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THE METABOLISM OF GA_9 IN MAIZE (*ZEA MAYS*)

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Key Word Index—*Zea mays*; Graminaceae; *dwarf 5* mutant; *dwarf 1* mutant; gibberellins; metabolism; plant growth hormones; non-early 3,13-hydroxylation pathway; early 13-hydroxylation pathway; GA_9.

Abstract—[17-^{13}C,$^{3}H_2$]GA_9 was administered to 4-week-old seedlings of normal, *dwarf 1* and *dwarf 5* seedlings at the 3–4 leaf-stage. After 12 or 24 hr incubation, shoots were harvested, extracted with methanol and the extracts purified by solvent partitioning and HPLC. The resulting radiolabelled fractions were analysed by full-scan GC mass spectrometry with Kovats Retention Indices (R_I). The major metabolite was [^{13}C]GA_{20}, with [^{13}C]GA_{51}, [^{13}C]GA_{69}, [^{13}C]GA_{70}, [^{13}C]3-*epi*-GA_4 and [^{13}C]3-*epi*-GA_1 being identified as minor metabolites. The metabolism of GA_9 to GA_{20} demonstrates the possibility of late convergence of the two biosynthetic branch pathways, the presumptive non-early 3,13-hydroxylation pathway and the early 13-hydroxylation pathway, both of which originate from GA_{12}. There was no evidence for the metabolism of GA_9 to GA_1, GA_4, GA_5, GA_3, GA_7, or 2,3-dehydro-GA_9. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Gibberellins (GAs) are a class of cyclic diterpenes that are of widespread occurrence in the plant kingdom. They were originally isolated from the fungus *Gibberella fujikuroi* [1–3], and shown to be present in higher plants more than 15 years later [4–6]; 108 GAs have now been chemically identified as native to higher plants and fungi [7]. Biological studies with plants suggest that gibberellins act as plant hormones, controlling a wide variety of responses, e.g. shoot elongation in dwarf mutants [8] and the release of α-amylase during seed germination [9]. The chemistry and biochemistry of these interesting compounds have been investigated extensively [10, 11] and the molecular biology associated with GA formation is now playing an increasing role in the analysis of the regulation of plant growth [12, 13].

The first evidence for the biosynthetic origin of GAs came from studies with the fungus, as the first organism shown to have these compounds and because the organism produced relatively large amounts of GAs which accumulated in the medium [14]. The results from these early studies served as models for similar studies in higher plants. GA-mutants have played an important role in unravelling the details of the pathways for both the fungus [15] and higher plants [16]. In the fungus it was shown that GAs originate from MVA via the standard isoprenoid biosynthetic pathway, a pathway that leads to the tetracyclic diterpene, *ent*-kaurene. *ent*-Kaurene is then stepwise oxidized with rearrangement of the B-ring to give GA_{12}-aldehyde, the common precursor for all known gibberellins. Two independent fungal pathways diverge from GA_{12}-aldehyde to give a series of C-20- and C-19-gibberellins. They are the early 3,13-hydroxylation pathway and the non-early 3,13-hydroxylation pathway. The subsequent identification of GAs in higher plants [4–6] led to a series of gibberellins not present in the fungus; these GAs have been shown to be members of a third branch pathway from GA_{12}-aldehyde, namely the early 13-hydroxylation pathway. It is now apparent that, depending on the species, higher plants have at least one to three branches, each originating from GA_{12}-aldehyde [11]. While the metabolic evidence from intact plants suggests independence of the three pathways, pathway convergence has been shown in the late steps, GA_9 to GA_{20} [17–19], GA_4 to GA_1 [17,19,20–22] and GA_9 to GA_4[17, 19, 23]. In contrast, studies with cell-free systems from pea, bean and pumpkin show metabolic crossover at several points early in the three branch pathways from GA_{12}-aldehyde [24–28] to give a grid that interconnects the three

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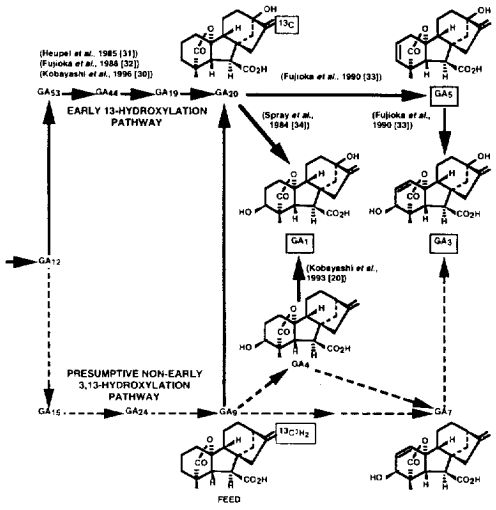

