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DISTRIBUTION OF *ENT*-KAURENE SYNTHETASE IN *HELIANTHUS ANNUUS* AND *MARAH MACROCARPUS*

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Key Word Index—*Helianthus annuus*; Compositae; *Marah macrocarpus*; Cucurbitaceae; *ent*-kaurene synthetase; natural inhibitors; distribution in tissues.

Abstract—Kaurene synthetases catalyse the biosynthesis of *ent*-kaurene, a precursor of the gibberellins. In 4-day-old dark- or light-grown *Helianthus annuus* seedlings, the cotyledons contained over 90% of the synthetase activity. The low enzyme activity in the seedling hypocotyls and roots is not a consequence of inhibitory factors in these tissues. The cotyledons not only have the highest kaurene synthetase activity, but also have the highest inhibitory activity. The differences in kaurene synthetase activities in the different tissues cannot be explained on the basis of the levels of inhibitor(s) in the extracts. The mature perennial root of *Marah macrocarpus* has very low kaurene synthetase activity, in contrast to the liquid endosperm of immature seeds of the same plant which is a rich source of the enzyme.

INTRODUCTION

Kaurene synthetase catalyses the biosynthesis of a gibberellin (GA) precursor, *ent*-kaurene (kaurene). This paper reports the distribution of this synthetase system in different parts of young *Helianthus annuus* (sunflower) plants and its activity in extracts of the perennial root of *Marah macrocarpus* (wild cucumber). It has been reported earlier that GA-like substances are localized in 10-week-old sunflower seedlings mainly in young leaves and root tips, but are present only in minor amounts in internodal segments of the plant axes [1].

The biosynthesis of kaurene involves the conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP), catalysed by kaurene synthetase A and the subsequent conversion of CPP to kaurene by synthetase B [2]. In sunflower seedlings, enzyme A is not readily detectable, due partly to the presence of inhibitors [3]. However, A activity can be enhanced by storing the seedlings in liquid nitrogen for several days [4]. In the present study, we stored sunflower seedlings in liquid nitrogen before enzyme preparation and then investigated the distribution of kaurene synthetase activity in dark- and light-grown cotyledons, hypocotyls and roots of 4-day-old sunflower plants and in the fresh mature roots of *Marah*. Liquid endosperm of the *Marah* seed is a known source of both kaurene synthetases A and B [5].

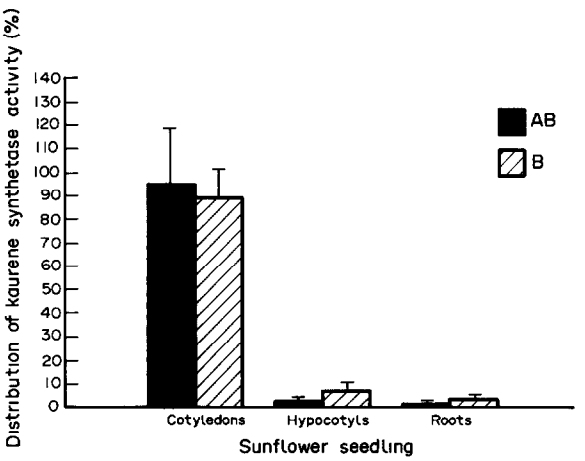

Fig. 1. Distribution, in per cent, of crude kaurene synthetase activity in 4-day-old, dark-grown seedlings of *Helianthus annuus* L. Weight contributions to the seedlings by cotyledon, hypocotyl and roots are, respectively, 51.3%, 27.4% and 21.2%. Seedlings were stored in liquid nitrogen for 7 days before enzyme preparation. Actual enzyme volume per incubation: cotyledon, 1 μl; hypocotyl and root, 5 μl each. Incubation time, 90 min. Four experiment, $n = 8$, vertical line above each bar denotes s.e. of the mean.

RESULTS AND DISCUSSION

We investigated the overall kaurene synthetase activities by incubating preparations of crude sunflower enzymes with labeled GGPP as substrate, and estimating the production of labeled kaurene. The B enzyme activity was determined separately, based on the production of [^{3}H]kaurene from labeled CPP. Figure 1 shows that, of the various plant parts sampled, the cotyledons of 4-day-old dark-grown sunflower seedlings contained the greatest amount of total enzyme activity. Of the measured AB activity 96% was localized in this tissue, whereas only minimal activity was observed in the dark-grown hypocotyls and roots. The relative amounts of kaurene synthetase activities in dark-grown cotyledon, hypocotyl and root tissue are presented in Table 1, on a tissue fr. wt basis and on a seedling fr. wt basis. These data, on the seedling basis, are those used in Fig. 1. Regardless of the method of expression, the highest enzyme activity is in the cotyledons. However, when the specific activities based on

