Differential Regulation of ethylene Biosynthetic Gene Expression in Higher Plants

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I. Introduction

A number of physiological processes during plant growth and development are regulated by the phytohormone ethylene. The production of this gaseous hormone in plant tissues in usually low, but is greatly induced at certain stages of plant development, such as seed germination, leaf abscision, and fruit ripening. Ethylene production can also be induced by auxin treatment or in response to a wide range of environmental stresses including wounding, anaerobiosis, drought, flooding or pathogen invasion (Yang and Hoffman, 1984; Theologies, 1992; Kende, 1993). It has been recognized that increased ethylene production, in turn, brings about many important physiological responses. The biosynthetic pathway of ethylene in higher plant is well established as follows: methionine (Met)——〉S-Adenosylmethionine (AdoMet)——〉1-aminocyclopropane-1-carbo-xylic acid (ACC)——〉ethylene (Adams and Yang, 1979). The last two steps are catalyzed by ACC synthase and ACC oxidase (also known as the ethylene forming enzyme), respectively. In fruit tissue, these two unique enzymes are induced during ripening and contribute to the regulation of ethylene biosynthesis (Yang and Hoffman, 1984; Kende, 1993). The induction of ethylene synthesis by a variety of factors is due to de novo synthesis of these enzymes so that a fundamental qustion arises: are there as many genes as inducing factors, or is there only one gene whose promoter recognizes all these factors? In view review we describe research progress that has occurred recently with regard to the molecular biology of ACC synthase and ACC oxidase.

II. ACC Synthase

1. Purification of ACC synthase

Since ACC synthase is unstable ($T_{1/2}$= 1 hr, Kim and Yang, 1992) and present in low concentration (Yang and Hoffman 1984; Theologies, 1992), even in those tissues where ethylene production is greatly induced, progress in the purification of this enzyme has been slow. Partial purification of the enzyme was carried out in ripe tomato fruits after wounding. Although monoclonal antibodies were prepared by Bleecker et al. (1986), they failed to recognize ACC synthase on Western blots. In winter squash fruits, ACC synthase was isolated after wounding (Nakajima et al., 1988). Van der Straeten et al. (1989) obtained several internal peptide sequence following tryptic digestion of their highly purified tomato ACC synthase preparation. Some of these sequences, however, were found to be homologous to that of enolase; three of them were not homologous to enolase sequences and were assumed to be putative ACC synthase sequences. ACC sythase in zucchini fruits was also partially isolated after induction by treatment with IAA, LiCl and aminooxyacetic acid in combinaton with wounding (Sato et al., 1989).

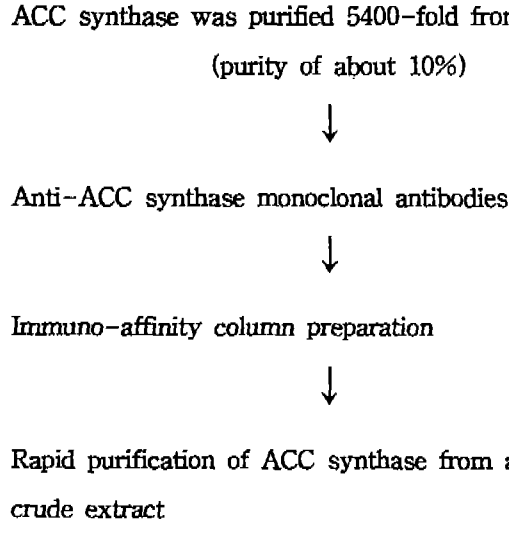

Fig. 1 The scheme of rapid purification of ACC synthase from apple fruits

This partially purified enzyme preparation was used directly for the production of polyclonal antiserum, which was subsequently used for cDNA screening of an expression library. In apple fruits, we have taken a different approach by which monoclonal antibodies (mAbs) were raised against a partially purified apple enzyme preparation. Specific mAbs against ACC synthase were then screened based on their ability to precipitate the enzyme activity (Dong et al., 1991b), and these were in

turn used to immuno-purify apple ACC synthase protein from crude extract of apple fruits (Yip et al., 1991). In addition, this immuno-affinity purified apple ACC synthase protein was subjected to trypsin digestion followed by reversed-phase HPLC separation (Dong et al., 1991a). Ten well-separated major fragments were sequenced. From these sequences, three partial sequences were chosen to construct corresponding degenerate oligonucleotides which were employed as probes for screening apple fruit cDNA library for ACC synthase cDNA clone. Figure 1 shows the scheme of rapid purification of ACC synthase from apple fruits. Figure 2 shows amino acid sequences of ten tryptic peptides and nucleotide sequences of three tryptic oligonucleotide probes prepared on the basis of the peptide sequences.

