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# Involvement of Negative Feedback Regulation in Wound-Induced Ethylene Synthesis in 'Saijo' Persimmon

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Wounding is one of the most effective stress signals to induce ethylene synthesis in persimmon (*Diospyros kaki* Thunb.). We found that wound-induced ethylene biosynthesis is subjected to negative feedback regulation in mature 'Saijo' persimmon fruit since ethylene production was enhanced by 1-methylcyclopropene (1-MCP) (an inhibitor of ethylene perception) pretreatment, which was approximately 1.8 fold of that in control tissues (without 1-MCP pretreatment). Wound-induced 1-aminocyclopropane-1-carboxylate (ACC) synthase activity and *DK-ACS2* gene expression were substantially increased by 1-MCP pretreatment after 12 h, which resulted in much higher ACC content in 1-MCP pretreated tissues than that in a control after 24 h. These results indicated that wound-induced *DK-ACS2* gene expression was negatively regulated by ethylene in mature persimmon fruit. However, 1-MCP pretreatment had no effect on *DK-ACO1* gene expression, suggesting the independence of wound-induced *DK-ACO1* on ethylene. Out of accord with *DK-ACO1* gene expression, ACC oxidase activity was enhanced 48 h after wounding in 1-MCP pretreated tissues, reaching a peak 1.5-fold higher than that in control tissues at 60 h.

**KEYWORDS: Negative feedback; wound; 1-methylcyclopropene (1-MCP); ethylene; persimmon**

## INTRODUCTION

Persimmon is usually classified as a climacteric fruit (*1*). Unlike typical climacteric fruit, very little ethylene is produced during maturation (*2*, *3*), which, however, could be induced by many external stimuli, such as de-astringency treatment (dry ice or alcohol treatment) (*4*), wounding (*5*), and water loss (*6*). Among them, wounding is regarded as one of the most serious stresses in the environment, which could rapidly induce ethylene synthesis. Eventually, the stress-induced ethylene accelerates persimmon fruit softening.

It has been established that ethylene is biosynthesized from methionine to *S*-adenosylmethionine (AdoMet) and ACC in higher plants. ACC synthase and ACC oxidase are two critical enzymes involved in the ethylene biosynthetic pathway (*7*). As demonstrated in other plants species (*8*), both ACC synthase and ACC oxidase are encoded by multigene families in persimmon fruit. Recently, three ACC synthase genes (*DK-ACS1*, *DK-ACS2*, and *DK-ACS3*) and two ACC oxidase genes (*DK-ACO1* and *DK-ACO2*) were cloned from young persimmon fruit (*6*). These genes were regulated by many factors; each gene is independently regulated and differentially expressed in response to various signals. Nakano et al. (*3*) reported that *DK-ACS1* is a ripening-related gene and *DK-ACS2* is a water-stress-induced gene. Previously, we have shown that *DK-ACS2* and *DK-ACO1* are wound-induced genes, *DK-ACS2* is the rate-limiting gene in wound-induced ethylene biosynthesis, whereas *DK-ACO1* was believed to be expressed constitutively (*5*).

Ethylene highly regulates its own biosynthesis through positive or negative feedback control on ACC synthase and/or ACC oxidase (*8*). Negative feedback control of ethylene production has been recognized in a variety of fruits and vegetative tissues (*7*). For instance, wound-induced ethylene production in avocados is enhanced by 1-MCP pretreatment since *PA-ACS2* is negatively regulated by ethylene (*9*). Kim et al. (*10*) reported that exogenous ethylene markedly increased transcript level of *VR-ACO1* and reduced that of *VR-ACS1* in mungbean hypocotyls, indicating that expression of *VR-ACO1* and *VR-ACS1* genes are under positive and negative feedback control by ethylene, respectively. In ripening tomato, positive and negative internal feedback regulation by ethylene of specific ACC oxidase and ACC synthase genes, respectively, has been demonstrated (*11*).

