(*Invited review paper*)

Recent progress in research of ethylene biosynthesis[1]

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Key words: Ethylene biosynthesis; ACC synthase; ACC oxidase; Molecular cloning; Ripening; Senescence; Transgenic plants.

Contents

Introduction

Ethylene was unwittingly used as a plant growth

Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid; AdoMet: S-adenosyl-L-methionine; AspAT: aspartate aminotransferase; EFE: ethylene-forming enzyme; IAA: indole-3-acetic acid; mAb: monoclonal antibody; MTA: 5'-methylthioadenosine; PLP: pyridoxal phosphate; Rubisco: ribulose-1,5-bisphosphate carboxylase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

regulator for a long time. The Chinese knew for hundreds of years that picked fruits would ripen more quickly if placed in a chamber containing burning incense. This effect is undoubtedly attributable to the presence of ethylene. Following the demonstration that ethylene was a plant hormone initiating fruit ripening, many other plant processes, ranging from seed germination to flower fading, leaf senescence and abscission, were recognized as being regulated by ethylene. The rate of ethylene production by plant tissue is normally low. As part of the normal life of a plant, an increase in ethylene production is induced during certain stages of growth. Ethylene production can also be induced by auxin application or environmental stresses, such as physical wounding and cutting, chilling, drought, and water flooding. It has been recognized that this in-

creased ethylene production, in turn, brings about many important physiological responses. It has been established that ethylene is synthesized in plant tissues *via* the following pathway: methionine → S-adenosyl-methionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4 (Adams and Yang, 1979). In this paper we describe research progress that has occurred recently with regard to ACC synthase and ACC oxidase.

ACC Synthase

In plant tissues ACC synthase (EC 4.4.1.14), which catalyzes the conversion of AdoMet to ACC and 5'-methylthioadenosine (MTA), is generally the rate-limiting enzyme in the ethylene biosynthetic pathway (Yang and Hoffman, 1984). ACC synthase is controlled by a variety of environmental and developmental factors, and has received a great deal of attention in relation to the regulation of ethylene biosynthesis. The enzyme has been characterized at both biochemical and molecular levels.

Substrate-Dependent Inactivation and Radiolabeling

ACC synthase is a pyridoxal phosphate (PLP)-requiring enzyme and is subject to substrate-induced inactivation during the catalytic reaction. Satoh and Yang (1988, 1989) incubated a partially purified tomato enzyme with Ado[3,4-^{14}C]Met in the presence of PLP and observed that ACC synthase was specifically radiolabeled as a 50 kD polypeptide on SDS-PAGE. They demonstrated that this AdoMet-dependent inactivation involved a covalent linkage of the 2-aminobutyrate portion of the AdoMet molecule to the active site of the enzyme. Such an AdoMet-dependent radiolabeling of ACC synthase protein has been employed as a tool for the confirmation of the identity of ACC synthase on SDS-PAGE (Van der Straeten *et al.*, 1989; Dong *et al.*, 1991a; Nakagawa *et al.*, 1991). A reaction scheme for the inactivation of ACC synthase by AdoMet is depicted in Fig. 1.

Since ACC synthase is a PLP-utilizing enzyme, it can also be radiolabeled with NaB^3H_4 by the reduction of the PLP-enzyme aldimine to the hydrolytically stable secondary amine. Privalle and Graham (1987) and Satoh and Yang (1988) showed that when a partially purified ACC synthase preparation isolated from wounded tomato pericarp was incubated with NaB^3H_4
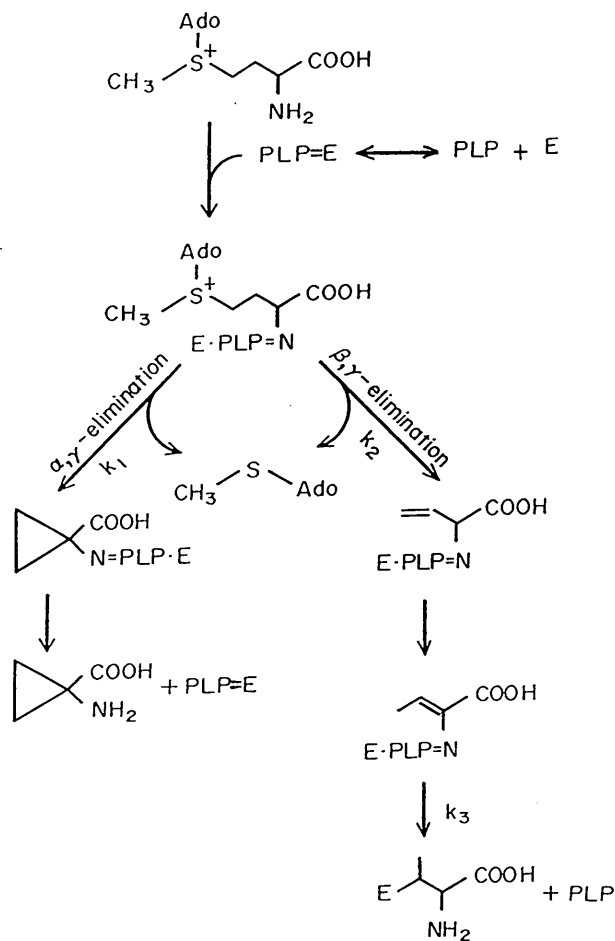
Fig. 1. A postulated scheme for the mechanism-based inactivation of ACC synthase by AdoMet. Ado, E, PLP=E and PLP=N-R stand for adenosine moiety, apo-ACC synthase, internal aldimine and external aldimine, respectively. The values of k_1 and k_2, and k_3 were estimated to be 300, 0.01 and 0.1 min^{-1}, respectively (Adapted from Satoh and Yang, 1989; Yip *et al.*, 1990).

