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ENDOGENOUS GIBBERELLINS FROM CALLUS CULTURES OF MAIZE

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Abstract—This paper reports the identification of gibberellin A_1(GA_1), GA_{17}, GA_{19}, GA_{20}, GA_{29}, and GA_{53} from extracts of normal (wild-type) maize callus using full-scan GC-Mass Spectrometry and Kovats retention indices (KRIs). In addition, evidence is presented for the presence of GA_8 based on its molecular ion at the appropriate KRI. In a preliminary experiment, the presence of GA_3, GA_5, and GA_{44} was suggested, based on GC-selected ion monitoring (SIM); however, the presence of these GAs was not confirmed by full-scan GC-Mass Spectrometry. The information represents the first identification of GAs from maize callus. All of the identified GAs are members of the early-13-hydroxylation pathway, a biosynthetic pathway that leads to bioactive GA_1. GA_1, GA_8, GA_{19}, GA_{20}, and GA_{29} were quantified from GC-SIM isotopic dilution data. GA_{17} and GA_{53} were quantified from GC-SIM data by comparing the ion intensity from each gibberellin to a known amount of a GA_{19} standard. The levels of the identified GAs ranged from 6 pg g^{-1} fr. wt to 210 pg g^{-1} fr. wt.

INTRODUCTION

Gibberellins (GAs) are a class of cyclic diterpenes that act as plant hormones in the control of growth, especially shoot elongation (for reviews see [1, 2]). Gibberellins were originally isolated as natural products from the fungus, *Gibberella fujikuroi*, and later from a variety of higher plants. At present, 94 GAs have been identified, 28 from fungi (Ascomycetes) and 82 from vascular plants (Angiosperms, Gymnosperms and Pteridophytes) (for reviews see [3, 4]). The numbers and classes of GAs vary depending on the source of plant material, e.g. the species, variety (cultivar), as well as specific organ [5–8].

A combination of chemical and genetic studies suggest that GA-dependent shoot growth in maize (*Zea mays*), pea (*Pisum sativum*), and rape (*Brassica rapa*) is controlled primarily by the presence and levels of the specific gibberellin, GA_1, which originates from GA_{20} *via* the early-13-hydroxylation pathway [9–11]. For maize, 17 GAs have been identified from vegetative shoots, 11 of which are members of the early-13-hydroxylation pathway (Fig. 1) [11-14]. This report concerns maize callus (masses of undifferentiated cells grown in culture) and the possibility that GAs may control the growth of these cells. Two questions are addressed: (1) are GAs endogenous to maize callus and (2), if GAs are endogenous, what are the kinds and levels produced by the callus? The presence of endogenous GAs in maize callus could suggest that GAs control the growth of the cells of these cultures.

RESULTS

Two experiments were designed using callus cultures in the log-phase of growth. The results from the first experiment provided preliminary evidence for the presence of GAs; bioassay data demonstrated the presence of GA-like substances and GC-SIM data suggested the presence of specific GAs. In the second experiment, six GAs were identified by full-scan GC-mass spectrometry and KRIs; GC-SIM was used to quantify these GAs.

Experiment 1

Maize callus cultures were extracted with aqueous methanol. After evaporation to an aqueous phase and solvent partitioning, the acidic ethyl acetate (AE) fraction was divided into two equal portions, each of which was passed through an immunoaffinity chromatography (IAC) column [15] to give 10 fractions. Each fraction from the first portion was bioassayed using the dwarf rice assay [16] ; fractions 1–4 were inhibitory. Fractions 5–10 were bioactive (Fig. 2). All fractions from the second portion were combined, then methylated and trimethylsilylated. GC-SIM analyses of the combined fractions

