GENETICS AND ULTRASTRUCTURE OF A CYTOPLASMICALLY INHERITED YELLOW MUTANT IN SOYBEANS[1]

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ABSTRACT

A chimeric plant was observed in the F_2 generation of a cross between a male-sterile line and a plant introduction homozygous for a chromosome interchange in soybeans [*Glycine max* (L.) Merr.]. F_3 progeny of this plant included one chimera, 36 yellow plants and 16 green plants. The yellow plants, which progressively turn green, were viable and fertile in field, greenhouse and growth-chamber environments. Reciprocal cross-pollinations were made between these yellow plants and four known nuclear yellow mutant plants, between these yellow plants and sibling green plants and between these yellow plants and unrelated green plants. Segregation data from F_1 and F_2 generations indicated cytoplasmic inheritance of the newly discovered yellow phenotype. Pollinations in which reciprocal F_1 hybrid plants were used as male or female parents were made with unrelated green plants. Observations in F_1 and F_2 generations substantiated the hypothesis of cytoplasmic inheritance. No interactions have been observed between this mutant and the various nuclear backgrounds. This is the first report of a cytoplasmically inherited mutant affecting plant color in soybeans. Exchange grafts were made between cytoplasmic yellow plants and sibling green plants and between cytoplasmic yellow plants and unrelated green plants. The phenotype was controlled by the scion, indicating that graft-transmissible agents were not involved. When grown in darkness, cytoplasmic yellow plants and normal green plants accumulated the same amount of protochlorophyllide. Cytoplasmic yellow plants grown in dim light accumulated slightly less chlorophyll than did their green siblings. Electron photomicrographs showed that the prolamellar body (a structure associated with synthesis of protochlorophyllide) and chloroplast ultrastructure were normal in the cytoplasmic yellow mutant. These observations led to the hypothesis that the synchrony involved in deposition of nuclear and cytoplasmic gene products during organelle development is impaired in this cytoplasmic mutant.

A chimeric plant, mostly normal green but with large yellow sectors, arose spontaneously in the F_2 generation of a cross of two green soybean plants. Progeny of this plant included one chimera, 36 yellow plants and 16 green plants. The yellow plants turned progressively greener and grew to maturity, at which

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time they were distinguished from normal green plants by reduced seed yield. Genetic tests indicated that this yellow phenotype was inherited uniparentally through the maternal parent. The objectives of our research were to study the genetics and the plastid ultrastructure of this cytoplasmic yellow mutant, designated *cyt-Y*$_2$, in soybeans, *Glycine max* (L.) Merr.

Understanding of the nuclear/cytoplasmic interaction has been facilitated by the existence of variants in cytoplasmically inherited genes (KIRK and TILNEY-BASSETT 1968; SAGER 1972; GRUN 1976; BANDLOW *et al.* 1977; GILLHAM 1978). Cytoplasmic inheritance includes traits not encoded by nuclear DNA. In eukaryotes, mitochondria and chloroplasts are the predominant carriers of extrachromosomal genetic information. For several reasons, attempts to obtain cytoplasmic mutants in higher plants by mutagenesis generally have been unsuccessful. (1) Numerous copies of the DNA are present in each organelle; mutagenic events, however, affect only one copy of the genetic information. (2) Once the defective genome becomes the only carrier of information by random segregation in an individual organelle, this defective organelle must segregate from its normal counterparts within the cell. (3) Many defects that alter organelle development prevent the defective organelle from competing effectively with normal cellular organelles. (4) When defective organelles segregate from the normal organelles, a sectored plant (chimera) results; only when the mutant sector encompasses the female gamete, however, is the trait transmitted. (5) Most perturbations altering organelle development prevent the progeny from growing to maturity, thus resulting in loss of the aberrant type. A number of such occurrences have been observed in our work (PALMER, unpublished data). Cytoplasmic variation, however, occurs frequently in nature. Inheritance of such variation is non-Mendelian; *i.e.*, these traits are transmitted uniparentally, principally, if not exclusively, through the female gamete.

Segregation patterns of chimeras manifest a direct relationship between sector phenotypes and cytoplasmic genotypes. When chimeric plants are crossed as the female parent, flowers borne on nonchimeric green regions produce all green progeny, flowers borne on sectored regions produce green, sectored, and fully mutant yellow progeny, and flowers borne on mutant regions produce all mutant progeny.

Several explanations have been offered regarding the nature of cytoplasmic defects. In Oenothera, for example, disharmony resulting from incompatibility between nuclear and cytoplasmic genomes generates a broad range of phenotypes from yellow to almost normal green (STUBBE 1964). Thus, combinations of normal nuclear and cytoplasmic components can result in abnormal plastid development and reduction in leaf pigment content (SCHOTZ 1970). In our study, a number of genetic backgrounds, as well as combinations of *cyt-Y*$_2$ and nuclear yellow mutants, were tested. No changes in *cyt-Y*$_2$ expression, however, were observed.

Epigenetic changes induced by nuclear gene mutants may occur, causing heritable defective phenotypes. Abnormal plastid differentiation induced by iojap (*ij*) in maize seems to be such a phenomenon (WALBOT and COE 1979). Heritable

loss of chloroplast ribosomes occurs seemingly without altering the chloroplast DNA. With iojap, it is speculated that all chloroplasts are affected, but that a variable number recover, resulting in degrees of variegation.

