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Full Length Research Paper

Expression of a 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene in peach (*Prunus persica* L.) fruit in response to treatment with carbon dioxide and 1-methylcyclopropene: possible role of ethylene

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In this study we investigated the effect of exogenous ethylene treatment on ethylene production, 1-aminocyclopropane-1-carboxylate (ACC) oxidase activity and expression of an ACC oxidase (*PP-ACO1*) gene previously cloned (Mathooko et al., 2001) in peach (*Prunus persica* L.) fruit. We also investigated the mode of action of CO_2 and 1-methycyclopropene (1-MCP) in the regulation of ethylene biosynthesis during peach fruit ripening. Fruits were treated with various concentrations of ethylene (0.1, 1, 10, 100, 500, 1000 ppm) and also with CO_2 and 1-MCP in the presence or absence of 500 ppm ethylene. Ethylene stimulated ethylene production at concentrations of 100 ppm and above while ACC oxidase activity was stimulated in a concentration-dependent manner. *PP-ACO1* was slightly constitutively expressed and exogenous ethylene stimulated accumulation of its mRNA transcript in a concentration-dependent manner up to 100 ppm after which the level remained constant. CO_2 and 1-MCP inhibited the ethylene-stimulated ethylene production, ACC oxidase activity and accumulation of *PP-ACO1* transcripts by about 50%. These results indicate that ethylene plays a key role in the regulation of ethylene production and ACC oxidase activity and its gene expression in peach fruit. Further the results indicate that CO_2 and 1-MCP regulate ethylene biosynthesis in peach fruit during ripening, at least in part, by antagonizing ethylene action.

Key words: ACC oxidase, carbon dioxide, ehylene, 1-methylcyclopropene, peach, *Prunus persica*.

INTRODUCTION

Fruit ripening is a genetically programmed event that is characterised by a number of biochemical and physiological processes that alter fruit colour, aroma, flavour, texture and its nutritional value. The onset of ripening in climacteric fruits is marked by a burst of ethylene production and exogeneous ethylene can induce ripening and concommitantly endogeneous ethylene production (Abeles et al., 1992; Yang and Hoffman, 1984). Enhancement of ethylene production serves as a signalling mechanism with profound physiological consequences and large losses of fruits and vegetables are incurred due to ethylene's effects on plant senescence (Theologis et al., 1992, Mathooko, 1996). The management and control of fruit ripening, therefore, is important for their successful transport and marketing. This can be achieved by use of ethylene action inhibitors such as CO_2 and 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997; Mathooko et al., 2001).

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For a long time 1-aminocyclopropane-1-carboxylate (ACC) synthase, the enzyme that catalyses the conversion of *s*-adenosyl-L-methionine (AdoMet) to ACC, the immediate precursor of ethylene had been considered the rate limiting enzyme in the ethylene biosynthetic pathway (Yang and Hoffman, 1984; Abeles et al., 1992; Kende, 1993; Mathooko, 1996). However, accumulated evidence indicate that ACC oxidase may also be limiting (Kende, 1993). Indeed the increase in ethylene production that is induced by developmental stages such as fruit ripening is accompanied by increase in the activities of both ACC synthase and ACC oxidase (Yang and Hoffman, 1984; Mathooko, 1996). The induction of these enzymes during fruit ripening (Rottmann et al., 1991; Nakatsuka et al., 1997, 1998; Lelievre et al., 1997; Mathooko et al., 2001) and following ethylene treatment (Woodson et al., 1992; Rottmann et al., 1991; Kim and Yang, 1994) is due to increase in their mRNAs. It is now clear that ACC oxidase (Barry et al., 1996; Lasserre et al., 1996; Tang et al., 1994; Mathooko et al., 2001) is encoded by more than one gene and that the genes induced by different stimuli are differentially regulated (Huang et al., 1991; Kende, 1993; Zarembinski and Theologis, 1994; Fluhr and Mattoo, 1996; Bouquin et al., 1997; Ruperti et al., 2001).

In peach fruit an ACC oxidase-related RNA (Callahan et al., 1992; Tonutti et al., 1997a,b; Ruperti et al., 2001) accumulate as the fruit ripens and more specifically when the fruit has softened to less than 40 N. The expression pattern of ACC oxidase gene during peach fruit development (Tonutti et al., 1997a) and during fruit ripening has previously been studied (Mathooko et al., 2001). Changes in gene expression are critical in the control of fruit ripening and these changes can be controlled at the level of transcription using appropriate postharvest techniques. This study examined the expression pattern of an ACC oxidase gene (*PP-ACO1*, Accession no. AB044711) which had been cloned earlier (Mathooko et al., 2001) in response to ethylene. It also examined the possible mode of action of 1-MCP and CO_2, both inhibitors of ethylene action, in the regulation of ACC oxidase activity and gene expression in peach fruit. Using CO_2 and 1-MCP, we recently demonstrated that the regulation of wound-induced *PP-ACO1* gene expression by CO_2 is through its antagonistic effect on ethylene action (Mathooko et al., 2001). Here we show that ripening related *PP-ACO1* is also regulated through a positive feedback mechanism.

