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Plastom Control of Chloroplast Development in Cotton, *Gossypium hirsutum* L.[1]

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ABSTRACT

Developing chloroplasts in a variegated cotton (*Gossypium hirsutum* L.) mutant were examined. This plastom mutation leads to a block in chloroplast development at the primary lamellar stage and no grana development occurs. The synthesis of reductive-pentose-phosphate-cycle enzymes was not impaired in this mutant.

Additional index words: **Grana development, Enzyme profile, Variegated cotton.**

NUCLEAR and plastom mutations are known to affect chloroplast development. However, the relative importance of nuclear DNA or chloroplast DNA in coding for chloroplast constituents is not completely understood. Smillie et al. (1967) have shown that the 70 S chloroplast ribosomes of *Euglena gracilis* are involved in the synthesis of a wide range of organelle proteins such as Calvin-cycle enzymes and photosynthetic-electron-transport proteins. Bovarnick, Freedman, and Schiff (1970) have shown that NADP-linked glyceraldehyde phosphate dehydrogenase is not synthesized on 70 S ribosomes but probably is synthesized on 87 S ribosomes of *E. gracilis*. Nuclear DNA may code or control this chloroplast enzyme. Mutations in nuclear genes have been shown to lead to an interruption in the formation of primary lamellae and thylakoids (von Wettstein, 1958, 1959, and 1960). Kawashima and Wildman (1970) suggest that all of the proteins of the thylakoids are synthesized from information coded in chloroplast DNA whereas information necessary for the synthesis of the mobile-phase proteins resides in the nuclear DNA. Eytan and Ohad (1970) have recently shown that the synthesis of chloroplast membranes requires proteins synthesized on the cytoplasmic and chloroplast ribosomes. Goodenough and Levine (1970), Levine and Paszewski (1970), and Togasaki and Levine (1970) have shown that the nuclear mutant *ac-20* of *Chlamydomonas reinhardi* can cause a reduction of chloroplast ribosomes, and this reduction in ribosomes was associated with a reduction in ribulose-1,5-di P carboxylase, cytochrome 559, Q (quencher of fluorescence of photosystem II), chlorophyll and carotenoids, and a lack of membrane organization. Other enzymes and constituents of the chloroplast were unaffected, which further demonstrates the involvement of chloroplast and cytoplasmic ribosomes in the protein synthesis of the chloroplast.

Little information has been published on the biochemical aspects of variegated mutants. Rédei (1967) has shown that variegated mutant expression in *Arabidopsis thaliana* was associated with increased ribonuclease activity. Shumway and Weier (1967) studied electronmicroscopically the plastids in a plastom mutant of maize. They were not able to detect ribosomes in aberrant chloroplasts, though their presence was obvious in cytoplasm of the cells, and they concluded that the grana-fretwork, the pigments, and probably the enzymes were absent due to the absence of the 70 S ribosomes.

In this report, we present structural details and an enzymatic profile of aberrant chloroplasts that result from a plastom mutation in cotton. We showed previously (Kohel and Benedict, 1971) that the plastom mutation in question resulted in aberrant chloroplasts with reduced Co_2 fixation potential and pigment concentrations, but they retained their ability to utilize C-2-acetate normally and to reproduce.

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MATERIALS AND METHODS

Plants

Variegated and normal green plants of Upland cotton, *Gossypium hirsutum* L. were grown in the greenhouse. The leaves of the variegated plants were characterized by green and white sectors. Expression of this mutant phenotype is determined by a plastom mutation (Kohel, 1967). The third or fourth leaf was used for electron microscopy and enzyme study.

Preparation of Soluble Leaf Protein

Mutant and normal green leaves were harvested, rinsed with distilled H_2O, blotted and weighed. The leaves were ground in a chilled mortar in 0.1*M* Tris buffer pH 7.5 containing 0.1 n*M* GSH (glutathione) and sand. The brei was squeezed through two layers of cheesecloth and centrifuged 30 min at 27,000 *g* in a Sorvall[3] refrigerated centrifuge. The soluble supernatant fraction was removed and used as a source of photosynthetic enzymes.