Some of the wound-induced genes could be regulated directly by the wound signal, while others could be controlled indirectly through the ethylene produced in response to wounding (*12*). In persimmon, it is clear that wound can induce ethylene biosynthesis and transcription of the encoding genes of ACC synthase and ACC oxidase, but the mechanisms of wound-induced gene transcription and ethylene synthesis remain

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unknown. Since the discovery of 1-MCP as an inhibitor of ethylene action (*13*), here, we treated the mature 'Saijo' persimmon fruit with 1-MCP preceding wounding and investigated the feedback regulation of ethylene biosynthesis and the gene expression of ACC synthase and ACC oxidase in wounded persimmon fruit.

## MATERIALS AND METHODS

**Plant Materials.** Mature persimmon (*Diospyros kaki* Thunb. cv. Saijo) fruit with uniform weight and shape and without visual defects were harvested in a local commercial orchard in October. Fruit were randomly divided into two groups: one for 1-MCP pretreatment and the other without 1-MCP pretreatment used as a control. Triplicates were applied for either group with 10 fruit used for each replication.

**Treatment.** After harvest, fruit were immediately treated with 0.5 $\mu L \cdot L^{-1}$ 1-MCP at 20 °C under 60% humidity for 16 h in a 117-L plastic container with a plastic lid sealed by a water moat. 1-MCP was applied as a gas generated by mixing 90.4 mg of Ethylbloc (Rohm and Hass) powder and 1.4 mL of distilled water in a 10-mL vial. 1-MCP gas was released from the vial into the plastic container. Control fruit were stored at the same conditions without 1-MCP. After 1-MCP pretreatment finished, both control and 1-MCP pretreated fruit were treated by wounding.

For wounding, the equatorial parts of 10 fruit were cut into 5 × 5 mm cubes and mixed together. All of the wounded tissues were incubated at 20 °C under 60% humidity. After ethylene production was assayed, the pulp tissues were frozen in liquid nitrogen and stored at −80 °C until use for extraction of ACC, ACC synthase, ACC oxidase, and total RNA. Data on ethylene production, ACC content, and the activities of ACC synthase and ACC oxidase were repeated three times, except RNA extraction.

**Determination of Ethylene Production and ACC Content.** Ethylene production was assayed every 12 h. About 5 g of wounded tissues was sealed in a 32-mL vial with a silicon rubber cap and incubated at 20 °C under 60% humidity for 2 h; 0.5 mL of gas headspace was withdrawn from the vial with a glass syringe and injected into a gas chromatograph (Shimadzu GC-14, Kyoto Japan) fitted with an activated alumina column and a flame ionization detector, and the temperatures of the column, injection, and detector are 130, 150, and 130 °C, respectively.

ACC in fruit samples was extracted in 80% ethanol according to the method described by Itamura (*14*), and the content was assayed by the method of Lizada and Yang (*15*).

**Extraction and Assay of ACC Synthase and in Vitro ACC Oxidase.** Both ACC synthase and ACC oxidase were extracted using the same poly(ethylene glycol) (PEG) acetone method as described by Zheng et al. (*16*). Five grams of frozen tissue was pulverized in liquid nitrogen with a mortar and pestle. The frozen powder was mixed with 15 mL of the PEG-added (1 w/v %, PEG) extraction buffer that contained 100 mM K-phosphate (pH 8.5), 10% (w/v) glycerol, 5 $\mu$M PLP, and 5 mM DTT; it was vacuum-infiltrated at 30 mmHg for 30 min and then homogenized with a Waring blender at 12 000 rpm for 5 min in an ice bath. To remove the excess PEG, the homogenate was mixed with two volumes of cold acetone (−20 °C) and centrifuged for 10 min at 3800*g* at −10 °C. After centrifugation, the precipitate was dissolved in 10 mL of extraction buffer that contained 0.2 g of insoluble poly(vinylpyrrolidone) and stirred at 4 °C for 1 h. The slurry was subsequently centrifuged at 18000*g* for 10 min at 4 °C. The supernatant was recovered and filtered through a 0.45 $\mu$m membrane filter (ADVANTEC DISMIC-25CS, Toyo Roshi Kaisha, Ltd. Japan); a 2.5 mL portion of the filtrate was desalted by passing through a Sephadex G-25 column (bed volume, 8.3 mL; Amersham Bioscience, Sweden) and equilibrated with the elution buffer. The components of the elution buffer were the same as those of the extraction buffer. A 3.5 mL aliquot was collected from the column and assayed for protein content and ACC synthase activity.

