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Changes of Endogenous Gibberellins in Tubers of Chinese Yam (*Dioscorea opposita*) during Storage Period

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ABSTRACT: For storage periods of tubers in Chinese yam, the levels of GA_{44} and GA_{20} was constant, meanwhile both GA_{53} and GA_{19} level were always higher than that of GA_{44} and GA_{20}. GA_9 content as precursor of GA_4 was not changed during storage. GA_{24} content was low to below 0.2 ng for 90 days after storage, GA_{36} content as precursor of GA_4 like GA_9 was about 6-8 fold higher than that of GA_9 during storage. GA contents of the two gibberellin biosynthetic pathways were gradually increased when storage periods were progressed. Bioactive GA_1 content as the GA members of an early C-13 hydroxylation was always constant, and its content was very low as below 0.1 ng per dry weight, meanwhile, bioactive GA_4 content as the GA members of non C-13 hydroxylation was drastically increased, also, its content was highest at 90 days after storage, and then decreased at 120 days after storage. Consequently, we suggest that GA_4 may be involved in controlling tuber sprouting in Chinese yam.

Keywords: Chinese yam, *Dioscorea opposita*, tubers, gibberellins (GAs), quantification, storage

Plants develop dormancy to survive adverse climatic conditions (Villiers, 1975). Application of gibberellins (GAs) is known to break dormancy of many organs of a large number of plant species (Pharis 1985; Stuart and Cathey, 1971). However, in contrast, applied GAs markedly inhibit sprouting of bulbils and subterranean organs of several species in the genus *Dioscorea* (Okagami and Tanno, 1993). Additionally, dormancy induction by GA application has been reported for various organs of several other species, namely, *Begonia evansiana* (bulbils [Nagao and Mitsui, 1959]), *Vitis vinifera* (winter buds [Weaver, 1959]), *Acer pseudoplatanus*, *Fagus sylvatica*, *Fraxinus excelsior*, *Liriodendron tulipifera*, and *Sorbus aucuparia* (vegetative and/or flower buds [Brian *et al.*, 1959]), *Spirodela polyrrhiza* (turions [Czopek, 1964]), and *Sedum kamchaticum* (seeds [Fujii *et al.*, 1960]). To date observations on the effect of GA application on the genus *Dioscorea* has been limited to several cultivated and wild species (Okagami and Nagao, 1971; Okagami and Tanno, 1993).

In bulbils and rhizomes of *Dioscorea*, because of sprouting promotion by application of inhibitors of GA biosynthesis (Okagami and Nagao, 1971; Tanno *et al.*, 1995), endogenous GAs are considered to be involved in the induction of dormancy under natural conditions. Furthermore, bulbil dormancy in *Begonia evansiana* (Okagami, 1972) and the genus *Dioscorea* (Okagami and Tanno, 1977) is also induced by endogenous and/or exogenous gibberellins. In Chinese yams, endogenous gibberellins in the dormant bulbils have been identified (Tanno *et al.*, 1992). It is shown that early C-13 hydroxylation and non C-13 hydroxylation, two possible biosynthetic GA pathways, may operate in the dormant bulbils of the Chinese yam.

Thus, in this context, we examined the changes of endogenous gibberellins of tubers in Chinese yam during storage periods.

MATERIALS AND METHODS

Plant growth and storage conditions

Tubers of *Dioscorea opposita* Thunb. cv. Tsukune were cut into three or six pieces (about 45-50 g, FW), which were pre-sprouted on a mixture of vermiculite and sand (1:1, v/v) in plastic pots ($0.5 \times 0.3 \times 0.15$ m [high]; total volume 2.3 m^3), and then kept in a chamber at 25°C in the dark. After 30 to 35 days of incubation, the sprouted tuber pieces were planted and grown in a glasshouse, following common cultural practice at the Institute of Bioresources at Kyongbuk Provincial Agricultural Technology Administration on June 15 in 2001. To determine the changes of endogenous gibberellins of tubers during storage, matured tubers which harvested at November 22 in 2001, were stored in a temperature-controlled chamber in the dark at 4±0.5°C at an approximate relative humidity of 85±3%. The contents of endogenous gibberellins in the stored tubers were determined periodically at 30 day interval and analyzed twice for each same samples.

