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Nuclear and Cytoplasmic Chloroplast Mutants Induced by Chemical Mutagens in *Mimulus cardinalis*: Genetics and Ultrastructure

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Summary. The modes of inheritance of chemically induced chlorophyll-deficient phenotypes in *Mimulus cardinalis* reveal that the chloroplast is controlled by the genome and the plastome. Three of the chlorophyll-deficient mutants in *M. cardinalis* are inherited through nuclear recessive genes and two are inherited through plastome genes. One chlorophyll-deficient mutant was sterile and could not be analyzed genetically. Ultrastructural analysis of the six mutant types reveals that each possesses a unique defective chloroplast type(s) in comparison to the genotypically and phenotypically normal chloroplasts. Based on plastid ultrastructure it seems reasonable to assume that the mutations, genome and plastome, are non-allelic or at least significantly different forms of the same allele. The isolation of these types of mutants provide suitable material needed to study the effects of specific biochemical blocks and the elucidation of developmental pathways leading to chloroplast biogenesis. The mutants also provide valuable information concerning the interrelationship between the nucleic acid of the genome and the plastome.

Introduction

In experiments designed to induce and isolate a spectrum of mutants in the angiosperm *Mimulus cardinalis*, several chlorophyll-deficient plants were detected (Travis and Wilson, 1970; Travis et al., 1971; Travis and Wilson, 1972a). Appearance of these mutants was not unexpected since chlorophyll mutants are important indicators of mutagenic activity and are among the most common type of mutations detected in higher plants (Baur, 1909; Correns, 1909; D'Amato et al., 1962; Genebach et al., 1970; Gichner and Veleminský, 1965; Monti, 1968; Murr and Stebbins, 1971; Rhoades, 1946; Stadler, 1928; Walles, 1971). Chlorophyll-deficient mutants may have their origin in nuclear gene mutations or extrachromosomal mutations (Levine, 1972; Preer, 1971; Sager, 1972; Walles, 1971; Wildman et al., 1973; von Wettstein et al., 1971). Phenotypically these mutant types range from white or pale yellow to variegated white or pale yellow. Many of the nuclear gene controlled chlorophyll-deficient mutants die in the cotyledon stage when the mutated loci are in the homozygous condition (Genebach et al., 1970; Veleminský et al., 1969). The lethal genes may be maintained, however, by isolating plants in the heterozygous condition. Chlorophyll-deficient mutants produced by extrachromosomal mutations can be maintained by self-pollinating appropriate flowers on variegated branches and collecting the mature seeds (Burk et al., 1964; Stewart, 1965). Although a large volume of information concerning the nature of pigment deficient mutants exists in the literature, the highly differentiated protoplasm of higher plant cells, with their system of particulate organelles, requires a great deal more understanding.

Chlorophyll-deficient plants provide favorable material for many types of genetic investigations (Boardman et al., 1971; Gibbs, 1971; Kirk and Tilney-Bassett, 1967; Sager, 1972). Biochemical mutants among chlorophyll-deficient plants can provide meaningful information on the biosynthetic pathways of specific metabolites. These types of chlorophyll-deficient mutants may respond to exogenous sources of specific metabolites (Boynton, 1966a; Li and Rédei, 1969; Murr and Stebbins, 1971; Rédei, 1965; Walles, 1963). The response of the biochemical mutants to metabolites allows for the localization of genetic blocks and thus elucidation of gene-controlled biosynthetic pathways. Biochemical mutations which lead to the abnormal development of chloroplasts in higher plants have been demonstrated to affect compounds synthesized only in the chloroplasts, e.g., carotenoids, chlorophyll, and some enzymes (Bachmann et al., 1967; Boynton and Henningsen, 1967; Heber and Gottschalk, 1963; Walles, 1965). Defective chloroplasts are also induced when mutations affect compounds which are synthesized in either the cytosol or other organelles, e.g., aspartic acid and thiamine

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(Boynton, 1966a; Langridge, 1955; Walles, 1963). Some chlorophyll-deficient mutants provide favorable material for investigating the gene-mediated differentiation of chloroplast development at the ultrastructural level (Boynton, 1966b; Rosinski and Rosen, 1972; von Wettstein, 1961). In addition to potentially providing information about metabolite biosynthesis and chloroplast development, chlorophyll-deficient mutants may provide exceptional material to investigate the molecular biology of DNA. Consequently, the controversy of chloroplast autonomy, in comparison to simultaneous genetic input from the nucleus, is being investigated by studying the relationship between chloroplast ultrastructure and its DNA (Wildman, 1971a; Wildman et al., 1973; Wong-Staal and Wildman, 1973). This relatively recent experimental pursuit requires a wide variety and an appropriate amount of chlorophyll-deficient material.