Several cytoplasmic traits have been attributed to alterations in chloroplast and mitochondrial DNA. LEVINGS and PRING (1976) and PRING and LEVINGS (1978) have shown that, in maize cytoplasm, the normal mitochondrial genome varies in its restriction pattern compared with the T, C and S mitochondrial genomes. The S cytoplasmic background also shows variation in the restriction pattern of its chloroplast DNA. On the basis of their results, they suggested that the cytoplasmic male sterility in maize may be due to alterations in the mitochondrial DNA. FRANKEL, SCOWCROFT and WHITFELD (1979) demonstrated an altered EcoRI restriction pattern in the chloroplast DNA's of two male-sterile tobacco lines. WONG-STAHL and WILDMAN (1973) provided evidence that, in a variegated tobacco mutant, chloroplast DNA had at least one extended region, 500–1000 nucleotides long, 1% higher in GC, that was different from the DNA of normal chloroplasts. Precise analyses have not been reported for this tobacco mutant.

It is not known whether the lesion in the soybean *cyt-Y₂* mutant represents an epigenetic alteration or a mutation in the chloroplast or mitochondrial DNA. On the basis of the ability to develop normal chloroplasts and grow to maturity, it seems that mutant plants are defective in coordination between nuclear and cytoplasmic components involved in organelle biogenesis in a manner that is largely correctable as the leaves mature. The purpose of this article is to characterize the genetics and to describe the ultrastructure of the plastids of the *cyt-Y₂* mutant in soybeans.

MATERIALS AND METHODS

Origin of cytoplasmic yellow mutant: A chimeric plant (A75–1165–117) was observed during 1975 in an F_2 generation of a cross between the Ames isolate of ms_1 and a "Clark" isoline homozygous for the chromosome interchange from Plant Introduction 101,404B. Plants heterozygous for this chromosome interchange have about 50% pollen grain and ovule abortion. Pollen grain abortion is determined microscopically with I_2KI staining. Ovule abortion is determined at maturity by observing seed set. A complete description of the different isolates of the male-sterile, female-fertile ms_1 mutant is given by PALMER, WINGER and ALBERTSEN (1978).

In the F_3 generation, progeny from the chimera segregated for yellow plants, green plants and one chimeric plant with small sectors. The yellow plants were viable and fertile, characteristics that have allowed maintenance of the mutant as a pure line. The F_3 yellow plants had normal chromosome structure or homozygous interchanged chromosomes or were heterozygous for the interchange. This mutant yellow line has been assigned a Genetic Type Collection Number (T275) and the gene symbol *cyt-Y₂* by the Soybean Genetics Committee. The normal green sibling line has been designated *cyt-G₂*.

Genetic studies: Standard soybean crossing techniques were used to obtain cross-pollinations (PASCHAL 1976). All genetic studies were conducted with field-grown plants. Chimera A75–1165–117 was crossed reciprocally with "Clark 63" by using flowers from branches of the chimera that contained a high percentage of yellow leaves.

Yellow mutant plants (*cyt-Y₂*) were crossed reciprocally with plants homozygous for the nuclear yellow mutants, y_{10}, y_{12} and y_{13}. These nuclear mutants also are viable as homozygous recessives and are maintained as pure lines by self-pollination. Another nuclear yellow mutant,

$y_{18}y_{18}$, is lethal under field conditions and is maintained as the heterozygote, $Y_{18}y_{18}$. Phenotypes of these 4 nuclear yellow mutants are discussed by BERNARD and WEISS (1973). Heterozygotes such as $Y_{18}y_{18}$ are green, but are distinguished from homozygous green $Y_{18}Y_{18}$ plants by progeny testing. Reciprocal cross-pollinations of $Y_{18}y_{18}$ plants with *cyt*-Y_2 plants were advanced to the F_2 generation. Mutant lines y_{10} and y_{18} have white flowers (w_1), and mutant lines y_{12} and y_{13} have gray pubescence (t). Yellow *cyt*-Y_2 plants are dominant for both purple flower (W_1) and tawny pubescence (T). Purple pigmentation is present on the hypocotyls of W_1W_1 and W_1w_1 seedlings, but is lacking on the hypocotyls of w_1w_1 seedlings (BERNARD and WEISS 1973).

In the F_2 generation of crosses of nuclear yellow mutants with *cyt*-Y_2, segregation of flower color and pubescence color genes gave unequivocal evidence of successful hybridizations. In certain crosses, we observed segregation of the chromosome interchange brought in by the *cyt*-Y_2 yellow mutant from Plant Introduction 101,404B. This chromosome aberration also gave positive evidence of successful hybridizations.

Yellow mutant plants (*cyt*-Y_2), green normal plants (*cyt*-G_2) and the cultivar "Minsoy" were crossed reciprocally in all combinations except Minsoy × Minsoy. Minsoy differs from *cyt*-Y_2 and *cyt*-G_2 in several readily identifiable traits, and cross-pollinations were easily distinguished from self pollinations. In reciprocal cross-pollinations between *cyt*-Y_2 and *cyt*-G_2 and in sibling crosses (*cyt*-G_2 × *cyt*-G_2 and *cyt*-Y_2 × *cyt*-Y_2), one parent was homozygous for the interchanged chromosomes, and the other parent was homozygous for the normal chromosome arrangement. The F_1 plants were heterozygous for the interchange and had about 50% pollen grain and ovule abortion, verifying their hybrid origin.

The F_1 plants of all 8 genetic combinations involving *cyt*-Y_2, *cyt*-G_2 and Minsoy were crossed reciprocally with OX281, which has white flowers (w_1); the hybrid parents had purple flowers (W_1). Segregation of hypocotyl color among F_2 seedlings confirmed that the respective families were hybrid.

