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Gibberellin Biosynthetic Pathway and the Physiologically Active Gibberellin in the Shoot of *Cucumis sativus* L.

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Abstract. $[^2H_2]$Gibberellin A_{24} (GA_{24}) and $[^2H_4]$-GA_9 were applied to the apices of normal-type cucumber (*Cucumis sativus* L. cv. Yomaki) seedlings treated with uniconazole, an inhibitor of GA biosynthesis. The metabolites from these feeds were identified by full-scan gas chromatography–mass spectrometry (GC-MS) to confirm the conversions of $[^2H_2]GA_{24}$ to $[^2H_2]GA_9$ and of $[^2H_4]GA_9$ to $[^2H_4]GA_4$. The results show that GA_4 is biosynthesized from GA_{24} *via* GA_9. In a cucumber hypocotyl elongation bioassay using cv. Yomaki, prohexadione (DOCHC), an inhibitor of 2-oxoglutarate-dependent dioxygenase, inhibited the hypocotyl elongation caused by application of GA_9, while DOCHC enhanced the elongation caused by application of GA_4. These results indicate that GA_4 is a physiologically active GA and that the activity of GA_9 is due to its conversion to GA_4 in cucumber shoots.

Evidence is accumulating that gibberellin A_1 (GA_1) is the main endogenous GA active per se in the control of shoot elongation in several species of higher plants (Fujioka et al. 1988, Ingram et al. 1986, Kobayashi et al. 1990). In shoots of these plants the early-13-hydroxylation GA biosynthetic pathway leading to GA_1 appears to be the predominant pathway.

On the other hand, we reported that a biosynthetic pathway of non-13-hydroxylated GAs leading to GA_4, the early-non-hydroxylation pathway, is operating in cucumber (*Cucumis sativus* L.) shoots (Nakayama et al. 1989). However, the early-non-hydroxylation pathway has not been well established in Cucurbitaceae, especially between GA_{24} and GA_4. In this report evidence is presented that GA_4 is biosynthesized from GA_{24} *via* GA_9 in cucumber shoots, and that GA_4 is the endogenous GA active per se in the control of shoot elongation of cucumber.

Materials and Methods

Chemicals

Prohexadione [3,5-dioxo-4-propionylcyclohexanecarboxylic acid (DOCHC); Nakayama I et al. 1990] was obtained by extraction with ethyl acetate (EtOAc) from an acidic aqueous solution (pH 3) of BX-112 (prohexadione calcium), which was supplied by Kumiai Chemical Industry Co., Japan. Preparation of [2,2,3α, 6-$^2H_4$]GA_9 was reported previously (Nakayama et al. 1989). [17-$^2H_2$]GA_{24} was synthesized at the Australian National University as described below.

Methyl oxalyl chloride (15 μl) was added slowly to a stirring mixture of GA_{19} dimethyl ester (GA_{19}-Me) (45 mg), triethylamine (25 μl), and 4-dimethylaminopyridine (3 mg) in dichloromethane (CH_2Cl_2; 1 ml) at 5°C under nitrogen gas stream, and stirring was continued for 1 h. The reaction mixture was then diluted with diethyl ether (50 ml), washed with brine (saturated aqueous sodium chloride), 2 N potassium dihydrogen phosphate, and then brine again. The organic layer dried with anhydrous sodium sulfate (Na_2SO_4) was concentrated to give GA_{19}-Me-13-methyl oxalate (57.2 mg): R_f value on silica gel thin-layer chromatography (TLC), 0.45 (*n*-hexane-EtOAc, 2:1); ^1H NMR (200 MHz, $CDCl_3$) δ 1.14 (s, 18-H_3), 2.25 (d, J = 12.7 Hz, 5-H), 3.63, 3.75 (2 × s, CO_2CH_3), 3.88 (s, $COCO_2CH_3$), 3.92 (d, J = 12.7 Hz, 6-H), 5.05, 5.18, (2 × s, 17-H_2), 9.69 (s, 20-H).

