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3-EPIGIBBERELLIN A_1: NATURAL OCCURRENCE IN PLANTS AND ARTEFACTUAL FORMATION FROM GIBBERELLIN A_1

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Key Word Index—*Zea mays*; Graminaceae; *Lactuca sativa*; Cruciferae; 3-epiGA_1; endogenous and artefactual; isotope dilution analysis; ^{12}C:^{13}C ratios.

Abstract—Whether 3-epigibberellin(3-epiGA_1) is endogenous in plants or is formed from GA_1 as an artefact, can be determined by adding [^{13}C]GA_1 to the plant material as an internal reference and measuring the ^{12}C:^{13}C ratio of 3-epiGA_1 and GA_1, recovered from the plant extract. We use this method to show that 3-epiGA_1 is not a natural constituent of vegetative tissues of *Zea mays* (maize) but is endogenous in *Lactuca sativa* (lettuce).

INTRODUCTION

3-epiGibberellin A_1 (3-epiGA_1) has been identified by GC-mass spectrometry in extracts of several plant species, e.g. the vegetative tissue of *Agrostemma githago* [1], *Pisum sativum* [2], *Zea mays* [3], *Brassica napus* [4] and *Lactuca sativa* [5–7]. In all cases 3-epiGA_1 was accompanied by the presence of GA_1. Since 3-epiGA_1 can be formed from GA_1 at high pH values and normal temperatures [8], the 3-epiGA_1 identified in extracts from plant material may not be endogenous to the plant but be an artefact formed from GA_1 during extraction, fractionation or GC-mass spectrometry.

As part of the first identification of 3-epiGA_1 from *A. githago*, Jones and Zeevaart [1] provided evidence that 3-epiGA_1 was not formed from GA_1 during extraction or fractionation of the extract and was therefore endogenous. These authors added [^3H]GA_1 to the plant homogenate and showed by TLC of the fractionated extract that there was no radioactivity in the zone at the R_f of 3-epiGA_1. Similarly, Hedden *et al.* [4] added [^3H]GA_1 to an 80% aqueous methanol extract from *B. napus* and found no evidence for the presence of labelled 3-epiGA_1 during solvent and HPLC fractionation.

When GC-mass spectral analysis is used 3-epiGA_1 could also be formed from GA_1 during derivatization or analysis. This possibility was suggested by Rood and Hedden [9]. Working with *B. rapa*, they identified 3-epiGA_4 by GC-mass spectrometry of an HPLC fraction that co-eluted with [^3H]GA_4. When the same HPLC fraction was re-derivatized and re-analysed by GC-mass spectrometry it was found to contain no 3-epiGA_4. Since 3-epiGA_4 would not be expected in the GA_4-containing HPLC fraction, Rood and Hedden suggested that the identified 3-epiGA_4 was formed from GA_4 during GC.

In this paper we describe a method to distinguish between 3-epiGA_1 that is endogenous to the plant and 3-epiGA_1 that is an artefact formed from GA_1 during fractionation, purification or GC-mass spectral analysis of plant extracts. The method uses GA_1, labelled with a stable isotope as an internal reference, followed by the measurement of the content of the stable isotope in the identified 3-epiGA_1 and GA_1. The procedure is illustrated using [17-^3H, ^{13}C]GA_1 as the internal reference. [17-^3H, ^{13}C]GA_1 is added to the plant material, or to the plant homogenate before extraction, in an amount close to the endogenous level of GA_1. 3-epiGA_1 and GA_1 are identified in the purified extracts by GC-mass spectrometry of their MeTMSi derivatives and the ^{12}C and ^{13}C content of both are determined by GC-SIM.

In the present study the GC-SIM analyses were performed using an isotope dilution programme [10] that subtracts the natural abundance of ^{13}C and the results are expressed as a ^{12}C:^{13}C ratio. Three types of ^{12}C:^{13}C ratios are possible: (1) if the ^{12}C:^{13}C ratio of 3-epiGA_1 is 100:0 no isomerization of GA_1 has occurred. All the identified 3-epiGA_1 is endogenous, i.e. 3-epiGA_1 is naturally occurring in the plant and is not an artefact of the methodology; (2) if the ^{12}C:^{13}C ratio of 3-epiGA_1 is not 100:0 and is the same as that of the recovered GA_1 then the identified 3-epiGA_1 is an artefact formed entirely from GA_1 as a result of the methodology; and (3) if the ^{12}C:^{13}C ratio of 3-epiGA_1 is between 100:0 and the ^{12}C:^{13}C ratio of the recovered GA_1 then the identified 3-epiGA_1 is a mixture of endogenous and artefactual 3-epiGA_1.

Using this method, the status of 3-epiGA_1 as an endogenous GA in vegetative tissue of maize of maize and lettuce has been examined.

