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QUALITATIVE AND SEMI-QUANTITATIVE ANALYSIS OF GIBBERELLINS

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Key Word Index—*Pharbitis nil*; Convolvulaceae; analysis of gibberellins; HPLC; GC/MS.

Abstract—A method for the qualitative and semi-quantitative analysis of gibberellins (GAs) was examined, and a systematic method consisting of six steps was established. By this method endogenous GAs in some organs of *Pharbitis nil* were quantitatively analysed.

INTRODUCTION

Gibberellin (GA) is a plant hormone with physiologically important roles in many aspects of plant growth. Through extensive surveys of GAs in higher plants, as many as 43 GAs have been identified from many species. Recently, the relationship between qualitative and quantitative changes of endogenous GAs and physiological processes in plant growth has been extensively investigated [1–4]. To facilitate further intensive research in this field, it is necessary to minimize the amount of plant material required and to shorten the analysis time. At present, the most sensitive and reliable method for the analysis of minute amounts of known free GAs is considered to be gas chromatography-selected ion monitoring (GC-SIM) which requires some purification of GAs in plant extracts. It has been shown that HPLC is an effective method for this purification [5, 6]. Jones *et al.* [7] and Barendse *et al.* [8] also reported the use of HPLC for purification of GAs. Thus, our plan was focused on the application of HPLC for purification prior to GC-SIM analysis and developing a systematic analysis of GAs which does not require bioassay.

RESULTS AND DISCUSSION

The procedure, consisting of the following six steps, was designed for the purification and analysis of GAs. (1) Solvent fractionation to obtain an ethyl acetate-soluble acidic fraction. (2) Sephadex LH-20 CC. (3) Reversed phase partition chromatography on Lichrosorb RP-18. (4) Gel permeation chromatography on Shodex A-801. (5) Chromatography on Nucleosil $N(Me)_2$. (6) GC–SIM analysis.

Steps 1–4 are designed to concentrate GAs, taking advantage of their common chemical similarities. Step 5 effectively groups GAs by differences in functionality. Step 6 is a GC-SIM method to identify and quantify GAs, in which as little as 20–100 pg of GA can be analysed. Recovery of GAs in purification steps 2–5 was examined using some of the representative GAs shown in Fig. 1.

Steps 1 *and* 2

Step 1 is the conventional solvent fractionation, by which most GAs are fractionated into an ethyl acetate-soluble acidic fraction [9]. In step 2, Sephadex LH-20 chromatography, several solvent systems such as MeOH, MeOH–Me_2CO (1:1), MeOH–$CHCl_3$ (1:1) and Me_2CO–$CHCl_3$ (1:1) were examined. When MeOH–Me_2CO (1:1) was used, most of all GAs tested were eluted in a narrow range (0.7–1.0) of V_e (elution volume)/V_t (total volume), and their recovery was reasonably high (Table 1). Step 2 works as rough gel permeation chromatography, and is effective in removing dusty material which would interfere with subsequent purification steps.

HPLC separation (*steps* 3, 4 *and* 5)

In step 3, two solvent systems (see Experimental) were tested for elution of all GAs in relatively small volumes of effluent. The recovery of GAs is shown in Table 2. By use of solvent system 1, GAs possessing hydroxyl group(s) were recovered in reasonable yield. However, GA_9 was not recovered, suggesting that this procedure is not suitable for purification of samples containing less polar GAs such as GA_9, GA_{12}, GA_{15} etc. On the other hand GA_9 and other GAs were recovered in good yield in the procedure employing solvent system 2. Therefore, solvent system 2 is preferable.

In step 4, a prepacked column, Shodex A-801 (500 × 8 mm), which was made for gel permeation chromatography, was used with THF as an eluent at a flow rate of 1.0 ml/min. R_t and the recovery of GAs in step 4 are listed in Table 3. GAs were eluted in a very narrow R_t range, showing that this column works in a gel permeation mode, and partition and adsorption on the gel is negligible. Step 4 effectively concentrates GAs with no recovery problems.