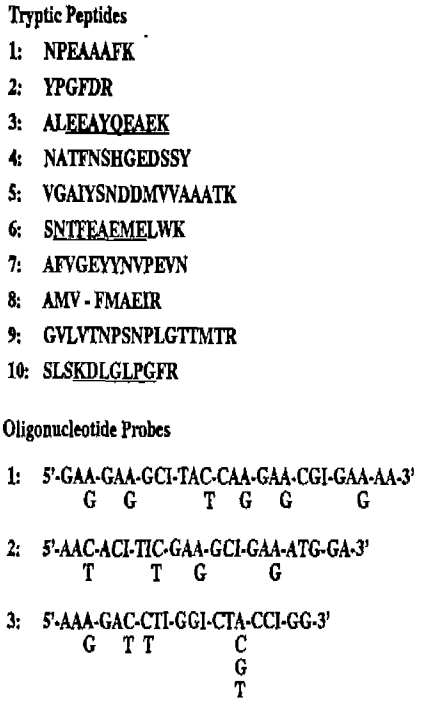

Fig. 2 Amino acid sepuences of ten tryptic peptides derived from immuno-purified apple ACC synthase and nucleotide sequences of three tryptic oligonucleotide probes prepared on the peptide sequences indicate regions used for the design of the oligonucleotide probes. I stands for the inosine substitute.

2. Screening and identification of apple ACC synthase cDNA clones

Primary screening of about 200,000 colonies with the oligonucleotide probes described above yielded one positive clone which hybridized to all three probes. This

putative ACC synthase clone, pAAS1, had a cDNA insert of only 1.3 kb, which is not long enough to encode the 48 kDa mature form of ACC synthase (Yip et al., 1991). In order to isolate a longer cDNA clone, an oligonucleotide complementary to the 5′-end of the pAAS1 clone was used as a probe to re-screen the library. Three clones were isolated. The longest clone, designated as pAAS2, had a 1.6 kb insert. The insert has one open reading frame encoding 404 amino acids residues. This reading frame did not begin with the initiation codon ATG, but ended with a poly A sequence. Since the deduced amino acid sequence contained sequences corresponding precisely to the 10 tryptic peptides, we concluded that the cDNA clone, pASS2, indeed encoded ACC synthase but lacked the 5′-end of the sequence.

3.Expression of the ACC synthase during the ripening of apple fruits

To study the expression of the ACC synthase gene during apple fruit ripening, we first analyzed the ACC synthase enzyme activity and ethylene production at different stages of fruit ripening. At stage I (pre-climacteric stage), ethylene concentration was as low as 0.17 ml/l and ACC synthase activity was undetectable. At the onset of ripening (stage II), both internal ethylene concentration and ACC synthase activity increased substantially. At a late ripening stage (stage III), internal ethylene concentration exceeded 300 ml/l and ACC synthase activity was about 40 units/mg protein (Fig. 3). We have analyzed poly(A)+RNA isolated from apple fruit at the preclimacteric (stage I) and late ripening stage (stage III) by RNA

