Gibberellins A_{19} and A_{24} from Yams, *Dioscorea bulbifera*, *D. pentaphylla* and *D. oppositifolia*

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Gibberellin A_{19} (GA_{19}) and GA_{24} were identified by gas chromatography-mass spectrometry from growing shoots of four strains of three species of *Dioscorea, D. bulbifera* var. *vera, D. pentaphylla* and *D. oppositifolia* (yams), which are distributed in the subtropical and tropical regions of Asia. An earlier report indicated the presence of several GAs including GA_{19} and GA_{24} in a cultivated temperate species of East Asia, *D. opposita;* these findings suggest possible coexistence of two GA biosynthetic pathways, early 13-hydroxylation and non-13-hydroxylation pathways commonly in Asian species of *Dioscorea.*

Introduction

Gibberellins (GAs) belong to a class of plant hormones which possesses basically an *ent*-gibberellane ring structure and are known to be prerequisite for growth and development of higher plants. Since GAs were originally identified as metabolites of the fungus, *Gibberella fujikuroi,* nearly 90 kinds of GAs have been clarified from higher plants as well as lower plants. Present knowledge on GA metabolism postulates the occurrence of two separate biosynthetic pathways of GAs in the higher plants (Graebe, 1987; Takahashi and Kobayashi, 1990).

It was previously shown that *Dioscorea opposita,* a cold temperate cultivated species, contained GAs that are considered to be as intermediates of early 13-hydroxylation and non-13-hydroxylation pathways and GA_4, an active form of the latter pathway (Tanno *et al.,* 1992). In the present work, we should examine whether these two biosynthetic pathways of GAs are present in general. We tried to identify endogenous GAs in three species of the genus *Dioscorea* (yams) from the tropics and the subtropics of the East Asia.

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Dormant state of various organs of the genus *Dioscorea* is induced by GAs (Okagami and Tanno, 1977, 1993). The features of biosynthetic pathways of GAs of *Dioscorea* obtained in the present study may contribute to understanding of involvement of GAs in GA-induced dormancy. The knowledge of endogenous GAs may improve the usage of GA application for post-harvest storage of *Dioscorea* tubers which are still important foods in the tropical and subtropical zones over the world and the temperate zone of the East Asia.

Materials and Methods

Plant materials

Species used in this study were *Dioscorea bulbifera* L. var. *vera, D. oppositifolia* L. and two strains of *D. pentaphylla* L. These species are distributed in the tropical or subtropical regions of the humid Asia (Prain and Burkill, 1936). Seeds of the former two species and one strain of the last species were introduced from Nepal by National Research Institute of Vegetables, Ornamental Plants and Tea. Bulbils of another strain of the *D. pentaphylla* were collected from naturally growing plants in Higashiyanaza, Okinawa Prefecture, Japan.

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Plants from the above mentioned seeds or bulbils were cultivated in the green house (15–35 °C) of Tohoku University, Sendai under natural daylight condition for more than 4 years. Growing shoots were harvested for extraction in the early September when vigorous growth of the shoots almost terminated.

Extraction and purification

The procedures described below were basically similar for each strain. Shoots (0.6–2.3 kg fresh weight) of these four strains of three species were homogenized with a mixer and extracted three times with aqueous acetone. The filterate of the extract was evaporated by a rotary evaporator to aqueous phase, which was mixed with equal volume of 1 M K_2HPO_4 (pH 8.2) and successively extracted three times with hexane and ethyl acetate (EtOAc). The residual aqueous phase, after adjusting the solution to pH 2.5 with a diluted HCl, was extracted three times with EtOAc. The combined organic phases were dehydrated through a column of anhydrous Na_2SO_4 and concentrated. The residue was solubilized in 0.1 M phosphate buffer (pH 8.2) and passed through a column of insoluble polyvinylpolypyrrolidone. The eluate was adjusted to pH 2.5, extracted three times with EtOAc. The EtOAc phases were dehydrated and evaporated. The residue was dissolved in methanol (MeOH) and loaded on a column of diethylaminopropyl silica gel, which was successively eluted with MeOH, and MeOH containing 0.75% acetic acid (HOAc). The latter eluate was concentrated *in vacuo* and purified with a Sep-Pak C18 cartridge (Waters Associates, Milford, MA, U.S.A.). The final eluate with MeOH was concentrated *in vacuo*, redissolved in 30% MeOH (0.1% HOAc) and centrifuged.

