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PLANT-LIKE BIOSYNTHESIS OF GIBBERELLIN A_1 IN THE FUNGUS *PHAEOSPHAERIA* SP. L487

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Key Word Index—*Phaeosphaeria* sp. L487; Loculoascomycete; fungus; biosynthesis; feeding study; gibberellin A_1.

Abstract—Gibberellin A_{12}(GA_{12}), GA_{15} and GA_{20} were identified by full scan GC–MS from a culture filtrate of *Phaeosphaeria* sp. L487, in addition to the previously identified GAs, GA_1, GA_4, GA_9, GA_{24}, and GA_{25}. The early-non-hydroxylation pathway from GA_{12}-7-aldehyde to GA_1 was shown to be present in the fungus by feeding studies of [1,2-$^3H_2$]GA_4, [17-^{14}C]GA_9, [17-^{13}C,^{3}H]GA_{12}-7-aldehyde and [17-^{13}C,^{3}H]GA_{20}. The fungus metabolized GA_9 to GA_1 via GA_4 and GA_{20}. Comparison of the conversion of GA_4 and GA_{20} to GA_1 based on HPLC analysis indicated that the conversion of GA_4 to GA_1 was more effective than that of GA_{20} to GA_1. The pathway from GA_9 to GA_1 via GA_4 and GA_{20} is novel in microorganisms and like that of some higher plants.

INTRODUCTION

Several species of fungi have been shown to produce gibberellins (GAs) [1]. In particular, *Gibberella fujikuroi* and *Sphaceloma manihoticola* produce large amounts of GAs [2, 3]. In 1989 we found a new species (strain code: L487) of *Phaeosphaeria* (Loculoascomycete) which produces GA_4 (**11**) and GA_9 (**13**), but the production of GA_4 and GA_9 was less than 1 mg l^{-1} [4]. The fungus is in a taxonomically different class from *G. fujikuroi* (Euascomycete) and *S. manihoticola* (Deuteromycete) [5]. The conversion of GA_9 to GA_4 in *Phaeosphaeria* sp. L487 was demonstrated by feeding of [17-$^2H_2$]GA_9 [6]. Recently, we have found that the fungus extracellularly produces large amounts of GA_1 (*ca* 50 mg l^{-1}) (**10**) by improvement of fermentation conditions but no detectable GA_3 (**15**). At the same time, we have shown the pathway from GA_9 to GA_1 via GA_4 by feeding studies of [17-$^2H_2$]GA_9 and [17-$^2H_2$]GA_4 [7]. In our recent paper, GA_{24} (**6**) and GA_{25} (**7**) were also isolated and identified from a culture filtrate of *Phaeosphaeria* sp. L487 [8]. We have isolated and identified GA_1, GA_4, GA_9, GA_{24} and GA_{25} from a culture filtrate of *Phaeosphaeria* sp. L487 grown in a GA production medium (YM medium) mainly consisting of yeast extract powder and maltose. We now report a reexamination of the identification of endogenous GA from culture filtrate of the fungus and elucidation of the GA biosynthetic pathway by feedings of [1,2-$^3H_2$]GA_4, [17-^{14}C]GA_9, [17-^{13}C,^{3}H]GA_{12}-7-aldehyde and [17-^{13}C,^{3}H]GA_{20}.

RESULTS AND DISCUSSION

To elucidate the GA biosynthetic pathways of *Phaeosphaeria* sp. L487, we carefully analysed minor GAs from the culture filtrate. The fungus was cultured in a chemically defined medium (CD medium) which does not contain a nitrogen source to allow the accumulation of GA_1 precursors. Well-developed mycelia of *Phaeosphaeria* sp. L487 from YM medium were harvested by filtration and then resuspended in the CD medium. The growth of the mycelia in the CD medium was slow, and the production of GA_1 was much lower than in the YM medium. After 24 hr of incubation, GA_{12} (**2**), GA_{15} (**4**), and GA_{20} (**14**) were identified from the ethylacetate-soluble acidic extracts by full scan GC–MS in addition to the previously identified major GAs. The MS data of the identified GAs and their relative amounts are shown in Table 1. Compared with GA_4, the relative amounts of GA_{12}, GA_{15} and GA_{20} isolated after the 24 hr incubation were 7, 2 and 4%, respectively. By contrast, minor GAs such as GA_{12} and GA_{15}, could not be detected from the culture filtrate of the YM medium. Since the YM medium is more favourable for growth, intermediates of GAs were probably metabolized to GA_4 and GA_1 effectively. With the exception of GA_{20}, all GAs identified from *Phaeosphaeria* sp. L487 belong to the early-non-hydroxylation pathway of GA biosynthesis [9]. GA_{12}-7-aldehyde (**1**),

