Correlations of Growth Rate and De-etiolation with Rate of Ent-Kaurene Biosynthesis in Pea (Pisum sativum L.)

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ABSTRACT

Biosynthesis of the gibberellin precursor ent-kaurene-4C from mevalonic acid-2-14C was assayed in cell-free extracts of shoot tips of etiolated and light-grown Alaska (normal) and Progress No. 9 (dwarf) peas (Pisum sativum L.). During ontogeny of light-grown Alaska peas, kaurene-synthesizing activity increased from an undetectable level in 3-day-old epicotyls to a maximum in shoot tips of 9-day-old plants and remained relatively constant thereafter until postanthesis. The capacity for kaurene synthesis in extracts from shoot tips of 10-day-old etiolated Alaska seedlings increased approximately exponentially during the first 12 hr of de-etiolation in continuous high intensity white light and remained relatively constant during the succeeding 24 hr of irradiation. Extracts from light-grown Alaska (normal) shoot tips possessed greater capacity for kaurene synthesis than did extracts from light-grown Progress No. 9 (dwarf) shoot tips. Extracts from shoot tips of either light-grown cultivar displayed greater kaurene-synthesizing capacity than was observed in extracts from their dark-grown counterparts. It is concluded that gibberellin biosynthesis in pea shoot tips is subject to partial regulation by factors controlling the rate of biosynthesis of kaurene.

Inherent differences or changes in rate of biosynthesis of gibberellins appear to be correlated with fundamental developmental phenomena in herbaceous annuals such as pea (Pisum sativum L.) as well as other species. However, assessments of apparent rates of GA biosynthesis generally have been restricted to extraction and diffusion methods or the use of exogenous GAs and synthetic inhibitors of GA biosynthesis (5, 6, 27).

The recent development of a cell-free system from pea shoot tips which incorporates mevalonic acid into ent-kaurene* (8) provides another experimental approach which should be a valuable addition to the techniques which have been used previously. Kaurene is a key intermediate in the GA biosynthetic pathway (19, 37), and it is plausible, from results with developing pea seeds (7), that the capacity of cell-free extracts from pea shoot tips to synthesize kaurene is indicative of the potential of shoot tips to synthesize GAs.

The purposes of the present investigations were to: (a) improve conditions for assaying kaurene synthesis in cell-free extracts of Alaska pea shoot tips so the system could be used for comparative studies; (b) determine whether the capacity of cell-free extracts of Alaska pea shoot tips to synthesize kaurene is correlated with the growth rate of shoots during the ontogeny of light-grown plants; and (c) compare dark-grown to light-grown plants and dwarf to tall (normal) plants with respect to the capability of cell-free extracts from their shoot tips to synthesize kaurene.

MATERIALS AND METHODS

Culture and Sampling of Plants. Peas (Pisum sativum L.) of the two cultivars Alaska (normal or tall) and Progress No. 9 (dwarf) from W. Atlee Burpee Company, Riverside, California, either were grown in a greenhouse, receiving a daily photoperiod of 16 hr at 20 to 23 C and an 8-hr dark period at 17 to 20 C, or were cultured in darkness as described later. The natural light intensity was supplemented with, and the natural photoperiod extended by, Gro-Lux fluorescent lamps; light intensity varied from 1200 to 1700 ft-c at the level of the plants. The vermiculite rooting medium was irrigated daily with water until the plants were 2 weeks old; thereafter irrigation was alternated with water or full-strength Hoagland's mineral nutrient solution.

Shoot tips of 14-day-old light-grown plants were used for most of the experiments directed toward optimizing conditions for the cell-free system. In studies to determine the relationship between kaurene-synthesizing activity and plant age, shoot tips of light-grown plants were harvested at 2- or 3-day intervals until the plants were 24 days old and once thereafter, over a period of several days, when the plants were 30 to 43 days old and had one or more fully open flowers.

The morphological unit called the "shoot tip" is, for plants older than 5 days, the portion of the shoot above, and including the uppermost node, and closed stipules of which were <1 cm in length. If the leaf at this node had elongated so that the leaflets were not in contact with the stipules, the leaf was removed. The entire epicotyl was harvested from 3-day-old plants, and the portion of the shoot above the first scale leaf was harvested from 5-day-old plants (Fig. 1).