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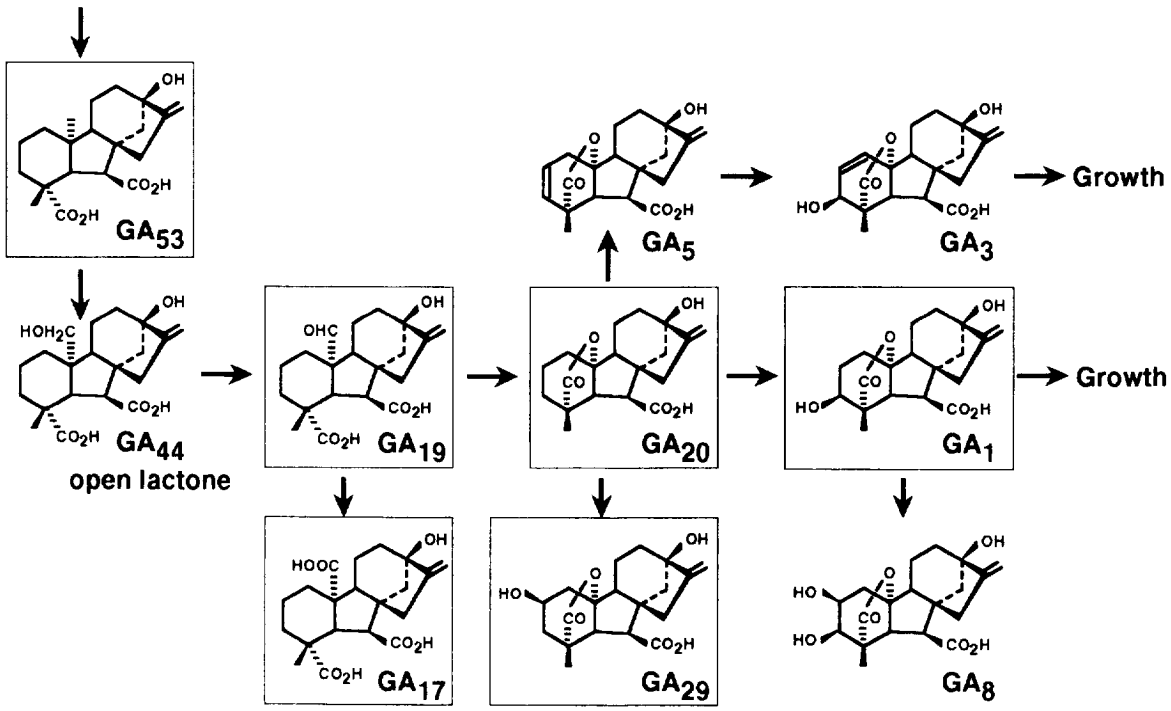

Fig. 1. Hypothetical early-13-hydroxylation gibberellin biosynthetic pathway for maize callus. GAs identified by full-scan GC-mass spectrometry are indicated by boxes.

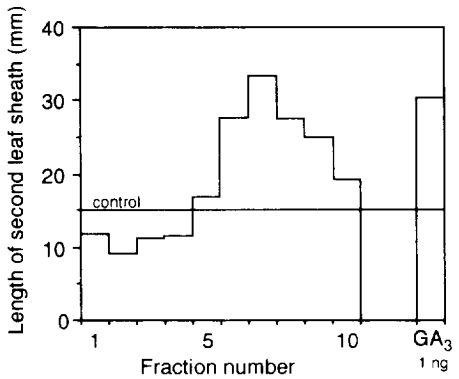

Fig. 2. Experiment 1: Response of the second leaf sheath of dwarf rice (cv. Tan-ginbozu) to immunoaffinity-purified maize callus extracts. Each column represents the average sheath length of six plants treated with the equivalent of 20.0 g fr. wt; the maximum standard error was ± 0.2 mm.

Table 1. Experiment 1: GC-SIM analysis of maize callus extract. Ions detected, their KRIs and GA assignment

GA*	KRI	Ion(s) m/z (response)†
GA_1	2668	506 (692); 448 (169)‡
GA_3	2691	504 (246)
GA_5	2479	416 (273)
GA_{17}	2575	492 (288); 432 (262)‡
GA_{19}	2596	434 (170)
GA_{20}	2483	418 (637)
GA_{29}	2683	506 (3453)
GA_{44}	2786	432 (281)

*The $[M]^+$ (for GA_{19}, $[M-28]^-$) was detected for each GA at the correct KRI. Assignments based on literature values [26].