Table 1. Kaurene synthesis by crude kaurene synthetase preparations from 4-day-old, dark-grown sunflower seedlings stored in liquid nitrogen for 7 days before enzyme preparation

Kaurene synthetase	Tissue (nmol/min · 10 g fr. wt)	Seedling (nmol/min · 10 g fr. wt)
AB		
Cotyledons	1.35±0.42	0.76±0.32
Hypocotyls	0.07±0.04	0.02±0.01
Roots	0.05±0.02	0.01±0.004
B		
Cotyledons	1.85±0.26	1.22±0.24
Hypocotyls	0.28±0.11	0.07±0.03
Roots	0.16±0.04	0.03±0.01

Actual enzyme volume per incubation: cotyledon, 1 μl; hypocotyl and root, 5 μl each. Incubation time 90 min. Weight contributions to the seedling by cotyledon, hypocotyl and roots are 51.3%, 27.5% and 21.2%, respectively. Four experiments, $n = 8$, ±s.e. of the mean.

Table 2. Comparison of specific activity of sunflower kaurene synthetase between 4-day-old, liquid nitrogen stored light-grown (L) and dark-grown (D) seedlings

Kaurene synthetase	Light (pmol/min · mg protein)	Dark (pmol/min · mg protein)
AB		
Cotyledons	0.66±0.12	3.96±1.45
Hypocotyls	0.61±0.28	1.49±0.82
Roots	3.96±0.84	2.86±1.71
B		
Cotyledons	6.70±1.20	5.32±0.45
Hypocotyls	12.8±2.44	6.24±2.62
Roots	4.71±1.00	7.77±4.57

Actual enzyme volume per incubation: cotyledon, 1 μl; hypocotyl and root, 5 μl each. Incubation time 90 min. Three experiments for L, $n = 6$; four experiments for D, $n = 8$, ±s.e. of the mean.

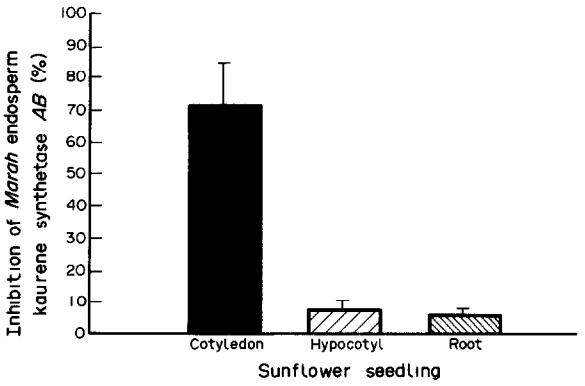

Fig. 2. Inhibition of kaurene synthetase AB of *Marah macrocarpus* endosperm by different crude enzyme preparations from 4-day-old, dark-grown *Helianthus annuus* L. seedlings (liquid nitrogen stored). The per cent of inhibition of AB is based on the total activity of individual sunflower tissue and individual *Marah* endosperm incubations. Seven μl of *Marah* endosperm enzyme [(7 μl) ammonium sulfate precipitated] was co-incubated with crude enzyme of either sunflower cotyledons (1 μl), sunflower hypocotyls (5 μl), or sunflower roots (5 μl). However, the per cent inhibitions expressed are corrected to those given by 1 μl of each of the three sunflower tissues. Total incubation time 90 min. Four experiments, $n = 8$; the vertical line above each bar denotes s.e. of the mean.

extractable protein are tabulated (Table 2), the differences in kaurene synthetase activity among the three tissues largely disappear. This is because of the great difference in the extractable protein content between the cotyledons and the other two tissues.

The amount of inhibition of AB activity occurring in the various dark-grown sunflower tissues sampled was determined by measuring the enzyme activity of *Marah* endosperm after it had been incubated in the presence of a sunflower preparation (Fig. 2). Because sunflower hypocotyls and roots contain very low enzyme activity, a greater enzyme volume (5 μl) was used in the incubations, as compared to that of the cotyledons (1 μl). In an earlier study [3], we found the inhibition of *Marah* endosperm AB activity to be proportional to the volume of the crude sunflower extract employed in the range 0–15 μl. (The inhibition of *Marah* AB activity by 0–15 μl of whole sunflower seedling extract was 0–72%.) These results indicate that the very low AB activity observed in the sunflower hypocotyls and roots does not appear to be due to the presence of inhibitors in these tissues; as shown in Figs 1 and 2, the cotyledons of these seedlings contain the highest measured enzyme activity, but also have the highest observed inhibitor content. However, the observed inhibition of *Marah* AB was demonstrably reduced by dialysing the crude sunflower enzymes. For the cotyledons, such dialysis resulted in a reduction of inhibition by 60% and for the hypocotyls and roots the dialysed preparations not only were not inhibitory but, in fact, slightly stimulated the *Marah* endosperm AB activity. The stimulation by dialysed hypocotyls was 16.2% and, by dialysed roots, 8%.

