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Gibberellin A_3 Is Biosynthesized from Gibberellin A_{20} via Gibberellin A_5 in Shoots of *Zea mays* L.[1]

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ABSTRACT

[17-^{13}C,^{3}H]-Labeled gibberellin A_{20} (GA_{20}), GA_5, and GA_1 were fed to homozygous normal (+/+), heterozygous dominant dwarf (*D8*/+), and homozygous dominant dwarf (*D8*/*D8*) seedlings of *Zea mays* L. (maize). ^{13}C-Labeled GA_{29}, GA_8, GA_5, GA_1, and 3-epi-GA_1, as well as unmetabolized [^{13}C]GA_{20}, were identified by gas chromatography-selected ion monitoring (GC-SIM) from feeds of [17-^{13}C, ^{3}H]GA_{20} to all three genotypes. ^{13}C-Labeled GA_8 and 3-epi-G_1, as well as unmetabolized [^{13}C]GA_1, were identified by GC-SIM from feeds of [17-^{13}C, ^{3}H]GA_1 to all three genotypes. From feeds of [17-^{13}C, ^{3}H]GA_5, ^{13}C-labeled GA_3 and the GA_3-isolactone, as well as unmetabolized [^{13}C]GA_5, were identified by GC-SIM from +/+ and *D8*/*D8*, and by full scan GC-MS from *D8*/+. No evidence was found for the metabolism of [17-^{13}C, ^{3}H]GA_5 to [^{13}C]GA_1, either by full scan GC-mass spectrometry or by GC-SIM. The results demonstrate the presence in maize seedlings of three separate branches from GA_{20}, as follows: (a) $GA_{20} \rightarrow GA_1 \rightarrow GA_8$; (b) $GA_{20} \rightarrow GA_5 \rightarrow GA_3$; and (c) $GA_{20} \rightarrow GA_{29}$. The *in vivo* biogenesis of GA_3 from GA_5, as well as the origin of GA_5 from GA_{20}, are conclusively established for the first time in a higher plant (maize shoots).

Our previous studies on the endogenous GAs[3] in vegetative shoots of maize (*Zea mays* L.) seedlings have provided evidence for the presence of the early-13-hydroxylation pathway, leading to GA_1 (7, 16, 18). We have also reported on the natural occurrence of GA_5 and GA_3 in normal, heterozygous *Dwarf-8*, and homozygous *Dwarf-8* seedlings of maize (8) and of trace amounts (less than 0.2 ng/100 g fresh weight) of GA_5 in *dwarf-5*, *dwarf-3*, and *dwarf-2* seedlings of maize (7).

This paper describes metabolic studies with [17-^{13}C,^{3}H]-labeled GA_{20}, GA_5, and GA_1, using normal and *Dwarf-8* seedlings, undertaken to establish the biogenetic origin of GA_5 and GA_3 in maize shoots.

MATERIALS AND METHODS

Double-Labeled Substrates

The [17-^{13}C,^{3}H]GA_{20} (0.915 atoms ^{13}C molecule^{-1}, 1.79 GBq mmol^{-1}) was synthesized as described by Ingram *et al.* (11); the [17-^{13}C,^{3}H]GA_5 (0.915 atoms ^{13}C molecule^{-1}, 1.51 GBq mmol^{-1}) and [17-^{13}C,^{3}H]GA_1 (0.915 atoms ^{13}C molecule^{-1}, 2.11 GBq mmol^{-1}) were synthesized by Fujioka *et al.* (7). The purity of the three double-labeled substrates was determined by GC-MS to be at least 99.5%.

Plant Material

The *Zea mays* L. (maize) seedlings used in this study came from genetic stocks (F2) that segregated in the ratio of 1 +/+:2 *D8*/+:1 *D8*/*D8*. Seeds were soaked in water on November 16, 1987. After 24 h the seeds were planted in a vermiculite:soil (1:1) mixture in the UCLA greenhouse. One week later the seedlings were transplanted to 2 gallon plastic pots (4 plants per pot) containing a vermiculite:soil (1:1) mixture and grown under natural light in the greenhouse. Feeds were made on December 21, 1987.

