Chemical and Plastome-Mutator Mutagenesis of *Oenothera*

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ABSTRACT

CHEMICAL AND PLASTOME-MUTATOR MUTAGENESIS OF *OENTHERA*

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In *Oenothera,* an elevated spontaneous mutation rate occurs when a *plastome mutator* allele (*pm*) is homozygous. Seeds of the *plastome mutator* line were treated with nitrosomethyl urea in an attempt to isolate antibiotic resistance mutations. The chemicals 9-aminoacridine hydrochloride, nalidixic acid, and novobiocin were used to test for synergism in the induction of chlorotic mutations. According to chi-squared analyses, acridine and nalidixic acid increased the *pm*-mutation rate in an additive fashion. In contrast, novobiocin had a synergistic effect on the mutation frequency of the *pm/pm* plant lines, while having little impact on the wild-type control. These results implicate subunit B of gyrase as the possible product of the *pm* gene.

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INTRODUCTION

Chloroplasts have a genome that codes for approximately 123 genes (Reviewed by Mullet 1988). The choroplast genome provides genetic information for components of the protein complexes present in the plastid and must rely on the nuclear genome to code for many of its components (Reviewed by Birky 1978 and Mullet 1988). The chloroplast depends on the nucleus to encode many gene products that are transported into the plastid. Should the nuclear DNA that codes for one of these gene products become mutated so that the gene product produced is damaged or lost it may severely disrupt chloroplast function. One such critical gene for the chloroplast genetic system is probably defined by the *pm*-locus of *Oenothera*.

In mutagenesis experiments performed by Mel Epp, *Oenothera hookeri* seeds were treated with ethylmethyl-sulfonate (EMS). He observed a plant with several different chlorotic sectors in the M2 generation (Epp 1973). Through reciprocal crosses and self-pollinations with this plant, Epp demonstrated that the mutations were initially induced by a recessive nuclear allele. However, the non-Mendelian inheritance of the variegated tissue indicated that the mutations occur in the

chloroplast genome (plastome). Once the chlorotic sector is induced, its expression is independent of the nuclear gene. This *plastome mutator (pm)* gene increases the frequency of spontaneous mutation in the plastome 200 - 1000-fold (Epp 1973; Sears and Sokalski, 1991).

The *plastome mutator* of *Oenothera* is known to induce mutations that appear as sectors having pigment deficiences. Sectors range from white to light green and may be mottled or solidly pigmented. These differences in pigmentation suggest that a variety of mutations are occurring, and are affecting a number of different loci. Studies by Blasko et al. (1988) and Chiu et al. (1990) have shown large deletions in chloroplast DNA (cpDNA) specific to the *plastome mutator* line. Two separate deletions studied by Blasko et al. (1988) occurred in a large open reading frame. Both *pm*-induced deletions were characterized from green, photosynthetically-competent plants. Chiu et al. (1990) reported that *pm* causes deletions at five hotspots on the cpDNA molecule including the site characterized by Blasko et al. (1988). Deletions at these sites occurred in green isolates as well as mutant lines. Thus, these separate studies concluded that the large deletions did not directly cause the mutant phenotypes and postulated that other small deletions or point mutations were responsible for the chlorotic sectors.

Sears and Sokalski (1991) investigated the *Oenothera plastome mutator* line by treating the seeds with nitroso methylurea (NMU), which has been shown to cause base substitutions in DNA (e.g., Richardson et al. 1987). They observed a synergistically high rate of mutation in the young seedlings. The interaction of the chemical mutagen and the *plastome mutator* line in the production of cpDNA mutations points to the likelihood that there is a defect in the cpDNA repair system. However, these data are not entirely consistent with the previous observations that the *plastome mutator* elevates spontaneous deletion frequencies in the cpDNA, since such deletions would likely result from a defect in a different cpDNA repair pathway. On the other hand, perhaps several types of mutation would result from erroneous replication or repair mechanisms from a defect in: replication bypass, fidelity of the replication system, specificity of the damage detection and removal systems, and DNA breaking and joining reactions. If a mutator allele codes for a disfunctional protein (or no protein at all) that is a component of any of these systems, it is conceivable that an increase in several types of mutations would be seen.

My goal was to further test the *plastome mutator* line by using the chemical mutagen, acridine, which induces DNA lesions different from those caused by NMU. If a synergistic response is again elicited, it would indicate that a defect

in a general cpDNA repair pathway is the most likely cause for the increased frequency of mutation induced by the *plastome mutator*. Similar experiments were performed with the gyrase inhibitors, nalidixic acid and novobiocin. If a change in the *pm*-induced mutation frequencies is observed with those chemicals, it would indicate that supercoiling of the cpDNA helix was involved in the generation of mutations. Data collected from these investigations may allow determination of a role for the *plastome mutator* gene product in replication or repair of cpDNA.

CHAPTER 1

NMU MUTAGENESIS AND SELECTION OF ANTIBIOTIC RESISTANCE SECTORS IN *OENOTHERA*

INTRODUCTION

Many of the plastome mutants of higher plants originated spontaneously or were induced by nuclear 'mutator' genes (Boerner and Sears 1986). The induction of mutations by chemical agents was originally investigated in studies of prokaryotic and eukaryotic systems, but was subsequently adapted to organelle genetic systems. Chemical mutagenesis with alkylating agents, particularly nitroso-compounds, has been the most effective means for inducing chloropast mutations in a wide range of plants, including *Antirrhinum* (Hagemann 1982), *Nicotiana* (Fluhr et al. 1985), *Brassica* (Walters et al.1990) and *Oenothera* (Sears and Sokalski 1991).

One of the common characteristics of chemical mutagens is their ability to form covalent products with DNA and thus interfere with the process of DNA replication and repair. Misreplication or misrepair of DNA can lead to incorporation of incorrect bases and the fixing of mutations in the DNA. The error produced by the chemical is either corrected or results in a permanent alteration in the DNA.

The damage by alkylating agents consists of the addition of alkyl groups (usually methyl or ethyl groups) to reactive sites on the bases or phosphates of the DNA. The methylation causes atypical base pairing during replication or repair.

Nitroso-compounds are well studied mutagens in many other genetic systems. Humans exposed to these types of chemicals exhibited liver damage and with prolonged dosage, hepatocellular carcinoma (Magee and Barnes 1956). Rats have shown chromosomal damage (carcinogenesis, chromosone aberration in somatic cells, polyploidy) after treatment (Monakhou et al. 1990). Genetic studies with bacteria and bacteriophages have shown that many point mutation induced by alkylating agents involve guanine to adenine transitions (Mutagenesis; Drake, Koch 1976; Loveless 1969; Richardson et al. 1987). One experiment showed that all of the NMU-induced mutations in *Escherichia coli* were GC:AT transitions and that of these transitions, 82% of the mutations occurred at the

middle guanine of the sequence 5'-GG(A or T)-3' (Richardson et al. 1987). The transitions are a consequence of alterations in the normal base pairing during replication due to the methylation of guanine at the N-7 position which is the most reactive (Lawley and Brooks 1961; Nagata, Imamura, Saito, Fukui 1963) or the O-6 position (Castellani 1987). Other oxygens and nitrogens can be methylated on the remaining bases, but they are not as highly reactive as the guanine base.

Prokaryotic and eukaryotic cells have repair systems to deal with damage to DNA. All of the systems use enzymes to repair the damaged bases, which are normally eliminated, bypassed or repaired by the organism. In addition, to deal with alkylation damage, prokaryotes have the enzyme O^6-methylguanine methyltransferase which recognizes the alkylated base in the DNA and removes the methyl group (Lawley 1961 and Castellani 1987). This changes the base to its original form.

NMU has been used in *Oenothera* to study mutation frequency (Sears and Sokalski 1991) and in *Brassica* to recover a cytoplasmic marker mutation, maternally inherited variegation (Walter et al. 1990). These mutational lesions were visualized as chlorotic sectors in the plant tissue. In *Nicotiana* (Fluhr et al. 1985) and other *Solanaceae* (McCabe et

al. 1989), the mutagen was used to induce antibiotic resistance. Antibiotic levels were used for selection that caused wild-type seedlings to exhibit chlorosis; the presence of antibiotic-resistant ribosomes in the chloroplast could be visualized as green sectors in the chlorotic background. Chloroplast ribosomes are similar to prokaryotic ribosomes, in terms of size and sensitivity to various drugs that inhibit protein synthesis. Many of the inhibitors of protein synthesis are familiar antibiotics, such as streptomycin, spectinomycin, and erythromycin. These antibiotics bind to sites on the ribosome that are defined by both the rRNAs and the ribosomal proteins. Thus, resistance to an antibiotic may occur by mutation in a gene for one of the rRNAs or for one of the polypeptides. All of the chloroplast rRNAs are encoded by the plastome; many of the ribosomal proteins are encoded in the nuclear genome while the remaining are encoded in the plastome (Shinozaki et al. 1986).

Antibiotics, such as streptomycin, chloramphenicol, tetracycline, and erythromycin inhibit protein synthesis in prokaryotes by different mechanisms. Streptomycin interacts with the site containing the protein S12 in the 30S ribosomal subunit. This antibiotic interferes with the initiation of protein synthesis and also causes the mRNA to be misread by allowing different amino acids to be incorporated into the polypeptide (Galili et al. 1989). Mutants in *E. coli* have

been obtained that show streptomycin resistance conferred by a C to U transition at position 912 of 16S rRNA (Montandon et al. 1986, Moazed and Noller 1987, Gauthier et al. 1988) and in *Nicotiana* there is a mutation in the 16S rRNA gene of the SPC23 line at position 860 (C to A) that confers streptomycin resistance (Etzold et al. 1987).

The antibiotic spectinomycin inhibits translocation of peptidyl-tRNA from the A site to the P site during early rounds of peptide bond formation. Resistance to spectinomycin has been shown to be conferred by mutations in ribosomal protein S5 and by a C to U transition at position 1,192 in the 3′ major domain of 16S rRNA (Moazed and Noller 1987). In *Nicotiana,* the SPC1 line had an A to U transition at position 1138 and the SPC2 line had a C to U transition at position 1139 both of which caused spectinomycin resistance and disrupted a conserved 16S rRNA stem structure (Svab and Maliga 1991). The SPC23 line had a mutation in the 16S rRNA at position 1333 (G to A) that caused spectinomycin resistance (Svab and Maliga 1991).

The investigation described in this chapter was designed to determine whether mutations caused by NMU could confer antibiotic resistance to *Oenothera* seedlings via mutations in the chloroplast genome. This experiment was modeled after that of Fluhr et al. (1985), in which antibiotic-resistant

mutants were induced and selected in *Nicotiana*. NMU has been used to successfully induce chlorotic plastome mutations in *Oenothera* (Sears and Sokalski 1991). My goal was to determine if NMU could also induce mutations that would result in antibiotic-resistance. Such mutants in *Oenothera* could be useful for inheritance studies and could be utilized for investigating aspects of the *plastome mutator* gene which are under intense study at the present time in our laboratory.

In my investigation, seeds of *Oenothera* were treated with the mutagen and placed on media containing an antibiotic. Because the chloroplasts are prokaryotic and their ribosomes are sensitive to the antibiotics, the plant tissue bleaches out or becomes chlorophyll-deficient under these circumstances due to the loss of the chloroplast's ability to translate RNA messages. If NMU induced mutations that caused the chloroplast ribosomes to become resistant to the antibiotic, green islands of plant tissue would appear on the bleached plant. This experiment has been successful in *Nicotiana*, where NMU mutagenesis of seeds results in a very high incidence of mutations to photosynthetic incompetence, and a definite (but low) frequency of streptomycin-resistant sectors on the seedlings' true leaves (Fluhr et al. 1985).

MATERIALS AND METHODS

Plant material. Homozygous plants of *Oenothera hookeri* strain Johansen containing plastome type II were constructed by Professor W. Stubbe (University of Duesseldorf). These lines were maintained by self-pollination.

Nitrosomethyl urea mutagenesis. Fungal contamination had to be controlled before proceeding with the experiment. Several modifications were incorporated: 1) placing the seeds in a sucrose solution to encourage fungal spore germination followed by daily surface sterilization with 50% bleach of the seeds, 2) using a 10% bleach solution on the germinated seedlings and 3) up to three daily surface sterilizations with 50% bleach solution before seed germination. The final method for the reduction of the fungus included an initial alcohol and flame sterilization of the seed capsule, followed by the three surface sterilizations.

