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GIBBERELLINS IN ENDOSPERM AND EMBRYOS OF *MARAH MACROCARPUS*

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Key Word Index—*Marah macrocarpus*; Cucurbitaceae; gibberellins; endosperm; embryo; homogenates; GC–mass spectrometry.

Abstract—Endogenous gibberellins (GAs) have been identified by GC-mass spectrometry in homogenates of the endosperm and embryos of *Marah macrocarpus* seeds, before and after fractionation of proteins by ammonium sulphate precipitation, followed by Sephadex G-25 filtration. The unfractionated endosperm homogenate contained GA_3, GA_4, GA_4-diacid, GA_7, GA_7-isolactone, GA_7-diacid, GA_{25}, GA_{37}, GA_{43}, GA_{46}, GA_{62} and the putative $2\beta,12\alpha$-dihydroxyGA_{25}, 12α-hydroxyGA_{43} and 2β-hydroxyGA_{84}; the amounts of these GAs were 2–100 $\mu g\ g^{-1}$, except for GA_3 and GA_{62}, which were present in much lower amounts. The fractionated endosperm homogenate still contained GA_4, GA_7, GA_7-isolactone, GA_{25}, $2\beta,12\alpha$-dihydroxyGA_{25}, and GA_{46}, but in reduced amounts ($\leq 1\ \mu g\ g^{-1}$ endosperm). The unfractionated embryo homogenate contained comparable amounts of the same GAs, as in the unfractionated endosperm homogenate, except for the absence of GA_7-diacid, GA_{62}, the putative $2\beta,12\alpha$-$(OH)_2GA_{25}$ and the putative 12α-OHGA_{43}; GA_1 and GA_{13} were also present. In contrast to the endosperm, no GAs were detected in the fractionated embryo homogenate.

INTRODUCTION

The study of gibberellin (GA) biosynthesis in cell-free enzyme systems began with the use of preparations from the endosperm of *Marah macrocarpus* (previously *Echinocystis macrocarpa* Greene) [1]. This prompted an interest in the endogenous GAs in the endosperm of *M. macrocarpus* [2,3], culminating in the identification of GA_4, GA_7, GA_{24}, GA_{25}, GA_{43} and GA_{46} [4] by GC–mass spectrometry and synthesis [5]. Our interest in the GA content of seeds of *M. macrocarpus* has been renewed by studies on the later steps of GA biosynthesis using cell-free enzyme preparation from these seeds [6, MacMillan *et al.*, unpublished].

Previously, we have shown [6] that an enzyme preparation from a mixture of endosperm and embryos from *M. macrocarpus* converts GA_9 and 2,3-dehydro-GA_9 into GA_7 and GA_5 into GA_3. In extending this study to investigate other GA-biosynthetic activities after GA_{12}-aldehyde in the endosperm and embryo of *M. macrocarpus* (MacMillan *et al.*, unpublished), we observed high dilution of the isotopic label in products from labelled substrates in enzyme preparations from both the endosperm and embryos. It was of interest to determine if the endogenous GAs were binding to proteins in these preparations. We have, therefore, identified the endogenous GAs in enzyme preparations from endosperm and embryos at different stages of purification.

RESULTS AND DISCUSSION

Seeds of *M. macrocarpus* of maturity index 50–90 [7] were used from the same collection as those used in the studies on enzyme activities (MacMillan *et al.*, unpublished). Homogenates of endosperm and embryos were prepared in 0.1 M Tris buffer (pH 7.3 at 5°) and fractionated by ammonium sulphate (AMS) precipitation, followed by filtration through Sephadex G-25. Aliquots of the unfractionated extracts, the AMS in Tris buffer, the AMS supernatant and the Sephadex G-25 eluate were acidified and extracted with ethyl acetate. The ethyl acetate extracts were methylated, trimethylsilylated and analysed by full-scan GC–mass spectrometry (Table 1). The identified GAs are listed in Table 2 for all four fractions from the endosperm. For the embryo fractions, no GAs were identified in the AMS precipitate, both before and after Sephadex G-25 filtration; only the GAs identified in the unfractionated embryo homogenate are shown in Table 2.

