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Pollination Increases Gibberellin Levels in Developing Ovaries of Seeded Varieties of Citrus[1]

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Reproductive and vegetative tissues of the seeded Pineapple cultivars of sweet orange (*Citrus sinensis* L.) contained the following C-13 hydroxylated gibberellins (GAs): GA_{53}, GA_{17}, GA_{19}, GA_{20}, GA_1, GA_{29}, and GA_8, as well as GA_{97}, 3-epi-GA_1, and several uncharacterized GAs. The inclusion of 3-epi-GA_1 as an endogenous substance was based on measurements of the isomerization rates of previously added [$^2H_2$]GA_1. Pollination enhanced amounts of GA_{19}, GA_{20}, GA_{29}, and GA_8 in developing ovaries. Levels of GA_1 increased from 5.0 to 9.5 ng/g dry weight during anthesis and were reduced thereafter. The amount of GA in mature pollen was very low. Emasculation reduced GA levels and caused a rapid 100% ovary abscission. This effect was partially counteracted by either pollination or application of GA_3. In pollinated ovaries, repeated paclobutrazol applications decreased the amount of GA and increased ovary abscission, although the pattern of continuous decline was different from the sudden abscission induced by emasculation. The above results indicate that, in citrus, pollination increases GA levels and reduces ovary abscission and that the presence of exogenous GA_3 in unpollinated ovaries also suppresses abscission. Evidence is also presented that pollination and GAs do not, as is generally assumed, suppress ovary abscission through the reactivation of cell division.

In seeded plants successful fruit set and subsequent development are dependent on pollination. Generally, ovary growth and cell division are temporarily reduced during the period of anthesis until pollination and fertilization occur, since the presence of fertilized ovules normally reactivates cell division, triggering fruit development (Gillaspy et al., 1993). It is widely accepted that reactivation of fruit development requires synthesis and action of growth regulators such as GAs and auxins (Nitsch, 1971; Goodwin, 1978; Pharis and King, 1985). Whereas auxins do not appear to be limiting factors controlling early fruit development in citrus (Talon et al., 1990d), current evidence suggests that GAs may be essential for that process. The support for this proposal comes from several reports that exogenous GA_3 considerably improves fruit set of self-incompatible genotypes in which the absence of cross-pollination results in negligible parthenocarpic fruit set (Soost and Burnett, 1961). Later, it was found that these genotypes also contain lower GA levels than seedless varieties that show natural parthenocarpy (Talon et al., 1992). In several species, such as pear and grapes, the tendency to naturally set parthenocarpic fruit has also been attributed to higher GA content (Pharis and King, 1985).

The endogenous GAs found in citrus fruits are mainly members of the 13-hydroxylation pathway (Goto et al., 1989; Turnbull, 1989; Talon et al., 1992) leading to GA_1, which is the bioactive GA regulating developmental processes (Phinney, 1984; Zeevaart et al., 1993). The GA_1 levels in developing ovaries are low just before and after anthesis and are increased approximately 2-fold at anthesis. This transitory increase in GA_1 levels can be detected in parental seeded genotypes, as well as in seedless mutants with high parthenocarpic ability (Talon et al., 1990a). From the above observations it follows that the increase in GA_1 detected in mature ovaries at the time of pollination may be part of the hormonal stimuli that reactivate cell division, triggering fruit development. The above results may also suggest that the GA_1 increases observed in the seeded genotypes are induced by pollination and/or fertilization. Conclusive data indicating a promotive effect of pollination on GA levels have been presented for only a few species such as peas (Garcia-Martinez et al., 1991), although circumstantial evidence has been reported for tomato (Sjut and Bangerth, 1981) and pear (Martin et al., 1982). In contrast, there was no correlation between pollination and high GA levels in walnut (Tadeo et al., 1994). Relevant data to establish hypothetical cause-effect connections between pollination and GA synthesis in citrus are lacking. Therefore, the main purpose of this work was to study the relationship among pollination, GA levels, and cell division in seeded citrus genotypes.

MATERIALS AND METHODS

Developing citrus ovaries and fruitlets from seeded Pineapple cultivars of sweet orange (*Citrus sinensis* L. Osb.) (Ortiz et al., 1988) were used in all of the experiments described in this work. This cultivar has been found to be somewhat proterandous (pollen maturing before the stigma), as are several other citrus varieties (Frost and Soost, 1968). It has great tendency for abundant self-pollination because anthers begin to shed pollen while they are still

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Abbreviations: DAA, days after anthesis; KRI, Kovats retention index; MeTMSi, methyl ester trimethyl silyl ether; PCB, paclobutrazol; SIM, selected ion monitoring.

pressed against the stigma in the unopened or opening flower, allowing pollination before anthesis. This genotype was also selected because of its absolute requirement for pollination to develop fruits, its elevated pollen viability, and its high seed production.

Emasculation and Pollination Treatments

Pollinated and unpollinated samples were obtained as follows. Terminal flowers were emasculated by carefully removing the stamens with a pair of tweezers at stage C (ovary at the stage of immature pollen), according to the description of Frost and Soost (1968). Emasculated and nonemasculated flowers were immediately isolated by covering them with paper and woven bags to avoid cross-pollination. Ten days to 2 weeks later, when flowers had reached the phenological stage of "style-fall," the bags were removed.

In the first experiment for GA determination carried out during the 1992 season, ovary and fruitlet samples from naturally pollinated flowers were periodically collected every 2 d for 2 weeks beginning −6 DAA. Emasculation was performed at −4 DAA, and unpollinated flowers were collected at 0, 2, and 6 DAA. The whole experiment was repeated the following year. After a sample was collected, growth parameters were measured and ovaries and fruitlets were frozen, lyophilized, and stored independently at −20°C until analysis. Endogenous GAs in mature pollen were determined by collecting unopened anthers at −2 DAA and separately analyzing anther tissues and pollen grains. The absolute amount of GA in pollen was calculated by estimating the average number of pollen grains per anther.