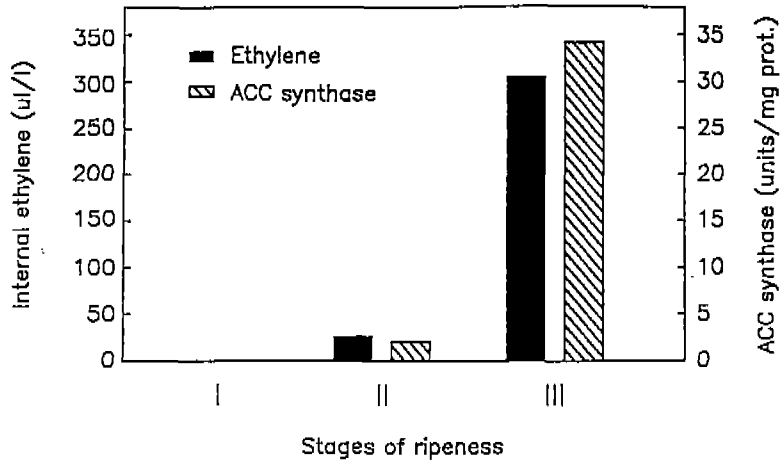

Fig. 3 Changes in internal ethylene concentration and ACC synthase activity of apple fruit at various ripening stages. Stages I, II and III represent the preclimacteric, clinacteric-rise and climacteric-peak stages.

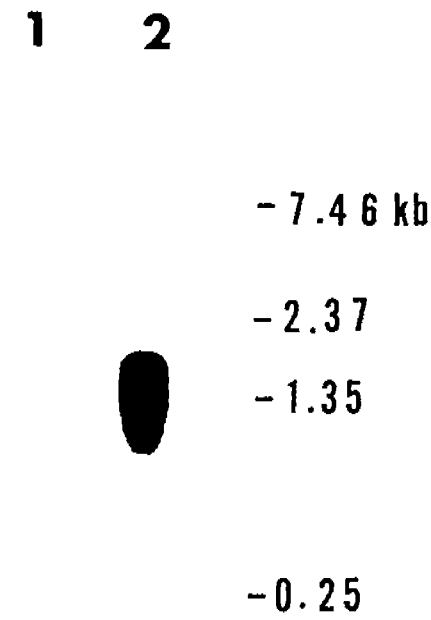

Fig. 4 Northern blot analysis of apple fruit poly(A)$^+$RNA. poly(A)$^+$RNA was obtained fromaldehyde-agarose gelelectrophoresis, and blotted onto a membrane filter. The blot was then probed with ^{32}p-labeled pAAS2 cDNA clone, washed and subjected to autoradiography.

blot hybridization using the pAAS2 cDNA clone as a probe. Figure 4 shows that the mRNA is about 2.0 kb in size and is detectable only in ripening apple fruit tissues. Even with prolonged exposure of the film, no signal was detected in preclimacteric fruit. Together, these data indicate that increased ethylene and ACC synthase activity in ripening apple is closely correlated with increased level of ACC synthase mRNA and the cDNA clone we have isolated is the product of ripening related gene.

4. Comparison of deduced amino acid sequences of ACC synthase

The deduced amino acid sequences of ACC synthase from apple, tomato and winter-squash fruits show a 52%, 53% and 62% sequence identity between apple and tomato enzymes, apple and winter-squash enzymes, and tomato and winter-squash enzymes. Although the amino acid sequence identity among all three species is only 43%, the overall similarity is as high as 83%. There are seven highly conserved regions, designated as regions 1 to 7, which contain at least eight amino acid residues and show greater than 95% identity (Fig. 5, boxed regions). We have previously identified region 5 as the active-site center, where K-264 is responsible

for binding its coenzyme pyridoxal 5′-phosphate and catalyzing the enzymatic reaction (Yip et al., 1990). We have isolated and sequenced the active-site center of both apple and tomato enzymes. While residue L-266 was found in the active-site peptide of apple enzyme, both methionine and leucine were found at this position in tomato enzyme. We have postulated that there are at least two ACC synthase isozyme in tomato fruit, one with leucine and the other with methionine in the active-site peptide. Besides the seven conserved regions, it is of interest that there is a divergent region near the N-terminus. Since these amino acid sequences are not conserved, one could speculate that this region of ACC synthase may not be directly related to enzyme activity.