Fig. 1. Metabolism of [17-^{13}C,^{3}H$_2$]GA$_9$ to [^{13}C]GA$_{20}$ in relation to the early 13-hydroxylation and presumptive non-early 3,13-hydroxylation pathways of maize. Solid arrows (⟶) are defined steps; dotted arrows (– →) are presumptive steps.

pathways. Information on the GA biosynthetic pathways has been detailed in a recent review by MacMillan [11].

In maize, 17 GAs have been identified as endogenous to vegetative shoots [29]; metabolic studies of these GAs have demonstrated the presence of eight sequential steps that belong to the early 13-hydroxylation pathway, the pathway that leads to the bioactive gibberellins, GA$_1$, GA$_3$ and GA$_5$ [30] (Fig. 1; [20, 30, 34]). In addition to the early 13-hydroxylation pathway in maize, the presence of a minor and parallel pathway can be presumed, based on the identification of GA$_{15}$, GA$_{24}$, GA$_9$, GA$_4$ and GA$_7$ as naturally occurring in vegetative shoots [29]. These gibberellins are members of the non-early 3,13-hydroxylation pathway involving the sequential steps: GA$_{12}$-aldehyde to GA$_{12}$, to GA$_{15}$, to GA$_{24}$, to GA$_9$ (Fig. 1). GA$_9$ may be converted via 2,3-dehydro GA$_9$ to GA$_7$ and/or 13-hydroxylated to give GA$_{20}$. We are testing this hypothesis and report here on the metabolism of GA$_9$ in vegetative shoots of maize.

RESULTS AND DISCUSSION

Seedlings of the 2 mutants, *dwarf 1* (*d1*) and *Dwarf 5* (*d5*) and wild-type plants were used in the studies reported here. Both mutants are recessive and non-allelic to each other [35]. The *d5* mutant blocks a step early in the GA-biosynthetic pathway, the cyclization of copalyl pyrophosphate to *ent*-kaurene [36]; the *d1* mutant blocks 3 steps late in the pathway, the oxidation of GA$_{20}$ to GA$_1$, GA$_{20}$ to GA$_5$ and GA$_5$ to GA$_3$ [37]. Endogenous GAs are low in the wild-type, and absent (or present in trace amounts) in the *d5* mutant [32]. In the *d1* mutant, GA$_5$, GA$_3$ and GA$_1$ are absent (or present in trace amounts) and GA$_{20}$ accumulates to levels more than 10 times that found in normals [32].

In this study—all three kinds of maize seedlings (*d1*, *d5* and normal) were used in order to test the effects of different levels of endogenous GAs accumulating following metabolism of [17-^{13}C,^{3}H$_2$], GA$_9$, that had previously been administered to maize seedlings by injection. After 12 or 24 hr metabolism, each group of seedlings was extracted with aqueous methanol and the extract was partitioned (see Experimental) to give an ethyl acetate-soluble acidic fraction. Following purification of the compounds of interest by HPLC, these were combined, and after methylation and trimethylsilylation, each was analysed by full-scan GC-mass spectrometry with Kovats Retention Indices (R_I). The recovered radioactivities and the GC-mass specgtroscopic data for the identification of the hydroxylated GA-metabolites are shown in Tables 1 and 2.

Table 1. Analysis of metabolites following administration of [17-^{13}C,^{3}H$_2$]GA$_9$* (1620 Bq/seedling) to normal, *d1* and *d5* seedlings of maize

Plant material	ODS-HPLC fraction	N(CH$_3$)2-HPLC fraction	Radioactivity Bq	Products†
Normal	15–16	11–15	652	GA$_{70}$
(58.6 g)	17–21	12–15	1930	GA$_{20}$
	22–25	12–15	335	3-*epi*-GA$_4$, GA$_{51}$
d1	9–12	14–18	83	3-*epi*-GA$_1$, GA$_{69}$
(28.0 g)	15–16	9–15	318	GA$_{70}$
	17–21	12–15	2150	GA$_{20}$
d5	13–14	14–19	655	GA$_{69}$
(29.8 g)	15–16	11–15	277	GA$_{70}$
	17–21	12–15	2650	GA$_{20}$
	22–25	12–15	483	3-*epi*-GA$_4$, GA$_{51}$

*The amounts of [17-^{13}C]GA$_9$ recovered from the seedlings following precursor administration were: 2,330 Bq for normal, 4,860 Bq for *d1* and 840 Bq for *d5*.

