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CONVERSION OF GERANYLGERANYL PYROPHOSPHATE TO *ENT*-KAURENE IN ENZYME EXTRACTS OF SONICATED CHLOROPLASTS*

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Key Word Index—*Pisum sativum*; Leguminosae; *ent*-kaurene; gibberellin biosynthesis; chloroplast; geranylgeranyl pyrophosphate; farnesyl phosphate.

Abstract—Farnesyl pyrophosphate-[^{14}C] and geranylgeranyl pyrophosphate-[^{14}C] were biosynthesized from mevalonic acid-[2-^{14}C] by cell-free enzyme extracts of pea (*Pisum sativum*) cotyledons containing $MgCl_2$, $MnCl_2$, ATP and AMO-1618. Maximum yields of farnesyl pyrophosphate were obtained after 30 min incubation while geranylgeranyl pyrophosphate was the primary product after 180 min. Biosynthesized geranylgeranyl pyrophosphate-[^{14}C] served as an efficient substrate for *ent*-kaurene biosynthesis in reaction mixtures containing cotyledon enzymes when AMO-1618 was omitted. Enzyme extracts from green pea shoot tips and chloroplasts also converted geranylgeranyl pyrophosphate to *ent*-kaurene in very low yields. *Ent*-kaurene production from mevalonic acid-[2-^{14}C] in extracts of pea shoot tips was also enhanced by addition of chloroplast enzymes. This evidence indicates that kaurene synthetase is present in pea chloroplasts and adds to the possibility that some gibberellin biosynthesis may be compartmentalized in those organelles.

INTRODUCTION

Evidence has accumulated over the past 7 yr that some GA‡ biosynthesis may be compartmentalized in chloroplasts. Stoddart [1] reported that approximately 16% of the GA-like activity in leaves of *Brassica oleracea* and *Hordeum vulgare* was associated with the chloroplast fraction and subsequently showed [2] that chloroplast preparations of *B. oleracea* converted *ent*-kaurenoic acid to a GA-like substance. More recently Murphy and Briggs [3] showed that *ent*-kaurenol and *ent*-kaurenoic acid were converted to *ent*-kaurenal and *ent*-hydroxykaurenoic acid, respectively, in preparations of barley leaf chloroplasts. Reid *et al.* [4] speculated that some process involved in GA biosynthesis might occur in chloroplasts on the basis of their observation that chloramphenicol, an inhibitor of plastidic but not cytoplasmic protein synthesis, inhibited a light-induced increase in extractable GA in etiolated barley leaf sections. Cooke and Saunders [5] have demonstrated a phytochrome-dependent increase in extractable GA-like activity in plastid preparations from etiolated wheat leaves.

In a previous paper [6] we reported that cell-free extracts from shoot tips of light-grown Alaska pea (*Pisum sativum*) seedlings had a 5-fold greater capacity for synthesizing *ent*-kaurene from MVA than extracts from shoot tips of etiolated seedlings of the same age. Upon continuous irradiation of 10-day-old etiolated seedlings with high-intensity white light, activity increased approximately exponentially between the 3rd and 12th hr, attaining a level equal to that of light-grown plants of the same age, and remained nearly constant during the succeeding 24 hr of development. Although the increase in capacity for *ent*-kaurene synthesis and chloroplast development occurred concurrently, there was no evidence that the two processes are related.

In previous studies [6–8] it was assumed that the synthesis of *ent*-kaurene from MVA does not require particulate enzymes. However, we noted [6] that the procedures we utilized to prepare enzyme extracts from pea shoot tips did not preclude contamination with enzymes or other substances of chloroplast origin. While this paper was in preparation, Simcox *et al.* [9] reported on the conversion of copalyl pyrophosphate to *ent*-kaurene in extracts of proplastids from etiolated pea shoot tips, developing castor bean endosperm and wild cucumber endosperm. Only the wild cucumber endosperm extracts were capable of converting GGPP to *ent*-kaurene [9].

