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The physiological role of lipoxygenase in ethylene formation from 1-aminocyclopropane-1-carboxylic acid in oat leaves*

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Abstract. In order to understand the physiological significance of the in-vitro lipoxygenase (EC 1.13.11.12)-mediated ethylene-forming system (J.F. Bousquet and K.V. Thimann 1984, Proc. Natl. Acad. Sci. USA **81**, 1724–1727), its characteristics were compared to those of an in-vivo ethylene-forming system. While oat (*Avena sativa* L.) leaves, as other plant tissues, preferentially converted only one of the 1-amino-2-ethylcyclopropane-1-carboxylic acid (AEC) isomers to 1-butene, the lipoxygenase system converted all four AEC isomers to 1-butene with nearly equal efficiencies. While the in-vivo ethylene-forming system of oat leaves was saturable with ACC with a K_m of 16 μM, the lipoxygenase system was not saturated with ACC even at 10 mM. In contrast to the in-vivo results, only 10% of the ACC consumed in the lipoxygenase system was converted to ethylene, indicating that the reaction is not specific for ethylene formation. Increased ACC-dependent ethylene production in oat leaves following pretreatment with linoleic acid has been inferred as evidence of the involvement of lipoxygenase in ethylene production. We found that pretreating oat leaves with linoleic acid resulted in increased ACC uptake and thereby increased ethylene production. A similar effect was observed with oleic acid, which is not a substrate of lipoxygenase. Since linoleic acid hydroperoxide can substitute for lipoxygenase and linoleic acid in this system, it is assumed that the alkoxy radicals generated during the decomposion of linoleic acid hydroperoxide are responsible for the degradation of ACC to ethylene. Our results collectively indicate that the reported lipoxygenase system is not the in-vivo ethylene-forming enzyme.

Key words: 1-Aminocyclopropane-1-carboxylic acid – *Avena* (ethylene synthesis) – Lipoxygenase.

Introduction

A number of cell-free ethylene-forming systems, which convert 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, have been reported during the past few years (Konze and Kende 1979; Mayak et al. 1981; Shimokawa 1983; Vioque et al. 1981; Vinkler and Apelbaum 1983). Although they were all isolated from plant materials and were capable of converting ACC to ethylene in vitro, their operation as in-vivo ethylene-forming enzyme has not been demonstrated. Based on results from in-vivo studies, two characteristics of the in-vivo ethylene-forming enzyme have been employed as criteria for evaluating the physiological relevance of these cell-free systems. The first is the possession of stereospecificity toward the four stereoisomers of 1-amino-2-ethylcyclopropane-1-carboxylic acid (AEC) as the precursor of 1-butene (Hoffman et al. 1982b). The other is to have a physiological K_m value for ACC (McKeon and Yang 1984). By using these criteria, McKeon and Yang (1984), Guy and Kende (1984) and Venis (1984) have examined all the above cell-free systems and found that none of them resembled the natural ethylene-forming enzyme. Thus far the only subcellular organelle that has been shown to possess all the characteris-

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Abbreviations: ACC = 1-Aminocyclopropane-1-carboxylic acid; AEC = 1-amino-2-ethylcyclopropane-1-carboxylic acid; Epps = N-(2-hydroxyethyl)-piperazine-N′-3-propanesulfonic acid; LH = linoleic acid; LOOH = linoleic acid hydroperoxide; pyridoxal-P = pyridoxal-phosphate

tics of the in-vivo ethylene-forming activity is the intact vacuole (Guy and Kende 1984; Mayne and Kende 1986).