In step 5, four different sizes of columns packed with Nucleosil $N(Me)_2$ were examined (Table 4). Methanol containing 0.05% acetic acid was used as an eluent. The R_t of GAs on column I are shown in Table 5, and the RR_t of GAs on columns I–IV are shown in Table 6, where the RR_t of GA_3 is 1.0. In general, introduction of hydroxyl group(s) into the *ent*-gibberellane skeleton causes an increase of R_t. However, introduction of 3β-hydroxyl groups in C_{19} GAs and C-10 aldehyde GAs decreases R_t. This ten-

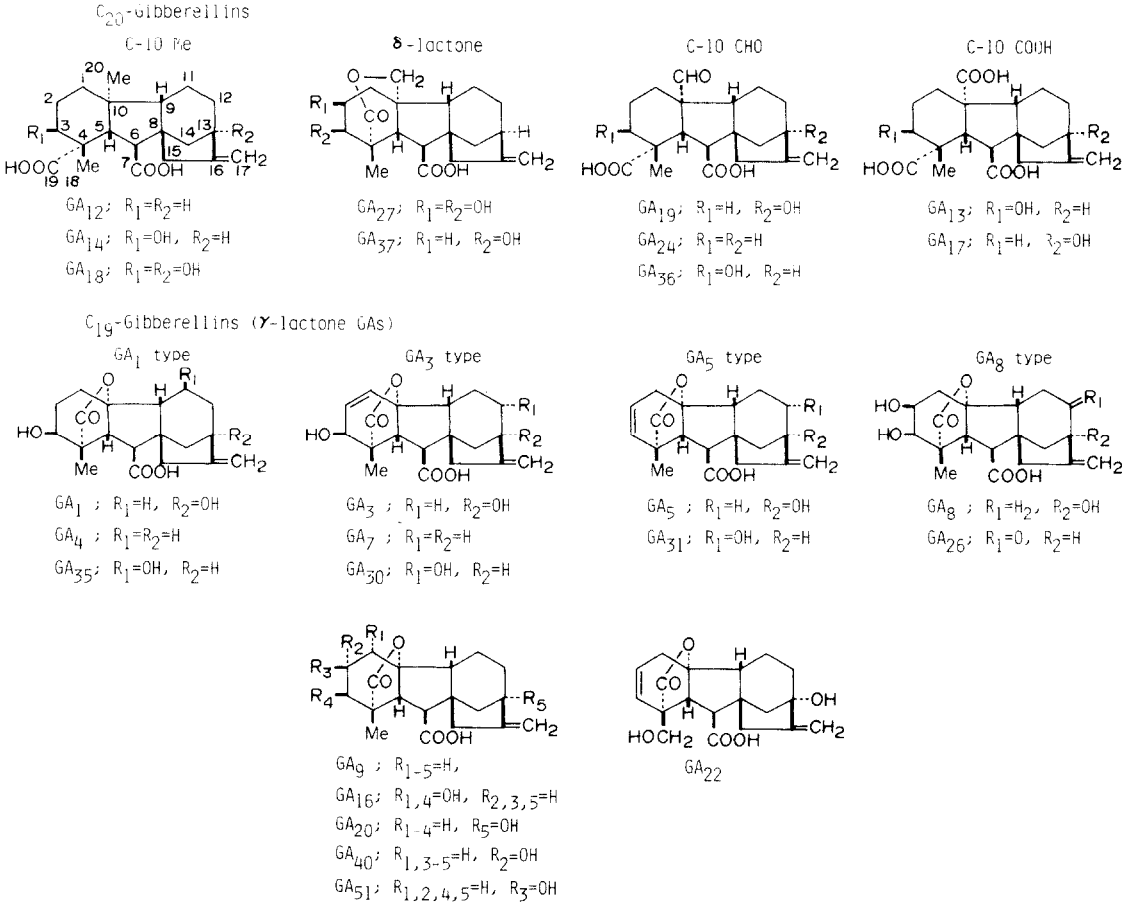

Fig. 1. Types of basic skeletons of GAs. GAs listed were used as authentic samples.