This communication reports evidence that kaurene synthetase occurs in pea chloroplasts. During these investigations we have also established: (a) a convenient method for the biosynthesis of substrate quantities of FPP-[^{14}C] and GGPP-[^{14}C]; (b) the conversion of GGPP to *ent*-kaurene in extracts of pea cotyledons; and (c) the possible presence of prenyl transferase in pea chloroplasts.

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‡ Abbreviations: AMO-1618, 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate; DTT, dithiothreitol; FP, farnesyl phosphate; FPP, farnesyl pyrophosphate; GA, gibberellin; GGP, geranylgeranyl phosphate; GGPP, geranylgeranyl pyrophosphate; MVA, mevalonic acid; S_{100}, 100000 *g* supernatant.

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RESULTS

Biosynthesis of FPP-[^{14}C] and GGPP-[^{14}C]. Cell-free enzymes from developing pea cotyledons were incubated with MVA-[2-^{14}C], $MgCl_2$, $MnCl_2$, ATP, AMO-1618 and phosphate buffer as described in the Experimental. The products of these reactions were extracted with collidine and purified by ion-exchange chromatography on Sephadex A 25 columns as described by Oster and West [10]. The results of one such experiment in which doubly labeled FPP and GGPP were formed from MVA-[2-^{14}C] and ATP-[γ-^{32}P] are illustrated in Fig. 1. These results were typical of all experiments, although most were done with MVA as the only labelled substrate.

The products in fractions 17–19 and 23–25 were identified as farnesyl phosphate (FP) and FPP by hydrolysis with alkaline phosphatase to yield farnesol and by TLC. The two peaks of radioactive substances representing fractions 28–29 and 31–34 both behaved chromatographically like geranylgeraniol after alkaline phosphatase treatment. Approximately 95% of the radioactivity migrated to the same position as authentic geranylgeraniol in solvent systems I, II and III. Both fractions also yielded two radioactive products on chromatograms developed in solvent system II after acid hydrolysis. One corresponded to the position of authentic geranylgeraniol which was co-chromatographed with it and the other corresponded to the published R_f value (0.14) of geranyllinalool [10]. The identification of GGPP was confirmed directly on thin-layer and paper chromatograms with authentic GGPP. The former peak (fractions 28–29) is considered to represent mostly geranylgeranyl phosphate (GGP) on the basis of its chromatographic behavior and the lower ^{32}P:^{14}C ratio in this product. The ratios of ^{32}P:^{14}C in FPP, GGP, and GGPP were 0.052, 0.037 and 0.054, respectively. The GGP fractions are thought to contain a small amount of GGPP since this material was converted to kaurene. However, the conversion was much less with this substrate (6.8%) than with the GGPP fractions (19.3%). No squalene was produced from either substrate.

A series of experiments was conducted to determine the optimum time of incubation for the production of FPP and GGPP. The results of these experiments (Fig. 2) indicate that FPP accumulates quickly in these reaction mixtures and then disappears as GGPP increases. After 30 min incubation 31% of the radioactivity was associated with FPP, while approximately 43% of the radioactivity which was extracted with collidine (76% of the radioactivity in phosphorylated products) was associated with GGPP after 180 min. This corresponds to 4.5–7.2 nmol of GGPP-[^{14}C] in two experiments. The comparative yields of these two products are relative, since Oster and West [10] have shown that they are not extracted with equal efficiency. It is noteworthy that the yields of FPP and GGPP are significantly reduced if the enzyme extracts are prepared by centrifuging at 100000 *g* for less than 2 hr. Under these conditions FP and GGP accumulate at the expense of the pyrophosphates due to particulate phosphatase.

