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COMPARISON OF *ENT*-KAURENE AND *ENT*-ISOKAURENE SYNTHESIS IN CELL-FREE SYSTEMS FROM ETIOLATED SHOOTS OF NORMAL AND *DWARF-5* MAIZE SEEDLINGS

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Key Word Index—*Zea mays*; Gramineae; *dwarf-5* mutant; *ent*-kaur-16-ene, *ent*-kaur-15-ene, squalene and phytoene biosynthesis; cell-free extract.

Abstract—An active cell-free system, prepared from young etiolated shoots of normal *Zea mays* seedlings, was shown to biosynthesize the terpenoid hydrocarbons *ent*-kaur-16-ene, squalene and phytoene from mevalonic acid. The biosynthesis of *ent*-kaur-16-ene from mevalonic acid was compared using cell-free systems obtained from normal and *dwarf-5* seedlings. *ent*-Kaur-16-ene was the predominant diterpene hydrocarbon synthesized by extracts from the normals; however, *ent*-kaur-15-ene was the major diterpene hydrocarbon synthesized by the *dwarf-5* mutants. *ent*-Kaur-15-ene and *ent*-kaur-16-ene were also produced as minor products in the normal and *dwarf-5* systems, respectively. The possible significance of the synthesis of the 'wrong' isomer (*ent*-kaur-15-ene) by the mutant is discussed.

INTRODUCTION

The *dwarf-5* (d_5) mutant of *Zea mays* is the result of a single gene recessive mutation which is expressed as a marked reduction in plant height from the seedling stage to maturity [1]. Mutant plants have shortened stems (and leaves) so that at maturity they are *ca* 1/5 the height of normals. *Dwarf-5* plants respond by normal growth to exogenously applied gibberellins (GAs) and to no other class of plant hormones [2, 3]. Also mutant plants contain little or no GA-like substances whereas such materials are present in normals [3]. Thus d_5 has been classified as a GA-less mutant, the mutated gene presumably blocking a specific step in the biosynthetic pathway leading to GAs [4]. Since the GA-precursors, *ent*-kaurene, *ent*-kaurenol and *ent*-kaurenoic acid give a GA-like growth response when fed to the mutant [5], the site of the presumed metabolic block has been thought to be prior to *ent*-kaurene in the biosynthetic sequence leading to the native GAs of maize.

Nothing has been published on the native GAs in maize seedlings, nor on their biosynthesis. However, Davies and Rappaport [6] reported no significant differences in the metabolism of GA_1-[^{3}H] by normal and d_5 plants and they concluded that the d_5 phenotype was not due to modification of GA_1 metabolism. Their data point to decreased biosynthesis rather than increased deactivation causing the apparent reduced levels of GA-like substances in d_5 plants.

In order to locate the position of the block in the GA-biosynthetic pathway, we have compared the *in vitro* biosynthesis of *ent*-kaurene in d_5 seedlings with that in normals. *ent*-Kaurene is formed from mevalonic acid (MVA) via a series of intermediates including farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). FPP and GGPP are also precursors of squalene and phytoene, respectively, which are intermediates in the sterol (squalene) and carotenoid (phytoene) biosynthetic pathways. The formation of *ent*-kaurene from MVA in cell-free extracts has been reported for a number of higher plants [7–13] and the fungus *Gibberella fujikuroi* [14, 15]. The enzymes catalysing the conversion of MVA into *ent*-kaurene, as well as into squalene and phytoene, occur in the high speed supernatant fraction.

RESULTS

Cell-free extracts were prepared from 6-day-old etiolated normal and d_5 *Z. mays* seedlings using shoots only, i.e. the coleoptiles and their enclosed young leaves. The 40 000 *g* supernatant from these preparations was incubated with MVA-[2-^{14}C] in the presence of ATP, NADPH, $MgCl_2$ and $MnCl_2$, and, after extraction, the hydrocarbon products were separated by TLC using solvent system 1 (see Experimental). Radioscanning of the TLC plates (Fig. 1) revealed three radioactive zones which co-chromatographed with authentic *ent*-kaurene, squalene and phytoene.

The material in zone I was identified as phytoene from both normal and d_5 incubations. The eluate from this zone was mixed with authentic phytoene and chromatographed sequentially in three different TLC systems with little change in specific radioactivity (Table 1).