After a four hour imbibition, seeds were surface-sterilized by incubation in a solution of 50% bleach and 0.1% SDS for 30 minutes, followed by one rinse with 0.01 N HCl and several rinses with sterile water. Nitroso methylurea (Sigma) was added at concentrations of 5-20 mM for 30 minutes. Since the NMU stock was dissolved in ethanol, the control seeds were soaked in 6.25% ethanol for a corresponding time period. This was followed by several rinses with sterile water before placing the seeds on seed media where they were allowed to

germinate. There were 50 seeds placed on each petri plate. When the second true leaves started to appear, approximately 2 weeks after germination, the seedlings were transferred to seed media containing streptomycin (400ug/ml), spectinomycin (10ug/ml) or lacking antibiotics. This should have allowed adequate time for mutations to sort out, since chlorotic secters can begin to be recognized 2-weeks post-NMU mutagenesis.

Seed media. Seed media was according to Nagata and Takebe (1971) with the following modifications: 0.5 x concentration of the major elements, 2% sucrose, and buffered with Hcl.

Maintenance and scoring of plants. The petri plates containing the seed media were sealed with parafilm, placed under broad spectrum lights(GE F40/PL/AQ), and checked periodically for seedlings with chlorotic sectors.

RESULTS

Optimization of seed sterilization procedures.

In the experiments performed with NMU, one-half of the seeds received a mock treatment with ethanol and were placed on three types of seed media: two types contained different antibiotics and one lacked antibiotics. The other half of the seeds were treated with the chemical, NMU, and also placed on the three media. In the first experiments the

fungal contamination was so pervasive that all seedlings were lost. Contamination rates ranged from 23.3% to 98%, comparisons between the control and the treated seedlings were not possible due to the extensive loss of some trials. Therefore, these experimental data are not included here.

As indicated in the materials and methods, three protocols were evaluated for control of fungal contamination. It was determined that the number of surface sterilizations was negatively associated with contamination. The final method for the reduction of the fungus included an initial alcohol and flame sterilization of the seed capsule, followed by the three surface sterilizations of the seeds.

NMU mutagenesis and selection for antibiotic resistance. *Oenothera* seeds were treated with NMU as described in the Materials and Methods section. After treatment, seedlings were placed on media containing the antibiotics streptomycin (400 ug/ml) or spectinomycin (10 ug/ml) and were allowed to grow. A dosage of 5mM NMU and the exposure time of 30 minutes was taken from previous trials, to optimize germination and seedling viability.

The seedlings were scored by examining the true leaves for green islands amidst bleached tissue. The plants on antibiotic media were bleached after approximately 25 days on

the media. Therefore, the scoring started at 25 days and continued for 60 days or until the seedlings were bleached completely and/or dead. It was observed that seedlings on streptomycin plates bleach slower than those on the spectinomycin plates.

Data on the effect of the mutagen on seed germination, viability, and green sectors are presented in Tables 1, 2 and 3. Although the treatment resulted in a high frequency of chlorotic sectors in the M1 seedlings on soil (data not shown), I recovered no bleached plants with persistent green sectors on the antibiotics.

Table 1. Sector frequency in seedlings treated with 5mM nitroso-methyl urea for 0.5 hours. Each trial contained 50 *wild-type* seeds.

			Seedling Leaf Coloration		
Seed media	Germination N %	Viability N %	Green N %	Bleached with green sectors	Bleached
control	50(100%)	49(98.0%)	49(100%)	0	0
w/strep[1]	50(100%)	46(92.0%)	0	0	46(100%)
w/spec[2]	50(100%)	29(58.0%)	0	0	29(100%)

[1] 400 ug/ml Streptomycin
[2] 10 ug/ml Spectinomycin

Table 2. Sector frequency in seedings treated with 5mM nitroso-methyl urea for 0.5 hours. Each trial contained 550 *wild-type* seeds, except for the control which contained 549 seeds.

			Seedling Leaf Coloration		
Seed Media	Germination N %	Viability N %	Green N %	Bleached with green sectors	Bleached N %
control	549(100%)	200 (36.4%)	200 (100%)	0	0
w/strep[1]	550(100%)	175 (31.8%)	0	0	175 (100%)
w/spec[2]	550(100%)	129 (23.5%)	0	0	129 (100%)

[1] 400 ug/ml Streptomycin
[2] 10 ug/ml Spectinomycin

Table 3. Sector frequency in the control seedlings treated with ethanol for 0.5 hours. Each trial contained 50 *wild-type* seeds. The plates containing the control and the streptomycin seeds were lost to contamination.

			Seedling Leaf Coloration		
Seed Media	Germination N %	Viability N %	Green	Bleached with green sectors	Bleached N %
control	50(100%)	0	0	0	0
w/ strep[1]	50(100%)	0	0	0	0
w/ spec[2]	50(100%)	50(100%)	0	0	50(100%)

[1] 400 ug/ml Streptomycin
[2] 10 ug/ml Spectinomycin

DISCUSSION

Experiments with *Nicotiana* have indicated that NMU induces plastome-encoded antibiotic resistant mutations, as well as chlorophyll-deficient mutations (Fluhr et al. 1985). In a previous study of NMU mutagenesis with *Oenothera*, sectors were recovered in up to 66% of the seedlings. My experiments were designed to test if a subset of mutations induced by NMU could cause the ribosomes of the *Oenothera* chloroplast to become antibiotic resistant.

It is known that NMU causes point mutations (Richardson et al. 1987), specifically transitions. Such a base substitution could change the nucleotide sequence of the rRNA or an amino acid in the sequence of a protein. Changes due to missense mutations are often minor; the protein with this damage will often fold up in its normal configuration or very close to it and retain its basic function. The significant effect concerning this change in amino acid sequence may be in the binding site for the antibiotics in the chloroplast ribosome, such that the antibiotic will not bind.

Although I was able to reproduce a high mutation rate from the application of NMU, I did not recover any antibiotic-resistant mutants. Four possibilities could explain this failure: 1) an inadequate sample size; 2) NMU does not target the right base to confer streptomycin- or

spectinomycin-resistance in *Oenothera;* 3) NMU does not cause base substitutions in *Oenothera* but causes other types of mutations, such as deletions; and 4) antibiotic binding sites of the *Oenothera* chloroplast ribosome are defined by many gene products, with an alteration in a single one not affecting antibiotic sensitivity.

To overcome these possible problems, I would suggest the following modifications to the experimental procedure: 1) a larger sample size, 2) trying a different mutagenic chemical that targets a different base in the DNA strand, 3) selection with different antibiotics.

CHAPTER 2

CHEMICAL MUTAGENESIS USING ACRIDINE, NALIDIXIC ACID AND NOVOBIOCIN

INTRODUCTION

Three chemicals were chosen to test as mutagens of the chloroplast genetic system: acridine, nalidixic acid, and novobiocin. These agents have been used by others to induce mutations and to perturb gyrase of higher plants. Studies on the mutagenic effects of 9-aminoacridine hydrochloride have used barley (D'Amato 1950; Eherenberg 1956), *Allium cepa* (D'Amato 1952, 1954; Nuti-Ronchi and D'Amato 1961), *Lycopersicum esculentum* (Buitti and Ragazzini 1966), and *Vicia faba* (Michaelis and Rieger 1963). Nalidixic acid and novobiocin experiments with *Solanum nigrum* (Ye and Sayre 1990), pea (Lam and Chua 1987), and *Nicotiania tabacum*

(Heinhorst et al. 1985) were done to inhibit gyrase and to observe its effects on chloroplast transcription.

Since the discovery of the *plastome mutator (pm)* in *Oenothera* by Mel Epp in 1973, researchers have been trying to unravel its mode of operation. Currently it is suspected that the *plastome mutator* interferes with repair or replication of chloroplast DNA. In keeping with this line of thought, several chemicals that target DNA metabolism in other organisms were used, to test whether the chloroplast genetic system of *Oenothera* would be susceptible, resulting in the appearance of chlorotic sectors in the seedlings.

The chemical mutagen, 9-aminoacridine hydrochloride, was first used on plants in 1950 by D'Amato, who treated barley seeds with it and saw a retardation of growth along with chlorosis. It is now known that this chemical intercalates between the stacked nitrogen bases at the core of the double helix (Nasim and Brychcy 1979).

The binding of 9-aminoacridine hydrochloride to DNA is non-covalent and its mutagenic action is vastly different than other acridines harboring bulky side-chains. 9-aminoacridine hydrochloride is a DNA-intercalating molecule that interacts preferentially with B-form DNA (Reviewed in Ferguson and Denny 1991; reviewed in Nasim and Brychcy 1979). Using X-ray

crystallography, Sakore et al. (1977; 1979) showed that 9-aminoacridine hydrochloride binds to the dinucleotide 5-iodocytidyl (3'-5') guanosine. The planar acridine molecules lie parallel to the plane of the base pairs. This intercalation of the molecule increases the length of the DNA helix and consequently reduces DNA twist at that site. This twist reduction unwinds the double helix and could affect the processes of replication, repair and/or transcription.

9-aminoacridine hydrochloride mimics base pairs, causing the deletion or addition of a base upon replication. Previous experiments with acridine in *Salmonella* (Hoffman et al. 1989; McCoy et al. 1981) and *E. coli* (Thomas and McPhee 1985; Pons and Muller 1989; Gordon et al. 1991) indicate that acridines induce single base-pair duplications and deletions that result in frameshifts. 9-aminoacridine hydrochloride has also been shown to be a frameshift mutagen in the lambda bacteriophage (Pons 1984). However, Levin et al. (1984) saw that 9-aminoacridine hydrochloride did not induce deletion revertants for *hisG428* by basepair substitution or small deletions of three to six basepairs. When 9-aminoacridine hydrochloride intercalates into the bacteriophage DNA it causes frameshifts, producing predominantly -1 frameshifts in runs of 3 or more identical G:C basepairs with 90% occurring at hot spots (Skopak and Hutchinson 1984).

Newton et al. (1972) synchronized chromosomal replication in *E. coli* and found that acridine-induced mutagenesis is associated with the DNA replication fork. Newton et al. also observed the same frequency of reversion in *wt*, *rec*A-, and *rec*B- strains. Similarly, Ferguson and MacPhee (1983) saw that in both *recA*+ and *recA*- strains of *Salmonella typhimurium*, 9-aminoacridine hydrochloride was an effective frameshift mutagen. Since the *recA* product is involved in both recombination and inducible error-prone repair these findings suggest that those processes are not involved in creating the mutations in response to the presence of 9-aminoacridine hydrochloride. Thomas and MacPhee (1985) included the *lexA* gene in their study, and concluded that it, too, does not impact the induction of mutations by 9-aminoacridine.

Regarding DNA repair, Ferguson and Denny (1991, pg. 144) state that the DNA damage caused by 9-aminoacridine hydrochloride does not appear to trigger common DNA repair systems (Podger and Hall 1984). Thomas and MacPhee (1985) examined the effects of *uvr*-dependent repair. They found that in *uvrB- E. coli*, 9-aminoacridine hydrochloride induced reversion of one *lacZ* frameshift mutation, but reduced the reversion frequency of a second *lacZ* frameshift mutation. They proposed that 9-aminoacridine hydrochloride stimulates a *uvr*-dependent excision, which then plays a role in the introduction of the frameshift mutations. Isamoto et al.

(1985) found that 9-aminoacridine hydrochloride did not induce mutations in mtDNA nor in nuclear DNA in *Saccharomyces cerevisiae*. Skopek and Hutchinson (1984) showed that a prophage is more susceptible to 9-aminoacridine hydrochloride mutagenesis in a *mutL* host that was deficient in mismatch repair than in a *wild-type* host. Using *Salmonella typhimurium* Hoffman et al. (1989) demonstrated by the presence of His^+ colonies and the lack of Trp^+ colonies on selective media that 9-aminoacridine hydrochloride was ineffective as an inducer of genetic duplications when a *hisC3076 uvrB*- strain was reverted.

In addition to determining if 9-aminoacridine hydrochloride could cause mutations in cpDNA, it was my goal to determine if distortions due to positive and negative supercoiling could be mutagenic. The rationale for this inquiry is that superhelicity influences many processes in repair and replication. Pommier et al. (1987) found that the 9-aminoacridine-induced DNA unwinding does not appear to inhibit mouse leukemia DNA topoisomerase II because 9-aminoacridine hydrochloride lacks bulky side chains.

