Interactions between Jasmonates and Absciscic Acid in Apple Fruit, and Stimulative Effect of Jasmonates on Anthocyanin Accumulation

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Summary

The interactions between jasmonates [jasmonic acid (JA) and methyl jasmonate (MeJA)] and absciscic acid (ABA) were investigated in 'Tsugaru' apple (*Malus pumila* Mill. var. *domestica* Schneid.). In Exp. 1, discs from pulp excised 93 days after full bloom (DAFB) (preclimacteric), 112 DAFB (climacteric), and 133 DAFB (postclimacteric) were collected and placed in petri dishes containing a solution of 0.4 M mannitol with a combination of MeJA, ABA, and aminoethoxyvinylglycine (AVG). Jasmonates, ABA, and ethylene levels in the discs were measured at 0, 24, 48, and 72 hr after the initiation of treatment. At the climacteric and postclimacteric stages, the ABA treatment increased endogenous JA concentration and decreased it when combined with AVG. This result suggests that ethylene may be related to the induction of endogenous JA by ABA. MeJA treatment decreased endogenous ABA concentration from 93 to 133 DAFB, whereas AVG had no influence. This result also demonstrates that jasmonates may influence endogenous ABA synthesis independently from ethylene. In Exp. 2 when discs with skins from the apple pulp samples from preclimacteric, climacteric, and postclimacteric stages were excised, cultured for 7 days under light on B5 media containing MeJA, and the combination of MeJA and AVG was investigated, MeJA and MeJA combined with AVG stimulated greater anthocyanin accumulation in both sampling stages compared to the untreated control; the combination of MeJA and AVG resulted in a lower concentration of anthocyanin than that of only MeJA at the preclimacteric and postclimacteric stages. Therefore, in most cases, jasmonates may be related to anthocyanin formation in apples in the presence and absence of ethylene.

Key Words: absciscic acid, anthocyanin, jasmonic acid, *Malus pumila*, methyl jasmonate.

Introduction

Jasmonic acid (JA), methyl jasmonate (MeJA), and absciscic acid (ABA) have been shown to promote leaf abscission (Miyamoto et al., 1997), stomatal closure (Riov et al., 1990), leaf yellowing (Tsai et al., 1996), and inhibition of plant growth (Tsai et al., 1997). The investigation of these substances and ethylene has shown that: 1) ABA treatment at lower concentrations promoted ethylene production in mature apple fruit (*Malus pumila* var. *domestica*), but higher concentrations inhibited it (Tan and Thimann, 1989); 2) MeJA application in tomato (*Lycopersicon esculentum* Mill.) stimulated ACC oxidase activity, and resulted in an increase of ethylene production, whereas the MeJA treatment of apple fruit after the climacteric stage inhib-

ited ACC oxidase activity and ethylene production (Saniewski et al., 1987a, b).

A lipoxygenase-like enzyme which is involved in the biosynthesis of ABA from carotenoids (Creelman et al., 1992a; Parry and Horgan, 1991) was enhanced by MeJA treatment of apple (Olias et al., 1992). Jasmonates may increase endogenous ABA concentration, but that possibility has not been explored with apple. Whether or not an interaction between jasmonates and ABA involves ethylene has also not been determined. Analyses of interactions among these substances which have similar functions in the fruit may explain their roles in fruit development and maturation.

We reported that JA and MeJA concentrations increased in maturing apple (Kondo et al., 2000) which demonstrates that jasmonates, with ABA and ethylene, may play a role in changes that occur during fruit maturation. MeJA application enhanced degreening in apple fruit (Fan and Mattheis, 1999). Although ethylene and ABA promote anthocyanin biosynthesis (Gaynor and Cowan, 1995; Morgan, 1992), little is known about the effect of jasmonates on anthocyanin accumulation. The objective of this study is to clarify the interactions

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between jasmonates and ABA at the preclimacteric, climacteric, and postclimacteric growth stages, and their effects on anthocyanin accumulation.

Materials and Methods

Plant material

‘Tsugaru’ apple fruit were obtained from fifteen randomly selected 11-year-old trees grafted onto Malling 26 (M. 26) rootstocks growing in the Hiroshima Prefectural University orchard.

