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Identification and Quantitative Analysis of Gibberellins in *Citrus*

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Abstract. Nine gibberellins (GAs) have been identified from tissues of Valencia orange (*Citrus sinensis* Osbeck) using gas chromatography–mass spectrometry and gas chromatography-selected ion monitoring of high-performance liquid chromatography (HPLC)-fractionated extracts. These GAs are GA_1, GA_3, GA_8, GA_{19}, GA_{20}, GA_{29}, 3-epi-GA_1, 2-epi-GA_{29}, and iso-GA_3. Selected-ion monitoring and stable-isotope dilution assays have been used to estimate levels of some of these GAs in vegetative and reproductive tissues. GA_{29} was found to be the most abundant GA measured. GA_1 was found in all samples examined, and there was always less 3-epi-GA_1 than GA_1. GA_{20} was present in most extracts. Leaves of developing inflorescence shoots contained six times more GA_{29} than did leaves of comparable vegetative shoots. Levels of GA_{29} increased during the early stages of fruit development. GA_{20} may be more abundant in growing fruitlets than in those about to abscise; however, there were no consistent differences in the relative amounts of the other GAs. No major differences were found between tissues of immature seeded and seedless fruit, and developing seeds did not contain high levels of any of the GAs measured. It is concluded that seed-produced GAs are not essential for normal fruit development in Valencia orange.

The genus *Citrus* includes several major fruit species including orange, mandarin, grapefruit, lemon, and lime. Despite the worldwide economic importance of these crops (and hence a substantial quantity of research), there are still large gaps in our understanding of the various physiological mechanisms that may directly or indirectly influence yield. There has been great interest in the interaction of plant and environment, and many attempts have been made, some successful, to alter plant responses using methods such as chemical manipulation with applied growth regulators. There is strong evidence that plant growth substances are involved in the control of many developmental processes in higher plants. In *Citrus*, there is already some information on the role

of gibberellins (GAs) in shoot extension (Muller and Young 1982; Aron et al. 1985; Randhawa and Singh 1986), flower induction (Monselise et al. 1981; Garçia-Luis et al. 1986; Lord and Eckard 1987), and fruit retention (Moss 1970, 1972; Southwick and Davies 1982; Agusti et al. 1982; Turnbull 1988). However, many of these reports describe responses to applications of growth substances to whole plants. Experiments of this type are difficult to interpret, partly because the physiology of woody perennials is inevitably more complex than that of herbaceous plants, and partly because of problems in controlling the uptake, transport, and metabolism of the applied growth substance. Thus, it is difficult to restrict the response(s) to the organ of interest. In addition, responses to applied compounds often vary considerably, depending on the species, variety, and geographical location.

One alternative is to study the endogenous growth substances. The essential first step here is to provide accurate data on the types and amounts of these compounds present throughout the plant. Although indoleacetic acid (Igoshi et al. 1971; Goldschmidt 1976) and abscisic acid (Goldschmidt et al. 1973; Goldschmidt 1976) have been conclusively identified from *Citrus* tissues, information on GAs and cytokinins is very sparse. In fact, only one definitive report exists of GA identification in *Citrus unshiu* by infra-red spectroscopy (Kawarada and Sumiki 1959). The present paper reports on the identification and quantification of several GAs from Valencia orange, using reliable and highly sensitive physicochemical methods.

Materials and Methods

Plant Material

Valencia orange (*Citrus sinensis* Osbeck) was the source of plant material for all experiments. Rooted cuttings and grafted trees (on Troyer Citrange rootstocks) were grown in pots in glasshouses at the Adelaide Laboratories of the Division. Liquid fertilizer and/or slow-release fertilizer granules were supplied at regular intervals. Average temperatures in the glasshouse were 28°C during the day and 14°C at night through the summer (November–April), and 22°C during the day and 9°C at night through the winter (May–October). Expanding leaves, developing fruit and seeds were sampled. Mature Valencia orange was obtained from Berrivale Orchards Ltd. (Adelaide, Australia). Material for extraction was excised and frozen in liquid N_2, and stored at -20°C or -70°C until required.