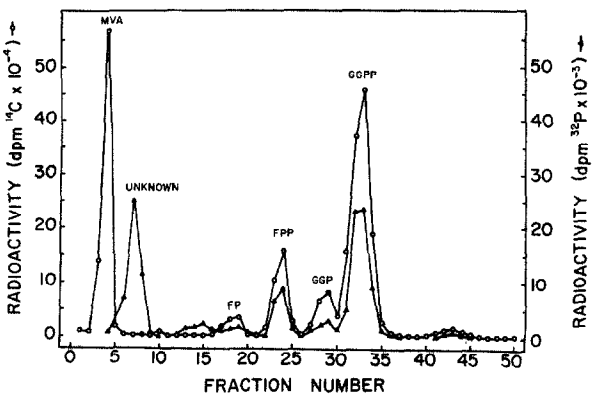

Fig. 1. Ion-exchange elution profile of doubly-labeled products biosynthesized from MVA-[2-^{14}C] and ATP-[γ-^{32}P]. Products were formed in cell-free enzyme extracts (S_{100}) of immature pea cotyledons and extracted with collidine; 10-ml fractions were collected from the ion-exchange column.

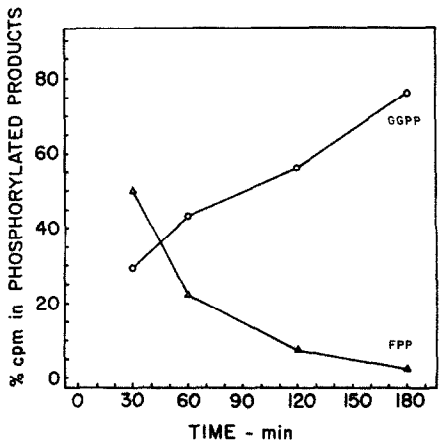

Fig. 2. Percentages of total radioactivity recovered in geranylgeranyl pyrophosphate and farnesyl pyrophosphate vs reaction time. Products were biosynthesized from MVA-[2-^{14}C] in cell-free enzyme extracts of immature pea cotyledons, extracted with collidine, and isolated by ion-exchange chromatography.

Biosynthesis of ent-*kaurene.* Several experiments were conducted to determine the incorporation of MVA-[2-^{14}C] into *ent*-kaurene in extracts of chloroplasts from pea shoot tips. The results of six such experiments showed an average of 6 dpm above background which migrated with authentic kaurene on TLC developed in solvent I. Extracts from whole shoot tips under the same conditions routinely incorporate between 500 and 2000 dpm.

The chloroplasts were prepared by a modification of the method of Walker [11]. After washing the chloroplasts with buffer, half of the resuspended chloroplasts were sonicated and the other half held in ice. At this stage electron micrographs of chloroplast preparations (Fig. 3) indicated the purity and integrity of non-sonicated chloroplasts and the nearly complete breakage of chloroplast membranes by sonication. Both preparations were then centrifuged 60–90 min at 100000 *g* yielding the chloroplast enzymes (chloroplast S_{100}).

Initial evidence for kaurene synthetase in chloroplasts came from two types of experiments which involved the conversion of endogenously formed terpenyl pyrophosphates to *ent*-kaurene. One such method utilized active shoot tip enzymes preparations (capable of producing terpenyl pyrophosphates and *ent*-kaurene) which were spiked with chloroplast enzymes in the absence of

Fig. 3. Electron micrographs of chloroplast pellets (A) before (×4300) and (B) after (×13800) resuspension and sonication. Samples of chloroplast pellets were embedded in 1% agar, then fixed in 1% *para*-formaldehyde–3% glutaraldehyde which was prepared in 0.2 M cacodylate buffer (pH 7.2) for 4 hr at 4°. Dehydrated samples were embedded in Epon and sectioned at 500–600 Å.

AMO-1618. The data from four representative experiments are presented in Table 1. These results revealed that a heat-labile substance from sonicated chloroplasts enhanced the quantities of *ent*-kaurene which normally accumulated in active shoot tip preparations alone.

Three of the experiments included reaction mixtures containing double doses of shoot tip enzymes to be sure the single dose was not saturating in enzyme concentration. Non-sonicated chloroplast extracts were ineffective in causing this stimulation. The apparent partial stimulation by boiled chloroplast extracts is considered to represent enzyme stabilization by the protein present. The same degree of stimulation was also observed when bovine serum albumin or PVP-40 was added to reactions containing shoot tip enzymes and could be eliminated by centrifuging the protein out before addition of boiled chloroplast S_{100}. It was also determined that the measured pH of all reaction mixtures was the same; thus added buffer or added chloroplast enzymes did not alter the pH of the reactions.