Experiment 1. Interactions between jasmonates and abscisic acid

Samples were collected at 93 DAFB (preclimacteric), 112 DAFB (climacteric), and 133 DAFB (postclimacteric). Discs (11 mm diam. and 3 mm thick), from apple pulp were excised with a cork borer. Groups of 20 discs were floated in petri dishes containing 10 ml 0.4 M mannitol, containing different combinations of: 10 μ M MeJA, 10 μ M ABA, 10 μ M MeJA with 20 μ M AVG (an inhibitor of ACC synthase) (Morgan, 1992), and 10 μ M ABA with 20 μ M AVG to determine the interactions between jasmonates and ABA. MeJA was used in experiments 1 and 2 because it more strongly inhibited plant growth than did JA when treated exogenously (Takeuchi and Kamuro, 1997). The control treatment contained only 10 ml 0.4 M mannitol. Discs from each fruit were randomly selected as replicates. There were 20 discs from three fruit in each petri dish and 30 petri dishes per treatment. Plates were sealed with parafilm and kept in the incubator at 20 °C in the dark. At 0, 24, 48, and 72 hr after the initiation of treatment, JA, MeJA, ABA concentrations, and ethylene production were measured.

Extraction and analysis of jasmonates

Extraction and quantification of JA and MeJA were performed with a modification of the method of Muller et al. (1993) previously reported by Kondo et al. (2000). Fruit discs (10 gFW; five replications of discs selected randomly) were homogenized with 1 μ g ($\pm$)-2-(2,3- 2 H₂) JA and ($\pm$)-2-(2,3- 2 H₂) MeJA as the internal standards in a 20 ml saturated NaCl solution, 1 ml 1 M citric acid and 50 ml diethyl ether containing 11.3 μ M butylated hydroxytoluene as an antioxidant. The ether phase was removed after centrifugation (10 min at 2000 $\times$ g); the aqueous layer was extracted a second time with 50 ml diethyl ether containing 11.3 μ M butylated hydroxytoluene. N₂ was then used to dry the pooled ether extracts. The residue was dissolved in 200 μ l chloroform/diisopropylethylamine, 1:1 (v/v), and derivatized 60 min at 50 °C with 10 μ l pentafluorobenzyl (PFB) bromide. The derivatized mixture was concentrated with N₂.

The residue was then dissolved in 5 ml *n*-hexane and added onto a silica gel column (5 mm i.d. $\times$ 14 cm) [250

mg of silica gel 60 (Merck KGaA, Darmstadt, Germany)]. The sample was eluted with 7 ml *n*-hexane/ether, 2:1 (v/v), dried under N₂, re-dissolved in 1 ml methanol, and applied to a sep cartridge carbograph column [150 mg in 3 ml (GL Sciences, Inc., Tokyo)]. The sample was concentrated under N₂, re-dissolved in 30 μ l methanol, and 1 μ l was analyzed by gas chromatography-mass spectrometry (GC-MS) [QP 5000; Shimadzu Scientific Instruments, Inc., Kyoto, Japan; column = CP-Sil 5 CB (Chrompack, Inc., Middelburg, Netherlands; 0.25 mm i.d. $\times$ 25 m, 0.25 μ m film thickness); linear He flow at 50.2 cm $\cdot$ sec⁻¹, column temperature step gradient, 60 °C for 2 min, 60 to 270 °C at 10 °C $\cdot$ min⁻¹, and 270 °C for 35 min; electron potential, 70 eV]. The quantitative analyses were carried out in the selected ion monitoring mode.