Unfortunately, in the majority of cases of detected abnormal chloroplast morphogenesis, resulting in chlorophyll-deficient mutants, the blocked biosynthetic pathways have not been identified. Ultrastructural analysis of defective chloroplasts under nuclear gene control has provided much data concerning their development. However, little information is available about the development of chloroplast mutants under the control of the plastome (chloroplast genome). Furthermore, in only one possible case, at the time of this writing, have the structural alterations of defective chloroplasts been directly attributable to mutated chloroplast DNA in comparison to wildtype chloroplast DNA (Wong-Staal and Wildman, 1973). Because of the incomplete understanding on the nature of chloroplasts, identification and classification of new plastid mutants continues to be important. New mutants may substantiate previously documented information and/or provide a more suitable material for future experiments on the nature of plant protoplasts.

In this paper, we report on the use of an organism which is highly advantageous in studies involving the structure and function of chloroplasts. This paper presents genetic and ultrastructural information on the nature of nuclear and cytoplasmic chloroplast mutants in the angiosperm *Mimulus cardinalis*.

Materials and Methods

The chlorophyll-deficient mutants discussed in this study were induced and isolated following treatment of *Mimulus cardinalis* seeds at the LD_{95} level in 0.01 M N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or following 30 minute treatment with 0.1 M nitrous acid (NA) (Travis and Wilson, 1970; Travis et al., 1971; Travis and Wilson, 1972a; Wilson, 1971). A strain of *Mimulus cardinalis* Douglas, culture number 5031, collected from Beaver Creek, Siskiyou County, California, was selected as the standard (Pollock et al., 1967). The seeds collected from the standard wildtype were used as the parental generation (P_1) and these seeds when treated with mutagens are referred to as M_1 generation seeds. The subsequent generation seeds are referred to as M_2 generation seeds. Standard wildtype plants were grown under greenhouse conditions, in a soilpeat mixture, and P_1 seeds were collected following self-pollination. Mutagen treated lines were maintained in the same manner. However, in some cases, M_1 mutant plants were propagated by rooting cuttings of various mutant branches that appeared as growth from lateral buds.

Inheritance studies began with observations and subsequent isolation of dominant variegated-chlorophyll mutants in mature M_1 generation plants. These mutants were tentatively classified as extrachromosomally (cytoplasmically) inherited chloroplast mutants. The search for recessive chlorophyll mutants was initiated by self-pollinating phenotypically normal M_1 plants. Self-pollinated M_1 plants which gave rise to approximately one-fourth achlorophyllous M_2 seedlings were tentatively classified as being heterozygous for nuclear recessive lethal genes. The various mutants isolated were self-pollinated and reciprocally crossed to substantiate their mode of inheritance. The M_1 and M_2 generation seeds were germinated in two manners, both of which gave similar results. In the first method, M_1 seeds, immediately after treatment with mutagen, were rinsed with sterile distilled water and sterilized for one minute in 10% sodium hypochlorite (commercial "Clorox") solution. After sodium hypochlorite treatment the seeds were rinsed twice in sterile distilled water and plated on Hoagland's medium enriched by addition of 1 ml of micronutrients per liter of solution (Arditti and Dunn, 1969; Henke et al., 1974). The plating medium was solidified by addition of Difco Bacto Agar in concentration of 2 per cent (w/v). Petri dishes containing about 30 ml of solidified sterile medium were overlayed with the treated seeds. The standard growth conditions were the incubation of seeds in a growth chamber, at 22°C. Light at about 900 ft-c was provided from cool-white fluorescent lamps under a 16:8-h light-dark regime. The alternative method was to allow the M_1 seeds to germinate on the surface of a soilpeat mixture under greenhouse conditions. M_2 seeds were always air dried after harvesting and germinated in the same manner as M_1 seeds. M_1 and M_2 plants were counted and classified in the cotyledon stage, as well as the mature plant stage, since seedlings totally deficient in chlorophyll do not survive beyond the cotyledon stage.

Cytological examinations of tissue from the standard wildtype and mutant plants were carried out to determine distribution of normal and abnormal chloroplasts. Freehand cross sections of fresh material mounted in water were examined using a Zeiss Nomarski differential interference microscope.

For electron microscopic examination, pieces of tissue, approximately 2×3 mm, were fixed for three hours with cold (ice bath) 5% glutaraldehyde in 0.2 M cacodylate buffer at pH adjusted to 7.2 with HCL. Following three rinses in cold 0.2 M cacodylate buffer, the material was postfixed for three hours in cold 1% unbuffered osmium tetroxide (OsO_4) or cold 2% unbuffered potassium permanganate ($KMnO_4$). The tissues were then washed twice in cacodylate buffer at about 23°C followed by dehydration in a graded acetone series of 20-40-60-80-100 p at minimum two hour intervals. The 100% acetone dehydration was repeated three times. The tissue

was then passed through 50% propylene oxide in acetone for 1 hour and finally placed in 100% propylene oxide before embedding. Both the postfixed OsO_4 and $KMnO_4$ material was embedded in Spurr's low viscosity medium (Spurr, 1969). Thin sections were cut on a Sorvall (Porter Blum) MT-1 ultramicrotome and post-stained with uranyl acetate and lead citrate for 5 minutes each. Ultrastructural examination was with an Hitachi HU-125 E electron microscope.

