Plant Physiol. (1972) 49, 972–976

Nuclear Gene Affecting Greening in Virescent Peanut Leaves[1]

Received for publication October 4, 1971

C. R. Benedict and D. L. Ketring
Department of Plant Sciences, Texas A&M University, College Station, Texas 77843

ABSTRACT

Chlorophyll synthesis induced by continuous illumination of dark-grown seedlings has been followed in wild-type and virescent peanut leaves. Compared to the wild-type leaves, chlorophyll synthesis in the virescent leaves shows a 72-hour lag period before the onset of a phase of rapid chlorophyll accumulation. The development of chloroplast grana and the activity of many enzymes of the reductive pentose phosphate cycle, phosphoenolpyruvate carboxylase, and malate dehydrogenase are reduced in the virescent leaves during the lag phase of chlorophyll accumulation. Although nucleic acid synthesis in the virescent leaves in normal, there is a distinctly lower rate of protein synthesis. The low level of protein synthesis during the lag period might limit the synthesis of a factor(s) essential for the development of both cell and chloroplast constituents.

Nuclear mutants have been described which show a classical Mendelian inheritance of aldolase (2), phosphoribulokinase (19), NADP-linked glyceraldehyde-P dehydrogenase (7) and which contain specific blocks in the synthesis of chlorophyll (4, 12, 15, 24), carotenoids (1, 13, 18, 28), photosynthetic electron transport proteins (19–21), and grana (5, 27). Goodenough and Levine (11) have described a nuclear mutant of *Chlamydomonas reinhardi* which affects the synthesis of 70S ribosomes. There is a poor development of chloroplast membranes, photosystem II activity, and RuDP carboxylase activity; and chlorophyll levels are low in this mutant. The mutation does not affect the activity of many of the enzymes of the reductive pentose phosphate cycle nor the development of the mitochondria. Huffaker *et al.* (17) have described a nuclear plant mutant which is defective in the synthesis of chlorophyll, carotenoids, and ribulose-1,5-diP carboxylase activity. The mutation does not affect the activity of glucose-6-P dehydrogenase, P-enolpyruvate carboxylase, or malate dehydrogenase. We describe a unique virescent peanut mutant which shows an altered development of cell and chloroplast constituents.

MATERIALS AND METHODS

Materials. 3-Phosphoglyceric phosphokinase, glyceraldehyde-3-P dehydrogenase, D-(−)-3-phosphoglyceric acid tricyclohexylammonium salt, D-fructose-1,6-diP tetracyclohexylammonium salt, ribulose-1,5-diP tetrasodium salt, NADH, NADPH, NAD, ATP, and GSH were obtained from Sigma Chemical Company.[2] Sodium ^{14}C-bicarbonate, ^{14}C-amino acid mixture, and uracil-2-^{14}C were purchased from New England Nuclear Corporation.

Plant Material. Seeds of virescent (T-811) and normal green (NC4-x) Virginia peanuts were obtained from Dr. Ray O. Hammons, Georgia Coastal Plain Experiment Station, Tifton, Georgia. Both of these peanuts were derived from irradiation research with the NC4 advanced breeding line and are true breeding. Dr. Hammons has shown that the virescence phenotype is inherited as a recessive Mendelian trait. The F_2 generation of a cross between virescent x green segregates in a 3:1 or 15:1 ratio of green to virescent plants depending on the specific varieties used in the crosses. Prior to planting, the peanut seeds were fumigated with the fungicide Panogen overnight in a large desiccator. Following the fumigation treatment, the seeds were planted in moist vermiculite and placed in a dark growth room at 25 C. After 6 days of growth the hypocotyls were about 5 cm long, and the primary leaves were 0.5 cm long. The greening experiments were initiated by placing the etiolated plants in an incubator at 30 C which contained two 20-w cool-white fluorescent bulbs which furnished about 200 ft-c of light at the plant surface. The levels of enzymes in the primary leaves and the incorporation of ^{14}C-amino acids and uracil-2-^{14}C into the leaves were determined following exposure of the seedlings to 24 hr of illumination. The majority of all of the experiments reported were repeated at least six times.