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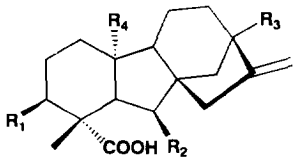
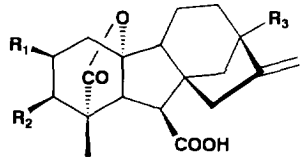
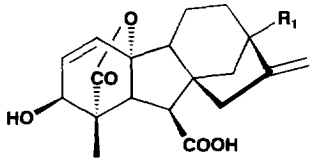


	R_1	R_2	R_3	R_4	
(1)	H	CHO	H	CH_3	GA_{12}-7-aldehyde
(2)	H	COOH	H	CH_3	GA_{12}
(3)	OH	CHO	H	CH_3	GA_{14}-7-aldehyde
(4)	H	COOH	H	CH_2OH	GA_{15} (open lactone)
(5)	H	COOH	OH	CHO	GA_{19}
(6)	H	COOH	H	CHO	GA_{24}
(7)	H	COOH	H	COOH	GA_{25}
(8)	H	COOH	OH	CH_2OH	GA_{44} (open lactone)
(9)	H	COOH	OH	CH_3	GA_{53}





	R_1	R_2	R_3	
(10)	H	OH	OH	GA_1
(11)	H	OH	H	GA_4
(12)	OH	OH	OH	GA_8
(13)	H	H	H	GA_9
(14)	H	H	OH	GA_{20}

(15) R_1=OH GA_3

which is a common intermediate in GA biosynthesis of both higher plants and fungi, was not isolated from culture filtrates of the fungus.

In order to elucidate the biosynthetic pathway from GA_{12}-7-aldehyde to GA_1 in *Phaeosphaeria* sp. L487, [17-^{13}C,^{3}H]GA_{12}-7-aldehyde was added to the culture. Hedden *et al.* demonstrated that cultures of *G. fujikuroi* containing plant growth retardants were useful for feeding studies in GA biosynthesis [10]. The effects of several plant growth retardants on GA production in *G. fujikuroi* and *S. manihoticola* were reported by Rademacher [11]. Uniconazole was used for our feeding studies because it inhibits the oxidation of *ent*-kaurene, a precursor of GA_{12}-7-aldehyde [12]. Uniconazole significantly inhibited GA_1 production in *Phaeosphaeria* sp. L487 at 10^{-4}–10^{-6} M. [17-^{13}C,^{3}H]GA_{12}-7-aldehyde was added to the CD medium containing the mycelia and 10^{-4} M uniconazole. The metabolites from the 18 and 66 hr incubations were purified by HPLC and analysed by full scan GC–MS (Table 2). [^{13}C]GA_4, [^{13}C]GA_9, [^{13}C]GA_{12}, [^{13}C]GA_{15}, [^{13}C]GA_{24} and [^{13}C]GA_{25} were identified as metabolites from the 18 hr incubation. From the 66 hr incubation, GAs in the early steps of GA biosynthesis, GA_{12} and GA_{15}, were not detected. By contrast, GAs from late steps of GA biosynthesis, such as [^{13}C]GA_1 and [^{13}C]Ga_{20} were identified. No polar GAs other than GA_1, e.g. GA_8 (**12**), could be detected by HPLC analysis. This is consistent with the finding that

Table 1. Gibberellins identified from a culture filtrate of *Phaeosphaeria* sp. L487 by GC–MS