in the presence of PLP, a number of proteins including a 50 kD protein were radiolabeled.

Purification of ACC Synthase

To understand the genetic regulation of ACC synthase in ethylene biosynthesis, it is necessary to clone the gene encoding the protein. Toward this end, basic approaches undertaken in various laboratories were similar. These included the purification of the enzyme followed by the preparation of the antibodies against it or by obtaining its peptide sequence from which the corresponding oligonucleotide probes were prepared. These antibodies or oligonucleotide probes

were used for gene/cDNA screening.

enzyme activity (Dong *et al.*, 1991a), and these were in

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                                                                        1
APPLE                    (------NAT)FNSHGEDSSYFLGWQEYEKNPYHEVLNTNGI[IQMGLAENQL]CFDL
TOMATO (1)    MDLETSEISNYKSSVVLRKL*S NRQ***N*P**D**KA*DND*F*L*N*L**V[*********]SV**
TOMATO (2)    MGFEI--AKTNSILSKL** NEE***N*P**D**KA*DSD*F*PLK*P**V[*********]*L**
W. SQUASH    MEFHQIDERNQALLSKI*V DDG***N*P**D**KA*DND*F*PED*PL*V[*********]S**M
ZUCCHINI     MGFNQIDERNQALLSKI*L DDGN**N*PV*D**KA*DND*FNPEN*PL*V[*********]S**M
                                                      2                                                         3
LESWLAKNPEAAAFKKNGESIFAELAL[FQDYHGLP]AFKKAMVDFMAKIRGNKVTFDPNHLVLTAGATSANETF[IFCLADPG]
I*E*IKR**K*SICIND*IES*RRI*N[*******]E*TN*IAK**E*T**G**K**AKRV*MAG***G****L[*L*****]
I*D*IKR**KGSICS-E*IKS*KAI*N[*******]E*R**IAK**E*T**GR*R***ERV*MAG***G****I[*******]
IVD*IR*H***SICTPK*LER*KSI*N[*******]E*RNGIAS**G*V**GR*Q***SRI*MGG***G*S**V[*******]
IVD*IR*H***SICTPE*LER*KSI*N[*******]E*RN*IAN**G*V**GR*K***SRI*MGG***G*S**V[*******]
                                                                   4
EAVLIPTPYYPGFDRDLKWRTGVEIVPIHCTSSNGFQITETALEEAYQEAEKRNLRVKGVLV[TNPSNPLGTT]MTRNELYLL
D*F*V*********N***R**S**QLL**S*K*C*N*K**IE*I****EKGQQA*VKI**LIL[***C*****I]LD*DT*KKI
D*F*V*S****A*N***R****QLI****E***N*K**SK*VK***EN*Q*S*IK***LIL[*********]LDKDT*KSV
D*F*V*S***AA*********RAQ*IRV**N***N**V*KA***I**KK*QEA*IK****II[*********]YD*DT*KT*
D*F*V*S***A*********RAQ*IRV**NG**N**V*KA***I**KK*QEA*MK****II[*********]YD*DT*KT*
                                                          5
LSFVEDKGIHLISDEIYSGTAFSSPSFISVMEVLKDRN---CDENSEVWQRVHVV[YSLSKDLGLPGFRVG]AIYSNDDMVVA
ST*TNEHN***VC***AA*V*N**K*V*IA*IINED*--CINKDL-----**I*[S*******F*****]IV**FN*D**N
***TNQHN***VC***AA*V*DT*Q*V*IA*I*DEQEMTY*NKDL-----**I*[*****M*******]I***FN*D**N
VT**NQHD****C*****A*V*KA*T***IAQIVEE--MEH*KKEL-----I*IL[*****M*******]I***YN*V**R
VT**NQND****C*****A*V*KA*T*T*IA*IVE-Q-MEN*KKEL-----INIL[*****M******F]II**YNDVVR
     6
AATK[MSSFGLVS]SQTQHLLSAMLSDKKLTKNYIAENHKRLKQRQKKLVSGLQKSGISCLNGNAGLFCWVDMRHLLRSNTFE
C*R*[*******]T*****AF****DEFVEEFLI*SA***RE*YE*FTR**EEI**K**ES***VY**M*L*S**KEA*LD
C*R*[*******]T***YF*A**P**E*FVD*FLR*SAM**GK*H*HFTN**EVV**K**KN******M*L*P***ES**D
R*RQ[*******]*******A*****EDFVDKFL***S***AE*HARFTKE*D*M**T***S***V*V*M*L*R**KDQ**K
RARQ[*******]*******A*****EDFVDKFL***S**VGE*HARFTKE*DSM**T***S**V*V*M*L*R**KDQ-**K
                                      7
AEMELWKKIVYEVHLNISPGSSCHCT[EPGWFRVCFAN]LPERTLDLAMQRLKAFVGEYYNVPEVNGGKQPFKPLKKTVAHKV
***S***L*IN**K**V*****FN*S[*V*********]IDDQ*MEI*LA*IRM*MDA*N**NKNGVM*NKHNGRGT*YDLTP
S**S**RV*IND*K**V*L***FE*Q[*********]MDDG*V*I*LA*IRR***-V-EKSGDKSSSMEK*QQW*KNNLRL
*****RV*IN**K**V*****F*V*[*********]MDDN*V*V*LN*IHS**ENI-DKK*D*TVAM*S*TRRRENKLRL
*****RV*IN**K**V*****FNV*[*********]MDDN*V*V*IN*IMS**ENI-DKK*D*TVAM*S*TRHRDNKLRL