Preparation of Soluble Leaf Protein. About 300 virescent and normal green leaves (wild-type leaves) were harvested, rinsed with distilled water, blotted, and weighed. The leaves were ground in a chilled mortar in 0.1 M tris buffer, pH 7.5, containing 0.1 mM GSH and sand. The brei was squeezed through two layers of cheesecloth and centrifuged for 30 min at 27,000*g* in a Sorvall refrigerated centrifuge. The soluble supernatant fraction was removed and used as the source of photosynthetic enzymes.

Enzyme Assays. Ribulose-1,5-diP carboxylase was assayed by the procedure described by Fuller and Gibbs (9). The reaction mixture contained, in μmoles: 100, tris buffer, pH 7.5; 10, $MgCl_2$; 2.5, GSH; 2.0 ribulose-1,5-diP; 50, potassium bicarbonate containing 10 μc of radioactivity; 50 μl of protein extract; and water to 1.0 ml. The reaction was incubated for 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 water and assayed for radioactivity.

Glyceraldehyde-3-P dehydrogenase was assayed in the reverse direction by the procedure of Gibbs (10). The reaction mixture contained, in μmoles: 100, tris buffer, pH 7.5; 10,

[1] This work was supported in part by a grant from the Texas A&M University Research Council and cooperative investigations of the Plant Science Research Division, Agricultural Research Service, United States Department of Agriculture and the Texas Agricultural Experiment Station, Texas A&M University.

[2] Mention of trademark name or proprietary product does not constitute endorsement by the United States Department of Agriculture or Texas A&M University and does not imply its approval to the exclusion of other products that also may be suitable.

$MgCl_2$; 10, ATP; 5, GSH; 0.25, NADH or NADPH; 0.5 mg of crystalline 3-phosphoglyceric phosphokinase in a 2.7 M $(NH_4)_2SO_4$ suspension; 10, glycerate-3-P tricyclohexylammonium salt; 0.1 ml of protein extract; and water to 3.0 ml. The decrease in absorbance at 340 nm was recorded for 5 min in a Beckman DK-2 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 a 0.25 M $(NH_4)_2SO_4$ suspension.

Fructose-1,6-diP aldolase was assayed by coupling the enzyme to glyceraldehyde-3-P dehydrogenase. The reaction mixture contained in μmoles: 100, tris buffer, pH 7.5; 0.25, NAD; 50, Na_2HAsO_4; 0.5 mg of crystalline glyceraldehyde-3-P dehydrogenase in a 2.7 M $(NH_4)_2SO_4$ suspension; 10, fructose-1,6-diP tetracyclohexylammonium salt; 0.1 ml of protein extract; and water to 3.0 ml. The increase in absorbance at 340 nm was recorded for 5.0 min.

Malate dehydrogenase activity was measured by the procedure of Ochoa (22).

Electron Microscopy. The ultrastructural analysis of the peanut leaves was as follows: the leaves were fixed in glutaraldehyde, postfixed in OsO_4, embedded in epoxy resin, and sectioned with glass knives on a MT-2 ultramicrotome. 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 virescent leaves was determined as previously described (3).

Measurement of Radioactivity. The amount of radioactivity in the aqueous samples was assayed in a Beckman liquid scintillation system. Each radioactive sample was added to 15 ml of scintillation fluid which contained 5 g of PPO, 100 g of naphthalene, 10 ml of H_2O, and dioxane to 1 liter. The scintillation vials were dark-adapted for several hours, and the counts were assayed with ±0.2% error.