COMPARISON OF PREDICTED AMINO ACID SEQUENCES OF ACC SYNTHASES

```
                                                                                1
APPLE                          (xxxxxxNAT)FNSHGEDSSYFLGWQEYEKNPYHEVLNTNGI IQMGLAENQL  41
TOMATO              MGFEI--AKTNSILSKL** NEE***N*P**D**KA*DSD*F*PLK*P**V **********
SQUASH              MEFHQIDERNQALLSKI*V DDG***N*P**D**KA*DND*F*PED*PL*V **********

                                                2
CFDLLESWLAKNPEAAAFKKNGESIFAELAL FQDYHGLP AFKKAMVDFMAKIRGNKVTFDPNHLVLTAGATSANET 117
*L**I*D*IKR**KGSICS-E*IKS*KAI*N ******** E*R**IAK**E*T**GR*R***ERV*MAG***G****
S**MIVD*IR*H***SICTPK*LER*KSI*N ******** E*RNGIAS**G*V**GR*Q***SRI*MGG***G*S**
          3                                                                  4
F IFCLADPG EAVLIPTPYYPGFDRDLKWRTGVEIVPIHCTSSNGFQITETALEEAYQEAEKRNLRVKGVLV TNPSN 193
I ******** D*F*V*S****A*N***R*****QLI****E***N*K**SK*VK***EN*Q*S*IK***LIL *****
V ********* D*F*V*S***AA*********RAQ*IRV**N***N**V*KA***I**KK*QEA*IK****II *****
                                                                           5
PLGTT MTRNELYLLLSFVEDKGIHLISDEIYSGTAFSSPSFISVMEVLKDRN---CDENSEVWQRVHVV YSLSKDL 266
***** LDKDT*KSV***TNQHN***VC****AA*V*DT*Q*V*IA*I*DEQEMTY*NKDL-----**I* ******M
***** YD*DT*KT*VT**NQHD****C*****A*V*KA*T***IAQIVE--EMEH*KKEL-----I*IL ******M
                                6
GLPGFRVG AIYSNDDMVVAAATK MSSFGLVS SQTQHLLSAMLSDKKLTKNYIAENHKRLKQRQKKLVSGLQKSGIS 342
******** I***FN*D**NC*R* ******** T***YF*A**P**E*FVD*FLR*SAM**GK*H*HFTN**EVV**K
******** I***YN*V**RR*RQ ******** *******A*****EDFVDKFL***S***AE*HARFTKE*D*M**T
                                                7
CLNGNAGLFCWVDMRHLLRSNTFEAEMELWKKIVYEVHLNISPGSSCHCT EPGWFRVCFAN LPERTLDLAMQRLKA 418
**KN*******M*L*P***ES**DS**S**RV*IND*K**V*L***FE*Q ********** MDDG*V*I*LA*IRR
***S***V*V*M*L*R**KDQ**K******RV*IN**K**V*****F*V* ********** MDDN*V*V*LN*IHS

FVGEYYNVPEVNGGKQPFKPLKKTVAHKVGFPAILR                                        454
***-V-EKSGDKSSSMEK*QQW*KNNLRLS*S--K*MYDES-VLSPLSSPIPPSPLVR
**ENI-DKK*D*TVAM*S*TRRRENKLRLS*SFSG*RYDEGNVLNSPHTMSPHSPLVIAKN
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Fig. 5 Comparison of deduced amino acid sequences of ACC synthase from apple, tomato (Van der Straeten et al., 1990) and winter squash (Nakajima et al., 1990) fruits. Amino acid residues are numbered based on the apple sequence starting with the residue F. *, Amino aced residues in tomato and winter wquash enzyme that are identical to apple ACC synthase;-, sequence gap.

5. Multigene family of ACC synthase and differential expression

In winter squash, the polyclonal antibody against wound-induced ACC synthase fails to cross-react with the enzyme from IAA-treated hypocotyls, suggesting that the primary structures of the wound-induced and auxin-induced enzymes are

