Identification of Endogenous Gibberellins by Feeding of [^{14}C] GA$_{12}$ in Chinese Yam, *Dioscorea opposite*

Sang-Kuk Kim*, Sang-Chul Lee**, Bong-Ho Lee*, Soo-Won Jang**, Tae-Shik Park*** and In-Jung Lee**,[†]

**Institute for Bioresources Research, Kyongbuk Provincial Agricultural Technology Administration, Andong 760-891, Korea,*
***College of Agricultural Life Sciences, Division of Plant Biosciences, Kyungpook National University, Daegu 702-701, Korea,*
****Division of Rice Physiology and Cultivation, National Crop Experiment Station, Rural Development Administration,*
Suwon 441-857, Korea

ABSTRACT: The metabolism of [^{14}C] GA$_{12}$ in the Chinese yam (*Dioscorea opposita* Thunb. var. Tsukune) was examined to determine the identification of endogenous gibberellins. [^{14}C] GA$_{12}$ was metabolized to GA$_{53}$, GA$_{44}$, GA$_{19}$, GA$_{20}$, GA$_1$, GA$_8$, GA$_{15}$, GA$_{24}$, GA$_9$, GA$_{36}$ and GA$_4$. Radioactivity of GAs in non C-13 hydroxylation route was five-fold higher than that of early C-13 hydroxylation in analyzed GA-metabolites. Radioactivity of GA$_4$ was always four times higher than that of GA$_1$ at every feeding time. GA$_1$ radioactivity has always a lower level to below 200 DPM. The major pathway of endogenous GA metabolism in seedlings of the Chinese yam might be the non C-13 hydroxylation pathway.

Keywords : Chinese yam, *Dioscorea opposita*, gibberellins, identification.

G ibberellins (GAs) play an important physiological role in the Chinese yam (*Dioscorea opposita* Thunb.) plants. They affect seed germination, tuber dormancy (Okagami and Tanno, 1993), and tuber enlargement (Kim et al., 2003). In particular, the application of gibberellins strengthens or deepens the dormancy of the tubers and bulbils in Chinese yams (Okagami and Nagao, 1971). Furthermore, bulbil dormancy in *Begonia evansiana* (Okagami 1972) and the genus *Dioscorea* (Okagami and Tanno, 1977) is also induced by endogenous and exogenous gibberellins. GA$_{12}$ is an early precursor in the GA biosynthesis and its biosynthesis pathway is important to elucidate the physiological role named to gibberellin induced dormancy in Chinese yam'. If the bioactive GAs, GA$_1$ or GA4, may be involved in the controlling the dormancy and sprouting of tubers and bulbils in the Chinese yam, several GA biosynthesis inhibitors such as ancymidol, mepiquat chloride and trinexapac-ethyl will be applied to the Chinese yams for modulating the sprouting and dormancy periods.

In general, the predominant GA biosynthesis pathway in

most vegetative plant tissues seems to be the early C-13 hydroxylation route (Sponsel, 1995), meanwhile intermediates of other pathways, especially the non C-13 hydroxylation pathway, are often also present (Zhu and Davies, 1991). Till now, several endogenous gibberellins have been identified in dormant bulbils of Chinese yam (*Dioscorea opposita* Thunb.) (Tanno *et al.*, 1992). However, it was not found that which GA biosynthetic pathway is operated and also dominant, GA biosynthesis pathway has not been understood in the Chinese yam plants.

Thus, this study focuses principally on the identification of endogenous gibberellins and biosynthesis pathways active in vegetative Chinese yam, as determined by the identification of endogenous GA-metabolites following the feeding of [^{14}C]GA$_{12}$ as a precursor of gibberellin in the Chinese yam seedlings.