In these experiments it was not possible to determine whether the increase in *ent*-kaurene accumulation in reactions containing chloroplast enzymes was due to additional kaurene synthetase from this source or simply due to increased terpenyl pyrosphosphates. Thus, the experimental design was modified slightly; the shoot tip enzymes were inactivated by heat after 1 hr incubation and prior to the addition of chloroplast enzymes which were subsequently incubated for a further 1 hr. At the end of 1 hr 2.84 pmol of *ent*-kaurene were formed, while 3.19 pmol had accumulated after addition of chloroplast enzymes. This represents an increase of 12% *ent*-kaurene. This experiment was similar to one reported by Anderson and Porter [12] in which pig liver enzymes were used to generate terpenyl pyrophosphates which were converted to phytoene by enzymes from higher plant plastids. Phytoene is also thought to be a product of the present system [7].

Conversion of geranylgeranyl pyrophosphate to kaurene. Since the experiments described above provide only indirect evidence for the presence of kaurene synthetase in pea chloroplasts, a more direct procedure was employed. Purified GGPP-[^{14}C], which was biosynthesized in extracts of developing pea cotyledons, was used as a substrate for reaction with enzymes from pea cotyledons, shoot tips and chloroplasts. The results of one of three such experiments are presented in Table 2. Although the yields of *ent*-kaurene were low, the results were reproducible and of very similar magnitude. The *ent*-kaurene activity in these preparations was determined by scraping the gel corresponding to the region of the plates where authentic *ent*-kaurene, which was co-chromatographed with the extracts, appeared after visualization with iodine vapours and counting by liquid scintillation in pre-counted vials containing toluene cocktail. In each experiment several plates were scraped over their entire length in 0.5 or 1 cm bands to confirm the presence of radioactivity in the region of *ent*-kaurene. A product other than kaurene was consistently observed at approximately R_f 0.2 on chromatograms and it was presumed to be phytoene. The radioactivity remaining at the origin was shown to be geranylgeraniol upon re-chromatography in solvent systems II and III. The activity of these preparations was tested over the range of pH values from 6.4 to 7.2 and the pH used in the described experiments (pH 7.1) gave the highest activity.

The activities observed in all preparations are heat labile and sensitive to AMO-1618. More than 95% of the activity in all reactions was eliminated by boiling the enzymes prior to the addition of substrate; and 85 and 95% of the activity was inhibited by 0.14 and 1.0 mM AMO-1618, respectively. The chloroplast extracts were at least as active in catalyzing the conversion of GGPP to kaurene as the shoot tip enzymes. But both of these systems were only about 3% as active as preparations from cotyledons.

As noted above, virtually all of the radioactivity recovered from these reaction mixtures was in the form of geranylgeraniol with substantial quantities of presumptive phytoene and very small amounts of *ent*-kaurene. After the original acetone/benzene extraction treatment of the reaction mixtures with 0.1 N HCl at 100° for 30 min did not release any significant radioactivity (geranyllinalool and geranylgeraniol) for subsequent C_6H_6/Me_2CO extraction. Using the cotyledon enzyme system as a model, preliminary attempts to separate phosphatase and kaurene synthetase on carboxymethyl cellulose and DEAE cellulose columns have been unsuccessful.