Feeding, Extraction, and Purification

Each substrate, dissolved in 10 μL ethanol:H_2O (1:1, v/v), was injected into the basal part (about 1 cm above the ground) of 5-week-old seedlings. A total of five seedlings were used for each feed. The plants were harvested 12 h later by separating the shoots from the roots. The shoots were immediately frozen in dry ice, and the roots were discarded.

The frozen plant material was homogenized and extracted with MeOH:H_2O (4:1, v/v). After removal of the MeOH, the resulting aqueous residue was partitioned as described by Fujioka *et al.* (7) to give the AE, NE, and AB fractions and AQ. The AE fraction was purified on a column of DEAE Sephadex A-25 as described by Gräbner *et al.* (10). The column (25 mL) was eluted with 25 mL portions of the following solvents: MeOH, 0.25 N HOAc in MeOH, 0.5 N HOAc in MeOH, 0.75 N HOAc in MeOH, 1 N HOAc in MeOH, 3 N HOAc in MeOH, 5 N HOAc in MeOH, and 10

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[3] Abbreviations: GA, gibberellin; +/+, homozygous normal maize; *D8*/+, heterozygous *Dwarf-8* maize; *D8*/*D8*, homozygous *Dwarf-8* maize; GC-SIM, gas chromatography-selected ion monitoring; MeOH, methanol; EtOAc, ethyl acetate; HOAc, acetic acid; Rt, retention time; KRI, Kovats retention index; MeTMSi, methyl ester trimethylsilyl ether; AE, acidic EtOAc; NE, neutral EtOAc; AB, *n*-butanol; AQ, aqueous residue.

N HOAc in MeOH. The radioactive fractions were combined, concentrated, and finally purified by HPLC on a column (10 cm × 10 mm i.d.) of Nucleosil 5 $N(CH_3)_2$, eluted with 0.1% (v/v) HOAc in MeOH at a flow rate of 3 mL/min. Fractions were collected every 1 min. After counting an aliquot of each fraction, appropriate fractions were combined, derivatized, and examined by GC-MS.

GC-MS

The GC-MS instrumentation, GC columns, and major operating parameters used in this study have been described for full scan GC-MS by Gaskin *et al.* (9) and for GC-SIM by Fujioka *et al.* (7). The methods for calculating the isotope ratios in the M^+ ion clusters and the fit factors have also been described by Fujioka *et al.* (7).

RESULTS

The percentage recoveries of the radioactivity from the feeds of [17-^{13}C,^{3}H]GA_{20}, [17-^{13}C,^{3}H]GA_5, and [17-^{13}C,^{3}H]GA_1 to seedlings of +/+, *D8*/+, and *D8*/*D8* maize are shown in Table I. The results of GC-MS and GC-SIM analysis of the derivatized fractions from the Nucleosil NMe_2 chromatography of the AE fractions are given in Tables II, III, and IV.

From the feeds of [17-^{13}C,^{3}H]GA_{20} (Table II), [^{13}C]GA_{29}, [^{13}C]GA_8, [^{13}C]GA_1, and unmetabolized [^{13}C]GA_{20} were analyzed by GC-SIM of the M^+ ion cluster. Trace amounts of [^{13}C]GA_5 and 3-epi-[^{13}C]GA_1 were also detected. The [^{13}C]GA_5, which cooccurred with [^{13}C]GA_{29} in the same HPLC fraction, was identified by GC-SIM of the M^+ ion cluster at the correct Rt in all three genotypes. The intensities of the M^+ ion cluster of the metabolite, [^{13}C]GA_5, in the normal and homozygous *D8* seedlings were too low to permit an accurate determination of the isotope ratio. However, for the heterozygous *D8* seedlings it was possible to show that there was no measurable dilution of the [^{13}C] label. [^{13}C]GA_3 was not detected as a metabolite from any of the [17-^{13}C,^{3}H]GA_{20} feeds.