Retention times of the PFB derivatives are as follows: *trans*-JA, 19.90 min; 2 H₂-JA, 19.90 min, *trans*-MeJA, 14.15 min; 2 H₂-MeJA, 14.15 min. Ions were monitored for JA / [2 H₂]($\pm$)-JA as follows: *m/z* 392, 390, 211, and 209. The concentration of JA in the original extract was determined from the ratio of peak areas for *m/z* 209 (2 H₀) / 211 (2 H₂). For MeJA / [2 H₂]($\pm$)-MeJA, with ions monitored as follows: *m/z* 226, 224, 153, and 151. MeJA concentration was measured from the ratio of peak areas for *m/z* 224 (2 H₀) / 226 (2 H₂). The fragmentation patterns were compared to those [*trans*-PFB-JA: *m/z* 390 (39) (M⁺), 322 (14), 209 (11), 191 (11), 181 (100), 141 (68); *trans*-PFB-MeJA: *m/z* 224 (24) (M⁺), 181 (88), 151 (39), 135 (43), 109 (29), 83 (100)] of the chemical standards to identify PFB-jasmonates within the samples.

Extraction and analysis of abscisic acid

Fruit discs (10 gFW; five replications of discs selected randomly) were homogenized with 1 μ g ($\pm$)-2 *cis*, 4-*trans*-*d*₆ ABA as the internal standards in 80% methanol. The extract was filtered and reduced to the aqueous phase in vacuo and adjusted to pH 2.5 with 0.1 M phosphoric acid. ABA was extracted with dichloromethane; the solvent was evaporated in vacuo and the residue re-dissolved in 4.8 M acetonitrile. ABA was quantified by high-performance liquid chromatography (HPLC) (Gulliver series; Japan Spectroscopic Co., Tokyo) as follows; column = Mightysil RP-18 (Kanto Chemical Co., Inc., Tokyo; 4.6 mm i.d. $\times$ 25 cm); mobile phase of acetonitrile with 20 mM acetic acid (4.8 M to 9.6 M acetonitrile over 30 min, then held at 9.6 M for 5 min); flow rate of 1.5 ml $\cdot$ min⁻¹; detector = UV 254 nm. The fraction responsible for the retention time of ABA standard was gathered, dried in vacuo, then methylated with diazomethane. The ABA-methyl ester was re-dissolved with methanol and quantified by GC-MS similarly with JA and MeJA. Retention times of the ABA-methyl esters are as follows: *cis*-ABA, 19.50 min; 2 H₆-ABA, 19.50 min. Ions were monitored for ABA / [2 H₆]($\pm$)-ABA as follows: *m/z* 194, 190, 166, and 162. The

concentration of ABA in the original extract was determined from the ratio of peak areas for m/z 190 ($^2\text{H}_0$) / 194 ($^2\text{H}_6$). To identify ABA-methyl esters in the samples, the fragmentation patterns were compared with those [m/z 278 (1) (M^+), 260 (3), 190 (100), 162 (45), 134 (52), 125 (57)] of the chemical standards.

Ethylene production

Five replications of six discs selected randomly (about 2 gFW) were rinsed with 0.4 M mannitol, then sealed in 20 ml flasks containing 2 ml 0.4 M mannitol for 2 hr at 20 °C. One ml headspace gas, containing ethylene, was analyzed by gas liquid chromatography (GLC) (GC-380; GL Sciences, Inc., Tokyo; column = Porapak Q, 2.2 mm i.d. $\times$ 2.0 m).

To confirm the growth stage of the fruit, internal ethylene concentrations during fruit development (80 DAFB to 133 DAFB) were measured from five fruit. One ml air sample from the core of the fruit was removed with a syringe and injected into GLC.

Experiment 2. The effect of jasmonates on anthocyanin accumulation

Similarly with experiment 1, samples were collected at 93 DAFB, 112 DAFB, and 133 DAFB. Apple fruit samples were bagged to avoid anthocyanin formation on the tree from 60 DAFB until each sampling date. Discs with skins, were excised with a cork borer as above. Ten discs on 30 ml B5 medium, containing 0.08 M sucrose as in experiment 1, were cultured in a petri dish. The control discs were kept isolated in 30 ml B5 medium containing 0.08 M sucrose. The 10 discs representing three fruit were randomly selected as a replicate, per petri dish; there were six dishes per treatment. Petri dishes were sealed with parafilm, and cultures were maintained at 20 °C under white fluorescent lights to give $120 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$. After 7 days, anthocyanin concentrations in the skins were measured. Skin samples were separated from the pulps and the anthocyanin concentration determined by extracting from skins of

Fig. 1. Changes of internal ethylene concentrations during 'Tsugaru' apple fruit development. Data are the means $\pm$ SE of five replications.

equal weight in 1% HCl-methanol and measuring absorbance at 530 nm by a spectrophotometer (U-2001; Hitachi Ltd., Tokyo). Anthocyanin is expressed as cyanidin 3-galactoside equivalents.