MATERIALS AND METHODS

Plant Material

Peach (*Prunus persica* cv. Hakuho) fruit at the preclimacteric stage (ethylene production <0.2 $nl.g^{-1}.h^{-1}$) were obtained from the Experimental Farm of Okayama University, Japan. The fruit were hand-picked and sorted with respect to defects, maturity, and uniformity of shape, size and degree of colour development. All handling procedures of the freshly harvested fruit were carefully undertaken to avoid any injuries.

Treatments with exogeneous ethylene, CO_2 and 1-MCP

Ethylene treatment was conducted in a static system. Fruit (six per treatment) were enclosed in plastic containers fitted with silicon rubber stoppers and ethylene (0.1, 1, 10, 100, 500 and 1000 ppm) was injected into the containers by means of a syringe and incubated at 24° C for 24 h. After the treatment the containers were flushed with air for 30 min in order to facilitate diffusion of any ethylene adsorbed on the fruit. In order to investigate whether the regulation of ethylene biosynthesis by CO_2 and 1-MCP is through their effects on ethylene action, fruit were treated with CO_2 or 1-MCP in the presence or absence of 500 ppm ethylene. Fruit were put in plastic containers and flushed with 20% CO_2 (balance 20% O_2 and 60% N_2) for 1 h, sealed and then ethylene was injected. For 1-MCP treatment, fruit were treated with 1-MCP for 15 h at 24°C, sealed in plastic containers and then ethylene was injected. Controls were treated with air, 20% CO_2 (balance 20% O_2 and 60% N_2), 1-MCP or ethylene only. The containers were incubated at 24°C for 24 h. The concentrations of CO_2 and ethylene were verified by gas chromatography. In the appropriate containers, small beakers containing 20% KOH and/or 0.25 M $Hg(ClO_4)_2$ were included to absorb CO_2 and ethylene respectively, released by the fruit (Liu et al., 1985). The 1-MCP used was synthesized according to the method of Magid et al. (1971) as a stable lithium derivative in ether solution and stored at -20°C until use. Intact fruit were enclosed in 10 L glass jars fitted with a silicon rubber stopper. 1-MCP gas was generated by aqueous neutralization of the lithium derivative as described by Nakatsuka et al. (1997) and injected into the containers. The 1-MCP concentration was estimated to be 10 – 20 nl/litre.

Measurement of ethylene production

Fruit were enclosed in 1.5-litre plastic containers fitted with silicon rubber stoppers for gas sampling and incubated at 24°C for 1 h. A 1-ml gas sample was withdrawn from the containers using a gas-tight hypodermic syringe. Ethylene concentration in the headspace gas was assayed by injecting the gas sample into a Shimadzu gas chromatograph (Model GC -4CM, Shimadzu Corp., Kyoto, Japan) equipped with an activated alumina column and a flame ionization detector.

Determination of ACC oxidase activity

ACC oxidase activity was assayed *in vivo* by measuring the capability of the tissue to convert administered ACC to ethylene as previously described (Mathooko et al., 1993).

RNA extraction

Total RNA was extracted using the hot borate method of Wan and Wilkins (1994) with minor modifications as previously described (Mathooko et al., 2001).

Plasmid DNA and radiolabeling

The cDNA fragment used in this study was cloned from peach fruit using reverse transcriptase polymerase chain reaction as previously described (Mathooko et al., 2001). The cDNA fragment for ACC oxidase was designated *PP-ACO1* (Accession no. AB044711).