The material in zone II was shown to be identical with squalene after elution and rechromatography using solvent system 3. A single radioactive band which co-chromatographed with authentic squalene was obtained for both normal and d_5 plants. The eluates from the radioactive bands from the two preparations were combined, mixed with squalene (75 mg) and, after formation of the hexahydrochloride (1537 cpm/μmol), recrystallized from acetone to constant specific radioactivity (1573, 1561, 1630 cpm/μmol, respectively for three crystallizations). The material from zone II for the preparation from normal seedlings was also examined by GC–RC and the radioactivity found to have the same retention time as authentic squalene.

The eluted material from zone III for preparations obtained from normal and d_5 plants was rechromato-

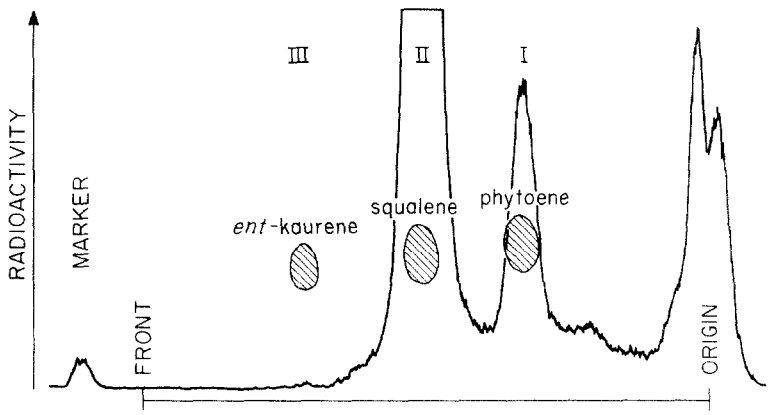


Fig. 1. Radiochromatogram of the products from the incubation of MVA-[2-^{14}C] with the cell-free extract of normal *Zea mays* shoots after TLC on Si gel developed with petrol. Non-radioactive *ent*-kaurene, squalene and phytoene were added to the incubation mixture as standards prior to extraction.

graphed using solvent system 2 (Fig. 2). For both preparations, two main regions of radioactivity appeared, a faster moving component which co-chromatographed with *ent*-kaurene and a slower moving component which co-chromatographed with *ent*-isokaurene (*ent*-kaur-15-ene). In the case of the preparation from normal seedlings, the major radioactivity was associated with *ent*-kaurene, whereas for d_5 the majority of the radioactivity co-chromatographed with *ent*-isokaurene. The identity of the faster moving radioactive material with *ent*-kaurene was confirmed for the cell-free systems from normal plants by elution of the material after TLC in solvent system 2, mixing with authentic *ent*-kaurene (5 mg, 492 cpm/mg) and recrystallizing from methanol to constant specific radioactivity (606, 580, 580 cpm/mg, respectively for three crystallizations). In addition, an aliquot of the radioactive material from zone III obtained from normal seedling preparations was found to have the same retention time as authentic *ent*-kaurene when examined by GC–RC. The identity of the slower moving component with *ent*-isokaurene was confirmed for d_5 by eluting the material after TLC in solvent system 2, mixing with authentic *ent*-isokaurene (136 cpm/mg) and recrystallizing from methanol to constant specific radioactivity (163, 221, 196 cpm/mg, respectively for three crystallizations). The material from the two minor zones, *ent*-isokaurene for the normal and *ent*-kaurene for the dwarf, were not identified further because of their low levels of radioactivity.

This difference in distribution of diterpene products from the cell-free systems obtained from normal and d_5 seedlings was seen also when GGPP-[^{14}C] or copalyl-pyrophosphate-[^{3}H] (CPP) were used as substrates. GGPP and CPP are the immediate precursors of *ent*-kaurene [14, 16]. Table 2 compares the radioactivity incorporated into *ent*-kaurene and *ent*-isokaurene from cell-free systems of normal and d_5 seedlings using MVA-[2-^{14}C], GGPP-[^{14}C] or CPP-[^{3}H] as substrates. The total incorporation into diterpenes was higher in the cell-free system from normal seedlings than in that from d_5, and also the ratio of radioactive *ent*-kaurene to *ent*-isokaurene was found to be much higher for the normal than for the dwarf.

