Plant Physiol. (1989) 91, 1255–1258
0032-0889/89/91/1255/04/$01.00/00

Received for publication April 19, 1989
and in revised form July 18, 1989

Communication

Further Identification of Endogenous Gibberellins in the Shoots of Pea, Line G2[1]

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ABSTRACT

To interpret the metabolism of radiolabeled gibberellins A_{12}-aldehyde and A_{12} in shoots of pea (*Pisum sativum* L.), the identity of the radiolabeled peaks has to be determined and the endogenous presence of the gibberellins demonstrated. High specific activity [^{14}C]GA_{12} and [^{14}C]GA_{12}-aldehyde were synthesized using a pumpkin endosperm enzyme preparation, and purified by high performance liquid chromatography (HPLC). [^{14}C]GA_{12} was supplied to upper shoots of pea, line G2, to produce radiolabeled metabolites on the 13-OH pathway. Endogenous compounds copurifying with the [^{14}C]GAs on HPLC were analyzed by gas chromatography-mass spectrometry. The endogenous presence of GA_{53}, GA_{44}, GA_{19} and GA_{20} was demonstrated and their HPLC peak identity ascertained. The ^{14}C was progressively diluted in GAs further down the pathway, proportional to the levels found in the tissue and inversely proportional to the speed of metabolism, ranging from 63% in GA_{53} to 4% in GA_{20}. Calculated levels of GA_{20}, GA_{19}, GA_{44}, and GA_{53} were 42, 8, 10, and 0.5 nanograms/gram, respectively.

The identification of endogenous gibberellins provides the necessary starting point from which metabolism and physiological studies can begin. The endogenous GAs of pea seeds and shoots have been extensively studied but evidence for some metabolic steps *in vivo* is still lacking (7, 14). In the G2 genetic line of peas (*Pisum sativum* L.) growth is indeterminate in short photoperiods while in long photoperiods the senescence of the plants takes place (12). Indeterminate growth is correlated with a higher level of biologically active GAs (13) which were subsequently associated with GAs identified by GC-MS as GA_{20} and GA_{19} (5). In addition, the presence of GA_{29} and GA_{29}-catabolite was shown in shoot extracts (5). When [^{14}C]GA_{12}-aldehyde is metabolized by G2 pea shoots many products are formed (4). The levels of the ^{14}C metabolites tentatively identified as GA_{53}, GA_{44}, GA_{19}, and GA_{20} were higher in shoots grown in SD than in those grown in LD. Since the products of [^{14}C]GA_{12}-aldehyde were tentatively identified from the HPLC retention time of endogenous gibberellins from pea cotyledons which were also products of [^{14}C]GA_{12}-aldehyde metabolism (9), we wished to confirm their identity and HPLC retention times using pea shoot extracts. In this paper, using GC-MS, we report the presence 13OH-GAs 53, 44, 19, and 20 from pea shoots, and also identify these GAs as products of [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} metabolism in pea shoots.

MATERIALS AND METHODS

[^{14}C]GA_{12}-Aldehyde and [^{14}C]GA_{12} Synthesis and Purification

All glassware used was baked in an oven at 500°C to remove any organic contamination and was silanized with Aquasil (Pierce, Rockford, IL). [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} were synthesized from [4,5-^{14}C]mevalonic acid using a cell-free system from the liquid endosperm of *Cucurbita maxima* Duchesne, cv Atlantic Giant and/or Big Max (3). Sixty pumpkin fruits with the correct color were sampled *in situ* to estimate the stage of seed development and were harvested at the 20% of maximum cotyledon length, reported to have the most activity. Liquid endosperm was mixed with an equal volume of buffer (40 mM Hepes, 0.6 M mannitol, 2.5 mM $MgSO_4$, 1 mM DTT, and 1 g/L BSA adjusted to pH 8.0 with KOH) per volume of endosperm and dialyzed three times against about 25 volumes of this buffer without BSA for 1, 0.75, and 0.75 h. The volume of endosperm recovered from each pumpkin differed from 15 to 65 mL. The extraction was made from 21 fruits to give a total enzyme extract of 956 mL. Extracts were separately assayed with mevalonic acid before a large scale reaction. The reaction volume was dependent on the volume of enzyme extract available. Per mL of reaction mixture (up to about 60 mL), 0.65 mL of enzyme was mixed with 0.05 mL of 0.1 M K-phosphate (pH 6.2) and with 0.2 mL of cofactor stock solutions to give the following final concentrations: ATP, 3.0 mM; NADPH, 0.5 mM; NADH, 0.5 mM; flavin adenine dinucleotide, 0.5 μM; flavin mononucleotide, 0.5 μM; $MnCl_2$, 1.0 mM; $MgSO_4$, 5.0 mM. Prior to adding the enzyme, the pH of the mixture of cofactors and buffer was adjusted to 6.2 (with 1 N KOH). Then the pH of the final reaction mixture was adjusted to 6.9 (with 1 N HCl), and the volume of reaction mixture was made up to the designated volume (including the volume of the added mevalonic acid) with water. The incubation mixtures were kept on ice until addition of [^{14}C]mevalonic acid to a final concentration of 100 μM. [4,5-^{14}C]Mevalonic acid lactone (110 mCi/mmol)

