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Changes in ABA, IAA and GAs Contents in Reproductive Organs of Satsuma Mandarin

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Summary

The endogenous levels of abscisic acid (ABA), indole-3-acetic acid (IAA) and gibberellins (GAs) in reproductive organs of satsuma mandarin (*Citrus unshiu* Marc. cv. Ozaki) were determined by gas chromatograph (GC)-electron capture detector, GC-mass spectrometry-selective ion monitoring and dwarf rice bioassay. Their probable roles are discussed.

1. As anthesis approached, IAA concentration in styles and stamens did not change, while ABA concentration of styles increased six-fold. Until 39 days after flowering (DAF), the fresh weigth of fruitlets increased dramatically, while that of 'sepals' which included floral disks, sepals and receptacles increased a little.

2. Fruitlets attained their maximum concentration of ABA 4 DAF and then declined rapidly. Its level remained much lower than that of 'sepals' from 7 DAF. IAA concentrations of fruitlets reached a maximum 7 DAF, and thereafter decrease ; that in the 'sepals' increased from anthesis to 31 DAF surpassing that of fruitlets about 15 DAF.

3. GAs concentrations of fruitlets continued to increase from 7 to 31 DAF, and decreased sharply 39 DAF.

4. Because there was a sequence of peaks of endogenous ABA, IAA and GAs levels in fruitlets, it seemed that these hormones may play a sequential and synergistic role in the retention and growth of fruitlets.

Introduction

Seedless fruit has long been desired in the citrus industry, thus elucidation of the mechanism of parthenocarpy is important. Fruit set and growth, which are related to parthenocarpy and believed to be regulated by several phytohormones (Schwabe and Mills, 1981), are dramatic reproductive processes.

Takahashi et al. (1975) reported the peaks of abscisic acid (ABA) and indole-3-acetic acid (IAA) concentrations in fruitlets of satsuma mandarin. Garcia-Papi and Garcia-Martinez (1984) suggested that different types of hormones may be involved in fruit-set based on studies of auxin- and GA-like substances and ABA of seeded and seedless mandarin. Talon et al. (1990) analyzed seedless mandarins of two related species for GAs and free- and conjugated-ABA and IAA, and suggested that the potential for setting parthenocarpic fruits was mainly influenced by the hormonal status.

As to parthenocarpy induced by phytohormone application, Naylor (1984) made a generalized statement that whereas auxins are most effective in inducing parthenocarpy in multiseeded fruits, gibberellins (GAs) are influential in fruits with a few ovules. Synthetic auxin application to the ovaries of satsuma mandarin did not affect fruit set, but did affect fruit growth (Guardiola and Lázaro, 1987). GA application promoted fruit set in mandarin (Brosh and Monselise, 1977). Powell and Krezdorn (1977) reported that GA_3 application in citrus flowers promoted mobilization of metabolites into young ovaries.

Recently, promotive effects of ABA in sink tis-

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sue (Hein et al., 1984 ; Schussler et al., 1984 ; Kojima, 1995 ; Kojima et al., 1995b) and in phloem unloading (Clifford et al., 1986 ; Ross et al., 1987) were reported, although ABA plays a negative role in reproductive development (Walton, 1980). However, roles of phytohormones in fruit set and development remain obscure and no prior studies have measured ABA, IAA and GAs simultaneously in fruitlets and the sepal region.

The present work traced the changes in ABA, IAA and GAs levels in fruitlets and the sepal region and proposes their possible relationships to fruit set and growth in satsuma mandarin which has a high degree of natural parthenocarpy.

Materials and Methods

Plant material

Three 20-year-old trees of satsuma mandarin (*Citrus unshiu* Marc. cv. Ozkai) grafted on trifoliate orange (*Poncirus trifoliata* Raf.) growing at the Kuchinotsu Branch were used for all the experiments. Flowers and fruitlets developing adjacent to the newly developing spring vegetative flush were used. Twenty flowers were tagged to observe fruit set and growth (diam.). For sampling and observation of parthenocarpic fruitlets, flower buds were covered with paper bags just before flowering. At least 60 organs for each analysis point were sampled −5, 0, 4, 7, 15, 23, 31 and 39 days after flowering (DAF) for the hormonal analysis. After sampling, the reproductive organs were immediately separated into parts. The 'sepals' include floral disks, sepals and receptacles. All separation procedures were performed in a chilling room (10 °C). The separated tissues were immediately frozen in liquid nitrogen, weighed, lyophilized and stored at −75 °C unitl analyzed.