It is conceivable that during the incubations *ent*-isokaurene is formed from *ent*-kaurene by isomerization rather than originating from CPP via a separate pathway. To test this possibility, *ent*-kaurene-[^{14}C] was incubated with preparations obtained from both normal and d_5 seedlings and the distribution of radioactivity between *ent*-kaurene and *ent*-isokaurene determined (Table 3). The same cell-free preparations were also incubated with MVA-[2-^{14}C] to ensure that *ent*-isokaurene formation did occur in the d_5 system. The results (Table 3) show that there was no isomerization of the incubated *ent*-kaurene

Table 1. Identification of phytoene from normal and d_5 seedlings

TLC system	Normal			*Dwarf-5*		
	Total dpm	Total wt (μg)	dpm/μg	Total dpm	Total wt (μg)	dpm/μg
(1)	220 780	1596	138	524 160	2184	240
(2)	119 416	945	127	241 164	1218	198
(3)	79 205	609	130	226 380	1155	196
(4)	39 725	343	116	82 350	450	183

The phytoene produced from MVA-[2-^{14}C] after incubation with cell-free preparations from normal and d_5 seedlings was purified by sequential TLC using the following systems: (1) Si gel developed with petrol (TLC system 1); (2) Al_2O_3 developed with petrol; (3) Si gel developed with 5% C_6H_6–petrol; (4) $AgNO_3$–Si gel (1:19) developed with 10% Me_2CO–C_6H_6. With systems 1, 2 and 3, phytoene was located by UV absorption at 254 mm; with system 4, phytoene was located by radioscanning.

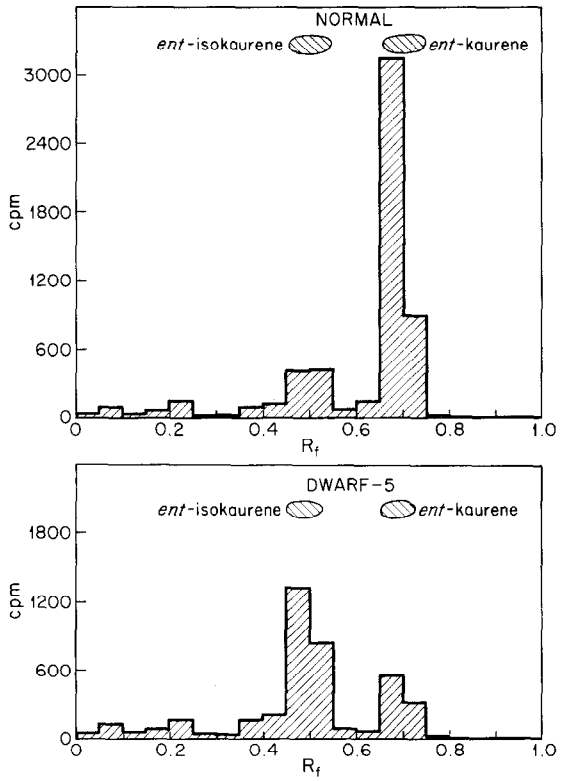


Fig. 2. Distribution of radioactivity between diterpene hydrocarbons after incubation of MVA-[2-^{14}C] with cell-free extracts of normal and d_5 *Zea mays* shoots. The material co-migrating with *ent*-kaurene after TLC on Si gel developed with petrol was rechromatographed on $AgNO_3$–Si gel (1:19) developed with C_6H_6. Non-radioactive *ent*-kaurene and *ent*-isokaurene were added as standards.

into *ent*-isokaurene in either system or during the isolation procedures.

DISCUSSION

The cell-free extracts from young shoots of normal maize synthesize *ent*-kaurene as the major diterpene hydrocarbon (Fig. 2). *ent*-Kaurene is known to be a precursor of GAs [16] and the cyclization step leading to *ent*-kaurene has been suggested as a limiting step in GA biosynthesis [17, 18]. This step is apparently controlled by the normal allele of the d_5 gene, since the mutant gene (d_5) results in a marked reduction in the synthesis of *ent*-kaurene. The reduction is observed whether MVA, GGPP or CPP is used as substrate, which indicates that the step between CPP and *ent*-kaurene (the B activity of *ent*-kaurene synthetase) is altered by the mutant gene. This idea is supported by the reversal of the *dwarf-5* growth habit by *ent*-kaurene, *ent*-kaurenol and *ent*-kaurenoic acid (although these compounds have relatively low bioactivity) [5].