All bacterial and eukaryotic cells possess topoisomerases that alter the topology of DNA by introducing supercoils

(topoisomerase II) and/or removing supercoils (topoisomerase I). Type II topoisomerase is also called DNA gyrase, and its activity is ATP-dependent.

Since chloroplast DNA (cpDNA) is a supercoiled molecule *in vivo*, for its replication to proceed, the DNA must be unwound at the replication fork by a helicase. The additional tension or supercoiling imposed on the cpDNA is released by the type I topoisomerase. To restore supercoiling to the closed, circular DNA molecule, the phosphodiester bond must be cleaved in both of the strands, the helix must be rewound, and closed again by repairing the broken bonds. This is the activity of the type II topoisomerases, represented by the bacterial gyrase (Reviewed by Cozzeralli 1977 and 1980). The chloroplast DNA gyrase resembles that of prokaryotes, which is composed of two subunits, A and B (Thompson and Mosig 1985; Lam and Chua 1987). Both subunits are required for the enzyme's activity. The A subunit contains a DNA binding site and mediates the ability to cut and rejoin double-stranded DNA. The B subunit binds ATP and is probably responsible for the introduction of negative supercoils into the double-stranded DNA (Lui and Wang 1978; Cozzarelli 1990).

DNA gyrase cleavage is highly site-specific (Sugino et al. 1977; Gellert et al. 1977). Analysis of sites showed that cutting between a TG doublet is common to nearly all gyrase

cleavages (Morrison and Cozzarelli 1979). When a gyrase cleaves a DNA strand it becomes covalently linked to a DNA phosphoryl group (Weintraub 1985; Wang 1986). Gyrase is thought to remain bound to DNA at discrete locations with ATP bringing about a conformational change in the enzyme.

Gyrase inhibitors interfere with both DNA replication and the initiation of transcription (Kubo et al. 1979). Because promoters are generally associated with palindromic DNA sequences it has been hypothesized that they loop out to form hairpin-like structures in supercoiled DNA (Gellert et al. 1976). In the absence of gyrase activity, supercoils cannot be generated, and hence the promoters would not be distinguishable. In contrast to these expectations, Thompson and Mosig (1987) found a *C. reinhardtii* chloroplast promoter activity stimulated by the DNA gyrase inhibitor novobiocin. Since novobiocin is known to reduce torsional stress in *E. coli* DNA (Thompson and Mosig 1985), they interpret their results to mean that their particular promoter is regulated by torsional stress in cpDNA.

Since Gellert's discovery of gyrase in 1976, the enzyme has been manipulated in a series of experiments using both novobiocin and nalidixic acid in various organisms, including *E. coli* (Gellert et al. 1976 & 1977; Sugino et al. 1977 & 1978), *Daucus carota* (Ciarrocchi et al. 1985), *C. reinhardtii*

(Thompson and Mosig 1985), and *Solanum nigrum* (Ye and Sayre 1990). With nalidixic acid treatment, a reduction in cpDNA synthesis was observed in *Euglena gracilis* (Lyman 1967), *N. tabacum* (Heinhorst et al. 1985), and *Solanum nigrum* (Ye and Sayre 1990). Novobiocin and nalidixic acid have not been used as mutagens in other organisms, but since gyrase plays such a critical role in DNA metabolism, I have attempted to determine if gyrase inhibitors may affect the mutation rate.

Gellert et al. (1976) found that novobiocin, an inhibitor of nucleic acid synthesis *in vivo*, is an inhibitor of DNA gyrase *in vitro*. Novobiocin interrupts ATP binding and may block ATP access without sharing binding sites (Cozzarelli 1990). When *Chlamydomonas reinhardtii* was treated with novobiocin (Thompson and Mosig 1984; Thompson and Mosig 1990) the superhelical tension in chloroplast DNA was reduced.

Nalidixic acid targets the A subunit of DNA gyrase in *E. coli*. This is accomplished through interference with the breakage-and-reunion component by stabilizing the enzyme-DNA cleavage complex (Gellert 1981). It is also likely that nalidixic acid does not just inactivate its target protein but corrupts it, converting it into a poison (Hsiang et al. 1985). The physiological effect of the inactivation of gyrase is the inhibition of replicative DNA synthesis, as observed after treatment with nalidixic acid in *Nicotiana*

tabacum (Heinhorst et al. 1985) and *E. coli* (Gellert et al. 1976; Cozzarelli 1977; Radl 1990).

Both chemicals, novobiocin and nalidixic acid (Gellert et al. 1977, 1976) have been shown to inhibit DNA gyrase *in vitro* in *E.coli*, and *in vivo* in *Daucas carota* (Ciarrocchi et al. 1985) and *Chlamydomonas reinhardtii* (Thompson and Mosig 1985, 1987). Thompson and Mosig (1985, 1987) saw changes in transcription with both agents. Novobiocin differentially affected transcripts; some increased and some decreased. Nalidixic acid caused many transcripts to become more abundant.

Ye and Sayre (1990) found that both novobiocin and nalidixic acid reduced cpDNA content of *Solanum nigrum* suspension culture cells. Neither chemical inhibited or reduced nuclear DNA content *in vivo*, indicating that a chloroplast localized gyrase is targeted.

The objective of the following experiments was to determine whether novobiocin or nalidixic acid would increase the frequency of spontaneous plastome mutations seen in wild-type *Oenothera*. In addition, the use of acridine, novobiocin, and nalidixic acid as mutagens might provide information relevant to the processes that affect the chloroplast genetic system.

Specifically, I hoped to determine if supercoiling of the cpDNA was essential for replication fidelity *in vivo*, and whether acridine could induce mutations in the chloroplast genetic system.

CHEMICAL TREATMENT

Materials and methods

Plant material. Homozygous plants of *Oenothera hookeri* strain Johansen containing plastome types I, II, and IV were constructed by Professor W. Stubbe (University of Duesseldorf). These lines were maintained by self-pollination.

9-aminoacridine hydrochloride mutagenesis. After a four hour imbibition, seeds were surface-sterilized by incubation in a solution of 50% bleach and 0.1% SDS for 30 minutes, followed by one rinse with 0.01 N HCl and several rinses with sterile water. To establish a dose curve, 9-aminoacridine hydrochloride (Sigma) was added at the specified final concentrations (see Results) for 30 minutes, 8 hours, 16 hours, and 32 hours. Since the 9-aminoacridine hydrochloride was dissolved in ddH_2O, the control seeds were soaked in ddH_2O for a corresponding time period. This was followed by several rinses with sterile water before placing the seeds in a beaker of ddH_2O where they were allowed to germinate.

Nalidixic acid mutagenesis. Seeds were surface-sterilized and germinated as described above. Since the nalidixic acid was dissolved in ddH_2O the control seeds were soaked in ddH_2O for a corresponding time period.

Novobiocin mutagenesis. Seeds were surface-sterilized and germinated as described under 9-aminoacridine hydrochloride mutagenesis. Since the novobiocin stock was dissolved in ddH2O the control seeds were soaked in ddH_2O for a corresponding time period.

Maintenance and scoring of plants. Following mutagenesis, seeds were surface-sterilized daily until they germinated. Seedlings from the chemical treatments were planted in multipots (Hummert Seed Co.), and the trays were placed under broad spectrum lights(GE F40/PL/AQ). Seedlings from crosses and self-pollinations were surface-sterilized for three days then placed on supplemental seed media. All seedlings were scored approximately every two weeks for chlorotic sectors until final scoring was completed at forty days after germination. Scoring consisted of checking the seedlings for chlorotic sectors in their true leaves.

Seed media. The supplemental seed media is identical to the seed media mentioned in Chapter 1.

Crossing Strategies. *Wild-type* plants with chlorotic sectors were transplanted to pots and placed in the greenhouse. Reciprocal crosses were performed with control plants to examine the inheritance patterns of the mutations. Self-pollinations were performed to provide an M2 population to test for nuclear recessive mutation. To induce bolting, plants ranging from 2 1/2 months to 6 months in age were sprayed for 15 out of 20 days with a solution containing 10^{-4} M Gibberillic acid in 0.005% Tween.

RESULTS

9-aminoacridine hydrochloride

Dosage trials

A dose curve was completed to determine the concentration and time interval that would result in the highest frequency of mutations. Six experiments were done to establish a dose curve (Tables 4 - 9). *Wild-type* (*wt*) and seeds homozygous for the *plastome mutator* allele (*pm/pm*) were treated concurrently, up until Table 5, where it was feared that samples of the *wt* and *pm/pm* seed may have been switched in the 2 ug/ml dosage at 0.5 hour treatment. At this treatment, the *wt* gave a high number of mutants while the treatment of *pm/pm* seeds (Chapter 3, Table 26) yeilded no plants with chlorotic sectors. After this time, experiments to determine the dosage curve for the *wt* seeds were undertaken separately from the experiments with the *pm/pm* seeds. 2 ug/ml acridine

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was chosen for further testing because the trials of Tables 1 and 2 suggested it might be the most mutagenic concentration. As shown in Table 6, increasing exposure times failed to show a steady increase in the mutation rate but the 16 hour exposure produced several mutations. As shown in Tables 4 - 10 , not every experiment produced mutants. Subsequently, 10 ug/ml acridine was tested on larger lots of seeds (Table 7). Sectors were observed in seeds treated for 16 hours. No consistent negative effect on seed germination was noted, but as indicated in the footnotes of these tables various transient developmental abnormalities were observed as a consequence of the acridine treatments.

In the concentration chosen, (2 ug/ml), the total number of plants containing chlorotic sectors was 22 out of a total of 3402 viable seedlings, giving a mutation frequency of 0.65%. Mutations were not recovered from seeds exposed to greater concentrations. At the 16 hour time trial, a total of 651 seeds produced 12 plants with chlorotic sectors, with a 1.84% mutation rate.

It is unknown why the increased exposure to the chemical did not appear to cause an increased level of lethality in the plants, nor a higher rate of mutagenesis.

Tables 4 - 10. Dosage trials for 9-aminoacridine hydrochloride.

Table 4. Sector frequencies for *wt* seedlings containing plastome II treated with varying doses of 9-aminoacridine hydrochloride. Each trial contained 50 seeds.

ug/mg	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	25(50.0%)	19(76.0%)	0
0	8	35(70.0%)	25(71.4%)[1]	0
0	16	17(34.0%)	13(76.5%)	0
0	32	32(64.0%)	18(56.3%)	0
1	0.5	37(74.0%)	31(83.8%)	0
1	8	39(78.0%)	31(79.5%)[2]	0
1	16	21(42.0%)	15(71.4%)	0
1	32	1(2.0%)	0	0
2	0.5	40(80.0%)	31(77.5%)	0
2	8	43(86.0%)	39(90.7%)	2(5.1%)
2	16	22(44.0%)	16(72.7%)	0
2	32	17(34.0%)	11(64.7%)	0
4	0.5	36(72.0%)	31(86.1%)[3]	0
4	8	26(52.0%)	21(80.8%)	0
4	16	11(22.0%)	6(54.5%)	0
4	32	28(54.0%)	15(53.5%)	0
8	0.5	23(46.0%)	15(65.2%)	0
8	8	36(72.0%)	26(72.2%)	0
8	16	28(56.0%)	21(75.0%)	0
8	32	19(38.0%)	13(68.4%)[4]	0
16	0.5	41(82.0%)	40(97.6%)	0
16	8	38(76.0%)	36(94.7%)[5]	0
16	16	34(68.0%)	26(76.5%)	0
16	32	46(92.0%)	22(47.8%)	0
32	0.5	46(92.0%)	33(71.7%)[6]	0
32	8	40(80.0%)	38(95.0%)[7]	0
32	16	20(40.0%)	15(75.0%)	0
32	32	28(56.0%)	23(82.1%)[8]	0

Table 4 (cont'd).

