Dormancy in Peach (*Prunus persica* L.) Flower Buds[1]

I. Floral Morphogenesis and Endogenous Gibberellins at the End of the Dormancy Period

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ABSTRACT

Flower buds of peach (*Prunus persica* L.) trees, cv Novedad de Cordoba (Argentina), were collected near the end of the dormant period and immediately before anthesis. After removal of scale leaves, morphological observations of representative buds, made on transverse and longitudinal microtome sections, showed that all verticils making up the flower are present in an undifferentiated form during the dormant period (June). Flower buds collected at the end of dormant period (August) showed additional growth and differentiation, at which time formation of two ovules was beginning in the unicarpelar gynoecium. Dehiscence of anthers had not yet occurred 10 days before full bloom, and the ovules were still developing. Free endogenous gibberellin (GA)-like substances were quantified by bioassay (Tan-ginbozu dwarf rice microdrop) after SiO_2 partition column chromatography, reversed phase C18-high performance liquid chromatography, and finally Nucleosil [$N(CH_3)_2$]high performance liquid chromatography. Bioactive fractions were then subjected to capillary gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM). Gibberellins A_1, A_3, and A_8 were tentatively identified in peach flower buds using GC-SIM and Kovat's retention indices, and relative amounts approximated by GC-SIM (2:8:6 for GA_1, GA_3, and GA_8, respectively). The highest concentration (330 nanograms per gram dry weight) of free GA_1/GA_3 was found in dormant buds (June) and diminished thereafter. The concentration free of GA_1/GA_3 did not increase immediately prior to bud break. However, high GA_1/GA_3 concentrations occurred during stages where rate of growth and cellular differentiation of (mainly fertile) verticils can be influenced.

Flowering is generally divided into two major steps: (a) the initiation of flower primordia and (b) the differentiation and development of these primordia into mature flowers that undergo anthesis. A clear distinction should be made between the various stages since they are not morphologically alike and may not react similarly to environmental and internal variables (4, 5, 9, 18).

Gibberellins are the PGRs[2] most likely to be causally implicated in at least several aspects of the flowering process in Gymnosperms and many Angiosperms, including woody perennials (22, 23, 27). However, changes in endogenous PGRs during floral development remain essentially unknown, especially for woody angiosperms. Similarly, the possible changes in meristem 'sensitivity' to these substances, as a function of the floral development stage, have received little attention in the past (3, 4, 9).

Dormancy can be considered as the period in which the growth of a differentiated organ is partially delayed. This assessment has been applied to both seeds and vegetative buds, although little is known about the relationship between the development of floral primordia and the dormancy status. Moreover, possible correlations of the various PGRs (mainly GAs) with floral bud dormancy and anthesis remain confused (23), and are in need of reexamination using unambiguous analytical techniques.

However, changes in PGRs during dormancy of floral buds of peach trees has been examined using bioassay (7, 11, and references therein). During peach flower bud dormancy there is an increase of GA-like substances (assayed by the barley α-amylase half seed bioassay) and a slight decrease of growth inhibitors (assayed by the wheat coleptile test) (7, 11). Unfortunately, these studies are flawed by a limited methodology for separation, identification and quantification of the PGRs.

Herein we examine the morphology of floral organogenesis in peach flower buds toward the end of the dormancy period, and investigate possible relationships between the morphological changes and endogenous levels of free GAs and putative GA conjugates.

MATERIALS AND METHODS

Plant Material

Flower buds were detached from year one limbs of peach (*Prunus persica* L.) trees, cv Novedad de Córdoba (8 years old), in an orchard at Rio Cuarto, Argentina. Collections were made on June 18 (middle of dormancy period), July 18 (end

[2] Abbreviations: PGR, plant growth regulator; AcOH, acetic acid; EtOH, ethanol; EtoAc, ethyl acetate; GA, gibberellin(s); GC-SIM, gas chromatography-mass spectrometry-selected ion monitoring; KRI, Kovats' retention index; MeOH, methanol; MeTMSi, methyl ester of trimethylsilyl ether derivative.

of bud dormancy), and August 20, 1984 (just prior to anthesis). Buds for GA extraction were weighed, immediately frozen in liquid N_2, and freeze-dried. Buds for morphological observations were stored in FAA (formaldehyde:AcOH: EtOH:H_2O; 30:5:50:15, v/v).