The oxalate prepared above (55 mg) was dissolved in toluene (2 ml), and to the solution added *n*-butyltin hydride (47 μl). To the solution at reflux, azobisisobutylnitrile (5 mg) in toluene (200 μl) was added slowly. The reaction mixture was refluxed (exter-

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nal temperature 130–140°C) with stirring for a further 15 min. After cooling, a saturated aqueous Na_2SO_4 (50 ml) was added and the mixture was extracted twice with diethyl ether (50 ml). The ether solution was washed with brine, dried with Na_2SO_4, and reduced to a pale yellow oil. This was chromatographed on silica gel, and GA_{24}-Me (29.5 mg, 70%) eluted with *n*-hexane-EtOAc (10:1) as a colorless gum which readily crystallized: melting point (mp) 94–95°C; R_f value on silica gel TLC, 0.45 (*n*-hexane-EtOAc, 5:1); ^1H NMR (200 MHz, $CDCl_3$) δ 1.14 (s, 18-H_3), 2.18 (d, J = 12.7 Hz, 5-H), 2.40 (dt, J = 15.2 Hz, 15-H), 2.62 (br.t, J = 6 Hz, 13-H), 3.64, 3.73 (2 × s, CO_2CH_3), 3.88 (d, J = 12.7 Hz, 6-H), 4.84, 4.91 (2 × br.s, 17-H_2), 9.67 (s, 20-H).

Ozonized oxygen was bubbled through a stirred solution of GA_{24}-Me (50 mg) and pyridine (1.2 ml) in CH_2Cl_2 (12 ml) at −78°C for 4 min. Dimethyl sulfide (250 μl) was then added, and after stirring for 10 min at −78°C the resulting mixture was allowed to warm to room temperature over a 10-min period. After evaporation of the solvent, the residue was chromatographed on silica gel. Elution with CH_2Cl_2-EtOAc (20:1) gave 17-nor-16-oxo-GA_{24}-Me (27.1 mg, 54%) as a colorless crystalline solid: mp 161–162°C; R_f value on silica gel TLC, 0.40 (CH_2Cl_2-EtOAc, 5:1); ^1H NMR (200 MHz, $CDCl_3$) 1.15 (s, 18-H_3), 2.21 (d, J = 12.7 Hz, 5-H), 3.65, 3.74 (2 × s, CO_2CH_3), 3.98 (d, J = 12.7 Hz, 6-H), 9.70 (s, 20-H).

Titanium (IV) chloride (52 μl) was added dropwise to a stirring suspension of zinc dust (138 mg) in [$^2H_2$]methylene bromide (50 μl) and tetrahydrofuran (1.5 ml) at −40°C over a 2–3-min period. After stirring at −40°C for 10 min, the mixture was allowed to warm to 5°C and stirring continued at this temperature for 20 h. Small portions of this slurry were then added to the ketone prepared above (3.8 mg) in CH_2Cl_2 (0.5 ml) at 0°C until TLC indicated complete reaction (total of 0.5 ml of reagent suspension over a period of 45 min). The reaction mixture was poured into a $NaHCO_3$ solution layered with ether, and the mixture was stirred until a clear ether layer was obtained. This was then separated, washed with brine, dried with Na_2SO_4, and reduced to dryness. The residue was chromatographed on silica gel and [17-$^2H_2$]GA_{24}-Me (2.4 mg, 63%) eluted with *n*-hexane-EtOAc (10:1).

[$^2H_2$]GA_{24}-Me (2.2 mg) was dissolved in methanol (MeOH) (0.1 ml), and 2 N sodium hydroxide (0.5 ml) was added. The mixture was refluxed for 7 h. After cooling and removal of solvent, the residue was suspended in MeOH (1 ml), and sufficient Dowex 50W resin (H^+ form) was added to lower the pH to 4. After filtration of the resin, the filtrate was concentrated and chromatographed on silica gel (elution with EtOAc) to afford [17-$^2H_2$]GA_{24} (1.8 mg, 88%). A small sample was methylated with etheral diazomethane to give [17-$^2H_2$]GA_{24}-Me, a ^1H NMR spectrum of which was identical to that of GA_{24}-Me, except for the absence of signals from the 17-methylene group.