Chemicals

[1,2-^{3}H]GA_1 (1.21×10^{12} Bq mmol^{-1}) was obtained from Amersham Australia Pty. Ltd. (Sydney, Australia). [2,3-^{3}H]GA_9 (1.7×10^{12} Bq mmol^{-1}) was a gift from Dr. A. Crozier, University of Glasgow, UK. GA_3 was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and GA_1 was synthesized from GA_3 using a modification of the method by Nadeau and Rappaport (1974). Sev-

eral other unlabeled GA standards were gifts from Professor N. Takahashi, University of Tokyo, Japan. [17-d_2]GA analogues of GA_1 and GA_{20} were purchased from Professor L. Mander, Australian National University, Canberra, Australia. The d_0 (unlabeled) contents of these standards were estimated from mass spectra to be 0.8% for GA_1 and 1.4% for GA_{20}.

Extraction

Tissue was homogenized either in a Waring blender or with a Polytron tissue disintegrator together with cold 80% methanol (10 ml/g fresh weight) and internal standards, if used ([^{3}H]GA_1 and [^{3}H]GA_9, 1670 Bq each and [d_2]GA_1 and [d_2]GA_{20}, 2 ng each). For GA identification, sample fresh weights were as follows: mature seeds, 100 g; mature leaves, 61 g; immature leaves, 3.6 g and 6.2 g; fruitlets, 13 g; immature fruit 30.3 g. Homogenization was continued for 1–2 min, and the extract was then cleared by centrifugation (1000 *g* for 5 min) or filtration. Insoluble material was reextracted with 80% methanol. The combined extracts were reduced to an aqueous residue by rotary evaporation at 40°C. Chlorophyll and other lipid material were removed by one of two methods, either freezing to −20°C followed by thawing and centrifugation at 12,000 *g* for 20 min, or adjusting the extract to pH 8.0 with NaOH and extracting four times with a one half volume of petroleum (40–60°C boiling range). The aqueous phase was then passed through a 5-ml column of insoluble polyvinylpolypyrrolidone (PVP) and eluted with 20 ml H_2O adjusted to pH 7. The eluate was evaporated further, then adjusted to pH 2.8 with 1 N HCl, and extracted five times with a one half volume of ethyl acetate. The ethyl acetate phase was washed with a one tenth volume of H_2O to remove excess acid, then rotary evaporated to dryness. Some larger extracts were then dissolved in 2 ml 0.05 M Tris-HCl buffer, pH 8.0, and passed through a column of DEAE-Sephacel equilibrated in the same buffer. The column was sequentially eluted with 60 ml buffer, 60 ml methanol, and 90 ml 1 M acetic acid in methanol. As judged by recovery of [^{3}H]GA standards, all GAs eluted in the first 30 ml of the acetic acid fraction, which was then evaporated to dryness. All extracts were methylated with a solution of diazomethane in diethyl ether, prior to high-performance liquid chromatography (HPLC).

HPLC

The HPLC system was comprised of a Varian 5020 ternary gradient pumping system, a Varian Varichrom VUV10 UV detector, and a Spectra Physics SP4100 computing integrator. The column used was a Shandon ODS 5-μm Hypersil, 250 × 4.6 mm. Mobile phase, pumped at 1 ml min^{-1} was a gradient of methanol in H_2O: either 20–100% methanol over 20 min, then 100% for 5 min; or 30% methanol for 4 min, 30–45% over 10 min, 45–100% over 10 min, 100% for 5 min. Methylated samples were dissolved in 900 μl of initial mobile phase, centrifuged at 10,000 *g* for 1 min, then injected via a Valco pneumatic injector fitted with a 1-ml sample loop. Fractions of 600 μl were collected from 6–30

min and evaporated to dryness in a Savant centrifugal sample concentrator. Retention times of standards were determined by monitoring UV absorbance at 206 nm or by an on-line radioactivity monitor. For the latter purpose, the eluate was directed, after mixing with Packard Picofluor 30 scintillant (3 ml min^{-1}), to a Berthold LB504 radioactivity detector equipped with a 1-ml spiral PTFE flow-cell.