Of the 14 GAs and GA-related products identified in the unfractionated endosperm homogenate, GA_4, GA_7, GA_7-19,2-lactone (*iso*GA_7), GA_{25}, GA_{46} and the putative $2\beta,12\alpha$-dihydroxyGA_{25} were still present in the AMS precipitate, before and after Sephadex G-25 filtration. Gibberellin A_4-diacid, GA_{43} and the putative 12α-hydroxyGA_{43}, first detected in the endosperm [8] and embryos [9] of *Cucurbita maxima* endosperm, were not present in the AMS precipitate. Gibberellin A_3,

Table 1. Representative GC-MS data for the GAs identified in endosperm and embryos of *Marah macrocarpus*

Compound	KRI	*m/z* (rel. int.)
GA_1	2663	506 (100), 491 (37), 448 (50), 376 (45)
GA_3	2686	504 (100), 475 (9), 387 (12), 370 (10), 283 (11), 238 (14), 208 (41)
GA_4	2493	418 (24), 386 (17), 358 (10), 289 (50), 284 (100), 225 (87), 129 (59)
GA_4-diacid	2425	432 (2), 415 (2), 400 (4), 342 (23), 310 (52), 282 (100), 223 (60)
GA_7	2519	416 (18), 384 (21), 356 (31), 298 (28), 282 (31), 222 (100),
GA_7-isolactone	2484	416 (14), 384 (41), 356 (64), 298 (50), 282 (44), 242 (25), 222 (100)
GA_7-diacid	2401	430 (16), 370 (28), 311 (52), 281 (100), 221 (62), 193 (29)
GA_{13}	2596	492 (1), 436 (14), 400 (41), 372 (14), 310 (51), 282 (32), 223 (24), 160 (33), 129 (100)
GA_{25}	2428	404 (1), 372 (24), 344 (2), 312 (73), 284 (100), 269 (4), 253 (9), 225 (57)
$2\beta,12\alpha$-$(OH)_2GA_{25}$ (putative)	2764	580 (5), 548 (57), 520 (20), 488 (43), 458 (72), 430 (100), 398 (54), 370 (53), 340 (63), 308 (43), 281 (68), 221 (75), 195 (35), 129 (51)
GA_{37}	2753	432 (19), 417 (6), 401 (6), 372 (4), 342 (20), 310 (46), 129 (100)
GA_{43}	2714	580 (7), 565 (3), 431 (100), 349 (15), 217 (27), 173 (38), 147 (22)
12a-$OHGA_{43}$ (putative)	2855	668 (7), 608 (9), 570 (15), 546 (11), 519 (100), 479 (20), 459 (18), 429 (69), 389 (40), 369 (36), 309 (22), 217 (38), 173 (46), 147 (57)
GA_{46}	2604	492 (15), 460 (52), 435 (15), 400 (64), 372 (23), 342 (63), 310 (41), 282 (100), 223 (70)
GA_{62}	2419	401 (1), 372 (3), 313 (10), 282 (38), 223 (75), 222 (100)
2β-$OHGA_{84}$ (putative)	2655	506 (100), 491 (42), 474 (24), 431 (72), 362 (24), 341 (24), 310 (60), 235 (78), 208 (84)

GA_7-diacid, GA_{37}, GA_{62} and the putative 2β-hydroxy-GA_{84} were detected only in the unfractionated endosperm homogenate. The amounts of the GAs in the endosperm Sephadex G-25 filtrate were estimated by incubating 2,3-dehydro-[17-^{14}C]GA_9 with 10, 20 50 and 89 μl of the filtrate. In each case, the substrate was completely metabolized to a single product that was isolated as the corresponding methyl ester by HPLC and shown to be [$^{14}C_1$]GA_7 by full-scan GC-mass spectrometry. The specific radioactivity of the GA_7 from each incubation was determined by GC–mass spectrometry (Table 3). From the averaged dilution of the label the amount of endogenous (unlabelled) GA_7 was calculated to be 0.75 ng μl^{-1} in the fractionated enzyme preparation (equivalent to 1.05 $\mu g\, g^{-1}$ of starting endosperm). Using this amount of GA_7 as a basis and the total ion response for each GA calculated from the intensity of a selected strong ion across the GC peak, the amounts of the GAs present in the original and fractionated endosperm homogenate were

Table 2. GAs identified in endosperm and embryos from *Marah macrocarpus* seeds (maturity index 50–90)

GAs identified	Crude endosperm homogenate	Endosperm AMS supernatant	Endosperm AMS ppt.	Endosperm G-25 eluate	Crude embryo homogenate
GA_1	–	–	–	–	+
GA_3	+	–	–	–	+
GA_4	+	+	+	+	+
GA_4-diacid	+	+	–	–	+
GA_7	+	+	+	+	+
$isoGA_7$	+	+	+	+	+
GA_7-diacid	+	–	–	–	–
GA_{13}	–	–	–	–	+
GA_{25}	+	+	+	+	+
$2\beta,12\alpha$-$(OH)_2GA_{25}$*	+	+	+	+	–
GA_{37}	+	–	–	–	–
GA_{43}	+	+	–	–	+
$12\alpha OHGA_{43}$*,†	+	+	–	–	–
GA_{46}	+	+	+	+	+
GA_{62}	+	–	–	–	–
2β-$OHGA_{84}$*	+	–	–	–	+

*Tentative structures.