†Identified by comparison with reference spectra and KRI of unlabelled compounds.

Table 2. Representatives GC-MS and R_I data used for the identification of GA metabolites (listed in Table 1) following, administration of [17-^{13}C,$^{3}H_2$]GA_9 to maize*

GA metabolite/ Reference Compound [ref.]	R_I		Ions (mass/intensity [Int.])											
[^{13}C]3-*epi*-GA_1	2783	Mass	208	377	417	432	449	460	479	492	507			
		Int.	(65)	(31)	(9)	(11)	(34)	(12)	(8)	(9)	(100)			
3-*epi*-GA_1 ref.	2788	Mass	207	376	416	431	448	459	478	491	506			
		Int.	(55)	(28)	(6)	(8)	(31)	(7)	(3)	(6)	(100)			
[^{13}C]3-*epi*-GA_4	2619	Mass	129	226	234	262	290	301	329	344	372	387	391	419
		Int.	(79)	(63)	(69)	(48)	(100)	(29)	(22)	(31)	(28)	(30)	(23)	(30)
3-*epi*-GA_4 ref.	2623	Mass	129	225	233	261	289	300	328	343	371	386	390	418
		Int.	(67)	(41)	(44)	(26)	(100)	(10)	(10)	(15)	(22)	(15)	(5)	(18)
[^{13}C]GA_{20}	2478	Mass	208	236	302	360	376	404	419					
		Int.	(30)	(13)	(15)	(18)	(62)	(11)	(100)					
GA_{20} ref.	2482	Mass	207	235	301	359	375	403	418					
		Int.	(30)	(8)	(12)	(12)	(46)	(16)	(100)					
[^{13}C]GA_{51}	2518	Mass	226	242	268	285	298	329	344	387	404	419		
		Int.	(100)	(24)	(81)	(94)	(21)	(23)	(6)	(24)	(5)	(2)		
GA_{51} ref.	2519	Mass	225	241	268	284	297	328	343	386	403	418		
		Int.	(95)	(40)	(84)	(100)	(32)	(36)	(10)	(32)	(8)	(3)		
[^{13}C]GA_{69}	2493	Mass	224	226	269	283	297	329	359	373	387	404	419	
		Int.	(100)	(43)	(94)	(53)	(82)	(41)	(24)	(22)	(17)	(23)	(19)	
GA_{69} ref.	2497	Mass	223	225	268	282	296	328	358	372	386	403	418	
		Int.	(89)	(35)	(100)	(44)	(85)	(31)	(19)	(17)	(12)	(14)	(13)	
[^{13}C]GA_{70}	2546	Mass	224	226	252	269	283	297	329	359	373	387	419	
		Int.	(100)	(42)	(24)	(86)	(40)	(61)	(22)	(15)	(15)	(9)	(10)	
GA_{70} ref.	2549	Mass	223	225	251	268	282	296	328	358	372	386	418	
		Int.	(96)	(41)	(28)	(100)	(48)	(86)	(32)	(20)	(19)	(14)	(13)	

*The discrepancies between the R_I values for the metabolites and reference standards are due to batch-to-batch variations in the GC columns used.

On the basis of radioactivity, the major metabolite from the three kinds of seedlings was [^{13}C]GA_{20}. The highest level was from the *d5* mutant which might be expected due to the absence of endogenous GA_{20} in that mutant. The high level of endogenous GA_{20} in the *d1* mutant had little effect on the level of the metabolite, GA_{20}, when compared to the wild-type. This is an interesting observation since feed-back control of GA biosynthesis has been reported in maize seedlings [38]. Our results clearly show that the presumptive non-early 3,13-hydroxylation pathway and the early 13-hydroxylation pathway converge via the hydroxylation of GA_9 to GA_{20}. The importance of this convergence is that it limits the number of 'bioactive' GAs, which in turn reduces the number of maize GA receptors that control GA-dependent growth. Our data support the results from an earlier small-scale feed of GA_9.