Incorporation of Radioactive Amino Acids into Proteins in Peanut Leaves. Following 24 hr of illumination, peanut leaves were excised and floated on 10 ml of water containing 10 μc of ^{14}C-amino acid mixture (250 μc/0.168 mg) at 30 C under 300 ft-c of light intensity for 4 hr. The uptake and incorporation of the amino acids into protein in the leaves were terminated by removing and rinsing the leaves in distilled water followed by grinding the leaves in 0.1 M tris buffer, pH 7.6, in a chilled mortar. The homogenate was centrifuged at 17,000*g* for 60 min. The soluble protein fraction was precipitated with 5% trichloroacetic acid. The pellet was collected by centrifugation and washed three or four times with 5% trichloroacetic acid. The original supernatant plus the trichloroacetic acid washings were assayed for radioactivity to determine the uptake of the ^{14}C-amino acids into the amino acid pools in the leaves. The protein pellet was dissolved in 0.05 N NaOH. An aliquot of this solution was assayed in a Beckman liquid scintillation spectrometer to determine the extent of ^{14}C-amino acids incorporated into the leaf protein in the light.

Protein Synthesis. The amount of soluble protein synthesized in developing peanut leaves during 96 hr of illumination was measured by the above procedure. The leaves were excised at the appropriate time interval, and the soluble protein was extracted with 0.1 M tris buffer, pH 7.5. The protein was precipitated with 5% trichloroacetic acid, collected by centrifugation, and dried at 80 to 100 C for several days. The protein pellet was weighed, and the amount of protein in the leaves was expressed as mg of soluble protein per leaf.

Incorporation of Uracil-2-^{14}C into Nucleic Acids in Peanut Leaves. Following 24 hr of illumination, peanut leaves were excised and floated on 5 ml of water containing 20 μc of uracil-2-^{14}C (50 mc/mmole) at 30 C under 200 ft-c of light intensity for 4 hr. The experiment was terminated by removing and rinsing the leaves in distilled water followed by grinding the leaves in phenol, glycine buffer (pH 9.5), sodium deoxycholate, and bentonite according to the procedure of Click and Hackett (8). The homogenate was centrifuged and separated into two layers. The nucleic acids were precipitated from the upper aqueous layer with 3 volumes of cold 95% ethyl alcohol. The precipitate was collected and washed several times with cold alcohol. The alcohol supernatant plus washings were added to the original bottom layer. The amount of radioactivity in these soluble fractions represents uracil-2-^{14}C uptake into the pyrimidine base, nucleoside, and nucleotide pools in the leaves. The amount of radioactivity into the nucleic acid

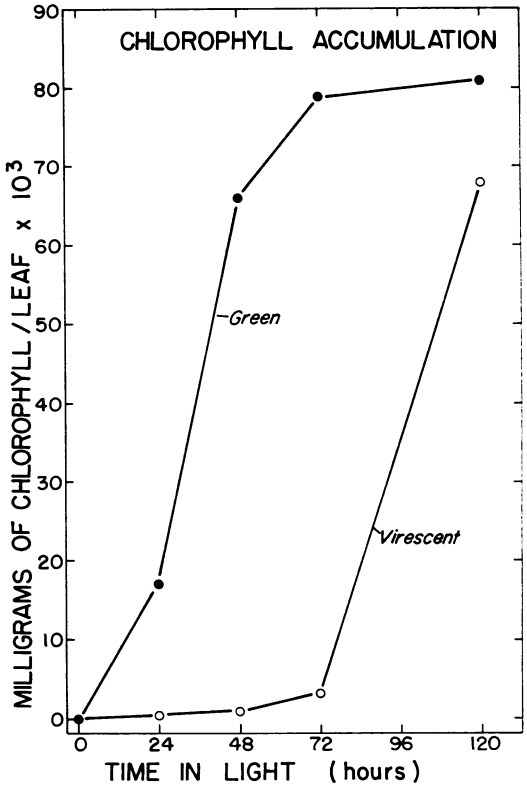

FIG. 1. The light-induced formation of chlorophyll in virescent and wild-type peanut leaves.