Results

Chlorophyll-deficient Mutant Phenotypes

The chlorophyll-deficient mutants discussed in this study are classified in a manner similar to the system used for barley mutants (Gaul, 1964). Six different chlorophyll-deficient mutant phenotypes were observed and studied following chemical mutagenesis (Table 1). The two basic chlorophyll-deficient phenotype patterns observed in the M_1 generation following seed treatment with MNNG are *"viridis"* and *"striata"* mutants. The *viridis* mutants have shoots with pale yellow-green variegated patterns. In addition to the variegated shoots, *viridis* mutant plants also possess phenotypically normal green shoots. The pale yellow-green variegated *viridis* shoots themselves persist with a life-span analogous to the wildtype standard. The *striata* mutants have shoots with white-green variegated patterns. *Striata* mutants also possess phenotypically normal green branches on the same plant. *Striata* type shoots survive for a maximum of two months. This is less than one-half the life of a normal shoot. Often, *striata* mutant plants give rise to shoots with stems and leaves that are completely or primarily *albino*, rather than basically variegated. The completely *albino* shoots persist for approximately two months, apparently due to efficient translocation of photosynthetic metabolite products from normal green shoots of the same plant.

In *viridis* and *striata* mutants, the variegated patterns of affected branches were conspicuous on leaves, stems, petioles and/or calyces. Frequently, variegated shoots of both mutant phenotypes give rise to shoots of all green tissue. New variegated branches often developed from plants where variegated shoots died or gave rise to all green tissued shoots.

These observations indicate that different plastome types somatically sort-out during normal growth and development (Burk et al., 1964; Stewart, 1965; Epp, 1973). Consequently, heteroplastidic plants, carrying wildtype and mutated plastomes, can produce, by virtue of segregation at the cell divisions in apical meristems, cell lineages of either heteroplastidic or homoplastidic nature. The heteroplastidic *striata* and *viridis* mutants may be also defined as periclinal chimeras inasmuch as their mosaic patterns sometimes completely segregate during mitosis to stable homoplastidic conditions, either wildtype or mutant (Tilney-Bassett, 1963).

The four basic patterns of chlorophyll-deficiency found in M_2 generation plants, segregating from phenotypically normal M_1 generation plants, are *"xantha"*,

Table 1. Characteristics of chemically induced chlorophyll-deficient mutants in *Mimulus cardinalis*

Chlorophyll-deficient mutant type	Chemical mutagen used	Generation mutant detected	Mutant tissue color	Distribution of mutant tissue	Distinguishing morphological mutant character(s)
Viridis	MNNG[a]	M_1	Pale yellow	Variegated patterns on stems, leaves, petioles, calyces	Mutant and normal shoots on mature plant
Striata	MNNG	M_1	White	Variegated patterns on stems, leaves, petioles, calyces and complete albino shoots	Mutant and normal shoots on mature plants
Xantha	MNNG	M_2	Yellow	Cotyledon stage	Condition is lethal; plants die in cotyledon stage
Albina-MG	MNNG	M_2	White	Cotyledon stage	Condition is lethal; plants die in cotyledon stage
Albina-NA	NA[b]	M_2	White	Cotyledon stage	Condition is lethal; plants die in cotyledon stage
Chlorotic	MNNG	M_2	Pale yellow	Entire mutant plant	Plant produces only dwarf shoots

[a] MNNG = N-methyl-N'-nitro-N-nitrosoguanidine.

[b] NA = nitrous acid.

"*albina*-MG", "*albina*-NA", and "*chlorotic*" mutants. *Xantha* mutants are completely yellow and die in the cotyledon stage of development. *Albina*-MG mutants and *albina*-NA mutants are completely white and also are lethal, dying in the cotyledon stage. *Xantha*, *albina*-MG and *albina*-NA mutants survive after germination for an estimated fourteen days. Foliar leaves never develop. The *chlorotic* mutants reach maturity and produce only mutant branches. The branches of the *chlorotic* mutants are completely pale yellow. In addition to the complete chlorosis, *chlorotic* mutant plants are morphologically abnormal. They vary from the standard wildtype in producing profuse lateral dwarf branches possessing extremely lanceolate leaves.

Inheritance Studies

The phenotype ratios of seedlings inherited from crosses involving the *striata* and *viridis* mutants depend on the pattern of variegation on the M_1 shoot and are transmitted only through the maternal parent. All seedlings grown from seed obtained from capsules taken from normal green shoots were green. All seedlings grown from seed obtained from capsules from the all *albino* shoots were white. Variable proportions of green, white, pale yellow, and variegated seedlings were obtained from selected capsules taken from white-green *striata* mutant shoots or pale yellow-green *viridis* mutant shoots. These observations are consistent with the concept that the subepidermal histogenic tissue layer, L II, gives rise to both mosaic leaves and calyces and continues into the reproductive tissue forming the ovules (Burk et al., 1964; Stewart, 1965; Satina and Blakeslee, 1941). Thus the phenotype of the subtending leaf is normally the phenotype of the reproductive cells. These assumptions are supported by our results since the heteroplastidic branches of *striata* and *viridis* mutants produced the expected offspring upon selfing. Thus, from selfpollinations and reciprocal crosses to the wildtype *Mimulus cardinalis*, the mode of inheritance of chlorophyll-deficiency in *striata* and *viridis* mutants was determined to be non-Mendelian (Table 2 and 3). Because the chlorophyll-deficient chloroplasts are transmitted in both mutants cytoplasmically, i.e., maternally, these chlorophyll variegations are assumed to be under the control of the plastome, the hereditary material localized in the plastids. The presence of nuclear mutator genes inducing defective chloroplasts is discounted, in part, because the chlorophyll variegations appear in the M_1 generation (Rédei, 1973; Epp, 1973).