GAs	Deriv. form	KRI	MS ion peaks (*m/z*, rel. int. %)	Relative amount (%)
GA_4^*	Me-TMSi	2533	418 [M]† (11), 328 (17), 284 (67), 233 (41), 225 (100)	100
GA_1^*	Me-TMSi	2682	506 $[M]^+$ (100), 491 (8), 448 (21), 376 (21), 207 (90)	53
GA_{24}^*	Me	2477	374 $[M]^+$ (2), 342 (10), 314 (84), 286 (44), 226 (100)	35
GA_{25}^*	Me	2474	404 $[M]^+$ (1), 372 (19), 312 (70), 284 (84), 225 (100)	
GA_9^*	Me	2346	330 $[M]^+$ (7), 298 (97), 270 (100), 243 (83), 227 (73)	34
GA_{12}	Me	2371	360 $[M]^+$ (7), 328 (20), 300 (100), 285 (22), 241 (37)	7
GA_{20}	Me-TMSi	2509	418 $[M]^+$ (100), 403 (17), 375 (70), 359 (20), 207 (59)	4
GA_{15}	Me	2653	344 $[M]^+$ (17), 312 (16), 298 (15), 284 (71), 239 (100)	2

GAs* were previously identified (see refs [5] and [7]). The relative amount of each GA is expressed as a percentage of GA_4. These data were obtained from the extracts cultured with a chemically defined medium for 24 hr at 28°.

Table 2. Metabolites of [17-^{13}C,^{3}H]GA_{12}-7-aldehyde identified by HPLC and GC–MS from *Phaeosphaeria* sp. L487

Metabolites	HPLC R_t (min)	Radioactivity (kBq)	MS (*m/z*, rel. int. %)	Isotope composition $^{13}C:^{12}C$
18hr-incubation				
[^{13}C,^{3}H]GA_4	31–32	0.5	419 $[M]^-$ (5), 329 (12), 285 (58), 234 (30), 225 (100)	55:45
[^{13}C,^{3}H]GA_{24}	33–34	0.7	375 $[M]^+$ (1), 343 (14), 315 (50), 287 (43), 226 (100)	89:11
[^{13}C,^{3}H]GA_9			331 $[M]^+$ (8), 299 (100), 271 (96), 243 (81), 227 (95)	89:11
[^{13}C,^{3}H]GA_{15}	34–35	2.1	345 $[M]^+$ (11), 313 (13), 299 (7), 285 (38), 240 (100)	90:10
[^{13}C,^{3}H]GA_{25}			405 $[M]^+$ (2), 373 (12), 313 (56), 285 (91), 226 (100)	71:29
[^{13}C,^{3}H]GA_{12}	39–40	2.8	361 $[M]^+$ (1), 329 (18), 301 (100), 286 (20), 242 (43)	90:10
66 hr-incubation				
[^{13}C,^{3}H]GA_1	16–19	0.9	507 $[M]^+$ (57), 492 (7), 449 (12), 377 (26), 208 (100)	52:48
[^{13}C,^{3}H]GA_{20}	25–27	1.2	419 $[M]^+$ (84), 404 (11), 376 (43), 360 (15), 208 (100)	85:15
[^{13}C,^{3}H]GA_4	31–32	5.6	419 $[M]^+$ (7), 329 (13), 285 (73), 234 (39), 225 (100)	78:22
[^{13}C,^{3}H]GA_{24}	33–34	2.8	375 $[M]^+$ (2), 343 (14), 315 (43), 287 (45), 226 (100)	75:25
[^{13}C,^{3}H]GA_9			331 $[M]^+$ (8), 299 (96), 271 (88), 243 (91), 227 (100)	78:22
	34–35	4.3		
[^{13}C,^{3}H]GA_{25}			405 $[M]^+$ (2), 373 (12), 313 (56), 285 (91), 226 (100)	67:33

The substrate (33 KBq, [^{13}C] composition = 99.9%) was incubated for 66 hr in a 3% glucose solution. After incubating for 18 hr, half of the culture filtrate was separated and the metabolites extracted. Mass ion cluster used for calculation of elemental composition in each [^{13}C]metabolite was as follows: [^{13}C]GA_1 and [^{13}C]GA_{20}: molecular ion clusters, [^{13}C]GA_4: 285, [^{13}C]GA_{24}: 315, and other [^{13}C]GAs: base ion clusters.

