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Relationship between Chloroplast Development and *ent*-Kaurene Biosynthesis in Peas[1]

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JOHN S. CHOINSKI, JR.[2] AND THOMAS C. MOORE
Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

Treatment of etiolated pea (*Pisum sativum* (L. cv. Alaska) seedlings with 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate (Amo-1618) prior to irradiation with white light inhibits photomorphogenesis and formation and stacking of thylakoid membranes in the chloroplasts, as well as (−)-kaur-16-ene (*ent*-kaurene)biosynthesis. Exogenous gibberellic acid also inhibits greening. A crudely determined action spectrum for the photoinduction of *ent*-kaurene biosynthesis shows two peaks, one in the blue region at 458 to 490 nanometers and another in the red region at 606 to 678 nanometers. The possible participation of phytochrome in the photoinduction of *ent*-kaurene biosynthesis is indicated by comparative effects of red, far red, and alternating red/far red irradiations on enhancement of enzyme activity. The activity of blue light as well as red shows a similarity of the photoinduction of *ent*-kaurene synthesis activity to the high irradiance responses, and indicates probable participation of a second photoreceptor. From these observations, it is concluded that photoinduction of *ent*-kaurene biosynthesis and chloroplast development in shoots are closely linked processes.

Photomorphogenesis of etiolated seedlings is characterized in part by a series of developmental changes involving etioplast membrane reorganization and Chl and protein synthesis (1). Concomitant with this process is the development of biosynthetic capacity for *ent*-kaurene[3] and presumably GAs. Intriguing connections between GAs and chloroplast development have been reported in recent years. Some evidence indicates that etioplasts and chloroplasts are compartments of GA biosynthesis during maturation (14, 24) and postgerminative growth (20). It has been shown in our laboratory that the biosynthesis of kaurene from MVA in cell-free extracts of pea is light activated (9). Evidence obtained by using a specific inhibitor of chloroplast protein synthesis indicates that one or more of these enzymes or their precursor proteins are synthesized in the cytoplasm (13).

Phytochrome involvement in GA biosynthesis and metabolism is indicated by reports of R/FR reversible efflux of GA-like substances from etioplasts (5, 10, 15, 27). Some phytochrome has been found to be specifically associated with chloroplast membranes (2, 10). There are reports also that R light induces GA biosynthesis in leaves (22), and conversion of GA_9 to other GAs (23). Abundant data also show substantial involvement of blue light, and presumably a second photoreceptor in chloroplast development (1, 26).

The purpose of this investigation was to attempt to determine whether chloroplast development and photoinduction of GA biosynthesis are closely dependent, or concurrent but independent, processes. The effect of light quality on the photoinduction of kaurene biosynthesis from MVA has been examined and compared with the known action of light on chloroplast development. An inhibitor of GA biosynthesis has been used to investigate the relationship between this hormone and Chl development and the photoconversion of etioplasts to chloroplasts during photomorphogenesis.

MATERIALS AND METHODS

Plant Material. Peas (*Pisum sativum* L. cv. Alaska) (W. Atlee Burpee Co., Riverside, Cal) were surface sterilized in 1.8% hypochlorite for 5–10 min, then presoaked for 4 h before being planted in moist Vermiculite. Plants were then maintained in darkness in growth chambers at 22 ± 1 C for 10 days. Shoot tips (all material above the fourth node, cotyledonary node counted as the first code) were excised after specific irradiations, and sorted in liquid N_2 until extraction and assay. All manipulations of material prior to irradiation were carried out under a green safelight (1 15-w white fluorescent lamp filtered through eight layers of amber and three layers of green cellophane, emitting total energy of less than 50 ergs cm^{-2} s^{-1}).

Preparation of Enzyme Extracts. Homogenization of shoot tip samples was as described previously (13). Briefly, frozen plant material was powdered in a mortar, and then further ground in PVP (0.5 g wet weight/g fresh weight tissue) and 100 mM K-phosphate (pH 7.1), 3 mM DTT, and 100 μM chloramphenicol (1.0 ml buffer solution/g fresh weight). Plant extracts were filtered through three layers of cheesecloth into cold centrifuge tubes and clarified twice: 10,800*g* for 10 min and 100,000*g* for 90 min. The 100,000*g* supernatant was used immediately as the enzyme source.