Table 1. Elution pattern and recovery (%) of GAs in step 2

V_e/V_t	0.6	0.7	0.8	0.9	1.0	1.1	1.2	Total
GA_3*	0	3.5	84.4	6.4	0	0		94.3
GA_5*	0	40.6	69.2	0	0	0		109.8
GA_9*	0	40.4	43.6	0	0	0		84.0
GA_{14}†	0	0	19.5	59.9	1.1	0		80.5
GA_{17}†	0	0	12.0	63.9	2.6	0		78.5
GA_{36}*	0	0	4.8	96.0	8.5	0		109.3
GA_{37}*	0	0	48.2	54.3	0.9	0		103.4

Column size; 260 × 16 mm; sample size: 10 μg of each GA.
*Analysed as methyl ester TMSi ether.
†Analysed as methyl ester.

dency seems to be common to δ-lactone GAs judging from the R_t of GA_{27} and GA_{37}, although GA_{15} and GA_{44} were not tested. This suggests that the 3β-hydroxyl group in these GAs supresses the dissociation of the C-6 carboxyl group [10]. However, the 3β-hydroxyl group in C-10 carboxyl GAs seems to increase R_t, as in C-10 methyl GAs, judging from the R_t of GA_{13} and GA_{17}. The RR_t of each C_{19} GA, C-10 methyl GA and γ- and δ-lactone GA shows almost constant values regardless of the column and particle size. The RR_t of C-10 aldehyde and C-10 carboxyl GAs vary with column size, suggesting that these GAs may have some affinity to the gel. Interestingly, pairs of GA_1 and GA_3, GA_4 and GA_7, and GA_5 and GA_{20}, which are difficult to separate by conventional methods, can be clearly separated in step 5. The recovery of GAs in step 5 is reasonable (Table 7). The elution pattern of GAs in column I is summarised in Fig. 2. Based on the R_t, GAs can be divided into three groups: group I(R_t, 10–18 min); group II (R_t,

Table 2. Recovery (%) of GAs in step 3

GAs	Solvent system 1	2‡
GA_1*	—	85.3
GA_3*	98.3	—
GA_5*	113.9	—
GA_8*	—	83.6
GA_9†	0	87.4
GA_{37}*	102.0	—

Column size: 200×8 mm; sample size: 5 μg of each GA.
*Analysed as methyl ester TMSi ether.
†Analysed as methyl ester.
‡*n*-BuOH was added prior to concentration.

purification methods for GC-SIM analysis of GAs. In some practical purification procedures, some of the steps can be omitted. For example, when the content of GAs in a sample is relatively high, as in immature seeds of *Pharbitis nil* [11] and *Phaseolus vulgaris* [12], and small sample size is enough for analysis, steps 2 and 3 may be unnecessary.

Practical application

This systematic method was applied to analyse GAs in some organs of *Pharbitis nil*, such as immature seeds, flower buds from which anthers were removed, pistils and anthers. As the sample size was small enough, step 2 was omitted in analysing all samples. In the analysis of GAs in pistils and anthers, step 3, which effectively removes pigments, was also omitted, because extracts of these samples were almost colorless compared to those of immature seeds and flower buds. The amount of sample used in each step is summarized in Table 8. Each sample was grouped into three categories by their R_t on columns

Table 3. R_t (min) and recovery (%) of GAs in step 4

	GA	Number of hydroxyls	R_t	Recovery
C_{19}–GAs	GA_9	None	13.7	—
	GA_5	Mono	12.9	93.9*
	GA_{20}		12.9	—
	GA_1	Di	12.2	102.5*
	GA_3		12.2	—
	GA_8	Tri	11.9	—
C_{20}–GAs	GA_{24}	None	13.0	—
	GA_{13}	Mono	12.0	110.9*
	GA_{14}		12.6	—
	GA_{19}		12.3	—
	GA_{36}		12.3	79.2*
	GA_{27}	Di	12.8	—

Column size: 500×8 mm; solvent: THF, 1.0 ml/min; detector: RI; sample size; 40 μg of each GA.
*Analysed as methyl ester TMSi ether.