The inheritance of *albina*-MG, *albina*-NA and *xantha* type mutants in the M_2 progenies, from phenotypically normal mutagen treated plants, fit classic Men-

Table 2. Inheritance of green, variegated, and white *Mimulus cardinalis* seedlings from backcrosses and self-pollinations of flowers on chlorophyll-deficient *striata* mutants

		Seedlings		
		Green	Variegated	White
Maternal plant	Paternal plant	Number	Number	Number
Striata (variegated)	× Self	131	2	128
Striata (variegated)	× 5031	194	0	137
5031	× *Striata* (variegated)	329	0	0
Striata (white shoot)	× Self	0	0	168
Striata (white shoot)	× 5031	0	0	366
5031	× *Striata* (white shoot)	381	0	0

Table 3. Inheritance of green, variegated, and pale yellow *Mimulus cardinalis* seedlings from backcrosses and self-pollinations of flowers on chlorophyll-deficient *viridis* mutants

		Seedlings		
		Green	Variegated	Pale yellow
Maternal plant	Paternal plant	Number	Number	Number
Viridis (variegated)	× Self	2	1	172
Viridis (variegated)	× 5031	6	0	243
5031	× *Viridis* (variegated)	264	0	0

delian ratios of 3 normal : 1 mutant (Table 4). Based on goodness-of-fit to a 3:1 ratio, we assumed that the genes responsible for the chlorophyll-deficiency in both *albina* and *xantha* mutants are nuclear recessive genes. Nuclear gene-induced plastome mutations are discounted because of the Mendelain ratios and the lack of variegated patterns.

One hundred per cent of the progeny which developed from seeds obtained from a single phenotypically normal (green) M_1 line plant gave rise to M_2 generation *chlorotic* mutants. *Chlorotic* mutants are sterile at maturity prohibiting genetic analysis.

Cytological Observations

The distribution of normal and abnormal chloroplasts within tissues and cell types in mutant and wildtype shoot material was determined by observations with a Zeiss Nomarski differential interference microscope (Table 1). The ellipsodial to oval shaped chloroplasts observed in all wildtype mesophyll cells and guard cells are assumed to have normal chlorophyll content, structure and function. The conspicuous places where chlorophyll is lacking in the wildtype plants is on the calyces and epidermis. The calyx suture areas, where sepals are fused, are white to pale green. Cytological examination shows apparent normal chloroplasts but occurring at a low frequency. Interestingly, the calyces of all white shoots derived from *striata* chimera mutants exhibit entirely green sutures where the sepals are fused, the direct antithesis of the wildtype situation. Chloroplasts were observed in the guard cells of the epidermis. Chloroplasts in other epidermal cells were not obvious in any plant examined.

Depending upon the tissue examined in either the *striata* or *viridis* mutants with the light microscope, cells with apparently normal green chloroplasts or cells with smaller yellowish to colorless plastids were observed. Single cells with a mixture of normal and abnormal plastids were observed in *striata* mutant tissue. Cells containing normal chloroplasts apparently adjacent to cells containing abnormal plastids are also frequently observed in sections of white tissue from the *striata* mutants. Normal green chloroplasts were consistently observed in guard cells contiguous to the all white *striata* tissues of the mesophyll layers. This observation supports our belief that *striata* mutants are heteroplastidic only in the subepidermal histogenic tissue layer, L II. No apparent normal chloroplasts were observed in any cells of the *albina*- MG, *albina*- NA, *xantha* and *chlorotic* mutants.