† Absolute area under GC peak of ion monitored.

‡ Two major ions [26] were detected for GA_1 and GA_{17}.

showed the presence of the $[M]^+$ (for GA_{19}, $[M\text{-}28]^+$) at the appropriate KRIs for GA_1, GA_5, GA_{17}, GA_{19}, GA_{20}, GA_{29} and GA_{44} (Table 1).

For experiment 1, the presence of high levels of interfering ions precluded identification of specific GAs by full scan GC-mass spectrometry. In order to minimize the presence of these ions, a second experiment was designed having additional purification steps.

Experiment 2

The AE fraction containing labelled internal standards was passed through BondElut DEA, BondElut C18, reverse-phase HPLC and IAC columns. The radioactive fractions (6–16) from the IAC column were combined, then methylated and trimethylsilylated. A full scan mass spectrum at the appropriate KRI was obtained for GA_1, GA_{17}, GA_{19}, GA_{20}, GA_{29} and GA_{53} (Table 2); for GA_8, only the $[M]^+$ was observed at the appropriate KRI. No

Table 2. Experiment 2: endogenous GAs identified from maize callus by GC-Mass Spectrometry and KRIs*

GA	Source	KRI	Characteristic ion (m/z) and relative abundance							
GA_1	Sample	2676	507 (100)	506 (35)	492 (21)	491 (1)	449 (24)	448 (16)	208 (33)	207 (13)
	^{12}C standard	2676		506 (100)		491 (9)		448 (18)		207 (23)
	^{13}C standard	2676	507 (100)		492 (9)		449 (17)		208 (22)	
GA_{17}	Sample	2584	492 (29)		460 (18)		433 (24)		208 (100)	
	^{12}C standard	2583	492 (43)		460 (23)		433 (26)		208 (100)	
GA_{19}	Sample	2608	436 (40)	434 (100)	404 (1)	402 (27)	376 (49)	374 (76)	210 (24)	208 (33)
	^{12}C standard	2608		434 (100)		402 (37)		374 (64)		208 (32)
	$^{2}H_2$ standard	2608	436 (100)		404 (36)		376 (67)		210 (36)	
GA_{20}	Sample	2495	419 (100)	418 (46)	376 (47)	375 (42)	360 (13)	359 (31)	208 (70)	207 (31)
	^{12}C standard	2496		418 (100)		375 (46)		359 (12)		207 (30)
	^{13}C standard	2496	419 (100)		376 (56)		360 (17)		208 (37)	
GA_{29}	Sample	2690	507 (100)	506 (51)	492 (32)	491 (16)	304 (46)	303 (15)	208 (87)	207 (31)
	^{12}C standard	2690		506 (100)		491 (11)		303 (20)		207 (39)
	^{13}C standard	2690	507 (100)		492 (9)		304 (20)		208 (28)	
GA_{53}	Sample	2505	448 (41)		389 (18)		214 (17)		208 (100)	
	^{12}C standard	2505	448 (63)		389 (32)		241 (34)		208 (93)	

* KRI and relative abundance of the characteristic ion [26] for each GA was obtained from purified callus extracts. Values are compared to [^{12}C] standards, and, where internal standards were added, to either [^{13}C] standards (GA_1, GA_8, GA_{20}, GA_{29}) or [$^{2}H_2$] standards (GA_{19}).

Table 3. Experiment 2: quantitative analysis of GAs from maize callus by GC-SIM. Replicated analyses were made for all GAs except GA_{53}, GA_{44}, GA_{17} and GA_3