Purification and fractionation of plant hormones

Hormonal fractionation was performed according to Kojima et al. (1995a) by adding soluble polyvinylpyrrolidone (K-30), ^{3}H-ABA and ^{13}C-IAA to the sample and homogenizing the mixture in 80 % ethanol. After the filtrate was evaporated to the aqueous phase, the pH was adjusted to 2.5. The acidified phase was filtered through membrane filters (0.22 μm pore size). The aqueous filtrate was partitioned 3 times against petroleum ether and then was partitioned 3 times against diethyl ether. The organic layer was evaporated to dryness.

Dried extract was dissolved in 25 % CH_3CN, and was fractionated with a high performance liquid chromatography (HPLC) system equipped with an ultraviolet and fluorescence detector. The temperature of the HPLC column (Inertsil ODS-2, 150×6.0 mm, GL Sciences Inc. Tokyo) was maintained at 40 °C. The sample was eluted with 25 % and 50 % CH_3CN solutions (20 mM acetic acid) at a flow rate of 1.6 m*l*/min. The starting solvent, 25 % CH_3CN was eluted isocratically for a retention time of 12 min, increased linearly to 50 % for 4 min, then held at 50 % for 30 min, decreased linearly to 25 % for 2 min and held at 25 % for 7 min. The effluent corresponding to the retention time of ABA and IAA was collected and was methylated with diazomethane. The remaining effluent was collected for 22 min for GAs analysis.

The water phase after the diethyl ether extraction was partitioned 3 times against ethyl acetate. The ethyl acetate layer was partitioned 2 times against 0.5 M K_2HPO_4. The pH of the aqueous phase was adjusted to 2.5 and was partitioned 2 times against ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 over night. The dried ethyl acetate layer and the effluent except for ABA and IAA fractions in the ether extract was combined and purified by a Sepralyte DEA column. The purified extract was injected into HPLC at the above mentioned conditions, then effluent was collected for 22 min for GAs analysis.

ABA and IAA analysis

The methylated ABA fraction was injected into a gas chromatograph (GC) equipped with a ^{63}Ni electron capture detector and a fused silica glass capillary column (Kojima et al., 1994b). A portion of methylated sample was injected into the HPLC system to isolate methylated ABA. The radioactivity of the collected fraction was measured in a scintillation counter. Data were corrected according to recovery rates.

The methylated IAA fraction was injected into GC equipped with mass spectrometry using the split-less technique using a fused silica capillary column (Kojima et al., 1994b). IAA content was calculated by the methods of Cohen et al. (1986) using [$^{13}C_6$] IAA as an internal standard.

Determination of GAs activity

The bioassay procedure used was similar to the 'modified micro-drop bioassay' (Nishijima and Katsura, 1989). Seeds of the dwarf rice cultivars (*Oryza sativa* L., cv. Tan-ginbozu) were sterilized and soaked in water, containing S-3307 for 24 hr at 30 °C. When the coleoptiles were ca. 2 mm in length after germination, seedlings were planted on 0.8 % (w/v) agar. Dry samples were diluted successively 3 times with 50 % acetone (Kojima et al., 1995a). After application of the standard (GA_3) and each of gradually diluted samples in 50 % acetone, seedlings were incubated under continuous irradiation for 48 hr at 32 °C. Ten seedlings were used for one determination of GAs activity. The highest value of the detectable levels obtained from gradually diluted samples was ascribed as the GAs value.