Chloroplast Ultrastructure

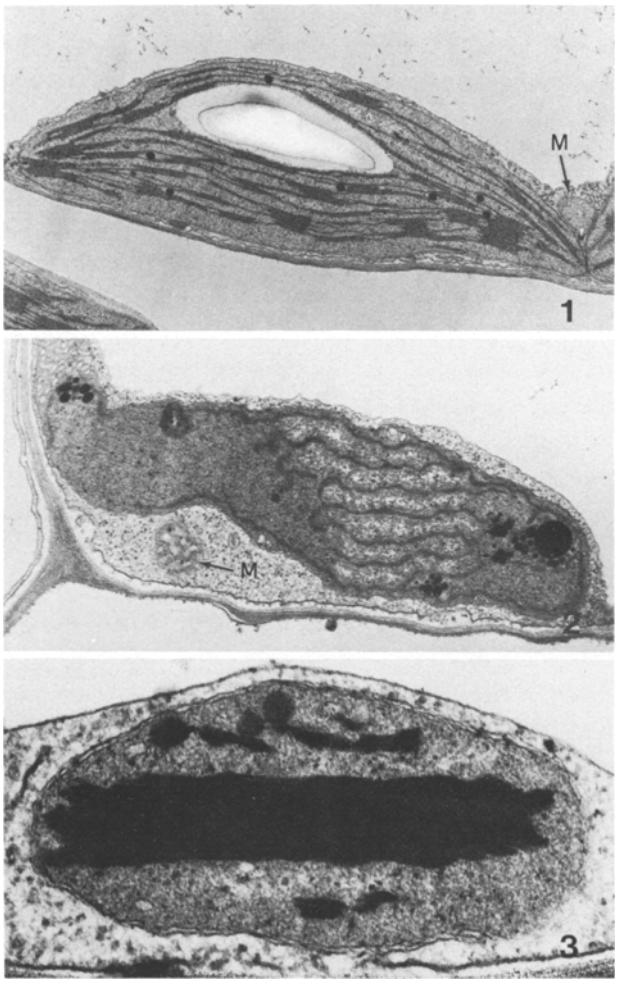
The ultrastructure of mutant chloroplasts is compared to standard wildtype chloroplasts. Chloroplasts, assumed to be genotypically and phenotypically normal, having the typical morphology previously reported for plastids of higher plants are found in standard wildtype *Mimulus cardinalis* mesophyll tissues (Fig. 1). The enclosure of the chloroplast by a limiting double membrane is frequently conspicuous. Internal lamellar (thylakoid) membranes, in which photosynthetic pigments are associated, rarely extend all the way across the chloroplast. Typically, the lipoprotein lamellar system is organized into stacks of fused discs (grana) interconnected by single discs (fretwork). Osmiophilic (lipid) globuli are ubiquitous in wildtype chloroplasts, varying in size and number. Often, one or two, ellipsoidal starch grains are present. Most of the remainder of the chloroplast is composed of the proteinaceous matrix called stroma, the location of enzymes involved in carbon fixation.

Electron microscopic examination of the white tissue of *striata* mutants, ascertained to be cytoplasmically

Table 4. Genetic analysis of lethal chlorophyll-deficient *albina*-MG, *albina*-NA and *xantha* mutant seedlings in *Mimulus cardinalis*

		M_2 seedlings				
		Green	White *(albina)*	Yellow *(xantha)*		
M_1 generation parent	Phenotype	Number	Number	Number	Chi-square[a]	P value[a]
Albina- MG × Self	Green	585	180	0	0.63	0.4 -0.5
Xantha × Self	Green	612	0	216	0.52	0.4 -0.5
Albina- NA × Self	Green	292	82	0	1.88	0.15-0.2

[a] Tested for goodness-of-fit to a 3:1 ratio.



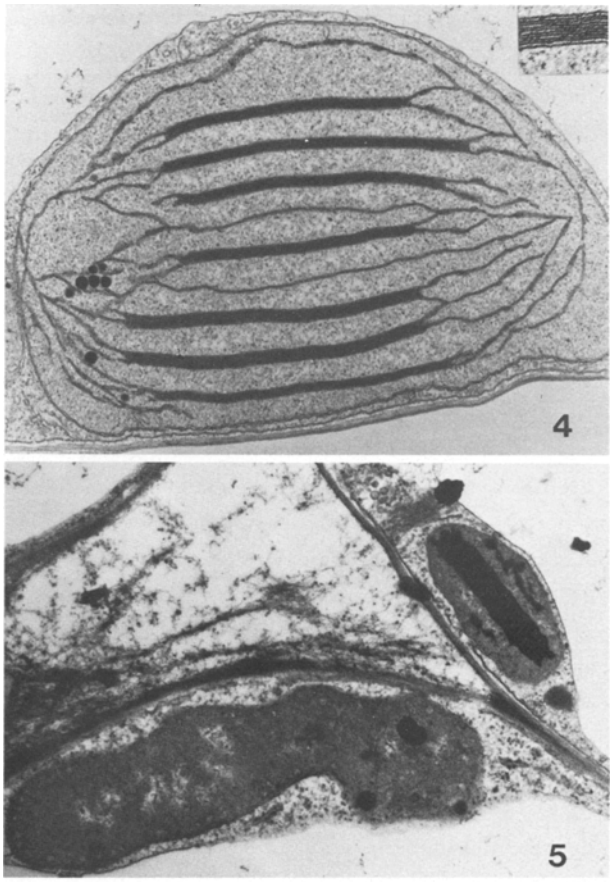


Fig.1. Wildtype chloroplast. M, mitochondrion. Glut-OsO_4. × 13,000
Fig.2. Mesophyll plastid of *xantha* mutant. M, mitochondrion. Glut-OsO_4. × 19,000
Fig.3. Mesophyll plastid of *xantha* mutant. Glut-OsO_4. × 33,500

