Ethylene Evolution and 1-Aminocyclopropane-1-carboxylate Oxidase Gene Expression during Early Development and Ripening of Peach Fruit

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ADDITIONAL INDEX WORDS. *Prunus persica*, fruit growth, softening

Abstract. The rate of ethylene biosynthesis was monitored throughout the four stages (S1, S2, S3, and S4) of peach (*Prunus persica* L. Batsch 'Springcrest') fruit development. The highest values of ethylene production were detected during the early S1 and at ripening. During S1, the increase in the evolution of ethylene was accompanied by high activity of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO). A weak accumulation of ACO mRNA was detected in developing fruitlets, indicating that ACO may play a specific role in modulating the rate of ethylene biosynthesis during the early growth stage. When fruitlets harvested at S1 were flushed with propylene (500 mL·L⁻¹) for 48 h, a two-fold increase of ethylene biosynthesis and a dramatic induction of ACO activity were observed. Treatment with the ethylene analogue greatly stimulated the expression of ACO gene(s). During ripening, the climacteric occurred when fruit had softened to ≈20 N. This process was preceded by an increase in ACC content and ACO activity in the mesocarp. ACO transcripts began to accumulate before the rise in whole-fruit ethylene biosynthesis with peak levels coincident with the climacteric when the highest values of ACO activity were detected. Propylene greatly enhanced ACO gene expression and stimulated the ripening-associated ethylene climacteric. ACO-related transcripts also accumulated in fruit treated with nitrogen for 72 hours.

An increase in ethylene biosynthesis is associated with a number of plant developmental events. After the elucidation of the ethylene biosynthetic pathway, the characterization of the two main enzymes involved in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate synthase (ACS) and oxidase (ACO), led to a better understanding of the molecular basis of the process. Initially it was claimed that ACS played the major regulatory role since ACO appeared to be constitutively in excess in several tissues (Yang et al., 1985). However, the role of ACO is being reconsidered as ACO enzyme activity and specific gene expression are dramatically affected by internal and external factors (Kende, 1993). ACO activity also has been linked to the regulation of physiological events as ethylene autocatalysis (Tang et al., 1994). In transgenic tomato (*Lycopersicon esculentum* Mill.) containing an antisense construct of ACO, the reduction of ACO gene expression and the concurrent lower rates of ethylene production appear to be physiologically significant since they are closely related to the extent of morphogenetic effects (English et al., 1995). Moreover, the onset and modulation of developmental processes such as leaf senescence and fruit ripening are affected in ACO antisense tomato (John et al., 1995; Picton et al., 1993) and melon (*Cucumis melo* L.) (Ayub et al., 1996). In tomato (Bouzayen et al., 1993), petunia (*Petunia hybrida* L.) (Tang et al., 1993), and broccoli (*Brassica oleracea* L.) (Pogson et al., 1995), ACO is encoded by a small multigene family, and it has been demonstrated that the expression of ACO genes is tissue specific and regulated by development and/or hormonal factors, including ethylene (Barry et al., 1996; Picton et al., 1993; Pogson et al., 1995; Tang et al.,

1994). In several fruit, ACO mRNA and activity rise during ripening, when the ethylene burst occurs. According to Tucker (1993), a control mechanism also involving the activation of ACO might be responsible for the transition from system 1 to system 2 (McMurchie et al., 1972), which leads to the autocatalytic ethylene production in climacteric fruit.

In peach (*Prunus persica*), an ACO cDNA clone has been isolated from ripening fruit, and ACO mRNA accumulates in response to wounding and during the softening stage of fruit ripening (Callahan et al., 1992). The highest rates of ethylene biosynthesis are detectable during early peach fruit growth (Miller and Walsh, 1990; Tonutti et al., 1991) and when fruit have softened to ≈20 N (Tonutti et al., 1996). At ripening, the appearance of ACO mRNA precedes the ethylene burst (Callahan et al., 1992), with the strongest transcript accumulation coinciding with the climacteric (Lester et al., 1994). The objective of our paper was to address some of the mechanisms of ethylene regulation during peach fruit development. On this subject, it is demonstrated that ACO mRNA also accumulates during the early growth phase and that propylene, an ethylene analogue, has a dramatic effect on ACO gene expression.