Bousquet and Thimann (1984) described an enzyme model system in which ACC was rapidly converted to ethylene in the presence of linoleic acid, pyridoxal-phosphate (pyridoxal-P), manganese ion and lipoxygenase. Several features of this system made it a tempting candidate for the physiological ethylene-forming enzyme. First, the yield of ethylene from this reaction was by far the highest among all the cell-free systems so far reported. Second, lipoxygenase is membrane-associated and its activity has been shown to be closely connected with the process of leaf senescence (Dhindsa et al. 1981). In parallel with this are the suggestions that the natural ethylene-forming enzyme is also associated with cell membranes (Yang and Hoffman 1984) and that its production increases during leaf senescence (Aharoni and Lieberman 1979; Gepstein and Thimann 1981). Third, ACC-dependent ethylene production in oat leaves was enhanced by the application of linoleic acid. All these features indirectly indicated that this system may function in vivo (Bousquet and Thimann 1984). The chemical basis for this model reaction was studied recently by Pirrung (1986), who showed that the lipoxygenase system had no selectivity for the stereoisomers of methyl-substituted ACC and the role of pyridoxal-P is not via Schiff's base but to chelate with manganese. Based on these discrepancies, he concluded that the lipoxygenase system is not the natural system.

In this study, we compared some characteristics of the lipoxygenase system with the in-vivo ethylene-forming system of oat leaves, and evaluated its physiological relevance.

Materials and methods

Plant material. Seeds of oat (*Avena sativa* L. cv. Gerry, Stanford Seed Co., Buffalo, NY, USA) were imbibed in aerated water for 6–8 h, planted in vermiculite, and grown in a growth chamber with a daily 16-h light period (540 μmol photosynthetically active radiation [PAR]$\cdot m^{-2} \cdot s^{-1}$ and 25/20° C day/night temperature. Primary leaves from 7- to 9-d-old plants were harvested for in-vivo feeding experiments.

Chemicals. 1-Aminocyclopropane-1-carboxylic acid was obtained from Calbiochem-Behring Corp. (La Jolla, Cal., USA). The AEC isomers were generous gifts from Professors S. Sakamura and A. Ichihara (Hokkaido University, Sapporo, Japan). The four diastereomers of AEC comprise two pairs of enantiomers: (1R,2S)- and (1S,2R)-AEC are (+)- and (−)-allocoronamic acid, respectively; (1S,2S)- and (1R,2R)-AEC are (+)- and (−)-coronamic acid, respectively. Lipoxygenase (EC 1.13.11.12) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Linoleic acid hydroperoxide (LOOH) was prepared according to the procedures used by Peiser and Yang (1978). Ethylene and 1-butene standards were from Matheson (East Rutherford, N.J., USA). All chemicals used were of reagent grade.

Lipoxygenase-mediated ethylene-forming system. The standard reaction mixture for the lipoxygenase system contains 1 mM ACC, 1 mM linoleic acid (Na salt in 0.03% Tween-20 [polyoxyethylene sorbitan monooleate]), 0.1 mM $MnCl_2$, 0.1 mM pyridoxal-P, 10 mM N-(2-hydroxyethyl)-piperazine-N′-3 propanesulfonic acid (Epps) buffer (pH 8.0) and 200 units of lipoxygenase (1 unit = an increase in A_{234} of $0.001 \cdot min^{-1}$) in a total volume of 1 ml as described by Bousquet and Thimann (1984). The reaction was carried out under ambient air in a test tube sealed with a serum cap and was initiated by the addition of lipoxygenase. After incubation for 1 h at 25° C, a 1-ml gas sample was taken for ethylene measurement. For the minus-oxygen treatment, air in the sealed reaction tube was evacuated and replaced with nitrogen gas three times and lipoxygenase was then injected into the tube to initiate the reaction. For the AEC experiment, ACC was replaced with 0.8 mM of one of the AEC isomers and 1-butene was measured after incubation. Ethylene and 1-butene were measured with a gas chromatograph equipped with an alumina column and a flame ionization detector. Gas concentrations were calculated against external standards of ethylene and 1-butene.