All plants used in the de-etiolation studies were grown in growth chambers. Dark-grown plants received 16 hr at 22 ± 1 C and 8 hr at 17 ± 1 C daily. Plants used for determining the effect of greening on the kaurene-synthesizing capability of shoot tip extracts were grown in the dark for 10 days and then...
exposed to continuous high intensity light (1600-1900 ft·c; 3 × 10^-10 - 4.4 × 10^9 erg cm^-2 sec^-1) provided by 12 8-foot cool white fluorescent lamps and 12 60-w incandescent lamps at 22 ± 1°C. Samples of shoot tips were harvested immediately before and at intervals during the period of continuous illumination.

All etiolated shoot tip samples were harvested under a green safelight (General Electric 15-w green fluorescent lamp covered with eight layers of amber and three layers of green cellophane).

Preparation of Enzyme Mixtures and Reaction Conditions.

General procedures were as described previously (8). Excised shoot tips were frozen and stored in liquid nitrogen. While still frozen, shoot tips were ground with a chilled mortar and pestle to a fine powder. As the tissue thawed, insoluble polyvinylpyrrolidone (0.5 g wet PVPP per g fresh weight of tissue) and 0.1 M KH_2PO_4-KHPO_4 buffer (pH 7.1; 1.0 ml/g fresh weight of tissue) containing 2 mM dithiothreitol and 133 µM chloramphenicol were quickly added, and the mixture was homogenized immediately. The homogenate was filtered once through four layers of cheesecloth, and the filtrate was centrifuged at 10,000 g for 20 min. The 10,000 g supernatant was centrifuged at 78,000 g for 60 min, and the resulting supernatant was the enzyme source. Both centrifugations were done at 0 to 4°C.

Following optimization of the reaction conditions, reaction mixtures routinely contained 0.70 ml of enzyme extract (7-8 mg of protein), 75 mM KH_2PO_4-KHPO_4 (pH 7.1), 1 mM MnCl_2, 1 mM MgCl_2, 3 mM ATP, 1.5 mM dithiothreitol, 100 µM chloramphenicol, and 19.4 µM MVA-2,4,6-3H (12.9 mc/mmole) in a total volume of 1.0 ml. Reaction mixtures were incubated at 30°C for 2 hr in an aerobic atmosphere. Each reaction was stopped by the addition of 2 ml of acetone containing 2.5 µg of non-radioactive kaurene. Acetone containing kaurene was added to controls prior to incubation. Each reaction mixture was extracted once with 1 ml of benzene and twice with 2 ml of acetone-benzene (2:1). The organic extracts from each reaction mixture were combined and evaporated to dryness under vacuum; the residue was extracted twice with 0.25 ml of acetone which was transferred to a thin layer of Silica Gel G for chromatography. Authentic kaurene and squalene were cochromatographed with each sample extract. Thin layer chromatograms were developed in n-hexane. The kaurene and squalene regions of the chromatogram were located by staining with iodine vapors and were removed for liquid scintillation spectrometry in a solution of toluene containing 3 g of p-terphenyl and 30 mg of 1,4-bis-2-(5'-phenylazoxy)benzene (POPOP) per liter. The methods used to identify the products isolated in this manner as kaurene and squalene have been described in detail previously (8). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, model 2425.

Preparation and incubation of reaction systems using cell-free extracts from etiolated and greening tissue were done under the green safelight. The chlorophyll content of greening shoot tips was estimated by the procedure of Arnon (1).

Protein content of 10% trichloroacetic acid-insoluble material in the enzyme extract was determined by the method of Lowry et al. (23), using bovine serum albumin as a standard.