Feeding Experiment

C. sativus L. normal-type cv. Yomaki seed were germinated on two layers of filter paper wetted with 20 ppm aqueous uniconazole solution (Izumi et al. 1984) in a petri dish at 25°C for 3 days. The seedlings were planted in vermiculite and grown at 25°C under continuous white light (approximately 3.2 W/m^2). Seven days after the sowing, [17-$^2H_2$]GA_{24} and [2,2,3α,6-$^2H_4$]GA_9 (1 μg/plant) were applied to the apices of 200 and 100 seedlings, respectively, in 5 μl 50% aqueous acetone solution. Twenty-four hours after the GA application, the shoots were collected by excising the roots at the coleoptilar node. The cotyledons were also removed and discarded. The shoot segments were combined, extracted three times with MeOH (300 ml), and fractionated to give an acidic ethyl acetate (AE) fraction. The AE fraction was dissolved in 1 ml 80% aqueous MeOH and loaded onto a Sep-Pak (ODS) cartridge, and eluted three times with 2 ml of 80% aqueous MeOH. The combined eluates were evaporated to dryness in vacuo, dissolved in 1 ml MeOH, and loaded onto a Sepralyte [diethylaminopropyl (DEA); 250 mg] column. The column was eluted with MeOH (5 ml), and 0.5% acetic acid (HOAc) in MeOH (5 ml). The MeOH eluate was evaporated to dryness in vacuo and repurified with a Sepralyte (DEA) column as described above. The eluates with 0.5% HOAc in MeOH from the first and second runs were combined, evaporated to dryness in vacuo, and subjected to ODS-high-performance liquid chromatography (HPLC) described previously (Nakayama et al. 1989). The eluates from the ODS-HPLC were analyzed by gas chromatography-mass spectrometry (GC-MS) using a JEOL DX-303 GC-MS system after derivatization as reported previously (Nakayama et al. 1989).

Cucumber Hypocotyl Assay

The assay was carried out according to the method described by Katsumi et al. (1965) with some modification. *C. sativus* L. cv. Yomaki seed were germinated on two layers of filter paper wetted with water in a petri dish at 25°C for 2 days. The seedlings were planted in vermiculite and grown at 25°C under continuous white light (approximately 3.2 W/m^2). Five days after planting, the hypocotyls were marked with ink 20 mm below the cotyledonary nodes. This 20-mm portion of the hypocotyl is called "hypocotyl unit." Fifty percent aqueous acetone solutions of DOCHC or GAs (10 μl/plant) were applied to the shoot apices of the cucumber seedlings. Six days after the application, the lengths of the hypocotyl units were measured. To test GA activity on the cucumber seedlings treated with DOCHC, each GA was applied 12 h after treatment with DOCHC.

Results and Discussion

With regard to biosynthesis of the non-13-hydroxylated GAs in the cucumber shoot, two possible pathways from GA_{24} to GA_4 could be considered. (1) Graebe et al. (1980) concluded that GA_4 is biosynthesized from GA_{24} *via* GA_{36} in immature seeds of *Cucurbita maxima* L., since GA_{24} was converted to GA_{36}, and GA_{36} to GA_4 in 5–10% yield in a cell-free system, the major metabolite of GA_{36} being GA_{43} which was formed *via* GA_{13}. Further evidence was provided by GC-MS analysis of endogenous GAs in the immature seed: GA_{24}, GA_{36}, and GA_4 were identified, but GA_9 was not detected at all. (2) Takahashi et al. (1986) showed that GA_4 was biosynthesized from GA_{24} *via* GA_9 in immature seeds of *Phaseolus vulgaris* L., since in a cell-free system GA_{24} was readily converted to GA_9 and GA_9 was converted to GA_4.

To clarify the biosynthetic pathway of the non-13-hydroxylated GAs in cucumber shoots, [$^2H_2$]-

Table 1. GC-MS data of Me or MeTMSi derivatives of authentic GAs.