In another experiment the effects of natural pollination, emasculation, and emasculation plus exogenous GA_3 on ovary growth and abscission were studied. Emasculated flowers were treated with GA_3 at two different stages: either immediately after emasculation (−6 DAA) or 6 d later, at anthesis (0 DAA). Growth and abscission were recorded weekly on batches of at least 200 tagged fruits per treatment for 1 month. The whole experiment was performed twice, in consecutive years.

To investigate the effect of reduced endogenous GA levels on the abscission of naturally pollinated fruits, 100 developing flowers (−5 DAA) were treated with PCB every other day for 2 weeks, and abscission was recorded as above. The flowers were distributed among 10 plants selected because of their low rate of fruit abscission. GA levels in naturally pollinated, emasculated, and PCB-treated ovaries were compared. In this experiment treatments were applied at −4 DAA, the PCB treatment was repeated for 3 consecutive days, and all samples were analyzed 6 DAA.

For microscopic observations, ovaries were emasculated at −2 DAA. Two-thirds of the emasculated flowers were either treated immediately with GA_3 or hand-pollinated with mature pollen; the remaining one-third did not receive any additional treatment. Nonemasculated ovaries were collected at −2 DAA, and samples from the three treatments were harvested 16 d later.

Chemical Treatments

GA_3 (20 μg/mL) was dissolved in 5% (v/v) aqueous ethanol containing 0.05% (v/v) Tween 20. One single application was made by dipping the emasculated ovary in the solution for 5 s, which delivered about 50 μL of solution per fruit. Control fruits were treated with water containing ethanol and Tween 20. The GA biosynthesis inhibitor PCB (ICI-ZELTIA, Pontevedra, Spain) was dissolved in 0.05% (v/v) Tween 20 (500 μg/mL), and 50 μL of the solution was applied with an adjustable pipette to the developing flowers. One petal of each flower was removed to allow discharge of the solution. Control flowers received only the Tween 20 solution.

Light Microscopy and Morphometrical Analyses

Samples from developing ovaries (−2 DAA) and emasculated, pollinated, and emasculated plus GA_3-treated ovaries (14 DAA) were fixed and embedded in London Resin White (London Resin, Woking, Surrey, UK) and stained with toluidine blue O (CI 52040, Merck, Darmstadt, Germany) according to the method of Tadeo et al. (1994). Morphometric analyses were performed on highly contrasted micrographs from transverse sections of ovary walls (The Morphometer, Electron Microscopy Sciences, Fort Washington, PA). The ovary wall width and cell number were measured on 15 cross-sectioned ovaries for each treatment. Cross-sectional cell areas were measured on 100 cells in 15 ovary walls for each treatment. The comparison of the morphometric parameters was performed using Duncan's test. Pollen grains were stained with aniline blue (CI 42755; Electron Microscopy Sciences) and examined under fluorescence microscopy in 15 independent preparations.

Identification and Quantitation of GAs

For GA identification 20 to 30 g dry weight of either vegetative shoots (four to six developing leaves per shoot) or developing flowers of *C. sinensis* were homogenized in 200 mL of cold methanol. The extracts were filtered and the residue was re-extracted with 200 mL of 80% methanol for 12 h at 4°C. The filtrates were combined, 40 mL of 1 M K-Pi buffer, pH 8.5, was added, and the extracts were reduced to the aqueous phase. The pH was adjusted to 8.5, the extract was centrifuged for 10 min at low speed, and the supernatant was partitioned against *n*-hexane (1×, 1:1, v/v) and then against diethyl ether (3×, 1:1, v/v). The pH of the aqueous extracts was readjusted to 2.5, the extracts were purified by charcoal (3 g):celite (6 g) adsorption chromatography, and the GAs were eluted with 200 mL of 80% acetone (Metzger and Zeevaart, 1980). The aqueous extracts were acidified to pH 2.5 and partitioned three times against ethyl acetate (1:1, v/v). The ethyl acetate was dried and the residues were redissolved in 1 M K-Pi, pH 8.5, and purified by a 2-g polyvinylpolypyrrolidone column, as described previously (Talon et al., 1992). The eluate of the column (80 mL) was readjusted to a low pH and partitioned against ethyl acetate, as described above. The dried residues were redissolved in a few drops of methanol, 5 mL

of distilled water, pH 8.0, was added, and the GAs were further purified with a 15-cm-long column of QAE-Sephadex A-25, as described by Talon et al. (1992). The acidified eluate was reduced to 5 mL, passed through a C_{18} Sep-Pak cartridge, and evaporated to dryness. The residue was subjected to reverse-phase HPLC (Waters HPLC equipped with an analytical column, 25 × 0.46 cm, packed with Hypersyl C_{18} attached to a C_{18} Guard-Pak precolumn). A 40-min linear gradient of 20 to 100% methanol in 1% aqueous acetic acid at a flow rate of 1 mL/min was used. The elution volumes of authentic GAs were first determined in preliminary experiments in which HPLC fractions were collected at 1-min intervals. Citrus samples were similarly fractionated, and the first seven fractions were discarded. The remaining 32 fractions were evaporated to dryness, methylated with ethereal diazomethane, and trimethylsilylated at room temperature for at least 1 h with 5 to 10 μL of bis-trimethylsilyl-trifluoroacetamide.