RESULTS

Partial Optimization of Assay Conditions. The preparation and assay conditions described in “Materials and Methods” gave optimal activity for kaurene synthesis. The following characteristics of the system are considered especially noteworthy: (a) reaction velocity was directly proportional to enzyme concentration up to approximately 8 mg of protein per 1.0 ml of reaction mixture, but reaction mixtures containing more than 10 mg of protein per 1.0 ml displayed submaximal activity; (b) the reaction velocity was constant through 120 min at 30°C; (c) the optimal pH was 7.1, and activity decreased abruptly at higher and lower pH values; (d) ATP at concentrations from 0.3 mM to 6.0 mM yielded maximal activity; and higher concentrations were inhibitory; (e) reaction velocity varied in predictable manner with substrate concentration, and 18 to 20 µM MVA was saturating; (f) Mn^2+ was a much better activator than Mg^2+ when either cation was used alone at concentrations below about 2.0 mM, but both cations together, each at 1.0 mM, yielded maximal activity; (g) omission of dithiothreitol resulted in 50% or greater reduction in activity; (h) kaurene synthesis was completely inhibited by 150 µM Amo-1618; and (i) contrary to the report of Coolbaugh et al. (8), activity in an aerobic atmosphere was equal to that in an anaerobic one. Optimal conditions for squalene synthesis were not determined.

Ontogenetic Variations in Capacity for Kaurene and Squalene Synthesis. Kaurene synthesis was not detectable in epicotyls of 3-day-old seedlings (Fig. 2). However, activity was detectable in extracts of plants older than 3 days and became maximal in shoot tips of 9-day-old plants. Thereafter kaurene-synthesizing activity remained relatively constant until the 24th day, during which interval the growth rate still was increasing. The same general trend can be seen when α-kaurene activity is expressed on a fresh weight basis. Mean dpm per gram fresh weight, based on three separate experiments, for ages of 3, 5, 7, 9, 12, 15, 18, 21, and 24 days were 0, 530, 470, 1010, 980, 970, 960, 850, and 1040, respectively. Three separate experiments using shoot tip preparations from postanthesis plants...
the shoot tips was used as an indication of the de-etiolation process.

The change in the morphological unit harvested during the 36-hr greening period is illustrated in Figure 3, and Figure 4 represents the results of a typical de-etiolation study. Four such experiments were performed and, although the absolute values for kaurene-synthesizing activity varied considerably among studies, the same general trend during the first 12 hr of illumination was observed in all the experiments. An approximately exponential increase in activity occurred between the 3rd and 12th hr of greening. Thereafter, activity was nearly constant for a succeeding 24 hr of illumination. Extracts of 12-hr illuminated shoot tips displayed activity comparable to that found in shoot tips of 10-day-old light-grown plants.

The data in Figure 4 and those from other identical studies suggested that light might initiate an endogenous rhythm in kaurene-synthesizing activity in shoot tips. To determine if such a circadian rhythm was present, plants were grown in the greenhouse receiving 16 hr of light and 8 hr of darkness per day. Beginning on the 10th day of growth, shoot tips were harvested at certain time intervals throughout the light and dark periods for 2 days. There was no indication of a circadian

### Table 1. Comparisons of Kaurene-synthesizing Capacities of Cell-free Extracts from Shoot Tips of Light- and Dark-grown Alaska and Progress No. 9 Peas

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme Source</th>
<th>Alaska, light-grown</th>
<th>Alaska, dark-grown</th>
<th>Progress No. 9, light-grown</th>
<th>Progress No. 9, dark-grown</th>
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<tr>
<td></td>
<td>dpm in kaurene/mg protein</td>
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<tr>
<td>1</td>
<td>59</td>
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<td>86</td>
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<td>3</td>
<td>88</td>
<td>22</td>
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<td>7</td>
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| | dpm in kaurene/g fresh wt |
|--------------------------|
| 1 | 760 | 530 |
| 2 | 1360 | 340 |
| 3 | 860 | 240 |
| 4 | 1240 | 1120 |
| 5 | 500 | 350 |

FIG. 3. Examples of morphological units from Alaska peas used for the determinations of variations in the capacity for kaurene synthesis in cell-free extracts with the period of de-etiolation. Sampling began with 10-day-old etiolated plants. Broken lines indicate the site of excision below the 4th node (node numbers begin with the cotyledonal node). All tissues above the 4th node were included in the samples. × 1.