MATERIALS AND METHODS

Plant material and feeding of [^{14}C]gibberellin A$_{12}$

Sprouted tubers of the Chinese yam (*Dioscorea opposita* Thunb. var. Tsukune) were grown in a sand-vermiculite mixture. After the seedlings had fully expanded leaves and grown for 4 weeks, and then they were fed to determine the GA biosynthesis pathway. The substrate of [^{14}C]GA$_{12}$ was purchased from L. N. Mander (Australian National University, Canberra, Australia). The compound was about 96% radioactive. Specific activity was 10 μCi mmol^{-1}. The substrate was dissolved in EtOH:water (1:1, v/v). 10 μl (370 Bq) for each twenty plant was fed to shoot tips and surface of whole leaves below apex of the plants. The application of [^{14}C]GA$_{12}$ was performed by injection using a microsyringe. After fed, seedlings were grown at 25°C in a constant temperature room with continuous light at 255 μmolm^{-2}s^{-1} provided by fluorescent lamps. Seedlings were harvested at 6, 12, 18 and 24 h after feeding (HAF). Seedlings were frozen in liquid nitrogen immediately after harvesting and stored at -70°C for analysis of GAs.

Extraction of endogenous gibberellins

Extraction and HPLC of GA-metabolites followed the reference (Lee *et al.*, 1998). Lyophilized tissue samples were ground to a fine powder in a mortar and pestle with the aid of acid-washed sea sand. The powdered tissue was extracted with 80% (v/v) methanol (MeOH). The 80% MeOH was removed by filteration, and the tissue was then extracted with 100% MeOH until the extract was clear. The volume of the 80% and 100% extracts were recorded, the two extracts were combined, and water was added to bring the combined MeOH extract concentration to 60%. This solution was chilled for 1 h at -70°C, and precipitated chlorophyll was removed by filtration through a GF/A filter. The extract was adjusted to pH 8.0-8.3 using 2N NH_4OH and passed through a 3 g column of Davisil C_{18} (90-130 μm, 60Å pore size, Alltech). The eluant was reduced to near dryness at 40°C *in vacuo*. The sample was dried onto 1 g celite and then loaded onto a 4 g SiO_2 (ICN Silica 32-100, active 60Å) partitioning column (deactivated with 20% water) to separate the GAs as a group from more polar impurities. GAs were eluted with 80 ml of 95:5 ethyl acetate (EtOAc): hexane saturated with formic acid. This solution was dried at 40°C *in vacuo*, redissolved in 4 ml EtOAc and partitioned 3 times against 4 ml of 0.1 M phosphate buffer (pH 8.0). Dropwise addition of 2N NaOH was required during the first partitioning to neutralize residual formic acid. Polyvinylpolypyrrolidone (PVPP) 1 g was added to the combined aqueous phases, and this mixture was slurried for 1 h. Following the removal of the PVPP by filtration, 6N HCl was added to reduce the pH 2.5. The extract was partitioned 3 times against equal volumes of EtOAc. The combined EtOAc fraction was dried *in vacuo*, and the residue was dissolved in 3 ml of 100% MeOH. This solution was dried on a Savant Automatic Environmental Speedvac (Model AES 2000). The dried sample was subjected to reverse-phase C_{18}-HPLC.

HPLC condition of endogenous gibberellins

The GAs were chromatographed on a 3.9×300 mm μ-Bonda Pak C_{18} column (Waters) and eluted at 1.5 ml min^{-1} with the following gradient: 0 to 5 min, isocratic 28% MeOH in 1% aqueous acetic acid; 5 to 35 min, linear gradient from 28 to 86% MeOH; 35 to 36 min, 86 to 100% MeOH; 36 to 40 min, isocratic 100% MeOH. Up to fifty fractions of 1.5 ml each were collected. Small aliquots (15 μ*l*) from each fraction were taken, and radioactivity was measured with liquid scintillation spectrometry (Beckman, LS 1801) to determine radioactivity and accurate retention times of each GA based upon the elution of ^{3}H-GA standards.