Enzyme Assays

Ribulose-1,5-di P carboxylase was assayed by the procedure described by Fuller and Gibbs (1959). The reaction mixture contained, in micromoles: 100 Tris buffer (pH 7.5), 10-$MgCl_2$, 2.5 GSH, 2.0 ribulose-1,5-di P, 50 potassium bicarbonate (containing 10 μc of radioactivity); plus 0.05 ml of protein extract and H_2O to make 1.0 ml. The reaction mixture was incubated 15 min at 37 C. The reaction was stopped by adding 1.0 ml of concentrated HCl to each tube. The tube contents were evaporated to dryness. The residue was dissolved in H_2O and assayed for radioactivity.

Glyceraldehyde-3-P dehydrogenase was assayed in the reverse direction by the procedure of Gibbs (1955). The reaction mixture contained, in micromoles: 100 Tris buffer (pH 7.5), 10 $MgCl_2$, 10 ATP, 5 GSH, 0.25 NADH (diphosphopyridine nucleotide) or NADPH (triphosphopyridine nucleotide), 10 PGA (phosphoglyciric acid) tricyclohexylammonium salt; plus 0.5 mg of crystalline 3-phosphoglyceric phosphokinase in a 2.7 *M* $(NH_4)_2$ SO_4 suspension; 0.1 ml of protein extract; and H_2O to make 3.0 ml. The decrease in absorbency at 340 nm was recorded for 5 min in a Beckman DK-2[3] recording spectrophotometer.

Glycerate-3-P kinase was assayed in the reverse direction. The reaction mixture was the same as the glyceraldehyde-3-P dehydrogenase mixture except the 3-phosphoglyceric phosphokinase was replaced with 0.5 mg of glyceraldehyde-3-P dehydrogenase in 0.25*M* $(NH_4)_2SO_4$ suspension.

Fructose-1,6-di P aldolase was assayed by coupling the enzyme to glyceraldehyde-3-P dehydrogenase. The reaction mixture contained, in micromoles: 100 Tris buffer (pH 7.5), 0.25 NAD, 50 Na_2HAsO_4, 10 fructose-1, 6-di P tetracyclohexylammonium salt; plus 0.5 mg of crystalline 3-phosphoglyceric phosphokinase in a 2.7*M* $(NH_4)_2$ SO_4 suspension; 0.1 ml protein extract; and H_20 to make 3.0 ml. The increase in absorbency at 340 nm was recorded for 5.0 min.

The activity of the following enzymes were measured as described in the corresponding references: PEP (phosphoenol pyruvate) carboxylase (Ting, 1968), NAD-linked malic dehydrogenase (Ochoa, 1955), NAD-linked isocitric dehydrogenase (Latzko and Gibbs, 1969), and glucose-6-P dehydrogenase (Kornberg and Horecker, 1955).

Chemicals

3-phosphoglyceric phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, D(-)3-phosphoglyceric acid tricyclohexylammonium salt, D-fructose -1, 6-diphosphate tetracyclohexylammonium salt, D-glucose-6-phosphate disodium salt, ribulose-1,5-diphosphate tetrasodium salt, NADH, NADPH, NAD, ATP, and GSH were obtained from Sigma Chemical Company[3]. Sodium ^{14}C-bicarbonate was purchased from New England Nuclear Corporation[3].

Electron Microscopy

The ultrastructure analysis of white sections of 75% expanded variegated leaves was as follows: the leaves were fixed in glutaraldehyde, post-fixed in OsO_4, embedded in epoxy resin, and sectioned with glass knives on a MT-2 ultra-microtome[3]. The thin sections were mounted on 400-mesh athene-type copper grids and stained with methanolic uranyl acetate and lead citrate.

Chlorophyll Analysis

The amount of chlorophyll in the normal and mutant leaves was determined as previously described (Benedict and Kohel, 1968).