The number of plants seen with developmental abnormalities (leaves growing together):

1,2,4,7	one plant
8	two plants
3,5,6	three plants

Table 5. Sector frequencies for *wt* seedlings containing plastome II treated with varying doses of 9-aminoacridine hydrochloride. Each test involved 50 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	29(58.0%)	22(75.9%)	0
0	7.5	6(12.0%)	4(66.7%)[1]	0
0	16	16(32.0%)	7(43.8%)	0
0	42	24(48.0%	19(79.2%)	0
1	0.5	18(36.0%)	14(77.8%)	0
1	7.5	28(56.0%)	25(89.3%)	0
1	16	14(28.0%)	6(42.9%)	0
1	42	31(62.0%)	31(100%)	0
2	0.5	17(34.0%)	17(100%)	7(41.2%)
2	7.5	11(22.0%)	11(100%)	0
2	16	7(14.0%)	1(14.3%)	0
2	42	28(56.0%)	24(85.7%)	0
4	0.5	32(64.0%)	32(100%)	0
4	8	18(36.0%)	18(100%)	0
4	16	24(48.0%)	13(54.2%)	0
4	42	11(22.0%)	7(63.6%)	0
8	0.5	32(64.0%)	32(100%)	0
8	8	23(46.0%)	20(87%)	0
8	16	2(4.0%)	0	0
8	42	6(12.0%)	3(50.0)	0
16	0.5	32(64.0%)	32(100%)	0
16	8	18(36.0%)	14(77.8%)	0
16	16	5(10.0%)	0	0
16	42	23(46.0%)	20(87.0%)	0
32	0.5	20(40.0%)	17(85.0%)	0
32	8	16(32.0%)	14(87.5%)	0
32	16	8(16.0%)	0	0
32	42	29(58.0%)	26(89.7%)	0

[1] one plant with abnormal leaf development.

Table 6. Sector frequency for *wt* seedlings containing plastome IV treated with 2 ug/ml of 9-aminoacridine hydrochloride. Each trial contained 100 seeds, except for the 16 hour trial which contained 200 seeds.

Exposure time (hours)	Germination	Viability	Seedlings with sectors
0.5	60(60.0%)	60(100%)	1(1.7%)
8	74(74.0%)	69(93.2%)	0
16	128(64.0%)	122(95.3%)	4(3.3%)
32	53(53.0%)	50(94.3%)	0

Table 7. Sector frequency for *wt* seedlings containing plastome II treated with 9-aminoacridine hydrochloride. Each trial contained 500 seeds, except the 1 hour trial, which contained 512 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	32	209(41.8%)	84(40.2%)	0
10	1	512(100%)	286(55.9%)[1]	0
10	6.5	363(72.6%)	194(53.4%)[2]	0
10	16.5	466(93.2%)	318(68.2%)[3]	3(0.9%)
10	32	403(80.6%)	239(59%.3)[4]	0

[1] ten stunted plants and one plant with abnormal leaf development,
[2] three plants with abnormal leaf development,
[3] three stunted plants, one plant with abnormal leaf development, one leaf fusion,
[4] seven stunted plants, two plants with abnormal leaf development, one plant with leaves fused midrib, five plants with two apical meristems.

Table 8. Sector frequency for *wt* seedlings containing plastome II treated with 9-aminoacridine hydrochloride. Each trial contained 500 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	32	460(92.0%)	[1]294(63.9%)	0
2	1	452(90.4%)	[2]379(83.8%)	0
2	6	451(90.2%)	376(83.3%)	0
2	16.5	376(75.2%)	[3]352(93.6%)	3(0.9%)
2	32	463(92.6%)	399(86.2%)	0

[1] Lost 26 plants to tray drying out, one plant with abnormal leaf,
[2] three plants with abnormal leaf development, one plant with double meristem, one stunted plant,
[3] one plant with abnormal leaf development and same plant as two apical meristems, one plant without trichomes on one-half of leaf margin.

Table 9. Sector frequency for *wt* seedlings containing plastome IV treated with 9-aminoacridine hydrochloride. Each trial contained 500 seeds with an exposure time of 16 hours.

(ug/ml)	Germination	Viability	Seedlings with sectors
0	224(44.8%)	[1]120(53.6%)	0
2	298(59.6%)	[2]160(53.7%)	0

[1] two plants with abnormal leaf development,
[2] two plants with holes in the leaf.

Table 10. Sector frequency for *wt* seedlings containing plastome I treated with 9-aminoacridine hydrochloride. Each trial contained 500 seeds with an exposure time of 16 hours.

ug/ml	Germination	Viability	Seedlings with sectors
0	245(49.0%)	134(54.6%)	0
2	236(47.2%)	[1]157(66.5%)	2(1.3%)

[1] three plants with abnormal shaped leaf margins.

Mutant Phenotypes resulting from acridine treatment

This chemical induced a wide spectum of colors and patterns in *Oenothera,* in contrast to NMU, which causes predominantly solid white sectors (Chapter 1 in this thesis). In the acridine treatments, I observed white, yellow, and light green sectors in the plant tissue, both as solid sectors and mottled.

Developmental anomalies were also observed. These ranged from abnormal leaf shapes/margins, leaves with fused midribs, leaves fused side-to-side, leaves with holes, and one plant without trichomes on half of its leaf margin. All appeared to be transitory and were never seen beyond the second set of true leaves. Two other aberrations present that were not transitory were stunted plants and plants with two apical meristems.

Using transmission electron microscopy, mottled mutant tissue was sampled from a 9-aminoacridine hydrochloride-induced sector from mutant A-E. The micrographs provided by the Center for Electron Optics indicated that the plastids in the white tissue were very large with few grana (Figure 1). The green tissue within the mottled sector contained chloroplasts with an excessive amount of densely stacked grana. In both pictures the mitochondria appear normal. Progeny from crosses of mutants were germinated on supplemented media, but

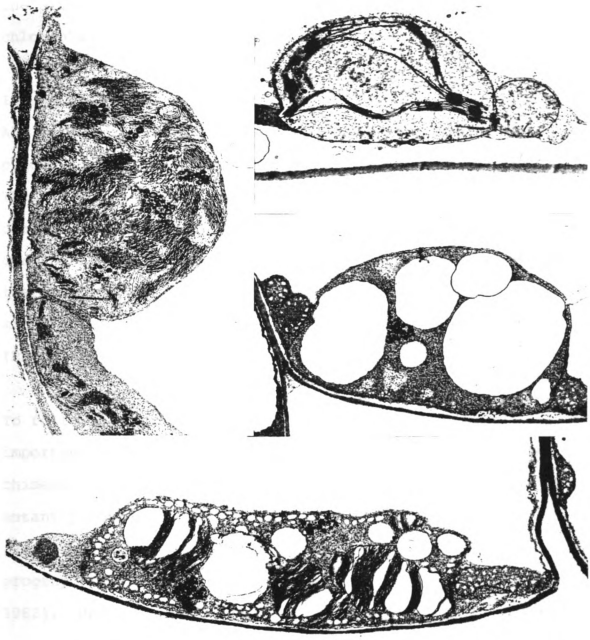

Figure 1. Transmission electron micrograph of mottled tissue from a 9-aminoacridine hydrochloride-induced mutant sector.

died within 45 days of their transfer to soil, where they were maintained under continuous light. This suggests that the primary mutational lesion affects the function of the chloroplast, not the mitochondrion.

Inheritance

All *wt* plants with acridine-induced chlorotic sectors were placed in the greenhouse and sprayed with gibberellic acid. These plants were then self-pollinated and crossed to determine the inheritance pattern of the mutation. In *Oenothera,* chloroplasts are transmitted from both parents to the progeny (Kirk and Tilney-Bassett 1978), while mitochondria are transmitted only from the female parent (Brennicke and Schwemmle 1984).

To follow patterns of non-Mendelian inheritance, it is important to know that in *Oenothera,* when a full periclinal chimera produces a flower, the germ line contains entirely mutant plastids. If this plant is chosen as the female parent, mutant plastids will predominate in this plant's progeny (Kirk and Tilney-Bassett 1978; Stubbe and Herrmann 1982). Unfortunately, the plants containing the new mutant sectors in these experiments never developed as complete periclinal chimeras. At some point in time, sectors did extend into the leaf margins of a few of the mutant plants so that a subset of the offspring received some mutant plastids.

This led to difficulty in establishing the mode of inheritance of the trait. Thus some uncertainty remains about whether the mutations were induced in the mitochondrial or chloroplast genome. However, non-Mendeliam inheritance and vegetative segregation could be demonstrated unequivocally.

The variegated plants obtained from the experiment shown in Table 3 were not used for crosses. The progeny from these plants had a bleached phenotype, reminiscent of a particular type of plastome-genome incompatibility. PCR assays kindly performed by Lara Steben indicated that the bleached leaves contained plastome III, indicating that the seed lot provided had been contaminated with seeds containing this somewhat incompatible plastome.

For analysis of the crosses, the entire seed capsule was germinated and resulting progeny scored. One plant, designated A-E, had one half of the leaf margin containing chlorotic tissue, and hence, the mutation. Since only half of the germ line would contain the mutation, when used as the female in a cross, approximately half of the progeny should inherit mutant plastids. In fact, when the mutation was carried by the maternal parent, (A-E), 103 out of 205 progeny contained sectors (See Table 11). There were 9 entirely white plants in which the mutation was inherited strictly

Table 11. Crosses of 9-aminoacridine hydrochloride induced mutants.

Parents[1]	# of seeds	F1 germinated	F1 viable	seedlings with sectors
White x Green A-B(II)[3]xIV[4] M-mutation-not in leaf margin P-green	385	365(94.8%)	355(97.3)	12(3.38%) M-1 V-11 G-0
White x Green A-E(II)xI M-1/2 leaf margin P-green	396	149(37.6%)	149(100%)	70(46.5%) M-2 V-68 G-0
Green x White IxA-E(II) M-green P-partial	390	342(87.7%)	342(100%)	0
White x Green A-E(II)xIV M-1/2leaf margin P-green	100	32(32.0%)	32(100%)	19(59.4%) M-2 V-17 G-0
White x Green A-E(II)xIV M-1/2leaf margin P-green	24	24(100%)	24(100%)	14(%) M-5 V-9 G-0
Green x White IVxA-E(II) M-green P-1/2leaf margin	109	100(91.7%)	100 (100%)	0
White x Green A-G(II)xI M-partial P-green	33	30(90.9%)	30(100%)	15(50.0%) M-4 V-11 G-0
Green x White IxA-G(II) M-green P-1/2leaf margin	358	49(13.69%)	49(100%)	0

Table 11 (cont.)

Parents[1]	# of seeds	F1 germinated	F1 viable	seedlings with sectors
White x Green A-F(II)xI M-poss. partial P-green	10	2(20%)	2(100%)	0
White x Green A-F(II)xI M-partial P-green	272	262(96.3%)	192(73.3%)	23(11.98%) M-5 V-18 G-0
Green x White IVxA-F(II) M-green P-poss. partial	200	176(88.0%)	176(100%)	2(1.14%) M-0 V-2 G-0

The flowers used as the maternal parent were deduced to contain varying amounts of mutant tissue, based on the abundance of the mutant tissue in the LII tissue layer of the subtending leaf.

[1] M = maternal parent P = paternal parent.

Notations about the leaf margin describe the prevalence of the mutant tissue as follows:

1/2 Leaf margin - chlorotic sector is contained in one-half of the leaf margin.

Partial - chlorotic sector is contained in less than half of the leaf margin.

[2] Seedlings with sectors:

M - seedling with solely mutant plastids
G -green progeny with no sectors
V -variegated seedlings

[3] Plants designation and roman numerals are the plastome types.

[4] When only a Roman numeral is listed, it refers to the *wild-type*, green plastome type in a nuclear background of *O. johansen*.

from the female. The 94 variegated plants could have inherited white plastids from the female and green plastids from the male or both types could have been contributed by the female. No variegation was seen in any offspring from A-E descendants when the male carried the mutation.

I observed similar results with plants A-B, A-F and A-G. When the mutation was carried by the maternal parent, variegation was seen in the progeny. The F_1 generation from A-B gave a total of 11 plants with sectors and 1 entirely white plant; A-G gave 11 sectored plants with 4 completely white plants; and A-F gave 18 variegated plants with 5 totally white plants.

Self-pollinations were done to determine whether recessive nuclear mutations had been induced by acridine. An attempt was made to self-pollinate flowers that did not contain the mutant tissue that had been noted previously, so that other traits could be observed among the progeny. However, plants A-A, A-C, A-D, and A-E had variegated offspring in the M_2 generation. This ranged from 9.5% to 37.8% as shown in Table 12. There were no other apparent nuclear anomalies observed.

If the mutation was carried in the *Oenothera* mitochondrial genome, which has been shown to be inherited solely from the

female (Brennicke and Schwemmle 1984), variegated plants should be observed only when the female carries the mutation and never when the male carries the mutation. In one instance, pollen contributed mutant plastids to the progeny. When the variegated plant A-F, was used as the pollen donor, I observed two of its progeny with sectors.

Table 12. Self-pollination of 9-aminoacridine hydrochloride induced mutants.