Assay for Kaurene Biosynthesis. The enzyme assay reaction mixture (13) contained, in a total volume of 1.0 ml: 0.7 ml enzyme extract, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 12 mM ATP, 75 mM K-phosphate (pH 7.1), and 30 μM [2-^{14}C]MVA (10.9 mCi/mmol). Samples were incubated for 2 h at 30 C. The reaction was stopped by adding 2.0 ml acetone containing 2.5 μg unlabeled kaurene. Each reaction mixture was then extracted twice in benzene-acetone (3:1). Pooled organic extracts were evaporated to dryness under N_2 and redissolved in 0.2 ml acetone. TLC and analysis of radioactivity were as described previously (7, 9, 20).

Photomorphogenesis and Growth Experiments. The effects of Amo-1618 and GA_3 on Chl development were tested as follows: under a green safelight, apical 6-cm segments of 10-day-old etio-

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[2] Present address: Department of Botany, University of Wyoming, Laramie, Wyoming 82071.

[3] Abbreviations: *ent*-kaurene: (−)-kaur-16-ene, referred to simply as kaurene; GA: gibberellin; MVA: mevalonic acid; R: red; FR: far red; Amo-1618: 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine-1-carboxylate; CCC: (2-chloroethyl)trimethylammonium chloride; Ancymidol: α-cyclopropyl-α-(*p*-methoxyphenyl)-5-pyrimidine methyl alcohol.

lated plants were excised and held upright with foil in 50-ml beakers each of which contained 8 ml of distilled H_2O, Amo-1618, GA_3, or Amo-1618 + GA_3 solutions (20–25 tips/beaker). The plants were then kept in the dark for another 30 min, and finally were transferred to growth chambers under continuous high intensity light (1.8×10^4 to 2.4×10^4 ergs cm^{-2} s^{-1}) for 16 h. The green portion of each tip was harvested, and then immediately assayed for total Chl content. In some cases, tips were frozen and stored in foil until assay. Total Chl was determined by boiling the shoot tips in 5 ml of 80% ethanol for 5 min and then reading the A of the extract at 652 nm in a Hitachi spectrophotometer. Specific activity was determined on the basis of A_{652} units per fresh weight of the original sample or total protein in replicate samples. Each data point was run in duplicate or triplicate, and confirmed in at least three separate experiments. Total protein was analyzed by the method of Lowry *et al.* (19) using 10% trichloroacetic acid precipitable material of shoot tip extracts. For this purpose, 20 shoot tips were homogenized in a ground-glass Potter-Elvehjem type tissue grinder with 1.0 ml of 50 mM K-phosphate (pH 7.1).

Experiments designed to test the growth inhibition of various inhibitors of GA biosynthesis were as follows: groups of 50 pea seeds were selected and presoaked in the dark for 4 h in 50 ml of distilled H_2O or appropriate concentrations of Amo-1618, CCC, or Ancymidol, all inhibitors of GA biosynthesis (6, 18). The seeds were then split into two groups and planted in moist Vermiculite. Light grown plants were maintained for 12 days in a greenhouse on a 16-h photoperiod (21 C day, 16 C night). Dark grown plants were grown, also for 12 days, as described under "Plant Material." Growth was scored by measuring epicotyl length starting in the cotyledonary node to the most newly formed internode (not including length of the uppermost expanded leaf).

Electron Microscopy. Segments of external leaves from shoot tips were fixed in 3% glutaraldehyde by the method of Frederick and Newcomb (12). Fixed samples were dehydrated in an acetone series, and imbedded in a mixture of low viscosity resins (25).

Irradiation Procedures. Long term irradiation experiments, plus procedures designed to test photoreversibility, were carried out with broad band R and FR light. The R and FR sources were 100-w incandescent bulbs filtered through sharp cut-off Corning glass filters (CS numbers 2-61 and 2-64). The filters exhibited 37% transmittance between 610 to 619 nm (R) and 648 to 725 nm (FR), and less than 0.5% at all wavelengths shorter than 590 nm and 620 nm, respectively. Additionally, the FR filter was covered with a sheet of blue cellophane. Both light sources had been previously tested and confirmed to give typical R/FR photoreversible responses using light-sensitive Grand Rapids lettuce seeds. The distances from the light sources to the surface of the peas were adjusted so that each source gave an equal irradiance of 3.0×10^5 ergs cm^{-2} s^{-1}. Light intensities were measured with a radiometer (Yellow Springs Instruments, Kettering model 65) with the probe placed at the level of the shoot tips.

