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GIBBERELLIN BIOSYNTHESIS IN ENDOSPERM AND COTYLEDONS OF *SECHIUM EDULE* SEEDS

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Key Word Index—*Sechium edule*; Cucurbitaceae; biosynthesis; hormones; gibberellins.

Abstract—Cell-free extracts prepared from endosperms and cotyledons of *Sechium edule* seeds at four stages of growth were incubated with [2-^{14}C]mevalonic acid to investigate their capacity for *ent*-kaurene. Both tissues synthesize *ent*-kaurene at each stage of seed growth. The rate of synthesis was higher in cotyledons than in endosperms and was the same at each stage examined.

Endosperm and cotyledon cell-free extracts prepared from seeds at the same stage of growth were incubated with ^{14}C-labelled *ent*-kaurene. In the absence of Fe^{2+}, dialysed extracts of both tissues metabolized *ent*-kaurene to *ent*-kaurenol, *ent*-kaurenal, *ent*-kaurenoic acid, *ent*-7 β-hydroxykaurenoic acid and GA_{12}-aldehyde. In the presence of Fe^{2+}, non-dialysed extracts were able to synthesize gibberellins. The radioactivity from *ent*-kaurene was incorporated in to the same products that accumulate endogenously both in endosperms and cotyledons, i.e. GA_4 and/or GA_7 and GA_9. Radioactive GA_{15} was also detected in the incubation mixtures.

INTRODUCTION

Biosynthetic studies performed with cell-free systems have shown that immature seeds are a site of gibberellin synthesis [1–4]. The occurrence and metabolism of endogenous gibberellins during seed growth have been investigated in beans [5–7] and peas [8, 9]. Nevertheless, very few investigations have been focused on the location of the biosynthetic capacity in various seed organs, most reports dealing with the whole immature seed [3] or a single tissue. Thus, GA biosynthesis has been extensively studied in endosperm of *Marah macrocarpa* [1, 10] and *Cucurbita maxima* [2, 11], while *ent*-kaurene synthesis has been located in cotyledons in peas [12]. Recently, the synthesis of gibberellins has been demonstrated in suspensor cell-free extracts of *Phaseolus coccineus* seeds [4].

We are currently investigating the distribution of hormonal substances and the regulation of their biosynthesis and metabolism in various tissues of the seed during development and maturation. As a part of this project, we have determined the occurrence of very high levels of gibberellins in endosperms and cotyledons of *Sechium edule* seeds at various stages of growth [13]. The biosynthetic origin of these gibberellins has been investigated using cell-free systems prepared from endosperms and cotyledons. The biosynthesis of GA_9 in cotyledons has been separately reported [14]. We now report the results of the whole investigation.

RESULTS

Cell-free extracts were prepared from endosperms and cotyledons isolated from seeds at various stages of development. The material was classified according to the lengths of the seed and the cotyledons [13]. The extracts were incubated with [2-^{14}C]mevalonic acid (MVA) to test for the presence of the enzyme systems needed for the synthesis of *ent*-kaurene. Preliminary results had shown that *ent*-kaurene synthesis was more efficient after dialysis of the crude enzymatic extracts; therefore only dialysed preparations were used for this part of the investigation.

Synthesis of *ent*-kaurene from [2-^{14}C]mevalonic acid was obtained with endosperm and cotyledon extracts prepared from seeds at each of the four stages of growth. These conclusions were based on the final identification of labelled *ent*-kaurene after purification on TLC and GC/MS of the extracts obtained from all the incubation mixtures. About 5% of the MVA supplied to cotyledons (based on the active isomer) was incorporated into *ent*-kaurene at all stages of growth. In endosperms, *ent*-kaurene synthesis increased from 2% in the early stages (A and B) to around 3.5% in the later stages (C and D) of seed growth.

The gibberellin pathway after *ent*-kaurene was then investigated in endosperm and cotyledon preparations from seeds of the same stage of growth. Seeds of class C were used for these investigations because they exhibited both the highest rate of *ent*-kaurene synthesis (based on MVA incorporation) and the maximum levels of endogenous GAs [13], in endosperms and cotyledons.