Table 3. HPLC and mass spectral data for metabolites of [17–^{14}C]GA_9 by *Phaeosphaeria* sp. L487

[^{14}C]metabolites	R_t (min)	% of total radioactivity	MS data (*m/z*, rel. int. %)	Isotope composition $^{14}C:^{12}C$
[^{14}C]GA_9 (recovered substrate)	36.7	24.5	332 ($[M]^+$, 12), 300 (100), 272 (98), 243 (71) and 228 (77)	95:5
[^{14}C]GA_4	33.9	59.5	420 ($[M]^+$, 14), 330 (20), 286 (100), 235 (33) and 227 (97)	99:1
[^{14}C]GA_{20}	28.3	5.2	420 ($[M]^+$, 100), 405 (22), 377 (54) and 209 (68)	100:0
[^{14}C]GA_1	20.8	8.2	508 ($[M]^+$, 100), 493 (11), 450 (19), 379 (18) and 209 (27)	95:5

Mass ion cluster used for calculation of elemental composition in each [^{14}C]metabolite was as follows; [^{14}C]GA_1 and [^{14}C]GA_{20}: molecular ion clusters, and [^{14}C]GA_4 and [^{14}C]GA_9: base ion clusters.

GA_1 is the end product in the fungus [7]. All GAs identified by GC–MS contained more than 50% of the ^{13}C isotope peaks showing that uniconazole is an effective inhibitor of GA-biosynthesis prior to GA_{12}-7-aldehyde.

Since GA_{20} was identified as a metabolite of GA_{12}-7-aldehyde, there are two possible pathways from GA_9 to GA_1 in the fungus, either via GA_4 or via GA_{20}. In the previous paper we confirmed the conversion of GA_9 to GA_1 via GA_4 using [17-$^2H_2$]GA_9 and [17-$^2H_2$]GA_4 as

