Elimination of the Lag Period in Chloroplast Development in a Chlorophyll Mutant of Peanuts

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ABSTRACT

The mutation of a nuclear gene in peanut (Arachis hypogaea L.) plants results in a reduced light-dependent development of chloroplast fine structure, soluble protein, ribulose-1,5-diP carboxylase, NADP-glyceraldehyde-3-P dehydrogenase, fructose-1,6-diP aldolase, glyceraldehyde-3-P kinase, phosphoenolpyruvate carboxylase, malate dehydrogenase, and dark respiration during the 72-hour lag period of chlorophyll synthesis in dark-grown leaves exposed to continuous light. The mutation has pleiotropic affects. Kinetic analysis shows there is also a 72-hour lag period in the light-dependent development of NADP-glyceraldehyde-3-P dehydrogenase and fructose-1,6-diP aldolase in the mutant leaves, whereas there is no lag in the development of NAD-malate dehydrogenase and dark respiration. There is minimal development of the chloroplast during the 72-hour mutationally induced lag period, but there is pronounced plastidic and mitochondrial activity during this phase. There is a 24-hour lag period in the light-dependent enlargement of the mutant leaves. At the completion of leaf enlargement, chloroplast differentiation is initiated. The mutation does not result in any chloroplast deletions, it only affects the timing of the synthesis of these components.

Elimination of the lag period in leaf enlargement and chloroplast development (potentiation) requires a preliminary 72- to 96-hour dark period before exposing the dark-grown leaves to continuous light. There is extensive development of the etioplasts during this dark period. These results establish that the nuclear gene mutation affects the early stages of plastid development and not the light-dependent synthesis of plastid components. The nuclear gene may code for the regulation of the synthesis of a component (nutrient) in the dark (or during the lag phase in the light) which is essential for the development of mesophyll cells and plastids. Although, the chloroplast is a semi-autonomous organelle, nuclear gene control of chloroplast differentiation may not be independent of cellular growth.

The lag period in Chl synthesis in higher plants can be abolished by a brief preillumination with red light followed by a dark period before exposing the dark-grown material to continuous light (15, 16). Holowinsky and Schiff (8, 9) have shown that preillumination with blue and red light followed by a dark period abolishes the lag period of Chl synthesis in the light in Euglena gracilis. Apparently, the brief illumination triggers the synthesis of externally supplied nutrients required for plastid development which are usually synthesized during the lag period. This idea is consistent with the abolishment of the lag period in Chl synthesis in higher plants with externally supplied sugars (11, 13, 17). In this paper, we describe the conditions for eliminating the mutationally induced lag period in chloroplast development in peanut leaves. A dark period, equal in length to the lag period, prior to exposing the dark-grown leaves to continuous illumination, leads to potentiation. These data implicate nuclear genes in the regulation of the synthesis of constituents (nutrients) required for the early stages of chloroplast development in the dark.

MATERIALS AND METHODS

Materials. d-(−)-3-Phosphoglyceric acid tricyclohexylmmonium salt, 3-phosphoglyceric acid phosphorylase, glyceraldehyde-3-P dehydrogenase, d-fructose-1,6-diP tetracyclhexylmnomium salt, NADP, NADH, GSH, and ATP were obtained from Sigma Chemical Company.

Plant Material. The inheritance of virescent (T-811) (mutant) and normal green (NC4-X) (wild type) Virginia peanuts (Arachis hypogaea L.) has been described (4). The virescent phenotype is inherited as a recessive trait. The seeds were dusted with Arasan —50 Red obtained from E. I. duPont de Nemours and Company2 planted in moist vermiculite and placed in a dark room at 28 C for 6 days. The plants were watered daily after 3 days in the dark. After 6 days in the dark, the seedling hypocotyls were 5 cm long, and the primary leaves were 0.5 cm in length.

Preillumination and Dark Period. After 6 days, the seedlings were preilluminated at 30 C for various periods of time. Illumination was furnished by two 20-w cool-white fluorescent bulbs which furnished about 200 ft-c of light at the plant surface. Following the preillumination period, the plants were placed back in the dark incubator at 28 C for varying lengths of time before exposing the plants to continuous light from the cool-white fluorescent bulbs at 30 C.

Kinetic Analysis. The kinetic analysis of the development of chloroplast enzymes, a soluble enzyme, dark respiration and Chl was followed in the mutant and wild type leaves. About 40 leaves of the two types of plants were harvested, rinsed with distilled H2O, and blotted. The leaves were ground in a chilled mortar in 0.1 M tris buffer, pH 7.5, containing 0.1 mM GSH

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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.
RESULTS