The AB activity observed in the tissues of liquid nitrogen stored, dark-grown seedlings, though high in the cotyledons, decreased upon dialysis. On the other hand, enzyme prepared from cotyledons of fresh (non-liquid nitrogen stored) dark-grown seedlings exhibited very low AB activity (cf. ref. [4]), but the observed activity was increased by dialysis (cf. ref. [3]). In contrast, enzyme B activity remained relatively unchanged, whether or not the dark-grown seedlings were stored in liquid nitrogen or dialysed. Furthermore, the B activity was unaffected by factors that inhibit AB activity.

Sunflower seedlings grown in a light–dark regime showed reduced AB activity in cotyledons in comparison with dark-grown seedlings, but little difference in the activity in hypocotyls and roots. On an equal fr. wt seedling basis, the amount of kaurene produced via AB activity was reduced from 0.76 nmol/min · 10 g fr. wt for dark-grown cotyledons (Table 1) to 0.14 nmol/min · 10 g fr. wt for light-grown cotyledons. A similar effect of light

had been observed earlier in whole seedlings [4]. The distribution of AB activity, on a fr. wt seedling basis, among the various light-grown tissues, is only slightly different from that of the dark-grown tissues, the ratio for light-grown plants being 79:4:17 for the cotyledons, hypocotyls and roots, respectively. Enzyme B activities and distribution patterns were essentially the same in these light-grown tissues as in the dark-grown plants.

We realize that direct comparisons of enzyme activities between seedlings grown in complete darkness and those grown in a light–dark regime may not be possible because seedlings grown in the two conditions differ in fr. wt and, probably, also in dry wt (not measured). On a fr. wt seedling basis, the dark-grown (D) are heavier than the light-grown plants (L); the L–D weight ratio is 1 : 2.8. The L–D weight ratios, based on individual tissues for cotyledons, hypocotyls and roots are, respectively, 1 : 2.5, 1 : 4.1, and 1 : 2.9. Nevertheless, a comparison of specific activities between L and D (Table 2) still shows that the cotyledons from dark-grown seedlings contain more AB activity than those from light-grown seedlings.

Although the liquid endosperm of *Marah macrocarpus* is a rich source of both A and B activity [5], its mature root contains little of either enzyme (Table 3). Dialysis of the crude enzyme did not enhance the activity. In the root this low activity is not a function of inhibitors, as shown by the data in Table 4 where *Marah* endosperm enzymes were incubated in the presence of *Marah* root enzymes. On the contrary, the dialysed root preparations enhanced the endosperm AB activity by 2.4-fold (Table 4). This stimulation was not seen in the control in which buffer replaced the root preparation. The B activity, on the other hand, was not affected by the presence of root enzymes.

Since *Marah* roots contain essentially no kaurene synthetase activity, the role of the stimulatory factor(s) is unclear. *Marah* root is a perennial organ, while its above-ground vines die back annually. It could be that, during vine emergence in the early spring, the giant underground root supplies factor(s) to the new shoots for rapid growth and reproduction.

In sunflower, GA-like substances have been found to be synthesized in young, rapidly growing plant parts, particularly in young leaves of 9-week-old seedlings and in the root tips of 2-3-day-old seedlings [1]. The biosynthesis of kaurene, a precursor of GA, had been detected mostly in the tissues of developing embryos [6] and seeds [7–9]. Coolbaugh [unpublished] recently examined the distribution of *ent*-kaurene synthesizing enzymes in 10-day-old green pea plants. They found that most of the enzyme activity was in the uppermost internode and its subtending leaves and essentially that there was no activity in the root tips. The present study shows that, in the early stages of sunflower growth, kaurene synthesis is largely limited to the rapidly enlarging cotyledons; other parts of such seedlings exhibit little measurable synthetase activity. This study also shows that inhibitors are not the only factors limiting the levels of kaurene synthetase activity in the different sunflower tissues. In *Marah*, the giant root contains essentially no kaurene synthetase activity, at least during the period of vine emergence.

Table 3. Kaurene synthetase activity in *Marah macrocarpus*

Liquid endosperm		Root	
AB	B	AB	B
(kaurene, nmol/min · 10 ml)		(kaurene, nmol/min · 10 g)	
0.84 ±0.19	3.09 ±0.38	0.021 ±0.013	0.096 ±0.022
$n = 5$		$n = 10$	

Crude enzyme volume, 7 μl; incubation time 90 min, ± s.e.

Table 4. Kaurene synthetase activity of *Marah macrocarpus* liquid endosperm (ammonium sulfate precipitated) co-incubated with preparations of *Marah* roots

Enzyme	AB (% total activity)	B (% total activity)
Endosperm + buffer	(100)	(100)
Endosperm + root (crude)	99.1 ± 23.3	99.4 ± 15.7
Endosperm + root (dialysed)	240 ± 40.4	94.3 ± 5.5

Enzyme volume, 7 μl each; incubation time, 90 min; $n = 4$; ± s.e.