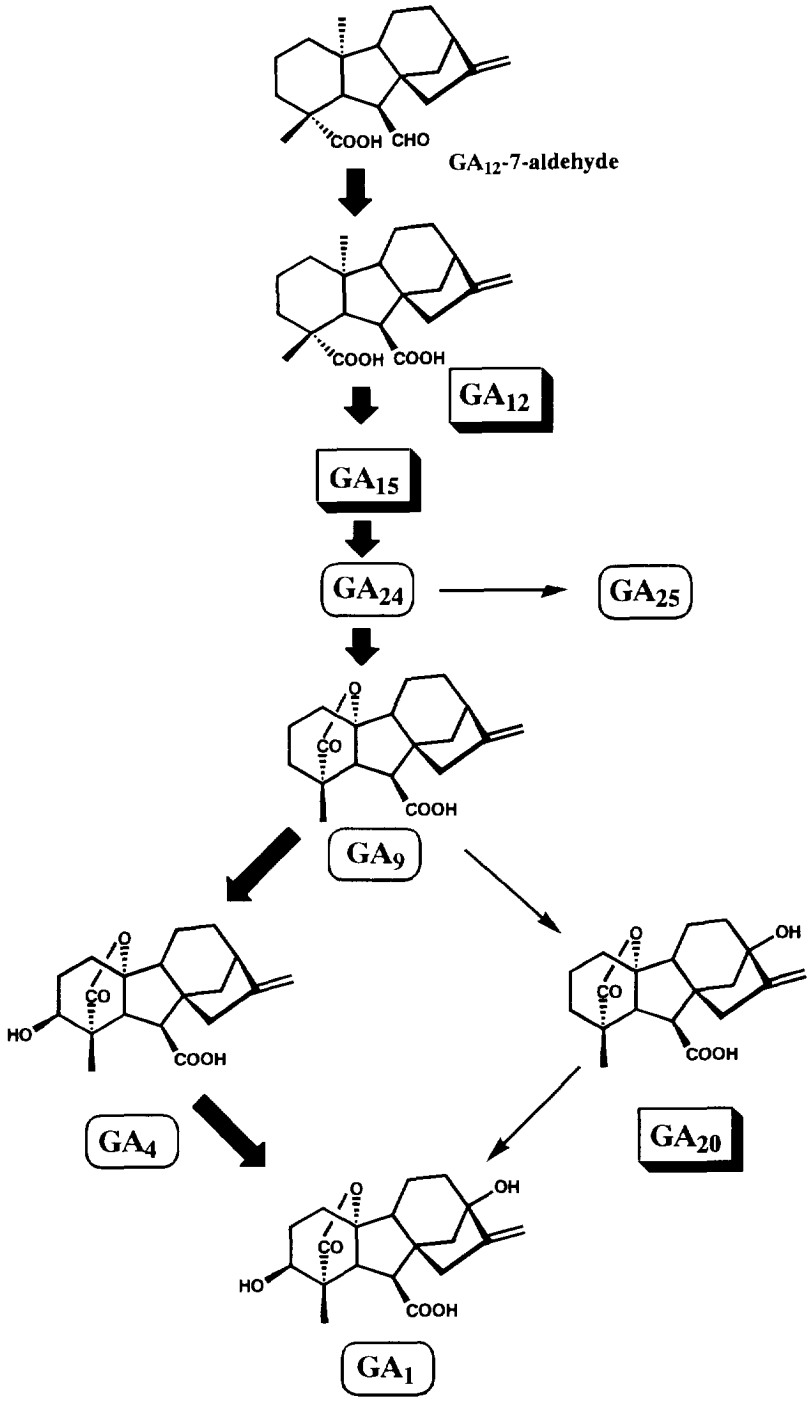


Fig. 1. The GA biosynthetic pathway from GA_{12}-7-aldehyde to GA_1 in *Phaeosphaeria* sp. L487 based on the feeding studies. GAs enclosed with shadow are identified by GC–MS in this paper.

substrates [7]. Since preliminary experiments showed that [17-^{14}C]GA_9 was more effectively metabolized in 3% glucose soln than in the CD medium, the 3% glucose soln was used for further feeding experiments. The metabolites converted from [17-^{14}C]GA_9 were purified by HPLC and analysed by GC–MS (Table 3). The conversion of GA_9 to GA_{20} was confirmed in the fungus, but the radioactivity of the [^{14}C]GA_{20} was 10 times lower than that of the [^{14}C]GA_4. Some of the substrate was recovered. This result indicates that the 3β-hydroxylation of GA_9 to form GA_4 is more effective than the 13-hydroxylation of GA_9 to GA_{20} in the fungus. However, these results did not necessarily suggest that GA_1 was predominantly formed from GA_4. If the metabolism of GA_{20} to GA_1 is rapid, GA_{20} will not accumulate during the incubation.

Before we compared the metabolism of GA_4 and GA_{20} to GA_1, it was necessary to demonstrate the conversion of GA_{20} to GA_1. [17-^{13}C,^{3}H]GA_{20} was incubated in the glucose solution containing uniconazole for 96 hr. Only one metabolite with a retention time corresponding to GA_1 was detected, with 6% of the total radioactivity estimated by HPLC. This product was identified as [^{13}C]GA_1 by GC–MS. When the CD medium was used for the incubation, the conversion of GA_{20} to GA_1 was not observed. Each equal amount of substrate, [1,2-^{3}H]GA_4 and [17-^{13}C,^{3}H]GA_{20} was separately incubated in the glucose soln for 96 hr. After incubation the comparison of the conversion of [^{3}H]GA_4 and [^{3}H]GA_{20} to GA_1 was done by HPLC analysis. From the data for these radio chromatograms, the conversion of [^{3}H]GA_4 to [^{3}H]GA_1 proceeded well (38% of total radioactivity recovered), while the conversion of [^{3}H]GA_{20} to [^{3}H]GA_1 was low (17% of total activity recovered). This result and the metabolism of [17-^{14}C]GA_9 (Table 3) clearly suggest that GA_4 is the major intermediate in the conversion of GA_9 to GA_1 in the fungus.

