Effect of Jasmonates Differed at Fruit Ripening Stages on 1-Aminocyclopropane-1-Carboxylate (ACC) Synthase and ACC Oxidase Gene Expression in Pears

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ABSTRACT. The effects of n-propyl dihydrojasmonate (PDJ), which is a jasmonic acid derivative, on 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase activities, their gene expressions, and ethylene productions in ‘La France’ pears (Pyrus communis L.) were investigated. The fruit was harvested 156 days after full bloom, stored at 4°C for 15 days, ripened at 20°C, and then dipped into 0.39 mM PDJ solution at the preclimacteric stage (0 day of ripening at 20°C) or the climacteric stage (9 days of ripening at 20°C). In the skin of the PDJ-treated fruit at the preclimacteric stage, the expressions of ACC synthase (ACS1) and ACC oxidase (ACO1) were higher than the expressions of those in the untreated control. Ethylene production also increased in the PDJ-treated fruit. In contrast, the accumulation of the ACS1 messenger RNA (mRNA) levels in the skin and an increase of ethylene production were observed in the untreated control fruit at the climacteric stage, although the levels of mRNAs hybridized with ACO1 were not different between the PDJ-treated fruit and untreated control. The endogenous jasmonic acid levels in the skin increased in the PDJ-treated fruit regardless of the application times of PDJ. These results indicate that ACS1 may be an ACC synthase gene that is induced by jasmonates in pears, and that system 2 ethylene may be regulated by jasmonates.

Ethylene is associated with various responses that plants show in germination, ripening, wounding, and environmental stress (Abeles et al., 1992). The synthetic pathway and signal transduction of ethylene have been investigated, and aminoethoxyvinylglycine (AVG), which is a 1-aminocyclopropane-1-carboxylate (ACC) synthase inhibitor (Lieberman, 1979), along with 1-methylcyclopentene (1-MCP), which blocks ethylene receptors and retards ethylene action (Blankenship and Dole, 2003), have been developed. Ethylene production is also influenced by other phytohormones. For example, the application of methyl jasmonate (MeJA) inhibited the ACC content, ACC oxidase activity, and ethylene production in grain amaranth (Amaranthus caudatus L.) seeds (Kepczynski et al., 1999), but increased it in sunflower (Helianthus annuus L.) seedlings (Emery and Reid, 1996). The interactions between ethylene and jasmonates have been also reported in fruit. Applying MeJA at the preclimacteric stage increased ethylene production in apples [Malus sylvestris (L.) Mill. var. domestica (Borkh.) Mansf.] (Saniewski et al., 1988), but production decreased when MeJA was applied at the climacteric stage (Miszczak et al., 1995).

Auxin had an effect on ACC synthase in the ethylene pathway. For example, auxin treatment increased ACS2 messenger RNA (mRNA) levels in melons (Cucumis melo L.) (Ishiki et al., 2000) and ACS3 and ACS5 mRNA levels in tomatoes (Lycopersicon esculentum Mill.) (Coenen et al., 2003). Furthermore, the effects of cytokinin on ACC synthase genes have also been shown (Coenen et al., 2003). Although jasmonates may influence ACC synthase or ACC oxidase genes because they can regulate ethylene production, there have been no studies confirming this. In addition, it is possible that jasmonate treatment, which has a different influence on fruit depending on the fruit ripening stage, can be used to regulate pear fruit ripening. MeJA, which is found in plants, has been used as an exogenous application for plants (Bialecka and Kepczynski, 2003; Chou and Kao, 1992), but it has been reported that n-propyl dihydrojasmonate, an n-propyl ester rather than a methyl ester jasmonic acid (JA) derivative, has a practical effect compared with MeJA (Koshiyama et al., 2006). n-Propyl dihydrojasmonate (PDJ) consists of four kinds of optical isomers including 1R, 2R; 1S, 2S; 1R, 2S; and 1S, 2R. Under field conditions, PDJ appears to have greater stability compared with MeJA. Currently in Japan, PDJ is applied before harvest to promote anthocyanin development in apples. However, the effect of PDJ application on the endogenous JA concentration is unclear. In this study, the effects of PDJ application at the preclimacteric or climacteric stage on the transcription of ACC synthase and ACC oxidase genes, their activities, ACC and 1-malonylamino cyclopropane-1-carboxylic acid (MACC) concentrations, and ethylene production were examined to clarify the roles of jasmonates on system 2 ethylene production in ‘La France’ pear fruit.