GFPAILR
QMGSTMKMLLA
S*S--K*MYDES-VLSPLSSPIPPSPLVR
S*SFSG*RYDEGNVLNSPHTMSPHSPLVIAKN
S*SFSG*RYDEGNVLNSPHTMSPHSPLVIAKN
```
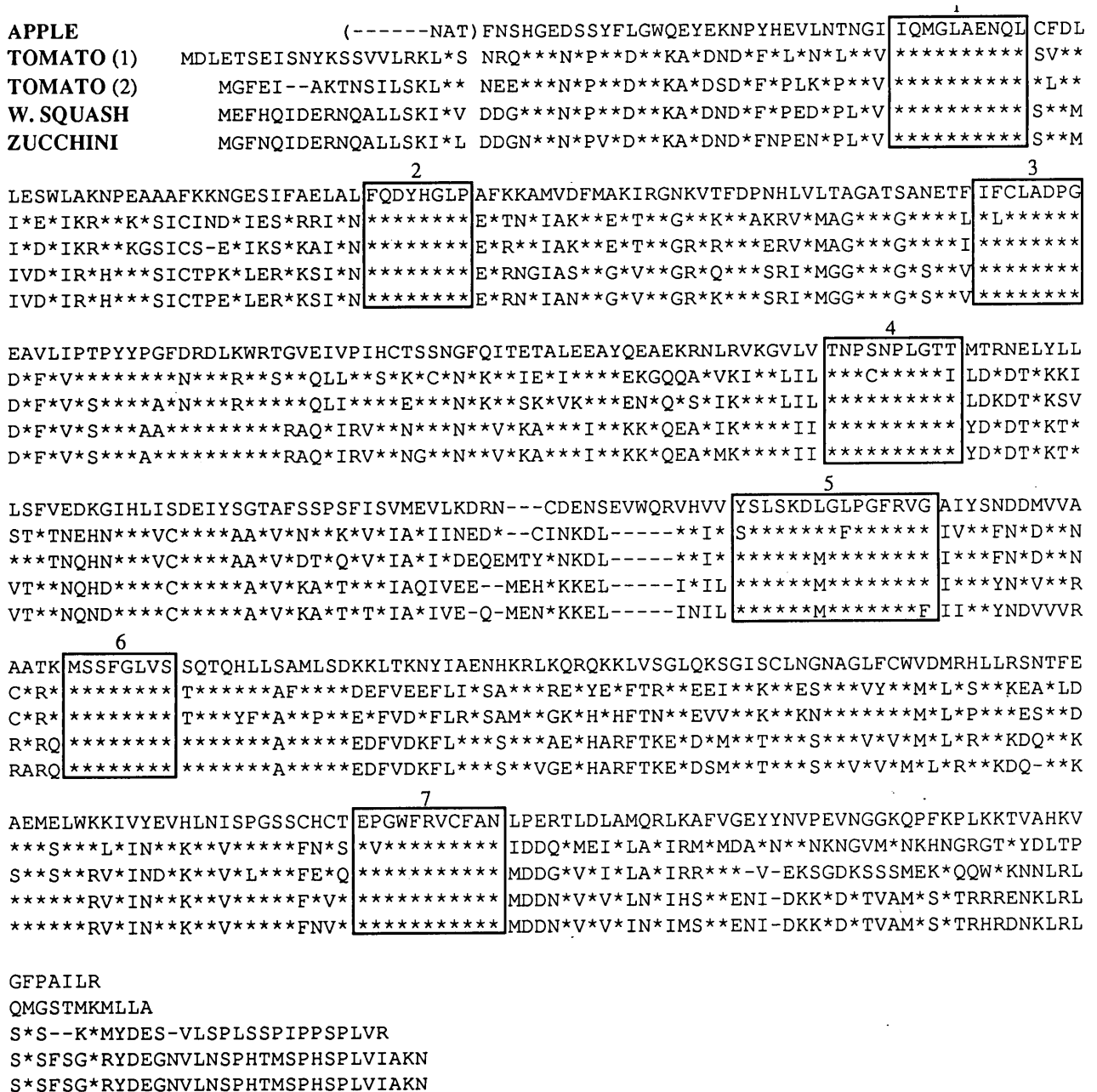
Fig. 2. Comparison of the deduced amino acid sequences of ACC synthases from apple (Dong et al., 1992b), tomato (Van der Straeten et al., 1990; Olson et al., 1991), winter squash (Nakajima et al., 1990) and Zucchini (Sato et al., 1991). *, amino acid residues that
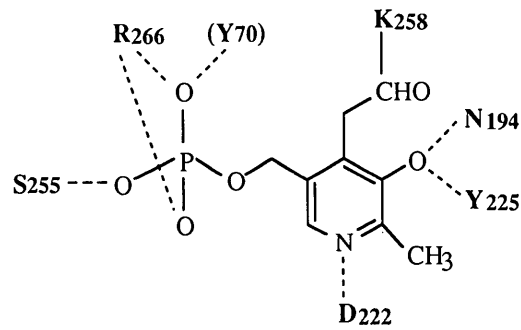are identical to that of apple ACC synthase; -, sequence gap. The seven highly conserved regions among all species are boxed in.

sequences from these species all contain the seven conserved regions.

PLP-Binding Sites

ACC synthase belongs to a superfamily of PLP-utilizing enzymes which catalyze a number of reactions including transamination, decarboxylation, racemization, elimination and replacement reactions. Among this superfamily, aspartate aminotransferase (AspAT) has been studied most extensively. Based on the X-ray structures and site-directed mutagenesis of catalytically important residues, six amino acid resi-

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                      194                         222 225                                           255   258     266
                      *                           *   *                                             *     *       *
AspAT     NPTGTDPTPDEWKQIAAVMKRRCLFPFFDSAYQG-------FASGNLEKDAWQVRYFVSEGF--ELFCAQSFSKNFGLYSER
Consensus NPlGT      l    f     h   DeiY  t                                         SlSKd G pg  R
Tomato 1  NPLGTTLDKDTLKSVLSFTNQHNIHLVCDEIYAATVFDTPQFVSIAEILDEQEMTYC-NKDL---VHIVYSLSKDMGLPGFR
Tomato 2  NPLGTILDRDTLKKISTFTNEHNIHLVCDEIYAATVFNSPKFVSIAEIINEDN---CINKDL---VHIVSSLSKDLGFPGFR
Tomato 3  NPLGTTLNRNELELLLTFIDEKGIHLISDEIYSGTVFNSPGFVSVMEVLIEKN--Y-MKTRVWERVHIVYSLSKDLGLPGFR
Tomato 4  NPLGTTMSRNELNILITFAMTKNIHIVSDEIYAGTVFDSPKFVSIIEALIDRK--LE-KSKMWNQVHIVSSLSKDLGLPGFR
Tomato 5  NPLGTLLDKDTLRDIVTFINSKNIHLVCDEIYAATVFDQPRFISVSEMVEE--MIEC-NTDL---IHIVYSLSKDLGFPGFR
Tomato 6  NPLGTLLDKDTLRDIVTFINSKNIHLVCDEIYAATVFDQPRFISVSEIVEE--MIEC-NTDL---IHIVYSLSKDLGFPGFR
Squash 1  NPLGTTYDRDTLKTLVTFVNQHDIHLICDEIYSATVFKAPTFISIAQIV--EEMEHC-KKEL---IHILYSLSKDMGLPGFR
Squash 2  NPLGTTMNRDELNLVFDFITSKGIHLISDEIYSGTVFGSPGFVSAMEVLKERSSEDEEVWKR---VHIVYSLSKDLGLPGFR
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Multigene Family of ACC Synthase and Differential Expression

