NUCLEAR GENE-INDUCED PLASTOME MUTATIONS IN *OENOTHERA HOOKERI*. I. GENETIC ANALYSIS*

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ABSTRACT

A nuclear gene mutation in *Oenothera hookeri* increases the frequency of variegated sectors. The gene is recessive; the variegation is cytoplasmically transmitted. Once variegation is induced, the mutant gene is not required for its continued expression. The induced sectors may differ one from another. The gene expresses unique patterns of penetrance and of maternal effect. The genetic data implicate the chloroplasts as the site for the expression of variegation. The chloroplasts of *O. parviflora* are also subject to the action of the nuclear gene. Possible mechanisms by which a gene might cause chloroplasts to mutate are discussed.

GENETIC systems with nuclear gene-induced plastome mutations have three features: (1) they contain a nuclear gene which increases the variegation frequency; (2) the induced variegation must be persistent through the sexual cycle as a cytoplasmically-inherited phenomenon; and (3) the induced variegation is independent of the nuclear gene for its continued expression. The term plastome encompasses the genetic material contained within the plastid.

Verification of the fact that plastids contain their own deoxyribonucleic acid established the material basis for the cytoplasmic transmission of plastids reported independently by CORRENS (1909) and BAUR (1909). Efforts to obtain cytoplasmically-inherited plastid mutations in higher plants by conventional methods of induction and selection have been predominantly unsuccessful (reviewed by KUTZELNIGG 1968; EPP 1972a). But an increasing number of examples in the literature on higher plant genetics indicate that, under the influence of specific mutant nuclear loci, the naturally low spontaneous rate of plastid mutations can be increased many fold. Spontaneous plastid mutations have been observed in 0.01–1.3% of the plants within control populations (MICHAELIS 1969).

Genes which increase the mutation rate of plastids have been isolated in a wide variety of angiosperms, including *Arabidopsis thaliana* (RÖBBELEN 1966; RÉDEI and PLURAD 1972), *Capsicum annuum* (HAGIWARA and OOMURA 1939), *Epilobium hirsutum* (MICHAELIS 1968), *Hordeum vulgare* (IMAI 1936; ARNASON, HARRINGTON and FRIESEN 1946; HAGEMANN and SCHOLZ 1962; WETTSTEIN and

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ERIKSSON 1965), *Nepeta cataria* (WOODS and DUBUY 1951), *Petunia hybrida* (POTRYKUS 1970), *Oryza sativa* (PAL and RAMANUJAM 1941), *Zea mays* (RHOADES 1943; STROUP 1970). A similar mutation has been isolated in *Oenothera hookeri* T. & G. (EPP 1972a,b). This mutation is of interest because (1) extensive studies on chloroplast genetics in the subgenus Euoenothera have been published; (2) the transmission of chloroplasts in Oenothera is biparental; (3) the mutation induces plastid mutations which differ in phenotypic expression; and (4) this mutation may provide new insights into nuclear-plastid interactions.

This paper presents genetic evidence that the nuclear mutation in Oenothera which increases the frequency of variegation is inherited as a single mendelian gene. Once induced, the variegation is thereafter not dependent upon the presence of the gene and is cytoplasmically inherited. Sectors arising independently of one another may differ phenotypically. The gene can also induce plastid mutations in a species of Oenothera different from the species within which the nuclear mutation was originally isolated. The mutant gene, when homozygous, has no effect upon the viability of seedlings.

MATERIALS AND METHODS

Stocks: Three laboratory strains of Oenothera subgenus Euoenothera (Onagraceae) were used for these experiments: (1) The Johansen strain is from the species *O. hookeri* T. & G. and has seven bivalents at meiosis. Johansen is a naturally cross-pollinating plant but has been maintained by single-plant self-pollinations for approximately thirty years; its genome appears to be lethal-free since no lethals have been recovered when genomes from control Johansen have been forced to homozygosity (EPP 1971). The plastids of *O. hookeri* are of type I (STUBBE 1959). (2) The Charlottesville strain, from the species *O. parviflora* L., is a true breeding complex-heterozygote. The chromosomes of Charlottesville form a circle of 14 during meiosis. Since the chromosomes normally segregate alternately, two types of gametes are formed. The α gamete is regularly transmitted through the egg cell and has its chromosome ends arranged so that when crossed with Johansen the hybrid will form a circle of 14 chromosomes at meiosis. The α genome contains lethals and therefore forms a genetic system for either forcing the Johansen genome to homozygosity or maintaining the Johansen genome in the heterozygous condition. The α-Charlottesville genome carries a dominant gene which imparts red pigmentation to the meristematic region and undersides of the cotyledon and causes the leaves to develop red pigment spots during the rosette stage. Seedlings homozygous for the Johansen genome are not pigmented during the cotyledon stage and develop only light pigment spots on the rosette leaves. In the adult stage these two genomically different plants can be identified morphologically as well as by meiotic chromosome analysis. Therefore, it is possible to distinguish between the homozygous Johansen plants and the hybrid α-Charlottesville/Johansen plants during each stage of development. This cross is diagrammed in Figure 1. The β-Charlottesville/Johansen hybrid can be identified morphologically in the seedling stage. This hybrid forms a circle of eight chromosomes with three bivalents at meiosis; the plant is weak and therefore has not been used in experiments. The plastids of *O. parviflora* are type IV. (3) Douthat 4b from the species *O. argillicola* Mackenz. is a seven-paired strain which, like Johansen, is naturally cross-pollinated but has been maintained for many generations by self-pollinating individual plants. Douthat 4b will be used here simply to test for plastid types. The plastids of *O. argillicola* are type V. The plastids native to each of these three species can be distinguished from each other on the basis of their nuclear-plastome interactions and their multiplication rates (reviewed by KIRK and TILNEY-BASSETT 1967; STUBBE 1971).