EXPERIMENTAL

Plant materials. Dehusked *Helianthus annuus* L. seeds were sterilized, rinsed and planted in a culture dish (18.5 cm diameter) which had been lined with a double layer of filter paper and moistened with 100 ml tap water before autoclaving and 20–30 ml sterilized water before planting. The dish was covered with a glass plate, placed in a growth chamber and maintained at 25 ± 1°. The seeds were germinated either in complete darkness or under a 14 : 10 hr light–dark regime [4]. Seedlings (4-day-old) were harvested and separated into cotyledons, hypocotyls and roots. Each batch of tissues was wrapped in Al foil and stored in a 20 l. liquid N_2 tank for 1 week prior to enzyme preparation. Immature seeds of *Marah macrocarpus*, collected in the canyons near Los Angeles in March 1982, had been stored at −20°. Two mature roots, weighing *ca* 41 and 30 kg, were dug by Paul Boyer of the UCLA Department of Chemistry and Biochemistry in 1983 and 1984, respectively. The roots were stored at 6° for 2 weeks to 1 month before enzyme preparation. Enzyme activity was tested in tissues located in the top, mid and tip sections of the root. No differences were noted between the different sections, hence, all data were pooled and tabulated.

Enzyme preparation. Fresh or liquid N_2 stored sunflower tissues, frozen immature *Marah* seeds and fresh *Marah* roots were used for enzyme preparations. Detailed methods of preparation are discussed in refs [2, 4]. In brief, crude enzymes of sunflower tissues were prepared in Tris buffer, pH 7.7. Individual fresh tissue (10 g) was homogenized with 1 g PVP (polyclar AT) in 25 ml Tris [100 mM, containing $MgCl_2$ (5 mM), DTT (0.5 mM), EDTA (0.1 mM) and 2% glycerol]. *Marah* roots were prepared either in Tris (as described above), or in imidazole buffer [pH 6.5, 10 mM, containing $MgCl_2$ (5 mM), KCl (50 mM) and EDTA (0.1 mM)]. No difference was noted in enzyme activity between the two preparations in the two buffers. The crude enzyme of *Marah* endosperm was prepared in the imidazole buffer as above, see also ref. [2] and, subsequently, ppted with $(NH_4)_2SO_4$ at 40% of satn and then dialysed. Dialysis of enzyme preparations (2–3 ml) was carried out overnight at 6° in the same buffer (1 l.) used for the enzyme preparation. The dialysed, $(NH_4)_2SO_4$ ppted enzyme of *Marah* endosperm was used in all

the tests for inhibition of AB activity by sunflower tissues or by *Marah* roots.

Enzyme assay. The overall enzyme AB activity (crude or dialysed) was estimated on the basis of the observed conversion of GGPP to *ent*-kaurene. The ^{3}H-labeled substrates were synthesized by Bruce Fellows and Robert Bruce in our laboratory, with labeling at either the 1-[^{3}H] or 4,17-[^{3}H] positions, and with sp. act. of 11.6 and 10 μCi/μmol, respectively. The B activity was assessed by measurement of 1-[^{3}H]CPP-derived *ent*-kaurene of sp. act. 33 μCi/μmol (synthesized earlier by John Duncan in this laboratory). The total incubation vol. was 500 μl for either sunflower or *Marah*, or co-incubation of *Marah* endosperm with either sunflower tissues or *Marah* root. The ingredients in the incubation tube consisted of enzyme (1 μl sunflower cotyledon; or 5 μl hypocotyl, or 5 μl root, or 7 μl *Marah* root or endosperm), 50 μl TES (40 mM), 50 μl $MgCl_2$ (50 mM) and substrate [25 μl GGPP (1.9 μM), or 20 μl CPP (1 5 μM)] made up to 500 μl with H_2O. Kaurene production was assessed by measuring the amount of radioactive product present at an R_f value coincident with authentic *ent*-kaurene on TLC silica gel plates impregnated with $AgNO_3$ (R_f 0.66, [4]). All data were corrected for background counting rates and the counting rates of control incubations carried out in the absence of added enzyme.

Inhibition measurements. Measurements of inhibition of AB in sunflower tissues and in root tissues of *Marah* were done by co-incubation of enzymes from those tissues with the dialysed $(NH_4)_2SO_4$ ppted enzyme of *Marah* endosperm (cf. 'enzyme assay' above and ref. [4]). In combined incubations, inhibition of AB, expressed in per cent, was based on the total activity of individual sunflower tissue and individual *Marah* endosperm incubations, or on that of individual *Marah* root and individual *Marah* endosperm incubations.

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