Ethylene Promotes the Capability To Malonylate 1-Aminocyclopropane-1-carboxylic Acid and D-Amino Acids in Preclimacteric Tomato Fruits

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ABSTRACT

When whole unripe green tomato fruits (Lycopersicon esculentum Mill, cv T3) were treated with ethylene (10 micromolars per liter) for 18 hours, the fruit’s ability to convert 1-aminocyclopropane-1-carboxylic acid (ACC) to N-malonyl-ACC (MACC) increased markedly and such an effect was also observed in fruits of mutant nor, which cannot ripen normally. The promotion of the capability to malonylate ACC by ethylene increased with the increasing ethylene concentration from 0.1 to 100 micromolars per liter and with increasing duration of ethylene treatment up to 8 hours; a longer duration of ethylene treatment did not further increase the malonylation capability. When ethylene was withdrawn, the promotion disappeared within 72 hours. Norbornadiene, a competitive inhibitor of ethylene action, effectively eliminated the promotive effect of ethylene. Ethylene treatment also promoted the fruit’s capability to conjugate d-amino acids and a-amino-isobutyric acid. Since the increase in the tissue’s capability to malonylate ACC was accompanied by an increase in the extractable activity of ACC and d-amino acid malonyltransferase, ethylene is thought to promote the development of ACC/d-amino acid malonyltransferase in unripe tomato fruits.

Ethylene is biosynthesized in higher plants via the following sequence: Met → SAM → ACC → ethylene (2, 29). Recently, Amrhein et al. (3) and Hoffman et al. (10) have demonstrated that ACC is conjugated to form MACC in many tissues. Thus, malonylation of ACC serves as a mechanism to dissipate excess ACC and participates in the regulation of ethylene biosynthesis (4, 28). Since the malonylation of ACC occurs widely in plant tissues, it is thought that the enzyme responsible for this process is largely constitutive (4, 10, 29). However, little is known about the regulation of ACC malonylation in plant tissues. Since exogenous ethylene is known to inhibit or promote ethylene production in various plant systems (1, 29), it is pertinent to investigate whether ethylene may regulate ethylene production by affecting the malonylation of ACC. Malonylations of ACC and d-amino acids have been demonstrated in vivo and in vitro, and it is thought that both reactions are catalyzed by the same enzyme system (4, 14, 17, 20). In the present study, we have examined the effect of ethylene on the development of the capability to conjugate ACC and d-amino acids in preclimacteric tomato fruits.

MATERIALS AND METHODS

Plant Materials and Treatments. As described previously (18), tomato (Lycopersicon esculentum Mill) cv T3 and mutant nor, which does not undergo ripening normally (27), were grown in greenhouses. Fruits were harvested at the mature green stage and kept at 20°C overnight before use. Only those fruits producing less than 0.2 nl g⁻¹ h⁻¹ ethylene were used in experiments. Intact fruits were enclosed in a 8.6-L jar containing a cup of 20% KOH solution to absorb CO₂ released from tissues. The appropriate amount of ethylene was injected into the jar by a syringe and its concentration was verified by GC. In controls, a cup of 0.25 m Hg (ClO₂)₂ solution was placed in the jar where the ethylene concentration was maintained at less than 0.02 μl l⁻¹ during the treatment period. In NDE treatment, 100 μl of NDE liquid was injected into a piece of filter paper hung in the jar to facilitate evaporation (25). The concentration of NDE in the gas phase was calculated to be about 2500 μl l⁻¹.

Determination of Tissue Capability To Form MACC. After intact fruits were treated with air or ethylene, discs were prepared from the pericarp. Some discs (1 g) were incubated in 1 ml of 2 mM ACC solution containing 50 mM Mes buffer (pH 6.1), 2% sucrose, 50 μg ml⁻¹ chloramphenicol, and 0.1 mM CHI, in a 30-ml Erlenmeyer flask at 20°C. CHI was employed to inhibit any new enzyme formation during the incubation period. As control, other discs (1 g) were incubated in the same solution but without ACC and CHI. After incubation for 6 h, the discs were rinsed with buffer and used for the assay of MACC content.