Gas Chromatography–Mass Spectrometry (GC-MS)

Dry HPLC fractions were derivatized with N-methyl-N-trimethylsilyltrifluoroacetanide (MSTFA) (3 μl) in sealed tubes at 60°C for 45 min. GC-MS and GC-selected ion monitoring (GC-SIM) were carried out on a Hewlett-Packard 5970B mass selective detector. Columns used were BP-1 (19 m × 0.22 mm i.d.) or DB-1 (30 m × 0.25 mm i.d.). Samples (0.4 μl) were injected on-column with a S.G.E. OCI-3 injector. The Helium carrier gas flow rate was nominally 1 ml min^{-1}. Temperature programs for BP-1 were 80°C for 1 min, then 20° min^{-1} to 220°C, then 4° min^{-1} to 280°C; for DB-1: 50°C for 1 min, then 20° min^{-1} to 250°C, then 4° min^{-1} to 280°C. The electron multiplier was set at 1400 or 1600 V. In full scan mode, scans were set at approximately 2 Hz. In SIM mode, up to 20 ions could be monitored. For a "scan" rate of 2 Hz, this gave ion dwell times of 20 ms. Data were recorded and processed on a Hewlett-Packard 59970A Workstation. Kovats retention indices were calculated using the formula of Kovats (1958). Source of n-alkanes (C_{20}–C_{32}) was "Parafilm" dissolved in hexane (Gaskin et al. 1971).

[17-d_2]GA_1 and [17-d_2]GA_{20} were used as internal standards to quantify the levels of these GAs in extracts. The recovery of [^{3}H]GA_1 was calculated after the HPLC stage. No internal standards were available for epi-GA_1 and GA_{29}, so detector responses relative to that of GA_1 were estimated using external standards. Thus, the levels of GA_1 calculated from the d_2 isotope dilution method were used as the base for estimation of these GAs. Therefore, values given for epi-GA_1 and GA_{29} were not as accurate as for GA_1 and GA_{20}, but allow comparison between samples.

Results and Discussion

Several different parts of the plant were used as sources of GA, including young expanding leaves, newly mature leaves, and fruits and seeds at various stages of development. The extent of purification required for detection of GAs depended on the type of tissue, but the limiting factors for all extracts were the sample capacities of HPLC and capillary GC columns, and of the mass selective detector. Many of the GAs were present in very low amounts, estimated to be well below 1 ng (g fresh wt)$^{-1}$. Therefore, full scan mass spectra could be recorded only for the more abundant GAs. For the others, the selected ion monitoring (SIM) mode was used, allowing detection of up to 20 ions, which with a dwell time of 20 ms, gave a net "scan" rate of 2 Hz. This resulted in several spectra for each GC peak, which were then averaged and background subtracted.

Eight different GAs were identified using the above GC-SIM and GC-MS methods. These GAs were GA_1, GA_3, GA_8, GA_{19}, GA_{20}, GA_{29}, 3-epi-GA_1, 2-epi-GA_{29}, and iso-GA_3. Of these, full scan spectra were obtained only for GA_3, GA_{29}, and iso-GA_3. Standards were run for all GAs except epi-GA_{29} and iso-GA_3. In these cases identification was based on GC-MS from other sources (Moore et al. 1986; Gaskin et al. 1985; P. Hedden, personal communication). All GAs were detected in both vegetative and reproductive tissues, except GA_{19} which was found only in fruits and seeds. The principal characteristic ions and Kovats Retention Indices (KRI) of these GAs are summarized in Table 1. Figure 1 shows a typical ion chromatogram, in this case at m/z 506, the molecular ion of GA_1, GA_{29}, and their epimers.

All GAs were 13-hydroxylated and it is therefore likely that, in this *Citrus* species at least, one biosynthetic route was via the so-called "early 13-hydroxylation" pathway (Sponsel 1983). The probable metabolic relationship of the GAs found was known from pathways in other species [e.g., *Zea mays* (Spray et al. 1984; Phinney et al. 1986), *Pisum sativum* (Kamiya and Graebe 1983; Ingram et al. 1984] and is illustrated in Fig. 2. Definitive data on the precursors of GA_3 and iso-GA_3 in higher plants are not yet available. There are also alternative routes for production of C_{19}-GAs, but until information is available on the identity of C_{20}-GAs other than GA_{19} in *Citrus* and on the metabolic pathways present, it is not possible to say which biosynthetic routes predominate. The same is true of the late stages of the pathway(s) which may involve GA glucosyl conjugates, none of which have yet been detected in *Citrus*.