Table I. *Enzyme Activities in Wild-type and Virescent Peanut Leaves during Greening*

	Green	Virescent	Percentage of Control[1]
	μmoles/hr·leaf		
Ribulose-1,5diP carboxylase	1.33	0.014	1.1
Glyceraldehyde-3-P dehydrogenase (NADP)	0.75	0.079	10.5
Fructose-1,6-diP aldolase	0.55	0.069	12.5
Glycerate-3-P kinase	57.0	10.5	18.4
Phosphoenolpyruvate carboxylase	0.23	0.066	28.6
Malate dehydrogenase (NAD)	46.7	16.3	34.9
Chlorophyll content (mg/g fr wt)	1.15	0.085	7.4

[1] Virescent ÷ green × 100.

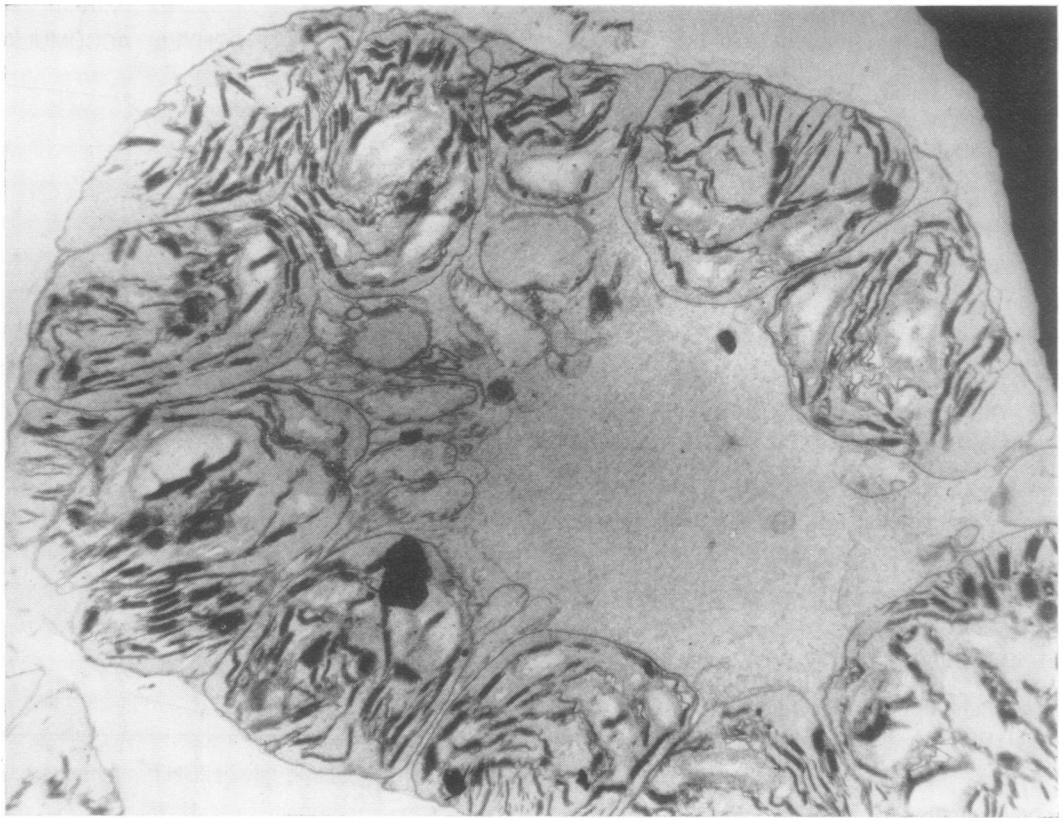

FIG. 2. An electron photomicrograph of a mesophyll cell from a wild-type peanut leaf. × 8500.