The GA biosynthetic pathway from GA_{12}-7-aldehyde to GA_1 in *Phaeosphaeria* sp. L487 based on the feeding studies is shown in Fig. 1. The conversion of GA_9 to GA_1 via GA_4 and GA_{20} is a novel pathway in fungal GA biosynthesis. Although GA_1 is produced from GA_4 in *G. fujikuroi* [13], GA_1 and GA_4 are not synthesized from GA_9 [14]. The first hydroxylation of the ent-gibberellane skeleton in *G. fujikuroi* occurs at C-3 position of GA_{12}-7-aldehyde to form GA_{14}-7-aldehyde (**3**) [15, 16]. The feeding experiment using [17-^{13}C,^{3}H]GA_{12}-7-aldehyde in *Phaeosphaeria* sp. L487 demonstrates that the early-non-hydroxylation occurs in this fungus. The 3β-hydroxylation of GAs in *Phaeosphaeria* sp. L487 occurs at the stage of GA_9 and GA_{20} as is the case in some higher plants [17]. If the same enzyme catalyses 3β-hydroxylation of GA_9 and GA_{20}, GA_9 must be a better substrate for the enzyme. The 13-hydroxylation (i.e. GA_4 to GA_1) of the fungus occurs at the C-19 GA stage which is different from most higher plants. In vegetative tissues of higher plants the early 13-hydroxylation pathway mainly operates: GA_{53} (**9**)–GA_{44} (**8**)–GA_{19} (**5**)–GA_{20}–GA_1. This is different from *Phaeosphaeria* sp. L487. The elucidation of the GA biosynthetic pathway in *Phaeosphaeria* sp. L487 may allow new studies on fungal GA biosynthetic enzymes. In particular, the 3β-hydroxylation of GA_9 and GA_{20} may be a useful model for the study of GA biosynthetic enzymes.

EXPERIMENTAL

Radioisotope substrates. [17-^{13}C,^{3}H]GA_{12}-7-aldehyde (740 MBq mmol^{-1}) and [17-^{13}C,^{3}H]GA_{20} (1.5 GBq mmol^{-1}) were gifts from Profs B. O. Phinney (UCLA, U.S.A.) and H. Yamane, (The University of Tokyo, Japan). [17-^{14}C]GA_9 (2.18 GBq mmol^{-1}) was a gift from Prof. I. Yamaguchi (The University of Tokyo, Japan). [1,2-^{3}H]GA_4 (15 GBq mmol^{-1}) was a gift from Prof. N. Murofushi (The University of Tokyo, Japan). The purity of radioactive substrate was checked by HPLC before use.

GC–MS analysis of GAs was carried out using an INCOS 50 mass spectrometer (Finnigan MAT Instruments) connected with a Hewlett-Packard 5890A gas chromatograph. Samples were methylated with ethereal diazomethane and then trimethylsilylated with *N*-methyl-*N*-trimethylsilylfluoroacetamide (80, 30 min). These methylated and trimethylsilylated derivatives (Me-TMSi) were injected (250°) into a DB-1 column (15 m × 0.25 mm i.d., J&W Scientific). The column temp. was maintained at 80° for 1 min, then increased at a rate of 30° min^{-1} to 245°, then at a rate of 5° min^{-1} to 300°. The flow rate of carrier He gas was 1 ml min^{-1}. MS were acquired scanning from *m/z* 200 to 600 at 70 eV.

HPLC analysis of the radioactive GAs was performed using a System Gold HPLC system (Beckman) with an Inertsil ODS-2 column (150 × 4.6 mm i.d., GL Science) and a 30 min gradient from MeOH–H_2O–HOAc (400:1600:1) to MeOH–H_2O–HOAc (3200:800:1). The eluate was collected in 1 ml portions per min into test tubes. A Tri-Carb 2000CA liquid scintillation analyser (Packard Instrument) was used to detect radioactive compounds. An aliquot of 50 μl from each tube was removed and the radioactivity measured in dioxane cocktail [120 g naphthalene, 5 g 2,5-diphenyloxazole and 0.5 g 2,2-*p*-phenylene-bis(5-phenyl oxazole) in 1 l dioxane].

