Endogenous Gibberellins from Three East Asian Stenophora Species of the Genus *Dioscorea* (Yams)

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Exogenous gibberellin A_3 (GA_3; 30 μM) promoted shoot elongation of germinated seeds of *Dioscorea nipponica*, an East Asian species of a taxonomic section Stenophora of the genus *Dioscorea* (yams). Endogenous gibberellins (GAs) were analyzed by combined gas chromatography-mass spectrometry (GC-MS) from growing shoots of three East Asian Stenophora species. Gibberellin A_4, GA_{12}, GA_{19}, GA_{24} and GA_{53} were identified by Kovats retention indices and by full spectra of GC-MS from *D. quinqueloba*. Gibberellin A_{19}, GA_{24} and GA_{53} were also identified from *D. nipponica* and *D. septemloba*. These results suggest that two separate biosynthetic pathways of GAs, early 13-hydroxylation and non-13-hydroxylation pathways are functioning in the growth of shoots of these thee species.

Introduction

Gibberellins endogenously control, like other classes of plant hormones, a variety of aspects of growth and development in higher plants. The genus *Dioscorea* (yams) consisting of 23 taxonomic sections (Burkill, 1960) possesses a variety of economically important species whose subterranean tubers or rhizomes and occasionally aerial bulbils are still utilized as food crops in the humid tropics and subtropics over the world and in the humid tropics to cold temperate region in the East Asia, and as medicinal sources. As commonly found with the genus *Dioscorea* including several East Asian species of the section Stenophora, application of GAs inhibits sprouting of tubers or rhizomes and bulbils, and induce the dormancy of these asexual organs (Okagami and Tanno, 1977, 1993) in contrast to usual plants where GAs promote seed germination and bud sprouting (Stuart and Cathey, 1971). On the other hand, it is unclear whether *Dioscorea* species always respond to exogenously applied GAs in a usual manner such as shoot elongation. In only one species, *D. opposita*, of the genus (section Enantiophyllum) (Tanno *et al.,* 1992a, 1994b), shoot elongation was proved to be promoted by exogenous GAs as in other plant species (Phinney, 1984). Both inhibitory and promotive actions of GAs in growth of *Dioscorea* may provide important information for understanding the physiological action mechanism of the hormone.

Although GAs are believed to be of general occurrence in higher plants, information on endogenous GAs in the genus *Dioscorea* is limited to some Asian temperate, subtropical and tropical species of three sections, Enantiophyllum, Opsophyton and Lasiophyton (Tanno *et al.,* 1992b, 1994a). The co-occurrence of two separate GA biosynthetic pathways, the early 13-hydroxylation and non-13-hydroxylation pathways (Graebe, 1987; Takahashi and Kobayashi, 1990) has been demonstrated in these three sections of East Asian *Dioscorea*. However, in any species of the section Stenophora, the major group of East Asian *Dioscorea* (Burkill, 1960), neither shoot elongation has been observed to be promoted by GAs, nor have endogenous GAs been identified.

In the present study, we will report shoot elongation by exogenous GA_3 in *D. nipponica*, and furthermore, the successful identification of

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several endogenous GAs by GC-MS from the shoots of *D. nipponica*, *D. septemloba* and *D. quinqueloba*, all East Asian Stenophora species.

Materials and Methods

Plant materials

Species used in this study were *Dioscorea nipponica* Makino, *D. septemloba* Thunb. ex Murray and *D. quinqueloba* Thunb. ex Murray, all of the section Stenophora of the genus *Dioscorea*. *D. nipponica*, *D. septemloba* and *D. quinqueloba* range from subarctic to cold temperate regions, in warm temperate region, and from warm temperate regions to subtropics, respectively, of East Asia (Okagami and Kawai, 1982).

Shoot elongation

Seed collection, storage and incubation procedure for germination were basically as described previously (Okagami and Kawai, 1982). The mature seeds of *D. nipponica* were collected in autumn from the plants growing in a natural habitat, and stored at room temperature in a desiccated condition until use for shoot elongation. The seeds were incubated at 23 °C in the dark on a thin layer of absorbent cotton moistened with distilled water in a 15-cm petri-dish. Then, just as their radicles protruded from the seed coats, 7 germinated seeds were incubated at 23 °C in the light (3 W m^{-2} from white fluorescent lamps) on absorbent cotton moistened with 30 μM GA_3 (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) or distilled water. The elongated shoot length was measured after the incubation for 20 days.