Light for the action spectrum was obtained by the use of Kodak Wratten gelatin filters (125 × 125 mm) Nos. 50, 70, 72B, 73, 74, 75 (dominant transmitted wavelengths 458, 678, 606, 576, 538, 490, respectively). For all filters the maximum transmittance occurred in a 30-nm bandwidth or less. Any IR light transmitted was minimized with 1.5 cm of 1% $CuSO_4$. The light source was a 150-w floodlight (General Electric Co.). Light sequences were regulated with a mechanical interval cam timer (Meylan Stopwatch Corp., New York).

Reagents. [2-^{14}C]MVA-lactone in benzene was purchased from Amersham/Searle. The lactone was hydrolyzed overnight in excess NaOH equivalents. Amo-1618 was purchased from Enomoto and Company (Redwood City, Cal.). CCC was a gift from American Cyanamid Co. Ancymidol was generously supplied by R. C. Coolbaugh.

RESULTS

Effects of Inhibitors of GA Biosynthesis on Chl Synthesis and Growth. Amo-1618 and Ancymidol markedly inhibited stem elongation in both etiolated and light-grown seedlings, whereas CCC was only slightly inhibitory (Fig. 1). Amo-1618 at 1 mM inhibited epicotyl elongation over 70% in both etiolated and green seedlings. Thus, Amo-1618 was the inhibitor of choice for studies of photomorphogenesis or deetiolation. This growth retardant had a substantial inhibitory effect on total Chl synthesis, causing approximately 60% inhibition at concentrations of 0.6–1.0 mg/ml (Fig. 2). However, the level of total Chl was never reduced to zero at any Amo-1618 concentration and eventually the level became constant at 30–35% of the control value at concentrations of 1.0 and 2.0 mg/ml of growth retardent. At concentrations greater than 1.0 mg/ml, a large portion (up to 60%) of the stems collapsed during incubation. Samples that collapsed in this manner often developed Chl levels similar to H_2O controls. The specific effect of Amo-1618 on Chl development occurred early during the irradiation period. If Amo-1618 was applied 8 h or more after the onset of light, Chl levels in treated samples were similar to shoots irradiated in water (Table I).

Ultrastructural Features of Pea Leaves Treated with Amo-1618. Further evidence for a relationship between chloroplast development and GA biosynthesis was provided by ultrastructural examination of shoot tip samples which had been treated with Amo-1618 (Fig. 3). Typical etioplast prolamellar body structures observed in thin sections of nonirradiated samples (Fig. 3A) were few or absent in sections from untreated cells after 16 h in white light (Fig. 3B). Numerous starch containing chloroplasts exhibiting characteristic thylakoid membrane stacking (grana formation) were also commonly observed in these profiles. Samples treated with Amo-1618 (1.0 mg/ml) (Fig. 3C), in contrast, showed more frequent profiles of prolamellar bodies. Very few thylakoid membranes were observed showing any stacking conformations. In many Amo-1618-treated cells, the chloroplasts appeared to be entirely agranal.

Effect of GA_3 on Chl Synthesis. GA_3 was found to have similar

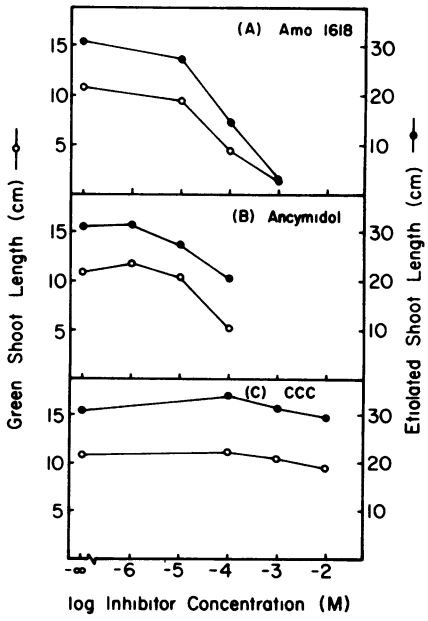

FIG. 1. Effects of three inhibitors of GA biosynthesis on stem elongation in etiolated and light-grown Alaska pea seedlings. Dry seeds were imbibed in the dark for 4 h in solutions of the inhibitors, and then grown in a dark growth chamber or greenhouse for 12 days. Each data point represents a mean of at least 15 determinations.