Earlier experiments by Nakagawa *et al.* (1988) have shown that the ACC synthase induced by wounding and by IAA treatment are immunologically different. Analysis of the amino acid sequence of the active site of tomato ACC synthase isolated from wounded ripe fruit revealed two sequences which differ in one amino acid (Yip *et al.*, 1990). These observations suggest that ACC synthase exists in isoforms which are derived from different genes. Indeed, Van Der Straeten *et al.* (1990) isolated two ACC synthase cDNAs from a cDNA library constructed from mRNAs isolated from wounded and ripe tomato fruit. Olson *et al.* (1991) demonstrated that one of the above sequences is expressed during ripening while the other is expressed after wounding. By using PCR-based amplification, Yip *et al.* (1992) isolated four fragments (about 300 bp) from tomato, two of which represented new ACC synthase transcripts.

The restriction maps and organization of five tomato ACC synthase genes have been reported by Rottmann *et al.* (1991) from a tomato genomic library. Two of the five genes are identical in structural sequences to the cDNAs isolated previously (Van der Straeten *et al.*, 1990; Olson *et al.* (1991). while one of

ACC Oxidase

Although the enzyme *in vivo* was characterized long ago, the isolation of ACC oxidase in cell-free extracts had encountered much difficulty. Unlike ACC synthase, ACC oxidase was first identified by its cDNA. The information obtained was then used to demonstrate the enzyme activity. and to isolate the protein in plant tissues.

Concerning the nomenclature, the enzyme was first named as ethylene-forming enzyme (EFE) by Yang and his colleagues (Yu and Yang, 1979; Yang, 1981), because the reaction mechanism was not known at that time. It was not until very recently that authentic EFE activity was demonstrated *in vitro*, and the enzymic reaction characterized. The enzyme requires ascorbate and oxygen as co-substrates and Fe^{2+} and CO_2 as cofactors. Based on these data EFE can be referred to as ACC oxidase.

Molecular Cloning and Identification of ACC Oxidase cDNAs

In order to study gene expression during fruit ripening, Davies and Grierson (1989) isolated a cDNA, pTOM13, by differential cloning techniques. The function of the polypeptide encoded by the cloned cDNA

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pTOM13    MEN---FPIINLEKLN---GDERANTMEMIKDACENWGFFELVNHGIP  42
pHTOM5    *EN---**IINLENLN---*D**AKTMEM*K***E*****EL***GIP  42
pAVOe3    *D---S**VINMEKLE---*Q**AATMKL*N***E*****EL***SIP  42
pSR120    *ANIVN**IIDMEKLNNYN*V**SLVLDQ*K***H*****QV***SLS  48
pAE12     *AT---**VVDLSLVN---*E**AATLEK*N***E*****EL***GMS  42
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H*VMDTVEKMTKGHTKKCMEQRFKELVASKGLEAVQAEVTDLDWESTFFLRHLPTSN  99
H*VM*T**KM**G*T**CM**R**ELVAS*GLEAVQAEVT*L*****FL**L*T**   99
V*LM*E**RL**E*Y**CM**R**ELMAS*-VEGAVVDAN*M*****FI**L*V**   98
H*LM*K**RM**E*Y**FR**K**DMVQT*GLVSAESQVN*I*****YL**R*T**105
T*LL*T**KM**D*Y**TM**R**EMVAA*GLDDVQSEIH*L*****FL**L*S**   99
```