Extraction and purification

Plants of these three species from the seeds collected in natural habitats in Japan were cultivated in the nursery garden of Tohoku University, Sendai under natural condition for more than 4 years. Growing shoots were harvested for extraction in early September when vigorous growth of the shoots almost terminated. The procedures described below were basically as previously reported (Tanno *et al.*, 1994a). Shoots (2.9 kg fresh wt.) of *D. quinqueloba* were homogenized with a mixer and extracted three times with 7 l acetone. The concentrated filtrate (700 ml) of the extract was evaporated by a rotary evaporator to aqueous phase, which was mixed with an equal volume of 1 M K_2HPO_4 (pH 8.2) and succesively extracted three times with 1 l hexane and 1 l ethyl acetate (EtOAc). The residual aqueous phase, after adjusting the solution to pH 2.5 with diluted HCl, was extracted three times with 1.3 l 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 polyvinylpolypyrrolidone (PVP; 50 g). The eluate was adjusted to pH 2.5 and extracted three times with 1 l EtOAc. The EtOAc phases were dehydrated and evaporated. The residue was dissolved in methanol (MeOH) and loaded on a column of diethylaminopropyl silica gel (DEA; 3 g), which was successively eluted with MeOH, and MeOH containing 0.75% acetic acid (HOAc). The latter eluate (105 ml) 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 for octadecylsilane (ODS)-high performance liquid chromatography (HPLC).

The shoots of *D. nipponica* (890 g fresh wt.) and *D. septemloba* (960 g fresh wt.) were similarly extracted and purified. However, some modification is required due to large amount of impurified residues from these two materials, where a 150 g of PVP column was used for *D. nipponica* and for *D. septemloba* were used a column of charcoal (3 g) before DEA purification.

HPLC

The supernatant was further purified by reversed phase ODS-HPLC on a Chemco Pak column (10 x 300 mm; Chemco Co., Ltd., Osaka, Japan) packed with Nucleosil C18 (7 μm; 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 35 fractions (6 ml/fraction) were collected. The biologically active fractions were further purified

on a Senshu-Pak dimethylamino silica [$N(CH_3)_2$] column (5 μm, 6 x 250 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 35 fractions (3 ml/fraction.).

Bioassay

The eluates from ODS-HPLC were tested by the dwarf rice, Tan-ginbozu, micro-drop assay (Murakami, 1968), which was referred to as a conventional assay. For eluates from $N(CH_3)_2$-HPLC seeds were used that had been pretreated with uniconazole because of elevating the sensitivity of the seedlings to GAs (a sensitive assay) (Nishijima and Katsura, 1989). The sensitive assay was also used for some eluates from ODS-HPLC. In both conventional and sensitive assays 5 seedlings were used in each fraction and a typical chromatogram is shown for each species (Tanno *et al.*, 1994a).

Table I. Identification of endogenous gibberellins by GC-MS as their methyl (Me) or methyl trimethylsilyl (MeTMS) derivatives from the growing shoots of three Asian Stenophora species of the genus *Dioscorea*. Biologically active fractions on ODS-HPLC (column 1) were further purified by $N(CH_3)_2$-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 the relative intensity of each diagnostic ion *(m/z)* normalized to the most intense ion in the full mass spectrum by GC-MS.