Results and Discussion

Growth and phytohormones of bud and flower

Table 1 shows fresh weights and ABA, IAA and GAs concentrations in the three parts of flower buds and flowers. Each part increased in fresh weight from −5 to 0 DAF. ABA concentration of the style increased six fold from −5 DAF to anthesis ; it was the highest of the three floral parts. ABA concentration in styles of parthenocarpic varieties of citrus was reported to increase toward flowering in 'Shamouti' orange (Goldschmidt, 1980) and in 'Washington' navel orange (Harris and Dugger, 1986). Our results confirm this tendency in satsuma mandarin. Although Goldschmidt (1980) suggested that the tendency may be related to the hypothesis that the style is a sensor organ and leads toward wilting of all floral organs after successful pollination (Gillissen, 1976), the exact role for ABA in the style is yet not clear.

There was no change in IAA concentrations of stamens from −5 to 0 DAF (Table 1). Kojima et al. (1994a) reported that in tomato IAA concentration of stamens increased more than four times from −5 to 0 DAF and suggested that the rapid increase in IAA might be related to maturation of the pollen within an anther. In Hyuganatsu (*Citrus tamurana* Hort. ex. Tanaka) which forms normal pollen, IAA concentration of stamens doubled from the bud to the flower stage (Kojima, 1996). Thus, the lack of change in IAA of stamens in satsuma mandarin may be due to aborted anthers. GAs concentration of styles decreased to one-fourth toward flowering (Table 1), but its physiological significance is not understood.

Growth of reproductive organ

The growth of citrus fruit can be divided into three stages (Bain, 1958): a) cell division and slow growth, b) cell enlargement and rapid growth and c) maturation stage. During this experimental period, the growth stage of satsuma mandarin corresponded to the first stage, cell division and slow growth. Figure 1A shows changes in the retention number and diameter of fruitlets. The fruitlets continued to drop until 39 DAF. Because only inflorescences with leaves were used in this experiment, no peak of the fruitlet drop was observed. The diameter and fresh weight of ovaries or fruitlets increased dramatically, but that of 'sepals' increased slightly (Fig. 1B).

Table 1. Fresh weights and ABA, IAA and GAs concentrations in three parts of flower buds 5 days before flowering (−5 DAF) and flowers at anthesis (0 DAF)

Part	FW (mg/organ)		ABA[z] (pmol/gFW)		IAA[z] (pmol/gFW)		GAs (pmol GA_3eq./gFW)	
	−5 DAF	0 DAF	−5 DAF	0 DAF	−5 DAF	0 DAF	−5 DAF	0 DAF
Styley	23	38	470 ± 10	3100 ± 190	72 ± 7	69 ± 6	6.1	1.6
Stamen	72	94	910 ± 30	700 ± 120	51 ± 6	52 ± 5	0.5	0.9
Petal	209	330	290 ± 9	410 ± 39	55 ± 16	38 ± 6	1.3	0.9

[z]All values of ABA and IAA levels are means ± SE of three determinations.
[y]Styles include stigmas.

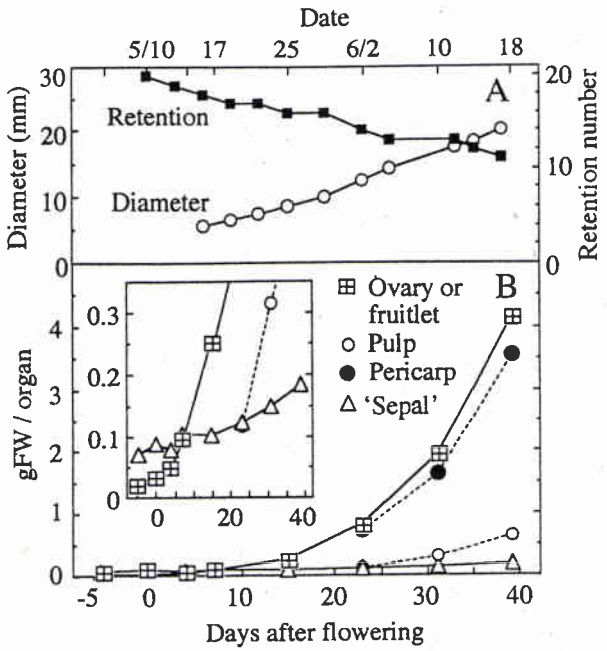

Fig. 1. Changes in retention number of fruit set (squares) and diameter (circles) of reproductive organs (A) and fresh weight of the developing reproductive organs (B) of sastuma mandarin. 'Sepals' include floral disks, sepals and receptacles. The upper horizontal axis shows sampling date.