Of the GAs found, not all will be biologically active. Based on data previously established for a number of GA bioassay systems (Reeve and Crozier 1975), 2β-hydroxylated GAs are generally inactive. This includes GA_8 and GA_{29} in *citrus*. Epi-GA_{29}, with a 2α-HO group may also be in this category. GA_1 and GA_3 are almost invariably very active, whereas activity of GA_{19} and GA_{20} depends on the test system used. These two GAs are thought to achieve activity, at least in shoot tissues, only after metabolic conversion through to compounds such as GA_1 (Phinney et al. 1986). The activity of epi-GA_1 is not known, but may be quite high due to its structural similarity to GA_1. Because epi-GA_1 is not a common endogenous GA, the possibility is considered that it is an artifact formed from GA_1 during the extraction procedure. However, this has been ruled out by examination of mass spectra from samples where [d_2]GA_1 was added as an internal standard. In these spectra, no enrichment of the d_2 ions of epi-GA_1 was ever seen. Typical peak area ratios of m/z 506/508 for (GA_1 + [d_2]GA_1) were 2159:5334, and for epi-GA_1, 1025:158. With a detection limit of 50 area units, a conversion of only 1% of [d_2]GA_1 to [d_2]epi-GA_1 would still have been detected as an increase in m/z 508 in epi-GA_1. Thus, it can be concluded that no such conversion occurred, and the same is likely to be true for native GA_1 and epi-GA_1. As for iso-GA_3, the possibility does remain that it was formed from GA_3 during extraction, especially as it was only found in samples where GA_3 was also present. Although samples here were never exposed to temperatures >40°C, iso-GA_3 is the major breakdown product of autoclaved GA_3 (Pryce 1973). There is also the possibility that GA_3 is a contaminating compound from other sources in the laboratory. However, precautions were taken to exclude all GAs from the glasshouse in which the plants

Table 1. Principal gas chromatography–mass spectrometry (GC-MS) characteristics of GAs identified from "Valencia" orange.

GA	Source	Characteristic ions in mass spectra M/z (relative intensity)											KRI[a]
GA_1	Seeds	313 (11)	375 (14)	376 (24)	377 (20)	447 (11)	448 (19)	491 (10)	506 (100)				2680
epi-GA_1	Seeds	313 (9)	375 (19)	376 (33)	377 (26)	447 (15)	448 (40)	491 (8)	506 (100)				2797
GA_3	Fruitlets	207 (13)	208 (30)	221 (4)	223 (2)	347 (14)	355 (5)	369 (8)	370 (10)	445 (7)	489 (8)	504 (100)	2716[b]
iso-GA_3	Fruitlets	207 (6)	208 (3)	221 (10)	223 (11)	355 (9)	369 (14)	370 (8)	445 (13)	475 (15)	489 (9)	504 (100)	2662[b]
GA_8	Leaves, fruitlets	238 (28)	375 (24)	376 (13)	448 (25)	535 (7)	579 (5)	594 (100)					2821
GA_{19}	Seeds	239 (44)	285 (22)	312 (10)	344 (14)	374 (65)	375 (65)	402 (46)	434 (100)	462 (4)			2621
GA_{20}	Seeds, leaves	207 (36)	301 (17)	359 (17)	375 (69)	403 (13)	418 (100)						2497
GA_{29}	Seeds, leaves	303 (36)	375 (18)	389 (12)	447 (11)	491 (12)	506 (100)						2696
epi-GA_{29}	Seeds	303 (43)	375 (19)	389 (15)	447 (9)	491 (11)	506 (100)						2684

[a] Kovats Retention Index. GC column was BP-1 except where marked ([b]), when DB-1 was used.

were grown, and glassware used for extraction was routinely washed in a chromic acid bath.