Measurement of Radioactivity

The amount of radioactivity in the aqueous samples was assayed in a Beckman[3] liquid-scintillation-system. Each radioactive sample was added to 15.0 ml of scintillation fluid (containing 5g of PPO (diphenyloxazole), 100g of napthalene, 10 ml H_2O, and dioxane to make 1 liter). The scintillation vials were dark adapted for several hours and the samples were counted with $\pm$ 0.2% error.

RESULTS AND DISCUSSION

Electron photomicrographs of white leaf sectors of variegated cotton leaves revealed the two types of aberrant chloroplasts (Fig. 1). Chloroplasts devoid of internal structure, except for a few vesicles, represent about 70% of the chloroplasts observed in the thin-sections (above). The remaining 30% of the chloroplasts show an extrusion of primary lamellae. The double membrane sheets extend from the prolamellar body. These chloroplasts do not develop any structure beyond the primary lamellar structure shown in Fig. 1 (below).

The aberrant chloroplasts of Fig. 1 (above) are representative of that stage reported by Shumway and Weier (1967) in the *iojap* variegated mutant of corn. However, the cotton chloroplasts of Fig. 1 below illustrate a stage of greater structural development, extrusion of primary lamellae, than that reported by Shumway and Weier (ibid.) in *iojap*.

Not all mutant tissue of the variegated cotton mutant is white when the leaf expands. Some of the mutant tissue is yellow and bleaches to white. Bleaching of the expanded leaf may represent a breakdown during a labile period of development. However, bleaching of the variegated mutant differs from streptomycin induced (Provasoli, Hutner, and Schatz, 1948) or U. V. induced (Lyman, Epstein, and Schiff, 1961) bleaching in *E. gracilis*. Cells of *E. gracilis* cultured in streptomycin or treated with U. V. not only bleach, but also lose their ability to form chloroplasts.

The data in Table 1 show an enzyme profile of some reductive-pentose-phosphate-cycle and non-photosynthetic enzymes in the white and green sectors of variegated cotton leaves. The same enzyme profile in these leaves has been found in six different samplings and determinations. It is evident that photosynthetic enzymes were synthesized in white leaf sectors. Enzymes of the reductive-pentose-phosphate-cycle and PEP carboxylase, malic dehydrogenase and isocitric dehydrogenase were present and displayed similar or enhanced activities in the white sectors as compared with the green. The lower amount of RuDP carboxylase and NADP-linked glyceraldehyde-P-dehydrogenase in the white sectors may indicate a decreased rate of synthesis of these enzymes in the aberrant chloroplasts. However, in older white leaf sectors we have obtained enzyme activities (values in parenthesis in Table 1) for RuDP carboxylase and NADP-linked glyceraldehyde-P-dehydrogenase which exceeded the activities of these enzymes in the green leaf sectors. It

