), whereas the cpDNA can be inherited from both parents (Stubbe and Herrmann 1982). Therefore, when the mutant plant is used as the source of pollen, the progeny should not inherit the mutation if it is in the mtDNA.

In *Oenothera* plants with non-Mendelian mutations, if a leaf containing a full periclinal chimera occurs at a floral node, the germline of the adjacent flower consists entirely of mutant organelles (Kutzelnigg and Stubbe 1974). However, the mutant plant A-E had a partial periclinal chimera with both wild-type and mutant organelles present in the germline. In crosses using mutant A-E as the maternal parent, 103 out of the 205 progeny inherited the mutant organelle, and most of the progeny were variegated (Table 2). In the reciprocal crosses with A-E as the male parent, no variegated progeny were recovered.

Co-segregation of the mutant phenotype analyses using molecular markers

Data from the TEM studies indicated that the chloroplast was the affected organelle in the 9AA treated mutant plant A-E, whereas the transmission patterns of the mutation in reciprocal crosses suggested that the 9AA-induced mutation could be in the mtDNA. However, in *Oenothera*, when a weak plastome type is used as the male parent in a cross, the plastids may be transmitted only or predominantly from the maternal parent (Chiu et al. 1988). Therefore it could not be determined with confidence which organelle genome carried the 9AA-induced mutation. In order to establish whether the mutation in A-E co-segregated with the cpDNA, the green and yellow sectors from variegated progeny were dissected and DNA was extracted separately, so that a co-segregation analysis could be conducted.

Chloroplast DNA marker

To determine whether the yellow sectors in the progeny from the cross (A-E × wild-type) co-segregated with the physical markers on the cpDNA, PCR amplification using primers flanking the *oriB* region was performed with DNAs from the green and yellow sectors of the progeny as well as from the wild-type and the mutant parent (A-E). Figure 2 shows that the cpDNA marker in the yellow tissue in the progeny plant (lanes 5 and 6) is identical to the band of plastome type (II) from the mutant parent A-E plant (lane 2). The green tissue from the progeny plant (lanes 3 and 4) showed heteroplasmy as indicated by the presence of PCR products similar to those from both the wild-type and the mutant parent (lanes 1 and 2, respectively). A middle band was seen in the DNAs amplified from the green sectors. This band was also observed from a mixing experiment using two different plastome types (not shown); it most likely represents heteroduplex molecules.

Table 2 Crosses to establish the inheritance pattern of the 9AA-induced organelle mutation in A-E. The table lists the number of seeds obtained and the fraction that germinated. The F_1 progeny that germinated showed 100% viability, but varied in the inheritance of the A-E mutation

Parents[a]		Number of seeds	Percentage germination of F_1	Progeny phenotypes			
Maternal	Paternal			Mutant	Variegated	Green	N
Variegated (II) 1/2 mutant 1/2 green	Green (I)	396	37.6	2 46.5%[b]	68	79	149
Variegated (II) 1/2 mutant 1/2 green	Green (IV)	100	32	2 59.4%[b]	17	13	32
Variegated (II) 1/2 mutant 1/2 green	Green (IV)	24	100	5 58.3%[b]	9	10	24
Green (I)	Variegated (II) <1/2 mutant >1/2 green	390	87.7	0 0%[b]	0	342	342
Green (IV)	Variegated (II) 1/2 mutant 1/2 green	109	91.7	0 0%[b]	0	100	100

[a] A variegated plant with a heteroplasmic germline transmitted the A-E organelle mutation; the other parent contributed the green wild-type plastids, with Roman numerals indicating the plastome types
[b] Progeny with mutant tissue