Determination of MACC, ACC, and Ethylene. Discs were extracted three times with 10 ml 80% ethanol at 70°C, and after removal of ethanol, the residue was dissolved in 2 ml water. An aliquot (20–200 μl) was employed for the ACC assay according to Lizada and Yang (21). Another aliquot (0.6 ml) was passed through the cation exchange resin (Dowex 50, H⁺ form, 2 ml bed-volume) to remove amino acids including ACC, and the effluent containing MACC was hydrolyzed in 2 N HCl at 100°C for 3 h to liberate ACC (10). Following neutralization with NaOH, the resulting hydrosylate was assayed for ACC as described above, and this ACC content was regarded as MACC content. Ethylene concentration was determined on a gas chromatograph equipped with an alumina column and a flame ionization detector.

Administration of Radioactive Chemicals. Each pericarp disc (1 cm diameter), cut from ethylene-treated or control (air-treated) fruits, was smeared uniformly on the internal side with 10 μl of 50 mM Mes buffer (pH 6.1) containing [2,3,4-¹⁴C]ACC (5 nmol, 8.5 nCi), [1-¹⁴C]AIB (5 nmol, 45 nCi), D-[3,4-¹⁴C]methionine

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2 Abbreviations: SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; NDE, 2,5-norbornadiene; AIB, α-aminoisobutyric acid; CHI, cycloheximide; MACC, l-(malonylamino)cyclopropane-1-carboxylic acid.
was ethanol through determined that expressed and concentrated as with water and extracted with 80% ethanol. The extract was concentrated under reduced pressure and the radioactive metabolites were separated by paper chromatography using 1-butanol:acetic acid:water (4:1:1.5, v/v) as the developing solvent. The radioactivities in paper chromatograms were determined with a radioscanner. For quantitative determination of radioactive amino acid conjugates, the tissues were extracted with ethanol and, after removal of ethanol, the extract was passed through an ion exchange resin column of Dowex 50 (H\(^+\) form) as described previously (17). The radioactivities in the extract and in the effluent were taken as the total extractable radioactivity and conjugate radioactivity, respectively. Radioactivity was determined with a scintillation counter.

Assay of Malonitriltransferase Activity. The extraction and determination of the malonyltransferase were essentially those described by Kionka and Arnehr (14) and Liu et al. (20). Percarp tissue (10 g) was homogenized with an equal weight of 0.1 M K-phosphate (pH 7.2) containing 0.1 M KC1 and 0.4 mM DTT by an ULTRA-TURRAX homogenizer. After centrifugation (12,000g, 30 min) the supernatant was fractionated with (NH\(_4\))\(_2\)SO\(_4\) and the precipitate at 30 to 60% saturation was redissolved in the buffer mentioned above. The enzyme extract was dialyzed overnight against 10 mM K-phosphate (pH 7.2) containing 10 mM KC1 and 0.2 mM DTT. The dialyzate extract was employed as the enzyme solution. Protein concentration was determined by the method of Bradford (6). The reaction mixture contained 0.1 mM KC1, 0.1 mM K-phosphate (pH 8.0), 0.25 mM [2,3-\(^14\)C]ACC (10 nCi), 1 mM malonyl-CoA and 0.1 mg protein in a total volume of 50 \(\mu\)l. After incubation at 35\(^\circ\)C for 1 h, the reaction mixture was passed through a small column (0.3 ml bed volume) of ion exchange resin Dowex 50 (H\(^+\) form). The radioactivity in the reaction mixture and the radioactivity in the effluent plus washing were taken as the amount of substrate and product (MACC), respectively. The enzyme activity was expressed as nmol MACC formed/mg protein·h.

