Plant Physiol. (1985) 77, 407–411
0032-0889/85/77/0407/05/$01.00/0

Promotion by Ethylene of the Capability to Convert 1-Aminocyclopropane-1-carboxylic Acid to Ethylene in Preclimacteric Tomato and Cantaloupe Fruits[1]

Received for publication May 22, 1984 and in revised form September 10, 1984

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ABSTRACT

The intact fruits of preclimacteric tomato (*Lycopersicon esculentum* Mill) or cantaloupe (*Cucumis melo* L.) produced very little ethylene and had low capability of converting 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. When these unripe tomato or cantaloupe fruits were treated with ethylene for 16 hours there was no increase in ACC content or in ethylene production rate, but the tissue's capability to convert ACC to ethylene increased markedly. Such an effect was also observed in fruits of tomato mutants *rin* and *nor*, which do not undergo ripening and the climacteric increase in ethylene production during the senescence. The development of this ethylene-forming capability induced by ethylene increased with increasing ethylene concentration (from 0.1 to 100 microliters per liter) and duration (1 to 24 hours); when ethylene was removed this capability remained high for sometime (more than 24 hours). Norbornadiene, a competitive inhibitor of ethylene action, effectively eliminated the promotive effect of ethylene in tomato fruit. These data indicate that the development of the capability to convert ACC to ethylene in preclimacteric tomato and cantaloupe fruits are sensitive to ethylene treatment and that when these fruits are exposed to exogenous ethylene, the increase in ethylene-forming enzyme precedes the increase in ACC synthase.

Climacteric fruits are characterized by a surge in ethylene production at the onset of ripening and ethylene treatment is known to hasten such a process. This phenomenon is referred to as autocatalytic ethylene production (1). The pathway of ethylene biosynthesis has been demonstrated to be: Met[4] → SAM → ACC → ethylene by Adams and Yang (2). Since preclimacteric (unripe) fruits lack both ACC synthase and EFE, a massive increase in ethylene production requires development of both enzymes (20). It has been shown that at the onset of ripening, ACC synthase activity increases which is accompanied by an increase in ACC content (3, 8, 10, 18). Since EFE has not been demonstrated in a cell-free system, its activity can be only measured *in vivo* (20). Although the capability to convert ACC to ethylene has been shown to be very low in preclimacteric fruits and high in climacteric fruits, the changes of this capability during ripening has not been studied. Recently, ethylene has been reported to promote the development of this capability in excised tissues of preclimacteric cantaloupe fruit (9), citrus leaves (15), and tobacco leaves (6). In this study we investigated the effect of ethylene on the development of EFE activity in intact preclimacteric tomato and cantaloupe fruits.

MATERIALS AND METHODS

Plant Materials and Treatments. Tomato (*Lycopersicon esculentum* Mill) used in the experiments were two normal ripening varieties, T_3 and Castlemart, and two non-ripening mutants, *nor* and *rin*, which are nonclimacteric fruits that do not undergo the normal ripening process (19). Greenhouse-grown tomato fruits were harvested at the mature green stage and kept at 20°C overnight before use. Preclimacteric cantaloupe (*Cucumis melo* L. cv Powdery Mildew Resistant No. 45) fruits were harvested from the field when the fruits weighing approximately 500 g were still green externally and internally, and the netting of the fruits was incompletely developed; they were stored overnight at 25°C before use. Only those fruits producing less than 0.2 nl g^{-1} h^{-1} ethylene were used in experiments. For ethylene treatments intact fruits (two or more) or discs, which were cut from pericarp tissue of tomato fruits with a cork borer (0.5 cm diameter) and placed on a moistened paper in a Petri dish, were enclosed in 8.6-L jars containing a cup of KOH solution for absorbing CO_2 released from tissues. The appropriate amount of ethylene was introduced by syringe and the concentration of ethylene was verified by gas chromatography. In ethylene-free air treatments a cup of $Hg(ClO_4)_2$ solution was placed in the jar; this kept the ethylene concentration below 0.02 μl l^{-1} during the treatment period. In NDE treatment, appropriate amount of NDE was injected with a syringe into a piece of paper hung in the jar to facilitate evaporation. The concentration of NDE in the gas phase was calculated from the amount of NDE injected.