The supernatant was further purified by reversed phase octadecylsilane (ODS)-high performance liquid chromatography (HPLC) on a Chemco Pak column (Chemco Co., Ltd., Osaka, Japan) packed with Nucleosil C18 (30 cm×10 mm; Nagel, Darmstadt, Germany). The mobile phase had a flow rate of 4 ml/min at 50 °C. The column was eluted with 45% MeOH (0.1% HOAc) for 23 min and then with a linear gradient to 70% MeOH (0.1% HOAc) for 8 min which was followed by elution with 70% MeOH (0.1% HOAc). A total of 40 fractions (6 ml/fraction) were collected. In ODS-HPLC only for *D. oppositifolia*, the column was eluted with 45% MeOH (0.1% HOAc) for 41 min and then with 70% MeOH (0.1% HOAc) to give 45 fractions. The biologically active fractions were further purified on a Senshu-Pak dimethylamino silica [$N(Me)_2$] column (25 cm×6 mm; Senshu Scientific Co., Ltd., Tokyo, Japan). The mobile phase was MeOH (0.05% HOAc) supplied at a flow rate of 2 ml/min at 50 °C. The eluate was collected every 1.5 min to give 40 fractions (3 ml/fraction).

Bioassay

The eluates from ODS-HPLC were tested by the dwarf rice, Tan-ginbozu, micro-drop assay (Murakami, 1968). Five seedlings planted on agar, to each of which 1 µl of the test solution was applied, were incubated in the light at 30 °C for 3 days. Then, the second leaf sheath length of 5 seedlings was measured and the mean values were shown in the figure. For eluates from $N(Me)_2$-HPLC were used seeds that had been pretreated with uniconazole because of elevating the sensitivity of the seedlings to GAs (Nishijima and Katsura, 1989). A typical chromatogram is shown for each species.

Gas chromatography-mass spectrometry (GC-MS)

The procedure has already been reported (Tanno *et al.*, 1992). Prior to GC-MS, samples were subjected to methylation with diazomethane, and then trimethylsilylation with N-methyl-N-(trimethylsilyl)-trifluoroacetamide. The Kovats retention indices of GAs were also determined as previously stated (Tanno *et al.*, 1992).

Results and Discussion

D. bulbifera var. vera

An acidic EtOAc (AE) extract from growing shoots of *D. bulbifera* var. *vera* (from Nepal) was separated into two biologically active fractions after ODS-HPLC (Fig. 1). Fraction 22 of ODS-HPLC (column 1 in Table I) was further purified into a single biologically active fraction (fractions 13 to 15) on $N(Me)_2$-HPLC (column 2 in Table I), from which GA_{19} was identified by its full mass spectrum and Kovats retention index (column 3 in

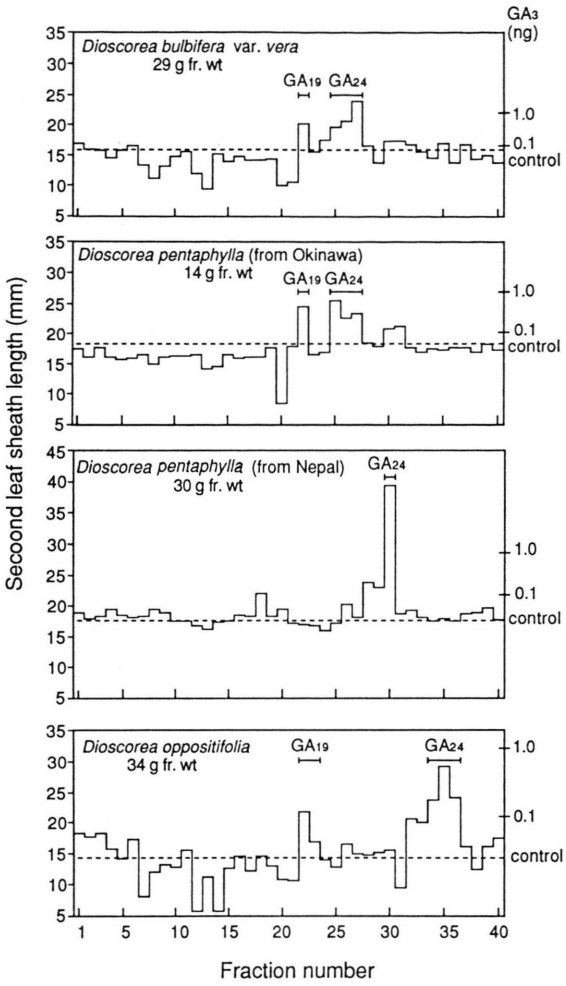

Fig. 1. ODS-HPLC profiles of biological activities of the acidic extracts from the shoots of tropical and subtropical wild species of *Dioscorea, D. bulbifera* var. *vera, D. pentaphylla* and *D. oppositifolia.* GAs were identified by GC-MS from the fractions which are marked in the figure. Standard concentrations of GA_3 were co-assayed with the samples, and the resultant length of the rice seedlings by the standard GA_3 is indicated in the right ordinate. The length corresponding to 10 ng GA_3 was beyond a scale of the figure (*e.g.* 42.2 mm for *D. bulbifera* and 50.6 mm for *D. pentaphylla* from Nepal) and are not shown in the figure.

Table I) of GC-MS (columns 3 to 5 in Table I). Fractions 25–27 on ODS-HPLC were further divided into four biologically active fractions, fractions 4–6, 8 and 9, 11 and 13, on $N(Me)_2$-HPLC, from the last of which, fraction 13, GA_{24} was identified by GC-MS (Table I).