Identification of endogenous gibberellins

Radioactivity for each concentrated GA fraction was counted for 15 min in liquid scintillation counter. Each GA fraction was redissolved in 100% methanol, transferred to a 1 ml vial and dried under N_2 at 40°C. The sample was dissolved in 35 μl of methanol, and the GA methyl ester was prepared with ethereal diazomethane. The sample was dried under N_2, redissolved in methanol and methylated one more time. The sample was dissolved in 35 μl pyridine, and silylated for 30 min at 65°C with the same amount of N, O-Bis (trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% TMCS (Pierce Chemical Co.). The sample was then reduced to dryness with N_2 and solublized in anhydrous dichloromethane. 1 μl of each sample was injected on-column on a 30 m×0.25 mm (i.d.), 0.25 μm film thickness DB-1 capillary column (J & W Co.). The GC (Finngan Mat GCQ) oven temperature was programmed for a 1 min hold at 60°C, then to rise at 15°C min^{-1} to 200°C followed by 5°C min^{-1} to 285°C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector with an interface and source temperature of 280°C, an ionising voltage of 70 eV and a dwell time of 100 ms. Full scan mode (the first trial) and three major ions of the supplemented [$^2H_2$]GA internal standards (the second trial) and the endogenous gibberellins were monitored simultaneously. Retention time was determined by using the hydrocarbon standards (C_{23}, C_{24}, C_{25}, C_{26}, C_{27} and C_{28}) to calculate the KRI (Kovats retention indices) value.

RESULTS AND DISCUSSION

To date, several endogenous GAs have been identified in the bulbils of the Chinese yam (Tanno *et al.*, 1992). Many metabolic products were formed following the feeding of [^{14}C]gibberellin A_{12} as the primary gibberellin precursor. To check authentic GA-metabolites in seedlings of Chinese yam, the deuterated GAs, KRI values, retention times and parent ion from the each HPLC fraction of seedlings were evaluated by GC-MS (Table 1).

Radioactivity of GA-metabolites, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, GA_{15}, GA_{24}, GA_9, GA_{36} and GA_4 was also showed in Table 3 and 4. [^{14}C] gibberellin A_{12} as substrate was rapidly metabolized for 18h after feeding to seedlings of the Chinese yam, otherwise, [^{14}C] gibberellin A_{12} was slowly metabolized at 18 h to 24 h in time-course feeding (Table 2).

Table 3 shows the radioactivity of endogenous gibberellins for an early C-13 hydroxylation (ECH) pathway. In the radioactivity of an early C-13 hydroxylation route, GA_{53} was one of the first compound committed to the GA pathway. After 6 HAF, GA_{19} was metabolized sharply, and then,

Table 1. HPLC fractions, KRI, relative ion intensity from acidic ethyl acetate fractions of gibberellins in seedlings of the Chinese yam.

Fraction no.	GAs	KRI[a]	Source		m/z (%, relative intensity of base peak)[b]			
6-8	GA$_8$	2818	^{12}C	594(100)	448(25)	379(20)	375(15)	238(28)
		2818	^{14}C	596(100)	450(24)	381(21)	375(11)	240(26)
12-14	GA$_1$	2674	^{12}C	506(100)	448(20)	313(17)	491(13)	377(12)
		2674	^{14}C	508(100)	450(19)	315(14)	493(11)	379(13)
24,25	GA$_{20}$	2485	^{12}C	418(100)	375(45)	403(14)	359(12)	301(13)
		2485	^{14}C	420(100)	377(45)	405(13)	361(10)	303(11)
26-28	GA$_{44}$	2789	^{12}C	432 (63)	238(41)	417(12)	373(17)	207(100)
		2789	^{14}C	434 (62)	240(39)	419(10)	375(16)	209(100)
26-28	GA$_{36}$	2600	^{12}C	284(100)	430(58)	312(47)	462(11)	402(38)
		2600	^{14}C	286(100)	432(56)	314(45)	464(10)	404(40)
29-31	GA$_{19}$	2600	^{12}C	434(100)	374(59)	402(41)	462(10)	375(57)
		2600	^{14}C	436(100)	376(57)	404(40)	464(9)	377(55)
32,33	GA$_4$	2506	^{12}C	284(100)	225(80)	289(70)	224(76)	418(26)
		2506	^{14}C	286(100)	227(76)	291(71)	226(75)	420(23)
34-36	GA$_{24}$	2444	^{12}C	314(100)	226(89)	286(77)	342(42)	374(4)
		2444	^{14}C	316(100)	228(87)	288(75)	344(40)	376(3)
37,38	GA$_{53}$	2450	^{12}C	448 (47)	251(30)	235(30)	389(25)	241(18)
		2450	^{14}C	450 (47)	253(28)	237(28)	391(25)	243(19)
37,38	GA$_9$	2305	^{12}C	298(100)	270(78)	227(48)	243(43)	330(6)
		2305	^{14}C	300(100)	272(77)	229(48)	245(42)	332(6)
39,40	GA$_{15}$	2608	^{12}C	239(100)	284(50)	344(23)	312(22)	298(13)
		2608	^{14}C	241(100)	286(48)	346(19)	314(17)	300(11)
42-44	GA$_{12}$	2335	^{12}C	300(100)	240(31)	328(31)	360(2)	285(19)
		2335	^{14}C	302(100)	242(32)	330(29)	362(2)	287(20)