The derivatized HPLC fractions were individually analyzed by full-scan GC-MS using a gas chromatograph (Star 3400 CX, Varian, Sunnyvale, CA) coupled to an ion-trap mass spectrometer (Saturn 3, Varian). The samples (1 μL) were automatically co-injected with an autosampler (8200 CX, Varian) with a series of hydrocarbons to obtain the relative KRIs (Gaskin et al., 1971) in the splitless mode into a fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness; DB-5MS, J&W Scientific, Folsom, CA); at an oven temperature of 50°C. After 1 min, the oven temperature was increased 30°C/min to 240°C and then 10°C/min to 280°C. The He inlet pressure was 60 kPa and the injector, interface, and manifold temperatures were 280, 290, and 170°C, respectively. Multiplier voltage, axial modulation amplitude voltage, automatic gain control, and emission current were 1200 V, 4 V, 35600 counts, and 36 μA, respectively. Ion-electron-impact mass spectra were acquired, scanning from 200 to 600 atomic mass units at 1 s/scan cycle. Identification of endogenous GAs was established by comparing mass spectra, KRIs, and HPLC elution volumes with authentic standards.

The extraction and purification procedures for GA quantitation were as above, except that batches of 2 to 3 g of dry material and reduced volumes of columns and partitioning solvents were used. For quantitation purposes [17-$^2H_2$]GA_{19} (95.9% enrichment), [17-$^2H_2$]GA_{20} (99.6%), [17-$^2H_2$]GA_{29} (99.8% enrichment), [17-$^2H_2$]GA_1 (99.6%), and [17-$^2H_2$]GA_8 (99.7%) were added to the methanolic extracts as internal standards. Fractions of HPLC containing the GAs of interest were derivatized as above and separately analyzed using a gas chromatograph (model 8000, Fisons, Danvers, MA) equipped with the above GC column and coupled to a quadrupole mass spectrometer (MD 800, Fisons). The samples (1–2 μL) were injected in the splitless mode, and the oven temperature program was identical to the one used for qualitative analyses. The He inlet pressure was 85 kPa and the injector, interface, and MS source temperatures were 250, 250, and 200°C, respectively. Ion-electron-impact masses at 70 eV were acquired in the SIM mode. Ions were monitored (dwell times = 80 ms) as follows: for GA_{29}/[$^2H_2$]GA_{29}-MeTMSi, m/z 508 and 506; GA_{19}/[$^2H_2$]GA_{19}-MeTMSi, m/z 436 and 434; GA_{20}/[$^2H_2$]GA_{20}-MeTMSi, m/z 420 and 418; GA_1/[$^2H_2$]GA_1-MeTMSi, m/z 508 and 506; and GA_8/[$^2H_2$]GA_8-MeTMSi, m/z 596 and 594. In each sample the identity of the endogenous/standard mixture of GAs under SIM analysis was previously confirmed by full-scanning analyses, with the instrument utilized for qualitative determinations as described above. Estimates of the GA levels were obtained from full-scan and SIM data in preliminary extractions without internal standards. For the quantitative analyses, various amounts of internal standards, based on previous estimates, were added to the extracts. This strategy was repeated until the amount of internal standards and the amount of endogenous GAs were approximately equal, which is a requirement of the GA calculation method used (Talon and Zeevaart, 1990). Once the amount of internal standard to be added was determined, a further quantification experiment was carried out. In addition, the whole experiment was repeated in two consecutive seasons. In all cases, at least two injections, which provided practically identical results, were made for each sample.

RESULTS

Identification of GAs

The results of the GA identification analyses showed that there were no qualitative differences between vegetative and reproductive tissues of *C. sinensis* cv Pineapple. Developing flowers and leaves contained the C-13 hydroxylated GA_{53}, GA_{17}, GA_{19}, GA_{20}, GA_1, GA_{29}, and GA_8 (data not shown), as has been found in several citrus varieties (Poling and Maier, 1988; Turnbull, 1989; Talon et al., 1992), in addition to GA_{97} and 3-epi-GA_1 (Table I). The identification of GA_{97} (2β-hydroxy-GA_{53}, Mander et al., 1996) and 3-epi-GA_1 as endogenous compounds in citrus is a novel finding. The presence of 3-epi-GA_1 in extracts from several species has been reported (Gaskin et al., 1995, and refs. therein). However, 3-epi-GA_1 can be formed from natural GA_1 during extraction, purification, and GC-MS analyses, which creates uncertainty about its origin. To determine the origin of 3-epi-GA_1 we followed a recent procedure (Gaskin et al., 1995) that uses stable, isotopically labeled GA_1 and further measurement of the stable isotope in the identified 3-epi-GA_1 and GA_1. Table II is a summary of the

Table I. *GAs newly identified by full-scan GC-MS of MeTMSi derivatives in developing leaves and flowers of C. sinensis cv Pineapple*

GA	HPLC Fraction	KRI	Ion m/z
3-Epi-GA_1	17–18	2802	M^+ 506 (100)[a], 491 (23), 459 (15), 448 (42), 376 (38), 208 (21), 207 (42)
GA_{97}	16–17	2690	M^+ 536 (83), 521 (23), 504 (33), 477 (47), 239 (22), 208 (43), 207 (100)

[a] Numbers in parentheses indicate relative abundance.