Table 4. Nucleosil $N(Me)_2$ columns and conditions in step 5

Column	Column size (mm)	Particle size (μm)	Flow rate (ml/min)	Column temp. (°)
I	250×6	10	2.0	50
II	50×8	5	2.0	40
III	100×4.6	5	1.1	40
IV	30×4.6	5	0.7	40

18–30 min); and group III (R_t, 30–52 min). Group I contains C-10 methyl GAs and δ-lactone GAs, group II C-10 carboxyl GAs and most of the C_{19} GAs, group III C-10 aldehyde GAs and C_{19} GAs such as GA_5 type in ring A, GA_{22} and GA_{26}. Such grouping is helpful in the selection of ions to be monitored in GC-SIM (step 6).

As described above, steps 2–5 were examined using authentic GAs and shown to work well as pre- of Nucleosil $N(Me)_2$ in step 5. Based on the species of GAs already characterized in immature seeds of *P. nil* [7, 11] and their biosynthetic pathway, GA_{27}, GA_{44} and GA_{53} were expected to be in group I, GA_1, GA_3, GA_8, GA_{17}, GA_{20}, GA_{34} and GA_{43} in group II, and GA_5, GA_{19} and GA_{26} in group III. Among these, GA_{43} was excluded from analysis because the authentic sample was not available. Thus, to analyse GAs as their methyl ester TMSi ethers, the ions at *m/z* 520

Table 5. R_t (min) of GAs on column I of Nucleosil $N(Me)_2$

Number of hydroxyls	Type of GAs				
	C-10 Me	δ-Lactone	C-10 CHO	C-10 COOH	γ-Lactone
None	GA_{12} 10.7	—	GA_{24} 32.4	—	GA_9 27.0
Mono	GA_{14} 11.4	GA_{37} 13.8	GA_{36} 31.4 GA_{19} 41.6	GA_{13} 28.0 GA_{17} 21.4	GA_4 20.0, GA_5 38.2, GA_7 27.8 GA_{20} 30.0, GA_{31} 37.6, GA_{40} 28.0 GA_{51} 30.2
Di	GA_{18} 11.7	GA_{27} 15.6	—	—	GA_1 22.0, GA_3 28.9, GA_{16} 25.6 GA_{22} 49.0, GA_{26} 48.2, GA_{30} 28.6 GA_{35} 22.5
Tri	—	—	—	—	GA_8 26.0

Sample size: 20 μg of each GA; detection: 205 nm.

Table 6. RR_t of GAs on columns of Nucleosil $N(Me)_2$ in step 5

Column	Type of GAs				
	C-10 Me	δ-Lactone	C-10 CHO	C-10 COOH	γ-Lactone
I	GA_{12} 0.37 GA_{14} 0.39	GA_{27} 0.54 GA_{37} 0.48	GA_{19} 1.44 GA_{24} 1.12	GA_{13} 1.00 GA_{17} 0.74	GA_1 0.76, GA_3 1.00 (R_t = 28.9 min) GA_5 1.32, GA_9 0.93
II	GA_{12} 0.39 GA_{14} 0.42	GA_{27} 0.59 GA_{37} 0.47	GA_{19} 1.85 GA_{24} 1.58	GA_{13} 1.43 GA_{17} 0.85	GA_1 0.78, GA_3 1.00 (R_t = 20.0 min) GA_5 1.30, GA_9 0.92
III	GA_{12} 0.36 GA_{14} —	GA_{27} 0.57 GA_{37} —	GA_{19} 1.78 GA_{24} 1.49	GA_{13} 1.08 GA_{17} 0.83	GA_1 —, GA_3 1.00 (R_t = 20.6 min) GA_5 1.33, GA_9 —
IV	GA_{12} 0.40 GA_{14} —	GA_{27} — GA_{37} 0.50	GA_{19} — GA_{24} 1.39	GA_{13} 1.06 GA_{17} 0.77	GA_1 —, GA_3 1.00 (R_t = 10.0 min) GA_5 1.30, GA_9 —