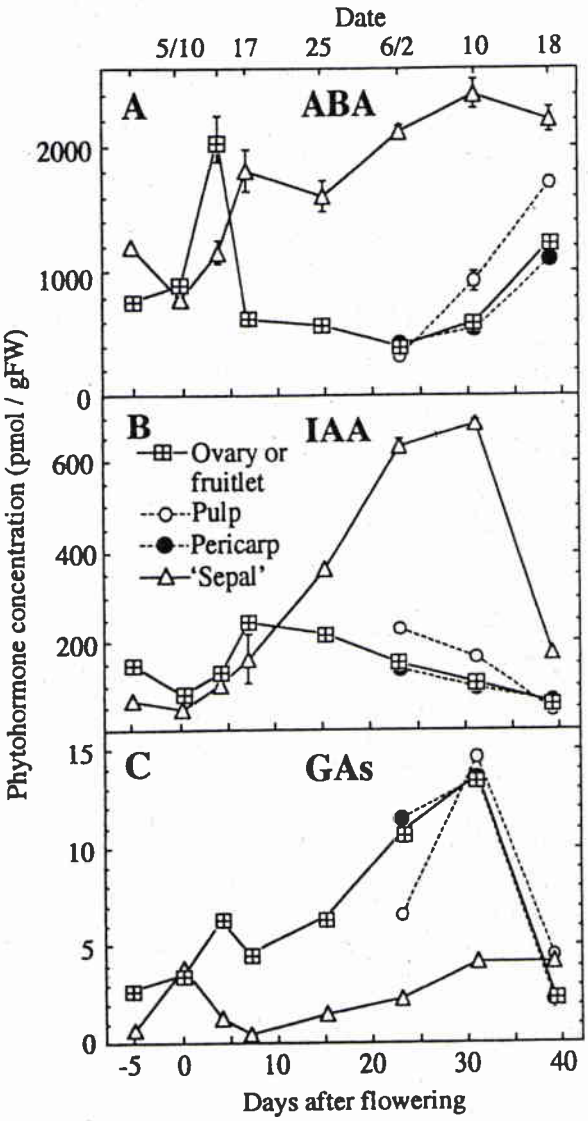

Fig. 2. Changes in concentrations of ABA (A), IAA (B) and GAs (C) of the developing reproductive organs. GAs are expressed as GA_3 equivalents detected by the dwarf rice bioassay. For ABA and IAA, means of three determinations and their SE are shown (n = 3), and where vertical bars are not shown the limits are within the dimensions of symbols. The fruitlet values are calculated using the pulp and pericarp values from 23 to 39 DAF.

Phytohormones of reproductive organ

ABA, IAA and GAs concentrations in the developing reproductive organs fluctuated (Fig. 2). ABA concentration of fruitlets was the highest 4 DAF, declined rapidly 7 DAF, thereafter maintained almost the same levels, and increase again to 31 DAF (Fig. 2A). ABA concentration in 'sepals' which increased from 0 to 7 DAF, maintained higher levels than in fruitlets from 7 to 39 DAF.

ABA has been considered to play a negative role in reproductive development (Walton, 1980). Fruit drop of citrus grown in the field increased after ABA injection proportional to the ABA concentration (Kojima et al., 1995b). In laboratory experiments with fruit explants, ABA also induced fruit drop (Rasmussen, 1974 ; Kojima et al., 1995b). However, no direct relationship between ABA levels in fruitlets and 'sepals' and fruitlet abscission was observed in this experimental (Fig. 1A and 2A). On the other hand, promotive effects of ABA in sink tissue (Hein et al., 1984 ; Schussler et al., 1984 ; Kojima, 1995 ; Kojima et al., 1995b) and in phloem unloading (Clifford et al., 1986 ; Ross et al., 1987) have been reported. Brenner et al. (1989) proposed that ABA functions as a promoter of sink activity and as a stimulator of unloading of assimilates from phloem into the sink. The fruitlets during cell division stage need substrates so that if this theory is applied, ABA may promote sink activity in fruitlets

4 DAF and after 31 DAF, thereby hastening accumulation of assimilates in 'sepals' from 7 DAF.

IAA concentration of fruitlets reached a maximum 7 DAF, and thereafter continued to decrease until 39 DAF (Fig. 2B). Takahashi et al. (1975) assayed auxin content of satsuma mandarin by Avena curvature test and reported that there was always a maximum peak of acidic auxins five to ten days after full bloom. Our results confirm this earlier report.