Materials and Methods

PLANT MATERIALS. Nine randomly selected 16- to 17-year-old ‘La France’ pear trees growing in an open field at the Prefectural University of Hiroshima, which had been grafted onto ‘Quince C’ (Cydonia oblonga Mill.) rootstock, were used in 2004 and 2005. Similar experiments were performed in 2004 and 2005, but the harvest dates varied between years. Because results from both years were similar, only the 2005 results are reported. Each

ACS1, ACS3, and ACO1.

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The membranes were washed twice in hybridization buffer of DIG Easy Hyb (Boehringer Mannheim) containing 150 mM NaCl and 0.39 mM PDJ (preclimacteric, 0 d of ripening at 20 °C and 90% RH). In the second group, after ethylene production began, fruit were dipped as stated for group 1 (climacteric, 9 d of ripening at 20 °C and 90% RH). In the third group, untreated control fruit were dipped into distilled water including 0.1% (v/v) Approach BI only. Fruit were air-dried after treatment. Thirty fruit were sampled at 3-d intervals after PDJ treatment. Skin samples were separated carefully with a knife, frozen immediately in liquid nitrogen, and then stored at −80 °C until analysis. Because a small amount of flesh remained on the skin, the skin sample should be considered the epidermal tissues of fruit. The ACC synthase and ACC oxidase gene expression, ACC synthase and ACC oxidase activities, ACC and MACC concentrations, ethylene production, fruit firmness, and JA concentrations in the skin samples were analyzed.

**NORTHERN BLOT HYBRIDIZATION.** The fragments used for probes were from base pair (bp) 142 to bp 1566 of the ACS1 clone with accession numberAY 388987, from bp 288 to bp 790 of the ACS3 clone with AY 388988, from bp 148 to bp 641 of the ACS4 clone with AF 386518, and from bp 1 to bp 1542 of the ACO1 clone with X87097 (El-Sharkawy et al., 2004; Lelièvre et al., 1997). The primers used for the probe amplification are as follows:

| ACS 1-F | 5′-AGCTCATGTTATCCAGAAACGCCA-3′ |
| ACS 1-R | 5′-TCATCTACCCTGGATAGACGCCG-3′ |
| ACS 3-F | 5′-TCGTGGCAAAACCCACTTGGA-3′ |
| ACS 3-R | 5′-AATGTACCCCTCTACCTCCTC-3′ |
| ACS 4-F | 5′-CTTGGTTGAAAGATGGTAGTTAG-3′ |
| ACS 4-R | 5′-AGATCAAGCCCTTGACAT-3′ |
| ACO 1-F | 5′-ACTCCATTGTCAAAACCTAG-3′ |
| ACO 1-R | 5′-AGCTCGTGATGGTTCAACAG-3′ |

The specificity of each probe was confirmed by southern blot analysis. Each complementary DNA (cDNA) was used as a probe on gel blots of plasmids cut with EcoRI and HindIII containing other cDNAs, but cross-hybridization was not observed (data not presented). Total RNA isolation and northern blot analysis were performed according to a previously reported method (Kondo et al., 2002). Total RNA of 20 µg was separated by electrophoresis in a 1.2% agarose gel with 0.66 M formaldehyde, and transferred to a nylon membrane. Using the PCR digoxigenin (DIG) probe synthesis kit (Boehringer Mannheim, Mannheim, Germany), the probes were labeled with DIG. Northern membranes were hybridized according to the manufacturer’s instructions. Hybridization was carried out for 16 h at 50 °C in a hybridization buffer of DIG Easy Hyb (Boehringer Mannheim). The membranes were washed twice in [2× SSC (a mixture of sodium chloride and sodium citrate dihydrate)] 150 mM NaCl and 15 mM trisodium citrate (pH, 7.0) and 0.1% (w/v) sodium dodecyl sulfate (SDS) at room temperature for 5 min each, and then washed twice in 0.1× SSC, 0.1% SDS at 68 °C for 15 min each.