D. pentaphylla

An AE extract from *D. pentaphylla* (from Okinawa) was separated into three biologically active fractions after ODS-HPLC (Fig. 1), two of which were further purified by $N(Me)_2$-HPLC. Fractions 25–27 on ODS-HPLC were separated into three biologically active fractions, fractions 4–6, 8 and 9, and 12–14, on $N(Me)_2$-HPLC. GA_{24} was identified from the last fractions by GC-MS (Table I). Fractions 21 and 22 on ODS-HPLC were purified by $N(Me)_2$-HPLC exhibiting a single biologically active peak (fractions 14 and 15). These fractions were analyzed by GC-MS to identify GA_{19} (Table I).

An AE extract from *D. pentaphylla* (from Nepal) was purified by ODS-HPLC, yealding a single biologically active peak (fractions 28–30). The biologically active fractions were divided into two peaks, combined fractions 8–10, and 12–14, on $N(Me)_2$-HPLC. Surprisingly, GA_{24} was identified from both samples (Table I). It has been known that GA_{24} forms a lactol form between C-19 carboxyl group and C-20 aldehyde group (Harrison *et al.*, 1968). Therefore, it is most likely that a lactol GA_{24} was separated from a free acid GA_{24} in $N(Me)_2$-HPLC.

D. oppositifolia

In this species elution profile of ODS-HPLC was slightly different from the cases of other species because of modified elution program of the mobile phase (Fig. 1). ODS-HPLC of the extract yielded biologically active fractions 22 and 23. Further, $N(Me)_2$-HPLC gave a fraction 17, in which GA_{19} was identified by GC-MS. Fractions 34–36 on ODS-HPLC were divided into two biologically active fractions, a fraction 9 and fractions 14 and 15, on $N(Me)_2$-HPLC, from both of which GA_{24} were identified by GC-MS (Table I) as seen in the Nepal strain of *D. pentaphylla.*

In summary, three tropical and subtropical species, *D. bulbifera, D. pentaphylla* and *D. oppositifolia* possessed GA_{19} and GA_{24} as endogenous GAs although only GA_{24} was identified from Nepal strain of *D. pentaphylla* (Table I). Recently, from the dormant bulbils of *D. opposita,* a cultivated northern cold temperate species of *Dioscorea,* eight GAs, GA_4, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, GA_{36} and GA_{53}, were unambiguously

Table I. Identification of endogenous gibberellins by GC-MS as methyl (Me) or methyl trimethylsilyl (MeTMS) derivatives from the growing shoots of four strains of three wild southern, tropical and subtropical species of *Dioscorea.* Biologically active fractions on ODS-HPLC (column 1) were further purified by $N(Me)_2$-HPLC, and the resultant active fractions (column 2) were subjected to be tested by GC-MS after methylation and trimethylsilylation. Per cent abundance (column 4) shows relative intensity of each diagnostic ion *(m/z)* normalized on the most intense ion in the full mass spectrum by GC-MS.

(1) Fraction on ODS-HPLC	(2) Fraction on $N(Me)_2$-HPLC	(3) Kovats retention index	(4) Diagnostic ions *(m/z)* (% abundance)	(5) Identified compound
D. bulbifera var. *vera* (Nepal)				
22	13–15	2593	462 (12) [M^+], 434 (100), 402 (23), 375 (32), 374 (35)	GA_{19}MeTMS
25–27	13	2448	374 (9) [M^+], 342 (55), 314 (100), 286 (82), 226 (80)	GA_{24}Me
D. pentaphylla (Okinawa)				
22	14/15	2594	462 (14) [M^+], 434 (100), 402 (15), 375 (24), 374 (30)	GA_{19}MeTMS
25–27	12–14	2447	374 (9) [M^+], 342 (50) 314 (100), 286 (77), 226 (68)	GA_{24}Me
D. pentaphylla (Nepal)				
28–30	8–10	2449	374 (5) [M^+], 342 (37), 314 (100), 286 (73), 226 (72)	GA_{24}Me
	12–14	2448	374 (8) [M^+], 342 (50), 314 (100), 286 (77), 226 (68)	GA_{24}Me
D. oppositifolia (Nepal)				
22/23	17	2591	462 (9) [M^+], 434 (100), 402 (17), 375 (23), 374 (24)	GA_{19}MeTMS
34–36	9	2447	374 (8) [M^+], 342 (48), 314 (100), 286 (79), 226 (62)	GA_{24}Me
	14/15	2447	374 (8) [M^+], 342 (48), 314 (100), 286 (59), 226 (73)	GA_{24}Me
Reference compounds				
GA_{19}MeTMS		2593		
GA_{24}Me		2447		

identified (Tanno *et al.,* 1992). These findings suggest that both early 13-hydroxylation and non-13-hydroxylation GA biosynthetic pathways are present in tropical and subtropical, and cold temperate Asian species of *Dioscorea.* Successful identification of GA_{19} and GA_{24} in tropical and subtropical species suggests that the pool sizes of GA_{19} and GA_{24} are larger than those of other GAs.

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