[2,3-^{14}C]ACC experiment. [2,3-^{14}C]ACC (20 nmol, $5.66 \cdot 10^3$ Bq; from Research Products International, Mount Prospect, Ill, USA) was added to a standard lipoxygenase reaction mixture containing 1 μmol unlabeled ACC as described above. A strip of filter paper wetted with 0.1 ml 0.2 M $Hg(ClO_4)_2$ was hung inside the enclosed tube to absorb the radioactive ethylene formed. The reaction mixture was incubated at 25° C for 20 h. Non-volatile radioactive products of ACC were separated by paper chromatography (1-butanol-acetic acid-water, 4:1:1 by vol.), cation-exchange column (Dowex-50, H^+ form; Sigma), and high-voltage paper electrophoresis (2.5% HCOOH:7.5% CH_3COOH, pH 1.9, and 20 mM potassium-phosphate buffer, pH 7.0) as described in Hoffman et al. (1982a). Radioactivity in the paper chromatogram and paper electrophoresis was detected with a radioscanner.

Assay for in-vivo 1-butene formation. Ten oat leaf segments excised 7 cm below the tip were incubated at 25° C in darkness with cut ends in 100 μl of 25 mM K-phosphate buffer (pH 6.8) and 4 mM of AEC isomer. Incubations were carried out in glass tubes, 16 mm diameter and 100 mm long, enclosed with rubber stoppers. Accumulated 1-butene were sampled and assayed after 6 h incubation as described above.

Assay for ACC and AEC content. Oat leaf segments which had been incubated in ACC or AEC solutions as described above were rinsed thoroughly with distilled water and extracted three times with 5 ml 80% ethanol at 70° C. The ethanol extracts were evaporated to dryness in vacuo and redissolved in 2 ml of water. The ACC and AEC contents were determined by chemical conversion to ethylene and 1-butene respectively by the method of Lizada and Yang (1979). The conversion efficiencies of AEC isomers to 1-butene were 2.5, 2.6, 4.3 and 3.5% for, in this order, (1R,2S)-, (1S,2R)-, (1R,2R)- and (1S,2S)-AEC.

Fatty-acids pretreatment experiment. Lots of ten oat leaf segments, 3 cm long excised at 1 and 4 cm below the apex, were

incubated in a Petri dish (100 mm diameter and 15 mm height) between two layers of filter paper wetted with 5 ml of the pretreatment media containing 10 mM Na Epps (pH 8.0), 0.1 mM Mn^{2+}, 0.1 mM pyridoxal-P and, where indicated, 1 mM linoleic acid or oleic acid (both as Na salts) as described by Bousquet and Thimann (1984). After 3 h, all leaf segments were similarly transferred between two layers of filter paper in Petri dishes containing 5 ml of 0.5 mM ACC. After incubation for 1 h, the leaf segments were rinsed, blotted dry, and sealed in test tubes for 1 h. Gas samples were taken for ethylene measurement and the leaf segments were extracted with 80% ethanol for ACC measurement.

Results and discussion

As described by Bousquet and Thimann (1984), the lipoxygenase-mediated ethylene-forming system (lipoxygenase system) effectively converted ACC into ethylene (Table 1). Our results are in agreement with their observation that all components of the reaction mixtures are necessary for maximal ethylene production.

Several studies have shown that among four stereoisomers of AEC, only the (1R,2S)-AEC is preferentially converted to 1-butene in a number of plant tissues (Hoffman et al. 1982b; McKeon and Yang 1984). Using this criterion, McKeon and Yang (1984) and Venis (1984) have reported that the cell-free ethylene-forming systems lack the stereospecific characteristic of the in-vivo system. Like those tissues (mungbean hypocotyls, apple fruit and pea epicotyls; Hoffman et al. 1982b; McKeon and Yang 1984), oat leaf segments also exhibited high stereospecificity toward AEC isomers, converting preferentially the (1R,2S)-AEC to 1-butene (Table 2). This difference could not be attributed to differential uptake of these isomers, because after 6 h of incubation, free-isomer contents in the oat leaf segments were: 74, 219, 252 and 278 nmol·g^{-1} for, in the order, (1R,2S)-, (1S,2R)-, (1S,2S)- and (1R,2R)-AEC. Thus, the (1R,2S)-AEC content was the lowest, but it caused the highest 1-butene production. By contrast, the lipoxygenase system lacked the stereospecificity toward AEC isomers (Table 2), as has been observed in other cell-free systems (McKeon and Yang 1984; Venis 1984). It should be mentioned that the pair of allocoronamic-acid enantiomers were converted to 1-butene in the lipoxygenase system at a 1.5–2 times higher rate than the coronamic-acid enantiomers. A complete lack of selectivity by the lipoxygenase for the stereoisomers of 2-methyl-substituted ACC was reported by Pirrung (1986).