The light-dependent development of fructose-1,6-diP aldolase and NADP-glyceraldehyde-3-P dehydrogenase activities in mutant and wild type peanut leaves is shown in Figures 1 and 2. In continuous light, there is a rapid synthesis of both of these enzymes of the reductive pentose phosphate pathway in the wild type leaves. There is a 72-hr lag period in the development and sand. The homogenate was centrifuged at 27,000g for 30 min in a Sorvall refrigerated centrifuge. The supernatant fraction was removed and used as the source of enzymes. Each day (24-hr intervals) fructose-1,6-diP aldolase, NADP-glyceraldehyde-3-P dehydrogenase, and malate dehydrogenase was measured in the leaf extracts by the procedure previously described (2, 4). For the measurement of dark respiration in the peanut leaves at 24-hr intervals, two replicates of 20 leaflets of the mutant and wild type seedlings were placed in 125-ml Erlenmeyer flasks containing 1.0 ml of H2O. To ensure darkness, the flasks were wrapped with aluminum foil. The flasks were flushed with CO2-free air, sealed with a serum stopper, and incubated at 25 C. After 2 hr of incubation, the microliters of CO2 evolved/leaflet-unit time were determined with the use of a Beckman GC-4 gas chromatograph equipped with a thermal conductivity detector and 6 feet X 1/8 inches stainless steel silica gel column. At 24-hr intervals throughout the developmental period, the leaves were ground in 80% acetone, and the Chl a+b content of the mutant and wild type leaves was determined by the method of Arnon et al. (1).

Electron Microscopy. The mutant leaves were examined by electron microscopy. The etiolated leaves were excised from the etiolated plant material and immediately fixed in cold 1.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2. The samples were then subjected to a phosphate-buffered osmium fixation, dehydrated with ethanol and embedded in Epon. The sections were stained with uranyl acetate and lead citrate and photographed with a Hitachi HU-11E electron microscope.

The light-dependent development of fructose-1,6-diP aldolase and NADP-glyceraldehyde-3-P dehydrogenase activities in mutant and wild type peanut leaves is shown in Figures 1 and 2. In continuous light, there is a rapid synthesis of both of these enzymes of the reductive pentose phosphate pathway in the wild type leaves. There is a 72-hr lag period in the development
of fructose-1,6-diP activity in the mutant leaves in continuous light. Following the 72-hr lag period, there is a rapid development of this enzyme activity which approaches the level of activity in the wild type leaves after 144 hr in the light. There is a slow rate of synthesis of the NADP-glyceraldehyde-3-P dehydrogenase in the mutant leaves (Fig. 2). After this lag of 96 hr, there is a rapid development of enzyme activity which approaches the level of activity in the wild type leaves after 120 hr of continuous illumination.

The development of NAD-malate dehydrogenase in the mutant and wild type leaves is shown in Figure 3. The development of this enzyme activity is rapid in the green leaves and reaches a plateau in 24 hr in the light. In the mutant leaves the development of this activity is slower than in the normal leaves but proceeds without any lag period. After 72 hr the level of malate dehydrogenase activity is the same in the two types of leaves. The development of dark respiration in these leaves is similar to the development of malate dehydrogenase (Fig. 4). Dark respiration in the wild type leaves is maximal following 48 hr of exposure of the seedlings to continuous illumination. In the mutant leaves the rate of development of dark respiration is slower than the wild type leaves but proceeds with no appreciable lag period.

The light-dependent development of the mutant and wild type leaves is shown in Figure 5. The wild type leaves are fully expanded in 72 hr. There is a 24-hr lag period in the light-dependent leaf expansion in the mutant leaves and leaf expansion is complete after 72 to 96 hr in the light.

The lag period in the development of some of the enzymes of the reductive pentose phosphate pathway is similar to the lag period in Chl synthesis in these mutant leaves (2). Many workers (8, 9, 15, 16) have shown that the lag period in Chl synthesis can be abolished by exposing the dark-grown material to a brief illumination followed by a dark period before placing the leaves or cells in continuous illumination. The results in Figure 6 show the rate of Chl synthesis in the mutant peanut leaves which had been exposed to a 2-hr preillumination period followed by a 70-hr dark period before placing the leaves in continuous light. In contrast to the lag in Chl synthesis in mutant leaves not given any pretreatment (non-potentiated) before exposing the leaves to light, the potentiated leaves show only a slight or negligible lag period in Chl synthesis.

The results in Table I show the effect of varying the light or dark period on the degree of potentiation for Chl synthesis in the mutant leaves. Varying the preillumination period from 0 to 2 hr (with a 72-hr intervening dark period) has no effect on the amount of Chl synthesized in the following light period. Varying the dark period from 0 to 96 hr (with no preillumination period) leads to a linear increase in Chl synthesis when these leaves are exposed to light. To establish potentiation for Chl synthesis in the light, no preillumination period is required, but a 72 to 96 hr dark period is necessary. The amount of Chl, fructose-1,6-diP aldolase activity, and NADP-glyceraldehyde-3-P dehydrogenase in the mutant and wild type leaves are shown in Table I.

![Fig. 4. Light-dependent development of dark respiration in mutant and wild type peanut leaves.](image1)

![Fig. 5. Light-dependent development of leaf enlargement in mutant and wild type leaves.](image2)
aldehyde-3-P dehydrogenase activity developed in the mutant leaves in continuous illumination following a 96-hr dark period is shown in Figures 7, 8, and 9. In each instance, the 96-hr dark period eliminates the lag period in the light-dependent development of this pigment and chloroplast enzymes. In separate experiments, we have shown that the conditions for potentiation also abolish the 24-hr lag period in the light-dependent leaf enlargement in the mutant leaves.