Table II. *Isotope dilution analysis by GC-SIM of the molecular ion cluster of MeTMSi derivatives of GA_1 and 3-epi-GA_1 from ovaries of C. sinensis cv Pineapple collected at different stages of development*

Sample	$[^2H_2]$GA	Relative Amounts	Ion m/z	
			506[a]	508[b]
1	GA_1	9	38	62
	3-Epi-GA_1	1	83	17
2	GA_1	30	40	60
	3-Epi-GA_1	1	83	17
3	GA_1	12	30	70
	3-Epi-GA_1	1	32	68
4	GA_1	15	49	51
	3-Epi-GA_1	1	50	50
5	GA_1	20	39	61
	3-Epi-GA_1	1	48	52
6	GA_1	14	38	62
	3-Epi-GA_1	1	55	45

[a] Endogenous. [b] Derived from $[^2H_2]$GA.

results of 6 of 16 analyses performed on different ovary and fruit samples. The results indicate that 3-epi-GA_1 is endogenous in *C. sinensis* cv Pineapple, since the isotope dilution analyses of samples 1 and 2 showed that no epimerization of GA_1 occurred. However, in samples 3 and 4 the 508:506 (ion m/z) ratio of 3-epi-GA_1 was equal to that of GA_1, which implied an artifactual origin. In samples 5 and 6, the data suggest that 3-epi-GA_1 was a mixture of both (Gaskin et al., 1995). Isomerization of GA_1 (≤6%) was occasionally observed, without previous extraction, during direct injection of authentic, derivatized GA_1, as was reported for GA_4 (Rood and Hedden, 1994). However, we were unable to determine the optimal GC conditions for isomerization, since this apparently occurred in a nonreproducible manner.

Vegetative and reproductive tissues of *C. sinensis* cv Pineapple contained various uncharacterized GAs (Table III), which, based on their molecular ions, were hydroxylated derivatives of GA_9, GA_{12}, GA_{15}, and GA_{24}. Two of these compounds, which have KRIs of 2760 and 2671, have previously been detected in Arabidopsis (Talon et al., 1990b) and spinach (Talon et al., 1991), respectively.

Effect of Pollination on GA Levels

In the first experiment the endogenous GA changes that occurred during pollination were determined in samples harvested in 1992. For each GA a minimum of two extractions were performed. The whole experiment was also repeated the following season, and data for both experiments were plotted together in Figure 1. Since pollination takes place before anthesis in cv Pineapple, the initial effects of pollination on the GA levels were observed shortly thereafter (at anthesis, 0 DAA). The levels of GA_{19} increased in pollinated ovaries at anthesis. After anthesis, GA_{20} levels declined continuously. In unpollinated ovaries GA_{19} and GA_{20} levels were progressively reduced during and after anthesis. Pollination induced a transitory GA_1 increase during anthesis (from 5.0 to 9.5 ng/g dry weight), which progressively declined thereafter (5.1 ng/g dry weight). In unpollinated ovaries GA_1 levels were low (4.1 ng/g dry weight) at anthesis and even lower later (2.7 ng/g dry weight). The inactive product of GA_1, GA_8, accumulated from anthesis in pollinated ovaries, whereas emasculation induced lower GA_8 levels. GA_{29} levels also increased at anthesis in pollinated ovaries and then remained constant, whereas in unpollinated ovaries levels of GA_{29} were also lower (Fig. 1). The above results show that pollination increased the levels of the 13-hydroxy-GAs, GA_{19}, GA_{20}, GA_{29}, GA_1, and GA_8, whereas emasculation reduced or did not modify the basal amounts found at the pre-anthesis stages.

Effect of Pollination and Exogenous GA_3 on Abscission

The effect of GA_3 on ovary abscission was studied during 2 consecutive years. The abscission trends observed in both experiments were practically the same, although the values for the controls varied from year to year, since *C. sinensis* cv Pineapple exhibits an alternate bearing habit. This behavior determines the relative intensity of abscission (Fig. 2), although the absolute fruit set in this cultivar is very regular (Hodgson, 1968). Emasculation caused 99% ovary abscission as soon as 14 DAA, whereas natural pollination reduced abscission (50–75%). In independent experiments it was shown that the effect of emasculation on abscission was not mediated by wound ethylene, since the removal of the anthers did not significantly modify the basal levels of ethylene found in flowers (data not shown). Application of GA_3 was more effective at the pre-anthesis stages (50–65% abscission) than at anthesis (88% abscission), indicating that exogenous GA_3 mimicked the effect of natural pollination, which also takes place before anthesis.

Table III. *Putative GA-related compounds detected by full scan of MeTMSi derivatives in developing leaves and flowers of C. sinensis cv Pineapple*

Putative Compound	HPLC Fraction	KRI	Ion m/z
Di · OH · GA_9	27	2615	M^+506(12)[a], 491(56), 446(100), 356(63), 326(8), 236(16)
Di · OH · GA_{24}	25	2670	M^+462(5), 447(38), 430(100), 402(26), 374(26), 312(65)
Di · OH · GA_{15}	23–24	2708	M^+520(77), 505(32), 489(49), 430(100), 358(9), 286(18)
Di · OH · GA_{12}	23–24	2437	M^+536(100), 521(47), 504(43), 477(26), 446(23), 208(33)
Di · OH · GA_9[b]	21	2760	M^+508(100), 493(58), 451(4), 418(52), 386(8), 329(11)
Di · OH · GA_{12}[c]	19–20.	2671	M^+536(65), 521(27), 504(37), 477(36), 239(41), 207(100)

[a] Numbers in parentheses indicate relative abundance. [b] Compound also detected in Arabidopsis (Talon et al., 1990). [c] Compound also detected in spinach (Talon et al., 1991).