As the incorporation of [2-^{14}C]MVA into *ent*-kaurene was relatively low, the later steps of the pathway were investigated by incubating the extracts with ^{14}C-labelled *ent*-kaurene prepared from [2-^{14}C]MVA with a cell-free system from *Phaseolus coccineus* cotyledons [4]. Incubation of enzymatic preparations with labelled *ent*-kaurene was performed using either dialysed or non-dialysed extracts. In the latter case Fe^{2+} was included in the medium. The acidic ethyl acetate extracts were purified on TLC. Unmetabolized *ent*-kaurene was separated by developing the plate with solvent system 1, then the labelled products were chromatographed using solvent system 2.

When dialysed endosperm and cotyledon preparations

were incubated with labelled *ent*-kaurene, conversion to all intermediates from *ent*-kaurene to GA_{12}-aldehyde was observed for both tissues. *Ent*-6β,7β-dihydroxykaurenoic acid was also detected in the incubation mixtures. Figure 1A shows the radio-TLC (system 2) of the ethyl acetate extract from the endosperm incubation. No incorporation was observed with boiled enzymes. Conclusive identification of the labelled metabolites was accomplished by GC/MS analysis of the TMSi derivatives of each fraction eluted from TLC. The expected isotopic abundances were observed in the mass spectra thus confirming the origin of the compounds from labelled *ent*-kaurene. Conversion of *ent*-kaurene was higher with the cotyledon preparation (> 50%) than with the endosperm preparation (< 30%).

Different results were obtained when non-dialysed extracts of endosperms and cotyledons were incubated with labelled *ent*-kaurene and $FeSO_4$ was added to the incubation mixture. In this mixture, although most of the previously identified intermediates were detected, no GA_{12}-aldehyde was observed. However, label was incorporated into some of the endogenous GAs of this seed ($GA_{4/7}$, GA_9). Figure 1B shows the radio-TLC of the acidic ethyl acetate extract of the non-dialysed incubation medium obtained from cotyledons.

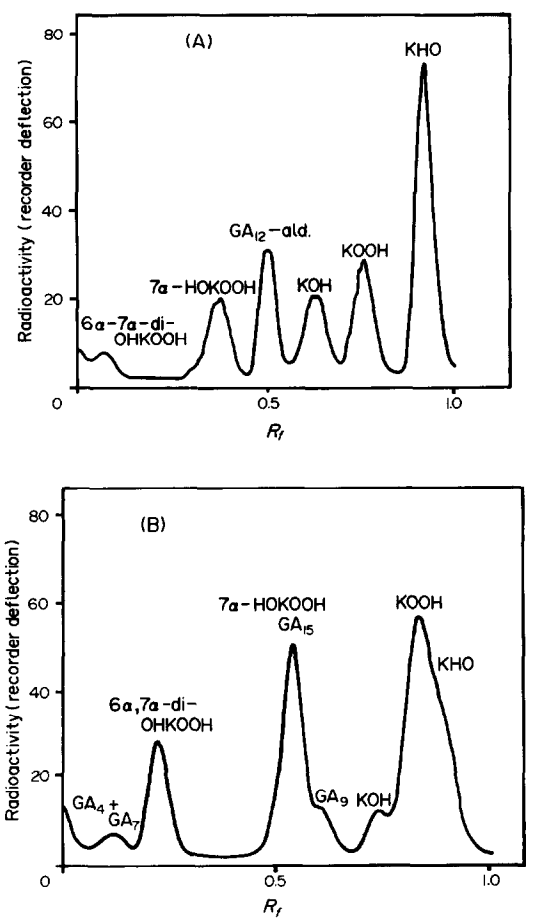

Fig. 1. Radio TLC of EtOAc extracts obtained from incubations with [^{14}C]kaurene. (A) endosperm, dialysed extract; (B) cotyledons, non-dialysed extract plus Fe^{2+}. KOH, kaurenol; KHO, kaurenal; KOOH, kaurenoic acid; HOKOOH, hydroxykaurenoic acid; diOHKOOH, dihydroxykaurenoic acid.