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RESULTS AND DISCUSSION

Maize

Phinney *et al.* [11] reported the presence and levels of GA_1 in the immature and mature stems and immature leaf sheaths of normal (+/+) maize; [17-^{3}H, ^{13}C]GA_1 was used as an internal standard and the ^{12}C:^{13}C ratios of the recovered GA_1 were published in that paper. We report here additional unpublished data from these studies on the detection and the ^{12}C:^{13}C ratios of 3-epiGA_1 from the three kinds of plant material (Table 1). Since the ^{12}C:^{13}C ratio of 3-epiGA_1 was close to the ^{12}C:^{13}C ratio of the recovered GA_1 for each kind of plant material, we conclude that the identified 3-epiGA_1 is an artefact. The fit factors are a measure of the reliability of the data.

The same conclusion that 3-epiGA_1 does not occur naturally in seedling shoots of maize can now be drawn from data, previously published by Fujioka *et al.* [12]. The data, shown in Table IV of that paper, had not been interpreted in terms of the origin of the identified 3-epiGA_1. In the paper by Fujioka *et al.* [12], 5-week-old normal (+/+), heterozygous Dominant dwarf (+/*D*8) and homozygous Dominant dwarf (*D*8/*D*8) maize plants were injected with [^{3}H, ^{13}C]GA_1. The seedlings were extracted 12 hr later and the ^{12}C:^{13}C ratio of 3-epiGA_1, GA_1 and GA_8 were determined by GC-SIM. Since the reported ^{12}C:^{13}C ratio of 3-epiGA_1 is very similar to the ^{12}C:^{13}C ratio of GA_1 for each kind of plant material we conclude that the identified 3-epiGA_1 was artefactual in origin.

Since the *dwarf*-1 (*d*1) mutant of maize contains very low levels of GA_1 [13], we have determined the ^{12}C:^{13}C ratio of 3-epiGA_1 and the ^{12}C:^{13}C ratio of GA_1 following feeds of [^{3}H, ^{13}C]GA_1 to the mutant. The experiment was conducted to look for the presence of 3-epiGA_1, in the absence of GA_1. *Dwarf*-1 seedlings were injected with [^{3}H, ^{13}C]GA_1 and the shoots were harvested 0, 2, 4 and 6 hr later. As a control [^{3}H, ^{13}C]GA_1 was added to untreated seedlings after homogenization in methanol–water (4:1). The ^{12}C:^{13}C ratio (Table 2), determined by GC-SIM, for 3-epiGA_1 and for GA_1 is very similar for each treatment. These results show that 3-epiGA_1 is artefactual, originating from the added GA_1.

In conclusion these isotope dilution data for (+ / +), (+ /*D*8), (*D*8/*D*8) and (+ /*d*1) maize genotypes show that 3-epiGA_1 does not occur naturally in shoots of maize. Its identification from maize shoots is the consequence of its artefactual origin from GA_1 at some stage in the analysis.

Lettuce

In a previous publication by Waycott *et al.* [5], GA_1 and 3-epiGA_1 were identified by GC-mass spectrometry in vegetative tissue of an early flowering cultivar, E-1, of lettuce and three GA-responding dwarfs, *dwf*1, *dwf*2 and *dwf*2^i. GA_1 was quantified by GC-SIM using [^{3}H, ^{13}C]GA_1 as an internal standard. Their data (Table II) were not analysed in terms of the origin of the identified 3-epiGA_1. This we now do and Table 3 shows the ^{12}C:^{13}C ratios for 3-epiGA_1 and GA_1 together with the fit factors and relative amounts. For both E-1 and *dwf*2^i the ^{12}C:^{13}C ratios for 3-epiGA_1 are close to 100:0; thus most of the identified 3-epiGA_1 is unlabelled and, therefore, endogenous in origin. For *dwf*2 the ^{12}C:^{13}C ratio of 3-epiGA_1 is intermediate between 100:0 and that of the recovered GA_1; thus the 3-epiGA_1 identified in extracts of *dwf*2 is both endogenous and artefactual in origin. Toyomasu *et al.* [14] have also shown that 3-epiGA_1 is endogenous in lettuce shoots by adding [2,2,3,6-$^2H_4$]GA_1 and showing that the identified 3-epiGA_1 contained no label.