Culture for identification of endogenous GAs in Phaeosphaeria *sp. L487.* A mycelial block (4 × 4 mm) maintained on a potato–dextrose–agar medium was inoculated into 100 ml of seed medium consisting of maltose (4 g) and Pharmamedia (0.4 g, made of cotton seed, Traders Protein, TX, U.S.A.). After incubating on a rotary shaker (150 rpm) for 6 days at 28°, the grown mycelia (5 ml) were transferred to 100 ml of GA production medium: maltose (8 g), yeast extract powder (0.4 g, Oriental Yeast Industry, Japan), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), and NH_4NO_3 (0.1 g) in a 500 ml-conical flask. The incubation was then continued under the same conditions for 10 days. Ten-day-old mycelia (2 g wet wt) were harvested by filtration and suspended in a 50-ml conical tube containing 10 ml of a chemically defined medium: glucose (40 g), KH_2PO_4 (5 g), $MgSO_4 \cdot 7H_2O$ (1 g), $ZnSO_4 \cdot 7H_2O$ (3.2 mg), $FeSO_4 \cdot 7H_2O$ (2 mg), $CuSO_4 \cdot 7H_2O$ (0.3 mg), $MnSO_4 \cdot 5H_2O$ (0.2 mg), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.2 mg) in 1000 ml H_2O adjusted to pH 5.6 with 5% $NaHCO_3$. After 24 hr cultivation, the culture filtrate (12 ml, pH 5.6) was adjusted to pH 8.8 with 5% Na_2CO_3 and partitioned against 15 ml of EtOAc. The H_2O layer was adjusted to pH 2.5 with 1 M HCl then extracted twice with 15 ml of EtOAc. The EtOAc fraction was washed twice with 1 ml of brine, and dried with Na_2SO_4. The dried EtOAc fraction was evapd *in vacuo* to obtain an EtOAc-soluble acidic fraction containing GAs. The fraction was analysed by GC–MS after derivatization. Relative amounts of endogenous GA were estimated from the data from the GC-total ion chromatogram.

Incubation of [17-^{13}C,^{3}H]*GA_{12}-7-aldehyde.* Ten-day-old mycelia grown in the YM medium were harvested by

centrifugation (4000 rpm, 5 min, 25°). The mycelia collected (wet wt 0.9–1 g) were washed ×3 with 20 ml of 3% glucose soln by centrifugation (4000 rpm, 5 min, 26°). The mycelia were suspended in a 50-ml conical tube containing 10 ml of 3% glucose soln and uniconazole (10^{-4} M) and incubated for 6 hr at 30°. The mycelia were washed ×3 with glucose soln by centrifugation. The mycelia washed were re-suspended in 10 ml CD medium containing uniconazole (10^{-4} M), and the substrate (20 μg dissolved in 20 μl MeOH, 33 kBq) was added. At 18 hr after the start of the incubation half of the culture soln was separated and GAs were extracted from the culture filtrate (total radioactivity 8.9 kBq). The remaining half was incubated for 66 hr and GAs then extracted (total radioactivity 20 kBq). Each culture filtrate (*ca* 15 ml) was passed through a Bond Elut C18 reverse-phase cartridge column (6 ml size, Varian). After washing the column with 5 ml of H_2O, substances retained on the column were eluted with 15 ml of MeOH. The MeOH eluate was passed through a Bond Elut DEA ion-exchange cartridge column (3 ml size, Varian). The GA fraction was eluted with 6 ml of 1 M HOAc in MeOH. The GA metabolites were purified by HPLC and then analysed by GC–MS after derivatization. Mass ion cluster used for calculation of elemental composition in each [^{13}C]metabolite [18] was as follows; [^{13}C]GA_1 and [^{13}C]GA_{20}: molecular ion clusters, [^{13}C]GA_4 and [^{13}C]GA_{24}: major ion clusters (*m/z* 285 and 315, respectively) and other [^{13}C]GAs: base ion clusters.

Incubation of [17-^{14}C]GA_9. Thirteen-day-old mycelia cultured in YM medium were washed and incubated with the glucose soln containing uniconazole as described above. The mycelia (0.9-1 g wet wt) were washed then re-suspended in glucose soln (10 ml) containing uniconazole (10^{-4} M), and the substrate (727 ng dissolved in 3 μl MeOH, 5 kBq) was added. After incubating for 24 hr, the metabolites were extracted by the cartridge columns, and purified by HPLC. The [^{14}C]GA_{20} (R_t 28.3 min), [^{14}C]GA_4 (R_t 33.9 min) and [^{14}C]GA_1 (R_t 20.8 min) converted from [^{14}C]GA_9 were confirmed by GC–MS. Mass ion cluster used for calculation of elemental composition in each [^{14}C]metabolite was as follows; [^{14}C]GA_1 and [^{14}C]GA_{20}: molecular ion clusters, and [^{14}C]GA_4 and [^{14}C]GA_9: base ion clusters.