ACC oxidase was extracted in a similar way for ACC synthase as above except that the extraction and elution buffers of ACC oxidase consisted of 100 mM K-phosphate (pH 7.2), 10% glycerol, 5 mM DTT, and 30 mM sodium ascorbate.

The activity of ACC synthase was assayed as described by Zheng et al. (*16*). In vitro ACC oxidase activity was determined as follows: 2 mL of ACC oxidase preparation was preincubated in a 19 mL vial with a silicon rubber screw cap, which contained 100 mM K-phosphate (pH 7.2), 10% (w/v) glycerol, 5 mM DTT, and 30 mM sodium ascorbate, at 30 °C for 15 min in a shaking bath. Thereafter, 0.2 mL of 20 mM ACC, 0.2 mL of 2 mM $FeSO_4$, and 0.2 mL of 300 mM $NaHCO_3$ were added to the above mixture, and the vial was incubated for an additional 1 h. Two milliters of headspace gas was injected into a gas chromatograph to determine ethylene production as described above.

Protein content in enzyme extracts was determined by the method of Bradford (*17*).

**In Vivo ACC Oxidase Activity Assay.** Approximate 2.5 g of fresh wounded tissues was soaked in 2.4 mL of ACC oxidase extraction buffer in a 19 mL vial with the addition of 0.2 mL of 20 mM ACC. The vial was immediately sealed with a silicon rubber cap after vacuum infiltrating at 30 mmHg for 3 min and then incubated at 30 °C for 1 h with shaking. One-half of a milliliter of gas headspace was withdrawn from the vial with a glass syringe and injected into a gas chromatograph to determine ethylene content.

**Probe Preparation and Northern Blotting Analysis.** For probe preparation, gene-specific probes containing the 3′-untranslated region were amplified using the corresponding cDNA provided by Dr. Nakano (Okayama University, Okayama, Japan) as template and further labeled with a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Germany) following the procedures of Zheng et al. (*5*).

Total RNA was isolated by the hot borate method (*18*). For Northern blot analysis, 20 $\mu$g of RNA from each sample was separated by electrophoresis on 1.2% (w/v) agarose gels containing 0.66 M formaldehyde in 1× MOPS buffer and then blotted onto positively charged nylon membranes (Hybond-$N^+$, Amersham Pharmacia Biotech, U.K.) and cross-linked by a UV autocross-linker (Amersham Pharmacia Biotech, U.K.). Membranes were prehybridized in DIG Easy Hyb (Roche Diagnostics, Germany) at 50 °C for at least 1 h. Hybridization was performed at 50 °C in the same prehybridization buffer supplemented with the DIG-labeled probes overnight. After hybridization, membranes were washed twice for 5 min in a 2× sodium chloride/sodium citrate (SSC) solution containing 0.1% (w/v) sodium dodecyl sulfate (SDS) at room temperature, then twice for 15 min in a 0.5 × SSC solution containing 0.1% (w/v) SDS at 58 °C. Signals were detected by chemiluminescent reaction using CDP-star (Tropix, U.S.A.) as the chemiluminescent substrate, and the membrane was exposed to X-ray film (Fuji Photo Film, Japan).