Self-pollinations	# of seeds	M2 germinated	M2 viable	seedlings with sectors
A-A	99	93(93.9%)	64(68.8%)	24(37.5%)
A-B	100	0	0	0
A-C	100	90(90.0%)	70(77.78%)	16(22.86%)
A-D	102	102(100%)	42(41.18%)	4(9.52%)
A-E	317	262(82.65%)	37(14.12%)	14(37.84%)
A-K	100	97(97.0%)	48(49.48%)	0
A-M	100	87(87.0%)	87(100%)	0
A-N	100	98(98.0%)	63(64.29%)	0
A-O	100	20(20.0%)	14(70.0%)	0
A-T	100	19(19.0%)	18(94.7%)	0

Nalidixic acid Results

Dosage trials

Initial experiments tested concentrations of 1 - 100 ug/ml nalidixic acid for 0.5 - 24 hours exposures (Tables 13 - 16). As shown in Tables 13 and 16, nalidixic acid did not induce mutations in every experiment. The mutants that did appear occurred almost entirely in the 10 ug/ml concentration at various lengths of exposure. Subsequent experiments utilized more seeds to further test the exposure time (Tables 14 - 15). Based on these experiments, the dosage and exposure time chosen was 10 ug/ml for 16 hours.

Mutant phenotypes resulting from nalidixic acid treatment

The phenotypes of the M_1 generation varied from white to pale green sectors with some mottling; in other plants aberrant leaf shapes were also seen. Many of the plants had leaves with developmental holes in them. These anomalies were seen in the first true leaves but never developed in any of the subsequent leaves, thus, they may have indicated transitory physiological damage, rather than mutations.

Other developmental anomalies observed included aberrently shaped leaves, stunting, presence of two apical meristems and presence of thin, bleached leaves.

Inheritance

In the concentration chosen, 10 ug/ml, a total of 1897 viable seedlings were examined, and 8 plants containing chlorotic sectors were observed. *Wild-type* sectored plants were transferred to the greenhouse and treated with gibberellic acid to induce bolting. The plants did not produce periclinal chimeric leaves but some of the mutant tissue did get to the leaf margin. Therefore, some mutant plastids could possibly be inherited by the progeny.

Due to limitations on the flower viability, number of control plants, and the placement of the sector, no crosses were performed in which the pollen carried the mutation. When a mutation was visible in one leaf's margin, the next leaf up did not always contain the chlorotic tissue in its leaf margin.

Out of 444 viable F_1 plants from crosses of N-C mutant as the female parent, 27 plants inherited solely mutant plastids, with no variegated individuals noted. An additional 26 plants displayed small clustered true leaves.

Two variegated M_1 plants were selfed, N-C and N-E, and only one plant from the offspring exhibited variegation.

Tables 13 - 19. Dosage trials for nalidixic acid.

Table 13. Sector frequency for *wt* seedlings containing plastome II treated with varying doses of nalidixic acid.

ug/ml	Exposure time (hours)	# of seeds	Germination[1]	Viability	Seedlings with sectors
0	0.5	50	25(50.0%)	22(88%)	0
0	6	47	10(21.3%)	3(30%)	0
0	24	50	8(16.0%)	2(25%)	0
1	0.5	42	9(21.4%)	7(78%)	0
1	6	50	1(2.0%)	0	0
1	24	50	1(2.0%)	0	0
10	0.5	50	15(30.0%)	14(93%)[2]	0
10	6	50	8(16.0%)	7(88%)	1(14%)
10	24	50	32(64.0%)	27(84%)	0
100	0.5	50	5(10.0%)	5(100%)	0
100	6	35	10(28.6%)	6(60%)	0
100	24	50	4(8.0%)	3(75%)	0

1 Unknown as to why germination was so poor.
2 two plants with leaf abnormalities.

Table 14. Sector frequency for *wt* seedlings containing plastome II treated with varying doses of nalidixic acid.

ug/ml	Exposure time (hours)	# of seeds	Germination	Viability	Seedlings with sectors
0	0.5	50	22(44.0%)	21(95.5%)	0
0	6	100	70(70%.0)	62(88.6%)	0
0	24	50	1(2.0%)	1(100%)	0
1	0.5	50	30(60.0%)	29(96.7%)	0
1	6	100	40(40.0%)	31(77.5%)	0
1	24	50	7(14.0%)	3(42.9%)	0
10	0.5	50	18(36.0%)	18(100%)	1(5%)
10	6	100	52(52.0%)	43(82.7%)	0
10	24	50	25(50.0%)	18(72.0%)	0
100	0.5	50	20(40.0%)	20(100%)	0
100	6	100	32(32.0%)	25(78.1%)[1]	0
100	24	100	12(12.0%)	12(100%)	0

[1] three stunted seedlings.

Table 15. Sector frequency for *wt* seedlings containing plastome II treated with varying doses of nalidixic acid. Each trial contained 50 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	16(32.0%)	5(31.3%)	0
0	6	21(48.0%)	20(95.2%)	0
0	24	14(28.0%)	7(50.0%)	0
1	0.5	9(18.0%)	3(33.3%)	0
1	6	16(32.0%)	12(75.0%)	0
1	24	6(12.0%)	3(50.0%)	0
10	0.5	11(22.0%)	9(81.8%)	0
10	6	24(48.0%)	18(75.0%)	0
10	24	27(54.0%)	25(92.6%)	0
100	0.5	5(10.0%)	2(40.0%)	0
100	6	16(32.0%)	14(87.5%)	0
100	24	12(24.0%)	3(25.0%)	0

Table 16. Sector frequency for *wt* seedlings containing plastome IV treated with 10 ug/ml nalidixic acid.

Exposure time (hours)	# of seeds	Germination	Viability	Seedlings with sectors
0.5	100	57(57.0%)	55(96.5%)	0
6	100	55(55.0%)	50(90.9%)	2(4.0%)
25	95	58(61.0%)	56(96.6%)	1(1.8%)

Table 17. Sector frequency for *wt* seedlings containing plastome IV treated with nalidixic acid. Each trial contained 500 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	32	209(41.8%)	84(40.2%)	0
2	1	287(57.4%)	179(62.4%)	0
2	6.5	373(74.6%)	157(42.1%)[1]	0
2	16.5	352(70.4%)	167(47.4%)[2]	0
2	32	345(69.0%)	122(35.4%)[3]	0

1 seven stunted plants,
2 four plants with longitudally cracked stems (all survived), two plants with abnormal leaves, six very stunted plants,
3 six plants with abnormal leaf development, eight stunted plants, three plants have double meristems.

Table 18. Sector frequency for *wt* seedlings containing plastome IV. Each trial contained 500 seeds.

Nalidixic Acid	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	32	451(90.2%)	413(91.6%)	0
10	1	449(89.8%)	214(47.7%)[1]	0
10	6.5	456(91.2%)	331(72.6%)[2]	0
10	16.5	475(95.0%)	379(79.8%)[3]	1(0.3%)
10	32	449(89.8%)	374(83.3%)[4]	0

1 one abnormal leaf, two plants with aberrantly shaped leaves,
2 lost twelve seedlings due to tray drying, one abnormal leaf two stunted plants, one plant with aberrantly shaped leaves,
3 three stunted plants, one plant aberrantly shaped leaves,
4 one plant with aberrantly shaped leaves.

Table 19. Sector frequency for *wt* seedlings containing plastome I treated with nalidixic acid. Each trial contains 500 seeds with an exposure time of 16 hours.

ug/ml	Germination	Viability	Seedlings with sectors
0	245(49.0%)	134(54.7%)	0
10	330(66.0%)	259(78.5%)[1]	2(0.8%)

[1] three plants with thin bleached leaves.

Table 20. Crosses of nalidixic acid-induced mutants.

Parents	# of seeds	F1 germinated	F1 viable	seedlings with sectors
White x Green N-C(IV)xIV M-1/2 leaf margin P-green	200	178(89.0%)	140(78.7%)	0
White x Green N-C(IV)xIV M-1/2 leaf margin P-green	200	183(91.5%)	183(100%)	27(14.75%) M-27(100%) V-0 P-0 (26 green plants with small clustered true leaves)
White x Green N-C(IV)xIV M-partial P-green	293	159(54.3%)	121(76.1%)	0

Table 21. Self-pollinations of nalidixic acid-induced mutants.

Parents	# of seeds	M2 germinated	M2 viable	seedlings with sectors
N-CxN-C	165	160(96.97%)	94(58.75%)	1(1.06 %)
N-ExN-E	100	54(54.0%)	45(83.3%)	0

Novobiocin Results

Dosage trials

For novobiocin, two experiments were done to test for an effective dose, using increasing numbers of seeds (Tables 22 and 23). Three variegated plants were obtained from these experiments, two of which came from the 1,000 ug/ml concentration. For these the largest time period was 32 hours, therefore, 1,000 ug/ml at 32 hours was chosen as the dose for a larger scale experiment (Table 24). Unfortunately, in the last experiment, seed germination was only about 50%, so the sample size was not as large as I would have liked.

Phenotypic alterations resulting from novobiocin treatment

Novobiocin-induced chlorotic sectors were quite small and pale green with some mottling in two plants while the third plant had white sectors. Double apical meristems, a swirled plant, ruffled leaves,and thin bleached leaves were some of the anomalies observed in the M_1 seedlings.

Using transmission electron microscopy a sample was removed from a small novobiocin-induced sector of the third plant. The micrographs provided by the Center for Electron Optics indicated that the cells were under physiological stress, since they were necrotic, had little cytoplasm, and had

vacuoles filled with debris. Many cells had cytoplasm that was very vacuolated. The chloroplasts had normal ultrastructure. However, the cells were not fixed well and thus the micrographs are of marginal quality. Therefore, the pictures are not included in this chapter.

Inheritance

Two of the three plants containing novobiocin-induced sectors were transferred to the greenhouse and treated with gibberellic acid. Crossing plants which contained sectors proved impossible because the sectors eventually disappeared. One plant, No-B, was sterile: when the No-B plant was used as the female, capsules didn't develop and pollen was not produced by the anthers. The other plant, No-A, never produced sectors in the margin of leaves and, thus, mutations could not be transferred to the progeny through the germ layer. Therefore, only self-pollinations of No-A were available to test for the presence of recessive nuclear mutations, and no variegated progeny resulted (Table 25).

Table 22. Sector frequency for *wt* seedlings containing plastome II treated with varying doses of novobiocin. Each trial contained 50 seeds.

ug/ml	Exposure time (hours)	# of seeds	Germination	Viability	Seedlings with sectors
0	0.5	50	35(70.0%)	26(74.3%)	0
0	1	50	44(88.0%)	44(100%)	0
0	24	50	45(90.0%)	40(88.9%)	0
0	32	50	40(80.0%)	35(87.5%)	0
10	0.5	50	29(58.0%)	28(96.6%)	0
10	1	50	45(90.0%)	36(80.0%)	0
10	24	51	51(100%)	40(78.4%)	0
10	32	50	13(26.0%)	9(69.2%)	0
100	0.5	50	41(82.0%)	38(92.7%)	0
100	1	50	27(54.0%)	[1]27(100%)	1(3.7%)
100	24	50	15(30.0%)	[2]12(80.0%)	0
100	32	50	39(78.0%)	30(76.9%)	0
1000	0.5	50	28(56.0%)	[3]24(85.7%)	0
1000	1	50	11(22.0%)	[4]10(90.9%)	0
1000	24	50	47(94.0%)	43(91.5%)	0
1000	32	50	39(78.0%)	[5]29(74.4%)	1(3.4%)

1 two plants with two apical meristems,
2 one plant with two apical meristem,
3 two plants with two apical meristem,
4 one plant appears swirled/deformed,
5 one plant with leaves ruffled.

Table 23. Sector frequency for *wt* seedlings containing plastome IV treated with varying doses of novobiocin. Each trial contains 100 seeds with an exposure time of 32 hours.

ug/ml	Germination	Viability	Seedlings with sectors
0	80(80.0%)	76(95.0%)[1]	0
10	98(98.0%)	89(90.8%)[2]	0
100	92(92.0%)	89(96.7%)[3]	1(1.1%)
1000	93(93.0%)	78(83.9%)[4]	0

1 two plants with thin bleached leaves,
2 two plants with thin bleached leaves,
3 nine plants with thin bleached leaves,
4 one plant with thin bleached leaves.