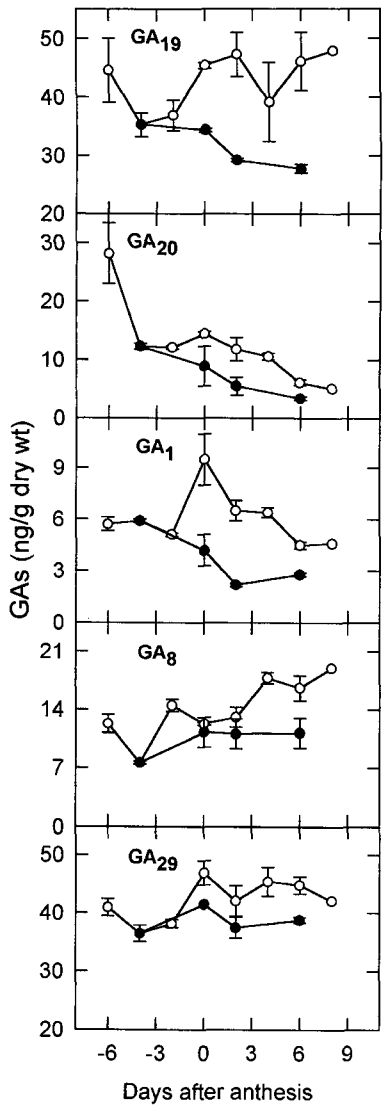

Figure 1. Effect of pollination on the 13-hydroxy-GA levels in developing ovaries of *C. sinensis* cv Pineapple. Emasculation was performed at −4 DAA, and pollinated (○) and unpollinated (●) ovaries were collected as indicated. Data are means ± SE; where the bars are not present the SE is smaller than the symbol. Dry wt, Dry weight.

Effect of Pollination and Exogenous GA_3 on Growth

Several growth parameters, such as ovary diameter, weight, and length, were recorded periodically on the emasculated, pollinated, and emasculated plus GA_3 samples collected in the two experiments described above. In addition, an independent but similar experiment was carried out in 1994. In this last experiment, natural pollination was substituted by hand-pollination. In none of these three experiments were differences in growth among the three treatments found (data not shown). An anatomical study was carried out on the width, cell number, and cross-sectional cell areas of ovary walls. The average ovary wall width increased during development similarly in all three treatments (from 466 to 635–666 μm) due to increases in both the total number of cells and the size of the mesocarpic cells (Table IV). The number of cells across the width increased between −2 and 14 DAA from 34 to 47 (emasculation), 46 (hand-pollination), and 39 (emasculation plus GA_3). The determinations of cell area in cross-sections of the exocarp and endocarp did not show any significant differences between the three treatments and the −2 DAA samples (data not shown). Cell enlargement occurred in the mesocarp, the target region for ovary growth during the earlier stages of development, according to Schneider (1968). The GA treatment slightly increased cell area in cross-sections, compared with the emasculation and pollination treatments (Table IV). Therefore, growth of the ovaries during this initial stage of development was due to both cell division and the radial increase of the mesocarpic cells.

Effect of PCB on Ovary Abscission and GA Levels

To study the effect of low GA levels on abscission of pollinated ovaries, 100 −5 DAA flowers were treated every 2 d with PCB (25 μg/flower) for 2 weeks. Four weeks after the beginning of the treatment, PCB had induced a 67% abscission rate, whereas nontreated plants had a 28% abscission rate (Fig. 3). Thus, PCB considerably increased ovary abscission but, unlike the emasculation treatments,

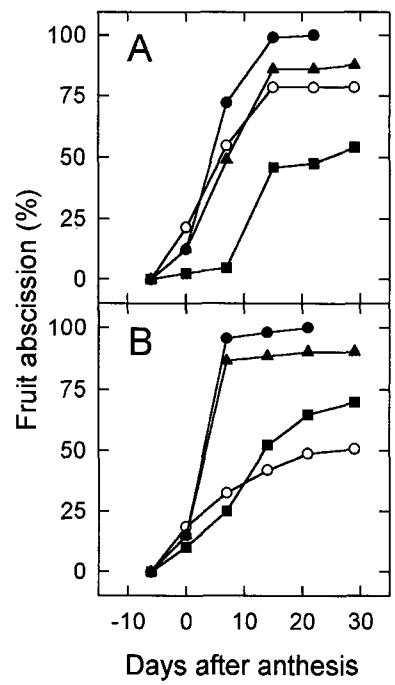

Figure 2. Effect of pollination and exogenous GA_3 (1 μg) on ovary abscission from *C. sinensis* cv Pineapple. Treatments were: ●, Emasculation at −6 DAA; ○, natural pollination; ■, emasculation at −6 DAA followed by immediate application of GA_3; and ▲, emasculation at −6 DAA and GA_3 application at 0 DAA. A, Experiment performed during 1992, higher fruit load and relative abscission. B, Experiment performed during 1993, lower fruit load and relative abscission. In both experiments a minimum of 200 ovaries per treatment was used.

Table IV. *Anatomical parameters obtained in cross-sections through ovary walls from C. sinensis cv Pineapple*

Treatments (emasculation, hand-pollination, and emasculation plus GA_3 [1 μg]) were carried out 2 d before anthesis and samples were collected this day and 16 DAA. Data are means ± SD (width, n = 5; cell area, n = 100; cell number, n = 15).

DAA	Sample	Width	Area of Mesocarp Cells	Cells across the Width
		μm	μm^2	n
−2	Developing flower ovaries	465.7 ± 38.9[a]	278.2 ± 83.0[a]	34 ± 3[a]
14	Emasculated ovaries	658.6 ± 96.3[b]	338.6 ± 104.8[b]	47 ± 8[b]
	Pollinated ovaries	666.3 ± 92.2[b]	346.5 ± 86.7[b]	46 ± 8[b]
	Emasculated ovaries GA_3-treated	634.5 ± 34.8[b]	447.1 ± 139.1[c]	39 ± 4[c]

[a–c] Values within a column bearing a common superscript letter are not significantly different ($P > 0.05$) from each other as determined by Duncan's test.

was not able to cause 100% abscission in 20 d (Fig. 2). The PCB treatment reduced GA levels in pollinated ovaries (Table V). This reduction was higher for GA_{19} (56%), GA_{20} (64%), and GA_1 (67%) and lower for the inactive 2β-OH GAs, GA_8 (17%) and GA_{29} (3%). The pattern of GA change induced by PCB, lower reduction of 2β-OH GA levels, followed the tendency observed in emasculated ovaries (Fig. 1; Table V). The effect of PCB on GA content may suggest that GAs are mostly synthesized in the ovaries, although contributions from vegetative tissues cannot be discounted.