Morphological Studies

To prepare buds for optical microscopy, the scale leaves were removed prior to dehydration of buds in a mixture of EtOH:Xylol at varying concentrations (16), and imbedding in paraffin. A series of transverse and longitudinal microtome sections 13 μm thick were obtained and triple-stained with hematoxilin, safranin, and fast green (16). The microtome sections were examined using Zeiss optical microscopy and schematic drawings were made.

Extraction Procedure for Gibberellins

After removal of bud scales, the equivalent of 1 g dry weight of peach flower buds was homogenized with 40 mL 80% MeOH and liquid N_2 in a mortar and pestle. The supernatant was removed and the residue reextracted with an extra 20 mL of solvent. The supernatants were pooled, filtered and passed through a preparative C_{18} column (19) at a flow rate of 2 to 4 mL min^{-1}. After adjusting the eluate to 50% MeOH with distilled H_2O, it was passed through a second preparative C_{18} column. The column was washed with additional 20 mL of 40% MeOH. The MeOH was evaporated in vacuo at 35° C, and the aqueous residue was frozen, and then freeze-dried. This final eluate will contain most of the free and putative GA conjugates (19).

SiO₂ Partition Column Chromatography

The residue containing free and putative GA conjugates was loaded onto a 'short' SiO_2 partition column (19) by using 0.5 g of celite (19). It was eluted first with a mixture of EtOAc:n-hexane (95:5) and then with 100% MeOH in order to separate the free highly EtOAc-soluble GAs from putative GA glucosyl conjugates and very polar free GAs, such as GA_{32} (8, 19). The MeOH fraction containing the highly water soluble substances was neutralized to pH 7.0 with 0.1 N NH_4OH. The solvents in both fractions were removed in vacuo and the residue chromatographed on a second 'long' SiO_2 partition column (14, 24), using a step-eluted (5% increments) gradient of 0 to 100% EtOAc in n-hexane. Twenty-four 10 mL fractions were collected, dried in vacuo and bioassayed (see below).

Enzymatic Hydrolysis

The putative GA glucosyl conjugate fraction from the short SiO_2 partition column was subjected to enzymatic hydrolysis in order to obtain the free GA moieties (25). In essence, the residue was dissolved in 0.4 mL of 0.2 M acetate buffer (pH 4.0), and 0.2 mL of a 1% cellulase (Calbiochem) solution was added. The mixture was incubated at 37° C for 16 h, centrifuged, and then partitioned against water-saturated EtOAc. The dried EtOAc-soluble residue (which will contain any free