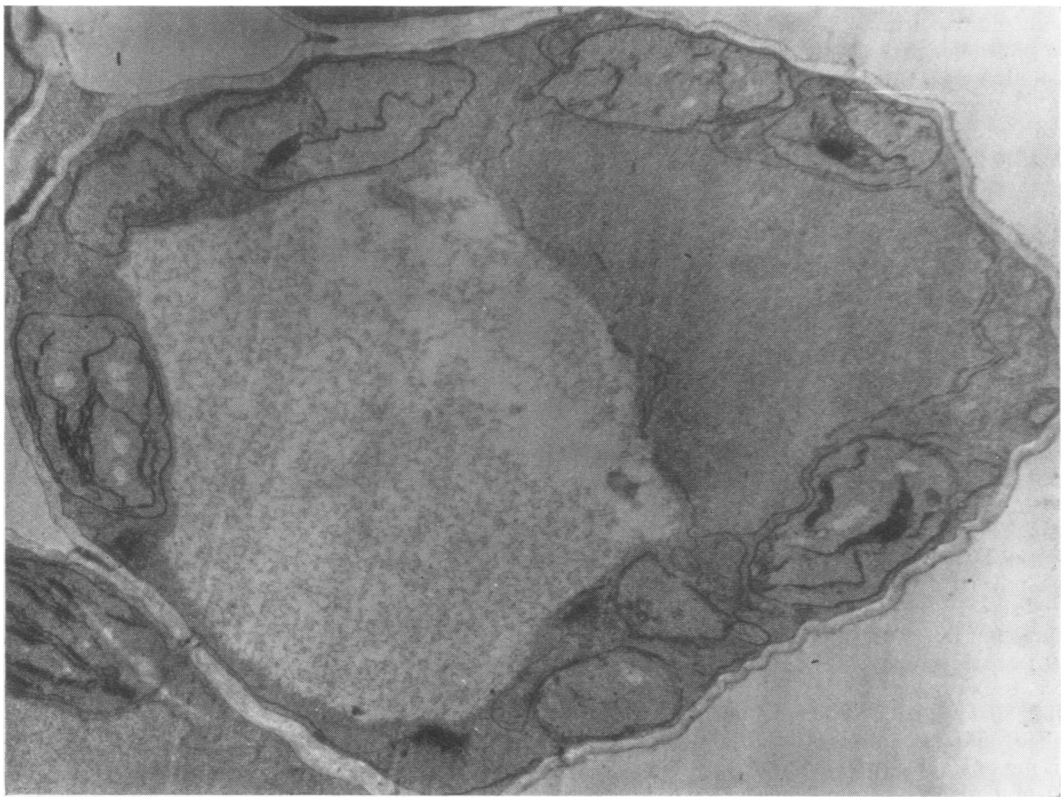

FIG. 3. An electron photomicrograph of a mesophyll cell from a virescent peanut leaf. × 9000.

precipitate was determined by dissolving the precipitate in dilute NaOH and assaying an aliquot in the scintillation counter. The results in Table II are the average of 6 experiments.

RESULTS

The rate of chlorophyll accumulation in leaves of dark-grown virescent and wild-type peanut seedlings which is induced by continuous illumination is shown in Figure 1. Chlorophyll formation in the wild-type leaves is complete after 48 to 72 hr of continuous illumination. In contrast, there is a long lag period in accumulation in the mutant leaves. In these leaves, the lag period lasts for 72 hr and is followed by a phase of rapid chlorophyll synthesis. This latter phase in the mutant leaves is similar to the rapid phase of chlorophyll accumulation in the wild-type leaves. The chlorophyll content of the two types of leaves is nearly equal after 120 hr in the light.