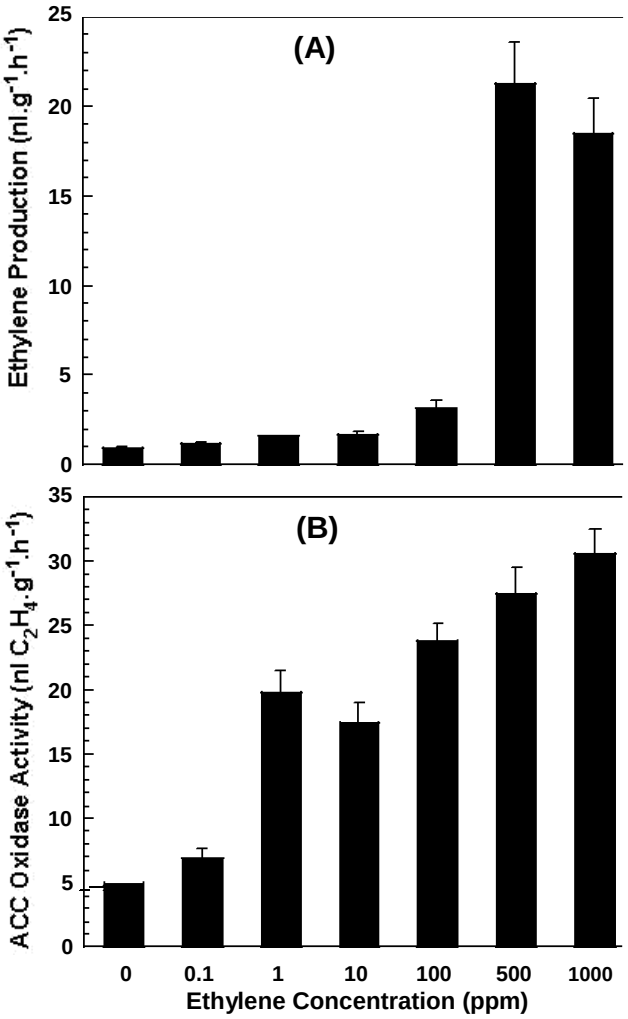

Figure 1. Ethylene production (A) and ACC oxidase activity (B) in preclimacteric peach fruits treated at 24°C with air (control) or various concentrations of ethylene. The vertical bars are mean ± SE of three replications.

RNA blotting and northern hybridization analysis

Total RNA (6 µg per lane) was subjected to electrophoresis on a 1.2% agarose gel containing 0.66 M formaldehyde and 1x MOPS (20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA) and were blotted overnight by capillary transfer to nylon membranes (Hybond N^+, Amersham International) in 20x SSPE [1x SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA (pH 7.4)] according to the manufacturer's instructions. After transfer, the membranes were baked at 80°C for 30 min and the RNAs were cross-linked to the membranes by UV irradiation using a UV cross linker (Amersham International). Northern hybridization analysis was then carried out as previously described (Mathooko et al., 2001). Actin was used as an internal control.

RESULTS

Effect of exogeneous ethylene treatment on ethylene production and ACC oxidase activity

Ethylene is known to regulate its own biosynthesis both positively and negatively. We, therefore, investigated the role of ethylene in the regulation of ethylene production and ACC oxidase activity. Fruit were treated for 24 h at 24°C with various concentrations of ethylene. Exogenous ethylene stimulated ethylene production at concentrations of 100 ppm and above (Figure 1A) while ACC oxidase activity (Figure 1B) was stimulated in a concentration-dependent manner from 1 ppm. At 1000 ppm ethylene stimulated ethylene production and ACC oxidase activity twentyfolds and sixfolds, respectively.

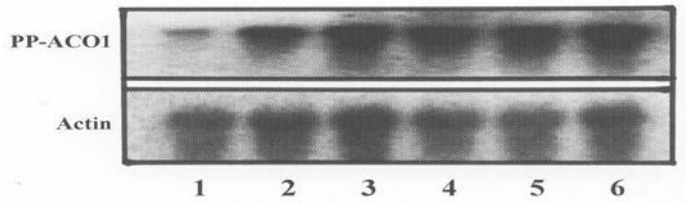

Figure 2. Expression of *PP-ACO1* gene in preclimacteric peach fruits treated at 24°C with air (control) or various concentrations of ethylene. Lane 1: air, Lanes 2, 3, 4, 5 and 6, 1, 10, 100, 500 and 1000 ppm of ethylene, respectively.

Effect of exogeneous ethylene treatment on PP-ACO1 gene expression

PP-ACO1 was slightly constitutively expressed in the fruit (Figure 2). However, the accumulation of *PP-ACO1* transcript increased with increase in ethylene concentration up to 100 ppm after which the transcript level remained constant. Based on this observation, we investigated the mode of action of CO_2 and 1-MCP in the regulation of ethylene biosynthesis and expression of the ACC oxidase gene.