Conversion of [17-^{13}C,^{3}H]GA_{20} *to* GA_1. Eight-day-old mycelia cultured in YM medium were used for this feeding experiment. The mycelia (0.9–1 g wet wt) washed and treated with uniconazole were re-suspended in glucose soln (10 ml) containing uniconazole (10^{-4} M), and the substrate (1.8 μg dissolved in 10 μl MeOH, 8.3 kBq) was added. After incubating for 96 hr, the metabolites were extracted by the cartridge columns, and purified by HPLC. Only one metabolite at R_t 21.5 min was observed. The presence of [^{13}C]GA_1 in the fraction was confirmed by GC–MS: *m/z* (rel. int); 507 $[M]^+$ of [^{13}C]GA_1 Me-TMSi (34), 506 $[M]^+$ of GA_1 Me-TMSi (36), 492 (6), 448 (13), 377 (32), 208 $[^{13}C^{12}C_{11}H_{19}OSi]^+$ (94), and 207 $[C_{12}H_{19}OSi]^+$ (100).

Incubation of [1,2-^{3}H]GA_4 *and* [17-^{13}C,^{3}H]GA_{20}. Thirteen-day-old mycelia cultured in YM medium were used for these feeding experiments. The mycelia (0.9–1 g wet wt) washed and treated with uniconazole were re-suspended in glucose soln (10 ml) containing uniconazole (10^{-4} M). The substrates [1,2-^{3}H]GA_4 (182 ng dissolved in 15 μl MeOH, 8.3 kBq) and [17-^{13}C,^{3}H]GA_{20} (182 ng dissolved in 15 μl MeOH, 830 Bq) were individually incubated for 96 hr. The metabolites obtained from each incubation were extracted with the cartridge columns, 0.4 kBq of each extract was analysed and estimated by HPLC. [^{3}H]GA_1 converted from [1,2-^{3}H]GA_4 (38% of total radioactivity at R_t 20.4 min) was detected. [^{3}H]GA_1 converted from [17-^{13}C,^{3}H]GA_{20} (17% of total radioactivity at R_t 20.2 min) was also detected.

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REFERENCES

1. Bruckner, B. and Blechschmidt, D. (1991) *Crit. Rev. Biotechnol.* **11**, 163.
2. Jefferys, E. G. (1970) *Adv. Appl. Microbiol.* **13**, 283.
3. Rademacher, W. (1992) *Phytochemistry* **31**, 4155.
4. Sassa, T., Suzuki, K. and Haruki, E. (1989) *Agric. Biol. Chem.* **53**, 303.
5. Haruki, E. and Mikawa, T. (1991) *Mitsubishi Kasei R&D Rev.* **5**, 40 (in Japanese).
6. Sassa, T. and Suzuki, K. (1990) *Agric. Biol. Chem.* **54**, 3373.
7. Kawaide, H. and Sassa, T. (1993) *Biosci. Biotech. Biochem.* **57**, 1403.
8. Sassa, T., Kawaide, H. and Takarada, T. (1994) *Biosci. Biotech. Biochem.* **58**, 438.
9. Crozier, A., Turnbull, C. G. N., Malcom, J. M. and Graebe, J. E. (1990) in *Gibberellins* (Takahashi, N., Phinney, B. O. and MacMillan, J., eds), pp. 83, 419. Springer, New York.
10. Hedden, P., Phinney, B. O., MacMillan, J. and Sponsel, V. M. (1977) *Phytochemistry* **16**, 1913.
11. Rademacher, W. (1992) *Plant Physiol.* **100**, 625.
12. Izumi, K., Kamiya, Y., Sakurai, A., Oshio, H. and Takahashi, N. (1985) *Plant Cell Physiol.* **26**, 821.
13. Pitel, D. W., Vining, L. C. and Arsenault, G. P. (1971) *Can. J. Biochem.* **49**, 194.
14. Bearder, J. R., Frydman, V. M., Gaskin, P., Hatton, I. K., Harvey, W. E., MacMillan, J. and Phinney, B. O. (1976) *J. Chem. Soc., Perkin Trans I* 178.
15. Hedden, P., MacMillan, J. and Phinney, B. O. (1974) *J. Chem. Soc., Perkin Trans I* 587.
16. Bearder, J. R., MacMillan, J. and Phinney, B. O. (1973) *Phytochemistry* **12**, 2173.
17. Graebe, J. E. (1987) *Ann. Rev. Plant. Physiol.* **38**, 419.
18. Sponsel, V. M. and MacMillan, J. (1977) *Planta* **135**, 129.