## RESULTS

**Effect of 1-MCP Pretreatment on Wound-Induced Ethylene Biosynthesis.** Wounding rapidly induced ethylene biosynthesis in mature 'Saijo' persimmon fruit and reached its peak (0.6 $nmol \cdot g^{-1} \cdot h^{-1}$) at 36 h, followed by a decrease thereafter (**Figure 1**), which is in line with wound-induced ethylene biosynthesis in mature 'Hiratanenashi' persimmon fruit reported previously (*5*). To further clarify the feedback regulation involved in wound-induced ethylene biosynthesis, we examined the effects of 1-MCP pretreatment on wound-induced ethylene biosynthesis in mature 'Saijo' persimmon fruit. Compared with control, the peak of wound-induced ethylene production in 1-MCP pretreated tissues was obviously postponed. A lower amount of ethylene was synthesized before 36 h; however, it increased markedly after 36 h and reached an even a higher maximum (1.1 $nmol \cdot g^{-1} \cdot h^{-1}$) at 60 h, approximately 1.8-fold of the maximal amount in control tissues without 1-MCP pretreatment, suggesting involvement of negative feedback regulation in wound-induced ethylene biosynthesis.

**Effect of 1-MCP Pretreatment on Wound-Induced ACC Content.** As the direct precursor of ethylene, ACC accumulation was effectively induced by wounding in mature 'Saijo' persimmon fruit. ACC content increased rapidly during the first 12 h, reached a peak (9.5 $nmol \cdot g^{-1}$) at 36 h, followed by a decrease

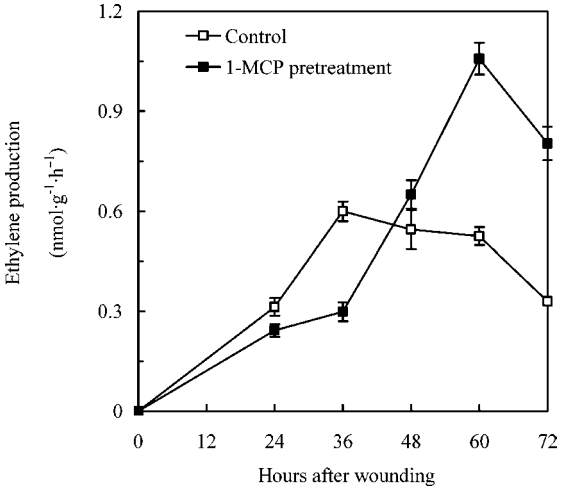


**Figure 1.** Time course analysis of ethylene production in wounded persimmon tissues after 1-MCP pretreatment or without 1-MCP pretreatment. 'Saijo' persimmon fruit were held either in air (control) or in the presence of 500 nL·L$^{-1}$ 1-MCP for 16 h at 20 °C under 60% humidity and then wound-treated by cutting into small pieces. Ethylene production was determined at the times indicated. Vertical bars represent the SE of three replications.

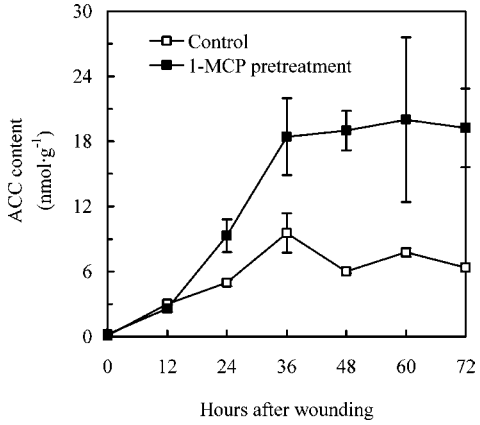


**Figure 2.** Time course analysis of ACC content in wounded persimmon tissues after 1-MCP pretreatment or without 1-MCP pretreatment. The samples were treated with the same methods as described in the legend of **Figure 1**. Vertical bars represent the SE of three replications.

during the subsequent time (**Figure 2**). 1-MCP pretreatment was shown to significantly enhance wound-induced ACC accumulation, although no enhancement was observed during the first 12 h. The ACC content in 1-MCP pretreated tissues reached its maximal amount of 18.4 nmol·g$^{-1}$ at 36 h, about two times more than that in the control, and remained constant thereafter (**Figure 2**).