Statistical analysis

Results were analyzed using the Newmen-Keuls test (SPSS, Inc., Chicago, USA)

Results and Discussion

Experiment 1. Interactions between jasmonates and abscisic acid

An internal ethylene concentration in the fruit was almost undetected at 93 DAFB, but it increased gradually after 101 DAFB. It reached a peak at 125 DAFB and then decreased (Fig. 1). From these observations, we selected three sampling dates; (A) the preclimacteric

Fig. 2. Effect of MeJA and ABA on ethylene production in 'Tsugaru' apple fruit discs at three stages, (A) preclimacteric, (B) climacteric, and (C) postclimacteric. The discs cultured in 10 μM MeJA (■), 10 μM ABA (◆), (●, control) were sealed in 20 ml flasks containing 2 ml 0.4 M mannitol for 2 hr at 20 °C. Data are the means $\pm$ SE of five replications. Data points within each time (24, 48, and 72 hr) with different letters show significant differences as calculated by Newmen-Keuls test, $P \leq 0.05$. Discs treated with 10 μM MeJA with 20 μM AVG and 10 μM ABA with 20 μM AVG produced no ethylene (data not shown).

stage, (B) climacteric, and (C) postclimacteric.

The effects of MeJA and ABA on ethylene production from fruit discs differed at each sampling stage. At the preclimacteric stage, discs incubated in solutions of MeJA had high ethylene production 24 hr later, but subsequently it decreased below the untreated control (Fig. 2A). ABA treatment increased ethylene production with incubation time. At the climacteric stage, MeJA greatly stimulated ethylene production only for the first 24 hr (Fig. 2B), whereas ABA had no influence on ethylene production for 48 hr, after which production was higher. At the postclimacteric stage, ethylene production from discs of each treatment decreased markedly at 24 hr (Fig. 2C). MeJA decreased ethylene production at 48 hr and 72 hr, compared to the untreated control, while ABA increased it. If plants are wounded, ethylene production increases (Saltveit, 1992). However, a rapid increase of ethylene from pulp discs floated in 0.4 M mannitol was not observed in the untreated control (Fig. 2). Thus, the effect of wound ethylene on

the pulp discs can be ignored. The effect of MeJA on ethylene production at each fruit development stage is similar to those previously reported by Saniewski et al. (1986, 1987a). ABA-induced ethylene production has also been observed in citrus leaves [*Citrus sinensis* (L.) Osbeck] and mature-green tomatoes (Riov et al., 1990). In each sampling stage, ethylene production was not detected in combinations of AVG and MeJA or AVG and ABA (data not presented).

Although ABA treatment had no influence on the *trans*-JA concentration of the discs prepared from preclimacteric fruit, it did enhance its synthesis from 24 to 72 hr at the climacteric stage, and at 24 hr in the postclimacteric period (Fig. 3). Combinations of ABA plus AVG decreased ABA-induced *trans*-JA concentration to the same levels as the untreated control. It has been reported that ABA increases the activity of ACC synthase, an enzyme which AVG inhibits (Riov et al., 1990). Therefore, system II (autocatalytic) ethylene may be connected with the regulation of JA concentrations. Changes in JA and ABA concentrations in apple fruit

Fig. 3. Effect of ABA on *trans*-JA concentrations in 'Tsugaru' apple fruit discs at three stages, (A) preclimacteric, (B) climacteric, and (C) postclimacteric. The discs were placed in a petri dish containing 10 ml 0.4 M mannitol (●, control) and with 10 μ M ABA (■) and 10 μ M ABA plus 20 μ M AVG (◆) and incubated at 20 °C in the dark. Data are the means $\pm$ SE of five replications. Data points within each time (24, 48, and 72 hr) with different letters show significant differences as calculated by Newman-Keuls test, $P \leq 0.05$.