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Extraction of endogenous gibberellins

Extraction and HPLC of gibberellins followed the reference (Lee *et al.,* 1998). Lyophilized tissue samples (ca. 1g, DW) 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 (Whatman International Ltd., England). 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 Associates) 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

Table 1. HPLC fractions, KRI, radioactive metabolites from acidic ethyl acetate fractions of gibberellins in tubers of the Chinese yam.

Fraction no.	GAs	KRI[a]	Source	*m/z* (%, relative intensity of base peak)[b]				
12-14	GA_1	2674	sample	506(100)	448(20)	313(17)	491(13)	377(12)
		2674	standard	508(100)	450(19)	315(14)	493(11)	379(13)
24,25	GA_{20}	2485	sample	418(100)	375(45)	403(14)	359(12)	301(13)
		2485	standard	420(100)	377(45)	405(13)	361(10)	303(11)
26-28	GA_{44}	2789	sample	432 (63)	238(41)	417(12)	373(17)	207(100)
		2789	standard	434 (62)	240(39)	419(10)	375(16)	209(100)
26-28	GA_{36}	2600	sample	284(100)	430(58)	312(47)	462(11)	402(38)
		2600	standard	286(100)	432(56)	314(45)	464(10)	404(40)
29-31	GA_{19}	2600	sample	434(100)	374(59)	402(41)	462(10)	375(57)
		2600	standard	436(100)	376(57)	404(40)	464(9)	377(55)
32,33	GA_4	2506	sample	284(100)	225(80)	289(70)	224(76)	418(26)
		2506	standard	286(100)	227(76)	291(71)	226(75)	420(23)
34-36	GA_{24}	2444	sample	314(100)	226(89)	286(77)	342(42)	374(4)
		2444	standard	316(100)	228(87)	288(75)	344(40)	376(3)
37,38	GA_{53}	2450	sample	448 (47)	251(30)	235(30)	389(25)	241(18)
		2450	standard	450 (47)	253(28)	237(28)	391(25)	243(19)
37,38	GA_9	2305	sample	298(100)	270(78)	227(48)	243(43)	330(6)
		2305	standard	300(100)	272(77)	229(48)	245(42)	332(6)
39,40	GA_{15}	2608	sample	239(100)	284(50)	344(23)	312(22)	298(13)
		2608	standard	241(100)	286(48)	346(19)	314(17)	300(11)
42-44	GA_{12}	2335	sample	300(100)	240(31)	328(31)	360(2)	285(19)
		2335	standard	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 was quantified with comparisons of peak area ratio of prominent ions.

fifty 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 radioactivity and accurate retention times of each GA based upon the elution of ^{3}H-GA standards.

Quantification of endogenous gibberellins

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 · 1 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 · 1 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 · 1 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. The endogenous GA contents of GA_{53}, GA_{12}, GA_{15}, GA_{44}, GA_{24}, GA_{19}, GA_9, GA_{20}, GA_{36}, GA_4 and GA_1 was calculated from the peak area ratios of 450/448, 302/300, 241/239, 209/207, 316/314, 436/434, 300/298, 420/418, 286/284 and 508/506, respectively (Table 1).

RESULTS AND DISCUSSION

Dormancy of reproductive organs, tubers and bulbils in Chinese yam was easily induced by exogenously applied gibberellins, GA_1, GA_3, and GA_4 (Okagami and Nagao, 1971). And also, application of gibberellin increases the batatasin content, particularly, batatasin-. We also described previously the biochemical alterations of endogenous ABA, batatasin and sugars in dormant tubers and bulbils during storage in Chinese yam (Kim *et al.*, 2002).