The laboratory strain Johansen with type IV plastids from Charlottesville was also used. The origin and utility of this strain has been described (STINSON 1960).

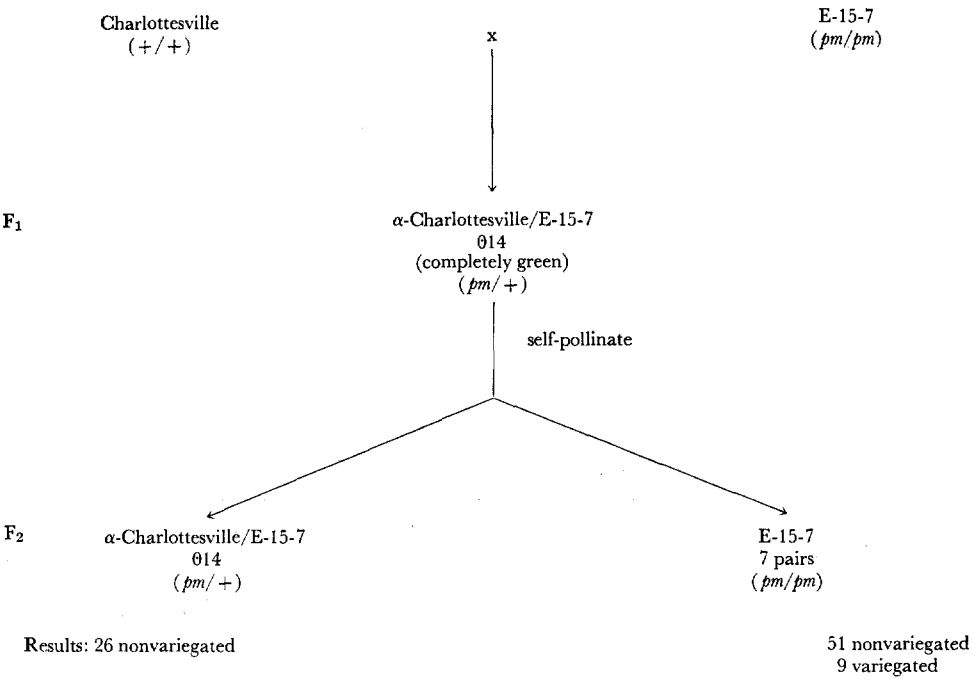

Figure 1.

FIGURE 1.—The crossing protocol and results indicating that the E-15–7 genome causes variegation. The seedlings were scored at the four-leaf stage. The putative genotypes are indicated.

Source of mutation: During the summer of 1969, a variegated plant, E-15–7, was isolated from a greenhouse-grown M_2 population of Johansen. This population was the progeny of a self-pollinated plant derived from a lot of mutagenized seeds. The seeds had been soaked in a 0.02 M ethyl methanesulfonate phosphate-buffered solution for 24 hours, 21–23°, pH 7.4. E-15–7 has seven pairs of chromosomes. It is phenotypically indistinguishable from control Johansen except that it develops variegated sectors. This paper describes the analysis that was worked out to ascertain the genetic basis of the variegation.

Cytoplasmic variegation in Oenothera: The cytoplasmic mutations studied in this paper must be maintained as variegated plants. Seedlings in the cotyledon stage survive only a few days if tissues contain the cytoplasmic mutations. But if the seedlings are variegated with tissue having normal chloroplasts, the normal tissue can support the development of the seedlings. Seedlings with enough supportive tissue can develop to maturity. They will be mosaic for normal tissue and tissue with cytoplasmic mutation. Crosses can be made with flowers on normal tissue or on mutant tissue.

Many plants develop branches with periclinal chimeras, i.e., one or more of the histogenic tissue layers contain the mutant cytoplasm and the other tissue layers will have normal cytoplasm. Periclinal chimeras are stable arrangements. Maintaining cytoplasmic mutations as periclinal chimeras in the subepidermal histogenic tissue layer is preferred over selecting completely mutant branches because periclinal chimeras support more vigorous growth of the mutant tissue. Mutant branches are weak and consequently more susceptible to disease, sunburn and early death.

Both the reproductive tissue and the margin of the leaf are derived from the subepidermal histogenic tissue layer, L II. The cytoplasmic phenotype of reproductive tissue of a flower is determined by observation of the margin of the subtending leaf. By extrapolation, the phenotype

of the leaf margin is also the presumptive cytoplasmic phenotype of the reproductive cells. As shown by many experiments over many decades, this procedure for identifying the cytoplasmic phenotype of the reproductive cells is remarkably accurate; however, it can give erroneous results, particularly in systems where the cytoplasmic phenotype is subject to frequent alterations. Small variegated sectors in the reproductive tissue might be a source of error; these would not be detected by observing the margin of the leaf that subtends a flower.

Cytoplasmic mutations which are transmitted to progeny from the parental generation will usually express themselves in the cotyledon stage. The first few leaves rarely show variegation if the cotyledons are normal green; but if the leaves are variegated, the variegation is contiguous with the apical meristem. The apical meristem would be chimeral containing a mutant sector.

Variegation which arises anew within a generation need not involve the apical meristem. The tissue involved and the size of the sector would reflect the time during the ontogeny of the plant that a stable cell lineage became established. The sizes range from chimeras involving a number of branches on a plant to a small sector on one leaf.

RESULTS

Is the variegation induced by a virus? Before completion of the genetic analysis, the possibility of a viral origin for the variegation was tested. Virus infections can cause patterns of variegation which at times simulate variegation caused by plastome mutations (KIRK and TILNEY-BASSETT 1967; EASU 1968); also, crystalline-like bodies were seen in the electron micrographs of *pm-1* and *pm-2* plastids.