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ISQVPDLDEEYRE-VMRDFAKRLKLEKLAEELLDLLCENLGLEKGYLKNAFYGSKG-153
I*QV***DEEY*E-V*RD**KRLRL*K*A*EL************G***NA*V*SKG-153
L*EI***TDEH*KNV*KE**EK--L*K*A*QV***********G***MA*A*TTTG153
I*EV***DDQY*K-L*KE**--AQI*R*S*QL***********A***NA*Y*ANG1157
I*EI***EEEY*K-T*KE**--VEL*K*A*KL***********G***KV*Y*SKG-151
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-PNFGTKVSNYPPCPKPDLIKGLRAHTDAGGIILLFQDDKVSGLQLLKDEQWIDVPP210
-*N***********D*I*****T****I*******K*S*******EQ*I****210
L*T***********R*E*F******T****L*******R*A*******GE*V****210
-*T***********K*D*I*****T****I*******K*S*******GH*V****214
-*N***********K*D*I******S****I*******K*S*******GE*V****208
MRHSIVVNLGDQLEVITNGKYKSVIHRVIAQTDGTRMSLASFYNPGSDAVIYPAKTL267
*R****V****L************M***I*****T***L*******N*AV*V**PSL267
*N****I*****V***********M***V****N***L*******S*AV*F**PAL267
*K****V****L************M***I*****N***I*******S*AV*Y**PTL270
*H****I*****I***********M***I*****T***I*******N*SF*S**PAV264
```

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VEKEAEESTQVYPKFVFDDYMKLYAGLKFQAKEPRFEAMKAMESD-----PIASA  317
I----*ESK*V******D***K**AG****P******A**AMEANVELVDQIASA  317
V**EA*EKKEV****-E***N**AG****A******V**MKAVETANLS*I-TT  320
V**E-*EKCRA*****FE***N**LK****E******A**AMETT----G*IPTA  321
L**KT*DAP-T*****FD***K**SG****A******A**AKEST-----*VATA  314
```

