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Endogenous Gibberellins in Clover Broomrape, a Parasitic Plant, and its Host, Clover: Dependency of the Parasite on the Host for Gibberellin Production

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Abstract. Endogenous gibberellins were analyzed from a parasitic plant, clover broomrape (*Orobanche minor* Smith), and its host, clover (*Trifolium repens* L.). Members of both the early-13- and the early-non-hydroxylation pathways were identified from both the parasite and the host (GA_{12}, GA_{24}, GA_9, GA_4, GA_{44}, GA_{19}, GA_{20}, and GA_1 from clover broomrape; GA_9, GA_4, GA_{44}, GA_{19}, GA_{20}, and GA_1 from clover). Quantitative analyses showed that GA_{44} was present at high levels in both host and parasite. The similarity in the gibberellins suggests the possibility that the major gibberellins in clover broomrape are transported from clover. However, gibberellins such as GA_{58}, GA_{38}, and notably GA_{47}, which was identified from a plant for the first time, were detected only from clover broomrape, suggesting that the parasite may have the ability to produce at least those gibberellins.

Parasitic plants depend on their hosts for the production of essential compounds. There are several reports demonstrating translocation of substances from host to parasite, including amino acids (Fer 1979; Renaudin and Larher 1981; McNally et al. 1983; Aber et al. 1983; Thalouarn et al. 1986), and carbohydrates (Hull and Leonard 1964). However, it is not clear if there is selectivity of substances translocated from host to parasite.

Since many parasitic plants can tap into the phloem of their hosts, they have access to the plant growth regulators produced by the hosts. At present we have little information on the ability of parasites to produce plant growth regulators. *Striga asiatica* required the addition of cytokinins and auxin in in vitro culture for normal seedling development (Yoshikawa et al. 1978), which suggested that the parasite was dependent on its host for those plant hormones. There is another report which suggests the dependency of parasites, *Amyema* mistletoes, on their hosts, *Eucalyptus*, for cytokinins, based on the resemblance of external morphology between host and parasite, and analytical results on endogenous cytokinins (Hall et al. 1987). These are the only reports on the dependency of parasites on their hosts for plant hormones.

In this study we focused on gibberellins, one of the classes of plant hormones. We analyzed the endogenous gibberellins in a parasitic plant, clover broomrape (*Orobanche minor* Smith), and its host, clover (*Trifolium repens* L.). Although the presence of gibberellin-like substances in *Cuscuta reflexa* and its host *Vicia faba* have been shown by bioassay, there is no report on the identification of endogenous gibberellins in parasitic plants by reliable methods such as GC-MS. Since over 80 structurally unique gibberellins have been identified from higher plants, and the kinds and amounts of endogenous gibberellins depend on the plant species, unambiguous identification and quantification of gibberellins in parasite and host will provide useful information on the dependency of the parasite on the host for gibberellin production.

Materials and Methods

Internal Standards

$[17,17\text{-}^2H_2]GA_{19}$ and $[17,17\text{-}^2H_2]GA_{24}$ were gifts from Prof. Mander of the Australian National University. The preparation of $[1,2,2,3,6\text{-}^2H_5]GA_1$, $[1,2,2,3,6\text{-}^2H_5]GA_{20}$, $[2,2,3,6\text{-}^2H_4]GA_9$

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and [2,2,3,6-$^2H_4$]GA_4 was reported by Endo et al. (1989), Nakayama et al. (1989), and Takayama et al. (1993), respectively.

Plant Materials

Clover broomrape (*O. minor* Smith) parasitizing red clover (*Trifolium pratense* L.) was harvested on the riverbed of the Watarase river, Tochigi; 770 g and 346 g of the aerial part were used for qualitative (*O*ql) and quantitative (*O*qn) analyses, respectively. Although the parasite was parasitizing red clover, white clover (*T. repens* L.) was used as a material of the host plant for analysis of gibberellins because white clover was easier to be harvested in large amounts than red clover, and clover broomrape parasitizing white clover could not be obtained, and it was considered that similar kinds and amounts of gibberellins were contained in the two clovers belonging to the same genus. Clover (*T. repens* L.) was harvested on the experimental farm of The University of Tokyo; 723 g and 445 g of the aerial part were used for qualitative (*T*ql) and quantitative (*T*qn) analyses, respectively. Materials were soaked in methanol (MeOH) immediately after harvest. Qualitative analyses were performed first. The amounts of the plant materials and internal standards used for quantitative analyses were based on the results of the qualitative analyses.