Leaves of variegated tissue were macerated with a sterile mortar and pestle by the techniques of Ross (1964) and MATTHEWS (1970). Some preparations were thinned with distilled water or phosphate buffer, others not. The slurry was applied with cotton swabs to leaves lightly dusted with carborundum. The plant species inoculated were *O. hookeri* strain Johansen, *Phaseolus vulgaris* (pinto bean UI111), *Nicotiana tabacum* (var. Xanthi N.C. and Turkish), *Chenopodium quinoa*, *Chenopodium amaranticolor*, and *Cucurbita pepo* (var. Early Prolific Straight-neck). (The seeds of Nicotiana and Chenopodium were the kind gift of DR. A. L. GRANETT, New York State Agricultural Experiment Station, Geneva, New York, and the Phaseolus seed, of DR. R. S. SANDSTED, Cornell University.) The leaves were scored 10 and 14 days after inoculation for the presence of local lesions; none were evident. The plants were scored again at four weeks for possible systemic infections; none were observed. This experiment was repeated twice. Electron micrographs of cross-sections of vascular bundles of Oenothera leaves containing only *pm-1* plastids also did not show any apparent virus infection or cytoplasmic changes and inclusions that might be indicative of virus infections. The crystalline-like body of *pm-1* and *pm-2* plastids may be analogous to the tubular structures that have been reported in dark-grown barley seedlings fed δ-aminoevulinic acid (WETTSTEIN *et al.* 1971). The lack of light energy may have prevented the barley chloroplasts from utilizing accumulated components. The Oenothera chloroplasts may be genetically incapable of using some components; these components may accumulate and subsequently aggregate.

Control Johansen scions were also grafted to an α-Charlottesville/Johansen hybrid plant expressing the *pm-1* phenotype in 75% of its tissue. Flowers on the control Johansen were self-pollinated. If the variegation were caused by a graft-

transmissible virus infection, the seedlings should express an increased frequency of variegation. The frequency of variegation remained at the spontaneous control rate. For control Johansen seedlings, 0.14% will express spontaneous variegation (EPP 1972a).

Variegation induced by nuclear gene: A protocol was designed to test for nuclear involvement in the initiation of variegated sectors. The first scheme tested for the involvement of the E-15–7 genome. Pollen from a flower on the original E-15–7 mutant sector was used to pollinate a flower on Charlottesville. Forty-three of the 99 resulting seedlings were variegated. This variegation was considered to have originated by cytoplasmic male transmission from E-15–7 since it was phenotypically similar to the sector on E-15–7. But to test for nuclear involvement in the induction of variegation, an F_1 plant was selected that was completely green, i.e., devoid of variegation, throughout its entire growing period. This was a precaution to minimize the possibility of any variegation being transmitted to the following generation and thus to ensure that any variegation among the progeny would be the result of newly induced cytoplasmic mutations. The diagram of this cross (Figure 1) indicates that only F_2 seedlings classified homozygous for the E-15–7 genome expressed variegation. Nine out of 60 such seedlings had variegated sectors in the four-leaf stage. The variegation scored in this experiment included any sector that followed cell lineages and was not normal green in color. These data indicate that the homozygous E-15–7 genome increases the frequency of variegation. The reason that only 15% of the homozygous E-15–7 seedlings expressed variegation at the four-leaf stage will be discussed below.

A second crossing scheme was used to test for the segregation of a mendelizing locus. Three α-Charlottesville/E-15–7 plants were pollinated with pollen from control Johansen, an E-15–7/Johansen hybrid, and E-15–7–1, a plant obtained by self-pollinating E-15–7. The three maternal plants were variegated with the variegation transmitted by E-15–7, but only flowers on branches with normal green tissue were used in these crosses. Only the seven-paired seedlings were scored at maturity. The seedlings containing α-Charlottesville were discarded because the E-15–7 genome would always be in the heterozygous condition. The cotyledons and first few leaves of these seedlings were not variegated; also much of the variegation scored here differed phenotypically from the original sector on E-15–7 and so are considered new inductional events.

The data in Table 1 show that whenever Johansen is the paternal parent none of the resulting progeny express variegation at maturity. But when the hybrid E-15–7/Johansen is used as pollen source, approximately half of the progeny are variegated. If E-15–7–1 is used, all the progeny express variegation. If a maternal parent is self-pollinated all of the progeny are also variegated.

These data support the evidence that the E-15–7 genome increases the frequency of variegation. The increased frequency of variegation is a recessive trait because it is observed only when the E-15–7 genome is homozygous and not when heterozygous with control Johansen. The approximation of a 1:1 segregation in the cross where the hybrid E-15–7/Johansen was used as the pollen parent indi-

TABLE 1

Mature plants scored for variegation

Maternal parent α-Charlottesville/E-15–7 Plant no.		Paternal parent	Mature plants Without variegation	Mature plants With variegation
6	×	Johansen	27	0
6	×	(E-15–7 × Johansen)	4	6
6	×	E-15–7–1	0	10
10	×	self-pollinated	0	19
10	×	Johansen	11	0
10	×	(E-15–7 × Johansen)	11	4
10	×	E-15–7–1	0	5
11	×	Johansen	15	0
11	×	(E-15–7 × Johansen)	6	7
11	×	E-15–7–1	0	8

Only the Johansen-type seedlings were scored among the progeny; only flowers on green tissue were used in these crosses.

cates a single mendelizing locus. The data also indicate that the original plant E-15–7 was homozygous for the mutation. Additional data will be presented below.

Designation of nuclear gene: The nuclear mutation has been designated *pm*, "plastome mutator". This name is intended not to imply a mechanism of action, but rather to describe a property. The rationale for inferring that mutations actually reside in the plastome will be detailed below. This nuclear mutation appears to be recessive. To date, no detectable increase in the variegation of plants heterozygous for *pm* has been observed.

Penetrance and maternal effect of pm: Not all seedlings homozygous for *pm* immediately express some form of variegation. The data in Figure 1 have shown that only 15% of the seedlings scored in the four-leaf stage expressed variegation. Another set of crosses focuses on the problem of penetrance of *pm*.