distinct (Nakagawa et al.,1988). As mentioned above, 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 results indicate that tomato ACC synthase exists in isoform 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 fruits. Olson et al. (1991) demonstrated that one of the above sequence is expressed during ripening while the other is expressed after wounding. By using PCR-based amplification, we isolated new 350 bp cDNA fragment encoding ACC synthase from cultured apple shoots and showed that its expression is induced by auxin-treatment (Kim et al., 1992). Furthermore, four cDNA fragments (about 350 bp) from tomato suspension culture were obtained by PCR amplification, two of which represented new ACC synthase transcripts (Yip et al., 1992). The restriction maps and organization of five tomato ACC synthase genes have been reported by Rottmann et al. (1991) froma tomato genomic library. Comparison of nucleotide and deduced amino acid sequences showed that 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 them corresponds to one of the PCR fragments reported by Yip et al. (1992). Thus, it can be concluded that in tomato genome, there are at least six different ACC synthase genes. Nakagawa et al. (1991) isolated an ACC synthase cDNA from winter squash seedling treated by IAA. This cDNA is homologous to that previously isolated from wound-induced winter squash fruit (Nakajima et al., 1990), but its transcript was differentially expressed by IAA treatment. These results support their previous notion that ACC synthase induced by wounding is different from that induced by IAA in the same species.

Ribonuclease protection assays were employed to examine the differential expression of four tomato ACC synthase PCR fragments under different conditions of enhanced ethylene production-namely, during fruit ripening, in response to mechanical wounding in fruit tissue, and auxin stimulation in vegetative tissue (Table 1). Transcripts of pBTAS1 (LE-ACC2) accumulated massively during ripening and wounding but only slightly in response to auxin treatment. Although pBTAS4 (LE-ACC4) was associated with fruit ripening, it was unresponsive to auxin treatment in vegetative tissue. In contrast, The expression of pBTAS2 (LE-ACC3) and pBTSA3 (LE-ACC5) was greatly promoted in auxin-treated vegetative tissue but was absent from fruit tissue. While the expression of pBTAS2 (LE-ACC3) was moderately dependent on wounding, pBTAS3 (LE-ACC5) was unresponsive to

wounding. Interestingly, the expression of pBTAS2 (LE-ACC3) was greatly induced in root tissue by anaerobiosis, while other three transcripts were not detectable. These data support the view that ACC synthase is encoded by a multigene family and that the members are differentially expressed in response to development, environmental, and hormonal factors.

Table 1. Differential expression of ACC synthase multigene family in tomato

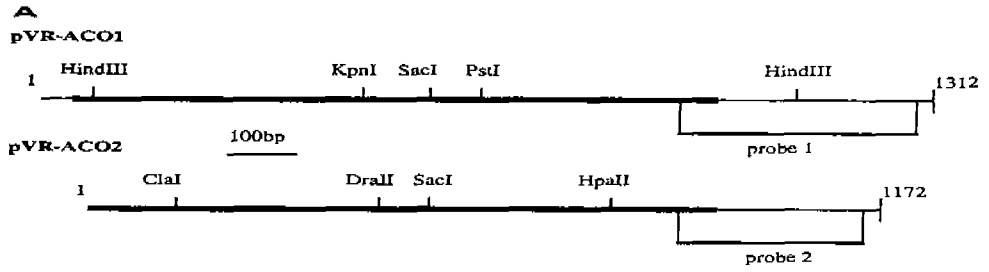
	ripening	wounding	auxin	anaerobiosis
pBTAS1 (LE-ACC2)	+++	++	+	−
pBTAS2 (LE-ACC3)	−	+	+	+++
pBTAS3 (LA-ACC5)	−	−	+++	−
pBTAS4 (LE-ACC4)	++	−	−	−

III. ACC Oxidase

1. Isolation and classification of mung bean ACC oxidase