GA_9 was conclusively identified in the cotyledon incubation mixture after GC and GC/MS analysis. The expected isotopic abundance was clearly observed in the mass spectrum. Thus dilution by unlabelled endogenous GA_9 was relatively low. In contrast, the high levels of dilution of the labelled GA_4 and/or GA_7 by endogenous compounds prevented observation of isotopic peaks due to the label. However, there is little doubt that GA_4 and/or GA_7 originates from the conversion of labelled *ent*-kaurene as radioactivity was associated with the GC peak identified as GA_4/GA_7 by mass spectrometry and no trace of other substances was observed in the mass spectrum.

GA_{15} was also conclusively identified in cotyledons incubation mixture after GC/MS analysis. The same gibberellins, GA_4 and/or GA_7, GA_9 and GA_{15}, were detected in the endosperm incubation mixture by GC/MS analysis. However, mass spectra peaks due to the label were only observed for GA_{15} in this case. Nevertheless, GC analysis demonstrated that the radioactivity was associated with the peaks cochromatographing with GA_9, $GA_{4/7}$. We therefore conclude that the endosperm as well as the cotyledons can convert *ent*-kaurene to GA_9, GA_4 and/or GA_7.

DISCUSSION

Endosperm and cotyledons have been shown to be sites for the synthesis for gibberellins, the most direct evidence coming from the work with cell-free systems. However, until this study there were no reported investigations on GAs synthesis in both tissues of the same seed.

By using cell-free systems, we have examined the potential for the biosynthesis of gibberellins in endosperms and cotyledons of *Sechium* during the growth and maturation of the seed. The research was developed in two steps: firstly, the ability to synthesize *ent*-kaurene from MVA in different stages of seed growth was demonstrated, then *ent*-kaurene metabolism was followed in endosperm and cotyledons at the same stage of seed development. The synthesis of *ent*-kaurene is considered a major regulation point in the pathway leading to gibberellins. The present study demonstrates that cell-free preparations from both endosperms and cotyledons can synthesize *ent*-kaurene from MVA. A good correlation was observed in endosperms between the endogenous amount of gibberellins and the rate of *ent*-kaurene synthesis in the cell-free systems, both increasing in the more advanced stages of seed growth. By contrast, in cotyledon extracts, where the rate of *ent*-kaurene synthesis was approximately constant, there was a marked reduction in the endogenous level of GAs in the last stage of growth. The reduction of the level of free GAs at this stage may be due to the formation of conjugates. This was not investigated in this study but the accumulation of conjugated forms has been frequently observed in maturing seeds [15, 16]. Alternatively, GAs may be exported from the seed to other tissues.

Incubation of labelled *ent*-kaurene with dialysed enzyme preparations from endosperms and cotyledons led to its conversion to all the known intermediates as far as GA_{12}-aldehyde i.e. *ent*-kaurenol, *ent*-kaurenal, *ent*-kaurenoic acid, *ent*-7β-hydroxykaurenoic acid. By using non-dialysed preparations and adding $FeSO_4$ to the

incubation mixture, GA_{12}-aldehyde was metabolized further and gibberellins were produced. GA_4, GA_7, GA_9 and GA_{15} were synthesized by both endosperm and cotyledon preparations.

GA_{15} was the only C_{20} gibberellin detected in the incubation mixtures. The absence of C_{20} GAs other than GA_{15} may indicate that GA_{15} is not an intermediate. It seems that GA_{12}-aldehyde is converted to the C_{19} GAs via open lactone forms of the C_{20} compounds [17]. No conversion to more polar gibberellins, like GA_1 and GA_3 which are endogenous in cotyledons, was observed under the experimental conditions used. In this connection, the metabolism of GA_4/GA_7 and GA_9 remains to be investigated.