RESULTS

Promotion of MACC Formation by Ethylene. Table 1 shows that pretreatment with ethylene resulted in little change in ethylene production of the discs, although the discs prepared from normal tomato cv T\(_3\) produced higher ethylene than those from the mutant nor. Ethylene treatment, however, resulted in lower ACC levels in both cultivars, but exerted little changes in MACC levels, which ranged between 1.2 and 1.9 nmol g\(^{-1}\). When ACC was administered to discs prepared from air-treated fruits, the MACC level increased only slightly (about 2 nmol g\(^{-1}\)) during the 6-h incubation period. This increase represented less than 1% of ACC within the tissues, indicating that the capability to convert ACC to MACC in those preclimacteric tomato fruit tissues was as low as their capability to convert ACC to ethylene (18; Table 1). The exogenous ethylene treatment promoted the tissues' capability to convert ACC to MACC more than 10-fold in both cultivars, although the magnitude of the promotion varied among different experiments. In parallel with the increase in MACC formation, the capability to convert ACC to ethylene also increased by ethylene treatment (18). Since CHI was present in the incubation solution, it can be assumed that there was little new synthesis of enzymes in the discs during the incubation period as observed by other investigators (5, 9, 30). Moreover, the quantity of ACC applied to the discs was so much greater than that of endogenous ACC that the changes in endogenous ACC resulting from excision would be insignificant. Hence, the marked increase in the capability to convert ACC to MACC observed in the present experiment is thought to result from the ethylene treatment on whole fruits, but not from excision. It should be noted that the endogenous level of ACC in the ethylene-treated discs is very low. Hence, the increased MACC formation in the tissue could not be observed until the tissue was administered with exogenous ACC.

Effects of Ethylene Concentration and Treatment Duration. The effect of ethylene concentration on the tissue capability to convert ACC to MACC is shown in Figure 1. When the discs which were cut from ethylene-pre-treated fruits were incubated without ACC, there was no increase in MACC content in all treatments, but when the discs were incubated with ACC, the MACC formation increased markedly (Fig. 1). The effectiveness of ethylene treatments in promoting the capability to malonylate ACC increased with increasing concentration of ethylene (Fig. 1). Ethylene at 1 \(\mu\)l l\(^{-1}\) resulted in slight promotion, whereas ethylene at 100 \(\mu\)l l\(^{-1}\) resulted in a 16-fold increase as compared to the air control. The concentrations of ethylene required in this response were much higher than those in other ethylene-mediated responses. When discs, instead of whole fruits, were similarly treated with ethylene, there was only a slight increase in the capability to malonate ACC (data not shown). Thus.

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Table 1. Effect of Ethylene Pretreatment in Tomato Fruits on their Ethylene Production, ACC Content, MACC Content, and the Capabilities To Convert ACC to Ethylene and MACC

| Pretreatment | \(C_2H_4\) | ACC | MACC | ACC→\(C_2H_4\) | ACC→MACC |
|--------------|-----------|-----|------|----------------|----------|
| Cv T\(_3\)   |           |     |      |                |          |
| Air          | 1.5 ± 0.1 | 1.6 ± 0.1 | 1.2 ± 0.1 | 7.2 ± 0.2 | 2.2 ± 1.0 |
| Ethylene     | 1.3 ± 0.2 | 0.4 ± 0.1 | 1.9 ± 0.1 | 47.1 ± 4.5 | 25.7 ± 2.0 |
| Cv nor       |           |     |      |                |          |
| Air          | 0.4 ± 0.1 | 1.1 ± 0.1 | 1.5 ± 0.2 | 2.8 ± 0.1 | 1.1 ± 0.4 |
| Ethylene     | 0.3 ± 0.1 | 0.5 ± 0.1 | 1.7 ± 0.1 | 11.4 ± 0.1 | 22.0 ± 1.0 |
Fig. 1. The effect of ethylene concentration on the tissues' capability to malonylate ACC in preclimacteric tomato fruits. Whole fruits were treated with various concentrations of ethylene for 16 h, and discs were then cut from pericarp tissue and incubated with or without ACC for 6 h. At the end of incubation, the MACC contents were determined. Each value represents the mean ± se of two replicates.

excised discs were not as responsive to ethylene as were intact fruits.

The effect of duration of ethylene treatment on the development of tissue capability to malonylate ACC is presented in Figure 2. The capability increase gradually with increasing treatment duration up to 8 h, after which it reached a plateau. To examine the liability of this ethylene-induced capability to malonylate ACC, fruits were first treated with ethylene for 16 h, and then transferred to air for various periods before their capabilities to malonylate ACC were determined (Fig. 3). Once the fruit was removed from ethylene treatments, the capability declined gradually, and approached a basal level after 72 h. The time during which this ethylene-induced capability declined to one-half was estimated to be about 15 h. Thus, it requires continuous presence of ethylene to sustain a high capability of malonylating ACC.

