



Research Paper

Identification of endogenous gibberellins operated in leaves of Kohlrabi (*Brassica oleracea* var. *gongylodes* L.) by feeding of [¹⁴C] GA₁₂

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ABSTRACT

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The aims of this study were first to determine the possible gibberellins biosynthetic pathway operated in the leaves of kohlrabi seedlings. Twelve gibberellins (GAs) were first identified and quantified in extracts of corm of the different skin colors of kohlrabi, *Brassica oleracea* var. *gongylodes* L. by GC-MS-SIM and Kovats retention indices. Six of these gibberellins are members of the early-C13-hydroxylation pathway (GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₈, and GA₁), and six are members of the non-C13-hydroxylation pathway (GA₁₅, GA₂₄, GA₉, GA₇, GA₃₆, and GA₄). Consequently, major gibberellin biosynthetic pathway operated in the leaves of kohlrabi seedlings was quantitatively an early C-13 hydroxylation route.

Key words: Kohlrabi, leaves, gibberellins, biosynthetic pathway.

INTRODUCTION

Kohlrabi (*Brassica oleracea* var. *gongylodes*) belongs to family *Brassicaceae* and is considered as a cole crop and its edible portion is swollen enlarged stem (corm). It is well known that, kohlrabi has enormous nutritional and medicinal values due to its high contents of vitamin C, potassium and antioxidant substances which prevent the formation of cancer causing agents (Beecher, 1994). There are several kohlrabi varieties with various skin colors; white, purple, and pale green. Recently, kohlrabi was introduced in Korea and its consumption is slowly increasing. Kohlrabi is mainly produced in Jeju island during winter to produce a high quality vegetable (Choi et al., 2010). Gibberellins (GAs) are essential phytohormones that control many aspects of plant developments, including seed germination, leaf expansion, stem elongation, flowering, and seed development (Kaur et al., 1998). The metabolism of GA has been deeply investigated and is quite well understood (Sponsel and Hedden, 2004). *Ent*-kaurene, synthesized from geranylgeranyl diphosphate by the action of two cyclases, is metabolized by the action of P450-dependent monooxygenases to GA₁₂ and/or GA₅₃, which in turn are metabolized by GA 20-oxidases and GA 3-oxidases, acting consecutively, to active GAs through two parallel pathways: the non-13-hydroxylation (leading to GA₄) and

the early-13-hydroxylation one (leading to GA₁ and GA₃ in some cases). Active GAs and their precursors can be irreversibly inactivated by GA 2-oxidases introducing a hydroxyl at the 2b position (Sponsel and Hedden, 2004). Gibberellins are also closely related to tuber enlargement of Chinese yam and gibberellin treatment increases tuber yield depending on their varieties (Kim et al., 2003a, b, c, d). There were no any studies on gibberellin metabolism of kohlrabi in relation to corm enlargement during growing season. Therefore, in this study, it focuses on the identification of endogenous gibberellins and possible biosynthetic pathway of gibberellins in the leaves of kohlrabi seedlings.

MATERIALS AND METHODS

Plant sample and feeding of [¹⁴C] gibberellin A₁₂

Purple kohlrabi (commercial cultivar: Purple king) seeds were germinated and grown in a sand-vermiculite mixture. When seedlings of kohlrabi showed fully expanded leaves and grown for two weeks, and then seedlings were fed to confirm the endogenous gibberellin biosynthetic pathways.

The substrate of [^{14}C] gibberellin A_{12} was purchased from L.N. Mander (Australian National University, Canberra, Australia). The compound was about 96.7% radioactive. Specific activity was $10 \mu\text{Ci mmol}^{-1}$. The substrate was dissolved in EtOH: water (1:1, v/v). Ten μl (about 370 Bq) for each fifteen seedlings was fed to shoot tops and surface of whole leaves below apex of the plants. The quantification of [^{14}C] gibberellin A_{12} was performed by injection using a micro syringe. After feeding, seedlings were grown at 25°C in a constant temperature room with continuous light at $289 \mu\text{mol m}^{-2}\text{s}^{-1}$ provided by fluorescent lamps. Seedlings were harvested at 6, 12, 18, and 24 h after feeding. Seedlings were frozen in liquid nitrogen immediately after harvesting and stored at -70°C for analysis of GAs (gibberellins).