**ACC SYNTHASE, ACC OXIDASE ACTIVITY, AND ACC AND MACC ANALYSIS.** 1-Aminocyclopropane-1-carboxylate synthase was measured with a modification of the method reported by Jiang et al. (1994). Skin samples were homogenized in 3 mL g⁻¹ ice-cold extraction buffer 50 mM Tris-HCl buffer with 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) at pH 8.2. The homogenated samples were centrifuged at 10,000 g for 20 min at 1 °C, then the supernatant was decanted and desalted using a Sephadex G25 column (PD-10; Amersham Biosciences, Uppsala, Sweden). For analysis of the ACC synthase activity, a desalted extract of 0.9 mL was placed in a glass tube with 4 mM pyridoxal phosphate, 50 mM s-adenosylmethionine, and 50 mM Tris-HCl buffer containing 1 mM DTT and 1 mM PMSF at a pH of 8.2 of 0.1 mL. 1-Aminocyclopropane-1-carboxylate synthase activity was determined by the formation of ethylene. For determination of ACC oxidase activity, 2-g skin samples were dipped into 15 mL of 10 mM phosphate buffer, 0.4 mM mannitol, and 5 mM ACC in a 50-mL flask. Ethylene in the sample was removed under low pressure for 5 min, then the flask was sealed with closure silicone and placed at 25 °C for 2 h. The ethylene in the head space of the syringe was measured by gas chromatography with flame ionization detection [GC-FID (model GC-380; GL Sciences, Tokyo); 2.2 mm i.d. × 2.0 m column (Porapak Q; Waters, Milford, Mass.); column temperature, 50 °C; He flow rate, 30 mL·min⁻¹]. The ACC concentrations in the skin [2 g fresh weight (FW)] were analyzed by GC-FID according to the method of Lizada and Yang (1979). 1-Malonylaminocyclopropane-1-carboxylic acid (MACC) quantification in the skin was performed using the method of Hoffman et al. (1983).

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**Fig. 1.** Changes of jasmonic acid concentrations and ethylene production in ‘La France’ pears during ripening at 20 °C and 90% relative humidity (RH). The fruit were ripened at 20 °C after storage at 4 °C and 90% RH for 15 d. Each arrow shows the initial date of n-propyl dihydrojasmonate (PDJ) treatment at the preclimacteric or climacteric stage. The data show the mean ± SE of three replications.
Ethylene analysis from whole pears was carried out according to the method previously described by Kondo and Takahashi (1987). Three pears per treatment (three replications) were sealed in glass jars (2 L). Fruit firmness was measured on two pared surfaces of each pear using a rheometer with a 1-mm probe (model NRM-2002J; Fudo, Tokyo).

**ANALYSIS OF JASMOLN ACID.** Determination of JA was performed as described previously by Kondo et al., (2000). The deuterium-labeled JA [(2H2)-JA; (±)-(9, 10-2H2)JA] was prepared from adipic acid through the catalytic semideuterio-ogenation of methyl (±)-9, 10-dehydrojasmonate, which was previously reported by Seto et al. (1996). Skin samples weighing 10 g FW were homogenized with 1 μg (2H2)-JA of the internal standard in 50 mL diethyl ether containing 11.3 μM butylated hydroxytoluene as an antioxidant, 20 mL saturated NaCl solution, and 1 mL 1 M citric acid. Homogenates were centrifuged, then dried. Extracts were dissolved in chloroform/ diisopropylethylamine, 1:1 (v/v), and “derivatized” for 60 min at 50 °C with pentafluorobenzyl bromide (PFB). Analysis of PFB-JA was conducted using GC-mass spectroscopy selected ion monitoring [QP 5000; Shimadzu, Kyoto, Japan; 25 m × 0.25 mm i.d. column (CP-Sil 5CB; Chrompack, Middelburg, The Netherlands); column temperature step gradient, 60 °C for 2 min, 60–270 °C at 10 °C-min⁻¹, and 270 °C for 35 min; linear He flow, 50.2 cm·s⁻¹; electron potential, 70 eV].

**STATISTICAL ANALYSIS.** Experiments were conducted using a randomized complete block design. The data represent mean values of three replications ±SE. The SAS analysis of variance procedure (SAS Institute, Cary, N.C.) was used to determine the treatment effects, and the mean separation was analyzed by Fisher’s least significant difference (P ≤ 0.05).