GAs liberated by enzymatic hydrolysis) was chromatographed on the long SiO_2 partition column as noted above.
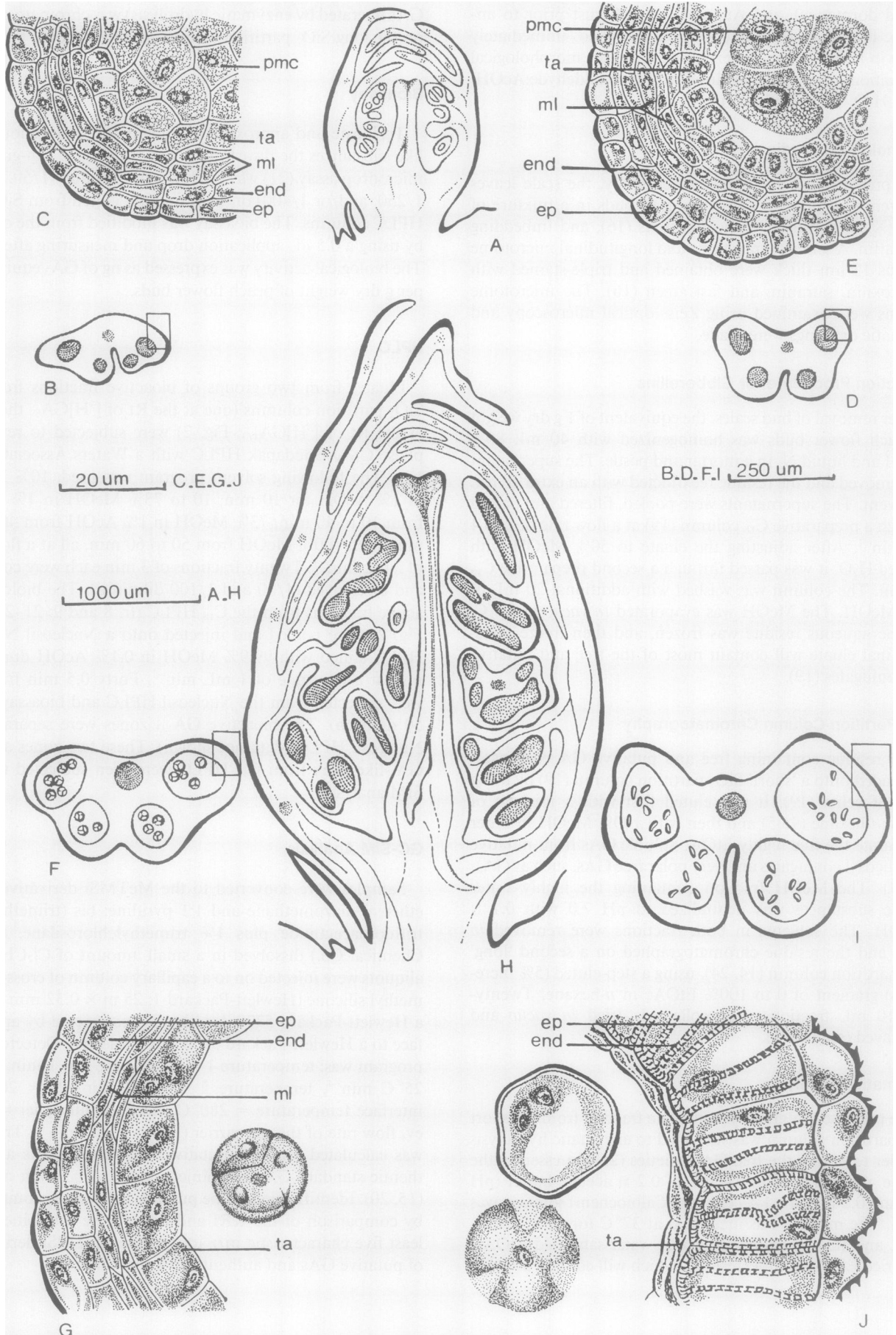
Bioassay

To locate and approximately quantitate endogenous GA-like substances the dwarf rice (Oryza sativa cv Tan-ginbozu) microdrop assay (21) was used in serial dilution (1/50, 1/100, 1/250, and/or 1/400) on each fraction eluted from SiO_2 and HPLC columns. The bioassay was modified from the original by using a 0.5 μL application drop and measuring after 48 h. The biological activity was expressed as ng of GA_3 equivalents per g dry weight of peach flower buds.
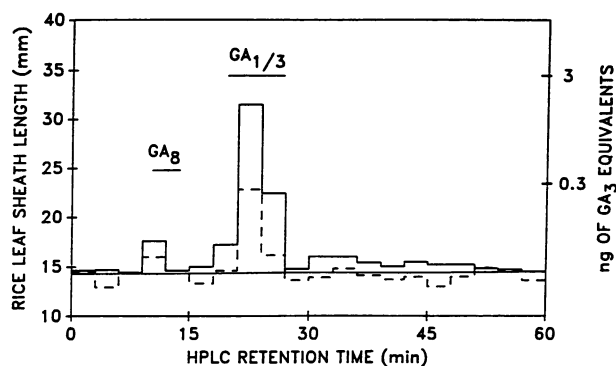
HPLC

Eluates from two groups of bioactive fractions from the SiO_2 partition columns (one at the Rt of [^{3}H]GA_8, the other at the Rt of [^{3}H]$GA_{1/3}$; Fig. 2) were subjected to reversed-phase C_{18} μ-Bondapak HPLC with a Waters Associates system. The following solvent program was used: 10% MeOH in 1% AcOH for 10 min, 10 to 73% MeOH in 1% AcOH from 10 to 40 min, 73% MeOH in 1% AcOH from 40 to 50 min, then 100% MeOH from 50 to 60 min, all at a flow rate of 2 mL min^{-1}. Twenty fractions of 3 min each were collected and bioassayed (1/50 and 1/100 dilutions). The biologically active fractions from the C_{18} HPLC (fr. 8 and 9; 21–27 min; Fig. 2) were pooled and injected onto a Nucleosil N(CH_3)$_2$ HPLC eluted with 99.9% MeOH in 0.1% AcOH during 20 min at a flow rate of 1 mL min^{-1}. Forty 0.5 min fractions were collected from the Nucleosil HPLC and bioassayed (1/50 dilution). The bioactive $GA_{1/3}$ zones were separated by Nucleosil HPLC (data not shown). These two zones and the GA_8-like peak from C_{18} HPLC were then subjected to GC-SIM analysis.