GA	Internal standard		Callus sample		
	Isotope ratio	Amount added (ng)	Isotope ratio	Fit*	Level calculated (ng 100 g^{-1})
GA_1	91.5 $^{13}C_1$:8.5 $^{13}C_0$	25	82.12 $^{13}C_1$:17.88 $^{13}C_0$	0.9657	0.57
		25	81.20 $^{13}C_1$:18.80 $^{13}C_0$	0.9727	0.63
GA_3	†	25	†	†	†
GA_8	92.0 $^{13}C_1$:8.0 $^{13}C_0$	25	76.16 $^{13}C_1$:22.94 $^{13}C_0$	0.9896	0.99
	—	25	77.33 $^{13}C_1$:22.67 $^{13}C_0$	0.9861	0.95
GA_{17}				—	21.69‡
GA_{19}	89.7 $^{2}H_2$:5.9 $^{2}H_0$	25	22.82 $^{2}H_2$:68.70 $^{2}H_0$	0.9905	13.21
		25	22.60 $^{2}H_2$:67.13 $^{2}H_0$	0.9884	13.03
		25	21.72 $^{2}H_2$:65.97 $^{2}H_0$	0.9934	13.33
GA_{20}	91.5 $^{13}C_1$:8.5 $^{13}C_0$	25	59.67 $^{13}C_1$:40.33 $^{13}C_0$	0.9942	2.67
		25	56.69 $^{13}C_1$:40.31 $^{13}C_0$	0.9944	2.83
GA_{29}	91.8 $^{13}C_1$:8.2 $^{13}C_0$	25	55.52 $^{13}C_1$:44.48 $^{13}C_0$	0.9718	3.27
		25	55.66 $^{13}C_1$:44.34 $^{13}C_0$	0.9861	3.25
GA_{44}	†	25	†	†	†
GA_{53}	—	—	—	—	8.55‡

* Defined in ref [26].

† Presence of interfering ions prevented the use of GA_3 and GA_{44} standards for quantification.

‡ Levels based on comparison of ion responses to GA_{19}.

evidence was obtained for the presence of endogenous GA_3, GA_5 or GA_{44}.

GA_1, GA_8, GA_{19}, GA_{20}, and GA_{29} were quantified from GC-SIM data by calculating the isotopic dilution of the added internal standards (Table 3). GA_{17} and GA_{53} were quantified from GC-SIM data by comparing their ion intensities with the ion intensity for the known amount of added GA_{19} internal standard. The GA levels ranged from 6 pg g^{-1} fresh weight for GA_1 to 217 pg g^{-1} fresh weight for GA_{17}.

DISCUSSION

Our first evidence for the presence of GAs in maize callus cultures came from the use of the rice seedling bioassay. In this assay, a putative GA-containing solu-

tion is added to the first seedling leaf; net elongation of the second leaf sheath is taken as evidence for bioactivity and, therefore, for the presence of GA-like substances [16]. Several fractions from the IAC column of experiment 1 exhibited bioactivity (Fig. 2) suggesting the presence of more than one GA. Specific GAs could not be identified from full scan GC-mass spectrometry because of the presence of ions from contaminating compounds present in the samples. However, using GC-SIM, the $[M]^+$ (for GA_{19} $[M-28]^+$) at the appropriate KRIs were detected for GA_1, GA_3, GA_5, GA_{17}, GA_{19}, GA_{20}, GA_{29}, and GA_{44} (Table 1). Since we base our definitive identification of specific GAs on the presence of at least three major ions, a second experiment was designed to identify specific GAs from full scan GC-mass spectrometry.

In experiment 2, the purification scheme was modified to omit the bioassay and to incorporate additional chromatographic steps to minimize contaminants, prior to GC-mass spectrometry. In addition, labelled GAs were added at the time of extraction as internal standards for quantification. Full scan mass spectra at the appropriate KRIs identified GA_1, GA_{17}, GA_{19}, GA_{20}, GA_{29}, and GA_{53} (Table 2). The levels of GA_1, GA_{17}, GA_{19}, GA_{20}, and GA_{53} (Table 3) were surprisingly low, being less than one tenth the levels found in intact vegetative tissues of maize [12]. Since the calli used in the experiments had been subcultured at least 10 times after isolation from the intact embryos, the identified GAs probably represent biosynthetic products produced by the callus, not carry-over GAs from the embryos.

Since the IAC columns selectively bind 13-hydroxy GAs [15], gibberellins lacking a 13-hydroxyl group, such as GA_4, GA_9 and their precursors, would have been lost during purification and, therefore, would not have been detected.