Grafting of cytoplasmic yellow anl green plants: Seedlings of *cyt*-Y_2, *cyt*-G_2 and Minsoy soybeans were grafted in all combinations except Minsoy on Minsoy. Seedlings were grafted 2 to 5 days after emergence by modifications of the techniques of BEZDICEK, MAGEE and SCHILLINGER (1972) and SANDERS and BROWN (1973). For each graft, the rootstock was cut with a new razor blade. Next, a short piece of plastic tubing that had been slit lengthwise and 2 small orthodontic rubberbands were slipped onto the stem of the rootstock. With a new razor blade, the scion apex was cut obliquely from the plant and trimmed to form a wedge. Tap water was applied to each cut face of the scion wedge; after the rootstock stem was slit and water was applied to the uppermost portion, the scion wedge was inserted. The graft was checked for alignment, and the tubing and rubber bands were raised to support and enclose the graft union. Water was applied again, and the edges of the tubing were sealed with lanolin paste.

Culture of plant material for electron microscopy and pigment determinations: Two growth chambers were used, one lighted and one dark, both at 26° ± 2°. Photosynthetic photon flux density in the illuminated chamber was 202 $\mu Em^{-2}sec^{-1}$ for 16 hr per day. Material grown in darkness was subjected, when necessary, to a dim green safelight (green Plexiglas #2093, λ minimum 525 to 530 nm, 55% transmittance, transmission range 480 to 600 nm). Plants were watered daily and were grown for 10 days.

Determination of pigment concentrations: In vivo spectra were determined with a Bausch and Lomb Spectronic 505 with a reflectance attachment. *In vitro* spectra were determined by extracting pigments from plants with 80% acetone, clearing by centrifugation and reading optical densities in a Beckman Acta C11 spectrophotometer. The millimolar extinction coefficient for protochlorophyllide at 628 nm (31.1) of KAHN, AVIVI-BIEISER and VON WETTSTEIN (1976) was used to approximate the protochlorophyllide concentration of plants grown in darkness. The equations of ARNON (1949) were used to estimate chlorophyll concentrations of plants grown in light.

Preparation of material for electron microscopy: Freshly harvested trifoliolate leaves were immersed in phosphate-buffered (pH 7.4, 0.05 M) 2.5% glutaraldehyde, sliced into 1- and 2-mm squares with a sharp razor blade and fixed for 2 hr at 4°. The tissue was postfixed in phosphate-buffered (pH 7.4, 0.05 M) 2% osmium tetroxide, dehydrated with ethanol and embedded in Epon

812. Sections were cut with an LKB 111 ultramicrotome, supported on 300-mesh uncoated grids, stained with uranyl acetate (20% aqueous, 15 min) and post-stained with lead citrate (10% methanolic, 3 min), and observed with a Hitachi HU-11C electron microscope.

RESULTS

Genetic studies: Seeds resulting from self-pollination were harvested from the original chimera (F_2 plant A75–1165–117), which was heterozygous for a chromosome interchange. Data from F_3, F_4 and F_5 generations from self-pollinations of the chimera, and from F_1, F_2 and F_3 generations from cross-pollinations with Clark 63 are presented in Table 1.

We observed one chimera, 16 green plants (three died) and 36 yellow plants (27 died) from self-pollination of the F_2 chimera. (A June hailstorm damaged many plants; the weaker yellow plants had a higher mortality rate.) Progeny of the F_3 chimera segregated 198 green:13 yellow plants in the F_4 generation. The 120 green and seven yellow F_4 progeny from the chimera that were progeny tested in the F_5 generation bred true for plant color. Progeny of the 13 F_3 green plants and the nine F_3 yellow plants that were tested in the F_4 and F_5 generations bred true for plant color (Table 1).

Among the 23 F_3 plants progeny tested for the chromosome interchange, five yellow plants and six green plants had about 50% pollen grain and ovule abor-

TABLE 1

Number of plants and their phenotypes in F_3, F_4 and F_5 generations obtained by self-pollination of the F_2 chimeric plant and in F_1, F_2 and F_3 generations derived from reciprocal cross-pollinations with Clark 63

Parents	F_3 generation	F_4 generation		F_5 generation	
	Number of plants and phenotypes*	Number of F_3 plants tested†	Number of plants and phenotypes	Number of F_4 plants tested	Number of plants and phenotypes
F_2 chimera, self-pollination	36 yellow (27 died)	9	139 yellow	46	1608 yellow
	16 green (3 died)	13	1667 green	100	4851 green
	1 chimera	1	13 yellow	7	380 yellow
			198 green	120	3497 green
	F_1 generation	F_2 generation		F_3 generation	
Parents	Number of plants and phenotypes*	Number of F_1 plants tested†	Number of plants and phenotypes	Number of F_2 plants tested	Number of plants and phenotypes
F_2 chimera × Clark 63	9 yellow (8 died)	1	4 yellow	4	138 yellow
	1 green	1	218 green	100	4734 green
Clark 63 × F_2 chimera	23 green (7 died)	16	1848 green	100	4698 yellow

* A hailstorm in June 1976 killed many seedlings. Yellow plants had a much lower survival rate than green plants. The surviving yellow plants were very weak and set fewer seeds than expected under normal growth conditions.

† Progeny tested for presence or absence of the chromosome interchange from Plant Introduction 101,404B.

tion, manifesting heterozygosity for the chromosome interchange. Four yellow plants, seven green plants and the chimera had completely fertile pollen and were homozygous either for the interchanged chromosomes or for the normal chromosome arrangement.

We observed one green and nine yellow (eight died) F_1 seedlings when the chimera was used as the female parent in cross-pollinations with Clark 63 (Table 1). The yellow plants and the green plants that were progeny tested in the F_2 and F_3 generations bred true for plant color, and both F_2 plants were heterozygous for the interchange, verifying hybrid origin.

When the chimera was used as male parent in cross-pollinations with Clark 63, all 23 (7 died) F_1 seedlings were green. The green plants that were progeny tested in the F_2 and F_3 generations bred true for plant color (Table 1). Among the 16 F_1 plants tested for the chromosome interchange, 10 were heterozygous for the chromosome interchange, verifying their hybrid origin. The remaining six plants had fertile pollen but were assumed to be hybrids.