Determination of Ethylene and ACC. A 1-ml gas sample was withdrawn from the head space of the incubating vessels with a syringe and ethylene was assayed with a gas chromatograph equipped with an alumina column and a flame ionization detector. For determination of ACC, discs were extracted with 80% ethanol as described by Liu *et al.* (11), and ACC content was assayed according to the method of Lizada and Yang (13).

Assay of EFE. EFE was determined *in vivo* by measuring the conversion of administered ACC to ethylene (9). After gas treatment, the pericarp of tomato fruit was cut into discs (0.5 cm diameter) using a cork borer. Discs weighing 1 g were incubated in 1 ml of solution containing 0.1 mM CHI, 50 mM Mes buffer

[1] Supported by a Research Grant (PCM-8414971) from the National Science Foundation.

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[4] Abbreviations: Met, methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AIB, α-aminoisobutyric acid; CHI, cycloheximide; EFE, ethylene-forming enzyme which catalyzes the conversion of ACC to ethylene; NDE, 2,5-norbornadiene; SAM, *S*-adenosylmethionine.

(pH 6.1), 2% sucrose, and 50 μg chloramphenicol in the presence and absence of 2 mM ACC, in an enclosed 30-ml Erlenmeyer flask for 6 h at 20°C with constant shaking. Cantaloupe discs, prepared as previously described (9), were immediately infiltrated with 10 μM CHI in the presence and absence of 1 mM ACC and enclosed in 6-ml syringes for 1 h at 25°C. CHI was employed to inhibit new enzyme (including ACC synthase) synthesis, induced by wounding, during the incubation period. The ethylene produced was assayed by GC.

Administration of Radioactive ACC and AIB. Each disc (1 cm diameter) cut from tomato pericarp tissue was smeared uniformly on the internal side with 5 μl of 50 mM Mes buffer (pH 6.1) containing [1-^{14}C]AIB (5 nmol, 45 nCi) or [2,3-^{14}C]ACC (5 nmol, 8.5 nCi) and two discs in each treatment were incubated in a 30-ml flask for 6 h. The radioactive ethylene was absorbed into a center well containing a slip of filter paper wetted with $Hg(ClO_4)_2$ solution. At the end of the incubation period the paper was soaked in Atomlight scintillation liquid and the radioactivity was determined with Beckman Liquid Scintillation Counter. The $^{14}CO_2$ produced from [1-^{14}C]AIB during the incubation period was absorbed into 150 μl KOH solution which was placed in a center well. At the end of incubation, the solution was transferred into a scintillation vial containing Atomlight liquid and the radioactivity was determined by scintillation counting. At the end of incubation the discs were rinsed with water, and the radioactive uptake was determined by the total radioactivity administered to discs minus the radioactivity remaining in the incubation medium and rinsing solution.

RESULTS

Effect of Ethylene on EFE. The intact preclimacteric tomato and cantaloupe fruit produced very little ethylene. Once it was cut into pieces, wound ethylene production was induced (4, 9, 24); pretreatment with CHI inhibited this wound ethylene production (9, 10, 24). Figure 1 and Table I show that when the tomato or cantaloupe discs were incubated in the presence of CHI, no increase in wound ethylene production was observed. In the present study, EFE was assayed by measuring the capacity to produce ethylene by discs which had been incubated with ACC and CHI. In control samples pretreated with air, the exogenously applied ACC only slightly increased ethylene production (Fig. 1; Table I) indicating that preclimacteric fruit, unlike vegetative tissues (5), have low EFE activity which is one of the factors limiting the rate of ethylene production (21). Under the present experimental conditions, ethylene treatment did not result in increased ACC level (Table I; Figs. 2 and 3). Ethylene treatment did promote EFE, as indicated by the marked increase in ethylene production following administration of external ACC. Although the magnitude of the promotion by ethylene varied among different fruits, the trends were identical. Ethylene has been shown to promote the development of EFE activity induced by wounding in excised tissues (6, 9, 15); the present study shows that ethylene also induces the development of EFE in intact, preclimacteric fruit. Figure 1 also shows that for the tomato system, ethylene production rate reached the maximum at 2 h after incubation with ACC and then declined gradually. When ACC was added in the medium again at 6 h, the ethylene production rates hardly change (data not shown), indicating that the decrease in ethylene production rates was due to the decline in EFE, but not due to the exhaustion of ACC. Assuming that the new synthesis of EFE was inhibited by the administered CHI, the apparent half-life time of EFE was estimated to be about 5 h, which is similar to that observed in mungbean hypocotyls by Yu *et al.* (23). Since the half-life time of ACC synthase is about 30 min in wounded green tomato (10) and in IAA-treated mungbean hypocotyl (22), it appears that EFE is turned over less rapidly than ACC synthase. The data obtained with variety