**Results**

Ethylene production gradually increased from 3 d of ripening at 20 °C and 90% RH (Fig. 1). In contrast, the endogenous JA concentrations decreased until 9 d of ripening, but increased toward 18 d of ripening. In the skin of the PDJ-treated fruit at the preclimacteric stage, the ACC synthase and ACC oxidase activities were higher than those of the untreated fruit skins.
1-Aminocyclopropane-1-carboxylate concentrations at 3 to 6 d after treatment, MACC concentrations at 6 to 9 d after treatment, and ethylene production at 3 to 6 d after treatment in the PDJ-treated fruit were higher than those in the untreated fruit.

n-Propyl dihydrojasmonate treatment also decreased fruit firmness at 6 to 9 d after treatment compared with the untreated control. At the preclimacteric stage, the expressions of ACS1 and ACO1 in the skin of the PDJ-treated fruit were higher than the expressions of those in the skin of the untreated fruit. In contrast, the expressions of ACS3 and ACS4 were not different between the PDJ-treated and untreated fruit. The expression of ACS3 was high at the start of both treatments.

In the skins of the PDJ-treated fruit at the climacteric stage, the ACC synthase activity at 6 to 9 d after treatment, ACC concentration at 3 d after treatment, and ethylene production were lower than those in the untreated fruit. ACC oxidase activity and MACC concentrations were not different between the PDJ-treated and untreated fruit. The endogenous JA concentrations in the PDJ-treated fruit skin were higher than those in the untreated fruit skin at both fruit growth stages (Fig. 4).

Discussion

The endogenous JA concentrations in the ‘La France’ pears increased after 15 d of ripening at 20 °C, 12 d later than the increase of ethylene, as shown in Fig. 1, although both jasmonates and ethylene increased at the almost same time in ‘Tsugaru’ apples (Kondo et al., 2000, 2001b). This result may have been incited by the endogenous ethylene production in ‘La France’ pears, which is lower than in ‘Tsugaru’ apples. In apples, combinations of MeJA and 1-MCP decreased internal ethylene concentrations compared with MeJA only, but increased them compared with 1-MCP only (Kondo et al., 2005). In addition, combinations of MeJA and AVG failed to...

Fig. 3. Northern blots, firmness, 1-aminocyclopropane-1-carboxylate (ACC) synthase activity, ACC and 1-malonylamino cyclopropane-1-carboxylic acid (MACC) concentrations, ACC oxidase activity, and ethylene production from ‘La France’ pear fruit skin at the climacteric stage. For RNA blot analysis, total RNAs (20 μg) were hybridized with ACS1, ACS3, ACS4, and ACO1. The analyses was repeated three times. The bottom panel shows the ethidium bromide (EtBr)-stained gel as a loading control. The data of firmness are the mean ±SE of 15 fruit. The analyses of ACC synthase activity, ACC and MACC concentration, ACC oxidase activity, and ethylene concentration were repeated three times. The data show the mean ±SE of three replications. ACC synthase and ACC oxidase activities, and ethylene production were based on fresh weight. ● = untreated control, ▲ = n-propyl dihydrojasmonate (PDJ) treatment.

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increase apple fruit ethylene production (Kondo et al., 2001). The increase of jasmonates was not observed in sweet cherries (Prunus avium L.) or grape berries (Vitis L.), and ethylene production did not increase at the ripening stages (Kondo et al., 2000, Kondo and Fukuda, 2001). These results may be indicative of interactions between jasmonates and ethylene or ACC synthase.