GC-SIM Analysis

Samples were converted to the MeTMSi derivatives with ethereal diazomethane and 1:1 pyridine: bis (trimethylsilyl) trifluoroacetamide plus 1% trimethylchlorosilane, (Pierce Chemical Co.) dissolved in a small amount of Cl_2CH_2, and aliquots were injected on to a capillary column of cross-linked methyl silicone (Hewlett-Packard-1; 25 m × 0.32 mm i.d.) in a Hewlett-Packard 5790 A series GC connected by an interface to a Hewlett-Packard 5970 Mass Selective Detector. The program was: temperature 1 = 60° C, time 1 = 1 min, rate = 25° C min^{-1}, temperature 2 = 270° C, time 2 = 20 min, interface temperature = 280° C, electron multiplier = 2600 ev, flow rate of the gas carrier (He) = 1 mL min^{-1}. The KRI was calculated for both putative endogenous GAs and authentic standard GAs by coinjecting a hydrocarbon mixture (15, 20). Identification of the putative GAs was accomplished by comparison of the KRI and the relative intensities of at least five characteristic m/z ions of the MeTMSi derivatives of putative GAs and authentic GA standards.

pmc
ta
ml
end
ep

C

pmc
ta
ml
end
ep

E

A

B

D

20 um ⌐ C,E,G,J

1000 um ⌐ A,H

B,D,F,I ⌐ 250 um

F

H

I

ep
end
ml

ta

G

ep
end

ta

J

Figure 2. A typical elution profile from C₁₈ HPLC of biologically active fractions (1/50 and 1/100 dilutions) from extracts of peach flower buds. The left side of the figure (e.g. [^{3}H]GA$_8$ Rt) represents the elution profile of bioactive fractions originating from very polar SiO$_2$ partition column fractions (22–23 min; Rt of [^{3}H]GA$_8$). The right side of the figure (e.g. [^{3}H]GA$_{1/3}$ Rt) represents the elution profile of bioactive fractions originating from relatively polar SiO$_2$ partition column fractions (17–19 min; Rt of [^{3}H]GA$_{1/3}$). Ordinate. Control = 14.4 mm; 0.1 ng GA$_3$ = 14.8 mm; 0.3 ng GA$_3$ = 17.9 mm; 1 ng GA$_3$ = 23.6 mm; 3 ng GA$_3$ = 32 mm. Abscissa. Twenty C$_{18}$ HPLC fractions of 3 min each.

RESULTS

Morphological Studies

Line drawings of the microscopic observations of peach flower buds collected at three different dates are shown in Figure 1.

On June 18, 1984 all verticils that eventually make up the peach flower bud were present, although they were not fully differentiated (Fig. 1A). The androecium was formed by many stamens with poorly developed filaments. Anthers were tetrasporangiate (Fig. 1B) with two locules in each of the two

lobes joined by connective tissue, in which the vascular bundle, mainly of a procambial nature, was situated. The microsporangium wall consisted of epidermis, endothecium, two to three middle layers, and tapetum. Sporogenous tissue was represented by the pollen mother cells (Fig. 1C). In the gynoecium, initiation of ovule formation was not yet observed.

In buds from July 18, 1984 (data not illustrated) morphological development was almost the same, except that abundant pubescence could be observed on the sepals, and the parenchyma between the two epidermal layers was thicker in the sepals than in the petals. Both sterile verticils were crossed by procambial elements, and there were also zones of very vacuolated parenchymatic cells with tannins inside.

Flower buds collected on August 8, 1984 (Fig. 1, D, E, F, and G) showed additional growth and differentiation, based on a longitudinal increase of all verticils. In the anther transsection, middle layers with their flattened cells and the tapetum (secretory type) with polyploid cells could be observed. Pollen mother cells (inside the loculus) had undergone meiosis, resulting in the formation of tetrahedral type tetrads. The formation of two ovules in the unicarpelar gynoecium had already begun.