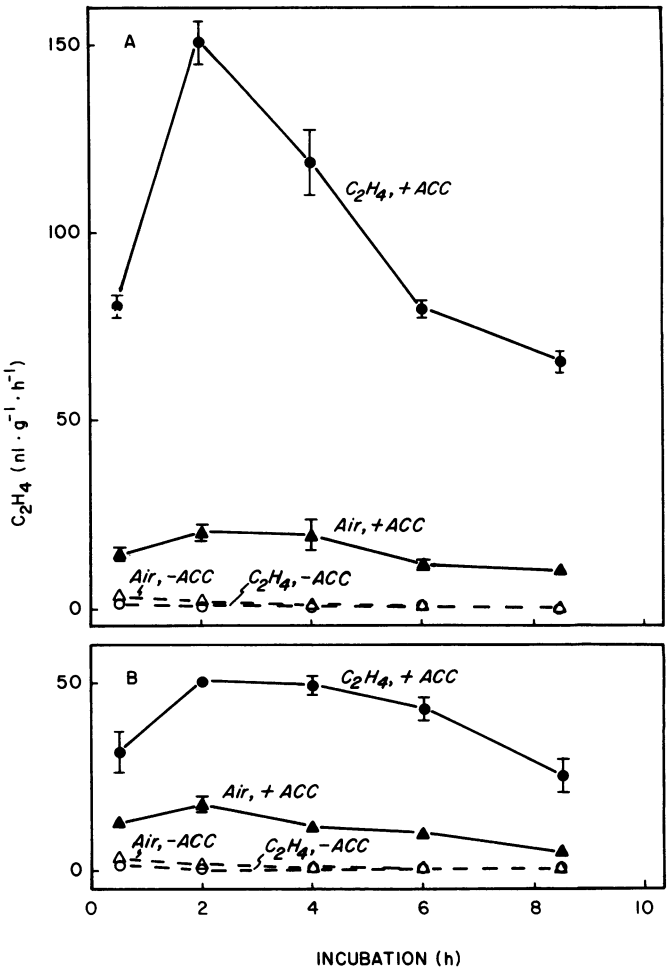

FIG. 1. Time course of ethylene production in tomato fruits. Fruits of variety T_3 (A) or *rin* (B) were treated with ethylene (10 μl l^{-1}) or air (control) for 16 h. The discs were cut from pericarp and incubated with CHI and with or without ACC. Ethylene production was determined at the indicated time. The flasks were flushed with air after each determination. Each value represents the mean ± SE of two replicates.

Table I. *Effect of Ethylene Treatment on ACC Content, Ethylene Production Rate, and the Capability to Convert ACC to Ethylene in Cantaloupe Fruit*

Intact immature cantaloupe fruits were exposed to 4 μl l^{-1} ethylene or air for 16 h. Discs were prepared and immediately vacuum infiltrated with 10 μM CHI and with or without 1 mM ACC. Discs (1 g) were then enclosed in 6-ml syringes, the ethylene produced between 20 and 80 min period was determined. Each value represents the mean ± SE of three replicate samples. Ethylene production by the discs with exogenously administered ACC was taken as the tissue's capability to convert ACC to ethylene.

Treatment	ACC	Ethylene	ACC → Ethylene
	nmol g^{-1}	*nl g^{-1} h^{-1}*	
Air	3.4	0.05 ± 0.01	0.26 ± 0.2
Ethylene	1.1	0.08 ± 0.02	11.4 ± 0.2

'Castlemart' (not shown) was very similar to that of variety 'T_3' which was presented in Figure 1.