The conversion of ACC to ethylene by various plant tissues was saturable at an internal ACC concentration between 100 and to 400 μM (Apelbaum et al. 1981; McKeon et al. 1982) with an apparent K_m for ACC of 66 μM in pea epicotyls (McKeon and Yang 1984). In the present study, we estimated the apparent K_m for internal ACC in oat leaf segments as described by McKeon and Yang (1984). As shown in Fig. 1A, the ethylene production by oat leaf tissue was saturated with 100 μM of internal ACC. The Lineweaver-Burk plot exhibits an apparent K_m of 16 μM with respect to internal ACC. By contrast, the lipoxygenase system produced ethylene at a measurable rate only at ACC concentrations above 100 μM (Fig. 1B). The ethylene-production rate increased with increasing ACC

Table 1. Ethylene production from ACC by the lipoxygenase system or by the LOOH system. A complete reaction mixture of the lipoxygenase system contains 1 mM ACC, 1 mM linoleic acid, 0.1 mM $MnCl_2$, 0.1 mM pyridoxal-P, 10 mM NaEpps (pH 8.0) and 200 units of lipoxygenase in a total volume of 1 ml. A complete reaction mixture of the LOOH system contains 1 mM ACC, 1 mM LOOH, 0.1 mM $MnCl_2$, 0.1 mM pyridoxal-P and 10 mM NaEpps (pH 8.0) in a total volumn of 1 ml. Ethylene produced during 1 h incubation was determined. Data are the mean ± SD of three replicates

Reaction mixture	Ethylene production (nl·h^{-1})
Lipoxygenase system:	
Complete	598 ± 96
−pyridoxal phosphate	114 ± 10
$-Mn^{2+}$	0.9
$-Mn^{2+}$, − pyridoxal phosphate	1.3
$-O_2$ (under nitrogen)	4.9
LOOH system:	
Complete	775 ± 19
−pyridoxal phosphate	178 ± 10
$-Mn^{2+}$	0.3
$-Mn^{2+}$, − pyridoxal phosphate	0.4
$-O_2$ (under nitrogen)	589 ± 24

Table 2. Conversion of AEC isomers to 1-butene by oat leaf segments and by the lipoxygenase system. Oat leaf segments and the lipoxygenase system were incubated with 4 mM and 0.8 mM AEC isomers, respectively. Incubation and measurement of 1-butene were as described in *Material and methods*. When AEC was replaced with the same concentration of ACC, oat leaf segments produced 7.33 nmol·g^{-1}·h^{-1} of ethylene, and the lipoxygenase system produced 19.5 nmol·h^{-1} of ethylene

AEC isomers	1-Butene production	
	Oat leaf segments (nmol·g^{-1}·h^{-1})	Lipoxygenase system (nmol·h^{-1})
(1R,2S)-AEC	1.91	18.2
(1S,2R)-AEC	0.14	13.9
(1S,2S)-AEC	0.005	10.6
(1R,2R)-AEC	0.01	8.8