Table 3. Distribution of radioactivity (dpm) between *ent*-kaurene and *ent*-isokaurene after incubating *ent*-kaurene-[^{14}C] (5000 dpm) with the cell-free extracts of normal and *dwarf-5* shoots. Also shown is the radioactivity incorporated into *ent*-kaurene and *ent*-isokaurene after incubating MVA-[2-^{14}C] with the same cell-free extracts

	Normal		*Dwarf-5*	
Substrate	*ent*-Kaurene	MVA	*ent*-Kaurene	MVA
Product				
ent-Kaurene	4226	263	3072	49
ent-Isokaurene	22	41	22	283

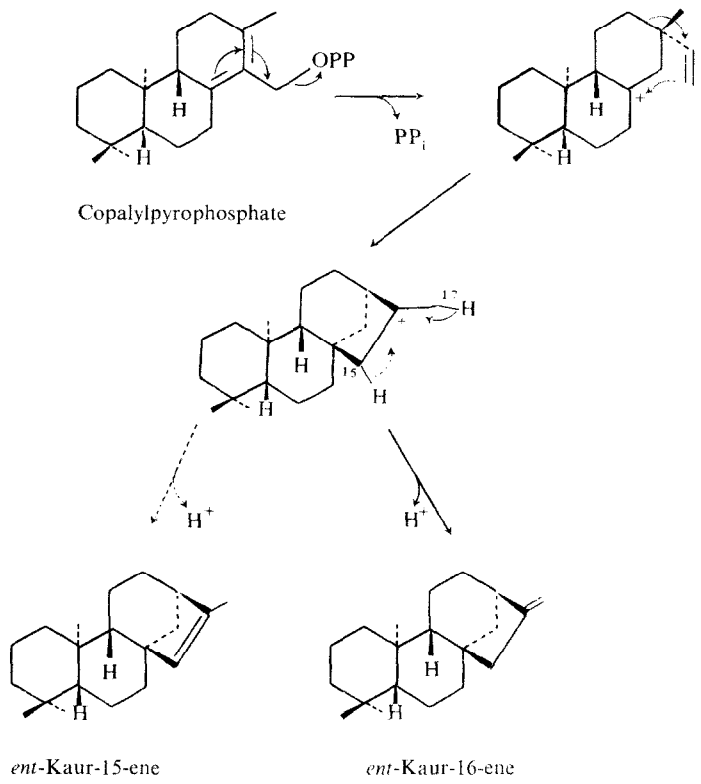
Some clues as to the nature of the mutation come from our observation that the lowered *ent*-kaurene synthesis by the cell-free system from d_5 plants is associated with a concomitant increased synthesis of *ent*-isokaurene, a metabolite which is not in the pathway leading to GAs [19]. It would appear that the mutated gene codes for an altered enzyme (B activity of *ent*-kaurene synthetase) which catalyses the formation of *ent*-isokaurene at the expense of *ent*-kaurene. *ent*-Isokaurene is probably not formed via *ent*-kaurene in the d_5 system, since no isomerization was observed in incubations of *ent*-kaurene using cell-free systems obtained from both normal and d_5 seedlings (Table 3).

A possible mechanism for the enzymatic formation of *ent*-kaurene and *ent*-isokaurene from CPP is suggested by a scheme originally proposed by Wenkert [20]. In this mechanism (Scheme 1), *ent*-kaurene is formed from CPP by the loss of a proton from C-17 of the carbonium ion; loss of a proton from C-15 gives rise to *ent*-isokaurene. It is possible that the loss of a proton from the carbonium ion could occur non-enzymatically in the mutant to give rise to a predominance of the thermodynamically favored product, *ent*-isokaurene. *In vitro* experiments have shown that the ratio of *ent*-kaurene to *ent*-isokaurene formed by treatment of either isomer with acid is 1:3 [21]. However, the corresponding ratio of products from the d_5 cell-free system using MVA as substrate is greater than 1:7 (see Table 2) which supports an enzymatic mechanism for the loss of the proton in the d_5 system. Thus any alteration of the enzyme such as a shift in the position of a

