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Short Communication

On the Reversible Conjugation of [17-D_2]GA_{20} in Seedlings of *Phaseolus coccineus* L.

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Abstract. After administering [17-D_2]GA_{20} to *Phaseolus coccineus* L. cv. Preisgewinner seedlings, [17-D_2]GA_{20}-O-glucoside was identified by liquid chromatography (LC)/ESI-tandem mass spectrometry (MS). Likewise, by LC/ESI-tandem MS the metabolic formation of [17-D_2]GA_{20} glucosyl ester was established. The application of both [17-D_2]-labeled GA_{20} 13-O-glucoside and GA_{20} glucosyl ester to *Phaseolus coccineus* L. seedlings resulted in free [17-D_2]GA_{20} by gas chromatography/MS. The results demonstrate that conjugation of GA_{20} and the reconversion of the glucosyl conjugates are concomitant processes in plants.

Key Words. *Phaseolus coccineus* L.—Gibberellin glucosyl conjugates—LC/ESI-MS—tandem MS—GC/MS

The phenomenon of conjugation of gibberellins (GAs) consists of the fact that GAs bind to sugar molecules to form glucosyl esters or glucosides (Schneider and Schliemann 1994). The outstanding feature of this process, however, is that conjugation of active GAs is accompanied by loss of activity and, vice versa, hydrolysis of glucosyl conjugates restores the biological activity of the parent GA. This information led to the hypothesis that the reversible conjugation of GAs may represent a means for the plant to precisely control GA pools. This model may apply not only in the case of storage or transport processes of GAs but also in homeostasis of the free hormone level (Kleczkowski and Schell 1995, Schneider and Schliemann 1994).

The metabolic formation of GA conjugates after application of free GAs (e.g., of GA_{20} to a series of plants like *Vicia faba* ([Lattke and Schneider 1985], *Phaseolus vulgaris* [Yamane et al. 1977], *Pisum sativum* [Sponsel and MacMillan 1978], and *Salix pentandra* [Rood and Junttila 1989]) has been described, although in most cases, only radioactive precursors have been used and the formed conjugates have been only tentatively identified by cochromatography or by hydrolysis of polar fractions. In a few experiments the metabolically formed GA-O-glucosides have been identified by mass spectrometry (MS) (e.g., in *Zea mays* [Schneider et al. 1987].

Little is known about the reconversion of gibberellin conjugates. There is indirect evidence on the reconversion of unidentified radioactive GA_{20} conjugates in *Zea mays* (Rood et al. 1983). On the basis of mass spectrometric methods only the reconversion of GA_{20} glucosyl ester has been shown by gas chromatography (GC)/MS in *Zea mays* (Schneider et al. 1992). In microbial cultures the hydrolysis of GA_{20} conjugates by *Azospirillum lipoferum* was reported by Picolli et al. (1997).

The cited metabolic studies used different plant systems at different developing stages. To demonstrate that both processes, the formation of GA_{20} conjugates and their reconversion, take place in the same plant system, we studied the metabolism of [17-D_2]-labeled GA_{20} and its glucosyl conjugates in *Phaseolus coccineus* L. seedlings, using mass spectrometric analyses.

Abbreviations: GA, gibberellins; MS, mass spectrometry; GC, gas chromatography; NF, neutral fraction; AF, acidic fraction; LC, liquid chromatography; SIM, selective ion monitoring; KRI, Kovats retention indices

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Table 1. Diagnostic ions (*m/z* (% abundance)) of GA_{20}-13-O-glucoside and of metabolically formed [17-D_2]GA_{20}-13-O-glucoside (*A*-AF-2/2) obtained by LC-ESI-tandem MS. (FS = full scan, SIM = selected ion monitoring; Dau = daughter ion scan); Ultrasep ES RP18 E, 1 × 100 mm, gradient of CH_3CN:H_2O (0.2% HOAc = 20:80 to 90:10 (20 min), 70 μL/min^{-1}.