Three plants were used: (1) the original plant E-15–7; (2) a hybrid between E-15–7 and control Johansen; and (3) control Johansen. Flowers on the plant E-15–7 were pollinated with pollen from either control wild-type Johansen or the hybrid E-15–7/Johansen; and the hybrid E-15–7/Johansen was pollinated with pollen from Johansen or E-15–7. Only flowers on phenotypically green tissue were used for these crosses. The progeny of these crosses were scored for variegation at the four-leaf stage; the results are given in Table 2.

These crosses were designed to test the hypothesis that a single gene is responsible for the induction of variegation. Since the penetrance pattern was not understood at the time the data in Table 2 were initially analyzed, the mode of inheritance of the induction of variegation could not be deduced. Therefore approximately 20 green seedlings from each cross shown in Table 2 were grown to maturity. Flowers on green branches were individually self-pollinated. Approximately 90 offspring from each such parent were scored at the four-leaf stage.

TABLE 2

Crosses to test hypothesis that a single gene is responsible for induction of variegation. The putative genotypes are indicated. See text for a discussion of the results

Crosses		Expected genotypes of progeny	Phenotype at 4-leaf stage (No. of plants) Observed		Expected	
Female	Male		Var	Green	Var	Green
1. E-15-7 (*pm/pm*)	E-15-7 × Johansen (*pm/+*)	1/2(*pm/pm*) 1/2(*pm/+*)	46	152	52	146
2. E-15-7 (*pm/pm*)	Johansen (+/+)	(*pm/+*)	20	179	0	199
3. E-15-7 × Johansen (*pm/+*)	E-15-7 (*pm/pm*)	1/2(*pm/pm*) 1/2(*pm/+*)	8	165	13	160
4. E-15-7 × Johansen (*pm/+*)	Johansen (+/+)	1/2(*pm/+*) 1/2(+/+)	0	201	0	201

All 14 plants tested from cross 2 were expected to be heterozygous for *pm*. The percentage of variegated seedlings on a per-plant basis ranged from 2.20% to 9.09%. This frequency of variegation was used to identify heterozygous plants in crosses 1, 3, and 4. Plants with progenies showing variegation at a frequency exceeding the cross 2 range by 1% or 2% were also classified heterozygous for *pm*. This formed natural groupings.

The plants from crosses 1 and 3 showed the breeding behavior of presumptive homozygous *pm* and heterozygous *pm* plants. The plants from cross 4 also fell into two distinct classes indicative of homozygous (+/+, all green plants on selfing) and heterozygous (*pm/+*) plants. The distribution of plants in the homozygous or heterozygous categories of crosses 1, 3, and 4 approximate a 1:1 ratio and support the evidence that *pm* is a single mendelizing locus.

It is also evident from Table 3 that not all seedlings homozygous for *pm* immediately express some form of variegation. Seedlings scored in the four-leaf stage express a level of penetrance which reflects the genetic composition of the parent. The overall mean percent of variegated progeny for all plants classified heterozygous *pm* in Table 3 is 4.65%. Since only 25% of the progeny of these plants would be expected to be homozygous for *pm*, the penetrance is 18.6%. But the overall mean for homozygous *pm* plants is 53.30%. Since all seedlings would be homozygous the penetrance is also 53.3%.

By using this information on penetrance, the data in Table 2 can be interpreted. In cross 1, one-half of the progeny are expected to be homozygous for *pm*. Since the maternal parent was also homozygous *pm* one would expect 53% penetrance or 52 variegated seedlings. This is in good agreement with the results because forty-six were actually observed. In cross 3 half of the progeny are expected to be homozygous for *pm* but since the maternal parent was heterozygous a penetrance of 18.6% is expected, or 15 variegated seedlings. Eight were observed. In crosses 2 and 4 no variegated seedlings would be expected since none of the seedlings would be homozygous *pm*. This is indeed the case in cross 4.

TABLE 3

Green seedlings from the crosses in Table 1 were grown to maturity and self-pollinated. The progeny of each plant was scored (four-leaf stage) for variegation. The data are presented on a per plant basis and are expressed as percentage of seedlings showing variegation. Approximately 90 offspring from each such parent were scored at the four-leaf stage. The putative genotypes of the plants are in parentheses

Cross 1		Cross 2	Cross 3		Cross 4	
(*pm*/*pm*) 1/2	(*pm*/+) 1/2	(*pm*/+)	(*pm*/*pm*) 1/2	(*pm*/+) 1/2	(*pm*/+) 1/2	(+/+) 1/2
48.48	1.74	2.80	50.43	5.36	1.05	0
20.00	4.17	6.45	50.00	6.42	5.08	0
36.56	2.86	4.11	64.95	11.50	7.69	0
47.79	4.17	4.81	59.16	6.14	2.94	0
58.00	1.75	3.51		4.21	3.30	0
57.66	7.76	2.20		1.75	0.91	0
73.63	2.52	2.38		3.64	5.26	
	2.61	3.13		6.67	7.22	
	4.49	9.09			0.80	
	8.79	6.32			4.29	
	3.00	5.00			7.14	
	11.01	5.13				
		3.16				
		5.13				

Only in cross 2 does the variegation frequency show a sizeable deviation from the expected frequency. This deviation is attributed to possible errors in selecting nonmutant maternal tissue. The maternal plant in cross 2 was homozygous for *pm*; an inductive event late in the development of the ovary could have caused a mutant sector involving 10% of the ovules. An event of this size would go unnoticed since it would not include the margin of the subtending leaf.

The maternal parent appears to determine the level of penetrance. This is evidenced in crosses 1 and 3 of Table 2. Cross 3 is the reciprocal of cross 1. If the maternal parent is homozygous *pm*, the penetrance in the progeny approximates the level observed when homozygous *pm* plants are self-pollinated. Similar levels of penetrance are observed for the progeny of a heterozygous maternal parent and the progeny of self-pollinated heterozygous plants.