Extraction of endogenous gibberellins

The corms harvested from different skin color of kohlrabi were immediately frozen in liquid nitrogen and stored at -80°C . When all the required materials for gibberellin analysis had been collected, the samples were lyophilized for 48 h. The extraction of endogenous gibberellins was followed as described by Lee et al. (1998).

High performance liquid chromatography

The gibberellins were chromatographed on a $3.9 \times 300 \text{ mm}$ $\mu\text{BondaPak C}_{18}$ column (Waters) and eluted at 1.5 ml min^{-1} with 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 50 fractions of 1.5 ml each were collected. Small aliquots (15 μl) from each fraction were taken, and radioactivity was measured with liquid scintillation spectrometry (Beckman, LS 1801) to determine accurate retention times of each gibberellin (GA) based upon the elution of ^3H -GA standards. The fractions were dried on a Savant Speedvac and combined according to the retention times of ^3H -GA standards and previously determined retention times of the labeled (deuterated) GA standards.

Identification of endogenous gibberellins

Each gibberellin fraction was redissolved in 100% methanol, transferred to a 1 ml vial and dried under nitrogen gas at 40°C . The sample was dissolved in 35 μl of methanol, and gibberellin methyl ester was prepared with ethereal diazomethane. The sample was dried under nitrogen gas, redissolved in methanol and methylated on 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 nitrogen gas and solubilized in anhydrous dichloromethane. 1 μl of each sample was injected on-column on a $30 \text{ m} \times 0.25 \text{ mm}$ (i.d.), 0.25 μm film thickness DB-1 capillary column (J&W Co.). The GC (Finnigan Mat GCQ) oven temperature was programmed for a 1 min hold at 60°C , then to rise at 15 min^{-1} to 200°C followed by 5 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 ionizing voltage of 70 eV and a dwell time of 100 ms. Full scan mode (the first trial) and five major ions of the supplemented [$^2\text{H}_2$] gibberellin 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 (Kovats, 1958).

Statistical analysis

The standard deviation was calculated using Sigma plot 2001 software (Jandel Scientific, San Rafael, CA, USA).

RESULTS AND DISCUSSION

Diverse metabolic products were formed following the feeding of [^{14}C] gibberellin A_{12} as the primary gibberellin precursor. To check authentic gibberellin metabolites in leaves of kohlrabi seedlings, the deuterated gibberellins, KRI values with those from a spectral library, retention times and parent ion from the each HPLC fraction of kohlrabi seedlings were analyzed by GC-MS (Table 1). The [^{14}C] gibberellin A_{12} was slowly metabolized at 12 to 24 h in time-course feeding (Figure 1). Radioactivity of gibberellin metabolites, GA_{53} , GA_{44} , GA_{19} , GA_{20} , GA_8 , GA_1 , GA_{15} , GA_{24} , GA_9 , GA_7 , GA_{36} and GA_4 were shown in Tables 2 and 3.

Table 2 shows the radioactivity of endogenous gibberellins for an early C-13 hydroxylation pathway (ECH). In the radioactivity of an early C-13 hydroxylation route, GA_{53} was one of the first compounds committed to the gibberellins biosynthetic pathway. Among these endogenous gibberellins, the GA_{53} , GA_1 and GA_8 were greatly metabolized at 18 h after feeding of [^{14}C] gibberellin A_{12} . And the GA_{19} and GA_{20} as intermediated metabolite of GA_1 were highly metabolized at 24 h after feeding of [^{14}C] gibberellin A_{12} . The GA_{44} having closed lactone form was only highly activated at 6 h after feeding of [^{14}C] gibberellin A_{12} . Gibberellin A_{12} -aldehyde is converted to GA_{53} , GA_{44} , GA_{19} and GA_{20} as main products and gibberellin A_{53} was metabolised mainly to the GAs GA_{44} and GA_{19} , with the GA_{20} , GA_1 and GA_8 as minor products (Lange et al., 2003).