Although the data presented here represent the first identification of GAs from a monocotyledonous callus, GA_1, GA_4, and GA_7 have been isolated from both carrot (*Daucus carota*) and anise (*Pimpinella anisum*) calli [17], and GA_1, GA_8, GA_9, GA_{17}, GA_{18}, GA_{19}, GA_{20}, GA_{23}, GA_{44} and GA_{53}, from tobacco (*Nicotiana tabacum*) calli [18–20]. The levels were quantified by GC-SIM in the studies using tobacco calli and were similar to those we report here for maize callus.

The demonstration of the presence of GAs in maize callus has interesting biological implications in terms of the control of growth. Chemical and biochemical analyses of the vegetative shoots of a series of dwarf mutants in maize have shown that each mutant blocks a specific step in the early-13-hydroxylation pathway [11, 12, 21]. It would be interesting to analyze callus cultures derived from these mutants to see if the pattern and relative levels of endogenous GAs in mutant calli match those from the intact plant.

EXPERIMENTAL

Initiation and growth of callus. Inbred lines, A188 and B73, of normal maize (*Z. mays* L.) were grown in the field at the University of Minnesota, St. Paul. Crosses were made to A188 with B73 as the male parent. Ten to 12 days after pollination, the developing ears were removed, husked, immersed for 20 min in 2.5% NaOCl soln containing 50 μg ml^{-1} PEX detergent. The ears were rinsed ($\times$ 3) with sterile H_2O. Young embryos (1–2 mm in length) were dissected from the ear as follows: the crown was removed from each caryopsis and the endosperm from the pericarp. The embryos were transferred, embryo axis down, onto solid medium [22] supplemented with 2.9 g l^{-1} L-proline, 1 mg l^{-1} 2,4-D, 100 mg l^{-1} casein hydrolysate, and solidified with 2.5 g l^{-1} Gelrite. The isolated embryos were incubated in the dark at 28°. Friable calli developed from the scutellar end of the embryos within one month. Thereafter, portions of the callus from each embryo were subcultured every 7–10 days. The calli used in these studies had been subcultured at least 10 times. They were friable and embryogenic [22]. Calli were harvested every 7–14 days, immediately frozen in liquid N_2, and stored at − 80° until extraction.

Labelled GAs. [17-$^2H_2$] GA_{19} and [6α-$^2H_1$] GA_{44} were gifts from Prof. L. N. Mander. [17-$^{13}C^3H$] GA_1 (7.85 GBq mmol^{-1}), [17-$^{13}C^3H$] GA_8 (5.50 GBq mmol^{-1}), [17-$^{13}C^3H$] GA_{20} (7.08 GBq mmol^{-1}), and [17-$^{13}C^3H$] GA_{29} (5.88 GBq mmol^{-1}) were prepared by Fujioka *et al.* [12]; [17-$^{13}C^3H$] GA_3 (3.76 GBq mmol^{-1}) was a gift from Dr C. L. Willis. [1β, 2β, 3β-$^3H_3$] GA_{20} (1110 GBq mmol^{-1}) was obtained from Amersham International. All int. standards were at least 99.5% pure by GC-MS analysis.

Extraction of endogenous GAs. Exp 1. Comb. frozen calli (340 g) were homogenized in 80% MeOH (1.5 l) and stirred overnight at 4°. The homogenate was centrifuged (3500 rpm, 30 min, 4°); the supernatant was removed and stored at 4° while the pellet was resuspended in 80% MeOH (0.5 l) for 2 hr at 4° and recentrifuged. The two supernatants were comb., the MeOH evapd *in vacuo* at 35° and the resulting aq. residue was partitioned ($\times$ 2) with hexane (1/4 vol.), acidified to pH 3 with 6 N HCl and partitioned ($\times$ 5) with EtOAc (1/5 vol.). The EtOAc frs were comb., washed ($\times$ 2) with H_2O (1/10 vol.) and evap to dryness to give the AE fr. which was dissolved in K-Pi buffer (0.1 M, pH 7.2; 1.5 ml).