(1) Fraction on ODS-HPLC	(2) Fraction on $N(CH_3)_2$-HPLC	(3) Kovats retention index	(4) Diagnostic ions *(m/z)* (% abundance)	(5) Identified compound
D. quinqueloba				
21/22	17–18	2593	462(9)[M^+] 434(100) 402(19) 375(32) 374(42)	GA_{19}MeTMS
25–27	5/6	2501	448(100)[M^+] 389(39) 251(22) 241(31) 235(21)	GA_{53}MeTMS
25–27	9	2504	418(37)[M^+] 289(15) 284(100) 225(61) 224(66)	GA_4MeTMS
25–27	13/14	2447	374(2)[M^+] 342(40) 314(100) 286(95) 226(93)	GA_{24}Me
30/31	5/6	2344	360(2)[M^+] 328(27) 300(100) 285(15) 240(22)	GA_{12}Me
D. nipponica				
19–22	13–15	2596	462(8.3)[M^+] 434(100) 402(22) 375(39) 374(46)	GA_{19}MeTMS
25/26	5/6	2500	448(100)[M^+] 389(27) 251(19) 241(15) 235(14)	GA_{53}MeTMS
25/26	12–14	2448	374(9)[M^+] 342(46) 314(100) 286(75) 226(78)	GA_{24}Me
D. septemloba				
19/21	15–18	2597	462(10)[M^+] 434(100) 402(23) 375(33) 374(42)	GA_{19}MeTMS
25/26	4–6	2500	448(100)[M^+] 389(39) 251(28) 241(27) 235(24)	GA_{53}MeTMS
25/26	14–16	2447	374(5)[M^+] 342(33) 314(100) 286(90) 226(100)	GA_{24}Me
Reference compounds				
GA_4MeTMS		2509		
GA_{12}Me		2348		
GA_{19}MeTMS		2593		
GA_{24}Me		2447		
GA_{53}MeTMS		2500		

GC-MS

The procedure has already been reported (Tanno *et al.*, 1992b). Prior to GC-MS, samples were methylated with diazomethane and then trimethylsilylated with N-methyl-N-(trimethyl-silyl)-trifluoroacetamide. The Kovatz retention indices of GAs were also determined as previously stated (Tanno *et al.*, 1992b).

Results and Discussion

Shoot elongation

Seven germinated seeds of *D. nipponica* with radicles just after protrusion were incubated with 30 μM GA_3 or distilled water. Exogenous GA_3 promoted shoot elongation of *D. nipponica* (ca. 190% of control) as seen in *D. opposita* (Tanno *et al.*, 1992a, 1994b). In addition to the dormancy induction by GAs commonly found in the genus, this finding suggests that shoot elongation by exogenous GAs is common in the genus *Dioscorea* as usual in plant species (Phinney, 1984).

Endogenous GAs

D. quinqueloba

An acidic EtOAc (AE) extract from growing shoots of *D. quinqueloba* was separated by ODS-HPLC into three biologically active fractions (Fig. 1). A fraction 21/22 of ODS-HPLC was further purified into a single biologically active fraction (fraction 17/18) on $N(CH_3)_2$-HPLC, from which GA_{19} was identified by its full mass spectrum and Kovats retention index of GC-MS (Table I). A fraction 25–27 on ODS-HPLC was further divided into three biologically active fractions, fractions 5/6, 9, and 13/14, on $N(CH_3)_2$-HPLC, from which, GA_{53}, GA_4 and GA_{24} were identified by GC-MS, respectively (Table I). A fraction 30/31 gave a single GA fraction, from which GA_{12} was identified.

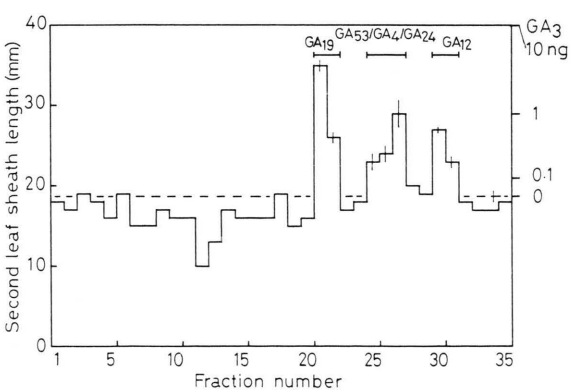

Fig. 1. A profile of biological activity on ODS-HPLC from the shoots of *D. quinqueloba* (49 g fresh wt. equivalent). Standard concentrations of GA_3 were co-assayed and the resultant length of the rice seedlings is indicated at the right ordinate. In control and some biologically-active fractions vertical bars indicate SD.