In our study, the GA_{19} was mainly metabolized for 24 h

Table 1. HPLC fractions, KRI, relative ion intensity from acidic ethyl acetate fractions of gibberellins in leaves of kohlrabi seedlings.

Fraction no.	GAs	KRI [†]	Source	m/z (% relative intensity of base peak) [‡]				
37, 38	GA ₅₃	2450	¹² C	448 (47)	251 (30)	235 (30)	389 (25)	241 (18)
		2450	¹⁴ C	450 (46)	253 (28)	237 (27)	391 (25)	243 (17)
43-46	GA ₁₂	2335	¹² C	300 (100)	240 (31)	328 (21)	360 (2)	285 (19)
		2335	¹⁴ C	302 (100)	242 (29)	330 (20)	362 (2)	287 (20)
39, 40	GA ₁₅	2608	¹² C	237 (100)	282 (30)	342 (22)	310 (20)	296 (11)
		2608	¹⁴ C	239 (100)	284 (28)	344 (23)	312 (22)	298 (13)
26-28	GA ₄₄	2789	¹² C	205 (100)	430 (67)	236 (40)	415 (11)	371 (18)
		2789	¹⁴ C	207 (100)	432 (63)	238 (41)	417 (12)	373 (17)
34-36	GA ₂₄	2444	¹² C	314 (100)	226(89)	286(77)	342(42)	374(4)
		2444	¹⁴ C	316 (100)	228 (85)	288 (76)	344 (43)	376 (4)
29-31	GA ₁₉	2600	¹² C	434 (100)	374 (59)	402 (41)	462 (10)	375 (57)
		2600	¹⁴ C	436 (100)	376 (55)	404 (40)	464 (11)	377 (53)
37, 38	GA ₉	2305	¹² C	298 (100)	270 (78)	227 (49)	243 (43)	330 (7)
		2305	¹⁴ C	300 (100)	272 (76)	229 (50)	245 (44)	332 (5)
33-35	GA ₇	2525	¹² C	222 (100)	356 (22)	223 (73)	416 (9)	384 (22)
		2525	¹⁴ C	224 (100)	358 (17)	225 (60)	418 (11)	386 (12)
24, 25	GA ₂₀	2485	¹² C	418 (100)	375 (45)	403(14)	359(12)	301(13)
		2485	¹⁴ C	420 (100)	377 (40)	405 (14)	361 (11)	303 (12)
26-28	GA ₃₆	2600	¹² C	282 (100)	428 (63)	310 (48)	460 (12)	400 (40)
		2600	¹⁴ C	284 (100)	430 (58)	312 (47)	462 (11)	402 (39)
32, 33	GA ₄	2506	¹² C	284 (100)	225 (80)	289 (70)	224 (76)	418 (27)
		2506	¹⁴ C	286 (100)	227 (78)	291 (68)	226 (75)	420 (26)
12-14	GA ₁	2674	¹² C	506 (100)	448 (20)	313 (17)	491 (13)	377 (12)
		2674	¹⁴ C	508 (100)	450 (17)	315 (16)	493 (14)	379 (11)
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)	377 (11)	240 (26)

[†]KRI, Kovats retention indices.

[‡]Gibberellin is identified with five ions and quantified by first ion with comparison of labeled standards and also identified as methyl ester trimethylsilyl ether derivatives by comparison with reference spectra and KRI data (Gaskin and MacMillan, 1991).

after feeding of [¹⁴C] gibberellin A₁₂. Each gibberellin operated in leaves of kohlrabi seedlings quantitatively differed from each time-course intervals. In particular, Radioactivity of each gibberellin-metabolite showed lowest at only 12 h after feeding of [¹⁴C] gibberellin A₁₂. And radioactivity of GA₈ as catabolite of GA₁ was always higher than that of GA₁. Radioactivity in gibberellin members of ECH route was below 500 DPM. This result was consistent

with gibberellin metabolism in vegetative tissues of most other plant species (Sponsel, 1995), including another member of the Solanaceae, *Lycopersicon esculentum* (Bohner et al., 1988) and the Dioscoreaceae, *Dioscorea opposita* (Kim et al., 2003). The greater radioactivity of each gibberellin for time-course was GA₁₉, GA₈, GA₄₄, GA₁, GA₅₃ and GA₂₀, in turn.