Yellow plants (F_4 generation) were crossed reciprocally with the nuclear yellow mutants, y_{10}, y_{12} and y_{13}, and the heterozygote, $Y_{18}y_{18}$ (Table 2). In the four genetic combinations in which *cyt-*Y_2 yellow plants were the female parent, all 14 F_1 plants were yellow, and their F_2 progeny were yellow. Phenotypes of y_{10}, y_{12}, y_{13} and y_{18}, which are distinguishable from *cyt-*Y_2, were evident among these F_2 plants, but no attempt was made to count nuclear yellow mutant plants that segregated. Segregation of nuclear markers T/t and W_1/w_1 fit a ratio of 3

TABLE 2

Phenotypes observed in F_1 and F_2 generations from reciprocal cross-pollinations between cytoplasmic yellow mutant cyt-Y_2 *and nuclear chlorophyll mutants* y_{10}, y_{12}, y_{13} *and* y_{18}

Parents		F_1 generation		F_2 generation			
Female	Male	Purple flower		Purple flower		White flower	
		Green	Yellow	Green	Yellow*	Green	Yellow*
Y_{18}–w_1w_1	× *cyt-*$Y_2W_1W_1$	4	0	237	0	101	0
$y_{10}y_{10}w_1w_1$	× *cyt-*$Y_2W_1W_1$	4	0	634	0	237	0
cyt-$Y_2W_1W_1$	× Y_{18}–w_1w_1	0	3	0	214	0	69
cyt-$Y_2W_1W_1$	× $y_{10}y_{10}w_1w_1$	0	4	0	591	0	201
		Tawny pubescence		Tawny pubescence		Gray pubescence	
		Green	Yellow	Green	Yellow*	Green	Yellow*
$y_{12}y_{12}t\,t$	× *cyt-*$Y_2T\,T$	4	0	383	0	121	0
$y_{13}y_{13}t\,t$	× *cyt-*$Y_2T\,T$	6	0	550	0	193	0
cyt-$Y_2T\,T$	× $y_{12}y_{12}t\,t$	0	5	0	431	0	138
cyt-$Y_2T\,T$	× $y_{13}y_{13}t\,t$	0	2	0	211	0	70

* When *cyt-*Y_2 was used as the female parent, all F_2 plants were yellow because of *cyt-*Y_2, but plants homozygous for y_{10}, y_{12}, y_{13} and y_{18} were evident. These four nuclear yellow mutants have phenotypes that can be distinguished from the yellow phenotype of *cyt-*Y_2 plants. When *cyt-*Y_2 was used as the male parent, the F_2 plants that were yellow had distinct phenotypes of y_{10}, y_{12}, y_{13} and y_{18}, respectively, (not phenotypes of *cyt-*Y_2 plants), and the data were not included in this table.

dominant:1 recessive. Among the 14 F_1 plants, eight different *cyt-Y₂* plants were used as female parents: three were heterozygous for the chromosome interchange, three were homozygous for normal chromosomes and two were homozygous for interchanged chromosomes.

When the *cyt-Y₂* yellow plants were used as male parents in genetic crosses with the four nuclear yellow mutants, all 18 F_1 plants were green. Progenies of the green F_1 plants did not segregate for the yellow plant color characteristic of *cyt-Y₂* (Table 2), but segregated only for y_{10}, y_{12}, y_{13} or y_{18} (data not included in Table 2). Segregation of the four nuclear yellow mutants fit a ratio of 3 green:1 yellow, and segregation of nuclear markers T/t and W_1/w_1 also fit a ratio of 3 dominant:1 recessive. Among the 18 F_1 plants, eight different *cyt-Y₂* plants had been used as male parents: four were heterozygous for the chromosome interchange, two were homozygous for normal chromosomes and two were homozygous for interchanged chromosomes.

Crosses of *cyt-Y₂* as the female parent with *cyt-G₂* or Minsoy produced yellow F_1 plants and F_2 plants (Table 3). In crosses of *cyt-Y₂* as the male parent with *cyt-G₂* or Minsoy, however, all F_1 and F_2 plants were green (Table 3). Sibling crosses between *cyt-Y₂* plants gave all yellow F_1 and F_2 progenies; sibling crosses between *cyt-G₂* plants gave all yellow F_1 and F_2 progenies; sibling crosses between *cyt-G₂* plants gave all green F_1 and F_2 progenies (Table 3). All F_1 and F_2 plants were green in reciprocal crosses between *cyt-G₂* and Minsoy (Table 3).

In the [(*cyt-Y₂* × *cyt-G₂*) × 0X281] and the [(*cyt-Y₂* × Minsoy) × 0X281] crosses, all F_1 and F_2 plants were yellow, while in the [(*cyt-G₂* × *cyt-Y₂*) × 0X281] and the [(Minsoy × *cyt-Y₂*) × 0X281] crosses, all F_1 and F_2 plants were green (Table 3). In the [(*cyt-Y₂* × *cyt-Y₂*) × 0X281] and [(*cyt-G₂* × *cyt-G₂*) × 0X281] crosses, all F_1 and F_2 plants were yellow and green, respectively (Table 3). In the [(*cyt-G₂* × Minsoy) × 0X281] and the [(Minsoy × *cyt-G₂*) × 0X281] crosses, all F_1 and F_2 plants were green (Table 3). All F_1 and F_2 plants were green in the three-way crosses with F_1 plants of the eight genetic combinations as male parents and 0X281 as female parent (Table 3). Segregation of hypocotyl color in all F_2 populations of the three-way crosses confirmed that the seedlings were hybrid.