Materials and Methods

PLANT MATERIAL AND TREATMENTS. Growth of peach [*Prunus persica* (L.) Batsch, 'Springcrest'] fruit was monitored at the Univ. of Padova research farm, northeastern Italy, beginning 27 d after full bloom (AFB) by measuring the cheek diameter of 200 fruit. To minimize the physiological fruitlet abscission, fruitlet thinning was performed 21 d AFB. Growth stages (S1, S2, S3, and S4) were defined by using the first derivative of the growth cumulative curve, according to Chalmers and van den Ende (1975). Flesh firmness was measured on fruit harvested at different ripening stages by inserting an Effegi (Alfonsine, Ravenna, Italy) penetrometer (8-mm probe) in the pulp. For each fruit, two determinations were performed and averaged.

Received for publication 5 Nov. 1996. Accepted for publication 14 May 1997. This research, done under the aegis of the Postharvest Working Group of the Italian Society for Horticultural Science (SOI), was supported by National Research Council of Italy, special project RAISA, subproject n.2. We thank A. Kanellis and J.C. Pech for critically reading the manuscript.

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Gas treatments were performed at 25 °C on fruit of the same size collected 27 d AFB (S1). Fruit were flushed with streams at saturated water vapor pressure (water temperature 20 °C) of air or air with propylene (500 mL·L⁻¹) for 48 h. Selected fruit, harvested at the preclimacteric stage (78 d AFB), were treated with air or air with propylene (500 mL·L⁻¹) for 24 or 48 h or with nitrogen (99.9%) for 24, 48, or 72 h. Immediately following ethylene (see below) and flesh firmness determinations, mesocarp (pericarp at S1) was frozen in liquid nitrogen and stored at -80 °C.

ETHYLENE, ACC CONTENT, AND ACO ACTIVITY DETERMINATIONS. Ethylene evolution and ACC concentration were determined on a fresh mass basis as described by Tonutti et al. (1991). ACO activity was determined in vitro as described by Ververidis and John (1991) with the following modifications: frozen tissue was ground and homogenized in extraction buffer [0.1 M tricine, pH 7.5; 10% (v/v) glycerol; 1% PVP; 5 mM DTT; and 0.1% (v/v) Triton]. The extract was filtered and centrifuged for 20 min at 20,000 *g*_n, and the supernatant (crude extract) was collected. All steps were performed at 4 °C. ACO activity was determined by incubating 400 µL of crude extract with 600 µL of a solution containing 0.1 M tricine (pH 7.5), 10% (v/v) glycerol, 1 mM ACC, 30 mM Na-ascorbate, 20 mM NaHCO₃, 0.3 mM FeSO₄, and catalase at 0.5 mg·mL⁻¹.

NUCLEIC ACID EXTRACTION. Total RNA was isolated from frozen tissue as described by Bonghi et al. (1992). Contaminating polysaccharides were removed from total RNA preparations by the method of Chang et al. (1993). Genomic DNA was extracted from young leaves according to the procedure described by Doyle and Doyle (1990). The only modification was an increase (5%) in the amount of β-mercaptoethanol in the CTAB buffer.

SOUTHERN AND NORTHERN BLOT ANALYSES. The homologous probe coding for ACC oxidase was obtained by PCR using primers (upstream 5'-CACACACAAAGAGAGAGA-3' and downstream 5'-TAAGTTTTACTAAGAAGCC-3') selected from pch313, a ripening induced cDNA (homologous to pTOM13)