Table 7. Recovery (%) of GAs in step 5

Column	GAs			
	GA_3	GA_5	GA_{13}	GA_{36}
I	99	92	91	82
II	103	82	89	84
III	102	97	88	96
IV	102	102	102	101

Sample size: 5 μg for each GA; analysed as methyl ester TMSi ether.

for GA_{27}, 373 and 432 for GA_{44} and 448 for GA_{53} in group I; the ions at m/z 506 for GA_1, GA_{29} and GA_{34}, 504 for GA_3, 580 and 594 for GA_8, 492 for GA_{17} and 418 for GA_{20} in group II; and the ions at m/z 416 for GA_5, 434 and 462 for GA_{19} and 520 for GA_{26} in group III were selected for monitoring.

The occurrence of GA_{53} was demonstrated by monitoring the ions at m/z 390 $[M-58]^+$, 417 $[M-31]^+$ and 448 $[M]^+$ of group I from immature seeds. The R_t of these ion peaks was identical to that of authentic GA_{53}, and the relative intensity of the ion peaks was *ca* 55 (m/z 390):20 (m/z 417):100 (m/z 448), affording enough data to confirm identification as GA_{53}. This is the first example of its occurrence in *P. nil*. The occurrence of GA_1 in immature seeds of *P. nil* was also confirmed by analysing the GA_1 fraction (R_t 14.0–18.0 min) from column III of the Nucleosil $N(Me)_2$ chromatography in step 5. In similar analyses of the GA_{29} fraction (R_t 22.0–24.0 min) and the GA_{34} fraction (R_t 18.0–22.0 min), GA_{29} was identified in immature seeds, but GA_{34} was not detected. The content of GA_1 was extremely low compared to that of GA_{29}. The occurrence of GA_{17} in immature seeds was noticed but the content was not determined because of the failure in centering the ion peak at m/z 492. The identification of GA_{44} by monitoring the ion peak at m/z 432 $[M]^+$ was unsuccessful due to high background interference. The results are summarized in Table 9.

Except for GA_{53}, the content in immature seeds is notably high compared to other organs. The content of GAs with high degrees of oxidation, such as GA_8 and GA_{26}, is particularly high. In contrast with this observation, GA_{53}, which is the first GA biosynthesized from steviol is found in high content in pistils and flower buds. In anthers, the content of GAs is very low, and GA_{26} and GA_{27}, which were detected in

Table 8. Amount of tissues of *P. nil* used in each step of the purification procedure

Organ		Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	
Immature seeds	A	220 g	—	5.0 g eq.	5.0 g eq.	2.5 g eq.	1.25 g eq.	(0.5–10%)†
	B	242 mg		3.2 mg	n.w‡	n.w		
Flower buds*	A	200 g	—	20.0 g eq.	20.0 g eq.	10.0 g eq.	5.0 g eq.	(1–20%)†
	B	111 mg		6.2 mg	n.w	n.w		
Pistils	A	3.1 g	—	—	3.1 g eq.	3.1 g eq.	0.4 g eq.	(1–20%)†
	B	5.7 mg			n.w	n.w		
Anthers	A	13.0 g	—	—	13.0 g eq.	6.5 g eq.	1.6 g eq.	(5–25%)†
	B	29.5 mg			n.w	n.w		

A, fr. wt equivalent submitted for purification.
B, wt of sample obtained after purification.
*Anthers were removed.
†Aliquot of sample (%) injected into GC/SIM at one time.
‡n.w, not weighed.

Table 9. Content of GAs in organs of *P. nil*

Organ	Content (μg/kg fr. wt)								
	GA_3	GA_5	GA_{17}	GA_{19}	GA_{20}	GA_{26}	GA_{27}	GA_{29}	GA_{53}
Immature seeds	19	100	(+)*	32	190	1200	176	40	0.8
Flower buds	0.2	0.6	(+)*	1	1	0.04	2	1	7
Pistils	0.6	5.2	(+)*	2	3	2.6	3.1	7.8	25
Anthers	(+)†	3.8	(+)*	0.2	0.8	(−)‡	(−)‡	0.4	(−)‡

*Detected but the content was not determined because of failure in peak centering.
†Detected but too little to determine the content.
‡Not detected.