Effect of NDE. NDE, a competitive inhibitor of ethylene action (25), has been shown to effectively inhibit the senescence of tobacco leaves (24), the ripening of tomato fruits (L. Su, unpublished), and the development of the capability to convert ACC to ethylene (EFE) in preclimacteric tomato and cantaloupe fruits (18). If the present ethylene-induced development of the capability to malonylate ACC is a general ethylene effect, it is expected that this promotive effect of ethylene can be reversed by the application of NDE. Table II shows that ethylene increased the capability from 1.1 to 6.3 n mole/g h, whereas NDE (2500 μl l⁻¹) eliminated completely such a promotive effect exerted by ethylene.

Formation of D-Amino Acid Conjugates. It has been well established that D-amino acids are conjugated to form malonyl-D-amino acids in vivo (7, 8, 12, 13, 15, 17, 23) and in vitro (14). These studies have suggested that malonylations of ACC and of D-amino acids are catalyzed by the same enzyme (14, 20). We have similarly examined the effect of ethylene treatment on the development of the capability to malonylate D-methionine or D-phenylalanine. Figure 4 shows the radiochromatograms of the extracts of tomato discs which were prepared from mature green tomato fruits pretreated with air or ethylene, and were incubated with labeled D-methionine or D-phenylalanine. It is clear that ethylene treatment resulted in an increase in malonylations of D-methionine and D-phenylalanine.

Activity of Malonyltransferase. The enzyme, which catalyzes the malonylations of ACC, AIB, and D-amino acids has been recently isolated and characterized (14, 20). AIB is an ACC analog, which has been demonstrated to be metabolized to form malonyl-AIB (17). We have therefore compared the effect of ethylene pretreatment on the discs' capability to malonylate ACC and AIB and on their respective extractable activity of ACC malonyltransferase isolated from the same discs. Ethylene treatment resulted in greater capability to conjugate ACC and AIB and resulted in correspondingly higher extractable malonyltransferase activity (Table III). These results indicate that the ethylene-promoted capability to conjugate ACC in the tissue resulted from the increase in ACC malonyltransferase activity and that the development of this enzyme can be induced by ethylene.

DISCUSSION

It has been shown that most plant tissues, noticeably vegetative tissues, are capable of metabolizing ACC to a malonylated con-
jugate, MACC, suggesting that the enzyme responsible for this process is constitutive and widespread (4, 10). However, the present study indicates that the preclimacteric tomato fruit is low in this capability, which can be greatly promoted by ethylene treatment. Most of the previous work on the malonylation of ACC was conducted with vegetative tissues (3, 10, 17). Hence, the view that the malonylation enzyme is constitutive is true in general, but not in all cases. Since a surge in ethylene production and in ACC level occurs when tomato fruits undergo ripening, we may expect that this surge in ethylene production would promote the development of ACC malonyltransferase enzyme, resulting in an increased level of MACC during ripening of tomato fruits. Indeed, Su et al. (26) have observed recently that the content of ACC conjugate in tomato fruits increased as ripening progressed. In this connection, it is pertinent to mention the ethylene-forming enzyme, which catalyzes the oxidation of ACC to ethylene. Based on the observation that application of ACC to most plant tissues results in a marked increase in ethylene production (29), it has been generally thought that the ethylene-forming enzyme is constitutive and widespread. However, there are exceptions, which include preclimacteric fruit tissues and immature flower tissues (29). It is interesting to note the parallel relationship between the development of ethylene-forming enzyme and ACC malonyltransferase in preclimacteric tomato fruit tissues. Activities of both enzyme systems are apparently very low in preclimacteric fruit tissues, but both increased markedly following ethylene treatment. While ACC malonyltransferase has