Grafting: In graft combinations of *cyt-Y₂* rootstock with *cyt-G₂* or Minsoy scion, the grafted scions and their progenies were green, but in reciprocal graft combinations, the grafted scions and their progenies were yellow (Table 4). Sibling graft combinations of *cyt-Y₂* gave all yellow plants among the grafted scions and their progenies, and sibling graft combinations of *cyt-G₂* gave all green plants among the grafted scions and their progenies (Table 4). All grafted scions and their progenies were green in reciprocal graft combinations between *cyt-G₂* and Minsoy (Table 4).

Pigment determinations: Yellow (*cyt-Y₂*) plants grown in the dark accumulated a small amount of protochlorophyllide that was converted to chlorophyllide after exposure to light (Figure 1). The amounts of protochlorophyllide accumulated by *cyt-G₂* green plants and by *cyt-Y₂* yellow plants grown in the dark were similar (Table 5). When plants were allowed to develop under the mild light and

TABLE 3

Phenotypes observed in F_1 and F_2 generations from reciprocal single cross-pollinations with cytoplasmic yellow mutant cyt-Y_2, *normal green* cyt-G_2 *and Minsoy, and three-way cross-pollinations involving these F_1 hybrids and OX281*

Hybrid combination*		Plant color and number of F_1 plants		Plant color and number of F_2 plants		Plant color and number of F_1 plants from three-way crosses				Plant color and number of F_2 plants from three-way crosses			
						F_1 hybrid as female†		F_1 hybrid as male‡		F_1 hybrid as female†		F_1 hybrid as male‡	
Female	Male	Green	Yellow	Green	Yellow	Green	Yellow	Green	Yellow	Green	Yellow	Green	Yellow
cyt-G_2	× *cyt-G_2*	6	0	587	0	5	0	4	0	473	0	381	0
cyt-Y_2	× *cyt-Y_2*	0	6	0	573	0	4	3	0	0	378	289	0
cyt-G_2	× *cyt-Y_2*	10	0	865	0	8	0	3	0	742	0	276	0
cyt-Y_2	× *cyt-G_2*	0	12	0	1093	0	7	2	0	0	686	181	0
cyt-G_2	× Minsoy	5	0	485	0	3	0	7	0	290	0	657	0
Minsoy	× *cyt-G_2*	6	0	533	0	4	0	5	0	375	0	479	0
cyt-Y_2	× Minsoy	0	9	0	857	0	6	6	0	0	572	557	0
Minsoy	× *cyt-Y_2*	4	0	381	0	5	0	4	0	468	0	378	0

* F_1 hybrids used as one parent in the three-way crosses.

† OX281, which has white flowers (w_1), was used as the male parent in each three-way cross.

‡ OX281, which has white flowers (w_1), was used as the female parent in each three-way cross.

TABLE 4

Graft combinations with cytoplasmic yellow mutant cyt-Y_2, *normal green* cyt-G_2 *and Minsoy and progenies of the grafted plants*

Combinations		Generation, plant color and number of plants*			
Rootstock	Scion	G_0		G_1	
		Green	Yellow	Green	Yellow
cyt-G_2	*cyt-G_2*	5	0	237	0
cyt-Y_2	*cyt-Y_2*	0	7	0	331
cyt-G_2	*cyt-Y_2*	0	8	0	372
cyt-Y_2	*cyt-G_2*	7	0	339	0
cyt-G_2	Minsoy	3	0	180	0
Minsoy	*cyt-G_2*	5	0	291	0
cyt-Y_2	Minsoy	6	0	161	0
Minsoy	*cyt-Y_2*	0	7	0	334

* G_0 is the generation of grafted plants; G_1 is the generation of progenies of the grafted plants.

temperature conditions of an illuminated growth chamber, leaves from yellow plants accumulated approximately 77% of the normal level of chlorophyll (Table 5). This represented a slight, but significant, reduction in the chlorophyll level. The chlorophyll a/b ratio was 2.9 in green seedlings and 2.6 in yellow seedlings grown in the growth chamber. The chlorophyll a/b ratio in the green plants was less in the field than in the growth chamber, but the chlorophyll a/b ratio in the yellow plants was greater in the field than in the growth chamber; these ratios, however, were in the normal range for young seedlings. Young, fully expanded trifoliolate leaves of field-grown green and yellow plants contained less total chlorophyll than did those of comparable growth-chamber-grown plants. Leaves of field-grown yellow plants, however, showed the more pronounced reduction,

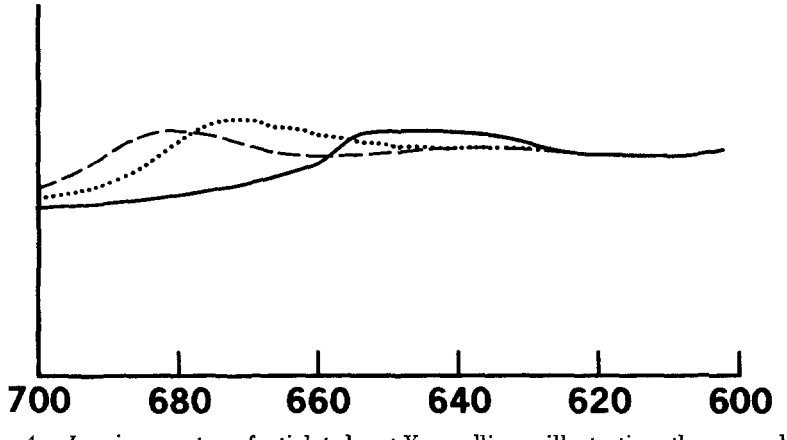

FIGURE 1.—*In vivo* spectra of etiolated *cyt-Y_2* seedlings, illustrating the normal pattern of conversion of protochlorophyllide to chlorophyllide after exposure to light. (——) dark-grown; (– – –) after exposure to light for 1 min; (···) after 1 hr in darkness following 1-min exposure to light.