[1] Supported by grant PCM-8304542 from the National Science Foundation (NSF). The GC-MS used in this research was funded by NSF (grant DMB-8505974) and the New York State College of Agriculture and Life Sciences at Cornell University.

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was custom synthesized by Amersham (Amersham, UK) and was converted to free mevalonic acid prior to addition by evaporating the toluene solvent from the mevalonic lactone, adding 100 μL (excess) 28% NH_4OH, heating at 50°C for 1 h, and evaporating off the ammonium hydroxide *in vacuo*. Reaction vessels (250 mL polypropylene bottles) were shaken at room temperature (*ca.* 22°C) for 5 h. At the end of incubation period the reaction mixture was stopped by addition of acetone to give a final concentration of 75%, and stored at −80°C until used.

To remove the large amount of unconverted mevalonic acid and other polar products, the reaction mixture was subjected to charcoal-celite adsorption chromatography. Ten g charcoal:20 g celite (with a void volume of 50 mL) were used per 100 mL of final dialyzed extract, in a 2.5 cm wide glass column. The charcoal-celite was prewashed with 100% acetone and then returned to 0.1 N acetic acid. The reaction mixture plus acetone was centrifuged at 5000*g* for 15 min, the supernatant withdrawn, and the acetone evaporated. The aqueous remainder was made 0.1 N in acetic acid and loaded into the column using nitrogen pressure at approximately 70 kPa. The column was washed with 0.1 N acetic acid (100 mL), 5% acetone (50 mL), 80% acetone (250 mL), and then 100% acetone (3 L). The [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} eluted in the 100% acetone fraction. (Some [^{14}C]GA_{12} was eluted in the 80% acetone fraction and could be recovered by HPLC of this fraction.) The 100% acetone fraction was evaporated *in vacuo* to about 0.5 mL (aqueous—from the 80% acetone remaining in the column). This was transferred to filter tubes with a 0.45 μm nylon membrane (Rainin, Woburn, MA) followed by 2 × 0.5 mL acetonitrile and 2 × 0.5 mL water rinses of the flask and was centrifugally filtered. A small sample was injected onto a preparative C_{18} column (1.0 × 15 cm containing 5 μm Spherisorb ODS-2 [Phase Separations, Norwalk, CT]), fitted with a 5 mL injection loop, and run using a 40% to 80% acetonitrile (containing 0.1 N acetic acid) gradient over 20 min at 4 mL/min. The elution times of the peaks were noted using an in-line radioactivity detector fitted with a 3 mm × 6 cm flow cell packed with solid scintillation beads (Trace 7140, Packard, Downers Grove, IL). The largest product was [^{14}C]GA_{12}-aldehyde at retention time (R_t) 16.0 min with less [^{14}C]GA_{12} at R_t 12.0 min. The [^{14}C]GA_{12} was preceded by another peak of similar size at R_t 10.40 min. (Virtually all the mevalonic acid and early eluting peaks [3] were removed by the water/5% acetone washes of the charcoal-celite column.) The entire sample was then run on the HPLC column disconnected from the detector, with the peaks manually collected by retention time directly at the end of the column. The [^{14}C]GA_{12} needed further purification on an analytical C_{18} column (5 μm, 0.4 × 25 cm, Microsorb, Rainin) run at 1 mL/min to remove traces of the material from the earlier peak. The identity of [^{14}C]GA_{12}-aldehyde and [^{14}C]GA_{12} was shown by GC-MS. The specific activities were 342 mCi/mmol for [^{14}C]GA_{12}-aldehyde and 351 mCi/mmol for [^{14}C]GA_{12} (15). The overall efficiency was 6.7% for [^{14}C]GA_{12}-aldehyde production and purification and 2.2% for [^{14}C]GA_{12} (though as only 50% of the mevalonic acid would be of the correct isomer, and therefore utilized in the reaction, the conversion from usable mevalonic acid would be twice these values).