GA Levels in Mature Pollen

The contribution of pollen to the absolute amounts of GA in the ovary was negligible (Table VI). Flowers of this seeded variety have a maximum of 40 anthers, each containing an average of 16,000 pollen grains, although during pollination no more than 20 pollen grains reach the embryo sac (data not shown). On a per-organ basis, individual anthers contained very low levels of GAs (0.3–24 pg) and pollen grains were at the femtogram level, whereas pollinated ovaries at anthesis had much higher amounts (444–2190 pg).

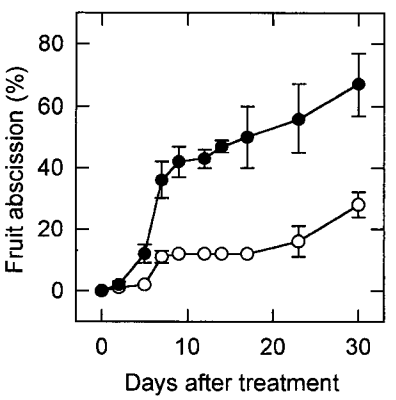

Figure 3. Effect of PCB on abscission of natural pollinated ovaries from *C. sinensis* cv Pineapple. The PCB treatment (25 μg) began at −5 DAA and was repeated every other day for 2 weeks. A minimum of 100 ovaries, distributed in 10 seedlings per treatment, was used. ○, Nontreated ovaries; ●, PCB-treated ovaries. Data are means ± SE; where the bars are not present the SE is smaller than the symbol.

DISCUSSION

It is generally accepted that the stimuli that trigger fruit development following pollination are of a hormonal nature (Nitsch, 1971; Goodwin, 1978; Pharis and King, 1985; Gillaspy et al., 1993). This concept is based mostly on three lines of evidence: (a) pollen contains plant hormones such as auxins and GAs (Nitsch, 1971); (b) exogenous auxin or GA results in fruit growth in the absence of fertilization (Gustafson, 1960); and (c) parthenocarpic ovaries contain increased amounts of auxins and GAs (Gil et al., 1972). Nevertheless, the support in the literature for a hypothetical stimulation of the hormonal synthesis promoted by pollination is scarce or indirect. Subsequent to these initial reports several studies of pears and tomatoes have suggested that pollination increases GA content (Sjut and Bangerth, 1981; Pharis and King, 1985), although conclusive evidence has been presented only in peas (Garcia-Martinez et al., 1991), in which emasculation was shown to reduce GA levels. However, this observation cannot be generalized, since it has also been shown that there is no correlation between pollination and GA levels in other species such as walnut (Tadeo et al., 1994). Whereas the auxin content does not appear to be a limiting factor controlling early fruit development in citrus (Talon et al., 1990c), several findings suggest that GAs are actively involved in this process.

It has been reported that the 13-hydroxylation GA pathway is the main route of GA synthesis in seedless citrus, which also contain at lower levels a few non- and 3-hydroxy-GAs (Poling and Maier, 1988; Goto et al., 1989; Turnbull, 1989; Talon et al., 1990a, 1992). In the present

Table V. *GA levels in developing ovaries from C. sinensis cv Pineapple*

Treatments were natural pollination, emasculation at −4 DAA, and PCB applications (25 μg) during 3 consecutive d beginning at −4 DAA. Samples were analyzed 6 DAA.

GA	Pollinated	Emasculated	PCB
	pg/ovary		
GA_{19}	2153	1297	948
GA_{20}	285	159	102
GA_1	210	131	70
GA_8	775	523	640
GA_{29}	2092	1812	2041

Table VI. *GA contents of pollinated ovaries at anthesis and in mature pollen from flowers of C. sinensis cv Pineapple*

Unopened anthers containing mature pollen were collected at −2 DAA. The amounts of GAs per individual pollen grain were calculated based on the average number of pollen grains per anther (16,000).

GA	Ovary	Anther	Pollen Grain
	pg		*fg*
GA_{19}	2120	2.57	0.14
GA_{20}	672	0.29	0.01
GA_1	444	0.34	0.01
GA_8	574	3.26	0.15
GA_{29}	2190	23.55	0.77

work we report that the endogenous GAs of seeded cultivars of citrus are also 13-hydroxylated GAs, so the presence of seeds does not appear to modify the qualitative GA content in citrus. We have also demonstrated that pollination increases levels of GA_{19}, GA_{20}, GA_1, GA_8, and GA_{29} in the developing ovaries (Fig. 1) and that this effect was not due to pollen contribution (Table VI). The 2-fold increase in the GA_1 content induced by pollination at anthesis (from 5.0 to 9.5 ng/g dry weight) appears to be of primary importance since this GA is thought to be the active GA of the 13-hydroxylation pathway (Phinney, 1984; Zeevaart et al., 1993). In other physiological processes that have been more extensively studied, such as stem elongation in long-day plants, it has been reported that the increase in the endogenous GA_1 level from 1.0 to 5.0 ng/g dry weight is the primary factor for induction (Zeevaart et al., 1993). In Arabidopsis a 3-fold reduction in the GA_1 content is correlated with dwarfism (Talon et al., 1990c). In citrus 2-fold increases in the GA_1 levels have also been observed in the ovaries of seeded and seedless mutants possessing higher parthenocarpic ability and lower rates of abscission (Talon et al., 1990a, 1992).