The present study demonstrates that endosperm and cotyledons synthesize gibberellins simultaneously in the seed of *Sechium*. This conclusion, based on the use of cell-free systems, is confirmed by the co-identity of the gibberellins synthesized by the enzymatic extracts and the gibberellins present endogenously in the tissues at the same stage of seed growth. The presence of GAs in both endosperm and cotyledons and their separate synthesis in both tissues suggest separate functions of these compounds in the two parts of the seed. In this regard, data on the presence and metabolism of gibberellins in the embryonic axis may add more information. At present this aspect is under investigation.

EXPERIMENTAL

Plant material. Fruits at different stages of growth were harvested from plants of *S. edule* grown outside during June–October 1981. The seeds were dissected, classified in relation to seed and cotyledon length, and cell-free systems prepared from endosperms and cotyledons separately.

Cell-free extracts. The material was homogenized with 0.05 M Pi buffer, pH 8, plus 2.5 mM $MgCl_2$ (1:1, w/v). After centrifugation at 10 000 *g* for 10 min at 4°, the supernatant was stored in liquid N_2 either before or after extensive dialysis against the homogenization buffer at 4° and used as the enzymatic source in all experiments.

Synthesis of ent-[^{14}C]*kaurene. ent*-[^{14}C]Kaurene was prepared enzymatically from [2-^{14}C]MVA (40 mCi/mmol) as described [4]. The labelled *ent*-kaurene was purified by HPLC (Licrosorb RP 18, 5 μ (15 cm × 1/4 in o.d.); MeCN, 1 ml/min; elution vol., 16 ml) and its identity confirmed by GC/MS analysis. The sp. act. calculated from the MS was 128 mCi/mmol which was finally diluted with unlabelled *ent*-kaurene to a sp. act. of 85 mCi/mmol.

Incubation mixtures, extraction and purification. The incubation mixtures for *ent*-kaurene synthesis experiments consisted of 5 mM ATP, 10 mM PEP, 7.5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.2 mM [2-^{14}C]MVA (22 mCi/mmol) and dialysed cell-free extract (0.9 ml) to a total vol. of 1 ml. After incubation for 4 hr at 30° the reaction was stopped by addition of 1 ml Me_2CO. The mixture was extracted with hexane and purified on TLC using hexane as solvent. Labelled *ent*-kaurene was finally identified by GC/MS. The incubation mixture for the *ent*-kaurene metabolism experiments contained 5 mM ATP, 10 mM PEP, 7.5 mM $MgCl_2$, 1 mM NADPH, 0.1 mM *ent*[^{14}C]kaurene (18×10^6 dpm) and cell-free extract (1.8 ml) to a total vol. of 2 ml. In experiments with non-dialysed extracts, $FeSO_4$ was added to the incubation mixture to give a final conc of 0.5 mM. The incubation was carried out for 4 hr at 30° and was stopped by adding 2 ml of Me_2CO. The pH was adjusted to 3.0 with HCl and the mixture was extracted with EtOAc. Purification of the extracts was performed by TLC and GC following procedures previously described [4]. Solvent systems used for TLC were (1) hexane, (2) $CHCl_3$–EtOAc–HOAc (85:15:1). GC conditions were: 2% OV1 on gas chrom Q in a glass column (150 cm × 0.4 cm i.d.); temp. programme from 200° to 300° at 4°/min; N_2 40 ml/min.

GC/MS. This was performed on a Hewlett–Packard 5992-B instrument equipped with a jet separator and using an ionization voltage of 70 eV. GC conditions were as previously described. The compounds were identified as TMSi derivatives by comparison of their MS with reference spectra obtained under the same conditions. The spectra of the following compounds showed isotopic peaks due to the incorporation of ^{14}C from labelled precursors: kaurene, kaurenol, kaurenal, kaurenoic acid, 7β-hydroxykaurenoic acid, 6β,7β-dihydroxykaurenoic acid, GA_{12}-aldehyde, GA_9 and GA_{15}. The incorporation of labelled precursors into GA_4 and/or GA_7, was proved by the association of radioactivity with the GC peak identified as GA_4/GA_7 by MS in the absence of contaminating compounds.

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