Table 1. Enhancement of *ent*-kaurene biosynthesis from MVA-[2-^{14}C] in chloroplast extracts prepared from pea shoot tips

Enzyme source	*ent*-Kaurene (pmol/reaction)			
	Exp. I	Exp. II	Exp. III	Exp. IV
Shoot tip S_{100} + buffer	2.04	5.54	4.84	5.51
Shoot tip S_{100} (× 2)	3.87	—	8.03	8.80
Shoot tip S_{100} + chloroplast S_{100}	5.51	7.23	6.15	7.62
Shoot tip S_{100} + boiled chloroplast S_{100}	4.32	5.70	4.11*	4.10*
Shoot tip S_{100} + Unsonicated chloroplast S_{100}	—	—	—	5.03

Reaction mixtures contained 1 mM $MnCl_2$, 1 mM $MgCl_2$, 3 mM ATP, 70 mM potassium PO_4 (pH 7.1), 0.03 mM MVA-[2-^{14}C] (sp. act. 18 mCi/mmol), 0.7 ml shoot tip S_{105} and 0.7 ml additional enzyme source or buffer. Reactions were incubated 60 min at 30°.

* Boiled chloroplast S_{100} centrifuged 5 min at 1000 *g* and the supernatant added to the reaction mixture.

Table 2. Biosynthesis of *ent*-kaurene from MVA-[2-^{14}C] and GGPP-[^{14}C] in cell-free extracts of pea tissues

Enzyme source and conditions	*ent*-kaurene yield* (pmol/reaction mixture)			
	GGPP as substrate		MVA as substrate	
	Mean†	(SE)	Mean	(SE)
Shoot tip S_{100}‡	0.273	(0.039)	5.554	(0.045)
Shoot tip S_{100} (×2)	0.273	(0.039)		
Shoot tip S_{100} (boiled)	0.025	(0)	0.114	(0.045)
Shoot tip S_{100} + AMO-1618	0.035	(0.019)		
Shoot tip S_{100} + Chloroplast S_{100}	0.266	(0.019)	7.228	(1.010)
Shoot tip S_{100} + Chloroplast S_{100} (boiled)	0.260	(0.052)	5.703	(0.500)
Chloroplast S_{100}‡	0.287	(0.020)		
Chloroplast S_{100} (×2)	0.246	(0.007)		
Chloroplast S_{100} (boiled)	0.017	(0.015)		
Chloroplast S_{100} + AMO-1618	0.044	(0.041)		
Cotyledon S_{100}‡	84.183	(10.750)	223.532	(14.500)

* *Ent*-kaurene was determined by scraping the gel corresponding to the region of the TLC where added authentic *ent*-kaurene co-chromatographed. † Mean values of two reaction mixtures. The data are from one of three experiments. ‡ Protein concentrations were: shoot tip S_{100} 19.0 mg/ml; chloroplast S_{100} 4.4 mg/ml; cotyledon S_{100} 17.5 mg/ml.

DISCUSSION

Recently several investigators have presented evidence that some GA biosynthesis may occur within chloroplasts of higher plants. All of these reports deal with reactions which occur in the later stages of this biosynthetic pathway: either the conversion of already oxidized intermediates to GA-like materials or to further oxidized intermediates.

That the enzymes catalyzing the biosynthesis of GGPP and phytoene are present in chloroplasts is well known. For example, Charlton *et al.* [13] and Buggy [14] have demonstrated that phytoene formation from MVA occurs in sonicated preparations of bean chloroplasts isolated by non-aqueous techniques. However, they also noted the failure of MVA and acetate to be converted to carotenoids by chloroplasts which were isolated in aqueous media. Stocking [15] has shown that some enzyme activities are lost from chloroplasts upon isolation in aqueous media. Very recently Green *et al.* [16] have demonstrated prenyl transferases in proplastids of developing castor bean endosperm. Although the incorporation of MVA into *ent*-kaurene in extracts of chloroplasts has not yet been demonstrated, three types of experiments are reported here indicate that kaurene synthetase is present in pea chloroplasts. The chloroplasts were prepared by the method of Walker [11]. Their purity and integrity were determined by electron microscopy. When pea shoot S_{100} was spiked with chloroplast S_{100}, an enhancement in *ent*-kaurene synthesis from MVA was consistently observed over the levels obtained in extracts of shoot tips alone. A substantial amount of this enhancement activity is heat labile and is not present in preparations obtained from non-sonicated chloroplasts. When the shoot tip enzymes were boiled prior to the addition of chloroplast S_{100}, a small amount (12%) of *ent*-kaurene synthesis occurred as compared to controls. That this *ent*-kaurene synthesis from endogenous prenyl pyrophosphates was less than the stimulation by combined active enzymes is understandable since GGPP probably does not accumulate in the absence of AMO-1618 either *in vivo* or *in vitro* (unpublished results).