Dwarf Rice Micro-drop Bioassay

Gibberellin-like activity was detected by the dwarf rice (*Oryza sativa* L. cv. Tan-ginbozu) micro-drop method (Nishijima and Katsura 1989). An aliquot equivalent to 10 g fr wt was applied to each assay plant.

Extraction and Purification

All materials (*O*ql, *O*qn, *T*ql, and *T*qn) were extracted three times with MeOH (6.2 L for *O*ql, 3.0 L for *O*qn, 5.3 L for *T*ql and 3.9 L for *T*qn), and the following internal standards were added to the samples for quantitative analysis: [$^2H_5$]GA_1 (100 ng for *O*qn and *T*qn); [$^2H_4$]GA_4 (300 ng for *O*qn and 100 ng for *T*qn); [$^2H_5$]GA_{20} (100 ng for *O*qn and *T*qn); [$^2H_4$]GA_9 (100 ng for *O*qn and *T*qn); [$^2H_2$]GA_{19} (100 ng for *O*qn and *T*qn); and [$^2H_2$]GA_{24} (100 ng for *O*qn and *T*qn). After filtration and removal of the MeOH, the aqueous residue was solvent-fractionated to give an acidic ethyl acetate (EtOAc) fraction (AE fraction) as described by Toyomasu et al. (1992). The AE fraction from each extract was successively purified by a polyvinylpolypyrrolidone (PVPP; 17 g for *O*ql, 11.6 g for *O*qn, 8.8 g for *T*ql, and 4.3 g for *T*qn) column (Toyomasu et al. 1992), Sepralyte (DEA) (Analytichem International) (2 g for *O*ql, 1.2 g for *O*qn, 3.5 g for *T*ql, and 2.4 g for *T*qn) (Endo et al. 1989), Sep-Pak (ODS) cartridge (Waters, Inc.) (Endo et al. 1989), and gel permeation chromatography (GPC) on a column of Shodex HF-2001 (50 cm × 2 cm i.d.) (Yamaguchi et al. 1990). Each purified gibberellin fraction was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a Senshu-Pak ODS 4253D column (25 cm × 10 mm i.d.) to give 32 fractions as described by Endo et al. (1989). Following the detection of the gibberellin-like activity in each fraction by the dwarf rice bioassay, the bioactive fractions were further fractionated on a Senshu-Pak $N(CH_3)_2$ 3151N column (150 mm × 8 mm i.d.), eluted with 0.05% HOAc in MeOH at a flow rate of 3 ml/min at 50°C. Fractions were collected every 2 min from injection to 50 min. The gibberellin-like activity in each fraction was detected by the dwarf rice bioassay.

GC/MS

The bioactive fractions obtained from *O*ql and *T*ql were methylated and trimethylsilylated as described by Takayama et al. (1993) and analyzed with a JEOL DX-303 GC/MS system, fitted with a fused-silica chemically bonded capillary column DB-1 (15 m × 0.258 mm i.d.). The analytical conditions were the same as those described by Endo et al. (1989).

GC/SIM

Under the same conditions as GC/MS analysis described above, the gibberellin fractions obtained from *O*qn and *T*qn were derivatized and analyzed by GC/SIM (*s*elected *i*on *m*onitoring). The amounts of GA_1, GA_4, GA_9, GA_{19}, GA_{20}, and GA_{24} were calculated from the peak area ratios of m/z 506/511, 284/288, 298/302, 434/436, 418/423, and 314/316, respectively. The approximate amounts of GA_{19} in clover and GA_{44} in both plants were determined by comparison of ion intensities at m/z 434 and 207, respectively, with those of authentic specimens.