Schwartzbach and Schiff (25) have shown that plastid differentiation in greening *Euglena gracilis* is dependent on synthetic events in the cytoplasm. Initial chloroplast differentiation following the lag period relies on synthetic events in the cytoplasm during the lag period. Since the mutation of the virescent gene in peanuts results in a lag in chlorophyll accumulation, it was of interest to establish the effect of the mutation on cell and chloroplast constituents. We have determined the effect of the mutation on the activity of several enzymes of the reductive pentose phosphate cycle, P-enolpyruvate carboxylase, malate dehydrogenase, and chloroplast structure. The activities of these enzymes in the virescent and wild-type leaves exposed to 24 hr of light are shown in Table I. The virescent leaves contain only 7% as much chlorophyll as the wild-type leaves. The enzymatic activities of ribulose-1,5-diP carboxylase, NADP-linked glyceraldehyde-P dehydrogenase, phosphoglyceric acid kinase, and fructose-1,6-diP aldolase are reduced to about the same extent as is chlorophyll in the virescent leaves. The activities of PEP carboxylase and malate dehydrogenase are reduced in the mutant leaves. Rocha and Ting (23) have shown that malate dehydrogenase activity in spinach leaves is due to soluble, mitochondrial, and microbody isozymes of malate dehydrogenase. The lower activities of malate dehydrogenase, P-enolpyruvate carboxylase, and several enzymes of the reductive pentose phosphate cycle indicate that some cell and chloroplast constituents are reduced in the virescent leaves.

The structure of chloroplasts in the wild-type and virescent leaves exposed to 24 hr of light is shown in Figures 2 and 3. The electron photomicrograph in Figure 2 shows that a mesophyll cell of the wild-type leaves contains large chloroplasts with prominent grana and starch grains. The electron photomicrograph in Figure 3 shows a mesophyll cell of the mutant containing only small chloroplasts with poorly developed grana. These results suggest that mutation may result in a limited synthesis of a factor necessary for cell and chloroplast differentiation. Therefore, we tested the rate of nucleic acid and protein synthesis during the lag period of chlorophyll accumulation.

The results in Table II show the relative amounts of incorporation of uracil-2-^{14}C and ^{14}C-amino acids in the light into virescent and wild-type leaves. Prior to this experiment both types of seedlings had been exposed to 24 hr of continuous illumination. The uptake of uracil-2-^{14}C and incorporation into nucleic acids are the same in the virescent and wild-type leaves. Compared to the wild-type leaves, the uptake of ^{14}C-amino acids and incorporation into proteins are reduced in the virescent leaves. As shown in Figure 4, the virescent leaves synthesize soluble protein at a slower rate than the wild-type leaves. Thus, the smaller uptake and reduced incorporation of ^{14}C-amino acids into protein in the virescent leaves are reflections of a lower rate of protein synthesis.

Table II. *Incorporation of Uracil-2-^{14}C and ^{14}C-Amino Acid Mixture into Virescent and Wild-type Peanut Leaves in the Light*

Three hundred leaves were used in these experiments.

	Uptake	Incorporation	
		Into nucleic acid	Into proteins
	cpm/leaf		
Uracil-2-^{14}C			
Green	6,551	2,246	
Virescent	5,817	2,880	
^{14}C-Amino acids			
Green	310,630		1,199
Virescent	169,565		693