Fig. 4. Comparison of the amino acid sequences deduced from pTOM13 (Hamilton *et al.*, 1991), pHTOM5 (Spanu *et al.*, 1991), pAVOe3 (McGarvey *et al.*, 1990), pSR120 (Wang *et al.*, 1991) and pAE12 (Dong *et al.*, 1992a). *, amino acid residues identical among all species. -, sequence gap.

and apple fruits (Dong *et al.*, 1992a; Ross *et al.*, 1992) and senescent carnation flowers (Wang and Woodson, 1991). These transcripts were found to be expressed during fruit ripening or flower senescence. As shown in Fig. 4, these sequences are highly homologous to those of tomato ACC oxidase cDNAs, sharing 85% identity at the amino acid level.

Characterization In Vivo

For more than a decade, unsuccessful attempts were made to demonstrate ACC oxidase activity *in vitro*. Hence, work on the characterization of ACC oxidase was carried out using an *in vivo* assay, in which exogenous ACC was fed into the tissue, and the ACC-dependent ethylene production was measured. Employing such an *in vivo* system, Hoffman *et al.*

(1982) examined the stereospecificity of ACC oxidase toward the four stereoisomers of the ethyl derivative of ACC, 1-amino-2-ethylcyclopropane-1-carboxylic acid, [(+)- and (-)-coronamic acids and (+)- and (-)-allocoronamic acids] as the substrate. They discovered that one of the four stereoisomers, (+)-allocoronamic acid, was preferentially converted into 1-butene. Since then, this stereospecificity has been employed as an important criterion for judging the authenticity of *in vitro* EFE systems. Other criteria for judging authentic EFE include a low K_m for its substrate ACC (Yang and Hoffman, 1984), inhibition by cobalt ions (Yu and Yang, 1979) and the production of equimolar amounts of ethylene, CO_2 and cyanide from ACC (Peiser *et al.*, 1984). Although a number of plant enzyme preparations were shown to be capable of oxidizing ACC to ethylene, these preparations lacked these characteristics. It was concluded that the following *in vitro* systems were not the authentic ACC oxidase that functions *in vivo* (Venis, 1984; Wang and Yang, 1987): IAA oxidase (Vioque *et al.*, 1981), peroxidase (Rohwer and Mäder, 1981), lipoxygenase (Bousquet and Thimann, 1984) and H_2O_2-generating oxidases (Legge and Thompson, 1983). It is likely that such systems generate various oxidants including "active-oxygen" species, which in turn react non-enzymatically with ACC to produce ethylene.

Intact vacuoles were the smallest entities of the cell exhibiting native EFE activity, although the total activity represented only 5% of that of the intact tissues (Guy and Kende, 1984). Because treatments modifying membrane structure (lipophylic compounds, osmotic shock, etc.) greatly reduced ethylene synthesis and caused the loss of the capability to convert ACC to ethylene, EFE was thought to require highly structured membrane integrity (Yang and Hoffman, 1984). However, this view is challenged by the recent demonstration of ACC oxidase activity in the cytosol. The primary structures displayed no membrane-binding domain, as determined by cDNA sequences.

Studies of the reaction products of ACC oxidase were instrumental in our understanding of the enzymic reaction mechanism. Based on the observation that 1-phenylcyclopropylamine is oxidized chemically by various oxidants to ethylene and benzonitrile via the intermediacy of the nitrenium ion, Adams and Yang (1981) proposed that ACC is oxidized *in vivo* to form the corresponding nitrenium ion or its equivalent, which is then degraded into ethylene (derived from C-2,3 of

ACC) and cyanoformic acid (derived from carboxyl and C-1 of ACC), the latter being further degraded spontaneously into HCN (derived from C-1 of ACC) and CO_2 (derived from the carboxyl group of ACC). Support for this hypothesis was provided by Peiser *et al.* (1984) who showed that in plant tissues the carboxyl carbon of ACC was liberated as CO_2, whereas the C-1 of ACC was recovered, not as free HCN but as cyanide conjugates, in an amount equivalent to that of the ethylene produced. When HCN was introduced into plant tissues it was similarly metabolized to β-cyanoalanine; β-cyanoalanine is then hydrated to asparagine. These results indicate that ACC is degraded into ethylene, CO_2 and HCN.

The requirement for molecular oxygen in the conversion of ACC to ethylene was demonstrated when Adams and Yang (1979) identified ACC as the immediate ethylene precursor, because ACC accumulated massively in apple fruit plugs when the tissue was incubated under N_2. The dependence of ethylene production on ACC and oxygen concentrations was later studied in a number of plant tissues (Yip *et al.*, 1988). The K_m value for O_2 varied greatly depending on the internal ACC content.

Purification and Characterization In Vitro

While progress in the isolation of cell-free EFE is slow, important advances in the molecular biology of this enzyme have been made recently, as described above. The nucleotide sequence of ACC oxidase reveals that its deduced amino acid sequence shares close homology with that of flavanone-3-hydroxylase (Hamilton *et al.*, 1990), and hyoscyamine 6β-hydroxylase (Matsuda *et al.*, 1991). Although Yang (1981) suggested that EFE may be an ACC hydroxylase, attempts to isolate and assay EFE as a hydroxylase were not pursued. Ververidis and John (1991) were the first to report the isolation of EFE activity *in vitro*, when they extracted and assayed under the conditions used for flavanone 3-hydroxylase (Britsch and Grisebach, 1986). EFE activity was shown to require Fe^{2+} and ascorbate for full activity and stability. Not only did the isolated ACC oxidase activity display the stereospecificity toward 2-ethyl-ACC isomers, but also it met the other criteria described above.