It is interesting to note that ethylene also induced the EFE development in *rin* (Fig. 1B) and *nor* (data not shown) mutant fruits, which are classified as nonclimacteric fruits (7, 14), although the magnitude of promotion was not as great as the normal tomato.

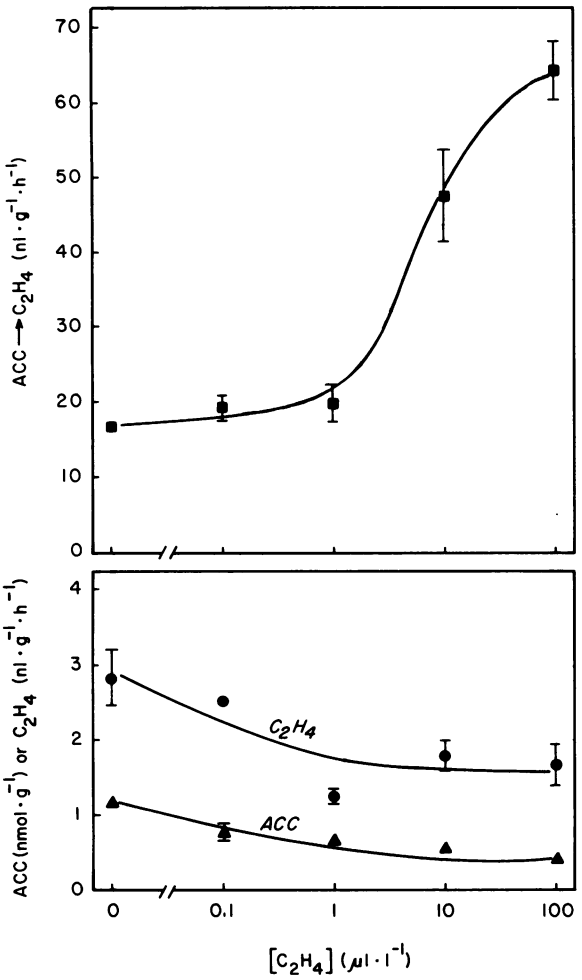

FIG. 2. Effect of ethylene concentration on ethylene production, ACC content, and capability to convert ACC to ethylene in tomato fruits. After fruits (T_3) were treated with various concentrations of ethylene for 16 h, discs were cut from pericarp and their ACC content and ethylene production in a 1-h incubation period without ACC were determined. ACC → ethylene was the ethylene production rates of discs incubated with ACC between 1 to 3 h. Each value represents the mean ± SE of two replicates.

Dependence of Development of EFE on the Concentration and Duration of Ethylene Treatment. The effect of various ethylene concentrations on ethylene production rate, ACC content and EFE development in intact T_3 tomato fruits are presented in Figure 2. The ethylene production rate and ACC content decreased slightly as ethylene concentration was increased from 0 to 100 $\mu l\ l^{-1}$. The promotion of EFE by ethylene markedly increased as the concentration of C_2H_4 was increased from 1 to 100 $\mu l\ l^{-1}$. Although ethylene treatment also caused similar effects when applied to tomato discs instead of intact fruit, the inhibition of ethylene production rate and ACC content was greater in discs than in intact fruit, whereas the promotion of EFE by ethylene treatment was much greater in intact tomato fruit than in discs (data not shown). It should be noted that the concentration of ethylene required to enhance the development of EFE is rather high (Fig. 2).

In many hormonal systems, continuous presence of the hormone is required for a sustained maximal effect. We pretreated the intact fruits with ethylene for different durations and then determined their resulting EFE capability (Fig. 3). The EFE increased progressively with increasing duration of treatment up to 24 h. However, the ACC content did not increase. Under