isolated from peach fruit (Callahan et al., 1993). First strand cDNA was reverse transcribed from DNA-free total RNA (10 µg) extracted from ripe mesocarp, using cDNA cycle kit for RT-PCR (Invitrogen) and an oligo (dT₁₇) as primer. The PCR reaction was performed in a volume of 50 µL with 1× PCR buffer, 1.5 mM MgCl₂, 0.8 mM deoxynucleotide triphosphates, 400 pM primers, 5 µL of cDNA synthesis reaction, and 1.5 units of *Taq* Polymerase. The reaction was cycled 35 times at 94 °C for 60 s, 48 °C for 90 s, and 72 °C for 120 s. The PCR fragment (1017 bp, including primers), identical to the clone isolated by Callahan et al. (1993), was subcloned into pCMTM II (Invitrogen) and sequenced by dideoxy chain termination method using Sequenase Version 1.0 (United States Biochemical, Cleveland, Ohio) with universal and reverse M 13 sequencing primers. For northern blot analysis, aliquots of total RNA (20 µg) were separated by electrophoresis in a 1.4% agarose gel containing formaldehyde and were blotted onto nylon membrane (Hybond N, Amersham). For quantifying total RNA, rRNA, detected using radish rDNA as a probe, was used. The membrane was hybridized to an ACO cDNA probe, labeled by random priming. Hybridization conditions were 40% formamide, 6× SSC, 5× Denhardt's, and 100 µg·mL⁻¹ herring sperm DNA at 42 °C. The last wash was 0.1× SSC at 50 °C. Southern blot analysis was performed with the same probe as described by Callahan et al. (1993).

Results

Ethylene evolution from peach fruit (Fig. 1) was 1.2 nL·h⁻¹·g⁻¹ (fresh mass basis) on the first sampling date (27 d AFB). In fruit with the highest relative growth rate, ethylene biosynthesis declined through S1 reaching a basal level that was maintained through S2, S3, and part of S4. In a separate trial, fruit characterized by slower rates of growth showed high levels of ethylene evolution at 33 and 47 d AFB, corresponding to the second and third (June drop) wave of abscission (data not shown). The rise of ethylene evolution started

Fig. 1. Diameter (open circle) and ethylene evolution (shaded square) of developing 'Springcrest' peach fruit. Stage transitions were determined according to Chalmers and van den Ende (1975). Each value is the mean of 10 (ethylene) and 200 (diameter) replicates. Bars indicate ±SE.

83 d AFB, and reached the value of $3.03 \text{ nL} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ at 88 d AFB, corresponding to the best eating quality of the fruit ($\approx 35 \text{ N}$). ACC content and ACO activity measured in mesocarp at 27, 70, and 88 d AFB (Fig. 2) showed that the amount of ethylene detected at 27 d AFB is associated with a low ACC content and moderate ACO activity. The basal level of ethylene measured at 70 d AFB (Fig. 1) is concurrent with the lowest values of ACC concentration and ACO activity (Fig. 2). The ethylene climacteric parallels the increases in ACC content and ACO activity (Fig. 2). The ethylene climacteric parallels the increases in ACC content and ACO activity.

When total RNA, extracted from fruit collected at 27, 70, and 88 d AFB, was probed with the ACO cDNA clone, a weak hybridization signal (1.4 kb) was detected at 27 d AFB, whereas ACO transcripts were undetectable at 70 d AFB (Fig. 3). The high levels of ethylene evolution and ACO activity measured at 88 d AFB were concurrent with a dramatic accumulation of ACO mRNA.

When the fruitlets collected at 27 d AFB were fumigated with $500 \text{ mL} \cdot \text{L}^{-1}$ propylene for 48 h, almost a 2-fold increase of ethylene evolution was observed (Fig. 4A). No significant difference from the control was detected in fruitlets treated with air for 48 h. ACC content remained unaffected by air and propylene treatments (data not shown). ACO activity was enhanced following air treatment and greatly increased when propylene was applied (Fig. 4B). In propylene-flushed fruitlets, ACO gene expression was markedly stimulated (Fig. 4C). A slight increase of ACO-related transcripts also was observed in fruitlets maintained for 48 h in air (Fig. 4C).

Detailed observations of ethylene physiology performed on a single-fruit basis through the ripening process (Fig. 5) reconfirmed that the climacteric occurs after fruit have already started to soften. Nevertheless, increases in mesocarp ACC content and ACO activity were detected at 80 d AFB when the fruit ethylene evolution was still almost undetectable and before any noticeable change in fruit firmness. The highest value of ACC content was observed 85 d AFB and declined thereafter; ACO activity dramatically increased at ethylene climacteric (90 d AFB). ACO transcripts were undetectable at the first sampling date (75 d AFB), whereas a hybridization signal appeared 80 d AFB, and the intensity increased through ripening (Fig. 5).