Effect of CO_2 and 1-MCP on ethylene production and ACC oxidase activity

Fruit were treated with air, CO_2 or 1-MCP in the presence or absence of 500 ppm ethylene. As expected ethylene stimulated ethylene production (Figure 3A) and ACC oxidase activity (Figure 3B). CO_2 and 1-MCP inhibited ethylene-induced ethylene production and ACC oxidase activity by about 50% compared to that of fruit treated with ethylene and air.

Effect of CO_2 and 1-MCP on ethylene-stimulated *PP-ACO1* gene expression

The effects of ethylene, CO_2 and 1-MCP on ethylene production and ACC oxidase activity paralleled their respective effects on the accumulation of *PP-ACO1* mRNA transcript. Ethylene stimulated the accumulation of *PP-ACO1* transcripts. However, CO_2 and 1-MCP

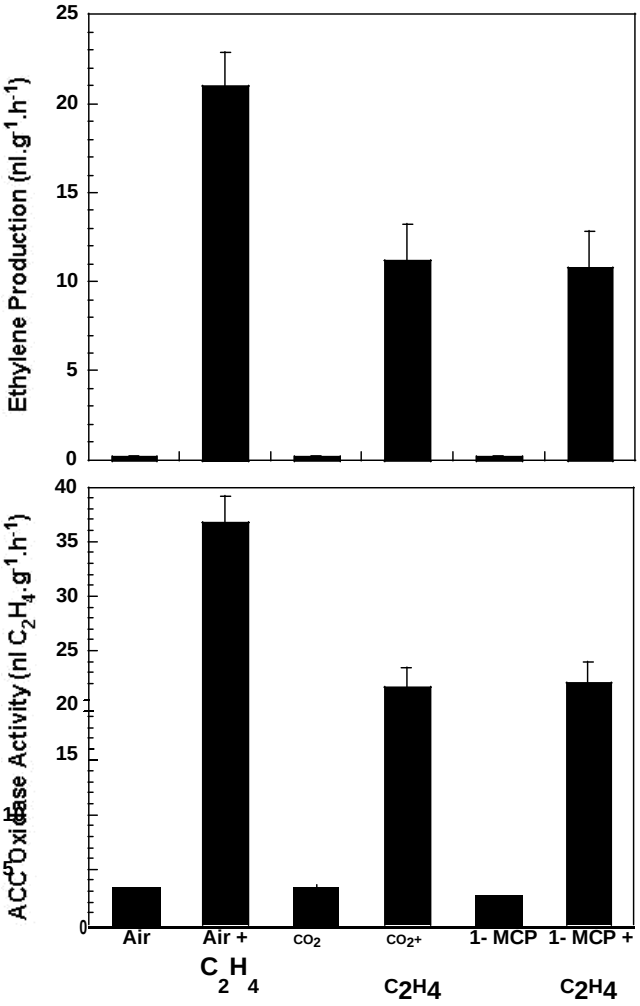

Figure 3. Ethylene production (A) and ACC oxidase activity (B) in preclimacteric peach fruits treated at 24°C with air, CO_2 or 1-MCP in the presence or absence of 500 ppm of ethylene. The vertical bars are mean ± SE of three replications.

inhibited this ethylene-induced accumulation of *PP-ACO1* transcript by 50 and 60%, respectively (Figure 4).

DISCUSSION

Preclimacteric fruits produce very little ethylene because of their low activities of both ACC synthase and ACC oxidase. However, when fruits are treated with exogenous ethylene, for a short period, an increase in the tissue capability to oxidize ACC to ethylene is observed (Liu et al., 1985). Ethylene stimulates the development of ACC oxidase activity in a number of plant tissues (Liu et al., 1985; Bufler, 1986; Kim and Yang, 1994). This stimulation is correlated with the accumulation of transcripts for ACC oxidase (Kim and Yang, 1994; Tang et al., 1994; Peck and Kende, 1995; Barry et al., 1996), thereby suggesting transcriptional regulation. Callahan et al. (1993) reported that treatment of preclimacteric peach

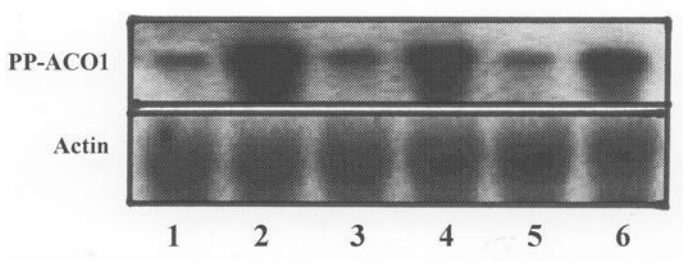

Figure 4. Expression of *PP-ACO1* gene in preclimacteric peach fruits treated at 24°C for 24 h with air, CO_2 or 1-MCP in the presence or absence of 500 ppm of ethylene. Lane 1:air; Lane 2: air + ethylene; Lane 3: CO_2; Lane 4: CO_2 + ethylene; Lane 5: 1-MCP and Lane 6: 1-MCP + ethylene.

fruit with ethylene had no effect on ACC synthase RNA accumulation although this treatment caused accumulation of ACC oxidase RNA.