†First reported in endosperm [8] and embryo [9] of *Cucurbita maxima*.

Table 3. Endogenous GA_7 concentration in the endosperm enzyme preparation of *Marah macrocarpus* after ammonium sulphate precipitation and Sephadex G-25 filtration. Values were obtained from dilution of specific radioactivity after incubation with 2,3-dehydro-[17-$^{14}C_1$]GA_9 (1.75 TBq mol^{-1})

Enzyme preparation (μl)	Specific activity of GA_7 product (TBq mol^{-1})	GA_7 concentration (ng μl^{-1})
5	1.32	0.75
10	1.09	0.90
20	0.77	0.74
89	0.56	0.72
	Average	0.75*

*Equivalent to 1.05 μg g^{-1} of starting endosperm.

estimated. The results are shown in Table 4. Gibberellin A_7, GA_{46} and the putative 2β,12α-$(OH)_2GA_{25}$ were present in highest amounts, both in the unfractionated and fractionated homogenates. The amounts of GA_3 and GA_{62} were too low to be determined.

The unfractionated homogenate from the embryo contained the same GAs as the crude homogenate from the endosperm, except for the presence of GA_1 and GA_{13}, and the absence of GA_7-diacid, GA_{62} and the putative 2β,12α-$(OH)_2GA_{25}$ and 12α-$OHGA_{43}$. The amounts of the GAs were comparable to those found in the unfractionated endosperm, except for GA_3 and GA_{25} which were higher and GA_{13}, which was the most abundant. In contrast to the endosperm preparations, the GAs in the unfractionated embryo homogenate were completely removed by AMS precipitation, followed by filtration through Sephadex G-25.

Our results indicate that there are proteins present in the endosperm, but not in the embryo, that bind GA_4, GA_7, GA_{25}, GA_{46}, GA_{46} and the putative 2β,12α-dihydroxyGA_{25} and 2β-$OHGA_{84}$.

EXPERIMENTAL

Plant material. Seeds of *M. macrocarpus* Kellogg were collected from the Santa Monica Mountains, California. The embryos and endosperm from seeds of maturity index 50–90 [8] were frozen in liquid N_2 and stored at −80°.

Homogenates. Endosperm and embryos, ground in liquid N_2, were separately homogenised at 5° in a ground glass homogenizer with 0.1 M Tris buffer (1 ml g^{-1}, pH 7.3 at 20°), containing 4 mM DTT (This buffer was used throughout). Each homogenate was pelleted in liquid N_2 and stored at −80°.

Purification of homogenates from endosperm. To thawed pellets of the homogenate (8 ml ≡ 4 g original endosperm) at 5°, AMS (3.75 g, 70% satn) was added during 1 hr with stirring. After centrifugation at 36 000 *g* for 1 hr, the pellet was dissolved in buffer (4 ml). A portion (2 ml) of the soln of the AMS ppt. and AMS supernatant (20 ml) were pelletted in liquid N_2 and stored at −80°. The other 2 ml of the soln of the AMS ppt. was made up to 2.5 ml with buffer and desalted using a Sephadex G-25 column (NAP-25, Pharmacia), pre-equilibrated and eluted with the same buffer (3.5 ml). The protein fr. was pelleted in liquid N_2 and stored at −80°.

Purification of homogenates from embryos. Thawed pellets of crude homogenate (4 ml ≡ 2 g embryos) were subjected to AMS pptn and Sephadex G-25 filtration as described in the preceding section. The fractionated protein fr. was pelleted in liquid N_2 and stored at −80°.