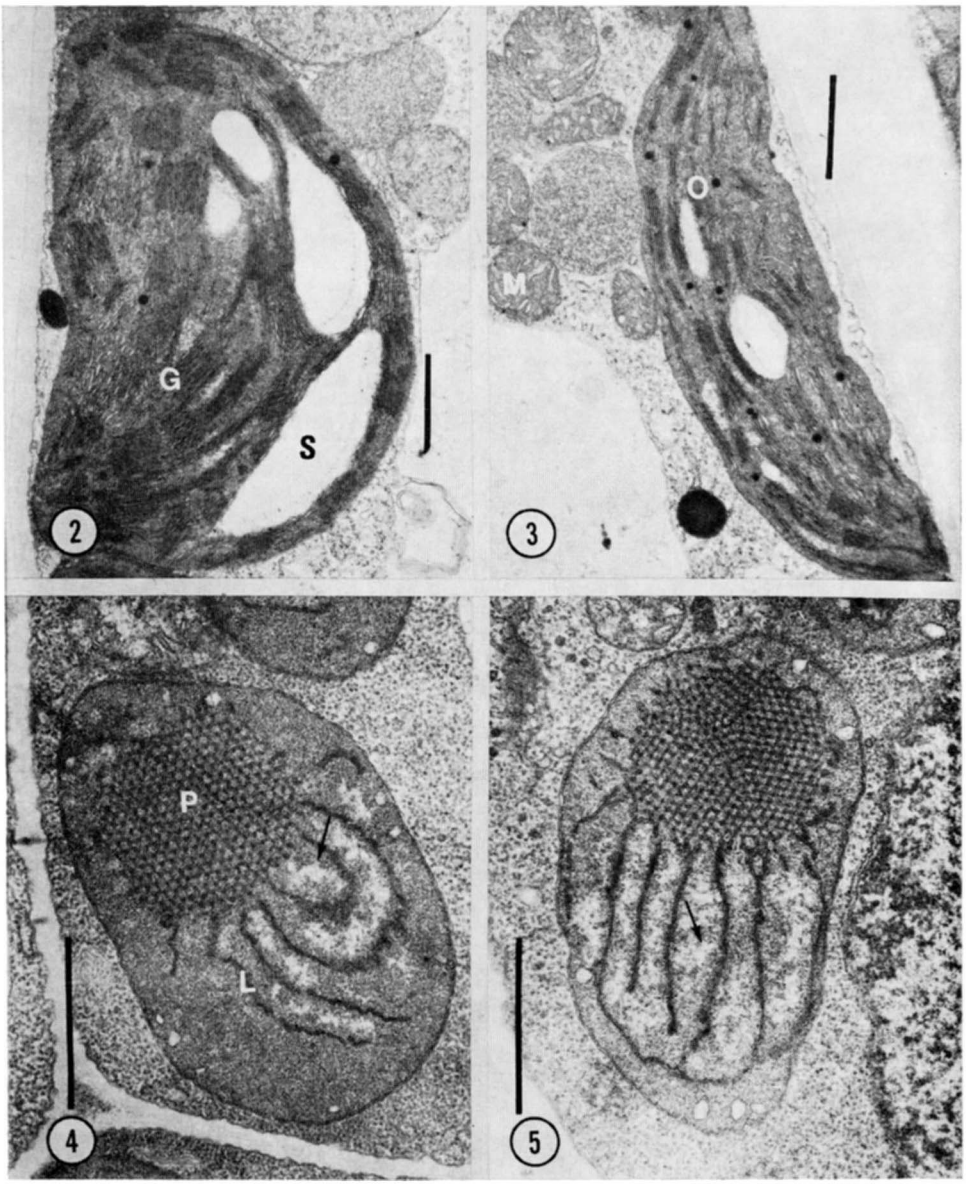

FIGURE 2.—Normal-appearing chloroplast of a normal green *cyt-G*$_2$ plant grown in the light in the growth chamber. Conspicuous are grana stacks and lamellae, starch grains, and osmiophilic globules. Also seen is a normal mitochondrion with invaginations of the internal membrane. 14,000×. FIGURE 3.—Chloroplast of cytoplasmic yellow mutant *cyt-Y*$_2$ plant grown in the same environment as the *cyt-G*$_2$ plant whose chloroplast is shown in Figure 2. The ultrastructure is indistinguishable from that of chloroplasts from green *cyt-G*$_2$ plants. 14,000×. FIGURE 4.—Etioplast of a normal green *cyt-G*$_2$ plant grown in the dark in the growth chamber, showing the paracrystalline prolamellar body, from which radiate a series of lamellar membranes. The background is packed with ribosomes; DNA fibers are evident (arrow). 23,500×. FIGURE 5.—Etioplast of cytoplasmic yellow mutant *cyt-Y*$_2$ plant grown in the same environment

TABLE 5

Pigment concentrations in cytoplasmic yellow mutant cyt-Y_2 *and normal green* cyt-G_2 *soybean lines*

Growing environment	Pigment	Concentration†		
		cyt-G_2	*cyt-Y_2*	*cyt-Y_2* as % of *cyt-G_2*
Growth chamber				
Dark	Protochlorophyllide	7.72 ± 1.39	8.17 ± 0.8	106
Light	Chlorophyll a	1571 ± 78	1177 ± 117	
	Chlorophyll b	537 ± 27	446 ± 47	
	Chlorophyll a/b	2.92 ± 0.05	2.64 ± 0.08	
	Total chlorophyll	2109 ± 104	1623 ± 162	77
Field grown				
	Chlorophyll a	928 ± 94	364 ± 38	
	Chlorophyll b	352 ± 42	123 ± 12	
	Chlorophyll a/b	2.64 ± 0.09	2.95 ± 0.07	
	Total chlorophyll	1280 ± 135	478 ± 50	38

† Concentrations are in nanomoles per gram fresh weight and are given as a mean value ± the standard deviation.

accumulating only 38% of the chlorophyll accumulated by those of field-grown green plants.