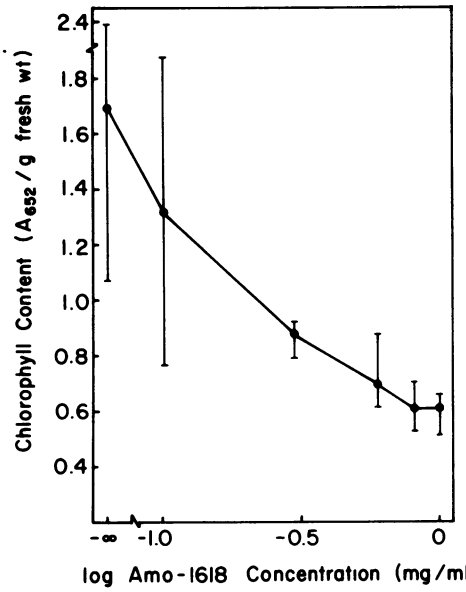

FIG. 2. Inhibition of greening of excised etiolated pea seedling shoots by Amo-1618. Chl was extracted in 80% ethanol from shoot tips after 16 h of illumination. Each point is the mean of at least three separate determinations.

Table I. *Effects of Amo-1618 (2.0 mg/ml) on Chl Formation during 16 h of Deetiolation of Excised Pea Shoots*

Each value is a mean of three separate determinations plus or minus the SE of the mean.

Time of Application of Amo-1618	Chl Content
h	*% of control*
0	38 ± 9
4	75 ± 1
8	91 ± 6
12	96 ± 8

inhibitory effects on Chl synthesis as did Amo-1618. Log linearity was observed with no additional inhibition at very high concentrations of GA_3 (Fig. 4). Samples treated with 0.1 mM GA_3 had only 63% of the total Chl (on a fresh weight basis) found in H_2O controls. This effect was not because of a mere dilution of Chl caused by GA-induced growth during the irradiation period. Fresh weight per tip did increase slightly as deetiolation proceeded in GA_3 (samples treated with 0.1 mM GA_3 weighed only 17% more than H_2O controls at 16 h), but not sufficiently to account for the observed Chl inhibition in treated shoots. Moreover, the amount of protein per unit fresh weight of tissue in samples treated with 0.1 mM GA_3 was only 13% less than in H_2O controls.

The effect of adding both Amo-1618 and GA_3 simultaneously to the uptake medium showed generally more inhibition than either treatment alone (Table II). The GA effect in this case may be explained by changes in fresh weight. No concentration of GA_3 was observed to overcome inhibition of Chl synthesis by Amo-1618.

Photoinduction of Kaurene Biosynthesis Activity by Continuous Broad Band Irradiation. Analysis of cell-free extracts prepared from shoot tips irradiated with equal total doses of broad band R, FR, and alternating R/FR light (4 min R followed by 8 min FR) showed a linear development of activity over a period of 16 h (Fig. 5). R-irradiated samples developed kaurene biosynthesizing activity to about twice the levels of the samples irradiated with FR light. It was also found that the effect of R light apparently could be partially reversed by FR while still maintaining the same total