**Effect of 1-MCP Pretreatment on Wound-Induced ACC Synthase Activity and Its Gene Expression.** Similar to our previous study (*5*), ACC synthase activity was not detectable at harvest time but rapidly induced by wounding in mature 'Saijo' persimmon fruit, which peaked at 24 h (0.134 nmol ACC·mg$^{-1}$ protein·h$^{-1}$) and declined during the following period (**Figure 3A**). Wound-induced ACC synthase activity was notably increased by 1-MCP pretreatment during the whole period of this experiment. ACC synthase activity in 1-MCP pretreated tissues was approximately 2-fold higher than that in control at 12 h and rapidly reached the maximum value at 24 h (0.271 nmol ACC·mg$^{-1}$ protein·h$^{-1}$); thereafter, it reduced continuously to 72 h (**Figure 3A**). The enhanced ACC synthase

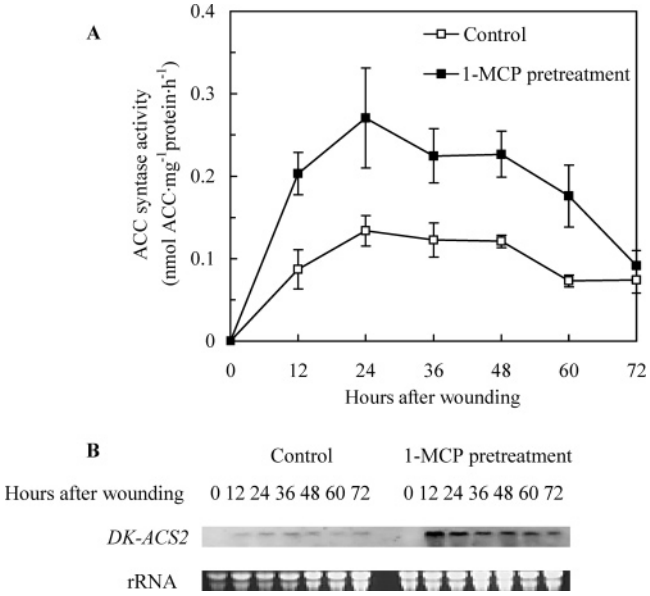


**Figure 3.** Time course analysis of ACC synthase activity and *DK-ACS2* mRNA expression in wounded persimmon tissues after 1-MCP pretreatment or without 1-MCP pretreatment. The samples were treated with the same methods as described in the legend of **Figure 1**. (**A**) Changes in ACC synthase activity. Vertical bars represent the SE of three replications. (**B**) Northern blot analysis of *DK-ACS2* gene. Each lane contains 20 $\mu$g of total RNA. Ribosomal RNA was used to normalize loading.

in 1-MCP pretreated tissues accounted for the increased ACC accumulation, as shown in **Figure 2**.

In control tissues, *DK-ACS2* transcription was rapidly induced by wounding at 12 h and almost remained at a similar level for the entire period except for a weak decrease at 60 and 72 h (**Figure 3B**). 1-MCP pretreatment showed significant effects on *DK-ACS2* transcription, although its mRNA accumulation showed a pattern similar to that in the control (**Figure 3B**). 1-MCP pretreatment led to much stronger *DK-ACS2* transcription during the entire period, which was consistent with the promoted ACC synthase activity measured with the same treated fruit. A rapid strong transcription of *DK-ACS2* was observed at 12 h, accounting for the maximal ACC synthase activity at 24 h. However, *DK-ACS1* gene expression was not detectable in this study (data not shown), as similarly reported by Zheng et al. (*5*). These results indicated an unambiguous negative feedback regulation of *DK-ACS2* transcription by ethylene.