Plant Material

Plants of genetic line G2 (10) of peas (*Pisum sativum* L.) were grown individually in a peat-vermiculite mixture in 4 L plastic pots. The seeds were grown in the greenhouse and the plants transferred 2.5 months later to a growth chamber with a photoperiod of 9 h and temperature of 19°C day and 17°C night. Vigorous growth continued under these conditions. After 35 d in the growth chamber, when the plant had leaves at node 33 with 12 to 15 pods and 6 to 7 flowers, the stem was cut 10 nodes below the apical bud, then a second cut was made just above the second node below the apical bud to leave the upper shoot, with one expanded and one half-expanded leaf, and the apical bud enclosed in ensheathing stipules.

Treatment Conditions

Each pea shoot prepared as above (mean weight = 2.9 g) was immediately placed individually in 50 μL water containing 0.05 μCi of [^{14}C]GA_{12} in a small plastic vial (1.2 × 2.5 cm i.d.) with a conical base. The vials were placed under a bank of cool-white fluorescent lights (144 $\mu E/m^2 \cdot s$) at 19°C. The treating solution was mostly taken up via the cut end of the shoot in about 6 min. Immediately after the solution was adsorbed, the vial was filled (about 1.2 mL) with distilled water and the water was replenished at about 30 min intervals. At 15 min, 30 min, 1 h, and 6 h after the start of the feeding treatment, the shoots were removed from the vials frozen in a liquid N_2 and stored at −18°C until extraction.

Extraction and Purification

Each frozen shoot was placed in 20 mL cold 80% methanol with 10 mg butylated hydroxytoluene per liter and immediately ground with a 2 cm head Polytron (Brinkman Instruments), rinsing the Polytron head with 2 × 10 mL of 80% methanol. The homogenate was then left at 4°C overnight prior to vacuum filtration. The solid material was rinsed with 3 × 10 mL of methanol. The resulting filtrate was evaporated at 33°C to the water phase (about 3–5 mL). An equal volume (3–5 mL) of hexane and 50 μL of 0.1 N NH_4OH were added and shaken for 5 min. Then the hexane was again evaporated so that the Chl was aggregated and retained on the wall of the flask, leaving the GAs in the remaining water solution. The water solution was then transferred onto a 2 cm thick layer of PVP (Polyclar AT, GAF Chemicals, Wayne, NJ) in a Büchner funnel and vacuum-filtered, rinsing with 3 × 10 mL of water containing 50 μL of 0.1 N NH_4OH. The resulting filtrate from 40 treated shoots was bulked, reduced to 100 mL *in vacuo*, acidified to pH 3 with acetic acid, and immediately loaded onto a charcoal:celite (2 g:4 g) column (2.5 cm diameter). The column was rinsed with water, eluted with 200 mL acetone overnight, and then eluted further with a 250 mL acetone. The eluate was evaporated to about 1 mL (aqueous) *in vacuo* at 33°C and centrifugally filtered through a nylon membrane (Rainin) pore size 4.5 μm.