[a]KRI, Kovats retention indices. [b]Identified as methyl ester trimethylsilyl ether derivatives by comparison with reference spectra and KRI data (Gaskin and MacMillan, 1991). Gibberellin is quantified with comparisons of peak area ratio of prominent ions.

Table 2. Reductive radioactivity of [^{14}C] gibberellin A$_{12}$ in time-course feeding in seedlings of the Chinese yam.

Time after feeding (h)	Radioactivity (DPM)
6	11,395 800
12	8,945 500
18	6,248 450
24	4,955 490

DPM: disintegration per 10 min. Data are means values of two replicates SE.

GA$_8$, GA$_{44}$, GA$_{53}$ and GA$_{20}$ were metabolized respectively. Radioactivity of GA$_{44}$ including GA$_{53}$, GA$_{19}$ and GA$_{20}$ was decreased greatly at 12 HAF after feeding except for GA$_8$. At 18 HAF, radioactivity of GA$_{19}$, GA$_{44}$ and GA$_{53}$ was increased dramatically, however, radioactivity of GA$_{20}$ was smaller than that of 12 HAF. At 24h as end feeding, GA$_{19}$ was only increased dominantly and radioactivity of GA$_{53}$, GA$_{44}$ and GA$_8$ was decreased compared to 18 HAF, GA$_{19}$ was the highest GA metabolite at 6 HAF, meanwhile, GA$_{20}$ as precursor of bioactive GA$_1$ was the lowest among GA

Table 3. Radioactivity of endogenous gibberellins for an early C-13 hydroxylation in time-course feeding of [^{14}C] gibberellin A$_{12}$ in the seedlings of Chinese yam.

GAs	Time after feeding (h)			
	6	12	18	24
	-----------DPM-----------			
GA$_{53}$	184±21	146±7	205±14	184±9
GA$_{44}$	233±9	130±11	270±23	197±14
GA$_{19}$	361±11	187±15	379±20	439±11
GA$_{20}$	147±15	138±18	127±16	159±26
GA$_1$	169±8	182±22	208±11	188±18
GA$_8$	255±24	266±10	301±25	257±23

DPM: disintegration per 10 min. Data are means values of two replicates±SE.

members of the ECH. Radioactivity of GA$_{20}$ was always lowest in time-course feeding except for 12 HAF. Radioactivity of GA$_{19}$ was linearly increased after 12 HAF suggesting one of the most abundant compounds in ECH route. The most abundant GA-metabolite in ECH route might be GA$_{19}$-

Table 4. Radioactivity of endogenous gibberellins for non C-13 hydroxylation in time-course feeding of [^{14}C] gibberellin A$_{12}$ in the seedlings of Chinese yam.

GAs	Time after feeding (h)			
	6	12	18	24
	-----------------------DPM-----------------------			
GA$_{15}$	292±33	227±13	286±20	377±11
GA$_{24}$	727±27	769±21	1,614±54	2,118±46
GA$_9$	606±19	221±11	812±28	378±13
GA$_{36}$	271±4	229±17	357±18	511±25
GA$_4$	463±21	353±5	535±26	612±10

DPM: disintegration per 10 min. Data are means values of two replicates±SE.

Table 5. Radioactivity of endogenous gibberellins for an early C-13 hydroxylation (ECH), non C-13 hydroxylation (NCH) and total endogenous gibberellins (TGA) in time-course feeding of [^{14}C] gibberellin A$_{12}$ in the seedlings of Chinese yam.