	t_R	MS mode	M-1	449/451	287/289	119 (*m/z*)
GA_{20}-13-O-Glc	7.20	FS	493 (100)			
	7.15	Dau *m/z* 493		449 (92)	287 (100)	119 (35)
A-AF-2/2	7.11	SIM	495 (100)			
	7.15	Dau *m/z* 495		451 (95)	289 (100)	119 (50)

Results and Discussion

In experiment *A* the [17-D_2]-labeled GA_{20} was injected between the first two expanded leaves in 9-day-old seedlings of *Phaseolus coccineus.* After 3 days, the material above the injection point was harvested, extracted, and analyzed (Senns et al. 1998). The extract was divided into the neutral fraction (NF) and the acidic fraction (AF) by anion exchange chromatography on DEAE Sephadex. The AF fraction, which is presumed to contain the free GAs and their glucosides, was purified and subjected to liquid chromatography (LC) MS. By selective ion monitoring (SIM) under negative ionization mode the M-1 ion at *m/z* 495 for [17-D_2]GA_{20}-13-O-glucoside could be detected at the expected retention time (t_R = 7.11 min). To verify the identity we ran the sample once again under LC/tandem-MS conditions. The $[M-H]^-$ ion was subjected to an additional collision, resulting in a characteristic spectrum of daughter fragments. The pattern of fragments appeared at the correct retention time (t_R = 7.14 min) and agreed precisely with that of the corresponding unlabeled standard compound (Table 1). Thus, we were able to conclusively identify the metabolically formed [17-D_2]GA_{20}-13-O-glucoside containing the deuterium label of the precursor.

Likewise, the NF fraction was subjected to LC/MS under negative ionization. In this case we were expecting an intense ion [M-162] at *m/z* 333 that results from the loss of the sugar moiety from the [17-D_2]GA_{20} glucosyl ester (Moritz et al. 1992, Schneider and Schmidt, 1996). The chromatogram showed a pronounced signal at t_R = 9.25 min, which was subjected to tandem-MS using parent ion technique, where the source ions of the *m/z* 333 fragment could be estimated. As shown in Table 2, the expected molecular ion at *m/z* 495 ($[M-H]^-$) and the ion of acetic acid adduct at *m/z* 555 ($[M-H + HOAc]^-$) was measured at the correct retention time and coincides with the standard substance (Table 2).

Thus, in *Ph. coccineus* seedlings, labeled [17-D_2]GA_{20} was shown by MS identification to be converted into both conjugate types, the gibberellin-O-glucoside and the glucosyl ester. This confirms former results obtained by feedings of radioactive precursors (see preceding). This is the first MS identification of the intact metabolically formed [17-D_2]GA_{20} glucosyl esters and was made possible by LC/tandem-MS technique.

Table 2. Diagnostic ions (*m/z* (% abundance)) of [17-D_2]GA_{20} glucosyl ester and of metabolically formed [17-D_2]GA_{20}-glucosyl ester (*A*-NF-2/2) obtained by LC/ESI-tandem MS. (FS = full scan, SIM = selected ion monitoring; parent ion scan); Ultrasep ES RP18 E, 1 × 100 mm, gradient of CH_3CN:H_2O (0.2% HOAc) = 20:80 to 90:10 (20 min), 70 μL/min^{-1}.

	t_R (min)	MS mode	333	495	555 *m/z*
[17-D_2]GA_{20} glucosyl ester	9.19	FS	(100)	(10)	(75)
	9.25	Parent *m/z* 333	—	(28)	(100)
A-NF-2/2	9.25	SIM	(100)	(15)	(100)
	9.22	Parent *m/z* 333	—	(25)	(100)

Comparison of observed signal intensities of the [17-D_2]-conjugates in the LC/MS with the standards (data not shown) suggest that the metabolically formed amounts of [17-D_2]GA_{20}-13-O-glucoside and [17-D_2]GA_{20} glucosyl ester range from 25–50 μg and 10–20 μg, respectively. Assuming losses during purification, the metabolic rate is estimated at about 10 and 5%, respectively.