Chloroplast ultrastructure: The chloroplasts in leaves of green plants and yellow plants developed similarly when the plants were grown under moderate light and temperature conditions of the growth chamber (Figures 2 and 3). Normal-appearing grana stacks, lamellae and starch were evident. Dark-grown normal plants and mutant plants developed typical etioplasts that were about 2 μm in diameter (Figures 4 and 5). These contained the paracrystalline prolamellar body (a structure associated with the normal synthesis of protochlorophyllide), lamellar membranes, DNA fibers and tightly packed ribosomes.

DISCUSSION

Predictions of the frequency of phenotypes expected in the F_1 of a cross involving a chimera having a cytoplasmic lesion are not possible because the phenotype depends directly on the genotype of the sector from which the egg developed. Chimeric plants have genotypically distinct tissues lying adjacent to one another (NEILSON-JONES 1969), and it is possible to relate leaf ontogeny to large sectors of green or yellow tissue. The size of the sectors may be limited by the number of mitotic divisions remaining before the leaf ceases growth. The amount of mutant tissue in chimeras can range from a number of branches on a plant to a

as the *cyt-G_2* plant whose etioplast is shown in Figure 4. Development in these etioplasts is indistinguishable from that of the normal green *cyt-G_2* types. Note DNA fibers (arrow). 23,500×. (G) granum; (S) starch; (L) lamellae; (M) mitochondrion; (O) osmiophilic globule; (P) prolamellar body.

small sector only on one leaflet. The cytoplasmic genotype of reproductive tissue of a flower usually is reflected by its phenotype. Equating phenotype with genotype for small chimeric regions, however, might be a source of error. We expected chimeras with a large yellow leaf area to have more yellow plants among their progeny than did chimeras with a small yellow leaf area. By extrapolation, flowers from branches with large yellow leaf areas should be used in pollinations to increase the likelihood of transmitting the "yellow factor" in the gametes. The high proportion of plants that were yellow (68%), resulting from self-pollination of chimera A75–1165–117, reflected the large leaf area that was yellow on the original plant and indicated that the "yellow factor" was transmitted to a large proportion of the gametes.

Observation of progeny rows descendent from chimera A75–1165–117 indicated that both yellow and green plants were true breeding (Table 1). There was only one chimeric plant descendent from chimera A75–1165–117; it produced 198 green plants, 13 yellow plants and no chimeric plants (Table 1). Even though the 198:13 ratio was a good fit to a 15:1 ratio, data presented in Tables 2 and 3 gave unequivocal evidence of cytoplasmic inheritance and the lack of nuclear inheritance for the yellow phenotype. The low percentage of yellow plants (6%) from the lightly chimeric plant reflected the small leaf area that was yellow.

We found no evidence that yellow plants or green plants produced chimeras in subsequent generations. This does not rule out the possibility that plants seemingly normal green may possess mutant plastids that may sort out in later generations, producing additional chimeras. Reciprocally, there is no evidence for reversion of the yellow phenotype to the normal green. In *Arabidopsis thaliana*, however, RÉDEI (1973) noticed green islands in white sectors, suggesting that back-mutation from white to green had occurred.

A number of reciprocal crosses was made to demonstrate the uniparental inheritance pattern of the *cyt-Y*$_2$ mutant. In the crosses of chimera A75–1165–117 by CLARK 63, and its reciprocal, single-gene markers were lacking, but the chimera was heterozygous for a chromosome interchange (Table 1). Half the F_1 hybrids were expected to have 50% pollen grain and ovule abortion, and half were expected to be fertile. Our results justified this prediction. In the chimera × Clark 63 cross, the yellow F_1 plant and the green F_1 plant each had 50% pollen and ovule abortion; they gave yellow plants and green plants, respectively, in the F_2 and F_3 generations. A plausible explanation was that the yellow plant was the result of cross-pollination with the yellow phenotype being inherited cytoplasmically. If the green plant was the result of a cross-pollination, the "yellow factor" was not transmitted in the female gamete, presumably because the flower was borne in a leaf axil that was not genotypically yellow.

In the cross of Clark 63 × chimera, 10 of 16 F_1 plants were heterozygous for the chromosome interchange and were of hybrid origin. All the progeny were green. Plausible explanations are that the green plants resulted either from cross-pollination with the yellow phenotype being inherited cytoplasmically or cross-pollination with the "yellow factor" not being present in the male gametes because the flower was borne in a leaf axil that was not genotypically yellow. The

data presented in Table 1 strongly support the hypothesis that the yellow mutant was inherited cytoplasmically.

Yellow plants descendent from the original chimera were used in allelism tests with known nuclear yellow mutants y_{10}, y_{12}, y_{13} and y_{18} (Table 2). Allelism tests were used to indicate whether our yellow mutant was identical with any of the four known nuclear mutants tested. This test also provides information about possible interaction between nuclear and cytoplasmically inherited genes. Yellow plants descendent from the original chimera also were used in a series of reciprocal cross-pollinations and three-way pollinations (Table 3). Analysis of reciprocal cross-pollinations is the basic means of testing for the cytoplasmic inheritance of a trait, and combined analyses of reciprocal cross-pollinations and three-way pollinations can help to determine whether male gametophytic incompatibility was operative.