Having established the identity of several of the GAs in *Citrus*, the next stage was to make accurate measurements of the changes in levels of these compounds in relation to developmental processes such as shoot growth, fruit set, and fruit drop. Table 2 gives the levels of GA_1, epi-GA_1, GA_{20}, and GA_{29} found in a range of organs.

The first point to note is that most of the levels measured were extremely low, often equivalent to <1 ng (g fresh wt)$^{-1}$, even in actively growing tissues. Presumably, this is simply a characteristic of *Citrus*. Young expanding leaves are known as a source of GAs so leaves from vegetative and reproductive shoots were compared. The shoot lengths were similar but at that stage of development, inflorescence leaves were twice the size of ones on vegetative shoots. No significance can be attached to the small difference in GA_1 levels; however, there was approximately six times more GA_{29} in the inflorescence leaves (Table 2). It might have been expected that since GAs are known to repress floral initiation (Monselise et al. 1981), GA levels in inflorescence shoots would be lower. However, the floral determination events took place in these shoots several weeks before this sampling and therefore any differences could well have disappeared. In any case, GA_{29} is not normally a biologically active GA.

In the early stages of fruit development, GA_{29} accumulated to high levels in both growing and abscising fruitlets. Between fresh weights of approximately 0.05 and 0.5 g, there was a 10- to 20-fold increase in this GA (Table 2). There is some indication that GA_{20} levels were higher in actively growing fruits than in ones that were about to abscise, whereas GA_1 levels remained relatively constant. GA_1 and GA_{29} are both direct products of GA_{20} metabolism (see Fig. 2) but because GA_{29} is biologically inactive, changes in its level do not directly affect the amount of active GA in the tissue. Any differences between GAs in growing and abscising fruits are in fact difficult to assess because the samples

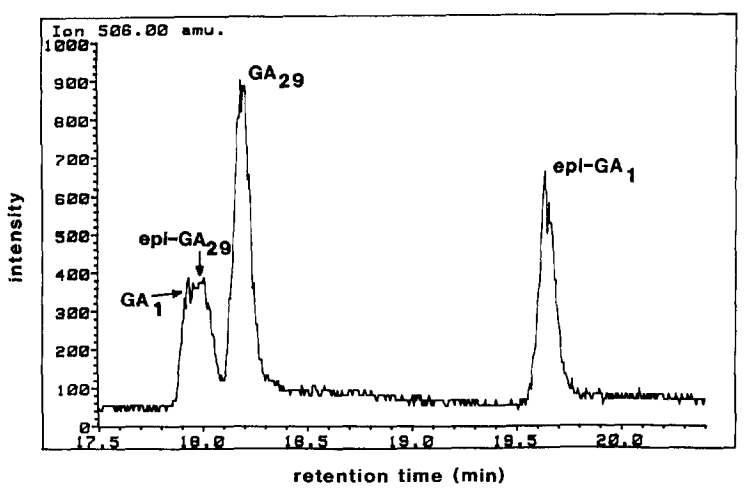

Fig. 1. Ion chromatogram of m/z 506 from GC-SIM analysis of GA_1/GA_{29} zone from HPLC fractionation of extract of mature seed of Valencia orange.