HPLC

The sample was loaded onto a preparative C_{18} (5 μm Spherisorb ODS-2) column (1 × 15 cm) using a 5 mL injection loop and run at 4 mL/min in 0.1 N acetic acid (A) to acetonitrile containing 0.1 N acetic acid (B) using the following gradient: 0 to 20% B over 2 min, 20 to 35% B over 15 min, 35 to 75% B over 15 min, 75 to 100% B over 2 min and holding at 100%. The column eluate was passed through an in-line radioactivity monitor and the data recorded at 6 s intervals with a counting efficiency of 29%. The main peaks had retention times of 7.1, 14.1, 15.2, 15.9, and 19.9 min. The last four were analyzed further. These four had retention times of 19.4, 20.6, 21.2, and 25.4 min on an analytical column (with a 1 mL injection loop) under similar conditions. Baseline separation from the preparative column was achieved for all peaks indicated. Each peak was automatically collected (on the basis of change of slope). One-tenth of each peak was evaporated to dryness *in vacuo*, dissolved in 0.5 mL methanol, and methylated with etheral diazomethane. (The remainder of the material was retained as markers for other work.) These were then run separately on an analytical ODS-2 column (0.4 × 25 cm), with solvents as above, but at 1 mL/min with a shallow gradient changing at 1% per 3 min starting at 38% B for the first three peaks and 45% B for the last peak. The peaks were automatically collected, dried *in vacuo*, transferred with methanol to 100 μL glass tubes held in a larger capped glass vial (Sun Brokers, Wilmington, NC), and taken to dryness under N_2 and then *in vacuo* over P_2O_5.

GC-MS Analysis

Samples of each HPLC peak fraction were trimethylsilated with 5 μL pyridine and 5 μL bis(trimethylsilyl) trifluoroacetamide with 1% trimethyechlorosilane at 60°C for 60 min. GC-MS analyses were done with the Hewelett-Packard (Palo Alto, CA) 5890A gas chromatography connected with a 5970B Mass Selective Detector. Samples (2 μL) were (splitless) injected onto a 25 m × 0.2 mm HP1 (methyl silicone) bonded phase silica capillary column with a helium velocity of 27 cm/s. After injection, the temperature was maintained at 105°C for 1 min, then increased at 30°C min^{-1} to 210°C and then at 4°C min^{-1} to 275°C. Mass spectra were taken from 100 to about 450 mass units (depending on the GA) at *ca.* 1 s per scan.

RESULTS AND DISCUSSION

Thirteen hydroxy GAs can be produced from either [^{14}C]GA_{12} or [^{14}C]GA_{12}-aldehyde, but as [^{14}C]GA_{12} produces only 13-OH GAs in pea shoots, whereas [^{14}C]GA_{12}-aldehyde produces many more products, [^{14}C]GA_{12} is the substrate of preference. Over 15 min to 6 h [^{14}C]GA_{12} is metabolized to several radiolabeled products with retention times of 10, 19.4,

Table I. *GC-MS Identification of Gibberellins in G2 Pea Shoots*

Trimethylsilated Gibberellin	Mol Wt	Retention Times		m/z Characteristic Ions (Relative Abundance)[a]								
		Analytical HPLC	GC	Published spectra		Standard (if available)		^{12}C Spectrum in G2 pea shoots		Representative ^{14}C ions in G2 pea shoots	
		min									
GA_{19}	462	19.4	20.7	434 (100)[b]	281 (34)			434 (100)	281 (23)	450 (13)	
				402 (35)	259 (17)			402 (36)	259 (24)	391 (17)	
				375 (58)	239 (45)			375 (54)	239 (29)	390 (11)	
				374 (60)	207 (72)			374 (61)	207 (35)		
				345 (24)				345 (27)			
GA_{20}	418	20.6	18.9	418 (100)[b]		418 (100)		418 (100)		434 (4)	
				403 (14)		403 (15)		403 (16)		215 (2)	
				375 (45)		375 (65)		375 (70)			
				359 (12)		359 (16)		359 (17)			
				301 (13)		301 (14)		301 (14)			
				207 (31)		207 (25)		207 (22)			
GA_{44}	432	21.2	24.1	432 (83)[c]	208 (47)			432 (66)	208 (37)	448 (10)	
				417 (18)	207 (100)			417 (13)	207 (100)	215 (15)	
				373 (21)	180 (12)			373 (21)	193 (9)		
				251 (9)				251 (7)	180 (11)		
				238 (36)				238 (34)	167 (9)		
GA_{53}	448	25.4	19.0	448 (34)[d],(69)[e]				448 (62)		464 (118)	
				419 (8)	235 (25)			419 (15)	235 (28)	217 (70)	
				416 (9)	209 (43)			416 (22)	209 (50)	216 (138)	
				389 (22)	208 (98)			389 (60)	208 (87)	215 (166)	
				329 (24)[e]	207 (100)			329 (27)	207 (100)		
				251 (30)	193 (23)			251 (35)	193 (30)		
				241 (16)	181 (72)			241 (58)	181 (41)		

[a] Excluding m/z = 73. [b] Ref. 2. [c] Ref. 6. [d] Ref. 1. [e] Ref. 9.