Direct evidence for kaurene synthetase in pea chloroplasts was obtained by the use of purified GGPP as substrate. This GGPP was biosynthesized in pea cotyledon extracts. Although a number of cell-free enzymes systems capable of synthesizing FPP and GGPP have been described [17 and references therein], the system reported here, derived from immature pea cotyledons, is relatively easily obtained and provides substantial yields (Figs. 1 and 2) The GGPP obtained from this source has proved to be an effective substrate for *ent*-kaurene biosynthesis in cotyledon extracts and is converted to *ent*-kaurene in low yields in pea shoot tip and chloroplast extracts. This substrate will certainly allow for more detailed studies on the regulation of this key enzyme than would be possible with MVA or other intermediates in the pathway.

Table 2 indicates that 0.27 pmol of *ent*-kaurene were produced from GGPP in extracts of chloroplasts and shoot tips, while cotyledon S_{100} was capable of converting 84.18 pmol. Thus the activity of *ent*-kaurene synthetase in pea chloroplasts as judged by these assays was very small.

Simcox *et al.* [9] reported the conversion of copalyl pyrophosphate to *ent*-kaurene (B activity of kaurene synthetase) in extracts of proplastids of peas, wild cucumber endosperm and germinating castor bean seeds. Although the plastids from wild cucumber also readily converted GGPP to *ent*-kaurene, those from peas and castor beans showed little or none of this activity. More recently Simcox and West (personal communication) have confirmed the very low activity from GGPP in pea plastid extracts as reported here.

The very low activity and the lack of stimulation of

these reactions by increased enzyme concentrations remain to be fully explained. The fact that MVA is converted to *ent*-kaurene at 20 times the rate of conversion of GGPP by shoot tip S_{100} indicates that the low kaurene synthetase activity, at least from this source, may be an artifact of the preparation. The amounts of substrate converted to *ent*-kaurene in these shoot tip extracts were actually 0.07% for GGPP and 0.29% for MVA. In cotyledon extracts, 20% of the GGPP was converted to *ent*-kaurene while only 12% of the MVA was incorporated.

Our data suggest that one possible explanation for the low activity might be the very active phosphatase activity in these reactions since no unreacted substrate was recovered. Another possible explanation is the channeled synthesis of intermediates in this pathway which has been reported previously [18,19].

The evidence presented in this paper demonstrates the presence of kaurene synthetase in extracts of chloroplasts of pea shoot tips. It is very likely that other enzymes catalyzing reactions in the GA biosynthetic pathway are also present. Indeed, the chloroplasts may contain the complete complement of enzymes required for GA biosynthesis. As noted previously, Charlton *et al.* [13] have demonstrated that phytoene synthesis from MVA occurs in sonicated preparations of bean chloroplasts isolated by non-aqueous techniques. Additional evidence for GA biosynthesis in chloroplasts is provided by the previously cited reports by Stoddart [1,2] and Murphy and Briggs [3]. While the contribution of chloroplast enzymes to the total GA synthesis in shoot tips remains to be determined, the evidence suggests that it could be very significant.

EXPERIMENTAL

Plant materials. Pea plants (*Pisum sativum* L. cv Alaska; W. Atlee Burpee Company, Riverside, California) were grown in a greenhouse equipped with supplemental lighting to provide a daily photoperiod of 16 hr at 20–23° (1200–1700 ft–c) and an 8 hr dark period at 17 to 20°. Plants were grown in vermiculite which was irrigated with mineral nutrient soln. Shoot tips (all of the shoot above the 6th node, including young leaves, leaf primordia and apical meristems) were harvested on the 13th day after planting. Developing cotyledons were excised from seeds which had attained approximately half maximum fr. wt. Plant materials were either used immediately after harvest or stored in liquid N_2 until needed.