Table 24. Sector frequency for *wt* seedlings containing plastome IV. Each trial contains 500 seeds with an exposure time of 32 hours.

Novobiocin ug/ml	Germination	Viability	Seedlings with sectors
0	245(49.0%)	134(54.70%)	0
1000	242(48.4%)	204 (84.30%)[1]	0

1 seven plants with thin bleached leaves.

*The control, 0 ug/ml, is the same as the acridine experiment dated 5/18/93.

Table 25. Self-pollinations of novoviocin induced mutants.

Parents	# of seeds	M2 germinated	M2 viable	seedlings with sectors
No-AxNo-A	100	99(99.0%)	95(95.9%)	0

Discussion

Several chemicals were used in an attempt to induce plastome mutations in *Oenothera*. The chemicals chosen were acridine (because it intercalates into DNA), novobiocin and nalidixic acid (because both chemicals inhibit gyrase). The seedlings were screened for mutations that would cause a visible phenotype, chlorosis, appearing in sectors of the M1 plants.

Treatment of *Oenothera* seeds with these chemicals resulted in some temporary morphological and physiological abnormalities in some seedlings, such as fused leaves, holes in leaves, missing trichomes on leaf margin, etc. This points to the chemical being toxic to the plant cell. Mutagenic compounds damage DNA, but some also damage other macromolecules (RNA or proteins) that are present in the cell during the treatment. This damage may cause aberrations but most of these would be transient (Ferguson and Denny 1991).

Conceivably, the mottled phenotype could be attributed to a leaky mutation or could represent a series of back mutations. Epp (1973), Stubbe and Herrmann (1982), and Sears and Herrmann (1985) attempted to select green progeny from mottled plastome mutants and were not successful, suggesting that mottling is not produced by back mutations.

Few pigment-deficient sectors were observed as a consequence of nalidixic acid and novobiocin. This may be attributed to any of several possibilities: 1) the chemicals not entering the plastid, 2) the gyrase binding the chemicals, but not becoming disfunctional, 3) gyrase being inhibited by the chemicals, but this not being mutagenic, as inferred in *Solanum nigrum,* where cell growth recovers after removal of the inhibitors (Ye and Sayre 1990), or 4) the existence of a novobiocin-insensitive bypass replication in the *Oenothera* chloroplast as seen in *Chlamydomonas reinhardtii* (Woelfle et al. 1993).

Our studies show that acridine can induce mutations that affect chlorophyll accumulation. Most sectors induced by acridine are mottled pale green and appear approximately 40 days after treatment. In contrast, NMU sectors are white and the majority can be recognized between 25 and 30 days after treatment. This may indicate that a different type of DNA lesion has been produced by acridine, than nitroso methylurea (NMU), which targets the plastome (Sears and Sokolski 1991). One possibility is that acridine may target mtDNA rather than cpDNA.

Precedence exists for mitochondrial mutations affecting chloroplast function. A mitochondrial mutation that indirectly cause chloroplasts to become disfunctional has

been characterized in *Zea mays*. The nonchromosomal striped 2 (NCS2) mutant of maize has a DNA rearrangement in the mitochondrial genome, whose abundance correlates with the severity of the albinism (Roussell et al. 1991; Hunt and Newton 1991), but no chloroplast DNA alterations were detected (Roussell et al. 1991). It appears that abnormalities in the mitochondrial genome can result in pleiotropic effects upon the chloroplast. In another investigation, severe stunting and striping in the nonchromosomal stripe 3 (NCS3) mutant was seen, but it was not determined if the chloroplast genome was altered (Hunt and Newton 1991).

In *Oenothera*, the crossing data should allow us to distinguish between chloroplast and mitochondrial mutations. The sectors are certainly non-Mendelian because vegetative segregation is observed in the progeny. In *Oenothera*, chloroplasts are inherited from both parents in most crosses (reviewed by Chiu and Sears 1993). In contrast, mitochondria are thought to be inherited solely from the maternal parent (Brennicke and Schwemmle 1984), based on the observation that restriction fragments of mitochondrial DNA in a number of hybrids have only the maternal mtDNA type. Since this investigation was not very rigorous, the occurrence of strictly maternal inheritance of mitochondria in *Oenothera* should be viewed with caution. However, if it is true that

the mitochondria are only maternally transmitted then my crosses point to a plastome-location of the acridine mutations because mutant A-F produced two variegated progeny when the male carried the mutation (Table 8).

In conclusion, evidence obtained from the crossing data and from chemical treatment data, suggests that acridine can act as a plastome mutagen.

CHAPTER 3

CHEMICAL MUTAGENESIS OF THE *PLASTOME MUTATOR* GENOTYPE

INTRODUCTION

Plastome mutator alleles that result in mutations in the cpDNA are present in a variety of plants. These *mutator* genes appear to differ in the types of mutations induced and the mode of induction. For example, in maize and barley they cause the same non-Mendelian albino defect to be expressed over and over again, while the *plastome mutator* of *Oenothera* (Epp 1973; Epp and Parthasarthy 1987), *Arabidopsis* (Redei and Plurad 1973), and petunia (Potrykus 1970) cause a variety of plastid mutations.

Crossing experiments verified that the *plastome mutator* trait found in *Oenothera* was a nuclear recessive allele (Epp 1973). Since the nucleus probably encodes most of the components of

the replication and repair processes necessary for chloroplast DNA (cpDNA), it is highly probable that one ofthese products is absent in the *pm* lines.

Studies of Sears and Sokalski (1990) using the *Oenothera plastome mutator* line treated with nitroso methylurea (NMU), reported numerous chlorophyll-deficient sectors in the young seedlings. The dramatically high mutation rate that they observed was interpreted to indicate that the two components, the chemical mutagen and the *plastome mutator* gene, interact synergistically. It appeared that the guanosine alkylation incurred by the NMU is not being repaired and suggests the the *pm* line has a defect in the alkylation-damage repair pathway. This interpretation seems to be in conflict with the finding that the *plastome mutator* causes deletions of short direct repeats in the cpDNA (Blasko et al. 1988; Chiu et al. 1990). Such a consequence would not be expected to result from the defective repair of alkylated bases. Rather, the occurrence of high frequency deletions involving short direct repeats would point to a process involving recombination or replication slippage. Conceivably, the *pm*-defect lies in a general aspect of cpDNA replication or repair that could increase the frequency of many sorts of mutational lesions. For example, if the exonuclease subunit of DNA polymerase was defective or absent, the proof-reading function would be missing. Alternatively, an elevated level

of error-prone repair could result in the fixation of many types of mutation. Another possibility is that an accessory enzyme for replication and repair, such as a helicase or topoisomerase is missing, causing stalling, replication slippage, and difficulties in making the DNA accessible for repair enzymes.

In order to elucidate which aspect of cpDNA metabolism is affected by the *plastome mutator*, it would be helpful to test the integrity of both the replication and repair processes in *Oenothera*. Thus, I chose to test for synergism of the plastome mutator genotype and a mutagen that causes a type of mutation that is quite different from those caused by NMU. Furthermore, I attempted to use inhibitors of gyrase to alter the extent of supercoiling of the cpDNA, to determine if that would affect the ability of the *plastome mutator* to induce mutations.

The mutagen 9-aminoacridine hydrochloride was chosen because it is known to cause frame-shifts in *Salmonella* (Hoffman et al. 1989) and *E. coli* (Thomas and McPhee 1985; Gordon et al. 1991). Acridine intercalates between the stacked nitrogen bases at the core of the double helix (Nasim and Brychcy 1979). It mimics base pairs causing the deletion or addition of a base upon replication, with hotspots occurring in runs of G-C basepairs (Skopek and Hutchinson 1984).

An increase in torsional stress of the cpDNA could be responsible for a higher frequency of replication slippage in the *pm*-line compared to *wild-type*. Such a situation could result if a topoisomerase I is absent, or if the supercoiling activity of topoisomerase II was elevated. In bacteria, gyrase activity seems to be intimately involved in creating deletions between direct repeats, since such deletions are *gyrA*-dependent, but *recA* independent (Saing et al. 1988; Maiura-Masuda and Ikeda 1990). To test whether an imbalance between relaxation and supercoiling results in *pm*-induced mutation, I attempted to inhibit gyrase activity.

Novobiocin and nalidixic acid have been shown to inhibit DNA gyrase *in vitro* in *E.coli* (Gellert et al. 1977, 1976), and *in vivo* in *Daucas carota* (Ciarrocchi et al. 1985) and *Chlamydomonas reinhardtii* (Thompson and Mosig 1985, 1987) and these experiments have also shown a concurrent decrease in DNA replication. Ye and Sayre (1990) found that both novobiocin and nalidixic acid reduced *Solanum nigrum* cpDNA content and that neither chemical inhibited or reduced nuclear DNA content *in vivo*. These findings suggest that an inhibitor-sensitive DNA gyrase participates specifically in cpDNA replication.

In vivo experiments using nalidixic acid and novobiocin have shown that these chemicals suppress DNA gyrase in the

chloroplast. If excessive supercoiling is responsible for *pm* mutations, then it is conceivable that the gyrase inhibitors would help relax the molecule and reduce the *pm* mutation frequency.

My objective was to first use wild-type *Oenothera* plants and determine if certain mutagenic chemicals (acridine, novobiocin, or nalidixic acid) would affect the frequency of spontaneous mutations in the plastome (Chapter 2). Then the goal was to apply these chemicals to *Oenothera* plants homozygous for the *pm* gene and determine if a synergistic response is observed.

Chemical Treatment

Materials and Methods

The Materials and Methods for the seeds homozygous for the *pm* gene are identical to the materials and methods used for the respective chemicals and the *wt* seeds (Chapter 2).

Results

9-aminoacridine hydrochloride effects on plastome mutator lines.

pm/pm IV genotype.

Since Sears and Sokalski (1991) had found that the combination of the *pm/pm* genotype and plastome IV gave a

spontaneous mutation rate that was 10% of the other plastome types, this was the initial plastome-genome combination of choice. As a control, *wt* seeds containing the same plastome type as the *pm* seeds were also treated with the chemical for comparison (Chapter 2 and 3, Tables 4 & 26; 5 & 27; 9 & 28; 10 & 29). The first experiment with the *pm/pm* seeds [containing plastome IV (Table 9 & 28)] produced an enormous number of sectors even in the *pm/pm* control with no chemical mutagen (Table 28). With these high numbers it would be extremely difficult to distinguish between an additive or a synergistic induction of mutation. Lacking a homozygous *pm/pm* line with a low mutation rate, I pursued inbred *pm/pm* lines with plastome II, while Dr. Sears performed the crosses necessary to produce an alternative *pm/pm* line with a low mutation rate.

To obtain a *pm/pm* line with a low background mutation rate, seeds with a reestablished *pm/pm* nucleus were used. These seeds were produced by crossing a *+/pm* line as the female parent with a *pm/pm* line as the pollen parent, resulting in an equal mixture of *pm/+* and *pm/pm* seeds. As Epp demonstrated (1973), such a newly restored *pm/pm* line has an initially low mutation rate, and maximally can be expected to reach 18.6%.

pm/pm II genotype.

The initial 9-aminoacridine hydrochloride dosage trials utilized stocks with plastome I (Chapter 2, Table 10), plastome II (Chapter 2, Tables 4, 5, 7, and 8), and plastome IV (Chapter 2, Tables 6 and 9) in the wild-type nuclear background. It was determined that an exposure to 2 ug/ml for 16 hours was optimal for the induction of mutations. *Wt* and *pm/pm* seeds were treated concurrently, up until Table 5 (Chapter 2), where it was feared that samples of the *wt* and *pm/pm* seed may have been switched in the 2 ug/ml dosage at 0.5 hour treatment. At this treatment, the *wt* gave a high number of mutants while the *pm/pm* sample did not give any plants with chlorotic sectors (Table 26). After this time, experiments to determine the dosage curve for the *wt* seeds were undertaken separately from the experiments with the *pm/pm* seeds. Hence the data in Tables 26 and 27 on *pm/pm* seedlings were generated concurrently with Tables 4 and 5 in Chapter 2, and were considered only in the establishment of the optimal dosage.

Mutagenesis of a mixed pm/pm, pm/+ line.

After the dosage tests, an experiment was done with *wt* seeds (Chapter 2, Table 10) for comparison with the new seeds (Table 29). Almost a two-fold increase in mutations occurred when the *pm* line was treated with 9-aminoacridine hydrochloride. The data obtained from the *wt* plants (Table

10) and the *pm* plants can be compared to determine if 9-aminocacridine hydrochloride caused a synergistic increase in the *pm* mutation frequency.