From the feeds of [17-^{13}C,^{3}H]GA_5 (Table III), [^{13}C]GA_3 and [^{13}C]GA_3 19,2-isomeric lactone were identified from all three genotypes by GC-SIM and Rt. (Pure GA_3-MeTMSi has been shown to be partially isomerized to the isomeric 19,2-lactone

Table I. *Recovery of Radioactivity from Feeds of [17-^{13}C,^{3}H]GA_{20}, [17-^{13}C,^{3}H]GA_5, and [17-^{13}C,^{3}H]GA_1 to Normal, Heterozygous Dwarf-8, and Homozygous Dwarf-8 seedlings of maize*

Genotype	MeOH ext.	AE	NE	AB	AQ
	(a) [17-^{13}C,^{3}H]GA_{20} feed (Total feed: 1.34×10^4 Bq, 2.49 μg)				
+/+	1.23×10^{4}Bq	0.75×10^{4}Bq	0.02×10^{4}Bq	0.43×10^{4}Bq	0.14×10^{4}Bq
	91.8%	55.9%	1.5%	32.3%	10.1%
		(56.0)[a]	(1.5)	(32.4)	(10.1)
D8/+	0.99×10^{4}Bq	0.70×10^{4}Bq	0.02×10^{4}Bq	0.21×10^{4}Bq	0.06×10^{4}Bq
	73.5%	51.9%	1.1%	15.3%	4.7%
		(71.1)	(1.5)	(21.0)	(6.4)
D8/*D8*	1.23×10^{4}Bq	1.08×10^{4}Bq	0.01×10^{4}Bq	0.15×10^{4}Bq	0.04×10^{4}Bq
	91.3%	80.6%	0.7%	11.1%	2.9%
		(84.6)	(0.7)	(11.6)	(3.0)
	(b) [17-^{13}C,^{3}H]GA_5 feed (Total feed: 1.32×10^4 Bq, 2.89 μg)				
+/+	1.26×10^{4}Bq	0.98×10^{4}Bq	0.01×10^{4}Bq	0.28×10^{4}Bq	0.10×10^{4}Bq
	95.8%	74.8%	0.9%	21.3%	7.5%
		(71.6)	(0.9)	(20.4)	(7.2)
D8/+	0.98×10^{4}Bq	0.81×10^{4}Bq	0.01×10^{4}Bq	0.14×10^{4}Bq	0.02×10^{4}Bq
	74.7%	61.2%	0.9%	10.3%	1.1%
		(83.3)	(1.2)	(14.0)	(1.5)
D8/*D8*	0.97×10^{4}Bq	0.78×10^{4}Bq	0.01×10^{4}Bq	0.16×10^{4}Bq	0.05×10^{4}Bq
	74.0%	59.3%	0.9%	11.9%	3.4%
		(78.5)	(1.2)	(15.8)	(4.5)
	(c) [17-^{13}C,^{3}H]GA_1 feed (Total feed: 1.26×10^4 Bq, 2.08 μg)				
+/+	1.10×10^{4}Bq	0.72×10^{4}Bq	0.02×10^{4}Bq	0.32×10^{4}Bq	0.09×10^{4}Bq
	87.1%	57.3%	1.6%	25.7%	7.2%
		(62.4)	(1.7)	(28.0)	(7.8)
D8/+	1.00×10^{4}Bq	0.75×10^{4}Bq	0.02×10^{4}Bq	0.2×10^{4}Bq	0.05×10^{4}Bq
	79.9%	59.5%	1.3%	15.8%	4.2%
		(73.6)	(1.6)	(19.6)	(5.2)
D8/*D8*	0.38×10^{4}Bq	0.24×10^{4}Bq	0.01×10^{4}Bq	0.08×10^{4}Bq	0.02×10^{4}Bq
	30.0%	19.2%	0.9%	6.0%	1.6%
		(61.3)	(3.2)	(21.7)	(5.8)

[a] Figures in parentheses = AE (ethyl acetate soluble) + NE (neutral ethyl acetate soluble) + AB (acidic butanol soluble) + AQ (aqueous residue) = 100.