Under similar conditions, Kuai and Dilley (1992) and Fernández-Maculet and Yang (1992) isolated and characterized ACC oxidase activity from apple fruit,

while McGarvey and Christoffersen (1992) isolated ACC oxidase from avocado fruit. As was reported for ACC oxidase from melon fruit (Ververidis and John, 1991; Smith et al., 1992), all of these enzyme preparations are from cytosol and require Fe^{2+} and ascorbate for activity. Unlike many other oxidases, which require 2-oxoglutarate as the co-substrate, ACC oxidase activity is independent of 2-oxoglutarate. The previous failure in isolating authentic EFE in vitro can be attributed to the loss of these factors during the enzyme extraction (Fernández-Maculet and Yang, 1992). A similar example is that extraction of flavanone-3-hydroxylase (Abbott and Udenfriend, 1974) was unsuccessful unless Fe^{2+} and ascorbate were added into the extraction buffer.

Recently, ACC oxidase from apple fruit has been purified to near homogeneity (Dong et al., 1992b; Dupille et al., 1992). The enzyme exists as a monomer with a molecular weight of 35 kD. Since an internal peptide sequence obtained from the purified enzyme precisely matched that predicted from the cDNA clone pAE12, and since the purified enzyme can be recognized on Western blotting by a polyclonal antibody raised against an oligopeptide whose sequence was derived from pAE12, the ACC oxidase protein that we isolated was thought to be encoded by pAE12 (Dong et al., 1992b). Using the purified ACC oxidase, we have determined the stoichiometry of the reaction catalyzed by ACC oxidase to be:

$$\text{ACC } (C_4NO_2H_7) + O_2 + AH_2 \xrightarrow{Fe^{2+}, CO_2} C_2H_4 + CO_2 + HCN + A + 2H_2O$$
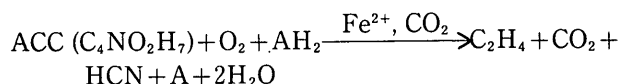
(where AH_2 and A stand for ascorbate and dehydroascorbate, respectively)

Ascorbate serves as a co-substrate, and is concurrently oxidized into dehydroascorbate in an amount equivalent to that of ethylene produced. The exact mechanism of the oxidation reaction catalyzed by ACC oxidase is unknown. If the enzymic reaction proceeds via the intermediacy of N-hydroxyl-ACC, EFE can be referred to as ACC N-hydroxylase as shown below:
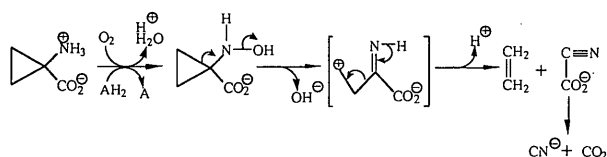
In the reaction scheme depicted above, N-hydroxy-

ACC is degraded into ethylene and cyanoformic acid by a stepwise mechanism rather than a concerted elimination mechanism. Such a stepwise mechanism is necessary to account for the fact that the hydrogen atoms in C-2,3 of ACC undergo scrambling, resulting in the loss of stereochemistry during its oxidation to ethylene (Adlington et al., 1983).

CO₂ Requirement

Ethylene production in photosynthetic tissues is inhibited by light when the experiment is carried out in a sealed container. Kao and Yang, (1982) were the first to demonstrate that the inhibitory effect of light in excised rice leaf segments resulted from a depletion in the endogenous CO_2 level by photosynthetic fixation, and that this inhibition was relieved by the addition of exogenous CO_2. They proposed that CO_2 stimulates ethylene production by direct modulation of the activity of EFE (Kao and Yang, 1982). The CO_2 stimulation of EFE activity in vivo was later observed by a number of investigators (Preger and Gepstein, 1984; Philosoph-Hadas et al., 1986; Tan and Thimann, 1989). To obtain direct evidence that CO_2 activates ACC oxidase in vitro, we characterized this phenomenon using purified ACC oxidase (Dong et al., 1992b). In our system both CO_2 and bicarbonate stimulated ACC oxidase activity markedly, but CO_2 seems to be the active species responsible for the activation. ACC oxidase activity is abolished when CO_2 is removed from the reaction tubes, indicating that CO_2 is an essential activator for the enzyme. The concentration of atmospheric CO_2 giving half maximal activity is estimated to be 0.5%. When ACC oxidase activity is assayed in the presence of ambient air where only 0.03% CO_2 is present, ACC oxidase activity was only about one-tenth of that measured in saturating CO_2 concentration. Since previous investigators assayed EFE activities in the ambient air, EFE activities must have been underestimated by them.

Ribulose-1,5-bisphosphate carboxylase (Rubisco) has been recognized as an enzyme that requires CO_2 for its activation (Lorimer, 1983). The demonstration of CO_2 requirement by ACC oxidase has added this enzyme as a second member of this family. In both cases CO_2 not only acts as an activator but is also involved in the reaction; Rubisco uses CO_2 as its substrate, whereas ACC oxidase releases CO_2 as one of its reaction products. In Rubisco it was demonstrated that

CO_2 forms a carbamate with the amino group of a lysyl residue of the enzyme, resulting in its activation, while decarbamation causes inactivation of the enzyme. It is interesting to know whether the activation of ACC oxidase also involves the formation of a carbamate complex between CO_2 and ACC oxidase.