D. nipponica

By a conventional bioassay an AE extract from *D. nipponica* showed only one biologically active fraction after ODS-HPLC. Further assay by a sensitive method showed that five groups of fractions, 5/6, 19–22, 25/26, 29/30, and 33–35, were biologically-active (Fig. 2). The fraction 19–22 on ODS-HPLC was further purified by $N(CH_3)_2$-HPLC to give a single large, active peak (fractions 13–15) from which GA_{19} is identified (Table 1). The fraction 25/26 on ODS-HPLC was separated into three biologically-active fractions, 5/6, 8–10, and 12–14, on $N(CH_3)_2$-HPLC. GA_{53} and GA_{24} were identified by GC-MS from the first and last fractions, respectively (Table I). No GAs were identified from other active fractions in ODS-HPLC.

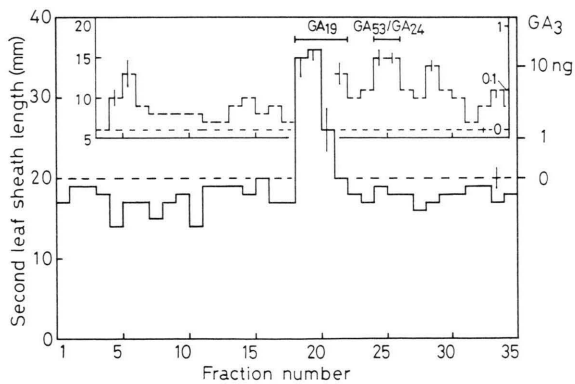

Fig. 2. A profile of biological activity on ODS-HPLC from the shoots of *D. nipponica*. The fractions which showed no GA activity by a conventional micro-drop assay (solid line: 30 g fresh wt. equivalent) were further surveyed by a sensitive assay, and the profile is outlined with a narrow, broken line (15 g fresh wt. equivalent) in the figure. The length by standard GA_3 is also shown as in Fig. 1. In control and some biologically-active fractions vertical bars indicate SD.

D. septemloba

The conventional bioassay detected two active peaks, fractions 19/20, and 26 from elution profile of ODS-HPLC. Further survey by the sensitive assay showed that fractions 16, 19–21, 25/26, 29 and 33 were also biologically active (Fig. 3). The fraction 19–21 on ODS-HPLC further gave by $N(CH_3)_2$-HPLC a single biologically active peak, fraction 15–18, from which GA_{19} was identified by GC-MS. The fraction 25/26 on ODS-HPLC was divided by $N(CH_3)_2$-HPLC into three biologically active fractions, fractions 4–6, 9–12, and 14–15 from the first and the last of which GA_{53} and GA_{24} were identified by GC-MS, respectively (Table I). No GAs have been identified from other active fractions in ODS-HPLC.

In summary, of three East Asian Stenophora species examined, *D. quinqueloba* possessed GA_4, GA_{12}, GA_{19}, GA_{24}, and GA_{53} as endogenous GAs, and both *D. nipponica* and *D. septemloba* also possessed GA_{19}, GA_{24}, and GA_{53}. Previously, from the dormant bulbils of *D. opposita*, a cultivated northern cold temperate Enantiophyllum species of *Dioscorea*, eight GAs namely, GA_4, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, GA_{36} and GA_{53}, were unambiguously identified (Tanno *et al.*, 1992b). Recently, GA_{19} and GA_{24} were identified from a tropical and subtropical Opsophyton species, *D. bulbifera*, Lasiophyton species, *D. pentaphylla*, and Enantiophyllum species, *D. oppositifolia* (Tanno *et al.*, 1994a). More recently, GA_{19}, GA_{20}, GA_{24}, and GA_{53} were identified from a wild, temperate East Asian Enantiophyllum species, *D. japonica* (unpublished data). These findings suggest that both early 13-hydroxylation and non-13-hydroxylation GA biosynthetic pathways are commonly present in tropical and subtropical, and cold temperate Asian species of *Dioscorea* including four sections, Stenophora, Enantiophyllum, Opsophyton, and Lasiophyton. In these species, the pool sizes of GA_{19} and GA_{24} are larger than those of other GAs by estimating the biological activities of GAs.