Table II. *GC-SIM Analysis of Feeds of [^{13}C,^{3}H]GA_{20} to Normal, Heterozygous Dwarf-8, and Homozygous Dwarf-8 Seedlings of Maize*

Genotype	HPLC Rt	Radioactivity	Products	GC Rt[a]	Ions	^{12}C:^{13}C	Fit Factor[b]
	min	*Bq*		*min*			
+/+	12–15	155	GA_1	15:22	506–511	11.7:88.3	0.9864
			3-epi-GA_1	16:54	506–511	11.8:88.2	0.9944
	17–20	5013	GA_{20}	16:22	418–423	10.8:89.2	0.9688
			GA_8	13:41	594–599	21.7:78.3	0.9875
	20–29	707	GA_{29}	15:31	506–511	10.1:89.9	0.9962
			GA_5	16:17	417–420	[c]	
D8/+	12–15	358	GA_1	15:18	506–511	45.8:54.2	0.9939
			3-epi-GA_1	16:50	506–511	46.7:53.3	0.9938
	17–20	5107	GA_{20}	16:22	418–423	13.0:87.0	0.9870
			GA_8	13:42	594–599	63.0:37.0	0.9896
	20–29	388	GA_{29}	15:27	506–511	18.1:81.9	0.9880
			GA_5	16:11	416–421	8.3:91.7	0.9585
D8/*D8*	12–15	408	GA_1	15:20	506–511	34.7:65.3	0.9934
			3-epi-GA_1	16:52	506–511	33.4:66.6	0.9882
	17–20	8525	GA_{20}	16:17	418–423	11.2:88.8	0.9127
			GA_8	13:43	594–599	56.8:43.2	0.9836
	20–29	407	GA_{29}	15:29	506–511	14.0:86.0	0.9882
			GA_5	16:12	417–420	[c]	

[a] GC temperature program for GA_5 and GA_{20} from 180°C at 5°C per min; for GA_1, GA_{29}, and 3-epi-GA_1 from 200°C at 5°C per min. [b] See Fujioka *et al.* (7) for definition of Fit Factor. [c] M^+ ion cluster intensities too weak for accurate determination of isotope content.

under the GC-conditions used.) In the case of the *D8*/+ seedlings, [^{13}C]GA_3 and [^{13}C]GA_3 19,2-isomeric lactone were identified by full scan GC-MS and KRI data. Isotope dilution was observed.

From the feeds of [17-^{13}C,^{3}H]GA_1 (Table IV), [^{13}C]GA_8 and 3-epi-[^{13}C]GA_1 were identified. [^{13}C]GA_5 and GA_3 were not detected. For both the recovered GA_1 and the metabolite, GA_8, dilution of the [^{13}C] label was highest in homozygous *Dwarf-8* seedlings and lowest in normal seedlings.

DISCUSSION

The present studies together with previous work by Fujioka *et al.* (8) establish the three branches from GA_{20}, shown in Figure 1, for maize seedlings. Each step has been defined by feeding ^{13}C-labeled substrates followed by identification of the immediate product by GC-MS and KRI or GC-SIM and Rt. All of the six GAs shown in Figure 1 are native to maize seedlings.