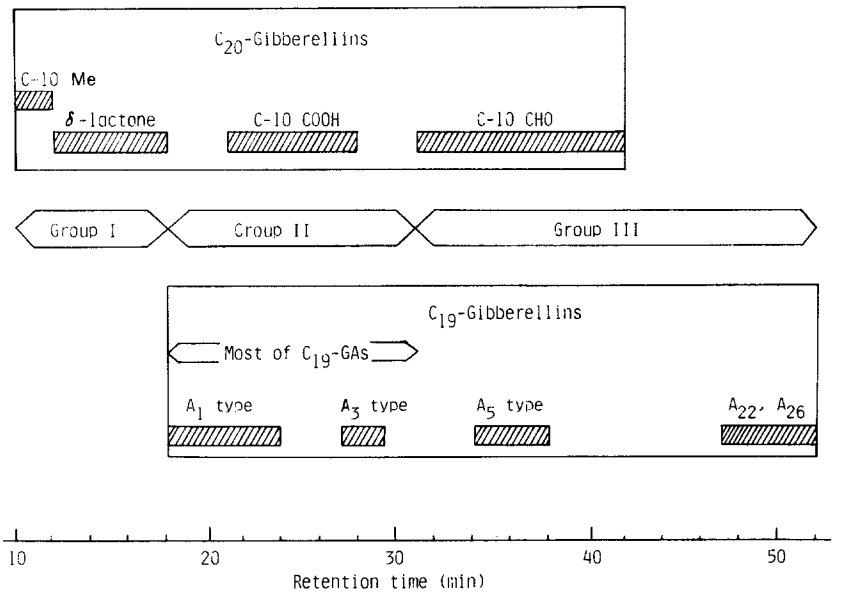

Fig. 2. Diagram of the elution pattern of GAs on column I in step 5. Column: Nucleosil $N(Me)_2$, 250 × 6 mm, 10 μm; solvent: MeOH containing 0.05% HOAc at 2.0 ml/min; Column temp.: 50°.

other organs, were undetectable. Murakami [13] reported that the content of GAs in floral organs of *P. nil* varies with the growth stage. Qualitative and quantitative analyses of some organs of *P. nil* at different growth stages are being continued, and details will be discussed elsewhere.

In this study, the effectiveness of this systematic analytical method for the known GAs was demonstrated. It is especially noteworthy that the amount of material required for analysis was greatly reduced compared to conventional method. Though it is preferable to use isotopically labelled internal standards for the accurate quantitative analysis, we could not use them, because of the limited number of isotopically labelled GAs available to us.

EXPERIMENTAL

Authentic gibberellins. Authentic GAs used in these expts were isolated from *Gibberella fujikuroi* or from plant sources in our laboratory.

HPLC procedures. Constant flow pump systems equipped with pulse dampers were used.

Step 3. The ODS column (200 × 8 mm, Lichrosorb RP-18, 10 μm) was packed using the same procedure described in our previous paper [14]. Two solvent systems were tested at a flow rate of 1.5 ml/min. In the procedure using solvent system 1, GAs dissolved in 60% MeOH were injected through a Reodyn loop injector onto the column in a flow of 5 mM Pi buffer (pH 3.2) containing 10% MeOH, and after 45 ml of effluent was collected, the eluent was switched to H_2O (20 ml), 60% MeOH (90 ml), and 100% MeOH successively. In the second procedure using solvent system 2, GAs in 60% MeOH were injected onto the column in a flow of 0.05 mM formic acid (pH 2.9) containing 10% MeOH, and after 45 ml of effluent was collected, the eluent was switched to 0.01 mM HCOOH (pH 5.2) containing 60% MeOH, and 100% MeOH (45 ml) successively. In both procedures effluent was monitored to determine the points of solvent exchange. Fractions eluted with solvents containing 60% MeOH were regarded as the GA fractions, and recovery of each GA was examined.