The data contained in Table 10 and 29 were analyzed by applying the chi-square goodness-of-fit test to the numbers of observed and expected mutations. The expected number of mutations is approximated by the formula (if the chemical mutagenesis and the genetic condition independently produce mutations):

$$(CN) + 1/2(NP)$$

where N is the population size of the *pm/pm* seeds, C is the frequency of mutations in the chemically treated wild-type plants, and P is the observed frequency of mutation of *pm/pm* alone (without chemical treatment). Because each *pm/pm* plant will be scored for a single mutation, when in fact, mutations would be caused by both acridine and the plastome mutator, this value must be halved. Thus, the expected number of mutations in the acridine-treated seeds of +/pm and pm/pm genotype is calculated as:

$$[(CN + 1/2(NP)] - 1/2[CP\ (1/2N)] = \text{Expected.}$$

A correction factor, 1/2[CP(1/2N)], is necessary because in half the population, (1/2N), of mixed seeds (those of the *pm/pm* genotype), both the chemical and the *pm* gene may cause mutation in the same plant. The likelihood that two

mutations occur in the same plant is the frequency of mutation seen in the chemically treated wild-type plant multiplied by the frequency of mutation in the *pm/pm* line (without chemical treatment).

Scoring after 40 days showed an increase in mutation frequency in *pm/pm* plants as a result of 9-aminoacridine hydrochloride treatment, but the differences in the final mutation frequency of treated and control *pm/pm* plants was not statistically significant ($0.05 < p < 0.10$).

Tables 26 - 29. 9-aminoacridine hydrochloride treatment of *pm/pm* seeds.

Table 26. Sector frequencies in *pm/pm* seedlings containing plastome II treated with varying doses of 9-aminoacridine hydrochloride. Each trial contained 50 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	31(62.0%)	16(51.6%)	12(75.0%)
0	8	37(74.0%)	21(56.8%)	11(52.4%)
0	16	30(60.0%)	14(46.7%)	6(42.9%)
0	32	19(38.0%)	3(15.8%)	1(33.3%)
1	0.5	41(82.0%)	13(31.7%)	3(23.1%)
1	8	35(70.0%)	12(34.3%)	6(50.0%)
1	16	32(64.0%)	15(46.9%)	6(40.0%)
1	32	6(12.0%)	0	0
2	0.5	34(68.0%)	11(32.4%)	6(54.5%)
2	8	35(70.0%)	9(25.7%)	3(33.3%)
2	16	28(56.0%)	13(46.4%)	6(46.2%)
2	32	10(20.0%)	6(60.0%)	4(66.7%)
4	0.5	28(56.0%)	26(92.9%)	14(53.8%)
4	8	38(76.0%)	26(68.4%)	16(61.5%)
4	16	16(32.0%)	3(18.8%)	2(66.7%)
4	32	11(22.0%)	4(36.4%)	3(75.0%)
8	0.5	29(58.0%)	28(96.6%)	10(35.7%)
8	8	33(66.0%)	30(90.9%)	17(56.7%)
8	16	34(68.0%)	32(94.1%)	18(56.3%)
8	32	31(62.0%)	24(77.4%)	14(58.3%)
16	0.5	28(56.0%)	22(78.6%)	13(59.1%)
16	8	33(66.0%)	29(87.9%)	9(31.0%)
16	16	28(56.0%)	23(82.1%)	15(65.2%)
16	32	27(54.0%)	25(92.6%)	16(64.0%)
32	0.5	31(62.0%)	13(41.9%)	6(46.2%)
32	8	32(64.0%)	23(71.9%)	12(52.2%)
32	16	39(78.0%)	28(71.8%)	17(60.7%)
32	32	39(78.0%)	32(82.1%)	15(46.9%)

Table 27. Sector frequency for *pm/pm* seedlings containing plastome II treated with varying doses of 9-aminoacridine hydrochloride. Each trial contained 50 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	11(22.0%)	10(90.9%)	5(50.0%)
0	7.5	27(54.0%)	8(29.6%)[1]	7(87.5%)
0	16	20(40.0%)	18(90.0%)	1(5.6%)
0	42	25(50.0%)	*	5
1	0.5	15(30.0%)	12(80.0%)	6(50.0%)
1	7.5	26(52.0%)	26(100%)	18(69.2%)
1	16	14(28.0%)	14(100%)	2(14.3)
1	42	20(40.0%)	*	4
2	0.5	31(62.0%)	26(83.9%)	0
2	7.5	24(48.0%)	5(20.8%)	2(40.0%)
2	16	9(18.0%)	7(77.8%)	0
2	42	23(46.0%)	*	1
4	0.5	11(22.0%)	11(100%)	4(36.4%)
4	8	21(42.0%)	21(100%)	7(33.3%)
4	16	9(18.0%)	8(88.9%)[1]	1(12.5%)
4	42	25(50.0%)	*	3
8	0.5	23(46.0%)	16(69.6%)	4(25.0%)
8	8	17(34.0%)	17(100%)	6(35.3%)
8	16	0	0	0
8	42	21(42.0%)	*	0
16	0.5	18(36.0%)	13(72.2%)	3(23.1%)
16	8	17(34.0%)	17(100%)	7(41.2%)
16	16	8(16.0%)	7(87.5%)	5(71.4%)
16	42	17(34.0%)	*	0
32	0.5	12(24.0%)	10(83.3%)	5(50.0%)
32	8	20(40.0%)	19(95.0%)	4(21.1%)
32	16	1(2.0%)	1(100%)	0
32	42	18(36.0%)	*	0

Table 27 (cont'd).

*These plant trays were not counted for the total number of viable plants, but were counted for the total number of sectors.
[1] Severe bug damage to plants was responsible for low viability.

Table 28. Sector frequency for *pm/pm* seedlings containing plastome IV treated for 16 hours with 9-aminoacridine hydrochloride.

ug/ml	# of seeds	Germination	Viability	Seedlings with sectors
0	508	558(100%)	337(60.4%)	175(51.9%)
2	500	476(95.2%)	342(71.8%)	193(56.4%)

Table 29. Sector frequency for seedlings containing plastome I treated with 9-aminoacridine hydrochloride. Seeds are mixed +/*pm* & *pm/pm*. Each trial contained 500 seeds that were treated for 16 hours.

ug/ml	Germination	Viability	Seedlings with sectors
0	468(93.6%)	332(70.9%)[1]	13(3.92%)
2	467(93.4%)	292(62.5%)[2]	22(7.53%)

[1] 40 plants with fused leaves, 5 stunted plants,
[2] 41 plants with fused leaves, 10 stunted plants.

Nalidixic acid effects on plastome mutator lines.

Preliminary tests with inbred pm/pm lines.

The initial nalidixic acid dosage trials utilized stocks with plastome I (Chapter 2, Table 19), plastome II (Chapter 2, Tables 13, 14,and 15), and plastome IV (Chapter 2, Tables 16, 17,and 18) in the *wild-type* nuclear background. Tables 30, 31, and 32 provide data on *pm/pm* seedlings, which were generated concurrently with Tables 13, 14, and 15 in Chapter 2. These data could not be used for the chi-square test due to size deficiencies in the chemical trials, and they were considered only in the establishment of the optimal dosage. It was determined that 10 ug/ml at 16 hours was optimal, in that occasional mutant sectors were observed at that concentration.

Nalidixic acid treatment of the mixed pm/pm, pm/+ line.

The data contained in Table 33 and Table 19 (Chapter 2) were analyzed by applying the chi-square goodness-of-fit test to determine if the mutation frequencies differed between the control and treated samples. Table 19 contained the data from the chemically treated *wild-type* seedlings that is necessary for the correction factor. Also, additional considerations, such as halving the population size, had to be made since the seed lot contained a mixture of seeds that were *pm/+* and *pm/pm* (see above).

Scoring after 40 days showed a slight increase in mutation frequency in *pm/pm* plants as a result of the nalidixic acid treatment, but the differences in the final mutation frequency of treated and control *pm/pm* plants were not statistically significant ($p > 0.5$).

Table 30. Sector frequency for *pm/pm* seedlings containing plastome II treated with varying doses of nalidixic acid. Each trial contained 50 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	26(52.0%)	21(80.0%)	6(28.6%)
0	6	3(6.0%)	3(100%)	2(66.7%)
0	24	8(16.0%)	2(25.0%)	0(<50%)
1	0.5	7(14.0%)	7(100%)	3(42.9%)
1	6	2(4.0%)	0	0(<0%)
1	24	3(6.0%)	1(33.3%)	0(<100%)
10	0.5	19(38.0%)	17(89.5%)	8(47.1%)
10	6	2(4.0%)	1(50.0%)	0(<100%)
10	24	37(74.0%)	26(70.3%)	13(50.0%)
100	0.5	16(32.0%)	11(68.8%)	6(54.5%)
100	6	30(60.0%)	21(70.0%)	10(47.6%)
100	24	45(90.0%)	40(88.9%)	14(35.0%)

Table 31. Sector frequency for *pm/pm* seedlings containing plastome II treated with varying doses of nalidixic acid.

ug/ml	Exposure time (hours)	# of seeds	Germination	Viability	Seedlings with sectors
0	0.5	50	21(42.0%)	14(66.7%)	6(42.9%)
0	6	100	33(33.0%)	20(60.6%)	10(50.0%)
0	24	50	25(50.0%)	19(76.0%)	6(31.6%)
1	0.5	50	11(22.0%)	10(90.9%)	8(80.0%)
1	6	100	34(34.0%)	27(79.4%)[1]	17(63.0%)
1	24	50	9(18.0%)	9(100%)	6(66.7%)
10	0.5	50	10(20.0%)	6(60.0%)	4(66.7%)
10	6	50	32(44.0%)	31(96.9%)	18(58.1%)
10	24	50	12(24.0%)	8(66.7%)	5(62.5%)
100	0.5	50	9(18.0%)	8(88.9%)	6(75.0%)
100	6	100	24(48.0%)	21(87.5%)	11(52.4%)
100	24	100	51(51.0%)	47(92.2%)	17(36.2%)

[1] two stunted plants

Table 32. Sector frequency for *pm/pm* seedlings containing plastome II treated with varying doses of nalidixic acid. Each trial contained 50 seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	25(50.0%)	17(68.0%)	2(11.8%)
0	6	29(58.0%)	18(62.1%)	4(22.2%)[1]
0	24	15(30.0%)	12(80.0%)	4(33.3%)
1	0.5	15(30.0%)	15(100%)	5(33.3%)
1	6	13(26.0%)	5(38.5%)	1(20.0%)[1]
1	24	15(30.0%)	13(86.7%)	5(38.5%)
10	0.5	26(52.0%)	22(84.6%)	10(45.5%)
10	6	18(36.0%)	9(50.0%)	2(22.2%)[1]
10	24	11(22.0.%)	10(90.9%)	6(60.0%)
100	0.5	24(48.0%)	24(100%)	9(37.5%)
100	6	24(48.0%)	12(50.0%)	3(25.0%)[1]
100	24	19(38.0%)	13(68.4%)	2(15.4%)

1 All seedlings had aberrantly shaped leaves.

2 All seeds from the six hour treatment came from the same seed lot, which was different then the rest of the trials.

Table 33. Sector frequency for *pm/pm, pm/+* seedlings containing plastome I treated with nalidixic acid. Each trial contained 500 seeds that were treated for 16 hours.

ug/ml	Germination	Viability	Seedlings with sectors
0	468(93.60%)	332(70.94%)	13(3.92%)[1]
10	483(96.60%)	361(74.74%)	20(5.54%)[2]

[1] 40 plants with fused leaves and 5 stunted plants,
[2] 24 plants with fused leaves and 6 bleached plants with thin leafs.

Novobiocin effects on plastome mutator lines.

Preliminary tests with inbred pm/pm lines.

The initial novobiocin dosage trials utilized stocks with plastome II (Chapter 2, Table 22), and plastome IV (Chapter 2, Table 23 and 24) in the wild-type nuclear background. The data on *pm/pm* seedlings in Table 34 was generated concurrently with Table 22 in Chapter 2, and was considered only in the establishment of the optimal dosage. It was determined that 1000 ug/ml at 32 hours was necessary to obtain an impact on chloroplast genetic system, since transient sectors were occasionally observed after such a treatment.

Novobiocin treatment of the mixed pm/pm, pm/+ line.