Table III. *Analysis of Feeds of [17-^{13}C,^{3}H]GA_5 to Normal, Heterozygous Dwarf-8, and Homozygous Dwarf-8 Seedlings of Maize*

Genotype	HPLC Rt	Radioactivity	Products	GC Rt[a]	Ions	^{12}C:^{13}C	Fit Factor[b]
	min	*Bq*		*min*			
+/+	16–19	175	GA_3[c]	15:39	504–509	13.0:87.0	0.9855
			GA_3-isolactone[c]	14:56	504–509	13.5:86.5	0.9882
	22–28	6718	GA_5[c]	12:59	416–421	7.8:92.2	0.9882
D8/+	16–19	273	GA_3[d]	15:37	504–509	25.7:74.3	0.9676
			GA_3-isolactone[d]	14:56	504–509	23.3:76.7	0.9865
	22–28	5893	GA_5[d]	12:59	416–421	8.4:91.6	0.9904
D8/*D8*	16–19	192	GA_3[c]	15:37	504–509	25.7:74.3	0.9942
			GA_3-isolactone[c]	14:56	504–509	23.3:76.7	0.9813
	22–28	5928	GA_5[b]	13:03	416–421	8.1:91.9	0.9978

[a] GC temperature program from 200°C at 5°C per min. [b] See Fujioka *et al.* (7) for definition of Fit Factor. [c] Identification by GC-SIM on M^+ ion cluster at correct Rt. [d] Identification by full scan GC-MS and KRI: isoGA$_3$ (minor) KRI 2634, MS, m/z values (relative intensities) 505(M^+, 100%), 446(27), 370(15); GA_3 (major) KRI 2692, MS, m/z values (relative intensities) 505(M^+, 100%), 461(9), 446(14), 388(10), 371(26), 356(15), 312(13), 209(89), 208(39), 194(38), 193(36), and 168(29).

Table IV. *GC-SIM Analysis of Feeds of [17-^{13}C,^{3}H]GA$_1$ to Normal, Heterozygous Dwarf-8, and Homozygous Dwarf-8 Seedlings of Maize*

Genotype	HPLC Rt	Radioactivity	Products	GC Rt[a]	Ions	^{12}C:^{13}C	Fit Factor[b]
+/+	13–16	5305	GA$_1$	15:24	506–511	8.7:91.3	0.9927
			3-epi-GA$_1$	16:56	506–511	8.9:91.1	0.9855
	19–23	268	GA$_8$	13:55	594–599	10.3:89.7	0.9898
D8/+	13–16	5373	GA$_1$	15:23	506–511	16.8:83.2	0.9935
			3-epi-GA$_1$	16:55	506–511	16.3:83.7	0.9902
	18–22	257	GA$_8$	13:58	594–599	26.2:73.8	0.9894
D8/*D8*	13–16	1553	GA$_1$	15:21	506–511	22.5:77.5	0.9923
			3-epi-GA$_1$	16:54	506–511	21.5:78.5	0.9930
	18–22	37	GA$_8$	13:56	594–599	48.7:51.3	0.9942

[a] GA$_1$ and 3-epi-GA$_1$ GC temperature program from 200°C at 5°C per min.; GA$_8$ GC temperature program from 220°C at 5°C per min. [b] See Fujioka *et al.* (7) for definition of Fit Factor.

The 2β-hydroxylation of GA$_{20}$ to GA$_{29}$ and 3β-hydroxylation of GA$_{20}$ to GA$_1$, reported here, were previously established by Spray *et al.* (18) in defining the position of the *d1* block in maize. The 2β-hydroxylation step from GA$_1$ to GA$_8$ in maize was previously reported by Davies and Rappaport (3, 4) for normal and *d5* seedlings, based on radio-TLC and GC-Rt data. Using similar data, Rood *et al.* (17) reported the formation of GA$_8$ from GA$_{20}$ in hybrid maize seedlings. The metabolism of GA$_1$ to GA$_8$ is confirmed here by ^{13}C-labeling and GC-SIM.