Half of the AE fr. was loaded onto a Sepharose-linked MAC-136 IAC column [15]. The column was washed with K-Pi buffer (0.1 M, pH 7.2; 10 ml) and then eluted with H_2O (flow rate 0.1 ml min^{-1}). Ten 1 ml frs were collected and bioassayed. The remainder of the AE fr. was similarly fractionated and the 10 frs were combined, methylated, trimethylsilylated and then analysed by GC-SIM. *Exp.* 2. Comb. frozen calli (500 g) were homogenized in 80% MeOH (1.5 l). Each of the int. standards (25 ng) [17-$^{13}C^3H$] GA_1, [17-$^{13}C^3H$] GA_3, [17-$^{13}C^3H$] GA_8, [17-$^2H_2$] GA_{19}, [17-$^{13}C^3H$] GA_{20}, [17-$^{13}C^3H$] GA_{29} and [6α-$^2H_1$] GA_{44} were added to the homogenate; [1β, 2β, 3β-$^3H_3$] GA_{20} (850 Bq) was also added to follow the GA-containing frs during the extraction and purification steps. The homogenate was stirred overnight at 4°, centrifuged (3500 rpm, 30 min, 4°) and the supernatant removed and stored at 4° while the pellet was resuspended in 80% MeOH (0.5 l) for 5 hr at 4° and recentrifuged. The supernatants were comb., the MeOH

evap *in vacuo* at 35° and the aq. residue was adjusted to pH 8 and a vol. of 100 ml with K-Pi buffer (250 mM, pH 8). PVP (20 g) was added and the mixt. stirred overnight at 4°. The slurry was filtered through a bed of Celite and the Celite washed (×2) with K-Pi buffer (250 mM, pH 8; 100 ml). The filtrate and washings were comb. and partitioned to give an AE fr. [12]. The AE fr. was dissolved in MeOH (500 μl) and loaded onto a BondElut-DEA solid phase ext. cloumn (500 mg) which was eluted as described in ref. [23]. Frs containing radioactivity were dried, redissolved in 10% MeOH (1 ml) and loaded onto a BondElut C18 column (1 g) [23]. Frs containing radioactivity were dried, redissolved in 50% MeOH (250 μl) and inj. onto a Nucleosil 5 μm C18 HPLC column (10 cm × 6 mm i.d.) [24]; 31 × 1.5 ml frs were collected. Frs 1–2 and 12–14 contained no radioactivity and were discarded. Frs 3–11 and 15–31, containing radioactivity, were comb. and passed through the IAC column (see Exp 1). After washing the column with K-Pi buffer (10 ml), the column was eluted with H_2O; 28 × 1 ml frs were collected. Frs containing radioactivity (6–16) were comb., methylated (CH_2N_2), trimethylsilylated (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) and then analysed by full-scan GC-MS for identification and by GC-SIM for quantification.

Bioassay. Callus exts were bioassayed for the presence of GA-like substances using a variation of the dwarf rice microdrop assay [16]. The bioassay was conducted under constant light (Phillips cool white, F48T12 CW/VHO; 80 μmol m^{-2} s^{-1}) at constant temp. (28°). The rice seed (*Oryza sativa* cv. Tan-ginbozu) was a gift from Dr M. Koshioka. Seeds were soaked in dist. H_2O for 24 hr, rinsed and soaked for an additional 24 hr. Germinating seed were selected for uniformity (coleoptile lengths of 1–2 mm) and planted upright in glass vials containing 1% agar (35 ml, 6 seedlings per vial). The vials were placed in a covered, clear plastic box and the seedlings grown for an additional 2 days. Each extract fr. to be bioassayed was dissolved in 50% EtOH; 1 μl from each fr. was added to one seedling, placing the aliquot between the first leaf and the coleoptile. After treatment, plants were grown for an additional 4 days and the length of the second leaf sheath of each seedling measured.

GC-MS and GC-SIM. The GC-MS instrument, the GC column and operating parameters used for analyses have been described previously [12, 25]. Quantitative data were determined as described in refs [12] and [26].

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