Complementary experiments were performed to demonstrate the reconversion of conjugates in seedlings of *Ph. coccineus.* [17-D_2]GA_{20}-13-O-glucoside (experiment *B*) and [17-D_2]GA_{20} glucosyl ester (experiment *C*) were applied to the seedlings as described previously and incubated 3 days.

The purified fractions of free GAs from these feedings were derivatized and subjected to GC/MS. Liberated [17-D_2]-labeled GA_{20} was identified after feeding of both [17-D_2]GA_{20} glucosyl ester and [17-D_2]GA_{20}-13-O-glucoside. The full-scan spectra of the derivatized [17-D_2]GA_{20} corresponded to the standard by fragmentation pattern, abundance of fragments, as well as by retention time (Table 3). Intensities of the molecular ions in the GC/MS suggest that the amounts of liberated [17-D_2]GA_{20} from [17-D_2]GA_{20} glucosyl ester and [17-D_2]GA_{20}-13-O-glucoside account for approximately 1–5 μg (1% metabolic rate) in both cases, whereby further metabolism to [17-D_2]GA_{29} or [17-D_2]GA_1 should be taken into consideration.

These data based on the MS identification of the metabolites confirm results obtained in *Vicia faba* (Lattke et al. 1987) and *Zea mays* (Rood et al. 1983). In Fig. 1 the

Table 3. Diagnostic ions (*m/z* (% abundance)) and retentions (KRI) obtained by GC/MS (full scan) of TMS-Me derivatives of [17-D_2]GA_{20} (standard, 6 ng) and [17-D_2]GA_{20} glucoside released from applied [17-D_2]GA_{20}-13-O-glucoside (*B*-AF-2/2, 1/200 equivalent) and [17-D_2]GA_{20} glucosyl ester (*C*-AF-2/2, 1/200 equivalent).

TMS-Me derivate of	KRI	[M]	[M-C_3H_7]	[$C_{12}SiOH_{17}D_2$]	Intensity (*m/z* 420)
[17-D_2]GA_{20} (6 ng)	2520	420 (100)	377 (60)	209 (60)	1.2×10^6
B-AF-2/2 (1/200 eq.)	2519	420 (100)	377 (77)	209 (56)	1.5×10^6
C-AF-2/2 (1/200 eq.)	2520	420 (100)	377 (80)	209 (60)	1.8×10^6

established metabolic pathways of conjugation and the reconversion of [17-D_2]GA_{20} are shown. The results demonstrate that both processes, formation of the glucosyl conjugates (O-glucoside and glucosyl ester) and their reconversion into the free GA, are concomitant in *Ph. coccineus* and support the model of reversible conjugation for controlling the actual pool of GA_{20}.

Experimental

Plant Material and Feeding

Seeds of *Phaseolus coccineus* L. cv. Preisgewinner were cultivated in sawdust under ambient glass house conditions for 9 days. Seedlings (ca. 40) were injected with 4 μL of a solution (2.5 mg/mL^{-1} EtOH) of [17-D_2]GA_{20} (experiment *A*), [17-D_2]GA_{20}-13-O-glucoside (experiment *B*) or [17-D_2]GA_{20} glucosyl ester (experiment *C*) into the epicotyl and cultivated for 72 hr. The [17-D_2]-labeled conjugates were synthesized from [17-D_2]GA_{20} according to Fuchs and Schneider 1996.