Fig.4. Mesophyll plastid of *viridis* mutant. Glut-OsO_4. × 28,500. Insert: Higher magnification of "super-extended" granum. × 51,500
Fig.5. Two types of mesophyll plastids from *xantha* mutant. Glut-OsO_4. × 11,000

inherited, revealed two types of abnormal chloroplasts ultrastructures. One of the defective chloroplast types is "dumbbell" shaped in cross section (Fig.9). The other defective chloroplast type is nearly amoeboid shaped containing a vacuole-like structure (Fig.10). Other than a small amount of stroma or matrix, no identifiable internal organization is detectable in *striata* defective chloroplasts. The characteristic chloroplast limiting double membrane is observable, however. Comparing their shapes and the presence internally of only matrix, it is reasonable to suggest one of the defective chloroplasts may be deteriorating or developing into the other defective type. We tend to believe the plastids are deteriorating, rather than differentiating, since amoeboid shaped plastids were never observed with internal structural fragments, etc. We have not seen in the literature any chloroplast mutants blocked in this stage of development. Interestingly, morphologically normal mitochondria were observed in the same cells possessing only *striata* mutant plastids. This does not preclude the possibility, however, of functional mitochondrial mutants.

In contrast to the *striata* mutants, the fine structural organization of the abnormal chloroplasts observed in the pale yellow-green tissue of *viridis* mutants is consistently the same (Fig.4). This suggests a single type of mutation may have taken place at a specific plastome locus. Furthermore, this single defective chloroplast type maintains its identity through cytoplasmic transmission. This situation is interesting since the same mutagen dose caused acute deleterious effects in

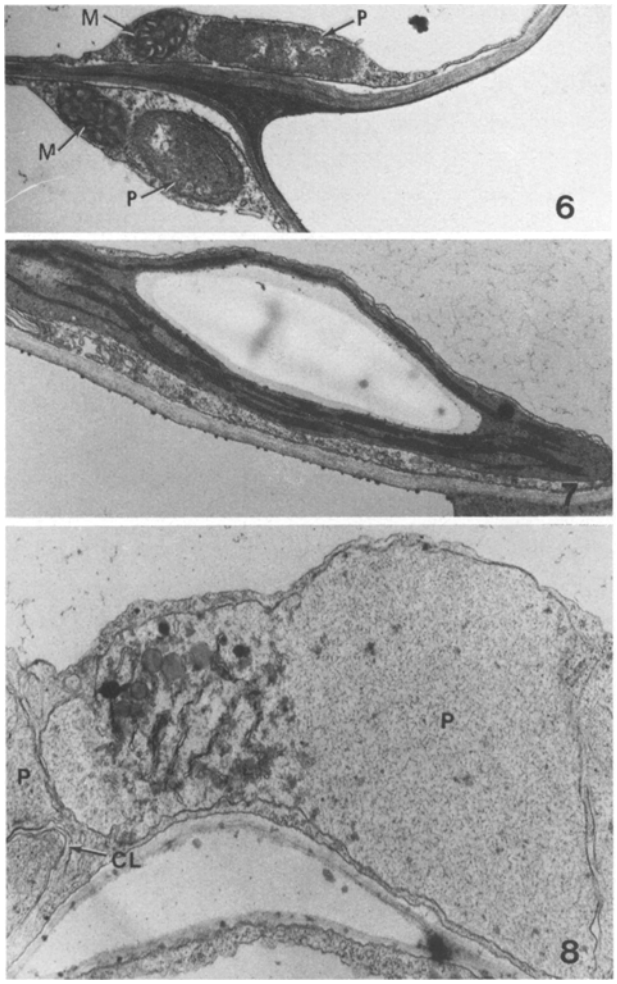


Fig.6. Cotyledon plastids from *albina*-MG mutant. M, mitochondria - P, plastids. Glut-OsO_4. × 20,500
Fig.7. Mesophyll chloroplast from *chlorotic* mutant. Glut-OsO_4. × 25,500
Fig.8. Cotyledon plastids from *albina*-NA mutant. P, plastids - Cl, concentric lamellae. Glut-OsO_4. × 15,000

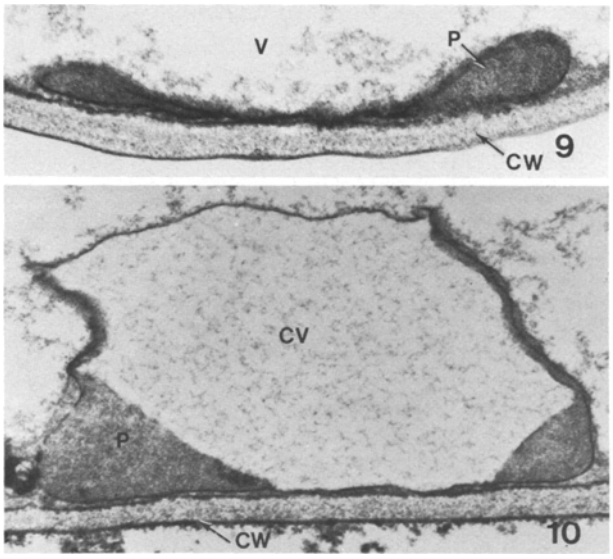


Fig.9. Cotyledon plastid from *striata* mutant. P, plastid - V, cell vacuole - CW, cell wall. Glut-$KMnO_4$. × 26,000
Fig.10. Cotyledon plastid from *striata* mutant. P, plastid - CV, vacuole-like plastid inclusion - CW, cell wall. Glut-$KMnO_4$. × 23,000

some chloroplasts in the *striata* mutants. Obviously the concentration of chemical mutagen taken in by seed embryos is unequal. The defective chloroplast of the *viridis* mutant exhibits several parallel primary lamellar layers and up to six "super-extended" grana extending almost the entire length of the chloroplast. Consequently, fretwork is nearly lacking. Osmiophilic globuli are present. Starch grains are absent under the same conditions in which they are present in the wildtype chloroplast. Apparently, synthetic enzymes, or their substrates, for starch synthesis are not present in this defective plastid type.