**Effect of 1-MCP Pretreatment on Wound-Induced ACC Oxidase Activity and Its Gene Expression.** A basal level of ACC oxidase activity was measurable at harvest time, which increased 12 h after wounding and progressively increased until 60 h (0.098 nmol $C_2H_4$·mg$^{-1}$ protein·h$^{-1}$), followed by a slight decrease at 72 h. Wound-induced in vitro ACC oxidase activity in 1-MCP pretreated tissues showed negligible difference from that in the control during the initial 36 h; however, higher in vitro ACC oxidase activity was observed from 48 h, with a maximum value of 0.147 nmol $C_2H_4$·mg$^{-1}$ protein·h$^{-1}$ at 60 h, which was 1.5-fold of that in the control (**Figure 4A**). To confirm the effect of 1-MCP pretreatment on ACC oxidase activity, we also determined in vivo ACC oxidase activity, and similar results were obtained (**Figure 4B**). In vivo ACC oxidase activity in 1-MCP pretreated tissues were higher than that in the control after 48 h of wounding treatment, reaching a peak at 60 h, which was 2-fold of that in control.

Unexpectedly, gene expression of ACC oxidase was not affected by 1-MCP pretreatment, especially during the late

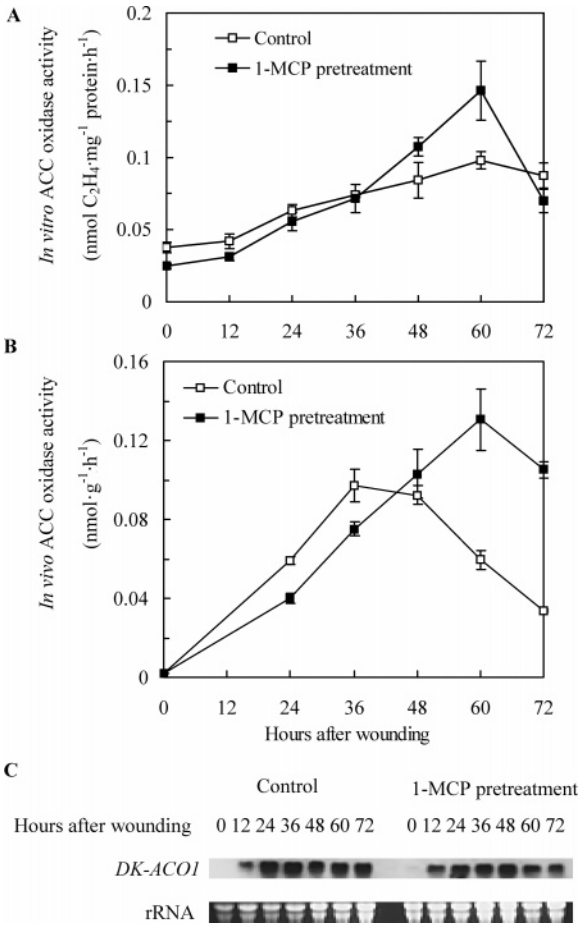


**Figure 4.** Time course analysis of in vitro and in vivo ACC oxidase activity and *DK-ACO1* mRNA expression in wounded persimmon tissues after 1-MCP pretreatment or without 1-MCP pretreatment. The samples were treated with the same methods as described in the legend of **Figure 1**. (**A**) Changes in ACC oxidase activity in vitro. (**B**) Changes in ACC oxidase activity in vivo. Vertical bars represent the SE of three replications. (**C**) Northern blot analysis of *DK-ACO1* gene. Each lane contains 20 $\mu$g of total RNA. Ribosomal RNA was used to normalize loading.

period as indicated by changes of the enzymatic activity (**Figure 4C**). In both 1-MCP pretreated and control tissues, *DK-ACO1* gene transcription initiated at 12 h, strengthened at 24 h, and remained steady thereafter. These results suggest the existence of posttranscriptional regulation of ACC oxidase and release of this regulation by 1-MCP pretreatment. As reportedly previously (*5*), *DK-ACO2* gene expression was not induced by wounding (data not shown).