The development of etioplasts in the nonpotentiated and potentiated mutant leaves is shown in Figures 10 and 11. The most pronounced difference in the nonpotentiated and potentiated mesophyll cells is the extensive development of the crystalline center in the plastids of the potentiated cells. There is the development of isolated lamellae in the chloroplasts of the nonpotentiated cells, but no development of a crystalline center. There does not seem to be any discernible difference in the mitochondria or other cytoplasmic inclusions in the nonpotentiated and potentiated mesophyll cells.

**DISCUSSION**

The mutation of a nuclear gene in peanut seedlings leads to a reduction in the light-induced development of chloroplast fine structure, soluble protein, ribulose-1,5-diP carboxylase, NADP-glyceraldehyde-3-P dehydrogenase, fructose-1,6-diP aldolase, glyceraldehyde-3-P kinase, phosphoenolpyruvate carboxylase, and malate dehydrogenase in the primary leaves during the lag phase of Chl synthesis (2). Clearly, the mutation of this gene has pleiotropic affects. The mutation induces a lag period in Chl synthesis in the light, possibly by nuclear gene regulation of cytoplasmic events during this period (2). A kinetic analysis of the development of chloroplast and non-chloroplast components in greening mutant and wild type leaves should give insight as to the nature of the mutation.

The light-induced developmental kinetics of fructose-1,6-diP aldolase and NADP-glyceraldehyde-3-P dehydrogenase activities is similar to the development of Chl in the mutant leaves. The development of all of these components in the light show a 72-hr lag period followed by a period of rapid synthesis. These results suggest that the mutation affects the

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**Table I. Effect of Varying the Preillumination Period and the Dark Period on Chl Synthesis in Mutant Leaves**

Leaves of 6-day-old dark-grown seedlings were exposed to varying lengths of preillumination followed by a 72-hr dark period prior to exposing the seedlings to continuous illumination. For the dark period experiment, leaves of 6-day-old dark-grown seedlings were given various lengths of an extended dark period prior to placing the plants in continuous light.

<table>
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<th>Preillumination Period</th>
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rate of chloroplast development and not just Chl synthesis. The development of enzymes of the reductive pentose phosphate cycle is different from the light-induced development of malate dehydrogenase and dark respiration in the mutant leaves. These latter components are reduced in the mutant leaves compared to the wild type leaves, but there is no lag period in their development. There is only a minimal development of the chloroplast during the lag phase; whereas there are high cytoplasmic and mitochondrial activities during this phase. Further, the mutation of the nuclear gene in peanuts, like the nuclear gene mutation in cotton (3, 4), affects only the rate of synthesis or timing, of most of these components. The mutation does not result in any deletion of chloroplast components which is characteristic of many nuclear mutants (5, 6, 10, 12). It has not been established how the nuclear gene mutation in these virecent plants affects the timing of these cytoplasmic and chloroplast components.
Fig. 11. Electron photomicrograph of a potentiated mutant peanut leaf. × 25,920.
Since it was known that preillumination followed by a dark period establishes chloroplast potentiation in higher plants (15, 16) and Euglena gracilis (9), it was established that similar conditions over-ride the mutationally induced lag period in chloroplast development in peanuts. In these mutant leaves, the conditions for establishing potentiation do not require a preillumination period with red light and as such does not involve phytochrome. Potentiation in peanuts does not require triggering of new synthesis; it requires only an extended dark period which must be equal to the lag period in chloroplast development in the light. The fact that the dark period equals the lag period probably means this is the amount of time necessary to synthesize components required for the rapid synthesis of the chloroplast in the light. These kinetic results together with the electron photomicrographs of nonpotentiated and potentiated cells establish that the nuclear gene mutation affects the early stages of plastid development and not the light-induced or light-dependent synthesis of chloroplast components.

Holowinsky et al. (7) and Smillie and Scott (14) have discussed chloroplast development in relation to cell differentiation in leaves. In an integrated developmental system, such as a leaf, light-dependent chloroplast development may not occur at a maximum rate until the mesophyll cells have reached a certain stage of development. An integrated timing system to control chloroplast development seems to be present in the peanut leaves. In the mutant leaves, the light-dependent development of chloroplasts exhibit a 72-hr lag period; whereas light-dependent leaf enlargement exhibits only a 24-hr lag period. However, chloroplast development is not initiated until the time leaf enlargement is complete. The mutation may result in a slowing of the synthesis of a component necessary for leaf development which ultimately delays chloroplast development in this coordinated system. The nuclear gene product controls the differentiation of leaf cells and plastids.

In agreement with Holowinsky and Schiff's (9) explanation of potentiation in Euglena gracilis, we conclude that the nuclear gene in peanut codes for the regulation of the synthesis of a component (nutrient, metabolite) in the cytoplasm in the dark (or during the lag period in the light in the absence of potentiation) which is required for the development of the mesophyll cell and the chloroplast. And, although the chloroplast is a semiautonomous organelle, nuclear gene control of chloroplast differentiation may not be independent of cellular growth.

**LITERATURE CITED**