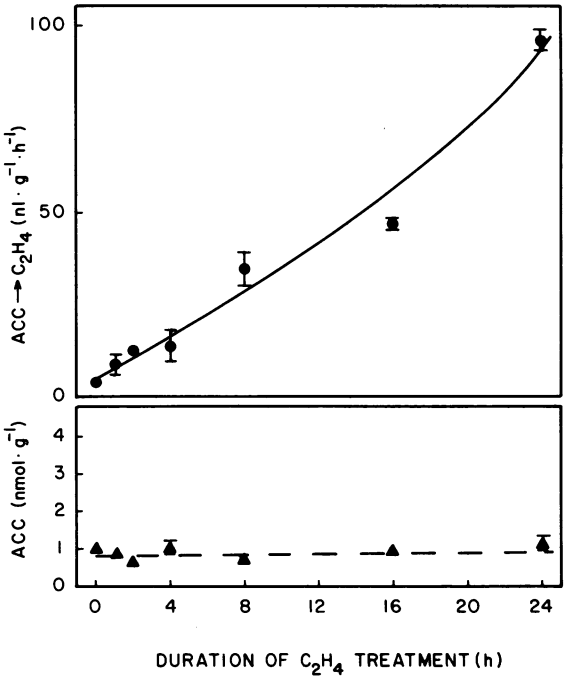

FIG. 3. Effect of duration of ethylene treatment on the capability to convert ACC to ethylene. Fruits were treated with 10 $\mu l\ l^{-1}$ ethylene for various durations as indicated. Discs were then cut and measurements were carried out as described in Figure 2.

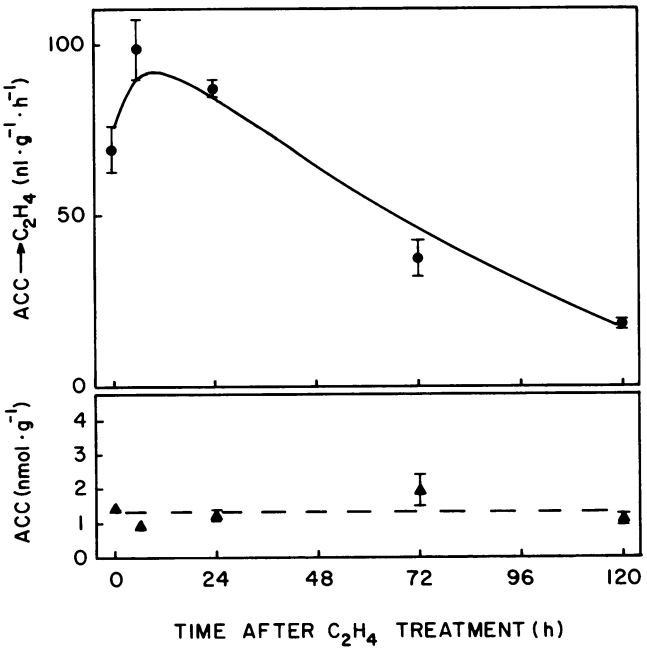

FIG. 4. Effect of time after ethylene treatment on the capability to convert ACC to ethylene. Fruits were treated with 11 $\mu l\ l^{-1}$ ethylene for 16 h and were then transferred to air for various durations as indicated. Discs were then prepared and measurements were conducted as described in Figure 2.

these experimental conditions, increased development of EFE requires the continuous presence of ethylene for maximal response.

After 16 h ethylene treatment the EFE activity remained high at least for 24 h and then declined gradually during the next 100 h experimental period. There was little change in the ACC content during this period (Fig. 4). These results indicate that