The measurements of penetrance appear to be reproducible phenomena. The penetrance level of all seedlings scored for new variegation at the four-leaf stage are summarized in Table 4. More than 5,000 seedlings from heterozygous maternal plants were scored in four experiments. The mean penetrance ranged from 9%–18%. In two experiments where the maternal parent was homozygous, the penetrance level approximated 50%.

The level of penetrance is primarily a reflection of events that occurred in the seedlings themselves. It does not reflect cytoplasmic variegation transmitted from the paternal generation. This finding is evidenced when samples of seeds were germinated on moist filter paper in petri dishes. These samples of seeds were

TABLE 4

Comparison of the penetrance of pm *in the progeny of homozygous and of heterozygous plants. Seedlings scored in the four-leaf stage.*

Source of progeny	Total number scored	Number of variegated progeny	Percent variegated progeny	Percent variegated plants expected	Penetrance
Heterozygous					
α-Charlottesville/E-15–7, self-pollinated, from Figure 1	60*	9	15.00	100.0	15.0
Total progeny of all plants classified heterozygous (*pm*/+), from Table 3	4109	191	4.65	25.0	18.6
Heterozygous (*pm*/+) × homozygous (*pm*/*pm*), from Table 2, cross 3	173	8	4.62	50.0	9.2
α-Charlottesville/E-15–7 with plastome IV, from Table 8	1056*	102	9.66	100.0	9.7
Homozygous					
Homozygous (*pm*/*pm*) × heterozygous (*pm*/+), from Table 2, cross 1	196	46	23.47	50.0	46.9
Total of all plants classified homozygous (*pm*/*pm*), from Table 3	1077	574	53.30	100.0	53.3

* Only the homozygous E-15–7 scored.

drawn from the same seed lots sampled for the experiment in Table 3. The germinated seedlings were scored for the presence of variegation under a stereoscopic dissecting microscope with a magnification of 10–35 X. Only nine seedlings of 8919 showed any cotyledon variegation; none was totally mutant. The nine variegated seedlings were found only among progenies of plants later classified homozygous *pm* on the basis of the results given in Table 3. These nine variegated seedlings might well have arisen by cytoplasmic transmission from the parent plants, but the frequency is too low to explain adequately the different levels of variegation among seedlings scored at the four-leaf stage from heterozygous or homozygous parents.

In an effort to learn more about the penetrance of *pm*, an experiment was designed to compare the levels of penetrance of sibling seedlings, some scored at the four-leaf stage and others at maturity. The seeds used were from self-pollinated plants classified homozygous *pm*; therefore, all the seedlings also should be homozygous *pm*. The results (Table 5) show that at the four-leaf stage penetrance ranged from 47.8% to 73.6%, but that by the time the seedlings were mature, 100% had expressed at least one variegated sector.

Designation of variegation: Independently arising variegated sectors were given designations of *pm-1*, *pm-2*, etc., signifying that the variegation arose under the action of the nuclear gene mutation. The genetics of six sectors has been studied. Some properties of these cytoplasmic mutations are detailed below. Each is inherited in a pattern consistent with the biparental transmission of cytoplasmically-inherited traits. Sectors *pm-1*, *pm-2*, and *pm-4* have arisen on the original E-15–7 plant or vegetatively propagated plants of E-15–7. Sectors *pm-3*,

TABLE 5

Comparison of the penetrance of pm *at the four-leaf stage and in mature plants in progeny of self-pollinated plants classified homozygous* pm *in the experiment of Table 3*

Plant no.	Four-leaf stage	Mature plants		
	Percentage of seedlings variegated	Without variegation	With variegation	Percent with variegation
1–7	47.8	0	27	100.0
1–8	58.0	0	42	100.0
1–13	73.6	0	28	100.0
1–19	48.5	0	21	100.0
3–1	50.4	0	18	100.0
3–7	50.0	0	32	100.0

pm-5, and *pm-6* have been identified among seedlings that had E-15–7 as the maternal parent and either Johansen or a hybrid E-15–7/Johansen as the paternal parent.

Since seedlings that are completely mutant with no green tissue are lethal, these cytoplasmic mutations are maintained as chimeras on plants having enough green tissue to sustain growth. The biparental transmission of these mutations aids in the routine perpetuation of chimeral plants.

Besides the above six cytoplasmic mutants, others have already been isolated and are being studied.

Cytoplasmic mutants differ one from another: An interesting feature of the *pm* system is that the *pm*-induced cytoplasmically-inherited mutations may have unique phenotypes. These phenotypes have been stable, and some have already been cytoplasmically-transmitted through as many as four successive generations. The general phenotypes of these mutants differ in their color during the cotyledon and mature leaf stages, in sensitivity to light intensity, and in effect on the expansion of the leaf lamina (Table 6).

The *pm-1* phenotype has a mottled white-green appearance due to different degrees of development of the plastids within adjacent cells; the development of all the plastids within a cell appears to be similar, but the green chloroplasts have an abnormal thylakoid formation, as shown by electron microscope studies. This mosaicism does not appear to be the result of several populations of different plastid types; there has never been a sorting-out of all white tissue. The original sector had this mottled phenotype, and it is developed anew every generation. The cotyledons containing *pm-1* are uniformly greenish-white initially and secondarily develop the mottled appearance.

The *pm-2* phenotype shares with *pm-4* and *pm-6* the characteristic that increased light intensities decrease the amount of pigment in the tissue. This can be demonstrated easily by shading portions of the greenhouse with whitewash or cheesecloth.