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

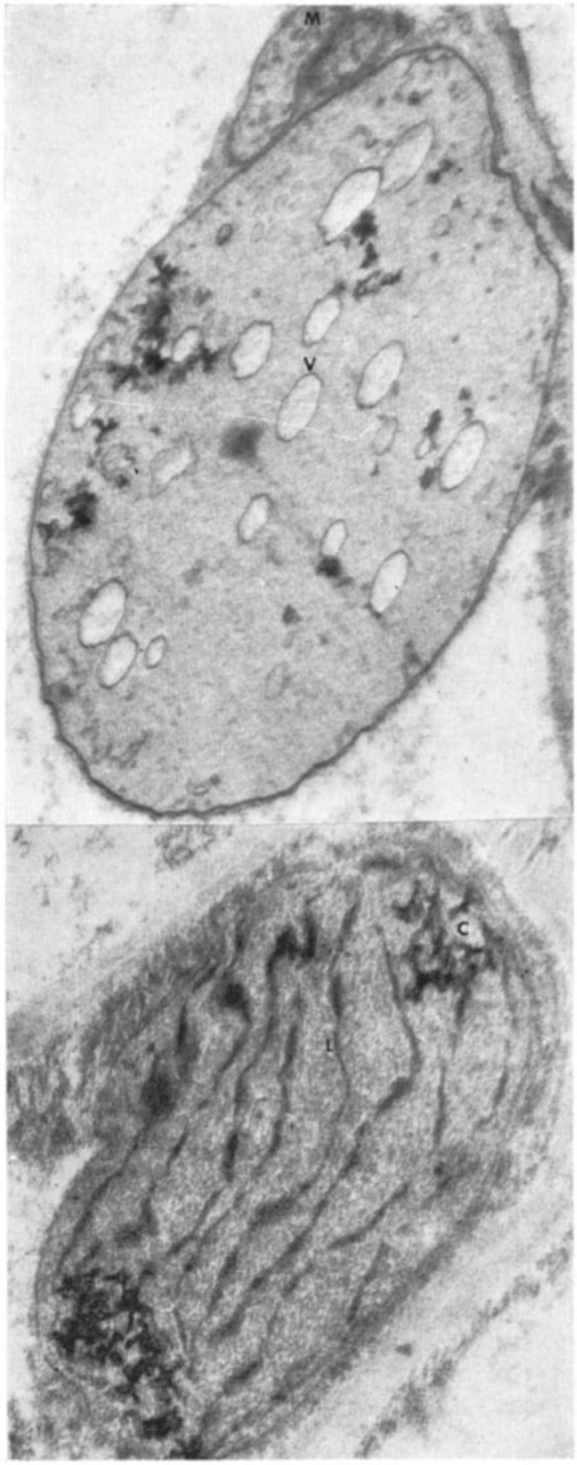

Fig. 1. Electron photomicrographs representative of the two types of chloroplasts found in white sectors of variegated cotton: (Above) Chloroplasts devoid of internal structure, except for the development of a few vesicles (V); these chloroplasts represent about 70% of those observed in the white sectors. (Below) The remaining chloroplasts show an extrusion of primary lamellae (L) and the remnants of the crystalline center (C). 8,333×

is difficult to obtain green and white sectors of the same physiological age even though the samples are taken from the same leaf. The difference in the physiological age of the leaf sectors may contribute to the variation in some of the enzymes included in this profile. In general, we conclude that the aberrant chloroplasts synthesize photosynthetic enzymes of the carbon reduction cycle at about the same or higher levels as do the green chloroplasts.

Table 1. Activities (umoles per mg protein per hour, 340 nm) of certain reductive-pentose-phosphate-cycle and non-photosynthetic enzymes and chlorophyll content in green and white leaf sectors of variegated cotton leaves.

Enzymes and chlorophyll	Green	White
Reductive-pentose-phosphate-cycle		
Ribulose-1, 5-di P carboxylase	24.4	11.4(26.1)*
NADP-linked glyceraldehyde-3-P dehydrogenase	4.7	1.3(7.2)
Glycerate-3-P kinase	145.6	179.8
Fructose-1, 6-di P aldolase	5.1	5.1
Non-photosynthetic		
Glucose-6-P dehydrogenase	0.5	2.1
NAD-linked glyceraldehyde-3-P dehydrogenase	2.7	3.2
NAD-linked malic dehydrogenase	107.2	179.8
NAD-linked isocitric dehydrogenase	0.5	2.1
PEP carboxylase	0.2	1.7
Chlorophyll content (mg chl/g fr wt)	0.57	0.009

* Values in parenthesis represent older white leaf sectors.

It is likely that the plastom mutation prevents the synthesis of proteins or other substances essential for thylakoid development. Togaski and Levine (1970) have shown a close association between the level of 70 S ribosomes and ribulose-1, 5-di P carboxylase activity in *C. reinhardi*. The presence of ribulose-1,5-di P carboxylase in the aberrant chloroplasts of variegated white leaf sectors of cotton indicates that 70 S ribosomes were present and active in protein synthesis. Our results indicate that a probable mutation in the chloroplast DNA results in a specific interruption in grana formation. The mutation stops chloroplast development at the primary lamellae stage, but it does not interfere with the synthesis of the enzymes analyzed.

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