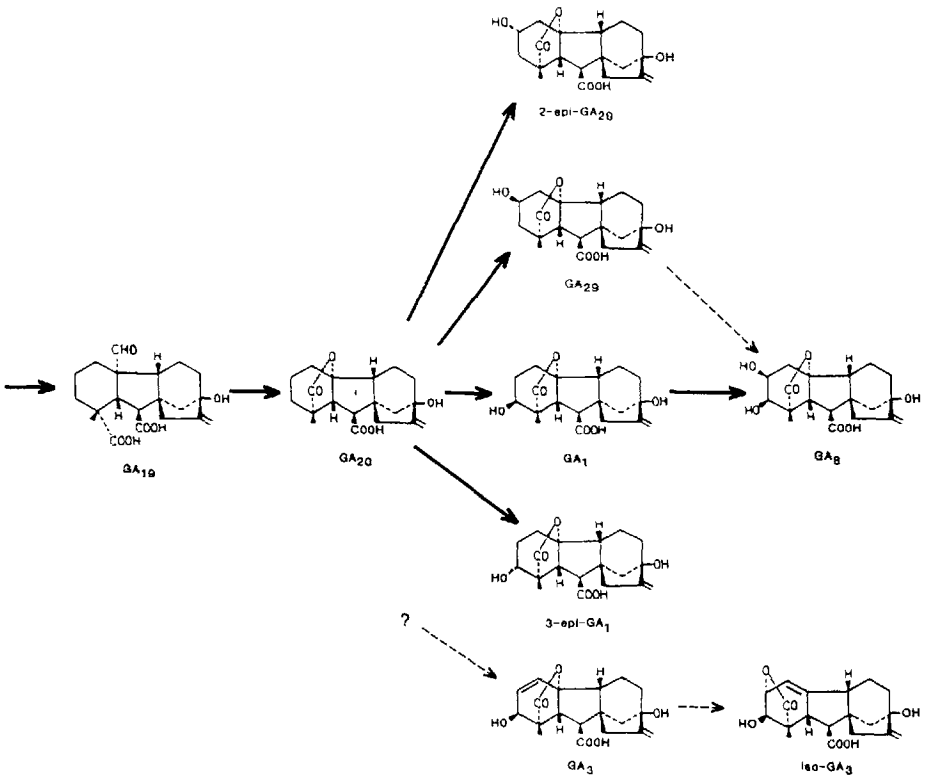

Fig. 2. Probable metabolic relationship of GAs identified from Valencia orange. Metabolic sequences were taken from data published for *Pisum sativum* and *Zea mays*. →, known metabolic route; - - ->, other possible conversions.

were necessarily taken after the start of the abscission process (slowing down of growth rate, etc.) and therefore changes in GA levels could be the effect rather than the cause. However, further data has come from experiments with applied GA_3 and paclobutrazol and measurements of endogenous GA_3 levels by radioimmunoassay, which showed a close relationship between GA content and fruit retention (Turnbull 1988). It may therefore be that certain GAs are involved in the fruit retention process, whereas others are not. This is the subject of further investigations.

There were no significant differences in GA levels from immature seeded and seedless fruit taken from a single tree (Table 2). The relative proportions of GAs in the seeds were somewhat different but the absolute amounts were not much higher than those in fruit tissues. Since the seeds represent less than 1% of the total fruit volume, they probably contribute only a minute proportion of the total GA present. This is in contrast to the massive amounts of GAs found in immature seed of many other species, particularly legumes (Crozier 1985). There remains the possibility that the seeds have high rates of GA biosynthesis and export which could result in a low steady-state level. However, in this variety of orange at least, the fact that trees can produce seeded and seedless

Table 2. Gibberellin levels in a range of organs of Valencia orange[a].

Source	Mean fresh wt per organ (g)	GA level (pmol [g fresh wt]$^{-1}$)			
		GA_1	epi-GA_1	GA_{20}	GA_{29}
Expanding leaves					
Vegetative shoot	0.084	2.07	0.98	3.24	6.4
Inflorescence shoot	0.164	3.48	1.26	3.05	38.6
Fruitlets					
Healthy, growing	0.047	1.90	0.64	1.56	1.2
	0.059	0.89	ND	NA	2.1
	0.109	0.72	ND	NA	12.6
	0.468	1.25	0.29	5.87	15.8
Nearly abscised	0.048	<0.6	ND	NA	0.7
	0.511	1.50	0.44	0.69	17.3
Fruits					
Immature					
Seedless	30.3	0.85	0.45	1.02	5.9
Seeded	30.3	1.07	0.50	1.01	7.5
Seeds from above	0.079	0.34	0.29	2.35	17.9

ND, not detected; NA, not assayed.

[a] Measurements of GA_1 and GA_{20} were made by GC-SIM using [d_2]GA internal standards and a stable-isotope dilution assay. Epi-GA_1 and GA_{29} were estimated by comparison of detector responses with that of GA_1 in the same sample.

fruit does suggest that fruit growth and retention are independent of seeds and seed-produced GAs.

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