In summary, the use of GA_1, labelled with a stable isotope, as an internal reference and a comparison of the stable isotope content in the recovered 3-epiGA_1 and GA_1 is a useful method to distinguish between endogenous and artefactual 3-epiGA_1, obtained from plant extracts. The data presented here show that 3-epiGA_1 is not endogenous in shoots of maize and is endogenous in vegetative tissue of lettuce. However, the method does not provide information on the specific stage in the isolation or identification procedures at which 3-epiGA_1 is formed as an artefact. The amount of 3-epiGA_1, formed from GA_1, is variable; for example the ratio of 3-epiGA_1 to GA_1 from *dwarf*-1 maize plants range from 2:1 to 1:16 (Table 2). This variability, and the difference in ratios of the amounts of 3-epiGA_1 formed from GA_1 from the

Table 1. Isotope dilution analysis by GC-SIM of the molecular ion cluster of the MeTMSi derivatives of GA_1 and 3-epiGA_1 from immature and mature stems and immature leaf sheaths of normal maize plants (the data for GA_1 are from Phinney *et al.* [11])

Sample	[^{13}C]GA	Relative amounts	^{12}C	^{13}C	Fit factor
Mature stem	GA_1	14.56	52.5	47.5	0.9961
	3-epiGA_1	1.00	49.8	50.1	0.9920
Immature stem	GA_1	3.04	63.6	36.4	0.9945
	3-epiGA_1	1.00	62.1	37.9	0.9900
Immature leaf sheath	GA_1	1.24	79.0	21.0	0.9950
	3-epiGA_1	1.00	77.3	22.7	0.9983

Table 2. Isotope dilution analysis by GC-SIM of the molecular ion cluster of the MeTMSi derivatives of GA_1 and 3-epiGA_1 from shoots of *dwarf*-1 maize plants treated with [17-^{13}C, ^{3}H]GA_1

Sample	[^{13}C]GA	Relative amounts	^{12}C	^{13}C	Fit factor
Homogenate	GA_1	1.05	8.8	91.2	0.9961
	3-epiGA_1	1.00	8.4	91.6	0.9948
Feed, 0 hr	GA_1	0.55	8.7	91.3	0.9923
	3-epiGA_1	1.00	8.4	91.6	0.9862
Feed, 2 hr	GA_1	10.02	8.4	91.6	0.9945
	3-epiGA_1	1.00	9.5	90.5	0.9901
Feed, 4 hr	GA_1	3.67	8.6	91.4	0.9929
	3-epiGA_1	1.00	8.7	91.3	0.9904
Feed, 6 hr	GA_1	16.05	8.7	91.3	0.9909
	3-epiGA_1	1.00	9.0	91.0	0.9908

Table 3. Isotope dilution analysis by GC-SIM of the molecular ion cluster of the MeTMSi derivatives of GA_1 and 3-epiGA_1 from lettuce (data derived from Waycott *et al.* [5])

Sample	[^{13}C]GA	Relative amounts	^{12}C	^{13}C	Fit factor
E-1*	GA_1	3.40	52.5	47.5	0.9909
	3-epiGA_1	1.00	97.7	2.3	0.9881
*dwf*2	GA_1	19.09	31.2	68.8	0.9936
	3-epiGA_1	1.00	77.0	23.0	0.9657
*dwf*2^{i}	GA_1	9.19	34.2	65.8	0.9934
	3-epiGA_1	1.00	90.4	9.6	0.9838

*Average of three determinations.

equilibrium ratio of 3:1 [8], support the previous evidence [3, 4] that 3-epiGA_1 is not formed during extraction or fractionation of the extract. 3-epiGA_1 may be formed from GA_1 during GC injection in GC-mass spectral analysis [9]; this possibility requires further investigation.

EXPERIMENTAL

[17-^{3}H, ^{13}C]GA_1 (0.915 atoms ^{13}C molecule^{-1}, 2.11 GBq mmol^{-1}) was prepared by Fujioki *et al.* [13].

Plant material. Maize *dwarf*-1 plants came from a selfed stock that is heterozygous for the *d*1 mutant. The origin of the stock is described in ref. [13]. Seeds were planted and grown in the UCLA greenhouse. Thirty-day-old plants were used.

Treatment, incubation, extraction and purification. Twelve *dwarf*-1 plants were each injected at the base of the stem with [17-^{13}C, ^{3}H]GA_1 (0.52 μg; 3.13×10^3 Bq), dissolved in EtOH–H_2O (1:1, 10 μl). Three of the treated plants were harvested at 0, 2, 4 and 6 hr after injection. The shoots were excised from the roots (discarded), frozen immediately on solid CO_2 and then homogenized in MeOH–H_2O (4:1). In addition, as a control, the shoots from three untreated seedlings were frozen and homogenized and [17-^{13}C, ^{3}H]GA_1 (1.56 μg; 9.4×10^3 Bq) was added to the homogenate. All homogenates were purified as described in ref. [13]. Radioactive frs from HPLC were methylated, trimethylsilylated and analysed by GC-MS. The conditions for GC-SIM and the determination of ^{12}C:^{13}C ratios by the isotope dilution fit programme were the same as those described in Method 2 by Croker *et al.* [10]. All $[M]^+$ ion clusters had the correct Kovats Retention Indices.

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