In the last sample of buds (August 20, 1984, Fig. 1, H, I, and J), just 10 d before anthesis, it was possible to distinguish the presence of chloroplasts in the sepals and a large number of mature vascular elements in the vascular bundles of both sterile verticils. The microsporangium wall (Fig. 1J) was modified relative to the previous sample; here it was formed by epidermis, differentiated endothecium with fibrous enlargement in the anticlinal and inner tangential walls, and flattened middle layers. The tapetum had not been totally consumed during microsporogenesis, and inside the loculus, tricolpate pollen grains and few young male gametophytes could be seen. Dehiscence of the anthers had not yet occurred (Fig. 1I),

Table I. Tentative Identification of Endogenous GA₁, GA₃, and GA₈ in Peach Bud Tissue by Capillary Gas Chromatography-Mass Spectrometry-Selected Ion Monitoring (GC-SIM) and Kovat's Retention Index (KRI)

HPLC Fraction	KRI	Actual Intensity and (Percentage Intensity) of Characteristic Ions					
		m/z					
Bud sample At GA₈ Rt GA₈		594	579	535	448	379	375
	2833	11 (100%)	1 (9%)	1 (9%)	2 (18%)	2 (18%)	2 (18%)
	2833	(100%)	(7%)	(6%)	(18%)	(16%)	(15%)
Bud sample At GA₃ Rt GA₃		504	489	473	445	414	
	2720	15 (100%)	2 (13%)	1 (7%)	2 (13%)	1 (7%)	
	2721	(100%)	(8%)	(2%)	(8%)	(2%)	
Bud sample At GA₁ Rt GA₁		506	491	448	377	313	
	2703	4 (100%)	1 (25%)	1 (25%)	2 (50%)	(0)	
	2703	(100%)	(10%)	(19%)	(26%)	(17%)	

Figure 1. A, Longitudinal section of a flower bud from June 18, 1984; B, transverse section by an anther of the same stage; C, detailed drawing of the sector shown in B; D, transverse section of an anther from August 8, 1984; E, detailed drawing of the sector shown in D; F, transverse section of an anther from August 8, 1984; G, detailed drawing of the sector shown in F, showing decussate and tetrahedral tetrads; H, longitudinal section of a bud from August 20, 1984; I, transverse section of an anther of the same stage; J, detailed drawing of the sector shown in I, showing a young male gametophyte and a tricolpated pollen grain. ep, epidermis; end, endothecium; ml, middle layers; ta, tapetum; pmc, pollen mother cells.

Table II. *Relative Concentrations (Fresh wt/Dry wt) of GA-Like Substances in Peach Flower Buds Collected from Trees Grown in Argentina on Three Different Dates*

Estimates are in ng of GA_3 equivalents, based on the microdrop dwarf rice cv Tan-ginbozu assay at the SiO_2 partition column stage of purification, of putative free $GA_{1/3}$ and GA_8, and the putative glucosyl conjugates of $GA_{1/3}$-like substances (after cellulase hydrolysis).

GA-Like Substance	Date of Sampling		
	June 18	July 18	August 20
Putative $GA_{1/3}$	161/330	22/48	8/24
Putative $GA_{1/3}$ glucosyl conjugates	4/8	0/0	3/9
Putative GA_8	0/0	5/11	0.3/1

and development of ovules still continued in the gynoecium, although their type was not identified.

Analysis of Gibberellins

Figure 2 represents a qualitative and semi-quantitative picture of free bioactive GA-like substances present in peach flower buds.

Analysis of flower buds collected on June 18 showed relatively high GA-like activity in fr 17, 18, and 19 (Rt of $GA_{1/3}$) of the SiO_2 column (Table II).

Samples collected on both July 18 and August 20 had reduced GA-like activity in the $GA_{1/3}$ zone (Table II).

From bioassays of the putative GA conjugates (after hydrolysis by cellulase) a low level of biological activity was found in fractions 17 and 18 of the long SiO_2 partition column, implying that putative conjugates of $GA_{1/3}$-like substances were present in the bud tissue. These putative $GA_{1/3}$-like conjugates were not detectable in the July collection, but were present at the August 20 harvest when the flower buds were almost at anthesis (Table II).