The *pm-3* phenotype has a unique pattern of localized greening. Leaves that are half mutant and half normal have a gradient of decreasing greenness extending about one-quarter inch from the normal tissue and fading into the *pm-3*

TABLE 6

General phenotype of pm-*induced cytoplasmically-transmitted mutations*

Phenotype	*pm-1*	*pm-2*	*pm-3*	*pm-4*	*pm-5*	*pm-6*
Color	white-green	yellow-green	white	light yellow	white	yellow-green
Mottled	yes	no	no	no	no	no
Color sensitive to light intensity	no	yes	no	yes	no	yes
Cotyledon color	greenish white	yellow-green	white	yellow-green	light yellow	light green
Restricted lamina development	no	slight to none	yes	slight	yes	slight
Greening if adjacent to normal tissue	none apparent	no	yes	no	no	no

tissues. Leaves that are totally *pm-3* have a greening at the junction of the primary lateral veins and the midrib of the leaf. Under low light intensities this greening can extend to the entire leaf but continues to be most intense near vascular tissue. Tissue with *pm-3* can nevertheless always be readily distinguished from tissue with wild-type plastids.

The mature leaves of *pm-4* have a light yellow color; *pm-5* is white. The *pm-5* phenotype appears much like *pm-3* but does not have the secondary greening behavior; these two mutants do share the common trait of restricting the laminal growth of a leaf. Restricted laminal growth is particularly evident in leaves that are divided along the midrib with one half containing the mutant and the other half normal wild-type plastids; the difference in growth rates causes these leaves to become asymmetric. The general phenotype of *pm-6* appears very similar to *pm-2*.

An initial study of the development of *pm-1*, *pm-2*, and *pm-3* plastids with electron microscopy (EPP 1972a) demonstrated that these three mutations have different developmental lesions. These mutants are different, and each can be identified by its unique fine structural phenotype.

Cytoplasmic inheritance of variegation: The six independently arising sectors mentioned above have been tested for cytoplasmic inheritance; the results are consistent with patterns of cytoplasmic inheritance for a plant species having biparental transmission of cytoplasm. The phenotype of each individual sector has been perpetuated intact in each successive generation. The data for the cytoplasmic mutants *pm-1*, *pm-4*, and *pm-6* are presented in Table 7. When a flower on cytoplasmic mutant tissue is self-pollinated, the progeny normally are all mutant. The self-pollinations of flowers on tissue bearing *pm-4* and *pm-6* are exemplary. When a flower on *pm-1* tissue was self-pollinated most of the seedlings were phenotypically *pm-1*, but several seedlings were variegated with normal green tissue and one seedling was completely green. The source of the green tissue is considered here to have come from the movement of cells between adjacent histogenic tissue layers. STEWART and BURK (1970) have studied periclinal chimeras in Nicotiana and showed that a low number of cells from the tissue layers adjacent to the subepidermal layer may become incorporated in the subepidermal layer. Evidence that this is occurring in Oenothera comes from the analysis of the progeny from individual adjacent capsules which shows that germinated seeds from some capsules give only mutant seedlings and others give mostly mutant plus a few variegated and green seedlings.

The cytoplasmic mutants described in Table 7 were crossed reciprocally to a control plant from the strain Johansen. Reciprocal cross differences are evident. Since plastids are inherited biparentally in Oenothera the differences are of degree rather than indicating absolute maternal transmission. The variegation observed among the progeny is always the unique phenotype characteristic of the cytoplasmic mutant involved in the cross. When the pollen source is a flower on cytoplasmically-mutant tissue, some of the mutant plastids are transmitted to the progeny. For the mutant *pm-4*, paternal transmission of the mutant could be demonstrated only when the maternal parent had a plastid with a slower multi-

TABLE 7

Cytoplasmic transmission of pm*-induced mutations. Cotyledon color was scored. The plastid compositions of the egg cells and pollen are indicated by superscripts above the nuclear pedigree. Since* pm*-induced mutations must be maintained as chimeras, the plastid type of the green supportive tissue is in parentheses*

	Number			Percent seed germination
	Green	Var*	Mutant†	
pm-1				
$\overset{pm\text{-}1}{(\text{E-15-7})(\text{I})}$ self-pollinated	1	5	82	59
Johansen$^{\text{I}}$ × $\overset{pm\text{-}1}{\text{E-15-7}}(\text{I})$	156	3	0	80
$\overset{pm\text{-}1}{\text{E-15-7}}(\text{I})$ × Johansen$^{\text{I}}$	6	134	84	53
pm-4				
$\overset{pm\text{-}4}{(\text{Johansen} \times \text{E-15-7})}(\text{IV})$ self-pollinated	0	0	243	60
Johansen$^{\text{I}}$ × $\overset{pm\text{-}4}{(\text{Johansen} \times \text{E-15-7})}(\text{IV})$	96	0	0	96
Johansen$^{\text{IV}}$ × $\overset{pm\text{-}4}{(\text{Johansen} \times \text{E-15-7})}(\text{IV})$	124	36	0	84
$\overset{pm\text{-}4}{\text{E-15-7}}(\text{I})$ × Johansen$^{\text{I}}$	0	7	59	86
pm-6				
$\overset{pm\text{-}6}{[\text{E-15-7} \times (\text{E-15-7} \times \text{Johansen})]}(\text{I})$ self-pollinated	0	0	96	96
Johansen$^{\text{I}}$ × $\overset{pm\text{-}6}{[\text{E-15-7} \times (\text{E-15-7} \times \text{Johansen})]}(\text{I})$	143	1	0	96
$\overset{pm\text{-}6}{[\text{E-15-7} \times (\text{E-15-7} \times \text{Johansen})]}(\text{I})$ × Johansen$^{\text{I}}$	3	23	121	98

* The seedlings were a mosaic of normal green tissue and tissue with the specific *pm*-induced cytoplasmic mutation used in the cross.