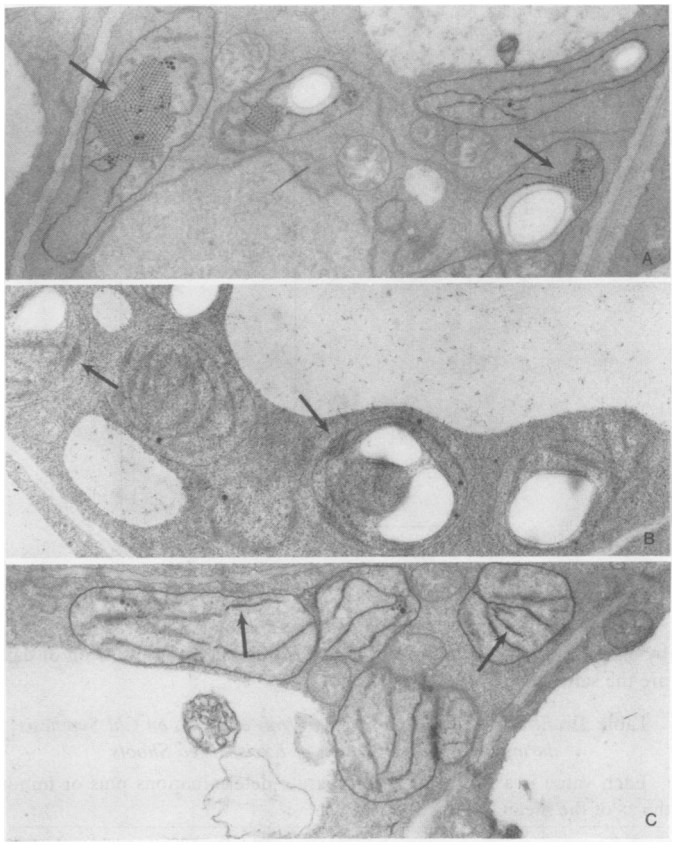

FIG. 3. Electron micrographs of leaf cells from excised 10-day-old pea shoot tips. A: etioplasts with characteristic crystalline prolamellar bodies (arrows) which are common throughout the cytoplasm of unirradiated tissue. B: chloroplasts from control tissue (16 h irradiation in H_2O) showing typical extensive granal development (arrows). C: chloroplast profiles from Amo-1618 (1.0 mg/ml) treated tissue after 16 h of illumination showing little or no grana formation. In some cases, two or three thylakoid membranes are appressed together (arrows).

incident dose (Fig. 5). That is, extracts from shoot tips irradiated with R light catalyzed substantially higher incorporation of label into kaurene than samples subjected to alternating R and FR light. This apparent partial photoreversal was observed throughout the irradiation period.

Action Spectrum for Photoinduction of Kaurene Biosynthesis. Kaurene biosynthesizing capacity of shoot tip samples was determined after 8 h irradiation using the monochromatic filters (Fig. 6). The irradiance was equalized for each filter to a level approximately 80% of that utilized in the broad band experiments. No filter transmitted light that developed greater activity in shoot tips than did samples irradiated with the broad band R source or the maximal recoverable activity previously noted in high intensity white light, 70 dpm/mg protein (9). Plants subjected to higher doses with the R and blue filters showed a linear increase in enzyme activity. From these data, we concluded that all doses were suboptimal for induction of kaurene biosynthesizing capacity. Two broad peaks were observed: in the blue region at 458–490 nm and another in the R region at 606 to 678 nm (Fig. 6). The efficiency of the most active filters was calculated to be 0.685 pmol kaurene biosynthesized/mg protein at 458 nm, and 1.03 pmol/mg protein at 678 nm.

DISCUSSION

Chloroplasts from tissues treated with Amo-1618 resemble organelles from mustard cotyledons treated with prolonged FR

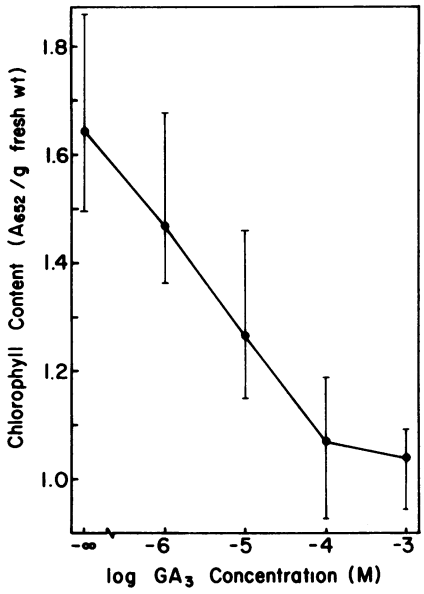

FIG. 4. Inhibition of greening of excised etiolated pea seedling shoots by exogenous GA_3. Conditions for Chl extraction and expression of data are the same as in Figure 2.

Table II. *Effects of Amo-1618 (0.3 mg/ml) and GA_3 on Chl Synthesis during 16 h of Deetiolation of Excised Pea Shoots*

Each value is a mean of three separate determinations plus or minus the SE of the mean

GA_3 Concentration	Chl Content	
	GA_3	GA_3 + Amo-1618
M	*% of water control*	
0	100	80 ± 6
10^{-6}	89 ± 11	54 ± 1
10^{-5}	77 ± 9	50 ± 1
10^{-4}	65 ± 7	54 ± 2
10^{-3}	63 ± 5	46 ± 2

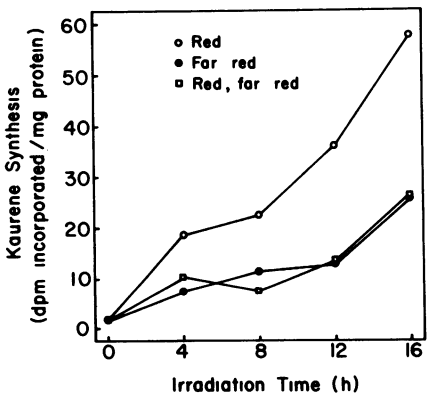

FIG. 5. Photoinduction of *ent*-kaurene biosynthesis activity by R, FR, and alternating R/FR light (4 min/8 min). Irradiance in all cases was 3.0 × 10^5 ergs cm^{-2} s^{-1}. Kaurene biosynthesizing activity was determined in 100,000*g* supernatants of tissue homogenates of shoot tips after specific irradiations. Each data point is the mean of two or three separate experiments.