The GC-SIM results obtained after sequential SiO_2 partition columns → C_{18}HPLC → bioassay → Nucleosil $N(CH_3)_2$ HPLC → bioassay are summarized in Table I. Three GAs $(GA_{1,3,8})$ were identified from fractions showing GA-like activity on the microdrop assay by GC-SIM based on KRI and relative intensities of highly characteristic m/z ions. GA_{32} could not be detected from bud extracts, although this GA was reported by Coombe (10) to be associated with ovary growth, during the post-fertilization stages.

Gibberellin-like substances were estimated using the dwarf rice microdrop bioassay (with several dilutions) at three different stages of HPLC purification—*e.g.* after the SiO_2 partition column, after C_{18} HPLC and after Nucleosil $N(CH_3)_2$ HPLC. The average $GA_{1/3}$ content was estimated at approximately 100 ng per g dry weight and the GA_8 content at 6 ng per g dry weight, both expressed in GA_3 equivalents. Conversion factors from bioassay amount to actual amount would be in the order of 1.0 × (GA_3), *1.2* × (GA_1) and *10* × (GA_8) (12). Finally, by comparison of the relative intensities of the respective parent ions (M^+) and the amount injected into GC-SIM, the proportion of GA_3, GA_1 and GA_8 can be calculated at about 8:2:6, respectively. The concentrations of putative

free $GA_{1/3}$ and GA_8 and their putative glucosyl conjugates (after cellulase hydrolysis) for each date are shown in Table II.

DISCUSSION

Earlier, Bottini *et al.* (8) suggested the presence of endogenous GA_8 in immature seeds of apricot *Prunus armeniaca* L., and this was recently confirmed by means of GC-SIM (G Abdala, R Bottini, D Pearce, R P Pharis, B Coombe, unpublished data). We also identified GA_1 by GC-SIM in the same immature seed material (8).

Herein we note the presence of free GA_1, GA_3, and GA_8 in peach flower buds using GC-SIM analysis. Somewhat surprisingly the highest level of GA_1/GA_3-like activity, on the basis of both dry weight and fresh weight of buds, occurred during the dormant period (June). This GA_1/GA_3-like bioactivity diminished thereafter. Hence, as was noted for *Solanum tuberosum* tubers (13) and garlic (*Allium sativum* L.) cloves (1, 2), the highest levels of endogenous bioactive GA-like substances occur well before bud dormancy begins to diminish. As the bud moves toward anthesis the concentration of bioactive free GA-like substances diminish (Table II).

The basic framework of the hormone theory of dormancy suggests that dormancy and its release depends on the interaction between naturally occurring growth-inhibiting and growth-promoting substances. More recently, the interaction has often been described as a balance between simultaneously occurring promotive hormones, such as GAs and cytokinins, versus the inhibitory compounds. However, recent reviews judge the experimental evidence on hand insufficient to justify the view that a balance of endogenous hormones controls dormancy and germination (6, 26). As Karssen and Lacka (17) have asked: have faulty techniques been applied or wrong hypotheses been formulated?

Our present results on peach flower buds indicate that absolute concentrations of GA_1/GA_3 do not increase immediately prior to bud break. However, high GA_1/GA_3 concentrations are present during floral stages where the rate of growth and cellular differentiation of (mainly fertile) verticils can be influenced. Thus, while flower buds are dormant and their development is still incomplete, there are relatively high concentrations of free GA_1 and GA_3.

Although there were detectable amounts of bioactive putative $GA_{1/3}$-like conjugates, levels are low, relative to free $GA_{1/3}$, and their hydrolysis *in situ*, (to yield free GAs) as the bud nears the end of the dormancy period, is not indicated by our results.

Our quantitative analyses of GA_1, GA_3, and GA_8 in peach buds are preliminary. Determination of endogenous free GA levels during bud dormancy → bud break using more definitive methods (*e.g.* GC-SIM with stable-isotope-labeled internal standards) is obviously needed. Quantification of GA conjugates remains a problem, one that will not be resolved until appropriate stable-isotope labeled internal standards of GA glucosyl conjugates are available. However, analysis of metabolism rates for ^{3}H-labeled native GAs may provide some insight into GA turnover as bud break approaches.

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