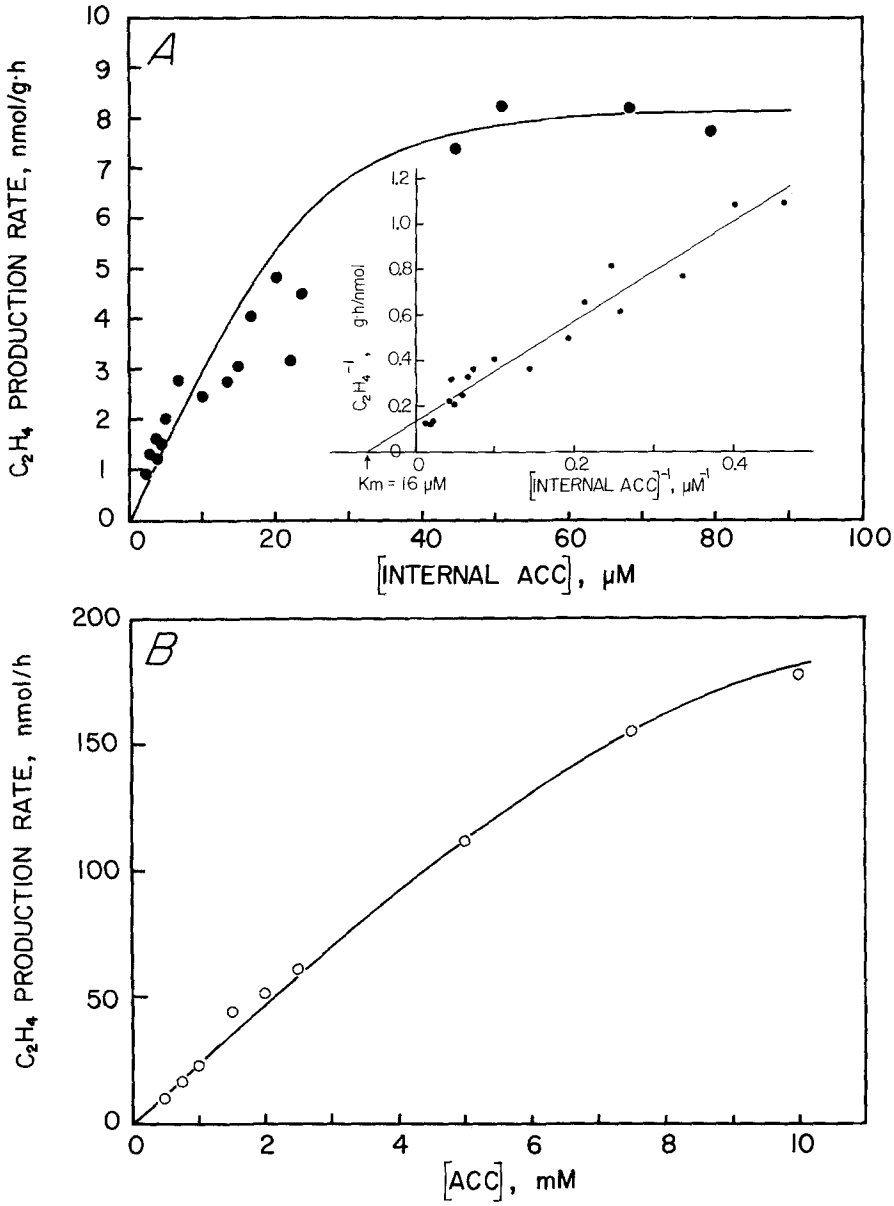

Fig. 1 A, B. Dependence of ethylene production on ACC concentration by oat leaf segments (**A**), and by the lipoxygenase system (**B**). *Insert:* Lineweaver-Burk plot of the oat leaf system. Lots of 10 oat leaf segments were preincubated with different concentrations of ACC (20–300 μM) in the presence of 0.03% Tween-80 for 1 h with shaking. After rinsing and blotting dry, the segments were sealed in a glass tube and incubated at 25° C. After measuring the ethylene production during the following 1 h, the segments were extracted with 80% ethanol for ACC measurement. Internal ACC concentration is expressed in μM (nmol·(g FW)$^{-1}$). Reactions of the lipoxygenase system were carried out with various ACC concentration as indicated

concentrations and was not quite saturated even at 10 mM of ACC.

The rate of ethylene production by the lipoxygenase system was highest during the first hour, gradually decreased thereafter, and essentially stopped after 24 h. The yield of ethylene from ACC by the lipoxygenase system was generally between 4 and 6% after 24 h. This low ethylene yield was not a result of destruction of ethylene during the reaction, because incubating a known amount of ethylene with the reaction mixture containing no ACC for 24 h did not affect the ethylene concentration. If no other reactions had occurred, the rest of the ACC should have remained unreacted in the solution, but when an aliquot of the reaction mixture was assayed for the ACC content by the method of Lizada and Yang (1979), only about 50% of the ACC was found in the reaction mixture (Table 3). This result indicated that during the lipoxygenase-mediated reaction in 24 h, 5% of ACC was converted to ethylene, whereas about 45% of the ACC was converted into other products. Thus, of the ACC reacted, only one-tenth was converted into ethylene.