GAs	Derivatization	KRI	Principal ions and relative abundance (% base peak)
GA_4	MeTMSi	2498	418(M^+,25), 386(18), 358(11), 328(30), 289(66), 284(100), 225(77)
GA_9	Me	2309	330(M^+,11), 298(100), 286(22), 270(90), 243(52), 226(61)
GA_{19}	MeTMSi	2589	462(M^+,9), 434(100), 402(28), 374(55), 345(22)
GA_{20}	MeTMSi	2480	418(M^+,100), 403(14), 390(7), 375(43), 301(13)
GA_{25}	Me	2437	404(M^+,1), 372(27), 312(77), 284(100), 225(64)
GA_{51}	MeTMSi	2515	418(M^+,5), 403(9), 328(30), 284(99), 268(77), 225(100)
GA_{69}	MeTMSi	2494	418(M^+,31), 403(25), 386(17), 372(28), 358(23), 328(52), 296(85), 282(51), 268(100), 223(87)
GA_{70}	MeTMSi	2547	418(M^+,25), 403(8), 386(19), 372(12), 358(25), 328(55), 296(92), 282(27), 268(100), 223(90)
12β-hydroxy-GA (tentative)	MeTMSi	2635	462(M^+,19), 430(15), 402(26), 374(12), 344(18), 312(100), 284(91)

Table 2. GC-MS analysis of $[^2H_4]GA_9$ and $[^2H_2]GA_{24}$ feedings to the cucumber seedlings.

Substrate	Rt on ODS-HPLC (min)	Metabolites	Derivatization	KRI	Principal ions and relative abundance (% base peak)
$[^2H_2]GA_{24}$	17–19	$[^2H_2]$12β-hydroxy-GA_{24} (tentative)	MeTMSi	2635	464(M^+,18), 432(21), 404(24), 376(14), 346(21), 314(100), 286(98)
	21–23	$[^2H_2]$monohydroxy-GA_{24}-like-compound	MeTMSi	2638	464(M^+,13), 432(12), 404(17), 376(13), 346(22), 314(100), 286(94)
	23–25	$[^2H_2]GA_{19}$	MeTMSi	2591	464(M^+,10), 436(100), 404(34), 376(51), 347(22)
	26–27	$[^2H_2]GA_4$	MeTMSi	2498	420(M^+,43), 388(25), 360(21), 330(33), 291(61), 286(100), 227(79)
	28–29	$[^2H_2]GA_9$	Me	2314	332(M^+,9), 300(100), 272(84), 243(51), 228(60)
	29–30	$[^2H_2]GA_{25}$	Me	2438	406(M^+,4), 374(39), 314(72), 286(100), 227(46)
$[^2H_4]GA_9$	17–19	$[^2H_4]GA_{69}$	MeTMSi	2492	422(M^+,50), 407(37), 390(24), 376(28), 362(38), 332(70), 300(77), 286(69), 272(100), 227(71)
	17–19	$[^2H_4]GA_{70}$	MeTMSi	2546	422(M^+,45), 407(10), 390(17), 376(48), 362(45), 332(65), 300(82), 286(75), 272(100), 227(77)
	19–21	$[^2H_4]GA_{20}$	MeTMSi	2479	422(M^+,100), 407(27), 393(12), 376(52), 304(21)
	22–23	$[^2H_3]GA_{51}$	MeTMSi	2517	421(M^+,13), 406(11), 389(36), 331(42), 287(100), 228(89)
	25–27	$[^2H_4]GA_4$	MeTMSi	2498	422(M^+,49), 390(31), 362(20), 332(33), 291(41), 288(100), 228(89)

GA_{24} and $[^2H_4]GA_9$ were separately fed to the normal-type cucumber seedlings. In these feeding experiments, cucumber seedlings treated with uniconazole were used to reduce the contents of endogenous GAs (M. Nakayama et al., unpublished observations) and to promote metabolism of exogenously applied GAs (M. Kobayashi, personal communication). The metabolites from these feeds were identified by full-scan GC-MS (Tables 1 and 2). $[^2H_2]GA_{24}$ was converted to $[^2H_2]GA_4$, $[^2H_2]GA_9$, $[^2H_2]GA_{19}$, $[^2H_2]GA_{25}$, $[^2H_2]$12β-hydroxy-GA_{24} (tentative), and a $[^2H_2]$monohydroxy-GA_{24}-like compound, while the conversion of $[^2H_2]GA_{24}$ to $[^2H_2]GA_{36}$ was not observed. $[^2H_4]GA_9$ was converted to $[^2H_4]GA_4$, $[^2H_4]GA_{20}$, $[^2H_3]GA_{51}$, $[^2H_4]GA_{69}$, and $[^2H_4]GA_{70}$ in the cucumber shoots (Fig. 1). In the cucumber shoots, GA_{24}, GA_9, and GA_4 were identified by GC-MS as endogenous GAs, whereas GA_{36} was not identified (Nakayama et al. 1989). The above evidence indicates that the following GA biosynthetic pathway, $GA_{24} \rightarrow GA_9 \rightarrow GA_4$, is probably the major one in the cucumber shoots