FIG. 2. Ontogenetic changes in shoot height and in kaurene-synthesizing capacity of cell-free extracts from Alaska pea shoot tips. Reaction mixtures each contained 0.70 ml of enzyme extract (7–8 mg of protein), 75 mM KH2PO4-K2HPO4 (pH 7.1), 1 mM MnCl2, 1 mM MgCl2, 3 mM ATP, 1.5 mM dithiothreitol, 100 μM chloramphenicol, and 19.4 μM MVA-2-14C (12.9 mC/m mole) in a total volume of 1.0 ml. Vertical bars indicate the standard deviations from the means based on pooled replicates from three separate experiments and reflect the variation among results from the separate experiments. Shoot height was measured from the cotyledonary node to the highest visible node.

Experimental results (30–43 days old) yielded a mean of 105 ± 25 dpm incorporated into kaurene per mg of protein; the ± value is the standard deviation from the mean based on pooled replicates from all experiments. The relatively large standard deviation reflects the variation among separate experiments, rather than variation among replicates in single experiments, which was minimal.

The incorporation of MVA-2-14C into squalene by the cell-free preparations displayed a pattern similar to that observed for kaurene during ontogeny. Maximal capacity for squalene synthesis appeared in preparations from shoot tips of 12-day-old plants and remained relatively constant thereafter. In general, the incorporation of MVA-2-14C into squalene was 10 to 20 times greater than its incorporation into kaurene. In the more active preparations, approximately 0.1% of the MVA-2-14C was incorporated into kaurene and 1 to 2% was incorporated into squalene. The comparisons are of limited value, however, since the assay conditions were optimal for kaurene and not for squalene synthesis. No exogenous NADPH was added, for example.

Increase in Kaurene-synthesizing Capacity of Shoot Tips during De-etiolation. It was found repeatedly that the capacity of cell-free extracts from shoot tips of 10- to 14-day-old, light-grown Alaska peas to synthesize kaurene was approximately 5 times greater than that of etiolated plants of the same age (Table I). Studies then were conducted to determine the rate of increase in kaurene-synthesizing capacity of shoot tips as 10-day-old etiolated plants were exposed to continuous high intensity illumination. The increase in chlorophyll content of...
rhythm in the capacity for kaurene synthesis in cell-free extracts from these shoot tip samples.

A major question concerning the increase in kaurene-synthesizing capacity of shoot tips during their exposure to light was whether it required continuous illumination of the shoot tips during the period in which it occurred, or whether it could be induced by a briefer illumination period and occur subsequently in darkness. To answer this, studies were conducted in which 10-day-old, dark-grown plants were exposed to 1, 6, or 12 hr (or more) of light (the illumination periods began simultaneously for all groups). At the end of the longest illumination period, shoot tips were harvested from each group, including a dark-grown control group. Cell-free extracts were prepared under a green safelight and assayed for kaurene-synthesizing activity. The results of two studies indicated that continuous illumination for at least 12 hr is required to obtain a maximal rate of kaurene synthesis in growing shoot tips.

**Comparison of Tall and Dwarf Cultivars.** Cell-free extracts from shoot tips of light-grown, 10- to 14-day-old Alaska peas consistently displayed a greater capacity for synthesis of kaurene than extracts from the dwarf cultivar Progress No. 9 grown under the same conditions (Table 1). Activity from Alaska shoot tips was 1.4 to 5 times greater than that from Progress No. 9 shoot tips, when expressed on a protein basis. Shoot tip extracts from light-grown plants of either cultivar, Alaska or Progress No. 9, possessed greater capacity for kaurene synthesis than that observed in extracts from etiolated counterparts. In a single experiment no difference was observed in the kaurene-synthesizing capacity of extracts from the shoot tips of etiolated Alaska and Progress No. 9 seedlings (Table 1). It should be noted that Alaska and Progress No. 9 are early-flowering cultivars and that analogous experiments with late-flowering cultivars yielded inconclusive results.