The present work provides the first *in vivo* example of the conversion of GA$_{20}$ to GA$_5$ and excludes the alternative biogenesis of GA$_5$ from GA$_1$, at least in maize seedlings. The *in vitro* conversion of GA$_{20}$ to GA$_5$ has been observed previously by Kamiya *et al.* (12), Takahashi *et al.* (19), Kwak *et al.* (15), and Albone *et al.* (2), using cell-free systems from *Phaseolus vulgaris* L. seeds, in which GA$_1$ and GA$_5$ occur naturally.

Previous identifications of GA$_3$ in plants have been uncertain because of the possibility of extraneous contamination by the widespread use of GA$_3$ as a reference compound. The identification of endogenous GA$_3$ in *D8* seedlings by Fujioka *et al.* (8), together with the demonstration of the biogenetic origin of GA$_3$ in these seedlings, clearly establishes GA$_3$ as native to maize shoots. The present identification of [^{13}C]GA$_3$ as a metabolite of [17-^{13}C,^{3}H]GA$_5$ by GC-MS and KRI data, reported in this paper for maize seedlings, is the first definitive example of the formation of GA$_3$ from GA$_5$ in any plant system (see also the following paper by Albone *et al.* [1]). The data demonstrate that the [^{13}C] label from the substrate, [17-^{13}C,^{3}H]GA$_5$, appears in the metabolite, GA$_3$. Previous reports (5, 6, 13, 14) on the conversion of GA$_5$ to GA$_3$ have used [^{3}H]GA$_5$ as substrate where the tritium label could not be detected in the metabolite by GC-MS or GC-SIM.

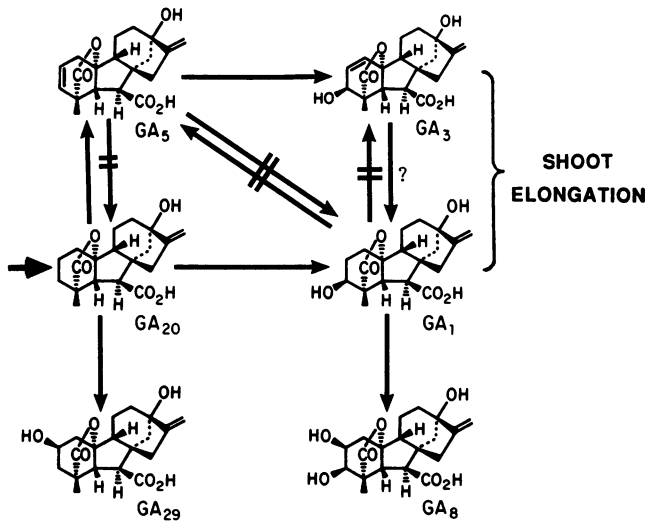

Figure 1. Later steps in the early-13-hydroxylation pathway in maize shoots. The plain arrow indicates observed metabolism in this study. The arrow with cross bars indicates absence of observed metabolism.

There is no evidence in this study for the metabolism of GA$_1$ to GA$_5$, of GA$_5$ to GA$_1$, or of GA$_1$ to GA$_3$. The possible, but improbable, conversion of GA$_3$ to GA$_1$ remains to be tested, *e.g.* using [17-^{13}C,^{3}H]GA$_3$. Thus, GA$_1$ and GA$_5$/GA$_3$ are members of separate branches from GA$_{20}$ as shown in Figure 1. Before the identification of the GA$_{20}$ → GA$_5$ → GA$_3$ branch pathway in maize seedlings, it was concluded by Phinney and Spray (16) that GA$_1$ was the primary, or only, native GA that was bioactive for stem elongation in maize. There is insufficient information available at present to assess the role of GA$_3$ in the control of stem elongation in maize. Finally, the 3-epi-[^{13}C]GA$_1$ formed in feeds of [17-^{13}C,^{3}H]GA$_{20}$ to normal, heterozygous *D8*, and homozygous *D8* seedlings may either be a true metabolite of GA$_{20}$ or an artifact from the [^{13}C]GA$_1$ also formed. This point is under investigation.

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