Treatments with air, propylene or nitrogen performed for 24 or 48 h on preclimacteric fruit harvested 78 d AFB, affected ethylene physiology differently (Fig. 6). In air, there was only a moderate stimulation of ethylene biosynthesis after 24 h, whereas a marked increment of ethylene evolution was monitored after 48 h. Propylene stimulated ethylene evolution and induced a dramatic accumulation of ACO transcripts after 24 h. A decline of ACO mRNA was observed at 48 h when fruit displayed the highest ethylene evolution. In N, no ethylene evolution was detected after 24, 48, and 72 h, although an accumulation of ACO transcripts after 48 and 72 h of incubation occurred.

Fig. 2. ACC content (open bars) and ACO activity (shaded bars) measured in 'Springcrest' pericarp [27 d after full bloom (AFB)] and mesocarp (70 and 88 d AFB). Values are means of three replicates $\pm$ SE.

Fig. 3. Northern blot analysis of total RNA extracted from 'Springcrest' pericarp, 27 d after full bloom (AFB), and mesocarp, 70 and 88 d AFB, and probed with the ACO cDNA clone.

Discussion

During peach fruit ('Springcrest') development, there was increased ethylene biosynthesis at the early stage (S1) of fruit growth and in late ripening. An increase of ethylene evolution in another peach variety was observed by Miller et al. (1988) in fruitlets characterized by slower growth rate and undergoing abscission. The presence of ACO transcripts and the high values of ethylene biosynthesis and ACO activity detected during early S1 suggest that ethylene might be involved, in association with other growth substances, in the modulation of cell division and/or enlargement processes. Ethylene regulation of growth has been described in vegetative tissues in which the increase of ethylene biosynthesis is induced by auxin through a stimulation of ACC synthase gene expression (Kim et al., 1992; Yip et al., 1992). ACO transcript accumulation appears to be unaffected by auxin in broccoli floral tissues (Pogson et al., 1995) and in orchid (*Phalaenopsis* spp.) ovary (O'Neill et al., 1993). However, in

orchid stigma and petals (O'Neill et al., 1993) and in geranium (*Pelargonium xhortorum* Bailey) leaves (Wang and Arteca, 1995), ACO gene expression has been induced by auxin. Peck and Kende (1995) demonstrated that in auxin-treated pea (*Pisum sativum* L.) seedling, the increase of ACO transcript and activity is mediated by ethylene formed as the result of the enhanced ACC synthase activity directly stimulated by auxin. Although the auxin content in peach fruitlets is high during S1 (Vizzotto et al., 1989), the hypothesis that an increase in endogenous IAA enhances ethylene evolution in peach fruit tissue has been discarded by Miller and Walsh (1990). Our data indicate that the relatively high values of ethylene evolution during early 'Springcrest' fruit development are mainly due to an enhancement of both ACO gene expression and enzyme activity, since the measurable ACC content remained at a basal level. Differently from Callahan et al. (1993), a weak

hybridization signal was detected when total RNA, extracted from 'Springcrest' fruitlets collected during early S1, was probed with ACO cDNA. This may be variety dependent. In fact, our attempt to detect the presence of ACO transcripts during S1 in 'Redhaven' fruit failed, even when low stringency conditions were used (data not shown).

In tomato, there is a differential expression of ACO gene family members in various tissues at different developmental stages (Barry et al., 1996). Considering that at S1, ACO activity is not well correlated with probe-specific mRNA accumulation, it might be hypothesized that functionally related genes are present and specifically expressed during early fruit development. This is consistent with the results of the Southern analysis (data not shown), which indicate that in "Springcrest" ACO appears to be encoded by a multigene family.

Fig. 4. Ethylene biosynthesis (A), ACO activity (B), and ACO transcript accumulation (C) in 'Springcrest' fruitlets, collected at 27 days AFB (CK) or flushed with air (AIR) or air with propylene (500 mL·L⁻¹) (PP) streams for 48 h. Values are means of 5 (ethylene) and 3 (ACO activity) replicates $\pm$ SE.