The three minor metabolites, [^{13}C]GA_{51}, [^{13}C]GA_{69}, and [^{13}C]GA_{70}, have not been identified as native to maize. They may have escaped identification in previous studies because of their very low endogenous levels. The other two minor metabolites, [^{13}C]3-*epi*-GA_4 and [^{13}C]3-*epi*-GA_1, have two possible origins. First, they may be artefacts, formed from the epimerization of the preformed metabolites, [^{13}C]GA_4 and [^{13}C]GA_1 as has been previously shown for 3-*epi*-GA_4 in maize [39]. If true, it is surprising that [^{13}C]GA_4 and [^{13}C]GA_1 were not detected as metabolites from these experiments. Secondly, and more likely, the two epimers could originate from the 3α-hydroxylation of GA_9 to 3-*epi*-GA_4, followed by 13-hydroxylation to 3-*epi*-GA_1. The latter explanation is favoured since the non-specific hydroxylation of the substrate, GA_9, is indicated by its metabolism to GA_{51} (2β-hydroxylation), GA_{69} (12β-hydroxylation), GA_{70} (12α-hydroxylation) and GA_{20} (13α-hydroxylation). In addition, the possible formation of GA_{40} (2α-hydroxylation) and GA_{34} (2β- and 3β-hydroxylation) was suggested by the presence of the correct R_f values. However, the mass spectra for these presumptive metabolites was ill-defined. We offer no explanation for the the presence of labelled 3-*epi*-GA_1 in the *d1* maize seedlings that had metabolised [17-^{13}C,^{3}H$_2$]GA_9; this metabolite was not identified from either normal or *d5* seedlings.

EXPERIMENTAL

Plant Material. Tall (wild-type), *d1* and *d5* maize (*Zea mays*) seedlings were used at the 3–4 leaf stage (4 weeks after germination). The plants were grown in the greenhouse.

Radiolabelled Substrate. [17-^{13}C,^{3}H$_2$]GA_9 (sp. act. 1.36 GBq mmol^{-1}) was obtained by NaOH hydrolysis of the methyl ester of [17-^{13}C,^{3}H$_2$]GA_9. The [17-^{13}C,^{3}H$_2$]GA_9 was freed of any radioactive contaminants by sequential HPLC purification with ODS (C_{18}) and NMe_2HPLC columns. The purified substrate used in the studies reported here was determined to be chemically pure by GC mass spectrometry.

Precursor Administration, Extraction and Purification. An initial small-scale precursor administration experiment (results not shown) was performed on 10 seedlings of the *d5* genotype with an incubation time of 12 hr. Subsequently, this was scaled up to 30 seedlings each of wild-type, *d5* and *d1* genotypes, with an incubation time of 24 hr (results in Tables 1 and 2). Each seedling was injected in the coleoptilar node with 2 μL of a MeOH soln containing [17-^{13}C,^{3}H$_2$]GA_9 (1.62×10^3 Bq, 1.36 GBq mmol^{-1}, 377 nanograms) using a microsyringe equipped with a 26S gaugeneedle (Hamilton 701 Microliter). After incubation, the seedlings were harvested, frozen with dry ice, and stored at -80°. The tissues were powdered in dry ice with a mortar and pestle, extracted $\times 2$ with MeOH–H_2O soln (4:1) using 10 ml per gram of tissue. After filtration, the MeOH was removed by rotary evapn with the aq. residue sequentially partitioned (EtOAc-H_2O) into H_2O-soluble (AQ), neutral EtOAc-soluble (NE), acidic EtOAc-soluble (AE), neutral *n*-butanol-soluble (NB) and acidic *n*-butanol-soluble (AB), respectivly as previously described [20]. In this respect, the MeOH extracts were combined for each genotype and concentrated *in vacuo* to give 3 aq. residues of 60 ml each. (Recoveries ranged from 90 to 100% depending on the experiment). Each aq. residue was partitioned to give, the 5 frs, NE, AE, AB, NB and AQ frs (combined recoveries ranged from 75 to 81%). Each AE fr. was purified further by sequential chromatographic steps employing Bond/Elute C-18, Bond/Elute DEA (with Sephadex A-25 overlay), ODS (C-18) HPLC, and NMe_2 HPLC columns as previously described [20, 37]. Radioactive frs were combined based on the R_fs of standards, and further purified by HPLC (Nucleosil 5 NMe_2 column). Radioactive frs were selected by scintillation counting, combined and analysed by GC-mass spectrometry.

GC Mass Spectrometry. All samples were methylated and analysed by GC mass spectrometry as described [20, 37, 40].

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