The above information suggests that the transitory GA_1 increase observed in nonpollination-requiring species is developmentally or constitutively regulated rather than induced by pollination or fertilization. Furthermore, we propose that in seeded varieties of citrus pollination induces an increase in the GA_1 ovary content and that this increase acts as a stimulus participating in the process of the transition from ovary to fruit. Supporting this proposal is the observation that exogenous GAs suppress the sudden 100% abscission observed shortly after anthesis in unpollinated ovaries, mimicking the effect of natural pollination on abscission (Fig. 2). However, our data do not support the idea that the GA effect is based mostly on the reactivation of cell division (Gillaspy et al., 1993), since pollinated and unpollinated ovaries grow in a like manner and both have similar rates of cell division and enlargement (Table IV). We have also shown that in pollinated ovaries the reduction of GA levels by repeated applications of high concentrations of PCB (Table V) considerably increased ovary abscission, although the pattern observed (continuous decline, Fig. 3) was different from that recorded in emasculated ovaries (sudden abscission, Fig. 2). This observation suggests that GAs are not the only factors triggering fruit development and that pollination likely generates other stimuli that may overcome, at least partially, the effect of a lack or shortage of GAs.

It is interesting to note that, although the rates of relative abscission of the pollinated ovaries varied according to the initial load (Fig. 2), this variety has a regular fruit set in absolute terms (Hodgson, 1968). Our data also showed that there was very little variability in GA levels from year to year (Fig. 1). These observations suggest that there is not a minimum threshold level of endogenous GAs preventing abscission; rather, it appears that the level increases as fruit load increases. This assumption is compatible with the idea that GAs are positive hormonal signals acting during the early phases of fruit development (Pharis and King, 1985). For successful fruit set, growth must be sustained by other essential factors, mostly nutrients (Gillaspy et al., 1993). In citrus the availability of nutrients strongly decreases with elevated fruit loads (Mehouachi et al., 1995). Our results also indicate that the rapid abscission of the emasculated fruits can be completely suppressed by pollination or by a single application of GAs. Fruit abscission in citrus can be induced by nutritional deficiency (Mehouachi et al., 1995) or by hormonal signals (Goren, 1993).

It has been shown in citrus that both pollination and exogenous GAs induce a stronger mobilization of ^{14}C metabolites to young ovaries, which appears to be essential for fruit growth (Powell and Krezdorn, 1977). Therefore, it has been suggested that the ability to increase the sink strength might be a complementary or fundamental function of GAs on the process of citrus fruit growth. However, in our system the carbohydrate supply appears to be normal, since growth of the emasculated ovaries was not arrested at any time (Table IV). Moreover, abscised, emasculated ovaries had a healthy, dark-green color, were of normal size, and did not exhibit senescence symptoms. Furthermore, a shortage of carbohydrates during anthesis in citrus results in a heavy abscission approximately 40 d later, during the June drop (Mehouachi, 1995). Thus, the data do not appear to support the view that the abscission of the emasculated ovaries was due to a carbohydrate shortage induced by the absence of pollination or lack of GAs.

On the other hand, evidence has accumulated suggesting that fruit abscission in citrus is promoted by hormonal signals involving ABA and ethylene. The main findings are (a) GAs increase at anthesis and ABA is low shortly thereafter in seeded and non-pollination-requiring species of citrus having low abscission rates (Garcia-Papi and Garcia-Martinez, 1984; Talon et al., 1990c, 1992); (b) GA levels are not enhanced at anthesis in self-incompatible species but show a prominent ABA transitory increase 6 to 10 DAA, followed by a heavy wave of abscission (Garcia-Papi and Garcia-Martinez, 1984; Talon et al., 1990c, 1992); (c) exogenous ABA increases ACC synthesis, ethylene production, and abscission in citrus fruit explants (for review, see Goren, 1993, and refs. therein); and (d) exogenous GAs suppress completely both postanthesis ABA increases and fruit abscission (Talon et al., 1992; Zacarias et al., 1995). It is possible that in unpollinated ovaries ABA may increase shortly after anthesis, just as it does in the self-incompatible species, and

that the GA increase induced by pollination (or the GA treatment) represses the ABA increase, thus blocking the hormonal sequence for ovary abscission.

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LITERATURE CITED

Frost HB, Soost RK (1968) Seed reproduction: development of gametes and embryos. *In* W Reuther, LD Batchelor, HJ Webber, eds, The Citrus Industry, Vol 2. University of California, Berkeley, pp 290–324

Garcia-Martinez JL, Santes C, Croker SJ, Hedden P (1991) Identification, quantitation and distribution of gibberellins in fruits of *Pisum sativum* L. cv Alaska during pod development. Planta **184:** 53–60

Garcia-Papi MA, Garcia-Martinez JL (1984) Endogenous plant growth substance content in young fruits of seeded and seedless Clementine mandarin as related to fruit set and development. Sci Hortic **22:** 265–274

Gaskin P, MacMillan J, Firn RD, Pryce RJ (1971) Parafilm: a convenient source of *n*-alkane standards for the determination of gas chromatographic retention indices. Phytochemistry **10:** 1155–1157

Gaskin P, MacMillan J, Spray CL, Suzuki Y, Phinney BO (1995) 3-Epigibberellin A_1: natural occurrence in plants and artefactual formation from gibberellin A_1. Phytochemistry **38:** 1–4

Gil GF, Martin GC, Griggs WH (1972) Fruit set and development in the pear: extractable endogenous hormones in parthenocarpic and seeded fruit. J Am Soc Hortic Sci **97:** 731–735

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell **5:** 1439–1451

Goodwin PB (1978) Phytohormones and fruit growth. *In* DS Letham, PB Goodwin, TJ Higgins, eds, Phytohormones and Related Compounds: A Comprehension Treatise, Vol 2. Elsevier-North Holland Biomedical Press, Amsterdam, The Netherlands, pp 175–213

Goren R (1993) Anatomical, physiological and hormonal aspects of abscission in citrus. Hortic Rev **15:** 33–46