**DISCUSSION**

Shoot tips were selected for these investigations on the basis of the evidence reported by Lockhart (21) for peas and Jones and Phillips (13) for sunflower that the shoot tip is the most active locus of GA biosynthesis in vegetative herbaceous shoots. The results reveal a direct correlation between the apparent rate of kaurene synthesis in pea shoot tips and the apparent rate of GA production, as assessed by indirect procedures in previous investigations (10, 24). Furthermore, the results for pea shoot tips are analogous to those for developing pea seeds, in which a large increase in capacity for kaurene production appears to precede slightly the period of maximal GA biosynthesis (7). It is not known at this time precisely to what extent these results obtained with cell-free enzyme extracts reflect actual rates of kaurene biosynthesis in vivo. Acknowledging this important limitation, the results presented here do nevertheless appear to be consistent with considerable data obtained by very different methods in other investigations.

The results of our investigations support the findings of previous physiological studies on the changing sensitivity of light-grown Alaska peas to growth-saturating levels of exogenous GA and AMO-1618 during ontogeny (10, 24). Three-day-old plants were most sensitive to exogenous GA (determined by growth enhancement compared to controls), and this sensitivity declined during ontogeny, with 9-day-old plants exhibiting practically no sensitivity to exogenous GA. It was proposed that endogenous GA increases to a growth-saturating level in shoots during the first 2 weeks of ontogeny, remains at that level throughout the period of maximal growth rate, and then declines during the period of decreasing growth rate and senescence (24). The sensitivity of Alaska peas (measured by a growth response) to applied GA or AMO-1618, an inhibitor of GA (19, 37) and sterol (9) biosynthesis, varied inversely with growth rate. That is, AMO-1618 produced its greatest growth-inhibiting effect during the first 2 weeks of ontogeny of the plants (10), corresponding to the period when kaurene-synthesizing capacity is developing to a maximum in shoot tips (Fig. 2). Barendse et al. (2) concluded, on the basis of similar investigations with AMO-1618, that the growth of pea seedlings does not depend on de novo GA biosynthesis during the first 2 to 3 days of germination. Our results, showing no detectable kaurene-synthesizing activity in 3-day-old pea epicotyls (Fig. 2), support this conclusion.

It appears that stem growth of light-grown Alaska peas during early ontogeny is regulated, to a considerable extent, by the rate of GA biosynthesis, which in turn may be controlled by the rate of kaurene formation in the shoot tip. Although a decline in growth rate occurs immediately after anthesis in Alaska peas (10), our results indicate no decline in the capacity to synthesize kaurene by extracts from shoot tips obtained from plants shortly after anthesis. However, shoot tips from postanthesis plants contain growing reproductive tissues the activity of which conceivably could compensate for the decline in activity which might occur in the vegetative tissues. Senescing shoot tips were not examined for their kaurene-synthesizing activity.

There are numerous reports of light-induced alterations in GA metabolism. In the case of etiolated wheat leaf sections, an increase in GA-like substances occurred shortly after a 5-min red light treatment which appeared to be the result of a release of GA from bound forms. GA biosynthesis, as well as RNA and protein synthesis, appeared to be implicated in an observed increase in GA-like substances which occurred subsequent to a 30-min red light treatment (4, 22). A transient increase in GA-like substances in etiolated barley leaf sections occurred within 15 min after a 30-min exposure to red light and also appeared to require GA biosynthesis (31) and RNA and protein synthesis (30). Additionally, Reid et al. (32) have reported that homogenates of etiolated barley leaves, when exposed to brief periods (20 min or less) of red irradiation, displayed an immediate increase in the levels of GA-like substances as compared to dark controls, and that the red light-induced increase in GA-like activity could be explained partially by the greater conversion of H-GA, into other GA-like substances with high biological activity.
None of the aforementioned investigations, in which quite rapid, light-induced increases in extractable GA-like substances were observed, seems directly comparable to our studies, in which a significant increase in the rate of kaurene synthesis (and potentially GA biosynthesis) by extracts from previously etiolated shoot tips required several hours of continuous illumination of the plants. However, there are previous investigations reported with which our observations agree quite closely. Köhler (16, 17) reported that exposure to red light (either continuous or intermittent [2.5 min/hr]) resulted in an increase in extractable GA from shoots of a normal (tall) pea cultivar, and that the light-induced increase in GA was a phytochrome-mediated process. Inhibition of the light-induced increase in GA by chlorocholine chloride (CCC) suggested the involvement of GA biosynthesis, and the increase in GA appeared about 1 day after light exposure. Ogawa (26) showed that several hours of irradiation of etiolated *Pharbitis nil* seedlings with white, red, or far red light resulted in an increase in extractable GA-like substances, and that the increase in GA-like substances was strongly correlated with the opening of the hypocotyl hook and expansion of cotyledons. On the basis of these several reports it seems certain that light affects diverse reactions involved in GA metabolism.