### Table II. Effect of NDE Treatment on the Tissue's Capability To Malonylate ACC in Preclimacteric Tomato Fruits

Table II presents the effect of NDE treatment on the tissue's capability to malonylate ACC in preclimacteric tomato fruits. Fruits were treated with air, ethylene (10 µl l⁻¹), or ethylene (10 µl l⁻¹) plus NDE (2500 µl l⁻¹) for 16 h and discs were prepared from pericarps. ACC, MACC, and ACC→MACC were determined as described in Table I.

| Treatment       | ACC | MACC | ACC→MACC |
|-----------------|-----|------|----------|
| Air             | 1.3 ± 0.1 | 1.5 ± 0.1 | 1.1 ± 0.1 |
| Ethylene        | 0.5 ± 0.1 | 1.4 ± 0.1 | 6.3 ± 0.4 |
| Ethylene + NDE  | 1.0 ± 0.1 | 1.3 ± 0.1 | 0.2 ± 0.1 |

### Table III. Effect of Ethylene Pretreatment on the In Vitro and the In Vivo Activities of ACC Conversion to MACC

Table III shows the effect of ethylene pretreatment on the in vitro and in vivo activities of ACC conversion to MACC. Preclimacteric fruits were treated with air or ethylene (27 µl l⁻¹) for 18 h. For the assay of ACC malonyltransferase activity, 10 g of pericarp tissue was cut after air or ethylene treatment, and employed immediately without incubation for the enzyme preparation. For the assay of the in vivo activity, discs were cut from the pericarp tissue and administered [2,3-¹⁴C]ACC (10 nmol, 17 nCi) or [1-¹³C]AIB (10 nmol, 90 nCi). After incubation for 6 h, the radioactive MACC or MAIB was determined and expressed as the percentage of extractable radioactivity.

| Treatment | In Vivo Activity | ACC Malonyltransferase |
|-----------|-----------------|------------------------|
| AIB→MAIB  | 1.0             | 8.4                    | 4.7 |
| ACC→MACC  | 4.5             | 35.7                   | 28.8 |
| MACC       |                  |                        |     |
been demonstrated in cell-free system (14, 20), the ethylene-forming enzyme, which appears to be associated with membrane, has not been well characterized in cell-free system (29).

The physiological significance of malonylation is thought to inactivate foreign and potential toxic substances such as D-amino acids or some herbicides which may otherwise be harmful to plants (11, 16). In this investigation, we have shown that ethylene promotes the development of ACC malonyltransferase, which functions to reduce the level of ACC, and hence reduce the ethylene production rate. Thus, ethylene is capable of feed-back regulation of its own production via regulation of ACC malonylation. It is well known that ethylene is produced in plants in response to various types of stress and it has been suggested that this stress-ethylene may play an adaptive role (1). If stress results in the production of toxic compounds, such as D-amino acids and related compounds, the promotion of malonylation capability by ethylene can be regarded as an adaptive response, which may enable the plants to increase their capability to cope with the adverse stress situation.