In 'sepals', IAA concentration increased from anthesis to 31 DAF, and then decreased sharply 39 DAF. To our knowledge this is the first report of changes of IAA in 'sepals'. Contrary to expectations that IAA concentrations in 'sepals' were always lower than in fruitlets, these results showed that IAA concentrations in 'sepals' were higher than those in fruitlets from 15 DAF. Huberman and Goren (1979) reported that auxin inhibited polygalacturonase and cellulase activities in abscission zones and postponed fruit drop. IAA applied via the peduncle markedly retarded the abscission between the fruitlet and 'sepal' of citrus explants (Iwahori et al., 1990). Thus there is a possibility that IAA from 'sepals' may inhibit hydolylase activity and hinder abscission.

GAs concentration of fruitlets increased slightly 4 DAF ; it thereafter continued to increase from 7 to 31 DAF, but decreased sharply 39 DAF (Fig. 2C). In 'sepals', GAs concentration showed a peak 0 DAF and thereafter tended to increase gradually from 7 to 39 DAF. Powell and Krezdorn (1977) reported that localized GA application on pistils of citrus flowers promoted the mobilization of assimilates into young ovaries. Thus GAs in fruitlets may play a role in the accumulation of assimilates.

As for endogenous production of hormones in relation to fruit development, Schwabe and Mill (1981) suggested that there might be either a sequence of phytohormone production, or different phytohormones peaked at different stages of development. In fruitlets of satsuma mandarin, there was a sequence of peaks of phytohormone levels: ABA and GAs, at 4 DAF ; IAA, at 7 DAF ; GAs, at 31 DAF ; and ABA, from 39 DAF. Thus it seems that endogenous phytohormones in fruitlets of satsuma mandarin may play a sequential and synergistic role in the retention and growth of fruitlets.

Regarding the distribution of phytohormones, ABA concentrations were higher in pulp than in pericarp from 31 DAF (Fig. 2A). Similarly, IAA concentrations were higher in pulp than in pericarp 23 and 31 DAF (Fig. 2B). However, GAs concentration was higher in pericarp compared to the pulp 23 DAF, but at 31 and 39 DAF it was slightly higher in the pulp than in the pericarp (Fig. 2C).

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ウンシュウミカンの生殖器官におけるABA，IAAおよびGAs含量の変化

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摘　要

温州ミカンの生殖器官の内生植物ホルモンの生理的役割を明らかにするため，アブシジン酸（ABA），インドール酢酸（IAA）およびジベレリン（GAs）を，それぞれ，ガスクロマトグラフ（GC）電子捕獲型検出器，GC－質量分析器（選択的イオンモニタリング）およびわい性イネ生物検定で測定した．

1，開花にむけて，花柱と雄ずいのIAA濃度は変化しなかったが，花柱のABA濃度は6倍に増加した．幼果の新鮮重量は39 DAF（開花後日数）まで，非常に増加し，'がく'（花盤，がく，花床を含む）はわずかに増加した．

2，4 DAFに，幼果は本実験中で最大のABA濃度のピークを示したが，7 DAF以降は'がく'よりも低い値であった．幼果のIAA濃度は，7 DAFに最高に達して，その後は低下し続けた．'がく'のIAA濃度は，0から31 DAFまで増加し，15 DAF以降は幼果よりもはるかに高い値であった．

3，幼果のGAs濃度は，7から31 DAFまで増加し続け，39 DAFに急に低下した．

4，このように幼果で，一連の内生ABA，IAAおよびGAs量のピークがあったので，これらホルモンが，順次におよび共働的に，幼果の保持と生長に役割をはたしていると思われる．