Fig. 4. Effect of ABA on *trans*-MeJA concentrations in 'Tsugaru' apple fruit discs at three stages, (A) preclimacteric, (B) climacteric, and (C) postclimacteric. The discs were placed in a petri dish containing 10 ml 0.4 M mannitol (●, control) and with 10 μ M ABA (■) and 10 μ M ABA plus 20 μ M AVG (◆) and incubated at 20 °C in the dark. Data are the means $\pm$ SE of five replications. Data points within each time (24, 48, and 72 hr) with different letters show significant differences as calculated by Newman-Keuls test, $P \leq 0.05$.

coincided with changes in respiration and ethylene production (Kondo et al., 2000; Lara and Vendrell, 2000). Jasmonates regulate the transition to autocatalytic ethylene production in climacteric fruit (Saniewski et al., 1986, 1987b). From these results, it can be inferred that the transduction pathway may occur in the following way; system I ethylene stimulates both JA and ABA which, in turn, regulate system II ethylene production, although it is unclear which of the substances, jasmonates or ABA, acts upstream. ABA has no influence on *trans*-MeJA concentration at all stages except for the first 24 hr in preclimacteric (Fig. 4A, B, and C). The combination of ABA and AVG decreased the ABA-induced *trans*-MeJA concentration for 24 hr in the preclimacteric stage. Accordingly, the effect of ABA which induces ethylene production on fruit discs (Fig. 2) may be more sensitive to JA than it is to MeJA because JA concentration was enhanced by ethylene (Creelman et al., 1992b). MeJA treatment decreased ABA concentration in discs for each stage, and the combination of

MeJA with AVG exhibited a similar result (Fig. 5A, B, and C).

Our results on the stimulation of endogenous ABA by the jasmonates supports the idea that the reaction between ABA and jasmonates induces enzyme activity related to ABA biosynthesis (Melan et al., 1993). However, it is not clear whether jasmonates directly regulate ABA levels or regulate them via ethylene. AVG completely blocked ethylene production induced by MeJA (data not presented), but not ABA concentrations (Fig. 5), and the effect of MeJA on ethylene production was different at each sampling stage (Fig. 2). Thus, it is possible that jasmonates may influence ABA biosynthesis unconnected to ethylene. Similarly, endogenous ABA concentrations decreased in MeJA-treated detached rice leaves (Wang and Kao, 1999). Fluridone, an inhibitor of ABA biosynthesis, reduced endogenous ABA levels just as JA failed to induce gene expression, demonstrating that JA required ABA as an intermediate (Hays et al., 1999). Higher concentrations of MeJA inhibited ethylene production in detached rice leaves, whereas a combination of low MeJA concentration and ABA yielded a similar result (Chou and Kao, 1992). Hence, MeJA and ABA may have a cooperative role in fruit development.

Experiment 2. The effect of jasmonates on anthocyanin accumulation

MeJA stimulated anthocyanin formation at the preclimacteric, climacteric, and postclimacteric stages (Fig. 6). Although the combination of MeJA and AVG reduced anthocyanin formation at the preclimacteric and postclimacteric stages, its concentration at the climacteric stage reached almost the same levels as the MeJA treatment alone.

Fig. 5. Effect of MeJA on ABA concentrations in 'Tsugaru' apple fruit discs at three stages, (A) preclimacteric, (B) climacteric, and (C) postclimacteric. The discs were placed in a petri dish containing 10 ml 0.4 M mannitol (●, control) and with 10 μ M MeJA (■) and 10 μ M MeJA plus 20 μ M AVG (◆) and incubated at 20 °C in the dark. Data are the means $\pm$ SE of five replications. Data points within each time (24, 48, and 72 hr) with different letters show significant differences as calculated by Newman-Keuls test, $P \leq 0.05$.

Fig. 6. Effect of MeJA on anthocyanin accumulation in the skin of 'Tsugaru' apple discs at three stages, (A) preclimacteric, (B) climacteric, and (C) postclimacteric. The discs on B5 medium with or without 10 μ M MeJA and 10 μ M MeJA plus 20 μ M AVG for 7 days at 20 °C under 120 μ mol·m⁻²·sec⁻¹ white fluorescent lights. Data are the means $\pm$ SE of six replications. Data points within each growth stage (preclimacteric, climacteric, and postclimacteric) with different letters show significant differences as calculated by Newman-Keuls test, $P \leq 0.05$.