Extraction and Purification

After harvesting, the root system was removed and the remaining plant material was extracted with MeOH and analyzed as described by Senns et al. (1998). By chromatography on DEAE-Sephadex A25 (HOAc gradient in MeOH) the extracts were separated into an NF and AF, purified on silica (MeOH/HOAc gradient in chloroform), and by preparative RP18-HPLC (MeOH: water containing 0.2% HOAc). The resulting fractions *A*-AF-2/2 and *A*-NF-2/2 were subjected to LC/MS; the fractions *B*-AF-2/2 and *C*-AF-2/2 were methylated by diazomethane, subsequently silylated by MSTFA, and subjected to GC/MS.

GC/MS

A Fisons quadrupole mass spectrometer MD 800 in combination with a GC 8000 equipped with a DB 5MS fused silica capillary column (15 m × 0.32 mm, film thickness 0.25 μm, phase ratio 250) was used (helium flow rate 1.3 mL/min^{-1}, 28 kPa head pressure, splitless injection, injector temp. 270°, temperature program 60° (1 min) to 270° (30° min^{-1}) to 290° (20° min^{-1}), interface temperature 300°, ion source temperature 200°, electron impact

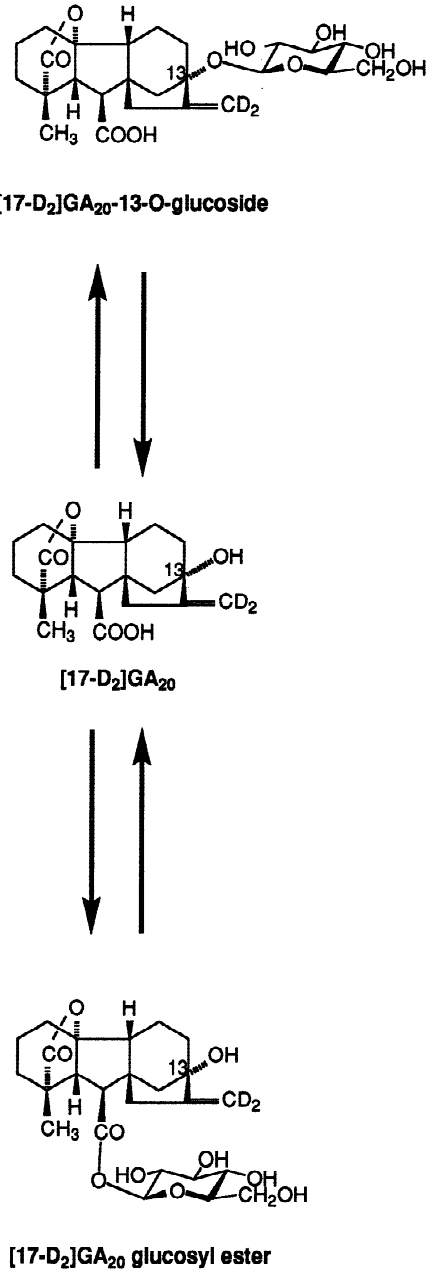

Fig. 1. Scheme of reversible conjugation of [17-$^2H_2$]GA_{20} in seedlings of *Phaseolus coccineus* L. cv. Preigewinner

energy 70 eV, mass range 50–650 *m/z* (full scan), dwell times 80 ms). The Kovats retention indices (KRI) were estimated by using parafilm.

LC/MS

A MicroTech HPLC fitted with an Ultrasep ES RP18 column (4 μm, 1 × 100 mm) was used with a gradient of CH_3CN:H_2O (containing 0.2% HOAc) from 20:80 to 90:10 (20 min) (flow rate 0.070 mL/min^{-1}; 0.5 μL injection volume). The HPLC was coupled to a Finnigan MAT TSQ 7000 instrument (electrospray voltage 3.5 kV, sheath gas nitrogen). The MS/MS experiments (daughter ion scan, parent ion scan and selected reaction monitoring) were performed under the following conditions: collision energy 40 eV for the O-glucosides, 15 eV for the glucosyl ester; collision gas: argon; collision pressure: 1.7×10^{-3} torr. All mass spectra were averaged and background substracted.

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