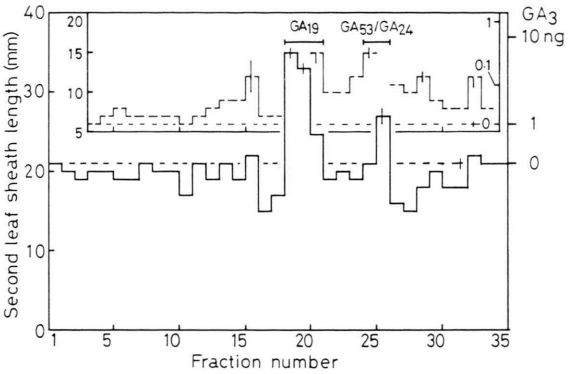

Fig. 3. A profile of biological activity on ODS-HPLC from the shoots of *D. septemloba*. The profiles outlined with solid line (16 g fresh wt. equivalent) and broken line (27 g fresh wt. equivalent) are as in Fig. 2. The length by standard GA_3 is also shown as in Fig. 1. In control and some biologically-active fractions vertical bars indicate SD.

These endogenous GAs may control the shoot elongation in the section Stenophora. The knowledge on these GAs may also contribute to understanding of physiological mechanism of dormancy induction by GAs in the section Stenophora.

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Burkill I. H. (1960), The organography and the evolution of *Dioscoreaceae*, the family of the yams. J. Linn. Soc. London (Bot.) **56,** 319–412.

Graebe J. E. (1987), Gibberellin biosynthesis and control. Annu. Rev. Plant Physiol. **38,** 419–465.

Murakami Y. (1968), The microdrop method, a new rice seedling test for gibberellins and its use for testing extracts of rice and morning glory. Bot. Mag. Tokyo **79,** 33–43.

Nishijima T. and Katsura N. (1989), A modified microdrop bioassay using dwarf rice for detection of femtomol quantities of gibberellins. Plant Cell Physiol. **30,** 623–627.

Okagami N. and Kawai M. (1982), Dormancy in *Dioscorea*: Differences of temperature responses in seed germination among six Japanese species. Bot. Mag. Tokyo **95,** 155–166.

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

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

Phinney B. O. (1984), Gibberellin A_1, dwarfism and the control of shoot elongation in higher plants. In: The biosynthesis and metabolism of plant hormones (A. Crozier and J. R. Hillman, eds.). Soc. Exp. Biol. Sem. Ser. 23, Cambridge Univ. Press, Cambridge, pp. 17–41.

Stuart N. W. and Cathey H. M. (1971), Applied aspect of gibberellins. Annu. Rev. Plant Physiol. **12,** 360–394.

Takahashi N. and Kobayashi M. (1990), Organ-specific gibberellins in rice: Roles and biosynthesis. In: Gibberellins (N. Takahashi, B.O. Phinney and J. MacMillan, eds.). Springer, New York, pp. 9–21.

Tanno N., Nakayama M., Yashima H., Sunaga K., Abe M., Okagami N. and Yokota T. (1994a), Gibberellins A_{19} and A_{24} from yams, *Dioscorea* bulbifera, *D. pentaphylla* and *D. oppositifolia*. Z. Naturforsch. **49c,** 399–403.

Tanno N., Tanno M., Yokota T., Abe M. and Okagami N. (1994b), Promotive and inhibitory effects of uniconazole and prohexadione on the sprouting of bulbils of Chinese yam, *Dioscorea opposita*. Plant Growth Regul., in press.

Tanno N., Yokota T., Abe M. and Okagami N. (1992a), Gibberellins induce the dormancy of bulbils of *Dioscorea opposita* Thunb. Proc. Plant Growth Regul. Soc. Amer. 19th Annu. Meet. pp. 187–192.

Tanno N., Yokota T., Abe M. and Okagami N. (1992b), Identification of endogenous gibberellins in dormant bulbils of Chinese yam, *Dioscorea opposita*. Plant Physiol. **100,** 1823–1826.