Based on the observation that transgenic tomato plants expressing atisense copies of pTOM13 exhibit reduced ACC oxidase activity and ethylene production in ripening fruit, it was first suggested that pTOM13 gene was related to ACC oxidase (Hamilton et al., 1990). Subsequent work confirmed that pTOM 13 or its homolog indeed confered ACC oxidase activity when expressed in yeast (Hamilton et al., 1991) or Xenopus Oocytes (Spanu et al., 1991). Several investigators have since isolated ACC oxidase cDNA clones from ripening avocado (McGarvey et al., 1990), apple (Dong et al., 1992b; Ross et al., 1992), peach (Callahan et al., 1992) and melon (Balague et al., 1993) fruits, senescent carnation (Wang and Woodson, 1991) and orchid (Nadeau et al., 1993) flowers, and pea seedling (Peck et al., 1993). These clones share about 85% identity at the amino acid level to the tomato ACC oxidase clone, and the level of these transcripts increased greatly during fruit ripening or flower senescence.

While there are many molecular studies on ACC oxidase from fruit tissues, information about this gene from vegetative tissues is limited. A mung bean hypocotyl cDNA library was screened using a combination of apple (Dong et al., 1992b) and tomato (Holdsworth et al., 1987) ACC oxidase cDNAs as probes. Twelve putative ACC oxidase clones were isolated (Kim and Yang, 1994). Restriction enzyme mapping reveals that these clones can be divided into two homology classes



B

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  1                                                CACAATCACATAGTTGTTTAAAGCGATAGAGAAGAAAGAAAGAA
 45 ATGGCAAACTTTCCAGTTGTTGACATGGGAAAGCTTAACACTGAAGAGAGAGGAACTGCC
      M  A  N  F  P  V  V  D  M  G  K  L  N  T  E  E  R  G  T  A  20
105 ATGGAAATGATAAAAGATGCTTGTGAGAACTGGGGTTTCTTTGAGTTGGTGAACCATGGT
      M  E  M  I  K  D  A  C  E  N  W  G  F  F  E  L  V  N  H  G  40
165 ATATCCATTGAGTTGATGGACACCGTGGAGAAGTTAACAAAAGAGCACTACAAGAAGACT
      I  S  I  E  L  M  D  T  V  E  K  L  T  K  E  H  Y  K  K  T  60
225 ATGGAGCAAAGGTTCAAAGAAATGGTGGCCAACAAAGGTCTTGAGTCAGTTCAGTCAGAA
      M  E  Q  R  F  K  E  M  V  A  N  K  G  L  E  S  V  Q  S  E  80
285 ATCAATGACTTGGACTGGGAAAGTACCTTTTTTCTGCGCCATCTTCCAGTCTCCAATGTT
      I  N  D  L  D  W  E  S  T  F  F  L  R  H  L  P  V  S  N  V  100
345 TCAGAGAACACAGATCTTGATCAAGACTACAGGAAGATAATGAAGCAGTTTGCAGAAGAA
      S  E  N  T  D  L  D  Q  D  Y  R  K  I  M  K  Q  F  A  E  E  120
405 CTGGAGAAACTTGCAGAGCATCTTCTTGACTTGCTGTGTGAGAATCTTGGACTGGAGAAA
      L  E  K  L  A  E  H  L  L  D  L  L  C  E  N  L  G  L  E  K  140
465 GGGTACCTGAAGAAGGTGTTCTATGGATCGAAGGGCCCAAATTTTGGCACGAAAGTTAGC
      G  Y  L  K  K  V  F  Y  G  S  K  G  P  N  F  G  T  K  V  S  160
525 AACTACCCTCCCTGTCCGACCCCTGATCTGATAAAGGGCCTAAGAGCTCACACTGATGCC
      N  Y  P  P  C  P  T  P  D  L  I  K  G  L  R  A  H  T  D  A  180
585 GGTGGCATTATCCTACTGTTCCAAGATGACAAGGTCAGTGGACTGCAGCTCCTCAAGGAT
      G  G  I  I  L  L  F  Q  D  D  K  V  S  G  L  Q  L  L  K  D  200
645 GACCAGTGGATCGATGTCCCACCAATGCGTCACTCCATTGTCATCAACCTTGGTGACCAA
      D  Q  W  I  D  V  P  P  M  R  H  S  I  V  I  N  L  G  D  Q  220
705 CTTGAGGTCATAACCAATGGCAAGTACAAGAGTGTCATGCACCGAGTCATTGCTCAGACG
      L  E  V  I  T  N  G  K  Y  K  S  V  M  H  R  V  I  A  Q  T  240
765 GATGGCACCAGAATGTCCCTGGCTTCCTTCTATAATCCCGGTGATGATGCTGTGATTTCT