Preparation of enzyme extracts. Enzyme extracts from excised developing cotyledons were prepared by grinding in a mortar and pestle with 0.1 M KH_2PO_4–K_2HPO_4 buffer (pH 7.1, 1 ml/g fr. wt) containing 0.1 mM chloramphenicol. The crude homogenate was centrifuged at 10,000 *g* for 15 min at 0 to 4°, and the resulting supernatant was centrifuged 60 to 120 min at 100000 *g* in a No. 65 angle head rotor in a Spinco Model L preparative ultracentrifuge. The supernatant (cotyledon S_{100}) was the enzyme extract, which was either used immediately or frozen in liquid N_2 for future use. Enzyme extracts from green pea shoot tips (shoot tip S_{100}) were prepared as described previously [6]. Chloroplasts were isolated by homogenizing fresh shoot tips in chilled KH_2PO_4–K_2HPO_4 buffer (pH 7.1, 3 ml/g fr. wt of tissue) containing 0.42 M sucrose, 2 mM DTT, 150 μM chloramphenicol, 1% $MgCl_2$ and 1% NaCl for 5 sec at top speed in a Sorvall Omnimixer. The homogenate was filtered 2× through 4 layers of cheesecloth, and the resulting filtrate was centrifuged 100 sec at 5000 *g*. The 5000 *g* pellet then was rinsed with buffer and recentrifuged for 100 sec at 5000 *g*. The final chloroplast pellet was resuspended in buffer (1 ml/g fr. wt of original tissue) using 4 complete strokes of a TenBroeck ground-glass homogenizer. The homogenized chloroplast suspension then was separated into two fractions. One was sonicated for 30 sec at 1.8 A in an MSE sonicator while the other was held in an ice bath. The sonicated or non-sonicated chloroplast suspensions were then centrifuged at 100000 *g* for 60–120 min, yielding the soluble chloroplast enzymes (chloroplast S_{100}) in the supernatant. Chloroplast S_{100} extracts contained approximately 26% of the chlorophyll present in each original sample of shoot tips.

Biosynthesis of GGPP-[^{14}C]. GGPP-[^{14}C] was synthesized biosynthetically from MVA-[2-^{14}C] using cotyledon S_{100} as the enzyme source. Reaction mixtures generally contained 37.5 ml enzyme extract, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM AMO-1618, 0.3 mM ATP and 0.03 mM MVA-[2-^{14}C] (sp. act. 17.5 or 18 mCi/mmol) in a total volume of 50 ml. The reaction mixtures were incubated at 30° for 30 to 180 min and stopped by adding sufficient 10 M NaOH to give a final concentration of 0.1 mM. The products of the reaction were extracted with collidine and purified by ion-exchange chromatography by the methods of Oster and West [10]. Aliquots (0.1 ml) of each 10 ml fraction from the columns were monitored for radioactivity by liquid scintillation spectrometry in 10 ml Bray's [20] soln. GGPP-[^{14}C], which was normally eluted in fractions 32–37 (approximately 0.35 M NH_4CO_3), was combined and evaporated to dryness and redissolved in a small vol of 0.1% (w/v) Tween 80 soln. The GGPP obtained in these expts was handled in silanized glassware prepared by treating with 1% dimethyldichlorosilane in C_6H_6 at 60°. GGPP was further identified by co-chromatography with authentic GGPP on S and S No. 509 paper strips developed in *n*-PrOH–NH_3–H_2O (6:3:1) and on Si gel F_{254} TLC developed with *iso*-PrOH–NH_3–1% EDTA (7.5:3:1). Acid hydrolysis and enzymic hydrolysis of GGPP and TLC of the resultant alcohols in hexane (System I), C_6H_6–EtOAc (9:1) (System II), and hexane–isopropyl ether–HOAc (2:1:1) (System III) with authentic standards were by the procedures of Oster and West [10]. Experiments using shoot tip S_{100} and chloroplast S_{100} as the enzyme sources for the production of GGPP-[^{14}C] were conducted with reaction mixtures containing 21 ml enzyme, 3.0 mM ATP, 1.0 mM $MnCl_2$, 1.0 mM $MgCl_2$, and 1.0 mM AMO-1618 in a total volume of 30 ml. The reaction products were isolated and identified as described for cotyledon extracts.