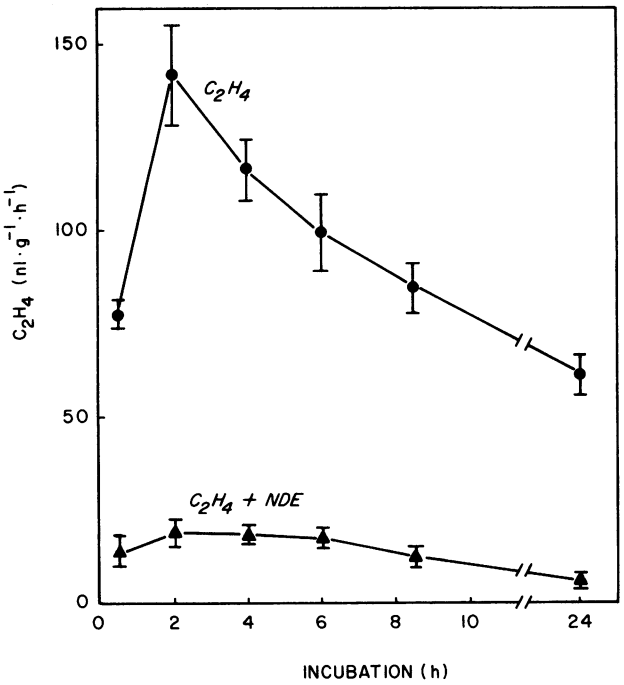

FIG. 5. Effect of norbonadiene treatment on the development of the capability to convert ACC to ethylene in tomato fruits. Fruits were treated with 15 μl l^{-1} ethylene or with 15 μl l^{-1} ethylene plus 2500 μl l^{-1} norbonadiene for 24 h. Discs were then prepared and measurements were conducted as described in Figure 2.

Table II. *Effect of Ethylene Treatment on the Capability to Convert ACC to Ethylene and to Decarboxylate AIB to CO_2 in Tomato Fruit*

After fruits were treated with or without ethylene (27 μl l^{-1}) for 18 h, discs were cut from the pericarp tissue and [2,3-^{14}C]ACC (5 nmol, 8.5 nCi) or [1-^{14}C]AIB (5 nmol, 45 nCi) was administered. The conversions of ACC to ethylene and of AIB to CO_2 were determined and expressed as the per cent of radioactivity uptaken by discs.

Treatment	[2,3-^{14}C]ACC → Ethylene	[1-^{14}C]AIB → $^{14}CO_2$
	% of uptake	
Air	5.6	0.5
Ethylene	16.8	3.0

the synthesis of EFE did not stop immediately after exposure to exogenous ethylene was terminated, as the EFE declined at a much slower rate than that estimated from the decline of EFE in the presence of CHI. This observation suggests that the messenger coded for EFE synthesis is induced by ethylene and decays slowly after ethylene is withdrawn.

Effect of NDE on the Development of EFE. NDE is a competitive inhibitor of ethylene action (17) and has been shown to inhibit the senescence of tobacco leaves (16) and the ripening of tomato fruits (L. Su, unpublished data). It is of interest to examine the effect of NDE on the promotion of EFE development by ethylene in tomato system. Figure 5 shows that ethylene treatment increased EFE activity but NDE eliminated such an effect, as expected.

Effect of Ethylene on Decarboxylation of AIB. Recently Liu *et al.* (12) have observed that the ability of mungbean hypocotyls to decarboxylate AIB to CO_2 is intimately related to its EFE activity. If oxidative decarboxylation of AIB is mediated by EFE, one would expect that ethylene should also promote the ability of tomato tissue to decarboxylate AIB. We have therefore compared the effect of ethylene on the conversion of [2,3-^{14}C]ACC to $^{14}C_2H_4$ and [1-^{14}C]AIB to $^{14}CO_2$ by the present tomato fruit tissue. Indeed, ethylene promoted both reactions as shown by the data of Table II.

DISCUSSION

Ethylene has been shown to stimulate the conversion of ACC to ethylene in excised tissues of preclimacteric cantaloupe fruit (9) and citrus and tobacco leaves (6, 15). In the present study we demonstrated that ethylene also induces the development of EFE in intact preclimacteric fruits of tomato and cantaloupe. Since preclimacteric (unripe) fruits lack both ACC synthase and EFE (20), a massive increase in ethylene production following ripening requires the induction of both ACC synthase and EFE. When preclimacteric fruits are exposed to ethylene, the ripening process is accelerated which results in a massive increase in ethylene production or autocatalysis of ethylene production. In the present experiments, where immature tomato or cantaloupe fruits were treated with ethylene for a short period, the tissue's ability to convert ACC to ethylene (EFE) was already stimulated. However, the tissue's endogenous ethylene production rates or ACC levels did not increase, but rather decreased slightly (Fig. 2). Once EFE is induced, it decays slowly (Figs. 1 and 4). The promotion of EFE synthesis by ethylene explains why application of ACC to intact preclimacteric tomato fruits effectively hasten the ripening process, even though EFE activity is initially low in the tissue (20). Our present data indicate that when preclimacteric fruit tissues are exposed to ethylene, the increase in EFE precedes the increase in ACC synthase. Whether or not this is also true during the natural ripening of fruits remains to be established.

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