Regulation and Genetic Engineering of Ethylene Biosynthesis in Ripening Fruits

Ethylene in Ripening Fruits

In climacteric fruits, one of the earliest events of ripening is the onset of ethylene production. Ethylene production in preclimacteric fruits is very low, but increases dramatically at the onset of ripening. Application of ethylene to pre-climacteric fruits such as tomato, banana, apple, and avocado hastens the ripening process. It is now recognized that ethylene plays an essential role in the ripening of climacteric fruits (Yang and Hoffman, 1984). Ethylene production in relation to the change in ACC content in ripening avocado fruit was studied by Hoffman and Yang (1980). Although the ACC content in preclimacteric avocado fruits was very low (<0.1 nmol g^{-1}), a large increase occurred at the onset of rapid ethylene evolution and ripening. Since the application of ACC to the preclimacteric fruits results in only a slight increase in ethylene, the fruit at the preclimacteric stage must lack the capabilities not only of synthesizing ACC but also of oxidizing ACC to ethylene.

When ethylene is applied to mature, preclimacteric fruits, the ripening process is triggered and accompanied by a massive synthesis of ethylene. The capability of tissues to synthesize large quantities of ethylene in response to ethylene is referred to as "autocatalytic regulation" of ethylene production. This ethylene effect was found to be due to the enhancement of both ACC synthase and EFE activities (Riov and Yang, 1982; Liu et al., 1985). Recent results establish that this increased synthesis of ethylene results from the expression of messenger RNAs for both ACC synthase and ACC oxidase (Oeller et al., 1991; Dong et al., 1992b). When preclimacteric fruit was treated with exogenous ethylene for a short period, ethylene production is not induced because there is no increased synthesis of ACC; this treatment, however, causes a marked increase in ACC oxidase transcript (Dong et al., 1992b). A similar result was observed by Ross et al. (1992) who treated apple fruit with exogenous ethylene and found

that the level of the transcript of an ACC oxidase homolog increased. These observations are in agreement with the view that during the ripening process the expression of ACC oxidase gene and the development of ACC oxidase activity precedes the expression of ACC synthase and the development of ACC synthase activity.

It is to be noted that ethylene production during fruit ripening and the autocatalysis of ethylene production are common features not only of ripening fruits, but also of other senescing organs and tissues.

Genetic Engineering of Ethylene Biosynthesis

The cloning of the genes involved in ethylene biosynthesis during fruit ripening has permitted scientists to use biotechnology to genetically engineer crop plants with reduced endogenous ethylene production and fruits with prolonged storage life. It is possible to reduce ethylene production by lowering the expression of either ACC synthase or ACC oxidase genes. Application of antisense RNA technology is such an example. In antisense RNA technique, a target gene (or cDNA) is inserted in the reverse orientation (antisense) to its parent gene in a plant through A. tumefaciens-mediated transformation. When both antisense and sense (parent) genes are expressed in the tissue, they tend to hybridize through sequence complementarity, and the subsequent translational process is prevented (Mol et al., 1990; Wintraub, 1990). Such an approach has previously been undertaken with success to inhibit the expression of polygalacturonase gene in tomato fruit (Smith et al., 1988; Sheehy et al., 1988).

So far, efforts to alter ethylene biosynthesis in plant tissue have been focused on ripening fruits. Hamilton et al. (1990) were the first to reduce ethylene biosynthesis in tomato plants by transformation with an antisense pTOM13 construct. Since ethylene production in the antisense-bearing plants was greatly reduced, they suggested that pTOM13 may be related to ACC oxidase. Ripening of the transgenic fruits was retarded and their storage life extended. A similar approach was undertaken by Oeller et al. (1991) who transformed tomato plants with the antisense ACC synthase genes. Fruits from transgenic plants bearing expressed antisense RNA produced little ethylene, and did not undergo ripening unless exogenous ethylene was provided. These results demonstrate that antisense technology can be successfully used to reduce ethylene

production and thereby to extend the shelf-life of fruits and vegetables. Alternatively, ethylene biosynthesis can be reduced by lowering the cellular level of ACC by introducing a bacterial gene encoding ACC deaminase (Sheehy *et al.*, 1991) into plants. Klee *et al.* (1991) cloned an ACC deaminase gene from *Pseudomonas* sp. and expressed it in tomato plants. Ethylene biosynthesis in the transformed plants was greatly reduced and ripening of fruits was similarly delayed. Research on ethylene biosynthesis is an excellent example illustrating that fundamental research into ethylene biosynthesis has not only helped in our understanding of how ethylene regulates plant processes, but also opened the prospect for improving through biotechnology the quality of crop products by extending shelf-life and reducing spoilage.

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