Step 4. Shodex A-801 is a prepacked column (500 × 8 mm) of a polystyrene–divinylbenzene polymer purchased from Showadenko Co. Ltd. This column is designed for gel permeation chromatography with an exclusion limit of MW *ca* 1000, and THF, DMF and $CHCl_3$ are usable as solvents. In this expt, THF for HPLC was used at a flow rate of 1.0 ml/min, and effluent was monitored by a RI detector.

Step 5. Four different sizes of Nucleosil $N(Me)_2$ columns (see Table 4) were examined. Column I was packed using a Varian 8500 pump system as follows. Into a mixture of 5 ml MeOH and 5 ml *iso*-PrOH, 5.5 g Nucleosil $N(Me)_2$ gel (10 μm, Nagel) was added, and the mixture treated in an ultrasonic bath. After 5 min, 12 ml C concentrate (Nagel) and 18 ml CCl_4 were added and the mixture again treated in an ultrasonic bath for 5 min to make a complete slurry. The slurry was transfered into a packer (40 ml) onto which a stainless steel column (250 × 6 mm) was connected. The slurry was pushed up by $CHCl_3$ until air inside the column was purged, and then the assembly was turned upside down. The flow of $CHCl_3$ was set to maintain the pressure at 400 kg/cm^2. After 100 ml $CHCl_3$ eluted, the column was washed with 100 ml MeOH at a flow rate of 4 ml/min. The column was disconnected from the packer and equilibrated with MeOH containing 0.05% HOAc at a flow rate of 2 ml/min. Columns II–IV were similarly packed with Nucleosil $N(Me)_2$ of particle size 5 μm. The expts were carried out at column temp. of 50° (column I) and 40° (columns II–IV) in an oven. Columns were eluted with MeOH containing 0.05% HOAc at flow rates shown in Table 4. Samples were injected in MeOH soln and effluent was monitored at 205 nm.

Determination of GA recovery. The recovery of GAs tested in each step was calc. by an int. standard correction method as follows. A test soln containing authentic GAs was prepared in each purification step. An aliquot of the test soln was chromatographed, and the same amount, which was not chromatographed, was used as ref. To both solns. chromatographed and non-chromatographed, equal amounts of another GA (GA_R) was added as int. standard. The amount of GA_R added (5–10 μg) was determined by the amount of each GA in the test soln. These mixtures were methylated and, if necessary, trimethylsilylated and submitted to GC analysis on a machine equipped with FID and an integrator. The recovery (%) of each GA was calc. by the following equation:

$$\text{Recovery (\%)} = \frac{\dfrac{(\text{Peak area of } GA_x)_{\text{chromatographed}}}{\text{Peak area of } GA_R}}{\dfrac{(\text{Peak area of } GA_x)_{\text{non-chromatographed}}}{\text{Peak area of } GA_R}} \times 100.$$

Analysis of GAs in Pharbitis nil. Conditions used for HPLC were the same as those described above. An RMU-6MG GC/MS spectrometer was used for GC-SIM analysis; the IE was 20 eV. The conditions of GC were as follows: column, 2% OV-1 on Chromosorb W (2 m × 3 mm); column temp., isocratic in the range 190–220°; carrier gas, He at 50 ml/min.