All F_1 plants and their F_2 progenies were yellow when *cyt-*Y_2 plants were the female parent (Table 2). In reciprocal crosses, all F_1 plants were green, and F_2 progenies were green plants and yellow plants characteristic of the respective yellow nuclear mutants used in the cross (Table 2). These data supported the hypothesis that yellow mutant *cyt-*Y_2 was not allelic to nuclear mutants y_{10}, y_{12}, y_{13}, and y_{18} and that the mutant reported herein was inherited cytoplasmically. Furthermore, in all backcrosses, *cyt-*Y_2 exhibited a consistent phenotype, indicating that the lesion does not show variable expression. The result also demonstrates the lack of interaction between the nuclear and cytoplasmic mutants; *i.e.*, all effects are independent.

The data in Table 3 from reciprocal crosses between *cyt-*Y_2 and *cyt-*G_2 and between *cyt-*G_2 and Minsoy confirmed that green plants were not capable of either restoring green pigmentation to yellow plants or inducing chimeras or yellow plants. That is, the plastome and genome were not uniquely capable of restoring or inducing phenotypic changes in plant color. A cytoplasmic-nuclear interaction like that of Oenothera (Epp 1973) was not responsible for the occurrence of A75–1165–117.

A possible genetic cause of uniparental inheritance, gametophytic incompatibility, had to be excluded to be certain of cytoplasmic inheritance. A recessive gene may be present in one of the lines, preventing certain gametes from functioning. Yellow F_1 plants and F_2 plants were observed when *cyt-*Y_2 was the female parent in the original crosses and in the three-way crosses. The source of the male parent (either *cyt-*G_2 or Minsoy) had no effect on expression of the yellow phenotype. The reciprocal crosses yielded only green plants. No transmission of the "yellow factor" was observed when yellow F_1 plants were used as male parents in crosses onto 0X281. In all F_2 populations of the three-way crosses, there was no indication that male gametophytic incompatibility was the cause of uniparental transmission of the yellow phenotype through the maternal line.

The failure of grafting of *cyt-*Y_2 rootstock with *cyt-*G_2 scion or with Minsoy scion to modify plant color of the grafted scions and their progenies indicated that graft-transmissible agents were not involved in the yellow plant color (Table

4). In reciprocal graft combinations, the grafted scions and their progenies were yellow, as expected (Table 4). Graft transmission of cytoplasmic male sterility, however, has been observed in several plants; *e.g.*, *Petunia hybrida* (EDWARDSON and CORBETT 1961; FRANKEL 1962); sugarbeet (*Beta vulgaris* L.) (CURTIS 1967) and alfalfa (*Medicago sativa* L.) (THOMPSON and AXTELL 1978). Failure of graft transfer of cytoplasmic male sterility has been reported for sugarbeets (CLEIJ 1967; THEURER, HECKER and OTTLEY 1968), wheat (Triticum spp.) (ZEVEN 1967), tobacco (Nicotiana spp.) (SAND 1960), pepper (*Capsicum annuum* L.) (EDWARDSON 1970), field bean (*Vicia faba* L.) (BOND, FYFE and TOYNBEE-CLARKE 1966) and *Crotalaria mucronata* (EDWARDSON 1967).

Growth-chamber studies were initiated to determine whether this cytoplasmic defect caused an alteration in organelle development and to determine the mutant's potential for normal development. Electron photomicrographs of thin sections from leaves of yellow and green plants, both grown in the dark, were indistinguishable. The paracrystalline prolamellar body was observed in etioplasts from both yellow and green plants, and protochlorophyllide accumulated at a normal rate, and development was similar for both genotypes. In the dark-grown plants, nourishment was provided by the cotyledons, and photosynthesis did not occur.

Light-grown yellow plants accumulated 200 times more pigment than did dark-grown yellow plants as the photosynthetic apparatus developed. Grown under conditions of low-intensity light and constant temperature, yellow plants accumulated slightly less chlorophyll than did green plants but seemed normal in internal structure. Grana stacks, starch grains and mitochondria also seemed normal. Therefore, we were unable to localize the defect to either the chloroplast or mitochondrial genome. These results were in contrast to those with plastids derived from disharmonious combinations of nuclear and cytoplasmic genomes of Oenothera (SCHOTZ 1970). Such incompatibility resulted in abnormal ultrastructure of the plastids.

Similarly, cytoplasmic chlorophyll-deficient mutants induced by *ij* in maize and *pm* in Oenothera were lethal and resulted in abnormal development of plastids (SHUMWAY and WEIER 1967; EPP 1973). Those of *ij* resulted in formation of abnormal prolamellar bodies and loss of chloroplast ribosomes (SHUMWAY and WEIER 1967; WALBOT and COE 1979). Apparent loss of ribosomes also can be seen in some of the electron photomicrographs by SCHOTZ (1970) of certain incompatible Oenothera combinations. In comparison with these effects, the lesions of *cyt-Y_2* are relatively minor. The *cyt-Y_2* soybean mutant does not have readily detectable abnormalities in cellular ultrastructure.

We found that, under more variable growing conditions in the field, emerging leaves of *cyt-Y_2* plants were yellow in comparison with leaves from normal green plants. As yellow leaves expanded, however, they became light green, and as they developed they became progressively greener. Leaves from yellow plants eventually became difficult to distinguish phenotypically from green plants, but seed yield per yellow plant was less than that per green plant.

These observations are consistent with the hypothesis that *cyt-Y*$_2$ mutant plants are defective in coordination between nuclear and cytoplasmic components involved in organelle biogenesis in a manner that is largely correctable as leaves mature. Studies at the molecular level may resolve the nature of the defect.

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