GA groups	Time after feeding (h)			
	6	12	18	24
	------------------------DPM------------------------			
ECH	1,349	1,049	1,490	1,424
NCH	2,359	1,799	3,604	3,996
TGA	3,708	2,848	5,094	5,420

DPM: disintegration per 10 min. Data for ECH, NCH and TGA are aggregated from Table 3 and 4.
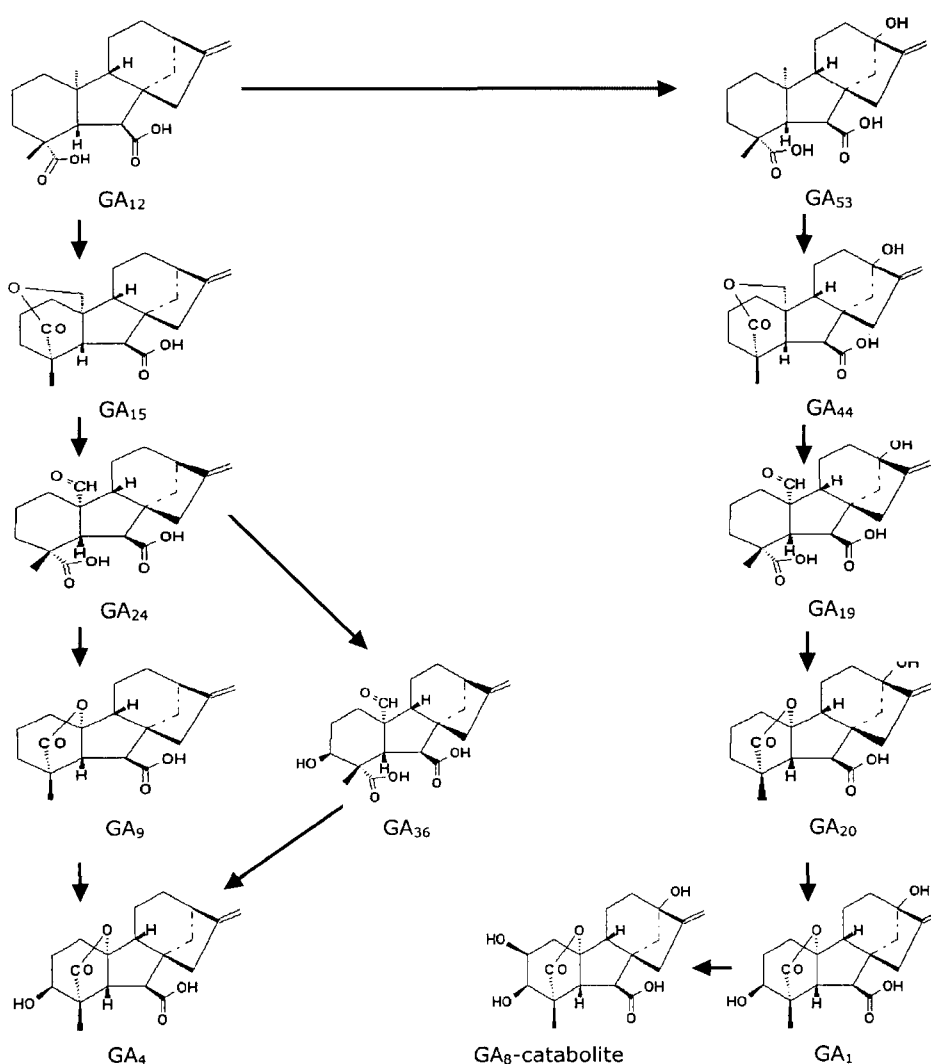
Fig. 1. Non C-13 (left) hydroxylation and early C-13 hydroxylation (right) routes as the two GA biosynthesis pathways operated in the Chinese yam plants.

metabolite. Radioactivity of GA$_8$ as catabolite of GA$_1$ was always higher than that of GA$_1$. Radioactivity in GA members of ECH route was below 450 DPM. This was consistent with GA metabolism in vegetative tissues of most other

plant species (Sponsel, 1995), including another member of the Solanaceae, *Lycoperisicion esculentum* (Bohner *et al.*, 1988).