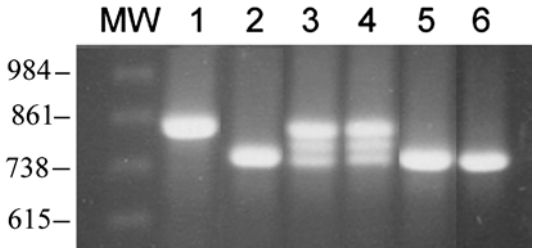

Fig. 2 PCR analysis of *16S rRNA-trnI* spacer region. From *left to right* on the gel, primers amplified through the *16S rRNA-trnI* region from the following *Oenothera* cpDNAs: *lane 1* green wild-type (wt) parent with plastome type I, *lane 2* mutant variegated parent A-E with plastome type II, *lanes 3 and 4* replicate samples of green tissue from a progeny of the cross A-E × wt, *lanes 5 and 6* replicate samples of yellow sectors from a progeny of the cross A-E × wt. The *lane MW* shows the DNA size markers (in bp) from the GIBCO-BRL 123-bp DNA ladder

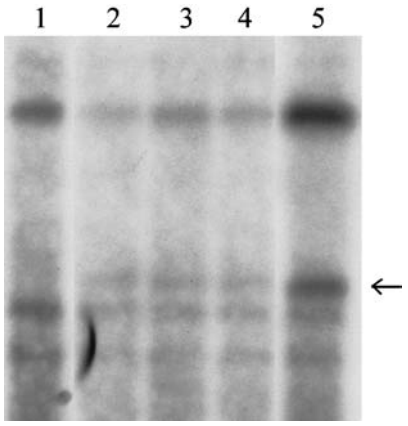

Fig. 3 Southern-hybridization analysis using DNA from the mitochondria-enriched fraction, extracted from the green and yellow sectors of the progeny and also from the parents of the A-E × wt cross. The *numbered lanes* contain *Bam*HI-digested DNAs from the following plants: *lane 1* green wild-type parent, *lanes 2 and 3* green sectors of the progeny from the cross, *lane 4* yellow sectors from the progeny, and *lane 5* the mutant variegated parent, A-E (5). The mtDNA of the two parents used for the cross is distinguished by a single band (indicated by an *arrow*) that is exclusive to the mutant parent A-E

Mitochondrial DNA marker

Southern analysis was conducted to test whether the unique mtDNA band of the mutant parent A-E (indicated by an arrow in Fig. 3) co segregated with the mtDNA of the yellow sectors in the progeny plants. For this purpose, DNAs from the mitochondrial-enriched fractions of the same samples used for the cpDNA analysis were extracted. These DNAs were digested and then blotted onto a membrane that was hybridized to a labeled mitochondrial cosmid probe (see Materials and methods). Southern-blot analysis showed no difference between the yellow and the green sectors from the progeny plant (lanes 2, 3, and 4), as both contained the band exclusive to the mtDNA of the maternal parent, A-E (lane 5), a result that is consistent with the strict maternal inheritance of the mtDNA. Although there was no RFLP between the mtDNA from the yellow and green sectors, the existence of a point mutation between them cannot be ruled out. However, the clear co-segregation of the cpDNA PCR product with the yellow sectors suggests that the A-E mutation is most likely on the cpDNA.

9-Aminoacridine hydrochloride dosage effects on the *plastome mutator* line

Seeds with a low background mutation rate were used to determine whether 9AA acts in synergism with *pm* to induce mutations in the cpDNA (see Materials and methods for details on how a *pm* line with low background mutation rate was produced). As a control, wild-type seeds containing the same plastome type as the *pm* seeds were also treated with the chemical for comparison. Mutant sectors were observed in both the 9AA-treated wild-type and the mixed *pm* seedlings (Table 3). Since the untreated *pm* line produces a high background of sectors, statistical methods were employed to assess whether the higher number of seedlings with sectors in the 9AA-treated *pm*-line reflected an additive or synergistic interaction of the two conditions (see Materials and methods). These analyses showed that the differences in the final mutation frequencies of the treated and control *pm*/*pm* plants were not statistically significant ($0.05<P<0.10$).