Our previous report showed that the application of the synthetic auxin 2, 4-dichlorophenoxypropionic acid (2, 4-DP) increased ethylene production and the ripening activity of ‘La France’ pear fruit on the tree (Kondo and Takano, 2000), and the levels of mRNAs hybridized with the ACS4 probe increased strongly in the 2, 4-DP-treated fruit (Kondo et al., 2006). These results show that ACS4 may be an ACC synthase gene that is induced by auxin in pears. Concerning the interaction between jasmonates and auxin in fruit development, the promotive effects of the combination of jasmonates and auxin on callus weight gain in apples (Kondo et al., 2001a) and the stimulation of indole acetic acid oxidase activity by jasmonate application in preclimacteric and climacteric apples have been shown (Masia et al., 1998). In our study, PDJ application increased ACC synthase activities significantly at the preclimacteric stage; in contrast, it decreased those activities at the climacteric stage. However, the expression of the ACS4 mRNA levels failed to show a clear tendency at either stage. This result suggests that the effects of PDJ on ethylene regulation in the fruit may not mediate auxin. In addition, the fact that the levels of mRNAs hybridized with ACS3 were higher before treatment may imply that ACS3 was not related to jasmonate treatment. In tomatoes (Shiu et al., 1998), the increase of the mRNA levels of ACS3 was associated with the increase of ethylene production that was incited by flooding. With regard to the increase of ethylene depending on the stress, the treatment of superoxide increased the expression of ACS1 in winter squash (Cucurbita maxima Duch.) (Sato and Mizuno, 2003). These reports suggest that the genes that react against the stress may differ depending on the kinds of stress or plant. In the current study, the ACC synthase activity, ACC concentration, ACC oxidase activity, and ethylene production increased in PDJ-treated fruit at the preclimacteric stage. These increases are considered to have possibly been incited by the increase of the ACS1 and ACCO1 mRNA levels, as shown in Fig. 2. However, the expression of ACS1 and the changes of ACC synthase activity, ACC concentration, and ethylene production failed to show the sequence of events. In tomatoes, ACC synthase cDNAs for ACS1, 2, 3, 4, 5, 6, and 7 have been confirmed (Sato and Mizuno, 2003). This report suggests that other ACS genes may also be associated with the ACC synthase activities in PDJ-treated fruit.

In ‘Passe-Crassane’ pear fruit, the expression of both the ACC synthase mRNA levels and ACC oxidase mRNA levels increased in rewarmed fruit after chilling treatment, but the accumulation of these levels was reduced in 1-MCP-treated fruit (Lelievre et al., 1997). Therefore, it is possible that the accumulation of the ACS1 and ACCO1 transcript levels may depend on the effects of either PDJ or stress ethylene incited by rewarming after chilling treatment. In contrast to the preclimacteric stage, the ACS1 mRNA levels and ethylene production were decreased by PDJ treatment at the climacteric stage. 1-Aminocyclopropane-1-carboxylate malonyltransferase is an enzyme that plays a role in the autoinhibition of the ethylene production of system 2 (Abeles et al., 1992). However, the MACC levels were higher in the PDJ-treated preclimacteric fruit than the untreated control. Furthermore, there was no significant difference in the MACC concentrations between the PDJ-treated and untreated control fruit regardless of the decrease of ethylene production in the PDJ-treated fruit at the climacteric stage. These results imply that jasmonates may not have a strong influence on ACC malonyltransferase in ethylene regulation. The results of PDJ treatment at the preclimacteric and climacteric stages suggest that ACS1 may be influenced directly by jasmonates, resulting in the regulation of ethylene production, although the effects of stress ethylene produced by rewarming after the chilling treatment on ACCO1 cannot be excluded. It has been shown that the ACS1 mRNA levels were increased by touch stimulation in tomato seedlings (Tatsuki and Mori, 1999). Furthermore, the jasmonate signal pathway is integrated in the regulation of the biotic or abiotic stress response and plant development (Turner et al., 2002). Thus, the effect of the PDJ application on the ACS1 is also considered to possibly be associated with a stress reaction. It has been reported that combinations of ethylene and MeJA synergistically induced groups of defense genes in tobacco (Nicotiana tabacum L.) (Xu et al., 1994). However, the different result between the preclimacteric and climacteric stages suggests that ACS1 mRNA levels and ACC oxidase activity may also be associated with the ACC synthase activity at the preclimacteric stage when ethylene production is low, but inhibits it at the climacteric stage when ethylene production is high.

At both the preclimacteric and climacteric stages, the PDJ application significantly increased the endogenous JA concentrations. In grape berries, it has been shown that PDJ was metabolized with hydroxylation of the pentyl side chain and cyclopentanon moiety, and then hydrolysis of the ester gave 5'-OH-PDJ and 4 or 5, 1' = 5'-diOH-deH-dihydrojasmonic acid as the main metabolite (Koshiyama et al., 2006). Furthermore, the metabolite is subsequently conjugated with glucose or
malonic acid. Therefore, it is considered that PDJ is not metabolized to endogenous JA or MeJA in the fruit. However, it has been reported that the applications of MeJA and PDJ stimulated the first enzyme of the octadecanoid pathway, allene oxide synthase (Ziosi, 2006). Our study and this report suggest that the increase of endogenous jasmonates based on PDJ application may influence ethylene production in system 2, including ACC synthase and ACC oxidase.

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