Table 2. Incorporation of radioactivity into *ent*-kaurene and *ent*-isokaurene from MVA-[2-^{14}C], GGPP-[^{14}C] and CPP-[^{3}H] incubated in cell-free extracts of normal and *dwarf-5* shoots

	Normal			*Dwarf-5*		
Substrate	MVA	GGPP	CPP	MVA	GGPP	CPP
ent-Kaurene	673	103	766	16	10	153
ent-Isokaurene	84	26	81	127	37	608
ent-Kaurene / *ent*-Isokaurene	8.0	4.0	9.5	0.13	0.27	0.25

Results are expressed in cpm per g fr. wt.



Scheme 1. Proposed scheme for the formation of *ent*-kaurene and *ent*-isokaurene from copalylpyrophosphate by a series of carbonium ion rearrangements.

proton-accepting group so that it becomes closer to C-15 than to C-17 would result in an increased production of *ent*-isokaurene at the expense of *ent*-kaurene. This could be the case for the d_5 mutant system.

EXPERIMENTAL

(3*R,S*)-[2-^{14}C]MVA (32 mCi/mmol) was purchased from Schwarz-Mann. Phytoene was isolated from carrot oil using a modification of the method of ref. [22]. *trans*-Geranylgeranyl-pyrophosphate-[^{14}C] (20 μCi/μmol) and *ent*-kaurene-[^{14}C] (20 μCi/μmol) were prepared biosynthetically from MVA-[2-^{14}C] using a cell-free system from endosperm of *Marah macrocarpus* [23]. Copalylpyrophosphate-[^{3}H] (20 μCi/μmol) was a gift from Dr. C. A. West.

Preparation of ent-*isokaurene*. The method used was similar to that described by Bearder *et al.* [24]. *ent*-Kaur-16-ene (300 mg) was dissolved in 2% CF_3CO_2H–C_6H_6 (10 ml). After 5 hr, 0.5 M phosphate buffer at pH 7.0 (10 ml) and EtOAc (5 ml) were added. After removal of the organic phase, the aq. phase was extracted with EtOAc (2 × 5 ml). After washing with H_2O (5 ml), the combined organic phases were evapd to dryness and redissolved in petrol. The soln was applied to a column of Si gel impregnated with 5% $AgNO_3$ which was eluted with petrol containing increasing amounts of C_6H_6. *ent*-Kaur-15-ene was eluted with 70–100% C_6H_6.

Plant material. The seeds used here were the F-2 generation of *Zea mays* L. that originated from a cross of homozygous *dwarf-5* (d_5/d_5) to normal (+/+). The F-1 (d_5/+) from these crosses were selfed to give F-2 seed which segregated into the ratio of 3 normals to 1 d_5 seedlings. The normals are of 2 genotypes (d_5/+ and +/+) but are phenotypically indistinguishable. The dwarfs are homozygous (d_5/d_5). These F-2 seeds were soaked in aerated distilled water overnight, planted in moist vermiculite and grown in the dark at 28° for 5–6 days.

Preparation of the cell-free extracts. Fifty coleoptiles, in which the first leaf had not yet emerged, were cut at the coleoptilar node and frozen in solid CO_2. This frozen material was ground to a fine powder with a mortar and pestle. PVP (0.5 g wet PVP per g fr. wt of tissue) and 0.1 M phosphate buffer at pH 7.0 (1.0 ml per g fr. wt), containing 5 mM mercaptoethanol, were added and the mixture was ground further until it was homogeneous. After it had warmed to 0°, the homogenate was filtered through 4 layers of cheesecloth and the filtrate centrifuged at 40000 *g* for 30 min. The supernatant was used as the source of enzymes.

Incubations with the cell-free extracts. Typically the incubation mixture contained MVA-[2-^{14}C] (20 μM), ATP (3 mM), NADPH (0.5 mM), $MgCl_2$ (1 mM), $MnCl_2$ (1 mM), phosphate buffer at pH 7.0 (100 mM) and 0.75 ml of the 40000 *g* supernatant in a total vol. of 1 ml. The incubations were carried out in test-tubes at 30° for 2 hr after which non-radioactive *ent*-kaurene, squalene and phytoene (*ca* 25 μg) were added as carriers and markers for TLC. The reaction was then stopped by the addition of Me_2CO (1 ml) and the mixture extracted with EtOAc (3 × 1 ml).