Incorporation of MVA into ent-*kaurene.* *Ent*-kaurene synthesis activity in cotyledon S_{100} enzymes was measured in reaction mixtures containing 0.7 ml enzyme, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.3 mM ATP, 70 mM PO_4, and 0.03 mM MVA-[2-^{14}C] in a total volume of 1.0 ml. Reactions were incubated for 60 min at 30°. The formation of *ent*-kaurene from MVA by extracts of pea shoot tips and chloroplasts was assayed in reaction mixtures containing 0.7 ml enzyme, 1.0 mM $MnCl_2$, 1.0 mM $MgCl_2$, 3.0 mM ATP, 70 mM PO_4 and 0.03 mM MVA-[2-^{14}C] in a total volume of 1.0 ml. In some cases, each component of the reaction mixture was doubled to give 2.0 ml reaction mixtures. Reaction mixtures were incubated for 60 min at 30°. Reactions were stopped by adding 1 ml Me_2CO containing 10 μg *ent*-kaurene and extracted with 3 × 1 ml Me_2CO–C_6H_6 (2:1). The organic extract from each reaction mixture was evaporated to dryness and transferred to the origin of a Si gel TLC plate (5 × 20 cm). Authentic unlabelled geranylgeraniol was applied at the origin and the plate was chromatographed 15 cm in hexane. After treating the top 5 cm of each plate with iodine vapour to visualize the *ent*-kaurene, the plate was then rechromatographed to 10 cm in C_6H_6–EtOAc (9:1). The authentic geranylgeraniol was then visualized in iodine vapour.

Conversion of GGPP to ent-*kaurene.* The capacities of S_{100} enzymes from cotyledons, shoot tips and sonicated chloroplasts to convert GGPP to *ent*-kaurene were measured in 1 or 2 ml reaction mixtures containing 0.5–1.4 ml enzyme, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 70 mM PO_4, and 0.14 to 0.21 μM GGPP-[^{14}C]. Reaction mixtures were incubated 60 min at 30°. The reactions were stopped by adding 1 ml Me_2CO con-

taining 10 μg *ent*-kaurene. *Ent*-kaurene-[^{14}C] and GGPP-[^{14}C] were determined as described above.

Radioactivity determinations. Radioactivity on TLC plates was detected either by scanning or by counting 0.5 cm bands of Si gel in 10 ml toluene containing 30 mg *p*-terphenyl and 0.3 mg of POPOP in which counting efficiency was 86%. Aq. samples (0.01 to 1.0 ml) were counted in Bray's soln [20]; the efficiencies for ^{14}C were from 65 to 85% and for ^{32}P were from 58 to 71%.

Protein and chlorophyll determinations. The protein content of enzyme extracts was determined by the method of Lowry *et al.* [21] using bovine serum albumin as a standard. Measurements of chlorophyll were by the method of Arnon [22].

Reagents. MVA-[2-^{14}C] lactone (sp. act. 17.5 or 18.0 mCi/mmol) in C_6H_6 was purchased from Amersham/Searle Corp. IL. Squalene, farnesol, chloramphenicol and DTT were purchased from Sigma. A sample of 2-*cis*/*trans*, 6-*trans*, 10-*trans* geranylgeraniol was kindly supplied by Hoffmann-LaRoche Switzerland. *Ent*-kuarene was a gift from Dr. Robert K. Clark, Jr. of Abbott Laboratories, North Chicago, IL. A sample of GGPP-[2-^{14}C] (1.08 mCi/mmol) was the generous gift of Dr. Charles A. West and Mr. David Simcox at the University of California at Los Angeles.

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