LITERATURE CITED

1. Abeles FB 1973 Ethylene in Plant Biology. Academic Press, New York
2. Adams DO, SF Yang 1979 Ethylene biosynthesis: Identification of l-amino-cyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc Natl Acad Sci USA 76: 170-174
3. Amrheim N, D Schneebeck, H Skorupka, S Tophof, J Stockiet 1981 Identification of a major metabolite of the ethylene precursor l-amino-cyclopropane-1-carboxylic acid in higher plants. Naturwissenschaften 68: 619-620
4. Amrheim N, F Breuing, J Eberle, H Skorupka, S Tophof 1982 The metabolism of l-amino-cyclopropane-1-carboxylic acid. In PF Wareing, ed. Plant Growth Substances 1982. Academic Press, London, pp 249-258
5. Boller T, H Kenne 1980 Regulation of wound ethylene synthesis in plants. Nature 286: 259-260
6. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72: 248-254
7. Fukuda M, T Tokumura, T Ogawa, K Sasakoa 1973 D-Alanine in germinating Pisum sativum seedlings. Phytochemistry 12: 2593-2595
8. Good NE, WA Andreae 1957 Malonyltryptophan in higher plants. Plant Physiol 32: 561-566
9. Hoffman NE, SF Yang 1982 Enhancement of wound-induced ethylene synthesis by ethylene in preclimacteric cantaloupe. Plant Physiol 69: 317-322
10. Hoffman NE, SF Yang, T McKeon 1982 Identification of l-(malonylamino)cyclopropane-1-carboxylic acid as a major conjugate of l-amino-cyclopropane-1-carboxylic acid, an ethylene precursor in higher plants. Biochem Biophys Res Commun 104: 765-770
11. Iwan J 1976 Miscellaneous conjugates-acylation and alkylation of xenobiotics in physiologically active systems. ACS Symp Ser 1976: 132-152
12. Kawai Y, T Ogawa, K Sasakoa 1982 Two pathways for formation of D-amino acid conjugates in pea seedlings. Agric Biol Chem 46: 1-5
13. Kieglicv D, B Ladesic, M Pokorny 1968 Biochemical studies in tobacco plants. IV. l-Nalonylmethionine, metabolite of D-methionine in Nicotiana rustica. Arch Biochem Biophys 124: 443-449
14. Konka C, N Amrheim 1984 The enzymatic malonylation of l-amino-cyclopropane-1-carboxylic acid in homogenates of mungbean hypocotyls. Planta 162: 226-235
15. Ladesic B, M Pokorny, D Kieglicv 1971 Metabolic patterns of L- and D-serine in higher and lower plants. Phytochemistry 10: 3085-3091
16. Lamoureux G, JM Gout, DG Davis, DG RUNN 1981 Pentachloronitrobenzene metabolism in peanut. 3. Metabolism in peanut cell suspension cultures. J Agric Food Chem 29: 996-1002
17. Liu Y, NE Hoffman, SF Yang 1983 Relationship between the malonylation of l-amino-cyclopropane-1-carboxylic acid and D-amino acids in mungbean hypocotyls. Planta 158: 437-441
18. Liu Y, NE Hoffman, SF Yang 1985 Promotion by ethylene of the capability to convert l-amino-cyclopropane-1-carboxylic acid to ethylene in preclimacteric tomato and cantaloupe fruits. Plant Physiol 77: 407-411
19. Liu Y, L Su, SF Yang 1984 Metabolism of D-alinosisobuturyic acid in mungbean hypocotyls in relation to metabolism of l-amino-cyclopropane-1-carboxylic acid. Planta 161: 439-443
20. Liu Y, L Su, SF Yang 1984 Stereoelectivity of l-amino-cyclopropane carboxylate malonyltransferase toward stereoisomers of l-amino-2-ethylcyclopropane carboxylic acid. Arch Biochem Biophys 235: 319-325
21. Lizada MCC, SF Yang 1979 A simple and sensitive assay for l-amino-cyclopropane-1-carboxylic acid. Anal Biochem 100: 140-145
22. POKORNY M, E MARCENKO, D KIEGLEVE 1970 Comparative studies of L- and D-methionine metabolism in lower and higher plants. Phytochemistry 9: 2175-2188
23. Rosa N, AC Neish 1968 Formation and occurrence of N-malonyl-phenylalanine and related compounds in plants. Can J Biochem 46: 797-806
24. Snider EC, A Pain 1973 Effect of ethylene and cyclic olefins on tobacco leaves. Tobacco Sci 17: 68-72
25. Snider EC, SF Yang 1984 Anti-ethylene effect of cis-2-butene and cyclic olefins. Phytochemistry 23: 2765-2768
26. Su L, T McKeon, D Grierson, M Cantwell, SF Yang 1984 Development of l-amino-cyclopropane-1-carboxylic acid synthase and polygalacturonase activities during the maturation and ripening of tomato fruits in relation to their ethylene production rates. HortScience 19: 576-578
27. T的時候aar EC, WB McGlasson, RW Buscher 1978 Genetic regulation of tomato fruit ripening. HortScience 13: 508-513
28. Yang SF, NE Hoffman, T McKeon, J Rov, CH Kao, KH Yung 1982 Mechanism and regulation of ethylene biosynthesis. In PF Wareing, ed. Plant Growth Substances 1982. Academic Press, London, pp 239-248
29. Yang SF, NE Hoffman 1984 Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155-189
30. Yu YB, SF Yang 1980 Biosynthesis of wound ethylene. Plant Physiol 66: 281-285