Goto A, Yamane H, Takahashi N, Hirose K (1989) Identification of nine gibberellins from young fruit of Satsuma mandarin (*Citrus unshiu* Marc.). Agric Biol Chem **53:** 2817–2818

Gustafson F (1960) Influence of gibberellic acid on setting and development of fruits in tomato. Plant Physiol **35:** 521–523

Hodgson RW (1968) Horticultural varieties of citrus. *In* W Reuther, LD Batchelor, HJ Webber, eds, The Citrus Industry, Vol 1. University of California, Berkeley, pp 431–589

Mander LN, Owen DJ, Croker SJ, Gaskin P, Hedden P, Lewis MJ, Talon M, Gage DA, Zeevaart JAD, Brenner ML, and others (1996) Identification of three new C_{20}-gibberellins: GA_{97} (2β-hydroxy-GA_{53}), GA_{98} (2β-hydroxy-GA_{44}), and GA_{99} (2β-hydroxy-GA_{19}). Phytochemistry **43:** 23–28

Martin GC, Horgan R, Nishijima C (1982) Changes in hormone content of pear receptacles from anthesis to shortly after fertilization as affected by pollination or GA_3 treatment. J Am Soc Hortic **107:** 479–482

Mehouachi J, Serna D, Zaragoza S, Agusti M, Talon M, Primo-Millo E (1995) Defoliation increases fruit abscission and reduces carbohydrate levels in developing fruits and woody tissues of *Citrus unshiu*. Plant Sci **107:** 189–197

Metzger JD, Zeevaart JAD (1980) The effect of photoperiod on the levels of endogenous gibberellins in spinach as measured by combined gas chromatography-selected ion current monitoring. Plant Physiol **66:** 844–846

Nitsch JP (1971) Perennation through seeds and other structures: fruit development. *In* Steward FC, ed, Plant Physiology, A Treatise, Vol 6A. Academic Press, London, pp 413–501

Ortiz JM, Zaragoza S, Bono R (1988) The major citrus cultivars in Spain. HortScience **23:** 691–693

Pharis RP, King RW (1985) Gibberellins and reproductive development in seed plants. Annu Rev Plant Physiol **36:** 517–568

Phinney BO (1984) Gibberellin A_1, dwarfism and the control of shoot elongation in higher plants. *In* A Crozier, JR Hillman, eds, The Biosynthesis and Metabolism of Plant Hormones, Cambridge University, Cambridge, UK, pp 17–41

Poling SM, Maier VP (1988) Identification of endogenous gibberellins in navel orange shoots. Plant Physiol **88:** 639–642

Powell AA, Krezdorn AH (1977) Influence of fruit-setting treatment on translocation of ^{14}C-metabolites in citrus during flowering and fruiting. J Am Hortic Sci **102:** 709–714

Rood S, Hedden P (1994) Convergent pathways of gibberellin A (1) biosynthesis in *Brassica*. Plant Growth Regul **15:** 241–246

Schneider H (1968) The anatomy of *Citrus*. *In* W Reuther, LD Batchelor, HJ Webber, eds, Vol 2. The Citrus Industry, University of California, Berkeley, pp 1–23

Sjut V, Bangerth F (1981) Effect of pollination or treatment with growth regulators on levels of extractable hormones in tomato ovaries and young fruits. Physiol Plant **53:** 76–78

Soost RK, Burnett RH (1961) Effect of gibberellin on yield and fruit characteristics of Clementine mandarin. Proc Am Hortic Sci **77:** 194–201

Tadeo FR, Talon M, Germain E, Dosba F (1994) Embryo sac development and endogenous gibberellins in pollinated and unpollinated ovaries of walnut (*Juglans regia*). Physiol Plant **91:** 37–44

Talon M, Hedden P, Primo-Millo E (1990a) Gibberellins in *Citrus sinensis*: a comparison between seeded and seedless varieties. J Plant Growth Regul **9:** 201–206

Talon M, Koornneef M, Zeevaart AD (1990b) Accumulation of C_{19}-gibberellins in the gibberellin-insensitive dwarf mutant *gai* of *Arabidopsis thaliana* (L.) Heynh. Planta **182:** 501–505

Talon M, Koornneef M, Zeevaart AD (1990c) Endogenous gibberellins in *Arabidopsis thaliana* and the possible steps blocked in the biosynthetic pathways of the semi-dwarf *ga4* and *ga5* mutants. Proc Natl Acad Sci USA **87:** 7983–7987

Talon M, Zacarias L, Primo-Millo E (1990d) Hormonal changes associated with fruit set and development in mandarins differing in their parthenocarpic ability. Physiol Plant **79:** 400–406

Talon M, Zacarias L, Primo-Millo E (1992) Gibberellins and parthenocarpic ability in developing ovaries of seedless mandarins. Plant Physiol **99:** 1575–1581

Talon M, Zeevaart JAD (1990) Gibberellins and stem growth as related to photoperiod in *Silene armeria* L. Plant Physiol **92:** 273–282

Talon M, Zeevaart JAD, Gage DA (1991) Identification of gibberellins in spinach and effects of light and darkness on their levels. Plant Physiol **97:** 1521–1526

Turnbull CGN (1989) Identification and quantitative analysis of gibberellins in *Citrus*. J Plant Growth Regul **8:** 273–282

Zacarias L, Talon M, Ben-Cheikh W, Lafuente MT, Primo-Millo E (1995) Abscisic acid increases in non-growing and paclobutrazol-treated fruits of seedless mandarins. Physiol Plant **95:** 613–619

Zeevaart JAD, Gage DA, Talon M (1993) Gibberellin A_1 is required for stem elongation in spinach. Proc Natl Acad Sci USA **90:** 7401–7405