† The seedlings were entirely of tissue with the phenotype of the specific *pm*-induced cytoplasmic mutation being tested.

plication rate (SCHÖTZ 1954). If the maternal parent carried the cytoplasmic mutant and the pollen source contained the normal wild-type plastid then the reciprocal gradient became apparent.

Induced variegation is independent of the nuclear gene for its continued expression: In experiments testing the transmission of *pm*, the inheritance of *pm* approximates mendelian ratios. The transmission of the variegation does not approximate mendelian ratios but rather each cytoplasmic mutant displays a characteristic frequency of transmission (EPP 1972a). Data from Table 7 show that, when the variegated plant used for self-pollinating a flower on *pm-4* tissue was heterozygous for the nuclear gene *pm*, all the seedlings were completely mutant despite the expectation that only one-fourth would be homozygous for *pm*. After the initial induction of cytoplasmically-inherited mutant sectors, the phenotype is not expressed in frequencies approximating mendelian ratios; this indicates that the expression of the cytoplasmically-inherited phenotype is not

dependent on the continued presence of the nuclear gene for expression. Additional experiments are in progress to verify specifically that a variegated plant can have a wild-type (+/+) genotype.

Is the action of pm *restricted to* O. hookeri *(type I) plastids?* The action of *pm* is not restricted to the *O. hookeri* plastids but can also induce variegation in the plastids from the species, *O. parviflora* (type IV). The experimental procedure leading to this result essentially follows the cross protocol outlined in Figure 1. A plant from the strain Charlottesville with its native plastid (type IV) was crossed as the maternal parent with the original plant E-15–7. The pollen used was from a flower on the sector later designated *pm-1*. Therefore the F_1 seedlings would be expected to have a nuclear composition as diagrammed in Figure 1, but all green plastids should be of type IV. Some plants would be variegated with the paternally transmitted *pm-1* plastids. Crosses were made to verify that the green plastids were type IV. Usually three or more consecutive flowers on an inflorescence were crossed to *O. argillicola* strain Douthat 4b as the pollen parent. On the basis of the extensive work of STUBBE (1959) characterizing the nuclear-genome interactions, if the egg cells carried plastids of type I the seedlings upon germination would be white or yellow. But if the egg cells contained plastids of type IV the seedlings would be green. The cotyledons would also on occasion be variegated with paternally transmitted plastids of type V from *O. argillicola*; these would be either yellow to yellow-green or periodically lutescent, depending upon the nuclear composition of the seedling—Johansen/Douthat 4b or α-Charlottesville/Douthat 4b, respectively. All branches tested did carry plastids from *O. parviflora.* Flowers that developed later than those used in the crosses described above were self-pollinated. The resulting homozygous E-15–7 seedlings would be homozygous for *pm* but carry type IV plastids. The data in Table 8 summarize the results from a total of 15 tested branches on eight different plants. Plastids of type IV are responsive to the action of *pm* and form variegated sectors at the frequency expected among seedlings derived from a heterozygous maternal parent and scored in the four-leaf stage.

Two of the four variegated seedlings scored α-Charlottesville/E-15–7 were variegated in the cotyledon stage and therefore may represent an event that occurred in the maternal parent since both seedlings were derived from the same capsule and had similar-appearing sectors. The other two were variegated in the leaves only and may represent the products of crossover events: (1) the dominant pigment marker identifying the α-Charlottesville genome may have crossed over to the Johansen genome; or (2) *pm* of the Johansen genome may have become

TABLE 8

The number of seedlings with variegation among the progeny of green α-Charlottesville/E-15–7 plants verified to carry plastid type IV. Seedlings scored in the four-leaf stage

α-Charlottesville/E-15–7		Homozygous E-15–7	
Green	Variegated	Green	Variegated
553	4	963	102

associated with the α-Charlottesville genome. From other experiments, the pigment marker is known to crossover into the Johansen genome at a frequency of about 1–2%. This would be sufficient to account for most of the crossing over even if it is assumed that the two variegated hybrid seedlings represent only 10% of one-half of the seedlings with crossover events (penetrance of *pm* approximates 10%, and only half of the crossover products could be identified). The seedlings were not grown to maturity for further evaluation. These data also indicate that the gene *pm* is located very near or proximal to the translocation breakage point. Crosses are in progress for mapping *pm* to chromosome, with strains of Oenothera having multiple translocations.

Viability of seedlings homozygous for the genome bearing pm: The Johansen seedlings scored in Table 8 represent the progeny of eight different α-Charlottesville/E-15–7 plants. By self pollinating these plants, the genetic content of eight pollen grains from E-15–7 were forced to homozygosity. Of the seedlings scored, 65.7% were classified as the homozygous Johansen and 34.3% as hybrid α-Charlottesville/Johansen. In a comparable experiment with genomes from control Johansen the frequency of the homozygous Johansen averaged 57.5% (EPP 1971). This information would indicate: (1) that the viability of seedlings homozygous for *pm* is not reduced and (2) that the genomes derived from E-15–7 do not harbor other undetected nuclear mutations which reduce seedling viability.

DISCUSSION

The results of the genetic analysis detailed above indicate that the original variegated plant E-15–7 was homozygous for a nuclear mutation which increases the frequency of variegation. It was also shown that the phenotypes of the variegated sectors were cytoplasmically-transmitted unaltered and intact and were not dependent on the nuclear gene for the expression of the mutant phenotype. The pattern of penetrance of *pm* indicates that the genomic composition of the maternal parent will influence the timing of the occurrence of variegation, but that all plants homozygous for the gene *pm* will have expressed some variegation at maturity.

Possible mechanisms whereby a nuclear gene causes components of the cytoplasm to become mutation-prone are indeed intriguing to contemplate. The evidence to date indicates that the variegation observed does emanate from altered plastids. This does not eliminate the possibility that other organelles are involved, but current evidence does not implicate other organelles. SURZYCKI and GILLHAM (1971) have shown that one must not assume that all cytoplasmically-inherited traits involve plastid DNA. Mitochondrial or plasmone changes have also reportedly been induced by several of the nuclear genes that induced plastome mutations (WETTSTEIN and ERIKSSON 1965; MICHAELIS 1968). Nevertheless, in electron micrographs of *pm-1*, *pm-2* and *pm-3* (EPP 1972a) the plastids are abnormal but mitochondria appear normal.