The *albina*-MG type mutant observed in M_2 generation in a 3:1 ratio, exhibited basically one type of abnormal chloroplast (Fig.6). No normal chloroplast were found. The tissue sample is from the cotyledon since the *albina*-MG mutant is not viable, dying in this stage. The ultrastructure of the defective *albina*-MG type chloroplast shows only slight evidence of primary lamellar structure, surrounded by a dense matrix, and no other identifiable internal structure. The *albina*-MG mutant is blocked in an early stage of development of chloroplast structure. They are not much larger than accompanying mitochondria of the same cell. They are, however, structurally unrelated to the *albina*-MG chloroplasts (Fig.6). The *albina*-NA mutant, like the *albina*-MG mutant, exhibited nuclear recessive inheritance. However, the ultrastructure of *albina*-NA plastids suggest their development to be more advanced than that of the *albina*-MG plastid. *Albina*-NA plastids are somewhat amoeboid shaped possessing osmiophilic globuli and primary lamellar structures (Fig.8). Some of the defective plastids had evidence of degenerative lamellar systems in the form of concentric thylakoids (Rosinski and Rosen, 1972). The nuclear recessive *xantha* mutant, which like the *albina* mutants dies in the cotyledon stage, exhibits three types of ultrastructural organization in its defective chloroplasts. One type of defective *xantha* chloroplast contains a dense "super-granum" made up of a fusion of almost all the thylakoids in the plastid (Fig.3, 5). A second *xantha* defective chloroplast shows osmiophilic globuli surrounded by the matrix with no other iden-

tifiable structures. The undulating shape of the whole chloroplast also sets itself apart from the normal phenotype (Fig.5). The last defective *xantha* chloroplast type possesses several lamellae extending parallel to one another, approximately one-third the length of the plastid. None of the thylakoids are stacked into grana. Osmiophilic globuli are present (Fig.2). The sterile chlorotic mutants exhibit nearly normal ultrastructure at the chloroplast level. One conspicuous difference is the presence of one to several abnormally large starch grains within the plastids (Fig.7). Consequently, the distortion of the chloroplast is probably due to the large amount of stored starch.

Discussion

The biogenesis and autonomy of chloroplasts are more easily investigated using chlorophyll-deficient mutants. Chlorophyll-deficient mutants are normally inherited following three types of mutation phenomena: (1) plastome mutation, (2) genome inducing-plastome mutation, and (3) genome mutation. The following basically illustrates these three mutation phenomena. Mutant chloroplasts, in some higher plants, are transmitted through the maternal, paternal, or biparental cytoplasm, segregate in non-Mendelian manner, and show no linkage to nuclear genes. The initial site of mutations in these type mutants is in the chloroplast DNA itself (Stewart, 1965; Burk et al., 1964). These chloroplast mutants are normally detected following observation of variegated plants in the F_1 generation. Not all variegated plants arise, however, from initial mutation at the chloroplast DNA level. This is illustrated by the genome inducing-plastome mutation phenomenon. Certain variegated *Zea mays*, *Arabidopsis*, and *Oenothera* plants pertain to this situation (Rédei, 1973; Epp, 1973; Rhoades, 1946). Probably the most thoroughly investigated is the inheritance of the "IOJAP" mutation in corn. In IOJAP the homozygous condition of the mutant gene causes ultrastructurally defective and colorless chloroplasts. The leaf variegated IOJAP mutants arise due to initial mutation in nuclear DNA, as ascertained from Mendelian segregation ratios and linkage to known chromosomal genes. Interestingly, the nuclear gene mutation somehow subsequently causes a permanent mutation in the chloroplast genome. The end result is that the mutant chloroplasts are inherited via the cytoplasm, in non-Mendelian ratios. Consequently, this type of cytoplasmically inherited chloroplast mutant arises through nuclear mutator genes and not plastome genes. Many explanations have been examined to explain the genome inducing-plastome mutation, or nuclear mutator gene, system. One suggestion is that chloroplast DNA-polymerases are coded in the nucleus and require chloroplast DNA as a template. Also, many chlorophyll-deficient mutants are inherited following true classical Mendelian genetics, i.e., three to one ratios. These are nuclear recessive inherited mutants and normally 25% of the offspring exhibit various degrees of chlorosis (Walles, 1965; von Wettstein et al., 1974).