Fig. 1 shows the quantitative changes of GA members for

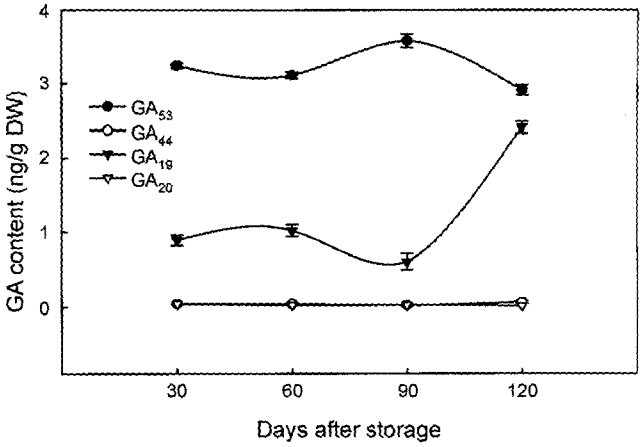

Fig. 1. Changes of endogenous GAs for GA members of an early C-13 hydroxylation pathway in the tubers of *Dioscorea opposita* CV. Tsukune during storage. Data presented are mean values of two replicates SE.

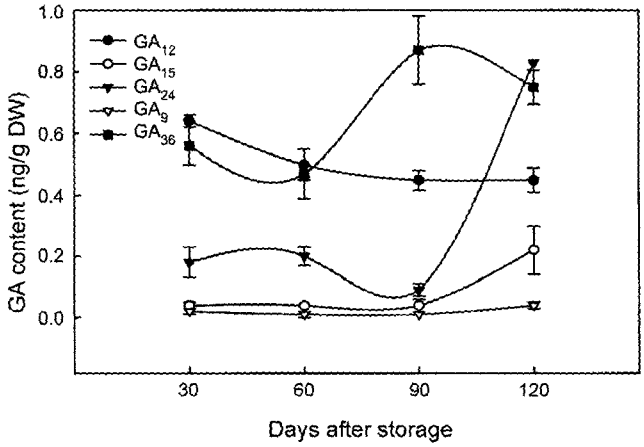

Fig. 2. Changes of endogenous GAs for GA members of non C-13 hydroxylation pathway in the tubers of *Dioscorea opposita* CV. Tsukune during storage. Data presented are mean values of two replicates SE.

an early C-13 hydroxylation pathway in stored tubers. During storage, the levels of GA_{44} and GA_{20} was constant and almost very low content. Meanwhile, both GA_{53} and GA_{19} level were always higher than that of GA_{44} and GA_{20}. GA_{53} level was always most highest during tuber storage. Interestingly, when GA_{53} content was decreased, GA_{19} was relatively increased during tuber storage.

Fig. 2 shows the quantitative changes of GA members for non C-13 hydroxylation pathway in tubers during storage. Intermediates of GA members for non C-13 hydroxylation pathways as GA biosynthetic route consist of five endogenous GAs named GA_{12}, GA_{15}, GA_{24}, GA_9, GA_{36}, and GA_4 (see Fig. 4 for GA_4). GA_9 content as precursor of GA_4 was not changed during storage. GA_{15} content as next metabolites of GA_{12} was gradually increased from 90 to 120 days

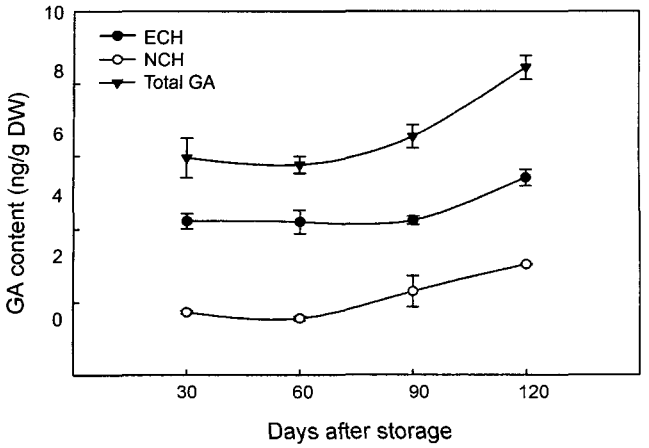

Fig. 3. Changes of each GA group and total GA contents in the tubers of *Dioscorea opposita* var. Tsukune during storage. ECH: early C-13 hydroxylation pathway; NCH: non C-13 hydroxylation pathway. Data presented are mean values of two replicates SE.