The data contained in Table 35 and Table 24 (Chapter 2) were analyzed by applying the chi-square goodness-of-fit test to determine if the mutation frequencies differed between the control and the treated samples. Table 24 contains data from the *wild-type* seedlings necessary for the correction factor. Additional considerations, such as the mutation rate, had to be made since the seed lot contained a mixture of seeds that were *pm/+* and *pm/pm* (see above).

Scoring after 40 days showed an increase in mutation frequency in *pm/pm* plants when compared to *wt* plants as a result of novobiocin treatment and the differences in the

final mutation frequency of treated and control *pm/pm* plants were statistically significant ($0.025 > p > 0.01$).

Table 34. Sector frequency for *pm/pm* seedlings containing plastome II treated with varying doses of novobiocin. Each trial contained 50 seeds except for trial 0 ug/ml at 32 hours which contained seeds.

ug/ml	Exposure time (hours)	Germination	Viability	Seedlings with sectors
0	0.5	25(50.0%)	8(32.0%)	4(50.0%)
0	1	18(36.0%)	11(61.1%)	7(63.6%)
0	24	22(44.0%)	9(40.9%)	2(22.2%)
0	32	20(52.6%)	20(100%)	13(65.0%)
10	0.5	22(44.0%)	6(27.3%)	2(33.3%)
10	1	25(50.0%)	8(32.0%)	2(25.0%)
10	24	25(50.0%)	5(20.0%)	2(40.0%)
10	32	22(44.0%)	17(77.3%)	8(47.1%)
100	0.5	14(28.0%)	3(21.4%)	0(<33%)
100	1	27(54.0%)	12(44.4%)	6(50.0%)
100	24	12(24.0%)	6(50.0%)	1(16.7%)
100	32	12(24.0%)	1(8.3%)	0(<100%)
1000	0.5	10(20.0%)	2(20.0%)	1(50.0%)
1000	1	8(16.0%)	4(50.0%)	2(50.0%)
1000	24	10(20.0%)	5(50.0%)	1(20.0%)
1000	32	28(56.0%)	3(10.7%)	2(66.7%)

Table 35. Sector frequency for a mixed population of *pm/pm* and *pm/+* seedlings containing plastome I treated with novobiocin. Each trial contained 500 seeds treated for 32 hours.

ug/ml	Germination	Viability	Seedlings with sectors
0	468(93.60%)	332(70.94%)	13(3.92%)[1]
1000	465(93.00%)	222(47.74%)	15(6.76%)[2]

1 40 plants with fused leaves, 5 stunted plants.
2 9 plants with abnormal leaf development.

DISCUSSION

Previous experiments with the *plastome mutator* gene of *Oenothera* have shown that the *plastome mutator* activity is determined by a nuclear recessive allele. Thus, it is likely that one of the elements for the chloroplast DNA repair and/or replication processes, which are encoded in the nucleus, is lost due to a null mutation.

Mutagenesis was used to test the plastome's repair and replication pathways, with chlorotic sectors identifying a newly mutated chloroplast. Acridine was used because it intercalates into the double helix causing frameshifts and addition/deletion events, and would verify the efficiency of the general repair processes in the *plastome mutator* system. The usage of novobiocin and nalidixic acid should interrupt the chloroplast DNA gyrase, and would thus test another element of the replication pathway.

Even though the number of visible sectors in wild-type *Oenothera* was low after treatment with the chemicals nalidixic acid and novobiocin, I still proceeded with the treatment of *Oenothera* plants homozygous for the *plastome mutator* gene for the following reason: if DNA damage was caused by these two chemicals, it may have been efficiently repaired in the *wild-type* plant lines, thus, giving a low mutation frequency. However, the *plastome mutator* defect

might render susceptible the chloroplasts of *pm*-plants. Other simultaneous investigations performed by Lara Steben (personal communication) using the restored *plastome mutator* (*pm/pm* and *pm/+*) line found late developing sectors when the plants were put into the field, consistent with the observations of Epp (1973). Conceivably, *plastome mutator* activity requires a great deal of dilution of the pm^+ product present in the egg cell. If this was the case, then the embryos exposed to chemical mutagenesis in the seed may not yet have possessed *plastome mutator* activity. However, one would expect that with the number of cell divisions that occur to produce the embryo, the pm^+ product would be adequately diluted such that the chloroplast genetic system would be susceptible to mutation. In any case, this is the only reasonable recourse to eliminate the tremendous starting mutation rate seen in seeds produced by *pm/pm* self-pollinations.

Scoring the seedlings for visible sectors showed an increase in the mutation frequency in the *pm/pm* plant line as a result of 9-aminoacridine hydrochloride treatment, but the differences in the final mutation frequencies of control and treated *pm/pm* seedlings were not statistically significant ($0.05 < p < 0.10$). In fact, neither acridine nor nalidixic acid could be demonstrated to affect the mutation frequency in the *pm* plant lines.

The difference between the control and acridine-treated *pm/pm* seedlings are not statistically significant, yet there were many chlorotic sectors observed in the *wt* seedlings. This suggests, along with the crossing data, that 9-aminoacridine hydrochloride is a potent plastome mutagen. The lack of a synergistic effect in inducing chlorosis in conjunction with the *pm/pm* plant line implies that the DNA lesion caused by 9-aminoacridine hydrochloride does not interfere with the *pm* defect. Hopefully, more trials with larger sample sizes will allow a clearer statistical determination.

From the experiments with nalidixic acid, as in the *wild-type Oenothera* experiments (Chapter 1) it appears that a functional gyrase subunit A is regenerated upon removal of the inhibitor, as inferred in *Solanum nigrum,* where cell growth recovers after the removal of the chemical (Ye and Sayre 1990) and the gyrase subunit A portion of the chloroplast replication system is not involved with the *pm* gene, either directly or indirectly.

Conversely, the novobiocin treatment of *pm/pm* seeds resulted in about twice the number of visible sectors as obtained without the chemical treatment. This also contrasted with the novobiocin treatment of *wt* seedlings which produced few chlorotic sectors. These differences in the final mutation frequencies of control and treated *pm/pm* seedlings were

statistically significant. This suggests that damage resulting from novobiocin is corrected in the *wild-type* plants but not in *pm/pm* plants. Many experiments show that novobiocin perturbs gyrase (Gellert et al. 1977, 1978; Sugino et al. 1977, 1978; Ciarrocchi et al. 1985; Thompson and Mosig 1985; Ye and Sayre 1990). If this is also true in *Oenothera*, it suggests that the *wild-type Oenothera* chloroplast may have a novobiocin-insensitive bypass replication as seen in *Chlamydomonas reinhardtii* (Woelfle et al. 1993). In that case, the *wt* seedlings would not show any effects or few effects from novobiocin while the *pm/pm* plants would show a synergistic increase in the induction of chlorotic sectors, if they lack the novobiocin-insensitive gyrase.

Further testing is needed to determine if topoisomerase II/gyrase is indeed the target for novobiocin. If the target is gyrase, it then becomes necessary to find out how transcription is affected in the *pm/pm* plant line and why no sectors were seen in *wt* seedlings when they were treated with novobiocin.

CONCLUSION

Previous investigations have shown that in the *plastome mutator* line of *Oenothera hookeri,* chloroplast DNA is the target of the increased spontaneous mutation rate. Significant cpDNA alterations and effects include: 1) *pm* induces a wide variety of mutations indicating that a number of loci can be mutated (Epp et al. 1987), 2) deletions of variable sizes occur at hot spots on the cpDNA molecule (Chui et al. 1990), 3) direct repeats are involved in the high frequency deletions (Blasko et al. 1988; Chiu et al. 1990), 4) the cpDNA deletions are separable from the mutant phenotypes (Chui et al. 1990) and 5) NMU mutagenesis interacts synergistically with the *plastome mutator* activity (Sears and Sokowski 1991). These findings and the inability to obtain antibiotic-resistance from *pm/pm* callus indicate that the mutations causing the chlorosis are likely to be very small deletions/insertions which eliminate gene function. Summarized, it appears that the *pm* gene mutates the cpDNA at many sites, causing small deletions/insertions of direct repeats and favoring certain sites.

Nalidixic acid and novobiocin have both been shown to inhibit gyrase in *Escherichia coli* (Gellert et al. 1976 and 1777; Sugino et al. 1977 and 1978; Drica and Snyder 1977; Lockshon

and Morris 1983), cultured carrot cells (Ciarrocchi et al.1985), cultured *Nicotiana tabacum* cells (Heinhorst et al. 1985), *Chlamydomonas reinhardii* (Thompson and Mosig 1984 and 1987), pea chloroplasts (Lam and Chua 1987), and cultured *Solanum nigrum* cells (Ye and Sayre 1990)). The synergism observed between novobiocin and the *pm* line implicates subunit B of gyrase as being involved in the *plastome mutator* defect or it may point to the possibility that a novobiocin bypass system similar to that found in *C. reinhardtii* (Woelfe et al. 1993) may be present in *Oenothera* but defective in the *pm* line.

Both nalidixic acid and novobiocin have been used on many organisms as mentioned above, to perturb gyrase. These chemical agents have been shown to bind gyrase, resulting in a secondary effect on transcription, with a decrease of some transcripts and a increase in other transcripts. Thus, gyrase seems to be utilized in transcription control, as well as in replication and repair. One hypothesis is that there are two different gyrases present in the chloroplast. One of these gyrases may be preferential and perform most or all of the supercoiling of the DNA in the chloroplast.

In this hypothesis the *plastome mutator* gene may encode a faulty B subunit for the preferred gyrase resulting in loss of its function. It now becomes necessary for the second

gyrase to perform all supercoiling in the chloroplast. This second gyrase may not be as well adapted to supercoiling as the perferred gyrase. This could result in an underwound or a highly supercoiled cpDNA molecule resulting in aberrant transcription, and in others, a high rate of mutation. With an underwound cpDNA, the replicating machinery might tend to slip ahead, deleting direct repeats or even a single base pair as suggested by Skopek and Hutchinson (1984).

An explanation as to why an increase in chlorosis is observed with the novobiocin treatment but not with the nalidixic acid treatment is due to the chemicals affecting different subunits of gyrase. When nalidixic acid is used to treat *Oenothera* seeds, the mutagen targets the A subunit of the preferred gyrase which in the *plastome mutator* line has lost function due to the mutator activity. Since the wild-type plants have a functional preferred gyrase, there would be little impact on their genetic system and a dramatic increase in mutation rate would not be observed. Conversely, when novobiocin is used to treat *Oenothera* seeds, the mutagen targets the B subunit of the second gyrase. The chloroplast of the *pm* line lack a functional preferred gyrase due to the *pm*-mutation, and contain a novobiocin-perturbed second gyrase leaving them with no functioning gyrase for the duration of the novobiocin-treatment. The consequence would be the synergistic increase in chlorosis seen in *pm/pm* plants.

The hypothesis presented could follow the premise introduced by Volff et al. (1993), who found that the occurrence of genetic instability, deletions and amplifications of DNA sequences in streptomycetes is specific to chemical treatments interfering with DNA replication, including gyrase inhibitors. Because the inhibition of DNA replication induces the SOS response causing deletions in *E. coli* and *Salmonella typhimurium* (Ishii and Kondo, 1975), Volff et al. (1993) suggested that either DNA gyrase is directly involved in genetic instability or that it may be part of an SOS-like system.

In addition to the above hypothesis, a gyrase-mediated recombination system was proposed by Ikeda et al. in 1981. The *plastome mutator* system may be similar in mode of action. If the gyrase cleaves DNA within each direct repeat in a molecule, single strands protruding at each end of the repeats would be complementary to each other and could pair. If the gyrase ligated both ends to join the two double strands, this would result in the formation of a deletion by eliminating the segment between the repeated sequences and one copy of the repeats. This would require that the gyrase cleavage site be within a repeat and may explain why one direct repeat suffers deletions over other direct repeats within the cpDNA. This gyrase-mediated recombination system, if faulty in the *plastome mutator* plant line, does not

account for the high rate of mutation frequency that is observed in *Oenothera*.

In summary, the data produced here lends supports to Epp's findings in 1973, that the *plastome mutator* is a null mutation. This null mutation may be a gyrase subunit and result in an underwound cpDNA molecule. The distortion of the cpDNA may cause the slippage of the replicating machinery and produce permanent alterations in the DNA of the plastids. This in turn, could affect a variety of loci and cause chlorosis as is observed in the *pm* plant line.

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