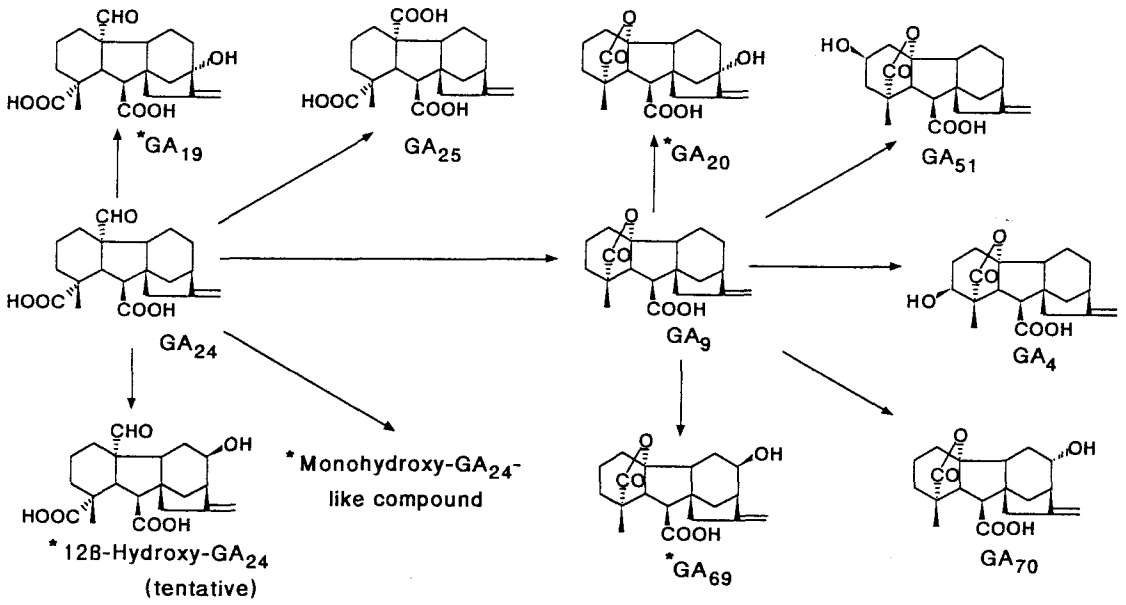

Fig. 1. Metabolism of GA_9 and GA_{24} in shoots of *C. sativus* L. cv. Yomaki. *GAs which were not identified from shoots of *C. sativus* L.

(Fig. 1), although the possibility that another pathway, $GA_{24} \rightarrow GA_{36} \rightarrow GA_4$, is also operating cannot be excluded.

These feeding experiments also provided evidence that GA_{25} was biosynthesized from GA_{24}, and that GA_{51} and GA_{70} were biosynthesized from GA_9 in cucumber. GA_{19}, GA_{20}, GA_{69}, 12β-hydroxy-GA_{24} (tentative), and the monohydroxy-GA_{24}-like compound were not detected in cucumber shoots (Nakayama et al. 1989). Such 12- and 13-hydroxylations might be ascribed to nonspecific metabolism due to high doses of the exogenously applied [^{2}H]GAs. The tentative identification of [$^2H_2$]12β-hydroxy-GA_{24} was based on comparisons of its retention time on ODS-HPLC and the KRI and full-scan mass spectrum of its methyl ester trimethylsilyl ether (MeTMSi) derivative with those of putative 12β-hydroxy-GA_{24} obtained from seed of *Raphanus sativus* L. (Nakayama M et al. 1990). The MeTMSi derivative of the [$^2H_2$]monohydroxy-GA_{24}-like compound showed very similar KRI and full-scan mass spectrum to those of the MeTMSi derivative of putative [$^2H_2$]12β-hydroxy-GA_{24}, but the retention time on ODS-HPLC of the [$^2H_2$]monohydroxy-GA_{24}-like compound was different from that of putative [$^2H_2$]12β-hydroxy-GA_{24} (Table 2). Since no [$^2H_2$]monohydroxy-GA_{24}-like compound was detected by GC-MS in the retention time fraction 20–21 min on ODS-HPLC, the possibility that the compound from the 21–23-min fraction was due to tailing of putative [$^2H_2$]12β-hydroxy-GA_{24} from the 17–19-min fraction is excluded. It seems likely, therefore, that the new metabolite is the 12α-epimer.