The transmission of the variegated phenotype also seems to indicate that the plastids carry the mutations. The plastids of different species of Oenothera have

been shown to have different intrinsic multiplication rates (SCHÖTZ 1954). In simplest terms the results have been interpreted to mean that the plastids of the different species can be ranked according to their multiplication rates when present together in the same cell. KIRK and TILNEY-BASSETT (1967) and STUBBE (1971) have reviewed this plastid characteristic; it appears to be determined primarily by the plastids.

This Oenothera plastid parameter, multiplication rate, can be used to strengthen the circumstantial evidence for true plastome mutations. If a common pollen source carrying a *pm*-induced cytoplasmic mutation were used to pollinate flowers on plants with a slow multiplying plastid and to pollinate flowers on plants with a fast multiplying plastid, and the amount of mutant tissue among the progeny were decreased relative to the multiplication rate of the plastids, the plastid would be implicated as the carrier of the mutation. The frequency of transmission of the cytoplasmic mutation would not be determined by the rate of plastid multiplication if some component other than the plastid were mutant. Evidence is accumulating that indeed the *pm*-induced cytoplasmic mutations are competitively transmitted relative to the multiplication rate of the wild-type plastids of Oenothera. This is illustrated in Table 7: *pm-4* was transmitted paternally only when type IV plastids were present in the egg cells, and not when the faster multiplying type I plastids were present. The nuclear composition of both maternal plants used was control Johansen. A more complete analysis of this competitive transmission is in progress, but preliminary analysis indicates also that the different *pm*-induced mutations differ in their rates of transmission to subsequent generations.

Since the genetic evidence indicates that placing the *pm*-induced mutations with a control nucleus (+/+) does not rectify the mutant phenotype, the cytoplasmic mutations appear to be permanent alterations in the DNA of the plastids. It is possible to envision that the gene *pm* codes for some component necessary for the replication of the plastid DNA. One such component may be the plastid DNA polymerase itself. There is circumstantial evidence from inhibitor studies with Chlamydomonas that the chloroplast DNA polymerase might be coded by a nuclear gene (SURZYCKI *et al.* 1970). There is evidence that the mitochondrial DNA polymerase is also coded by a nuclear gene (ASHWELL and WORK 1970). The work initiated by SPEYER (1965) suggests that some temperature-sensitive mutants of the T4 phage gene 43, the structural gene for T4 DNA polymerase, may be mutagenic in that increased levels of replication errors occur. In the process of inserting replication errors, these temperature-sensitive mutations can induce reversions of other known mutants within the T4 genome (DE VRIES, SWART-IDENBURG and DE WAARD 1972). Therefore, both new mutations and reversions can be induced by these mutant polymerases.

Several other possible mechanisms have been suggested for nuclear-induced plastome mutations. The gene product may have episome-like properties and become integrated into the plastome (RÖBBELEN 1966). In the integrated position the gene product would regulate the integrity of the DNA replication. A low molecular weight RNA species has been shown to elicit pathogenic responses in

several plants (SEMANCIK and WEATHERS 1972). The evidence obtained from *pm* indicates that controlling elements are probably not involved because the genetic behavior of the gene is the same when tested in crosses with either newly arisen variegated sectors or green tissue from the same plant. Also suggested has been the JACOB-MONOD model of gene regulation (POTRYKUS 1970). Again the *pm*-induced mutations do not support such a model because the cytoplasmic mutations persist even when *pm* has been replaced by its wild-type allele.

If *pm* does introduce errors into the DNA, other intriguing problems develop. Since the DNA of plastids appears to be extensively reiterated (reviewed by KIRK 1971), would a replication error be expressed? Can the penetrance pattern described above adequately account for the process of recombination and genomic rearrangement within the plastid which might be necessary before a mutation in the plastome would be expressed? Since *pm* induces many phenotypes, are different functional genetic units of the plastome being altered? These are some of the basic considerations as work continues with *pm*.

The action of *pm* appears to be restricted to mutating the plastids. The nuclear genes do not appear to mutate at rates above the spontaneous level even in plants homozygous for *pm*. No aberrant growth patterns have been observed which might indicate plasmone mutations.

Experiments are being planned to study not only the nuclear-plastome interaction but also the differences between the plastid mutations themselves. It is hoped that as more analyses of these cytoplasmic mutations are made, some concept will develop about the genetic units within the plastome which are being altered.

Since Oenothera plastids of both type I and type IV are influenced by *pm*, the action of *pm* involves a property of Oenothera plastids shared by type I and type IV and not a property unique to either. It has not yet been ascertained whether mutants of type I differ from mutants of type IV plastids.

The patterns of penetrance of *pm* seem to indicate a time factor, an induction period required for the action of *pm*. This suggests that the gene product of the wild-type allele at the *pm* locus is transmitted to the seeds while still attached to the maternal plant. This influence must be diluted out before the genotype of the embryo or seedlings can be expressed. By maturity the maternal influence is no longer evident, and the ability to variegate approximates mendelian ratios.

At present it is impossible to make any definitive statement about the mechanism of induction with reference to whether a single plastid initially is rendered mutant and then subsequently sorts out to establish a homoplastidic cell lineage, or whether by some mechanism all plastids in a cell are rendered mutant. By the time a sector is identified, a homoplastidic cell lineage has already been established. Inherent in this problem is the actual number of plastomes involved in the process of perpetuating the plastome from one cell generation to the next. The fewer plastomes that are actively reproduced, the more readily a mutant line can be established.

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