Chloroplast biogenesis in *Mimulus cardinalis* is genetically controlled by both the nuclear genome and the plastome. This is clearly evidenced by the results of this study which show the chemical induction of chloroplast mutants exhibiting different ultrastructures and modes of inheritance. In our experiments only plastome mutants and genome mutants were detected. Nuclear mutator system mutants were not isolated. The observation that *Mimulus* chloroplasts are partially autonomous is consistent with the data in the literature for other higher plants (Wildman, 1971a; von Wettstein, 1961; von Wettstein et al., 1971). Furthermore, from a perusal of the literature, the ultrastructure of some of the defective chloroplasts described in this paper appear to be unique. This suggests, as von Wettstein had predicted (1961), and others have shown (Boynton, 1966b), that past surveys of chloroplast mutants cannot be considered exhaustive. Our mutants appear to add to the growing list.

Evidence on the development of chloroplasts lending, or not lending, support concerning the degree of independent extranuclear continuity of plastids can be obtained by use of mutant chloroplast systems, such as the ones obtained in this study. For example, von Wettstein et al. (1974) recently demonstrated that nuclear genes hold a decisive control over chloroplast biogenesis in barley. In at least three of the *Mimulus* chlorophyll-deficient mutants, the nuclear genes serve as the controlling factor for normal chloroplast inheritance. Based on plastid ultrastructure it seems reasonable to assume that the mutations are non-allelic or at least different forms of the same allele. In two of the mutants, inheritance is clearly cytoplasmic with the structure of the abnormal chloroplasts due to specific heritable defects in the plastome. The initial recognition of cytoplasmic genes (chloroplast genes) in *M. cardinalis* followed the observation of phenotypes with leaf variegation. Substantive identification of chloroplast gene mutants followed the outcome of reciprocal crosses and observation of somatic sorting-out phenomena. Based on crosses using *striata* mutants

the pattern of chloroplast inheritance is via the maternal cytoplasm. Maternal inheritance, in lieu of paternal and/or biparental inheritance, was ascertained when only white progeny resulted from crosses of all white *striata* female parents and normal green male parents.

As ascertained by Mendelian segregation ratios, both *albina* and the *xantha* type mutant plastids are under the control of nuclear genes. The *striata* and *viridis* mutants, which exhibit sorting-out phenomena in various tissues, give rise to progeny in non-Mendelian ratios and possess defective plastids which are under plastome control. Furthermore, evidence that the biogenesis of chloroplasts in *Mimulus* is under both plastome and genome control, is seen in two situations. First, in the wildtype plant green chloroplasts are found in the suture areas of calyces arising from all green shoots but greater numbers of green chloroplasts are observed in the suture areas of calyces arising from all *albino* shoots of the *striata* mutant. In the second case, virtually no normal plastids are observed in specific mesophyll cells of the *striata* mutant; normal chloroplasts are found in the guard cells contiguous to them. Certainly, however, neither of these last two situations exemplify a sorting-out phenomenon. It seems, rather, they may represent altered development or regulatory functions. If these are such mutants, the mutant genes could act by over production or limiting the production of some necessary metabolite.

The epidermis in *Mimulus* and many other plants lack conspicuous normal chloroplasts, while guard cells and sutures of the calyx in *Mimulus* have chloroplasts. A possible explanation of the normal plastid containing cell is that the original mutation arose in a subepidermal meristematic cell and that all of the epidermal cells have the genetic potential to develop chloroplasts. The significant increase in number of chloroplasts in the sutures may indicate that some type of feedback mechanism controlling the number of plastids is compensating for lack of photosynthate from the subepidermal tissues. Normal epidermal tissue with genetically determined white mesophyll underneath should provide a very sensitive test for assaying potential developmental regulators.

The presence of mixed plastid types in the same cell, which has previously been reported for other plants, leads to the suggestion that (1) they are different final products, or (2) they are different stages in development, i.e., one giving rise to the other. It was not possible to distinguish between these hypotheses using available techniques but it seems reasonable to assume that each hypothesis may be valid, depending on the mutant being studied.

Evidence does exist, therefore, for strict nuclear and cytoplasmic control of chloroplasts within particular tissues of the *Mimulus* mutants. Since the activity of the mutant gene or genes in two of the mutants is strictly cell limited, an altered regulatory function is hypothesized. Thus, there is in our *Mimulus* chloroplast mutants the possibility of studying the effect of specific biochemical blocks, the elucidation of developmental pathways leading to chloroplast biogenesis, and gene regulation.

One significant enigmatic situation concerning these mutants, as well as all other chlorophyll-deficient mutants, does exist. Although we assume the cause for a chloroplast abnormality must reside in the nuclear DNA or chloroplast DNA, unequivocable evidence at the biochemical level is lacking. It is now reasonable to expect that comparative biochemical studies, which have already begun (Wong-Staal and Wildman, 1973; Travis, 1974; Travis et al., 1972b), using wildtype and abnormal chloroplasts, will provide evidence locating mutations at the genome or plastome DNA level. If location of mutations at the DNA level is to be accomplished a variety of chlorophyll-deficient mutant systems, similar to ours, with nuclear and cytoplasmic inheritance, must be isolated and investigated.

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