Immature seeds. Step 1. Immature seeds (220 g) were homogenized and extracted with Me_2CO (× 3). After removal of Me_2CO *in vacuo*, the aq. residue was fractionated to give an EtOAc-soluble acidic fraction which was concd to an acidic gum (242 mg). *Step* 2. Omitted. *Step* 3. Aliquots (5.5 mg) of the acidic gum obtained in step 1 were dissolved in 200 μl 60% MeOH and injected onto an ODS column (200 × 8 mm, Lichrosorb RP-18, 10 μm). Solvent system 2, described above, was used and the GA fraction (90 ml) was concd with 40 ml *n*-BuOH and yielded 3.2 mg of a concentrate. *Step* 4. The concentrate (3.2 mg) obtained in step 3 was dissolved in 2.4 ml THF and submitted (400 μl/injection) to GPC on Shodex A-801 (500 × 8 mm). The effluent of R_t 11.5–14.0 min was regarded as the GA fraction and concd to an acidic gum (not weighed). *Step* 5. The acidic gum obtained in step 4 was dissolved in 200 μl MeOH. Half of the MeOH soln was injected onto column IV of Nucleosil $N(Me)_2$, listed in Table 4, and three effluent groups were collected: group I (R_t 2–7 min), group II (R_t 7–13 min) and group III (R_t 13–22 min). Each group was treated with CH_2N_2 and concd. *Step* 6. The methylated samples obtained in step 5 were trimethylsilylated and submitted to GC–SIM. Calibration curves were made in the ranges of 100–1000 pg for GA_{27}, GA_{44} and GA_{53}, 125–2000 pg for GA_3, GA_8, GA_{17}, GA_{20} and GA_{29}, and 40–2000 pg for GA_5, GA_{19} and GA_{26}. The R_ts of each GA and column temp. were as follows. Group I; GA_{27} 6.0 min (220°), GA_{44} 4.6 min (220°), GA_{53} 3.8 min (190°). Group II; GA_3 8.0 min (200°), GA_{17} 6.0 min (200°), GA_{20} 4.6 min (200°), GA_1 6.3 min (210°), GA_8 9.6 min (210°), GA_{29} 6.5 min (210°) and GA_{34} 6.3 min (210°). Group III; GA_5 3.3 min (210°), GA_{19} 4.5 min (210°) and GA_{26} 9.6 min (210°).

Other organs. The analysis of GAs in other organs was

carried out in almost the same manner as for immature seeds. Flower buds were collected *ca* 18 hr before blooming, and anthers were removed. Both of the anthers and the residual flower buds were used as materials. Pistils were obtained from flower buds *ca* 18 hr before blooming. Anthers stored in Me_2CO at 2° were extracted as follows. Into the cake obtained by filtration of the Me_2CO, 10 ml. H_2O was added, the mixture frozen at −40°, melted at 30°, then 20 ml Me_2CO was added and filtered. This procedure was repeated twice to break crusts of pollens. In step 5, column IV was used for flower buds and pistils, and column II for pollens. The amount of samples used in each step is shown in Table 8.

Identification of GA_{53}. A portion (10%) of group I GAs of immature seeds, obtained in step 5, was analysed as their MeTMSi derivatives. The ions at *m/z* 390, 417 and 448 $[M]^+$, characteristic of a GA_{53} derivative, were monitored and these ion peaks were observed at R_t 3.8 min (190°) in the ratio of 55:20:100. Authentic GA_{53} showed these ion peaks at R_t 3.8 min in the ratio of 55:18:100.

Identification of GA_1 *and* GA_{29}. A portion (10%) of the acidic gum of immature seeds, obtained in step 4, was chromatographed on column III of Nucleosil $N(Me)_2$ (see Table 4). Three fractions, GA_1 (R_t 14.0–18.0 min), GA_{34} (R_t 18.0–22.0 min) and GA_{29} (R_t 22.0–24.0 min) were obtained. Each fraction was analysed as the MeTMSi derivatives by GC-SIM, monitoring the ions at *m/z* 506 $[M]^+$ and 491 which are characteristic of the derivatives of GA_1, GA_{29} and GA_{34}. The GA_1 fraction showed these ion peaks at R_t 5.3 min (column temp. 220°) in the ratio of 3 (*m/z* 491):100 (m/z 506) which were identical with those of authentic GA_1. Similarly, the GA_{29} fraction gave a GC-SIM chromatogragh identical with that of authentic GA_{29}, but no ion peaks at *m/z* 491 and 506 were observed in analyses of the GA_{34} fraction.

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