Discussion

The study described in this paper was undertaken to determine whether the *pm*-encoded gene product repairs the damage caused by frameshift mutagens, represented by 9AA, in order to delineate the role of the *plastome*

Table 3 Effect of *plastome mutator* on the sector frequency in seedlings containing plastome I treated with 9AA. Each trial contained 500 seeds with an exposure time of 16 h. Sectors were scored by the four-leaf stage

Nuclear background	µg/ml of 9AA	Percentage Germination	Percentage Viability	Number of seedlings with sectors (mutation frequency[a])
Wild-type	0	49.0	54.6	0
Wild-type	2	47.2	66.5	2/157 (1.3%)
Mixed +/*pm* and *pm*/*pm*	0	93.6	70.9	13/332 (3.9%)
Mixed +/*pm* and *pm*/*pm*	2	93.4	62.5	22/292 (7.5%)

[a] Mutation frequency determined using the formula described in Table 1

mutator in the replication and/or repair of cpDNA. To determine whether 9AA acts as a plastome mutagen in *Oenothera*, initial mutagenesis studies tested for conditions that would produce the maximum number of mutations, identified by the appearance of chlorotic sectors. Incubation of seeds in 9AA caused a low frequency of chlorotic sectors in the seedlings. Dosage trials established that increased exposure times or concentrations of the mutagen did not affect the germination or viability of the treated seedlings (Table 1), nor did those conditions produce a higher frequency of chlorotic sectors. These two characteristics may indicate that the mutagen lacks good penetration or potency.

The inheritance studies produced progeny with sectors indicating both vegetative segregation and non-Mendelian inheritance of the mutation. Therefore, the mutation was located in either the chloroplast or mitochondrial genome. In order to clarify which genome was targeted by the mutagen, several approaches were taken. TEM studies showed that the ultrastructure of the mitochondria in the mutated tissue was normal whereas the plastids were affected in the yellow sectors (Fig. 1). These observations indicated that the chloroplast was the likely target organelle of 9AA, although instances are known where mtDNA mutations affect plastid development and ultrastructure (for example, the NCS mutations in maize: Gu et al. 1993; Hunt and Newton 1991).

It is possible to distinguish between chloroplast and mitochondrial mutations in *Oenothera* by crosses: mutations that are carried by the cpDNA can be inherited from both parents, while mtDNA shows purely maternal inheritance (Brennicke and Schwemmle 1984). Initial data from crosses of one mutant (A-E) indicated maternal inheritance of the 9AA-induced mutation (Table 2); however, since the mutation only occupied a portion of the germ line, the data from the crosses remained inconclusive.

Because the crossing data were inadequate for establishing the target organelle of 9AA, co-segregation analysis using molecular markers was performed. As shown in Figs. 2 and 3, the mtDNA is inherited uniformly from the maternal parent, whereas in contrast, the yellow sectors co-segregated with the cpDNA from the mutant parent (A-E). The green sectors, on the other hand, contained chloroplasts from both the maternal and paternal parents. These data are most consistent with the plastome as the site of the mutation.

Since the data suggested that 9AA targets the cpDNA of *Oenothera*, its ability to act synergistically with the *plastome mutator* was explored. In the wild-type line, 1.3% of the seedlings showed mutant sectors after exposure to 9AA, whereas in the seeds that were half *pm*/*pm* and half heterozygote, a six-fold higher mutation frequency was observed (Table 3). However, most of these mutations can be credited to the *plastome mutator*, and our chi-square analyses using the adjusted expected number of mutations (See Materials and methods), indicate no statistical significance. This lack of synergistic effect implies that the *pm*-encoded gene product does not act upon the DNA lesions caused by 9AA. Because studies with prokaryotes have shown that the methyl-directed mismatch repair system (MMR) is involved in correcting 9AA-provoked mismatches (Miller 1998; Skopek and Hutchinson 1984), we conclude that the *pm*-encoded gene product is not likely a component of the cpDNA mismatch repair system.

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