      D  G  T  R  M  S  L  A  S  F  Y  N  P  G  D  D  A  V  I  S  260
825 CCAGCACCAGCCTTGGTGAAGGAATCGGATGAAACAAGCCAAGTATACCCGAAATTTGTG
      P  A  P  A  L  V  K  E  S  D  E  T  S  Q  V  Y  P  K  F  V  280
885 TTTAATGATTACATGAAGCTCTATGCTGGTCTCAAGTTTCAGGCTAAAGAACCAAGGTTT
      F  N  D  Y  M  K  L  Y  A  G  L  K  F  Q  A  K  E  P  R  F  300
945 GAAGCTATGAAGGCCGTTTCAAGCGTTGATGTGGGGGCCATAGCTACAGTTTGAGCCTCA
      E  A  M  K  A  V  S  S  V  D  V  G  A  I  A  T  V                317
1005 TAAATTTATATATATAAGTATGGGAACGTATGTGTGTTTATGTTAATAATAAACAAAGGC
1065 TACTCAGCTCAGCGTATAGTGTTTGATGCCCTTGATAATTGGTATGGAAGCTTGTGCAGT
1125 ATAATCAAACCTTATATATATAATATTTATTAGTTAAAAAATAAAGTATTAGGTTGATCT
1185 TAGTTGGTGTGTGTGTGATCAGAGATGTCGTCTGTGATATGATGGTCAAGTTAATGCGAT
1245 GTAGTGATACACTGTTTTAGAAAGTAACCTCCTCTTGCCCATTTTACAAGAAAAAAAAAA
1305 AAAAAAAA    1312
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Fig. 6 (A) Restriction enzyme map analysis of two mung bean ACC oxidase cDNA clones. Solid bars indicate the coding regions. Solid bars indicate the coding regions.The positions of the hybridization probes generated PCR are presented by solid lines. While pVR-ACO1 is a full lenghth clone, pVR-ACO2 lacks a portion at 5'-end. (B) Nucleotide and deduced amino acid sequences of pVR-ACO1. The putative translational initiation, termination and polyadenylation signals are shown as bold letters. The PCR prmers used for the generation of gene specific probes 1 are underlined.

B

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   1                    CACAATCACATAGTTGTTTAAAGCGATAGAGAAGAAAGAAAGAA
  45 ATGGCAAACTTTCCAGTTGTTGACATGGGAAAGCTTAACACTGAAGAGAGAGGAACTGCC
      M  A  N  F  P  V  V  D  M  G  K  L  N  T  E  E  R  G  T  A  20
 105 ATGGAAATGATAAAAGATGCTTGTGAGAACTGGGGTTTCTTTGAGTTGGTGAACCATGGT
      M  E  M  I  K  D  A  C  E  N  W  G  F  F  E  L  V  N  H  G  40
 165 ATATCCATTGAGTTGATGGACACCGTGGAGAAGTTAACAAAAGAGCACTACAAGAAGACT
      I  S  I  E  L  M  D  T  V  E  K  L  T  K  E  H  Y  K  K  T  60
 225 ATGGAGCAAAGGTTCAAAGAAATGGTGGCCAACAAAGGTCTTGAGTCAGTTCAGTCAGAA
      M  E  Q  R  F  K  E  M  V  A  N  K  G  L  E  S  V  Q  S  E  80
 285 ATCAATGACTTGGACTGGGAAAGTACCTTTTTTCTGCGCCATCTTCCAGTCTCCAATGTT
      I  N  D  L  D  W  E  S  T  F  F  L  R  H  L  P  V  S  N  V  100
 345 TCAGAGAACACAGATCTTGATCAAGACTACAGGAAGATAATGAAGCAGTTTGCAGAAGAA
      S  E  N  T  D  L  D  Q  D  Y  R  K  I  M  K  Q  F  A  E  E  120
 405 CTGGAGAAACTTGCAGAGCATCTTCTTGACTTGCTGTGTGAGAATCTTGGACTGGAGAAA
      L  E  K  L  A  E  H  L  L  D  L  L  C  E  N  L  G  L  E  K  140
 465 GGGTACCTGAAGAAGGTGTTCTATGGATCGAAGGGCCCAAATTTTGGCACGAAAGTTAGC
      G  Y  L  K  K  V  F  Y  G  S  K  G  P  N  F  G  T  K  V  S  160
 525 AACTACCCTCCCTGTCCGACCCCTGATCTGATAAAGGGCCTAAGAGCTCACACTGATGCC
      N  Y  P  P  C  P  T  P  D  L  I  K  G  L  R  A  H  T  D  A  180
 585 GGTGGCATTATCCTACTGTTCCAAGATGACAAGGTCAGTGGACTGCAGCTCCTCAAGGAT
      G  G  I  I  L  L  F  Q  D  D  K  V  S  G  L  Q  L  L  K  D  200
 645 GACCAGTGGATCGATGTCCCACCAATGCGTCACTCCATTGTCATCAACCTTGGTGACCAA
      D  Q  W  I  D  V  P  P  M  R  H  S  I  V  I  N  L  G  D  Q  220
 705 CTTGAGGTCATAACCAATGGCAAGTACAAGAGTGTCATGCACCGAGTCATTGCTCAGACG