Table 4 shows the radioactivity of endogenous gibberellins for non C-13 hydroxylation (NCH) pathway. In radioactivity of NCH, radioactivity of GA members except for GA_{24} was lowest at 12 HAF, in addition, radioactivity of GA members, GA_{15}, GA_9, GA_{36} and GA_4 was always lower than that of GA_{24} in all time-course feeding. Radioactivity of GA_{24} was not changed at 6 and 12 HAF, however, GA_{24} was highly increased at 18 and 24 HAF. GA_{15} and GA_{36} were smaller radioactivity than GA_{24} and GA_9 except for 24 HAF. In addition, radioactivity of GA_9 was always higher than that of GA_{36}.

We also compared the change of bioactive GAs, GA_1 and GA_4 during time-course feeding and also compared GA members of each ECH and NCH and total GAs in time-course feeding (Table 3, 4 and 5). Radioactivity of GAs in NCH group was five-fold higher than that of ECH in analyzed GA-metabolites. It was a major difference between the metabolites of two GA metabolic pathways obtained by feeding of [^{14}C] gibberellin A_{12}. In comparing the bioactive GAs, GA_4 and GA_1, radioactivity of GA_4 was always four times higher than that of GA_1 at every feeding time. GA_1 radioactivity has always a lower level to below 200 DPM.

Thus, we concluded that otherwise most other species which have been studied, the major pathway of endogenous GA metabolism in seedlings of the Chinese yam is the non C-13 hydroxylation pathway, and the early C-13 hydroxylation pathway is also presented (Fig. 1). Furthermore, detection of most of the members of the early C-13 hydroxylation (ECH) route, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1 and GA_8, and the member of the non C-13 hydroxylation (NCH) route, GA_{15}, GA_{24}, GA_9, GA_{36} and GA_4, strongly suggested that two GA metabolic pathways active in the Chinese yam.

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REFERENCES

Bohner, J, P. Hedden, E. Bora-Haber, and F. Bangerth. 1988. Identification and quantification of gibberellins in fruits of *Lycopersicon esculentum*, and their relationship to fruit size in *L. esculentum* and *L. pimpinellifolium*. Physiol. Plant. 73 : 248-353.

Gaskin, P. and J. MacMillan. 1991. GC-MS of the gibberellins and related compounds: Methodology and a library of spectra. Cantock's Enterprises, Bristol, UK.

Kim, S. K., S. C. Lee, D. H. Shin, S. W. Jang, J. W. Nam, and I. J. Lee, 2003. Quantification of endogenous gibberellins in leaves and tubers of Chinese yam, *Dioscorea opposita* Thunb. cv. Tsukune during tuber enlargement. Plant Growth Regul. 39(2) : 125-130.

Lee, I. J., K. R. Foster, and P. W. Morgan. 1998. Photoperiod control of gibberellin levels and flowering in *Sorghum*. Plant Physiol. 116 : 1003-101.

Okagami, N. 1972. The nature of gibberellin-induced dormancy in aerial tubers of *Begonia evansiana*. Plant Cell Physiol 13 : 763-771.

Okagami, N. and N. Tanno. 1993. Gibberellic acid-induced prolongation of the dormancy in tubers or rhizomes of several species of East Asian *Dioscorea*. Plant Growth Regulation 12 : 119-123.

Okagami, N. and M. Nagao. 1971. Gibberellin-induced dormancy in bulbils of *Dioscorea*. Planta 101 : 91-94.

Okagami, N. and N. Tanno. 1977. Dormancy in *Dioscorea*: generality of gibberellin induced dormancy in asexual dormant organs. Plant Cell Physol 18 : 309-316.

Sponsel, V. M. 1995. Gibberellin biosynthesis and metabolism. *In*: Davies PJ (ed.): Plant Hormones: Physiology, Biochemistry and Molecular Biology, Kluwer, Dordrecht, The Netherlands, pp. 66-97.

Tanno, N., T. Yokota, M. Abe, and N. Okagami. 1992. Identification of endogenous gibberellins in dormant bulbils of chinese yam, *Dioscorea opposita*. Plant Physiol.. 100 : 1823-1826.

Zhu, Y. X. and P. J. Davies. 1991. The identification and metabolism of GA_9 and its metabolites in seed coats and shoots of pea, line G2. Plant Growth Regulation. 10 : 13-26.