MeJA has been found to promote chlorophyll degradation in tomato peel (Saniewski et al., 1987b). Our results indicate that MeJA promotes not only chlorophyll disappearance but also anthocyanin accumulation. Growth regulators have been used as effective tools to improve fruit coloration (Kondo and Hayata, 1995), and jasmonates may have a similar effect. In apple fruit, ethylene is closely related to anthocyanin formation. The inhibition of endogenous ethylene concentration in the fruit prevents anthocyanin formation (Kondo and Hayata, 1995). In our study, however, the combination of MeJA and AVG decreased anthocyanin accumulation compared to MeJA alone, but did not greatly inhibit it, which indicates that MeJA may act on anthocyanin formation independent of ethylene. This conclusion is also supported from the result that MeJA inhibited ethylene production of the discs at the postclimacteric stage (Fig. 2). A similar result on anthocyanin accumulation was observed with tulip leaves (Saniewski et al., 1998). Furthermore, MeJA stimulated anthocyanin accumulation, despite the decrease of ABA, which demonstrates that MeJA may affect it independently from ABA and ethylene (Figs. 5, 6).

In summary, ABA enhances *trans*-JA accumulation in apple pulp while the combination of ABA and AVG cancels this effect; the stimulation of endogenous JA by ABA may be caused by ethylene. There is a slight depression of endogenous ABA concentration evoked by MeJA treatment. That the effect of MeJA on ethylene differed at each fruit growth stage indicates that jasmonates may influence the pathway of ABA synthesis independent of ethylene. MeJA generally stimulates anthocyanin accumulation in the skin related to ethylene action because a combination of it and AVG (in the absence of ethylene) increases anthocyanin concentration, compared to the untreated control.

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リンゴ果実におけるジャスモン酸類とアブシジン酸の相互関係およびアントシアニンの蓄積に及ぼすジャスモン酸類の促進効果

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摘 要

リンゴ 'つがる' 果実 (*Malus pumila* Mill. var. *domestica* Schneid.) 中のジャスモン酸類 [ジャスモン酸 (JA) およびジャスモン酸メチルエステル (MeJA)] とアブシジン酸 (ABA) の相互関係について検討した。満開後 93 日 (ブレイクマクテリック), 満開後 112 日 (クライマクテリック) および満開後 133 日 (ポストクライマクテリック) に果肉ディスクを採取し, MeJA, ABA およびこれらとアミノエトキシビニルグリシン (AVG) の組合せを含む 0.4 M マンニトール溶液中に静置した。果肉ディスク中のジャスモン酸類, ABA およびエチレンを, 処理開始時, 24, 48 および 72 時間後に分析した。クライマクテリックおよびポストクライマクテリック果実で, ABA 処理は内生 JA 濃度を増加させ, そして ABA と AVG の組合せはそれを減少させた。この結果は, エチレンが ABA による内生 JA の合成促進に関連している可能性を示唆している。MeJA 処理は果実の発育段階に関わらず, 満開

後 93 日から 133 日まで内生 ABA 濃度を減少させ, また AVG との組合せも同様の結果であった。この結果は, ジャスモン酸類がエチレンとは無関係に内生 ABA 合成に影響する可能性を示す。

ブレイクマクテリック, クライマクテリックおよびポストクライマクテリックのリンゴ果実から果皮の付着した果肉ディスクを採取し, 照明下で 7 日間, MeJA および MeJA と AVG の組合せを含む B5 培地上に置床し, アントシアニン蓄積に及ぼす MeJA の影響を検討した。ブレイクマクテリックおよびポストクライマクテリックの果実で, MeJA と AVG の組合せは MeJA のみに比べてアントシアニン濃度をやや低下させたが, いずれも無処理に比較してアントシアニン蓄積を促進した。ジャスモン酸類はリンゴのアントシアニン生成に, エチレンの大きな影響を受けずに関連している可能性が示唆された。