In order to examine the fate of ACC in the lipoxygenase system, [2,3-^{14}C]ACC was added to the system and its products were analyzed. Of the total radioactivity supplied, 4% was converted into ethylene after 20 h of incubation. The ACC assay indicated that 40% of the initial ACC was des-

Table 3. Relationship between ACC destruction and ethylene formation in the lipoxygenase system. Reaction was carried out as described in *Material and methods*. At the end of each time period, the total ethylene formed was determined and an aliquot of the reaction mixture was assayed for ACC by the method of Lizada and Yang (1979). Results expressed as percent of the initial ACC content (1000 nmol)

Time of reaction (h)	ACC lost (%)	C_2H_4 formed (%)
1	20.7	1.78
2	35.1	3.23
3	38.7	4.08
4	45.3	4.51
24	47.6	6.42

Table 4. Effects of pretreatment with linoleic acid and oleic acid on ACC uptake and ethylene production in oat leaf segments. Incubation conditions were described in *Material and methods*. Control medium contained 10 mM Epps (pH 8.0), 0.1 mM Mn^{2+} and 0.1 mM pyridoxal-P. Data are the mean ±SD of three replicates

Pretreatment medium	C_2H_4 ($nl \cdot g^{-1}$)	ACC ($nmol \cdot g^{-1}$)
Control	54.7±2.9	63.8± 2.5
+linoleic acid	69.5±2.0	80.5± 5.5
+oleic acid	77.3±9.6	120.0±14.6

troyed during the reaction. Paper radiochromatography of the reaction mixture showed the presence of two major radioactive peaks; one that corresponded to ACC, and one unknown peak with R_f 0.46. When the reaction mixture was passed through a cation-exchange Dowex-50(H^+) column, 62% of the radioactivity was retained in the column, and could be eluted with 2 N NH_4OH. The radioactivity in the eluate was identified to contain only ACC. The effluent fraction contained the unknown peak ($R_f = 0.46$) and several small peaks. Since the radioactive compounds in the effluent were not retained by the cation-exchange resin, they either possessed no amino group or the amino group was derivatized in such a way that the amino-N was no longer cationic in nature. Paper electrophoresis of the effluent fraction at pH 7.0 and 1.9 showed that it contained both neutral and anionic compounds. Further work to identify these peaks was not attempted. These results indicate that in this lipoxygenase system the ACC reaction was not specific for ethylene production, because ACC was extensively reacted into other products. By contrast, except for the conjugation of ACC to form the metabolite N-malonyl-ACC (Amrhein et al. 1981; Hoffman et al. 1982a), no metabolic product other than ethylene has been recognized in plant tissue.

Bousquet and Thimann (1984) have shown that ACC-dependent ethylene production by oat leaf segments was markedly promoted when linoleic acid, a substrate of lipoxygenase, was applied. This observation was interpreted to indicate that the lipoxygenase system may actually be operating in vivo. It should be pointed out that ACC-dependent ethylene production from plant tissues is dependent upon not only the activity of the ethylene-forming enzyme but also the endogenous ACC concentration. Since the ethylene-forming enzyme is thought to be a pre-exisiting enzyme in leaf tissues, it seemed possible that the enhanced ethylene production following linoleic-acid treatment of the tissue could be the consequence of an enhancement of ACC uptake. We have therefore compared the effect of linoleic acid and oleic acid on ACC-dependent ethylene production and on ACC uptake by oat leaf segments. Oleic acid, which is not a substrate for lipoxygenase, was found, as shown in Table 4, to be as active as linoleic acid in promoting ethylene production from oat leaf segments. Measurement of ACC content after incubation showed that the treatment with either fatty acid had resulted in a higher ACC content in the tissue. These findings showed that both linoleic acid and oleic acid enhanced ethylene production in oat leaves by enhancing ACC uptake, explaining their promotive effect on ethylene formation by the lipoxygenase system.