Results and Discussion

Endogenous Gibberellins in Clover Broomrape

The appropriate HPLC fractions obtained from *O*ql were analyzed by GC-MS. The results are shown in Table 1. The evidence for the natural occurrence of each gibberellin is based on KRI data and fragmentation patterns in full-scan GC/MS. All members of the early-non-hydroxylation pathway ($GA_{12} \rightarrow GA_{15} \rightarrow GA_{24} \rightarrow GA_9 \rightarrow GA_4$) except for GA_{15} were identified. All members of the early-13-hydroxylation pathway ($GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$) except for GA_{53} were also identified. These results suggest that both the early-13- and the early-non-hydroxylation pathways are operating in clover broomrape. In addition, $GA_{58/47}$ and GA_{38} were detected (putative metabolites of GA_4 and GA_{44}, respectively). These are uncommon gibberellins in plants. The identification of GA_{47} is the first report of a 2α-hydroxy gibberellin from higher plants. 2β-Hydroxy gibberellins are commonly observed in plants; 2β-hydroxylation is considered to be an inactivation process of active gibberellins. However, α-hydroxy gibberellins such as GA_{47} (2α-hydroxy-GA_4) and GA_{40} (2α-hydroxy-GA_9) have relatively high activities in a number of bioassay systems (Sponsel et al. 1977). The presence of GA_{47} may suggest a special mechanism to regulate gibberellin activity in the parasite. The presence of

Table 1. Gibberellins identified by GC-MS analysis of their MeTMSi (methyl ester trimethylsilyl ether) derivatives in clover broomrape

Rt on HPLC (min)		Identified		Principal ions and relative abundance
ODS	N $(CH_3)_2$	GA	[a]KRI	(% base peak)
11–15	8–12	GA_{38}	2904	520 (M^+, 40), 515 (22), 461 (22), 207 (100)
	16–20	GA_1	2666	506 (M^+, 100), 491 (14), 448 (26), 376 (20)
		GA_{58}	2727	506 (M^+, 30), 416 (100), 384 (85), 356 (100)
19–21	16–18	GA_{47}	2623	506 (M^+, 100), 459 (11), 431 (5), 288 (10)
	20–24	GA_{20}	2489	418 (M^+, 100), 403 (15), 375 (50), 301 (25)
21–25	14–20	GA_{44}	2771	432 (M^+, 75), 417 (30), 238 (40), 207 (100)
	36–40	GA_{19}	2591	462 (M^+, 7), 434 (100), 402 (22), 374 (38)
25–28	14–16	GA_4	2502	418 (M^+, 38), 386 (25), 289 (60), 284 (100)
	18–22	GA_9	2310	330 (M^+, 10), 298 (100), 270 (90), 243 (55)
	32–40	GA_{24}	2466	374 (M^+, 6), 342 (43), 314 (100), 286 (85)
28–32	6–10	GA_{12}	2347	360 (M^+, 3), 328 (55), 300 (100), 285 (45)

[a] Kovats retention index.

GA_{38} is also suggestive to special metabolic regulation of active gibberellins in the parasite. GA_{38} has been identified from only four plant species (Hiraga et al. 1974; Frydman et al. 1974; Albone et al. 1984). It has been suggested that GA_{38} is active *per se* and can show its activity without conversion of the δ-lactone ring to a γ-lactone ring, which active gibberellins like GA_1 and GA_4 possess (Kamiya et al. 1991).

Endogenous levels of GA_1, GA_4, GA_9, GA_{19}, GA_{20}, and GA_{24} were calculated from the peak ratios of specific ions for endogenous gibberellins and those for internal standards in the GC/SIM analyses of the appropriate HPLC fractions obtained from *O*qn (Table 2). Endogenous level of GA_{44} was also semiquantified by comparison of specific ion intensities between endogenous and authentic GA_{44}. The level of GA_{44} was estimated to be 20 times higher than that of any other gibberellin.

Table 2. Endogenous levels of major gibberellins in clover broomrape and clover (ng/g fr wt)

GA	Clover broomrape	Clover
GA_1	0.33	0.14
GA_4	0.28	0.05
GA_9	0.67	0.10
GA_{19}	0.52	[a]46
GA_{20}	0.08	1.8
GA_{24}	0.26	—
GA_{44}	[a]13	[a]18

[a] Amounts recovered in the finally purified fractions were determined by comparison of ion intensities with those of authentic samples.