Table 3 shows the radioactivity of endogenous

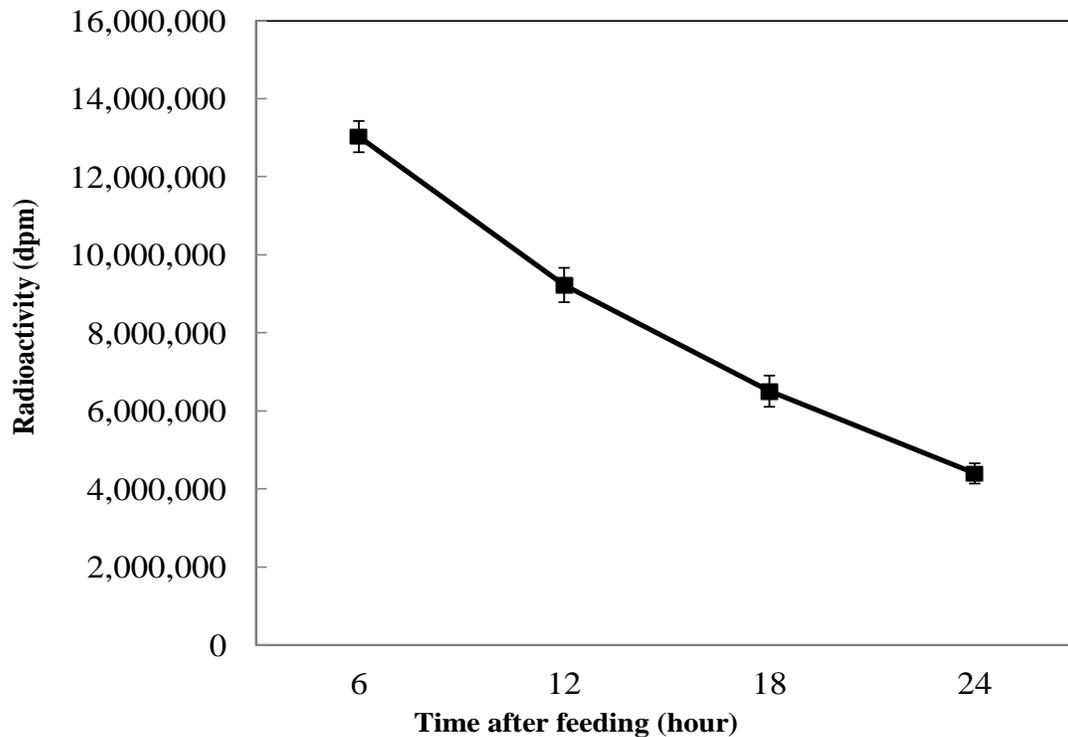


Figure 1. Radioactivity (unit: DPM) of [¹⁴C] gibberellin A₁₂ in time-course feeding in seedlings of kohlrabi. Data are means values of three replicates ± SE.

Table 2. Radioactivity(unit: DPM) of endogenous gibberellins for an early C-13 hydroxylation in time-course feeding of [¹⁴C] gibberellin A₁₂ in the leaves of kohlrabi seedlings.