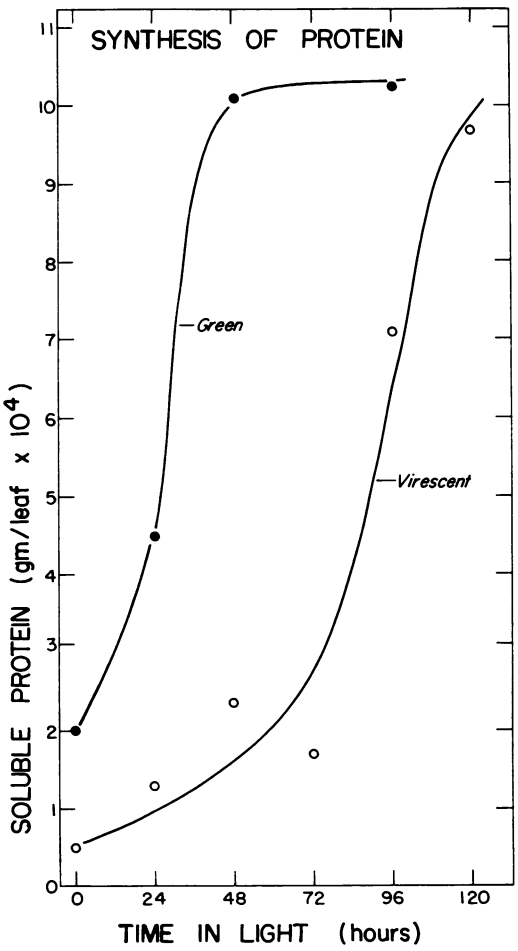

FIG. 4. The light-induced formation of soluble protein in virescent and wild-type peanut leaves.

DISCUSSION

The results in this paper show that there is a lag period in the greening of virescent peanut leaves. In the lag period of

chlorophyll accumulation there is an alteration in both cell and chloroplast constituents. The lag period in the virescent leaves is similar to the lag period in chlorophyll accumulation in greening leaves of higher plants (26) and cells of *Euglena gracilis* (16), except that the lag period lasts for 72 hr in the mutant leaves. In greening *E. gracilis*, Schiff and co-workers (16, 25) have shown that chloroplast development is dependent on synthetic events developed in the cytoplasm during the lag period. The low level of protein synthesis during the lag period in the virescent leaves might limit the synthesis of a factor(s) essential for the development of both cell and chloroplast constituents.

Many nuclear mutants of higher plants and of algae contain reduced levels of specific chloroplast enzymes, pigments, and lamellae (1, 2, 4–7, 12, 13, 15, 18–21, 27). Other mutants such as the ac-20 strain of *Chlamydomonas reinhardi* (11) and the temperature-sensitive chlorophyll mutant of *Medicago sativa* (17) contain reduced levels of many chloroplast constituents but contain normal levels of cytoplasmic constituents. The peanut mutant differs from all of these mutants in that both cell and chloroplast constituents are altered in the virescent leaves.

Many of the same chloroplast constituents are altered in this mutant and in the chlorophyll mutant of *M. sativa* (17), but the enzymatic activities of P-enolpyruvate carboxylase and malate dehydrogenase are only reduced in the virescent leaves. Many of the same chloroplast constituents are also altered in virescent leaves and the ac-20 strain of *C. reinhardi* (11); yet the activity of many more enzymes of the reductive pentose phosphate cycle and cell constituents are reduced in the virescent leaves. The alteration of chloroplast constituents in a *C. reinhardi* mutant has been found to be due to a specific reduction in the number of 70S ribosomes (11). The authors concluded that the normal activity of glyceraldehyde-3-P dehydrogenase, phosphoglyceric acid kinase, and fructose-1,6-diP aldolase in the mutant means that the synthesis of these enzymes does not depend on the levels of 70S ribosomes. This implies that these enzymes are synthesized in the cytoplasm.

The lag period in chlorophyll accumulation in the virescent peanut leaves could be the result of a deficient cofactor essential in cell metabolism. In this respect, it is important to emphasize that the virescent gene mutation does not result in an elimination in chlorophyll synthesis. The mutation merely results in a lag in chlorophyll accumulation. A deletion or suppression of the synthesis of a cofactor would cause the eventual cessation of all growth phenomena. This is not the case with the virescent leaves. Further, the mutation does not lead to an alteration in nucleic acid synthesis during the lag period of chlorophyll accumulation. (Heyes and Dale [14] have also shown that nucleic acid levels are not altered in virescent bean leaves.) It seems reasonable to conclude that the mutation does not lead to an alteration of cell and chloroplast development by limiting a cofactor involved in the synthesis of nucleic acids. Rather, the slow development of the cell and chloroplast constituents probably results from a low rate of protein synthesis.

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