In studies of GA biosynthesis, GA-deficient single gene dwarf mutants have been extensively used. To clarify which GA was physiologically active in *Zea mays*, *Oryza sativa*, and *Pisum sativum*, single gene dwarf mutants in which GA_{20} 3β-hydroxylation is blocked have provided definitive evidence that the introduction of a 3β-hydroxyl into GA_{20} is the final step to produce the active GA (GA_1) in the control of shoot elongation in these plants. Though all the identified GAs in cucumber shoots were non-13-hydroxylated GAs, their structures showed a good correspondence to those of endogenous GAs in *Z. mays*, *O. sativa*, and *P. sativum* except for the lack of a 13-hydroxyl group. We, therefore, speculated that the introduction of a 3β-hydroxyl into GA_9 (13-deoxy-GA_{20}) might be the final step for the production of active GA, GA_4 (13-deoxy-GA_1), in cucumber shoots. Since GA 3β-hydroxylase-deficient mutants are unknown in cucumber, we used DOCHC, an inhibitor of 2-oxoglutarate-dependent dioxygenase in GA biosynthesis, to clarify which GA was active in the control of shoot elongation of cucumber. Recently, Nakayama I et al. (1990) reported that DOCHC inhibited the shoot elongation caused by the application of GA_{20} in *O. sativa*, while it enhanced that caused by the application of GA_1. It was considered that the inhibition was due to the inhibition of conversion of GA_{20} to

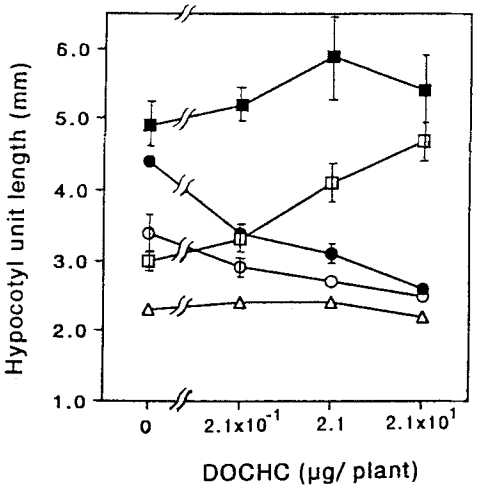

Fig. 2. Effects of DOCHC on hypocotyl elongation caused by exogenously applied GA_4 and GA_9 in *C. sativus* cv. Yomaki. Vertical bars represent the SEM of 10 replicates. SEs at the points without SE bars were less than 0.12 mm. –△–, control without GA; –○ –, with GA_9 (dose of 0.1 μg/plant); –●–, with GA_9 (dose of 1 μg/plant); –□–, with GA_4 (dose of 0.1 μg/plant); –■–, with GA_4 (dose of 1 μg/plant).

GA_1 (3β-hydroxylation; activation step), and that the enhancement was due to inhibition of conversion of GA_1 to GA_8 (2β-hydroxylation; inactivation step).

As shown in Fig. 2, DOCHC inhibited cucumber hypocotyl elongation promoted by application of GA_9 at 0.1 and 1 μg/plant, and showed clear enhancement of that by application of GA_4 at 0.1 μg/plant. Since DOCHC inhibits both 2β- and 3β-hydroxylation as mentioned above, DOCHC is considered to inhibit conversion of GA_9 to GA_4 (activation step) and that of GA_4 to GA_{34} (inactivation step). Though the enhancement by DOCHC in the application of GA_4 at 1 μg/plant was small, this is probably due to nearly saturated elongation by the application of a high dose of GA_4 (Nakayama et al. 1989). These results indicate that GA_4 is active per se in the control of shoot elongation in the cucumber, and that GA_9 is active as a result of its conversion to GA_4.

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