Endogenous Gibberellins in Clover

The gibberellins identified from clover are shown in Table 3. Members of both the early-13- and the early-non-hydroxylation pathways were identified, which suggested that both pathways were operating in clover, as well as in the parasite. Table 2 shows the results of the quantitative analysis. The endogenous levels of GA_{19} and GA_{44}, were semiquantified by external calibration curves. The levels of GA_{19} and GA_{44} were much higher than those of the other gibberellins. The level of GA_{19} was more than 25 times higher than that of GA_{20}, suggesting that the step from GA_{19} to GA_{20} is regulated so that the level of GA_1 is controlled in clover.

Dependency of Clover Broomrape on Clover for Gibberellin Production

Figure 1 shows the gibberellins identified from clover broomrape and clover, and their hypothetical biosynthetic pathways. All gibberellins shown in the figure except for GA_{15} and GA_{53} were identified from clover broomrape, and the gibberellins enclosed in the frame were identified from clover. Members of both the early-13- and the early-non-hydroxylation pathways were identified from both the parasite and the host. Quantitative analyses showed that GA_{44} was present at high levels in both the parasite and the host. The similarity in the gibberellins between the parasite and the host suggests that the major gibberellins detected from clover broomrape are possibly transported from clover. However, the presence of the uncommon gibberellins in clover broomrape (GA_{47}, GA_{58}, and GA_{38}) which were not detected from clover, suggests that

Table 3. Gibberellins identified by GC-MS analysis of their MeTMSi derivatives in clover

Rt on HPLC (min)		Identified		Principal ions and relative abundance
ODS	N $(CH_3)_2$	GA	[a]KRI	(% base peak)
11–14	16–20	GA_1	2658	506 (M^+, 100), 491 (20), 448 (34), 376 (30)
18–24	10–12	GA_{44}	2785	432 (M^+, 80), 417 (24), 238 (47), 207 (100)
	16–18	GA_{20}	2480	418 (M^+, 100), 403 (14), 375 (40), 207 (37)
	20–26	GA_{19}	2586	462 (M^+, 8), 434 (100), 402 (30), 374 (40)
24–29	10–12	GA_4	2498	418 (M^+, 30), 386 (30), 289 (65), 284 (100)
	14–16	GA_9	2312	330 (M^+, 15), 298 (100), 270 (85), 243 (50)

[a] Kovats retention index.

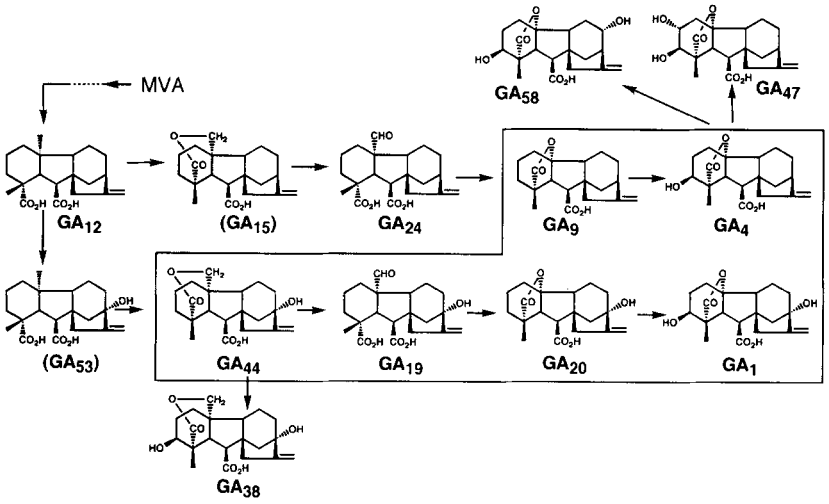

Fig. 1. Gibberellins identified from clover broomrape and clover, and their hypothetical biosynthetic pathways. All the gibberellins except for GA_{15} and GA_{53} (shown in *parentheses*) were identified from clover broomrape. The gibberellins *enclosed in the frame* were identified from clover.

the parasite has the ability to produce at least these three gibberellins.

Our hypothesis on the dependency of the parasite on the host for gibberellin production will be investigated by both metabolic and transport studies, in which isotope-labeled gibberellins and their precursors are fed to both the host and the parasite.

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