The results of our studies indicate that maximal kaurene-synthesizing activity occurs in etiolated shoot tips about 12 hr after the beginning of the illumination period and that light is required throughout this 12-hr period. Although the increases in the capacity for kaurene synthesis and chloroplast development occur concurrently in the etiolated shoot tips (Fig. 4), at present we have no evidence to suggest that the two processes are directly related. Chloramphenicol, reportedly an effective inhibitor of plastidic, but not cytoplasmic, protein synthesis (28, 33, also see 20), inhibited the light-induced increase in extractable GA in etiolated barley leaf sections, which led Reid and Clements (30) to speculate that some process involved in GA production might occur in the chloroplasts. Moreover, Stoddart has found GA-like activity associated with the chloroplast fraction of leaf homogenates of *Brassica oleracea* var. *apechala*, and that chloroplast preparations could incorporate ent-kaurenoic acid into GA (34, 35). The synthesis of kaurene from mevalonate does not require particulate enzymes (19, 37); however, the procedure utilized in our investigations does not preclude contamination of the enzyme extract with enzymes or other substances of chloroplast origin.

The role of *de novo* GA biosynthesis in stem elongation of dark-grown plants has been questioned (15, 27), and Paleg (27) proposed that growth in such plants may depend primarily upon the utilization of GA reserves in the seed or seedling. We have found relatively low, but quite detectable, kaurene-synthesizing activity in extracts from shoot tips of 10-day-old dark-grown plants, which shows that the potential for *de novo* GA synthesis is present. The fact that AMO-1618 inhibits elongation of etiolated pea stems (14) is evidence for the dependence of stem growth on *de novo* GA synthesis in dark-grown peas.

Dwarfism in varieties and mutants of several species has been correlated with a lower content of extractable GA compared to that found in normal (tall) varieties (11, 25, 29, 36). However, in the case of peas, the situation is complex. Kende and Lang (14) found no difference in the extractable GA content in light-grown and dark-grown dwarf peas. Furthermore, Jones and Lang (12) detected no significant difference between tall and dwarf peas or between light-grown and dark-grown peas with respect to their extractable or diffusible GA contents. These authors have described dwarfism in peas in terms of a light-induced difference between tall and dwarf cultivars in their sensitivity to a certain endogenous GA. However, an explanation for dwarfism in peas is complicated by reports of Köhler, who found that light-grown tall peas contained much more of a certain extractable GA fraction than did light-grown dwarfs, and that the extractable GA content of light-grown tall or dwarf peas was considerably greater than that of the tall cultivar grown in the dark (15, 16, 18). Our results are more in accord with those of Köhler, in that we have found that cell-free extracts from light-grown shoot tips of either the tall or the dwarf cultivar exhibited greater kaurene-synthesizing activity than was found in such extracts from etiolated shoot tips. Furthermore, cell-free extracts from shoot tips of light-grown Alaska peas were found repeatedly to possess greater kaurene-synthesizing activity (1.4–5 times) than that found in extracts from light-grown Progress No. 9 shoot tips (Table I). Our results also agree closely in certain important respects with those of Barendse and Lang (3), who determined the amounts of bound and free GAs in extracts from dwarf and tall strains of *Pharbitis nil* grown in darkness or in light during the first 18 days following germination. The dwarf strain was found to contain less GA than the tall strain, and dark-grown plants of both strains contained less GA than light-grown ones, although in this case the differences in GA content were reportedly attributable mainly to differences in levels of bound GA.

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**Literature Cited**