irradiation (1) by having crystalline prolamellar bodies and underdeveloped grana. The low level of kaurene-synthesizing activity induced in FR light (Fig. 5), coupled with similar structural

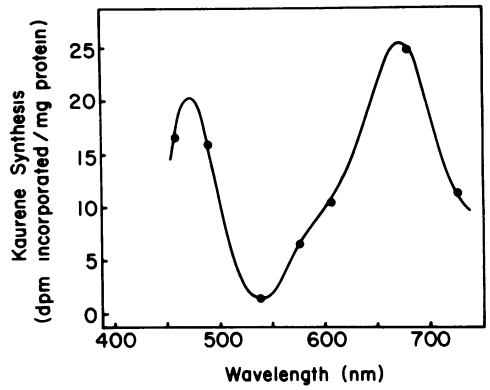

FIG. 6. Action spectrum for photoinduction of *ent*-kaurene biosynthesis. Total light doses were equal in all cases: irradiance at 2.4 × 10^5 ergs cm^{-2} s^{-1} for 8 h. Kaurene biosynthesizing activity was determined as per Figure 5. The data point at 720 nm was obtained using a Corning glass filter.

anomalies in Amo-1618 treated material, indicates an essential role of the GA-biosynthesizing system, or products thereof, in organizing chloroplast membrane structure. However, the experiments with Amo-1618 and exogenous GA otherwise yielded only equivocal results concerning that important point. Whereas Amo-1618 inhibited chloroplast development, GA_3 did not have a counteracting effect, as has been reported frequently for other Amo-1618 and GA effects (3, 18) and a previous study of greening in seedlings (21). In fact, exogenous GA_3 itself inhibited greening. Structural abnormalities of chloroplasts also have been observed in pea seedlings treated with a relatively very high concentration (10 mM) CCC (28), which like Amo-1618 inhibits GA biosynthesis. Yet neither growth retardant is a specific inhibitor of GA biosynthesis; both also inhibit sterol biosynthesis (8), for example. It cannot be said whether the effects of Amo-1618 observed in this study are attributable to inhibition of GA biosynthesis. Neither can the inhibitory effect of exogenous GA_3 on greening be explained. The suggestion that the GA effect might be due to end product inhibition of kaurene synthetase (11) seems unlikely from the results of preliminary experiments.

Both the broad band irradiation experiments and the action spectrum measurements indicate the involvement of phytochrome in the photoinduction of kaurene biosynthesizing capacity. However, because long-term irradiations (up to 8 h) are required to induce maximal activity, conventional methods of determining phytochrome involvement (particularly testing the criterion of photoreversibility) were not possible. Presumably, on the basis of the findings by Clarkson and Hillman (4), the amount of Pfr present during prolonged irradiation with FR light, and with alternating R and FR light, was lower than the amount of Pfr which was present in constant R light. Although direct correlations between Pfr level and physiological response to light have been noted only infrequently, Klein *et al.* (17) found that hypocotyl hook opening in *Phaseolus vulgaris* seedlings was correlated directly with the amount of Pfr in the tissue.

The uncertainty of R/FR photoreversibility and the action spectrum showing peaks of activity in both blue and R light, raise the possibility that induction of kaurene biosynthesizing capacity is a type of high irradiance reaction (HIR). However, the low induction capacity observed with FR light (Figs. 5 and 6) does not coincide with published spectra for some HIR; for example, lettuce hypocotyl lengthening showing a sharp action maximum at 720 nm (16). The substantial effect of blue light in this system (67% of the R) further suggests the involvement of a photoreceptor other than, or in addition to, phytochrome. Of the abundant evidence which has been published showing the photoinductive capability of blue light, many responses are associated specifically

with the chloroplasts (1, 26). Photosynthetic capacity, Chl concentration, grana formation, and etioplast fatty acid metabolism are all enhanced at greater levels or occur earlier in blue as compared with R irradiated material (26).

In summary, we conclude from all available evidence that chloroplast ontogenesis and development of maximum GA biosynthetic capacity in shoots are closely linked processes. Further elucidation of cause and effect relationships between the two processes is the objective of continuing investigations.

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