Separation of products. In order to separate the products from the incubation 3 TLC systems were used. System 1: Si gel developed with petrol (40°–60°). System 2: $AgNO_3$–Si gel (1:19) developed with C_6H_6. System 3: Si gel in which the top 15 cm of the plate were impregnated with $AgNO_3$, developed with C_6H_6. The EtOAc extract was chromatographed using either

system 1 or 2 depending on the experiment. When system 1 was used, the products were located both by radioscanning and visualizing in I_2 vapor. When system 2 was used, the plate was sprayed with 2,6-dichlorofluorescein/rhodamine and the products located through their fluorescence under UV light. In all cases the Si gel corresponding to the products was removed and counted by liquid scintillation.

Identification of products. In order to prepare *ent*-kaurene and squalene in sufficient quantity for identification, 5 ml incubations with cell-free systems from both normal and d_5 seedlings were carried out in a 125 ml Erlenmeyer flask using the same concns of co-factors as in the smaller incubations. After incubation of MVA-[2-^{14}C] with the cell-free extracts, non-radioactive *ent*-kaurene (700 μg) and squalene (2 mg) were added to each, and the extraction was carried out as before. The extracts were chromatographed using TLC system 1, the *ent*-kaurene and squalene were located by radioscanning, and the Si gel corresponding to these regions removed and eluted with EtOAc. The *ent*-kaurene was rechromatographed using solvent system 2, after which the radioactivity was detected by radioscanning and recovered by elution with EtOAc. The eluate for the normal system was combined with *ent*-kaurene (5 mg) and recrystallized from MeOH to constant sp. act.

The squalene from both systems was chromatographed using TLC system 3. The single radioactive bands at R_f *ca* 0.45 were eluted with EtOAc and combined together with squalene (75 mg). The squalene was converted to the hexahydrochloride by passing dry HCl gas through an Me_2CO soln [25] and the product was recrystallized to constant sp. act. from Me_2CO.

Phytoene was identified as a product from MVA in separate 5 ml incubations. At the end of the incubation, phytoene (5 mg) was added and the mixtures extracted as described above. The extracts were chromatographed using TLC system 1, the phytoene located by its UV absorption at 254 nm and eluted from the Si gel with EtOAc (3 × 1 ml). An aliquot of the eluted phytoene was examined for phytoene content spectrophotometrically from its adsorption at 285 nm ($E^{1\%}$ = 915) and then counted for radioactivity. The sp. act. for samples were then calculated. This procedure was repeated after TLC on 3 further systems (see Table 1).

Identification of ent-*isokaurene.* The radioactive material separating from *ent*-kaurene on the $AgNO_3$–Si gel system was identified as *ent*-isokaurene from a large-scale incubation (14 ml) with the system from d_5 shoots (which produces relatively large amounts of *ent*-isokaurene compared to *ent*-kaurene). The co-factor and MVA-[2-^{14}C] concns were as previously described. After incubating overnight at 30°, *ent*-kaurene (10 μg) and *ent*-isokaurene (4.7 mg) were added as carriers and the mixture was chromatographed on Si gel in petrol and the *ent*-kaurene/*ent*-isokaurene band was eluted with EtOAc and the material rechromatographed using solvent system 2. The *ent*-kaurene (400 cpm) and *ent*-isokaurene (1760 cpm) were eluted with EtOAc, the recovered wt of *ent*-isokaurene being 2.8 mg (60% recovery). The eluted *ent*-isokaurene was then mixed with non-radioactive *ent*-isokaurene to give 12.9 mg in total, which was recrystallized to constant sp. act. from MeOH.

GLC conditions for GC–RC were as follows: 2 m × 3 mm glass column packed with 2% OV-210. Flow rate, 67 ml/min, argon. Temp. programs, 130–200° at 5°/min for *ent*-kaurene, 150–200° at 5°/min for squalene. Injector temp. 300°; FID temp. 250°.

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