20.6, 21.2, and 25.4 min on a C_{18} analytical column under our conditions (JW Lee, A Halinska, PJ Davies, unpublished results). These metabolites are a subset of the metabolites of GA_{12}-aldehyde (4). The last four peaks have been shown to vary with photoperiod and have been designated G, I, J, K, respectively, in order of increasing polarity (4). These are the peaks studied in this investigation.

By following the radioactivity in the peaks the endogenous materials are copurified (9). GC-MS provided full scan mass spectra of each peak. Peak G is GA_{53}, peak I is GA_{44}, peak J is GA_{20}, peak K is GA_{19} (Table I). These identifications confirm the designations (derived from GC-MS identification on pea seeds [9]) assigned to these peaks in our previous work (4). There was no overlap of GA_{20} into other peaks as detected by Maki *et al.* (9), neither was any GA_{17} detected in the examined peaks, so GA_{17} may be exclusive to pea seeds (5, 8, 9). When the mass spectra were taken, they included the corresponding ^{14}C ions. These showed that the GA_{53} detected was predominantly [^{14}C]GA_{53} (Table II). This is not surprising given the rapid metabolism of GA_{53} (4) (JW Lee, A Halinska, PJ Davies, unpublished results). However, the GA_{53} detected by the mass spectrum had a lower content of ^{14}C than did the original [$^{14}C/^{12}C$]GA_{12} applied to the shoots (63% *versus* 70%, respectively) (15) so that this is a reasonable indication of the presence of endogenous GA_{53} in pea shoots, even though the vast majority of the detected GA_{53} was the product of metabolism of the applied [$^{12}C/^{14}C$]GA_{12}. The proportion of ^{14}C in the GA_{44} and GA_{19} was considerably lower (Table II), and that in GA_{20} was almost entirely diluted out by [^{12}C]GA_{20} from the plant, as might be expected given that GA_{20} is the most prevalent GA in the shoots (5).

The levels of endogenous GA_{20} in G2 shoots as determined by GC-MS with [^{2}H]GA_{20} internal standard is about 42 ng/g (Y-X Zhu, PJ Davies, A Halinska, unpublished results). From the relative amounts of [^{14}C]GAs in the tissue (Table II), the percent of ^{14}C in each GA, and subtracting for any [^{12}C]GA produced from the fed [$^{14}C/^{12}C$]GA_{12}, the endogenous levels of the other GAs are calculated to be about 8 ng/g for GA_{19}, 10 ng/g for GA_{44}, and 0.5 ng/g for GA_{53}. The present and previous work where we found GA_{19}, GA_{20}, GA_{29}, and GA_{29}-catabolite (5) demonstrates that all the GAs of the 13-hydroxy pathway exist in shoots of G2 peas. To our knowledge, this is the first time GA_{53} has been demonstrated in pea shoots, which is not surprising given its low level and rapid metabolism (4). Its detection became feasible only by following the [^{14}C]GA_{53} produced from [^{14}C]GA_{12}. GA_{44} was recently demonstrated in pea shoots by Potts (11). The definitive identification of the endogenous gibberellins represented by HPLC peaks which change in amount during the metabolism of [^{14}C] GA_{12}-aldehyde and [^{14}C]GA_{12} enables further studies on GA metabolism in pea shoots.

Table II. *Percentage of ^{14}C in Supplied [^{14}C]GA_{12} and Its Metabolites in G2 Pea Shoots*

Determined by GC-MS monitoring of the base peak ion, and the amount of radioactivity in each metabolite relative to GA_{20}.

Gibberellin	m/z Ions Monitored	^{14}C	Relative Radioactivity
		%	
GA_{12}	300/316	70	
GA_{53}	207/215	63	127
GA_{44}	207/215	13	67
GA_{19}	434/450	11	61
GA_{20}	418/434	4	100

ACKNOWLEDGMENTS

We thank Paul Birnberg and Sonja Maki for comments on [^{14}C] GA_{12}-aldehdye and [^{14}C]GA_{12} biosynthesis and constructive criticism, and Roger Horgan for providing a preparative HPLC column.

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