A reaction mechanism for the lipoxygenase system was proposed by Pirrung (1986). This mechanism involves a two-stage reaction for the lipoxygenase system to convert ACC to ethylene. The lipoxygenase first catalyzes the oxidation of linoleic acid (LH) to linoleic hydroperoxide (LOOH). The LOOH was cleaved by Mn^{2+} to form linoleic alkoxy radical (LO), which then oxidized ACC non-enzymatically to ethylene and other products. In this study, we also obtained evidence to support this proposed mechanism. First, LOOH could substitute for lipoxygenase plus linoleic acid in the lipoxygenase system (Table 1), and the LOOH-Mn^{2+} system did not require oxygen for the reaction, indicating that the primary role of lipoxygenase in this system was to form LOOH. Second, bisulfite is known to cause the homolytic cleavage of LOOH into LO· (Davis 1961, pp. 183–189; Peiser and Yang 1978, 1979). If the alkoxy radical is responsible for the oxidation of ACC to ethylene, then the LOOH-bisulfite system should also be effective in initiating ethylene production from ACC. Indeed, as shown in Table 5, ACC was

Table 5. Comparison of the conversion of ACC to ethylene by the bisulfite-LOOH system and by the lipoxygenase system. The bisulfite-LOOH system consisted of 20 mM Na-acetate, pH 7.5, 0.2 mM LOOH, 0.5 mM $NaHSO_3$, 65% ethanol and 1 mM ACC. The reaction was initiated by injecting sodium bisulfite into the reaction tube. The lipoxygenase system was as described in Table 1. Data are the mean ± SD of three replicates

Systems	C_2H_4 production (nl)	
	5 min	1 h
Bisulfite-LOOH	368 ± 23	568 ± 24
Lipoxygenase	3 ± 1	574 ± 7

also oxidized to ethylene to the same extent as in the lipoxygenase system. Since according to the above reaction mechanism, lipoxygenase does not catalyze the oxidation of ACC directly, it becomes apparent why the lipoxygenase system has a low or no affinity for ACC and lacks stereodiscrimination towards different AEC isomers.

The microsomal membranes of carnation petals (Mayak et al. 1981) represent one of several cell-free preparations that were shown to be capable of converting ACC to ethylene. Recently, Lynch et al. (1985) have provided evidence showing that the ethylene-forming activity of the microsomal system from carnation petals can be attributed to its membrane-associated lipoxygenase activity. Since, the pattern of in-situ ethylene production by intact flowers was different from that of in-vitro ethylene production by the isolated microsomes, Lynch et al. (1985) concluded that this in-vitro lipoxygenase system was not the native ethylene-forming enzyme.

Ethylene is a plant hormone produced by higher plants at certain stages of their life cycle as the consequence of developmental processes and environmental stresses. The biosynthesis of ethylene has been shown to be under rigid regulatory control via the methionine-ACC pathway (see review by Yang and Hoffman 1984). Since it was observed that lipid peroxidation increases when plant tissues are subjected to cellular damage (Galliard 1978) or undergoing senescence (Dhindsa et al. 1981), there have been suggestions that the lipoxygenase activity is involved in the biosynthesis of ethylene under situations like senescence (Bousquet and Thimann 1984), wounding or low-temperature stress (Kacperska and Kubacka-Zebalska 1985). It should be noted that in all these cases the induced ethylene production resulted primarily from the induction of the synthesis of ACC, rather than the promotion of the conversion of ACC to ethylene (Yang and Hoffman 1984). Although lipoxygenase may increase during the above-mentioned situations, considering the lack of affinity of the lipoxygenase system for the ACC and the low ACC concentration present in the plant tissue (<0.1 mM), it is very unlikely that the lipoxygenase system may produce ethylene to any substantial extent in vivo.

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