Fig. 5. Ethylene evolution and flesh firmness (A), ACC content and ACO activity (B), and ACO transcript accumulation (C) in 'Springcrest' fruit harvested at different stages of ripening. All determinations have been carried out on single-fruit basis.

During the last developmental stage, ACO mRNA starts to accumulate before textural changes occurred. John et al. (1995) observed that in tomato the expression of ACO takes place in green leaves before any visible sign of senescence. Our data show that a marked accumulation of ACO transcripts occurs in a preclimacteric stage, although the strongest hybridization signal is present late during peach fruit ripening when flesh firmness values are low (≈ 20 N) and in correspondence of the ethylene climacteric, as observed by Callahan et al. (1992, 1993) and Lester et al. (1994). Thus, it appears that peach fruit ACO gene expression is under developmental control and ethylene regulated. Considering the kinetics of ethylene evolution, ACC content and ACO activity, and transcript accumulation at the preclimacteric and climacteric stages (Fig. 5), it might be suggested that the probe-related ACO gene is not constitutively expressed, rather it is induced during ripening. The same data would indicate that ACC oxidase might play a regulatory role on ethylene biosynthesis.

Our results indicate that the propylene-induced ACO transcript accumulation occurs during early S1 and ripening, but the strongest effect is observed during the latter stage when the ethylene autocatalytic pathway is activated. An ethylene-induced stimulation of ACO gene expression has been demonstrated in reproductive tissues of carnation (*Dianthus caryophyllus* L.; Woodson et al., 1992), orchid (O'Neill et al., 1993), and tomato (Picton et al., 1993) and in vegetative tissue of mung bean (*Vigna radiata* L.; Kim and Yang, 1994) and pea (Peck and Kende, 1995). A promotion of ACO activity by ethylene has been demonstrated in the peel of apple (*Malus domestica* Borkh.) and in preclimacteric tomato and muskmelon (*Cucumis melo* L.) fruit (Liu et al., 1985; Bufler, 1986). We have shown that the stimulation of ethylene biosynthesis induced by propylene in preclimacteric peaches is concurrent with a strong accumulation of ACO mRNA (Fig. 6). Although ACO activity is markedly stimulated, the propylene-induced ethylene evolution is much lower at S1 (Fig. 4) than at ripening (Fig. 6). This might be the consequence of the low ACC content, which is unaffected by the ethylene analogue. Thus, ACC appears to be the limiting factor in ethylene biosynthesis in fruit during S1. It must be noted that the assay of ACO activity and the extraction of total RNA in fruitlets harvested at 27 d AFB have been performed on the whole pericarp. Difference among fruit tissues in terms of ethylene physiology have been described in peach fruit at ripening (Tonutti et al., 1991, 1996), and it is likely that this also occurs in developing fruitlets. As reported for petunia (Tang et al., 1994) and broccoli (Pogson et al., 1995), it is possible that in peach fruit, one or more of the ACO genes is/are regulated by ethylene and is/are responsible for the propylene-stimulated ethylene production at S1 and the autocatalytic biosynthesis at ripening. Whether the ethylene-regulated gene(s) is/are the same in the different developmental stages remains to be elucidated.

When preclimacteric peach fruit were incubated up to 72 h in a nitrogen atmosphere, a strong inhibition of ethylene evolution was

Fig. 6. Ethylene biosynthesis and ACO mRNA accumulation in 'Springcrest' fruit harvested at 78 d after full bloom (control) and treated with air or air with propylene (500 mL·L⁻¹) for 24 and 48 h or nitrogen (99.9%) for 24, 48, and 72 h at 25 °C. Determinations have been performed on single-fruit basis.

monitored throughout the experimental period and a reduction of ACO transcript accumulation occurred. These results, in agreement with those reported by Gorny et al. (1996) for apple maintained for longer periods in hypoxia, indicate that oxygen deprivation delays the induction of ACO gene expression in preclimacteric peach fruit.

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