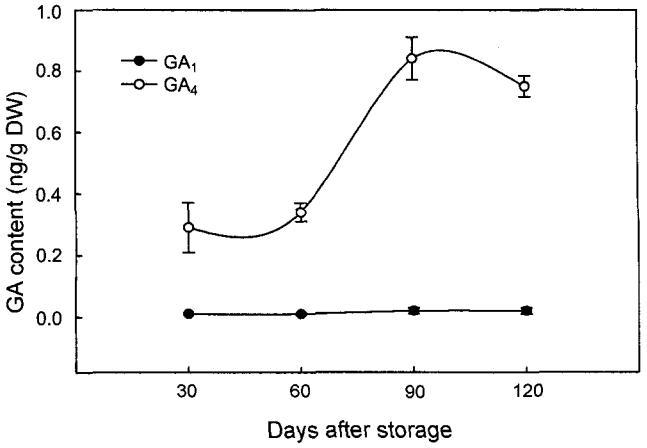

Fig. 4. Changes of bioactive GAs, GA_1 and GA_4 in the tubers of *Dioscorea opposita* var. Tsukune during storage. ECH: early C-13 hydroxylation route; NCH: non C-13 hydroxylation route. Data presented are mean values of two replicates SE.

after storage. GA_{24} content was low to below 0.2 ng for 90 days after storage, however, GA_{24} content was sharply increased at 120 days after storage. GA_{12} content as GA member of the first step of GA biosynthetic metabolism was gradually decreased as storage period was progressed. Its content during storage was ranged from 0.4 - 0.6 ng. GA_{36} content as precursor of GA_4 like GA_9 was about 6-8 fold higher than that of GA_9 during storage. Above results, the occurrence of higher GA_{36} content compared to the GA_9 content in stored tubers of Chinese yam suggests the presence of two non C-13 hydroxylation pathways, in which GA_4 might be converted from GA_{24} via GA_{36} rather than GA_9 quantitatively.

We also evaluated the quantitative changes of gibberellin for an early C-13 hydroxylation (ECH), non C-13 hydroxylation (NCH), and total GA content aggregated from Fig. 1 and 2 during storage (Fig. 3). GA contents of ECH were about 1.6-2 fold higher than that of NCH during storage, in particular, GA contents of the two gibberellin biosynthetic pathways were gradually increased when storage periods were progressed. In addition, total GA content was also gradually increased with prolonged storage periods. Apart from this study, in sprouting tests of stored tubers to determine dormancy periods, each tuber which stored for 30, 60, 90, and 120 days was incubated at 25°C in the continuous dark condition, sprouting was not observed in these tubers for long periods (data not shown). As a result, an imposed dormancy caused by low temperature condition in tubers may be resulted in a steady increase of endogenous gibberellins. Furthermore, an increase of total GA content might be triggered to inhibit the sprouting of tubers. This was consistent with the results and an early assumption, so called gibberellin-induced dormancy in Chinese yam plants (Okagami, 1971). Bioactive GAs, GA_1 and GA_4 were also quantified during tuber storage (Fig. 4). Bioactive GA_1 content as the GA members of ECH was always constant, and its content was very low as below 0.1 ng per DW, meanwhile, bioactive GA_4 content as the GA members of NCH was drastically increased, in particular, its content was highest at 90 DAS, and then decreased at 120 DAS. In a previous study (Kim *et al.*, 2003), GA_1 content was always constant during tuber growth, otherwise GA_4 content was highest in leaves and tubers of Chinese yam during tuber enlargement. This result shows that GA_4 rather than GA_1 might be involved in the regulation of tuber development. Endogenous GA_4 levels also plays some roles in controlling alteration of dormancy and sprouting in the stored tubers of Chinese yam.

From the present results, two GA biosynthetic pathways were operated, also major GA biosynthetic pathway would be an early C-13 hydroxylation routes in stored tubers. Consequently, we suggest that GA_4 might be involved in controlling tuber sprouting in Chinese yam.

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