## DISCUSSION

Since ethylene biosynthesis is often regulated by itself (*8*), 1-MCP, as an effective inhibitor of ethylene function, is very useful to clarify whether induction of ethylene biosynthesis is mediated by the action of ethylene and elucidate the feedback mechanism in ethylene biosynthesis (*13*). In this study, wound-induced ethylene biosynthesis was not increased by 1-MCP pretreatment during the initial 36 h, whereas after that the induction was effectively enhanced by 1-MCP pretreatment. The maximal wound-induced ethylene production in 1-MCP pretreated tissues was approximately 1.8-fold higher that in the control (**Figure 1**). These results indicated that the first increase of ethylene production in both cases was directly in response to wound signal and that the continuous increase in 1-MCP pretreated tissues resulted from the release of negative feedback regulation of endo-ethylene; this part of ethylene inhibited further ethylene synthesis in control tissues by negative feedback regulation. The involvement of negative feedback regulation in wound-induced ethylene biosynthesis has also been reported in etiolated pea (*19*), citrus peel (*20*), and avocados (*9*).

Wounding promotes ethylene production by inducing expression of genes for wound-inducible ACC synthases (*5*, *21*, *22*). Similar to the results of our previous study, *DK-ACS2* transcription was rapidly induced by wounding in the control and 1-MCP pretreated tissues, whereas a much more abundant mRNA amount in 1-MCP pretreated tissues was observed during the whole experiment (**Figure 3B**). As a consequence, wound-induced ACC synthase activity was also much higher in 1-MCP pretreated tissues than that in the control (**Figure 3A**). These results suggested that wound-induced *DK-ACS2* gene expression was under negative feedback regulation. Riov and Yang (*20*) reported that autoinhibition of ethylene production in wounded citrus peel was attributed to the suppression of ACC formation due to the inhibition of ACC synthase formation and activity. Our results indicated that regulation of ACC synthase gene expression and enzymatic activity also played important roles in ethylene feedback regulations. In avocados, wound-induced *PA-ACS2* transcription was also subjected to negative feedback regulation (*9*).

However, wound-induced *DK-ACO1* gene transcription was not affected by ethylene feedback regulation in persimmon fruit as demonstrated in **Figure 4C**. Similar observations have been reported in avocados (*9*) and tomato (*12*). The wound-induced transcription of *PA-ACO*, an avocado's ACC oxidase gene, and of *LE-ACO1* from tomato were unaffected by 1-MCP treatment (*9*, *12*). However, different from gene transcription, in vitro and in vivo ACC oxidase activity was enhanced by about 1.5-fold and 2.0-fold by 1-MCP pretreatment 48 h after wounding, respectively (**Figure 4A** and **4B**), just preceding the prompted ethylene production peak in 1-MCP pretreated tissues at 60 h, which was approximately 1.8-fold of that in the control (**Figure 1**). It was proposed that wound-induced ACC oxidase activity might be subjected to posttranscriptional/translational regulation and/or enzyme turnover, which might be related to ethylene feedback control. These results also suggested that ACC oxidase played an exclusive role in wound-induced ethylene biosynthesis in the presence of enough ACC content. It has been reported that ACC formation and its conversion to ethylene are the two main limiting steps in ethylene biosynthesis (*7*, *23*) and also in ethylene feedback regulations (*11*, *24*).

In conclusion, with the application of 1-MCP pretreatment, we found that wound-induced ethylene biosynthesis was autoinhibited in mature 'Saijo' persimmon fruit tissue. Wound-induced *DK-ACS2* gene expression was negatively regulated by ethylene, whereas *DK-ACO1* gene expression was independent of ethylene feedback control.

## ABBREVIATIONS USED

ACC, 1-aminocyclopropane-1-carboxylate; AdoMet, *S*-adenosylmethionine; 1-MCP, 1-methylcyclopropene; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; SSC, sodium chloride/sodium citrate.

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