Extraction. Aliquots, equivalent to 2 g tissue, were used from the crude homgenates, the soln of the AMS ppts, the AMS supernatants and Sephadex G-25 filtrates. Each of the 8 aliquots were extracted as follows. Thawed pellets were acidified to pH 2.5–3.0 with 2 N HCl and extracted with EtOAc (3 × equal vol.). The evapd extract, in H_2O–MeOH (4:1, 1 ml), was ex-

Table 4. Estimates* of the GA content in endosperm cell-free extracts before and after ammonium sulphate precipitation of protein and Sephadex G-25 filtration

GA	Crude homogenate (μg g^{-1} endosperm)	Sephadex G-25 filtrate (μg g^{-1} endosperm)
GA_4	10.3	0.17
GA_4-diacid	1.2	0
GA_7	65.0	1.05
isoGA_7	9.7	0.08
GA_7-diacid	1.57	0
GA_{25}	3.1	0.02
2β,12α-$(OH)_2GA_{25}$†	25.0	0.24
GA_{37}	1.9	0
GA_{43}	7.7	0
12α-$OHGA_{43}$†	2.2	0
GA_{46}	43.7	0.26
2β-$OHGA_{84}$†	5.9	0.27

*From the amount of GA_7 in the Sephadex filtrate, obtained by isotope dilution, and the total ion current response values, obtained from the intensity of a selected ion across the GC peak.

†Tentative structures.

tracted with *n*-hexane (3 × 1 ml), which was then discarded. The MeOH was evapd from the H_2O–MeOH soln, and the aq. soln made up to 1 ml and added to a Bond Elut C_{18} column (1 g). The column was eluted with H_2O (2 ml) at pH 3 and MeOH (2 × 2 ml). The MeOH eluates were combined and evapd to dryness. The residue, in EtOAc (1 ml), was washed with H_2O (3 × 1 ml) and evapd to dryness. The residue, dissolved in MeOH (200 μl), was methylated with CH_2N_2–Et_2O. The soln was evapd and the residue trimethylsilylated with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide, immediately before GC-MS.

Incubation of 2,3-dehydroGA$_9$ with fractionated enzyme preparation from endosperm. Endosperm prepn (10, 20, 50 and 89 μl), 5 μl 2,3-dehydro-[17-$^{14}C_1$]GA_9 (MacMillan *et al.*, unpublished) (31.4 ng, 1.75 TBq mol^{-1}, 10 000 dpm in 50% aq. MeOH) and 5 μl of co-factor soln, giving a final concn of 4 mM 2-oxoglutarate, 0.5 mM $FeSO_4$, 4 mM ascorbate, 4 mM DTT, 2 mg ml^{-1} BSA and 1 mg ml^{-1} catalase, were made up to 100 μl with Tris buffer (pH 7.3 at 20°). The mixt. was incubated at 30° for 1.5 hr with shaking in a water bath. HOAc was added and the mixt. was evapd to dryness. The residues from each incubation, in MeOH (200 μl), were methylated (CH_2N_2) and each product in 50% aq. MeOH (250 μl) was injected by an autosampler on to a guard column (RP-18, 5 μm, 3 × 0.21 cm), which was eluted with H_2O containing 50 μl HOAc l^{-1} (5 ml) for 5 min. By means of a switching device the guard column was connected to a C_{18}-column (5 μm, 250 × 0.46 cm) and an on-line, solid-scintillant, radioactivity monitor. The column was eluted with the following gradient of H_2O, containing 50 μl HOAc l^{-1} and MeOH: isocratic, MeOH-H_2O (3:1) for 15 min; exponential to 100% MeOH over 4 min; isocratic 100% MeOH for 6 min. The radioactive peak (R_t 12 min) was collected from each incubation and evapd to dryness. The residues were separately trimethylsilylated and analysed by GC-MS.

GC-MS. Derivatized samples were subjected to GC on a column (25 m × 0.2 mm) coated (0.10 μm) with BP1 (SGE). Samples were injected on to the column at 35° in the Grob splitless-mode and the injector purge was activated after 180 sec. The column temp. was taken rapidly to 60° and, after a 2 min isothermal hold, was programmed at 10° min^{-1} to 150° and then at 3° min^{-1} to 300° with a 10 min isothermal hold at the end of the programme. The pressure of the He carrier gas was 80 kPa. The column effluent was led directly to the ion source of a computerized MS with a source temp. of 220° and an interface temp. of 280°. The ionizing potential was 24 eV and the MS were recorded at 1 Hz.

Identification of gibberellins. Full-scan GC-MS was used; a mixt. of *n*-alkanes (C_{16}–C_{36}, *ca.* 1–15 ng of each) was co-injected with each sample to provide Kovats retention indices (KRIs) for each GC peak. The ^{14}C-specific radioactivities of GA_7 were determined [10] from the intensities of the $[M]^+$ ion cluster of the Me-TMSi derivative. GAs was identified by comparison of their KRIs and full-scan MS (Table 1) with published data [11].

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