It has been suggested that the expression of ACC oxidase mRNA is regulated by low basal level of ethylene (Oetiker and Yang, 1995). In the present study an ethylene concentration of between 0.1 and 100 ppm stimulated ACC oxidase activity without any significant change in ethylene production. Liu et al (1985) reported that when preclimacteric tomato and cantaloupe fruits were treated with ethylene, ACC oxidase activity increased without concomitant increase in ACC content or ethylene production indicating that the increase in ACC oxidase activity precedes that of ACC synthase. The accumulation of *PP-ACO1* increased with increase in ethylene concentration up to 100 ppm and the level remained constant thereafter. The presence of CO_2 and 1-MCP counteracted the effects of ethylene on the accumulation of *PP-ACO1* mRNA transcript by about 50 and 60%, respectively, in parallel with their effects on ethylene production and ACC oxidase activity. Carbon dioxide only slightly inhibits ethylene -promoted ACC oxidase activity and this is related to the concentration of ethylene used, being less effective when ethylene concentration is high (Bufler, 1986). NBD eliminates completely ethylene-promoted ACC oxidase activity in cantaloupe (Liu et al., 1985). In preclimacteric apple fruit (Dong et al., 1992, Ross et al., 1992) and in peach (Tonutti et al., 1997a), both ACC oxidase activity and transcript are undetectable but are markedly induced by an exogeneous ethylene treatment indicating that ethylene-dependent expression of the ACC oxidase gene is regulated at the transcriptional level. This is in agreement with observations made by Ruperti et al. (2001) who reported that accumulation of *PP-ACO1* mRNA in peach fruit is promoted by treatment with propylene. Indeed, a primary ethylene-responsive element (PERE) similar to that of tomato E4 is present in the promoter region of *PP-ACO1* (Ruperti et al., 2001) as is the case in the promoters of several ethylene-regulated genes (Solano et al., 1998).

When preclimacteric apple fruit are treated with exog-

eneous ethylene, ACC oxidase activity and accumulation of its transcript increase progressively with increasing duration of ethylene treatment (Dong et al., 1992). In peach fruit treated with ethylene, the ACC oxidase transcript level was not correlated to ethylene production (Tonutti et al., 1997b). In 'Andes' and 'Earl's Favorite' melon fruit ethylene greatly stimulates the accumulation of ACC oxidase transcript in both cultivars while it only slighlty induces accumulation of ACC synthase transcript in 'Andes' and not in 'Earl's Favorite' (Shiomi et al., 1999). This may be taken to imply that the effects of ethylene may also be cultivar dependent. The inhibition of ethylene-induced ACC oxidase activity and accumulation of its transcript by CO_2 and 1-MCP indicates that in ripening peach fruit, the regulation of ethylene biosynthesis is, at least in part, through their antagonistic effects on ethylene action. Furthermore, transgenic technology has demonstrated a requirement for ethylene synthesis and action for fruit to ripen normally (Theologis, 1992). By studying the expression pattern of mRNAs for phytoene synthase, an enzyme regulated by ethylene, Rothan et al. (1997) concluded that CO_2 may regulate ethylene biosynthesis in tomato fruit by interfering with ethylene signaling besides other mechanisms (de Wild et al., 1999). 1-MCP has been shown to have direct effect on ethylene perception (de Wild et al., 1999) particularly through its effect on the expression of ethylene receptor genes such as *Pp-ERS1* in peach fruit (Rasori et al., 2002).

We (Mathooko et al., 2001) have previously found that exogenous ethylene stimulated wound-induced ACC oxidase activity and accumulation of its mRNA. These effects were, however, counteracted by CO_2 and 1-MCP. The contrary was true for wound-induced ACC synthase (Mathooko et al., 2001). This and our present observation indicate that both wound-induced and ripening-related ACC oxidase activity and mRNA accumulation in peach fruit are regulated through a positive feedback mechanism. It is plausible, therefore, that the *PP-ACO1* mRNA constitutively present in peach fruit (Mathooko et al., 2001) could be regulated by endogenous ethylene present in the tissue.

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