Gibberellins (GAs)	Time after feeding (h)			
	6	12	18	24
GA ₅₃	199 ± 16	158 ± 7	229 ± 13	176 ± 17
GA ₄₄	284 ± 22	142 ± 11	248 ± 11	182 ± 10
GA ₁₉	289 ± 15	192 ± 8	302 ± 14	487 ± 10
GA ₂₀	137 ± 10	119 ± 13	167 ± 14	192 ± 13
GA ₁	184 ± 9	197 ± 10	222 ± 16	202 ± 12
GA ₈	287 ± 19	265 ± 7	347 ± 10	290 ± 16

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

gibberellins for non C-13 hydroxylation pathway (NCH). In the radioactivity of non C-13 hydroxylation route, among these endogenous gibberellins, the GA₁₅ and GA₇ were greatly metabolized at 18 h after feeding of [¹⁴C] gibberellin A₁₂. And the GA₉ and GA₃₆ as intermediated metabolite of GA₄ were highly metabolized at 24 h after feeding of [¹⁴C] gibberellin A₁₂. Otherwise the GA₂₄ and GA₄ showed independently higher radioactivity as 226 and 486 DPM at 6 h and 12 h after feeding of [¹⁴C] gibberellin A₁₂, respectively. In the time-course of radioactivity, bioactive GA₄ was pronouncedly the most activated among these gibberellins during time-course of [¹⁴C] gibberellin A₁₂ feeding. Otherwise GA₃₆ showed the lowest radioactivity for

time-course of [¹⁴C] gibberellin A₁₂ feeding. Table 4 represents the radioactivity of endogenous gibberellins for an early C-13 hydroxylation (ECH), non C-13 hydroxylation (NCH) and total gibberellins (TGA) in time-course feeding of [¹⁴C] gibberellin A₁₂ in the leaves of kohlrabi seedlings. The radioactivity of ECH gibberellin pathway was gradually increased except for 6 h after feeding of [¹⁴C] gibberellin A₁₂. Otherwise, the radioactivity of NCH gibberellin pathway was slightly elevated in proportion to time-course proceed. In the comparison of these two gibberellin biosynthetic pathways, radioactivity of non C-13 hydroxylation (NCH) gibberellin pathway was only higher metabolized in 12 h after feeding of [¹⁴C] gibberellin A₁₂. Radioactivity of an

Table 3. Radioactivity(unit: DPM) of endogenous gibberellins for non C-13 hydroxylation in time-course feeding of [¹⁴C] gibberellin A₁₂ in the leaves of kohlrabi seedlings.

Gibberellins (GAs)	Time after feeding (h)			
	6	12	18	24
GA ₁₅	132 ± 10	118 ± 13	165 ± 23	154 ± 6
GA ₂₄	226 ± 13	100 ± 8	203 ± 16	179 ± 12
GA ₉	232 ± 11	135 ± 14	248 ± 14	351 ± 8
GA ₃₆	103 ± 12	101 ± 18	135 ± 20	169 ± 2
GA ₄	229 ± 15	486 ± 10	293 ± 13	278 ± 5
GA ₇	207 ± 11	238 ± 23	305 ± 17	233 ± 11

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

Table 4. Radioactivity (unit: DPM) of endogenous gibberellins for an early C-13 hydroxylation (ECH), non C-13 hydroxylation (NCH) and total gibberellins (TGA) in time-course feeding of [¹⁴C] gibberellin A₁₂ in the leaves of kohlrabi seedlings.

Gibberellin routes	Time after feeding (h)			
	6	12	18	24
ECH	1,380	1,073	1,515	1,529
NCH	1,129	1,178	1,349	1,364
TGA	2,509	2,251	2,864	2,893

DPM: disintegration per 10 min. Data for ECH, NCH, and TGA are aggregated from Table 2 and 3.

early C-13 hydroxylation (ECH) gibberellin pathway was always greater metabolized in 6, 18 and 24 h after feeding of [¹⁴C] gibberellin A₁₂. The presence of all six non-C13-hydroxylation gibberellins (GA₁₅, GA₂₄, GA₉, GA₃₆, GA₄ and GA₇) and six early-C13-hydroxylation gibberellins (GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₁ and GA₈) are commonly operated in the leaves of kohlrabi seedlings. Consequently, the result suggests that major

gibberellin biosynthetic pathway operated in the leaves of kohlrabi seedlings is under non C-13 hydroxylation (NCH) gibberellin pathway. The radioactivity of total gibberellins including ECH and NCH gibberellin pathways was also gradually increased with proceeding of time-course.

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