      L  E  V  I  T  N  G  K  Y  K  S  V  M  H  R  V  I  A  Q  T  240
 765 GATGGCACCAGAATGTCCCTGGCTTCCTTCTATAATCCCGGTGATGATGCTGTGATTTCT
      D  G  T  R  M  S  L  A  S  F  Y  N  P  G  D  D  A  V  I  S  260
 825 CCAGCACCAGCCTTGGTGAAGGAATCGGATGAAACAAGCCAAGTATACCCGAAATTTGTG
      P  A  P  A  L  V  K  E  S  D  E  T  S  Q  V  Y  P  K  F  V  280
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      F  N  D  Y  M  K  L  Y  A  G  L  K  F  Q  A  K  E  P  R  F  300
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      E  A  M  K  A  V  S  S  V  D  V  G  A  I  A  T  V              317
1005 TAAATTTATATATATAAGTATGGGAACGTATGTGTGTTTATGTTAATAATAAACAAAGGC
1065 TACTCAGCTCAGCGTATAGTGTTTGATGCCCTTGATAATTGGTATGGAAGCTTGTGCAGT
1125 ATAATCAAACCTTATATATATAATATTTATTAGTTAAAAAATAAAGTATTAGGTTGATCT
1185 TAGTTGGTGTGTGTGTGATCAGAGATGTCGTCTGTGATATGATGGTCAAGTTAATGCGAT
1245 GTAGTGATACACTGTTTTAGAAAGTAACCTCCTCTTGCCCATTTTACAAGAAAAAAAAAA
1305 AAAAAAAA   1312
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Fig. 7 Comparison of the deduced ACC oxidase amino acid sequences from mung bean (pVR-ACO1), tomato (Holdsworth et al., 1987), apple (Ross et al., 1992), avocado (McGarvey et al., 1990), carnation (Wang et., 1991) and pea (peck et al., 1993). -, gaps to align the amino acid sequences; *, amino acid residues identical among all six sequences.

(Kim and Yang, 1994). Figure 6A shows the restriction enzyme map analysis of pVR-ACO1 and pVR-ACO2 which contain the longest insert among each homology class. Among the 12 isolated clones, 10 belonged to the class of pVR-ACO1, and the others to pVR-ACO2. The pVR-ACO1 clone is 1312 bp long comprising a 44-bp 5′uncoding region, a 951-bp long coding region encoding 317 amino acids and a 317-bp 3′ uncoding region (Fig. 6). The 3′untranslated region of pVR-ACO1 is enriched for A-T nucleotides and contains two putative polyadenylation signals (AATAAA) (Fig. 6B). pVR-ACO2 is a partial cDNA clone consisting of a 924-bp long coding region (encoding 308 amino acids) and a 248-bp 3′untranslated region which is also highly enriched for A-T nucleotides and contains one potential polyadenylation signal (AATAAA). The nucleotide sequence homology between pVR-ACO1 and pVR-ACO2 is 81%. Since th-eir 3′uncoding regions are divergent, we selected these regions to construct gene specific probes by PCR (Fig. 6 and see below). The deduced amino acid sequences of pVR-ACO1 and pVR-ACO2 show 83% sequence identity, and the overall similarity is as high as 90%. The predicted molecular mass of the protein encoded by pVR-ACO1 is 35.8 kD which is similar to the molecular size of purified apple ACC oxidase polypeptide measured by SDS-PAGE (Dong et al., 1992a; Dupille et al., 1993). The predicted amino acid sequence of pVR-ACO1 shows 86%, 81%, 80%, 74%, and 73% identity to ACC oxidases from pea seedling (Peck et al., 1993), tomato fruit (Holdsworth et al., 1987), apple fruit (Ross et al., 1992; Dong et al., 1992b), avocado fruit (McGarvey et al., 1990) and carnation flower (Wang and Woodson, 1991), respectively. While sequence conservation is found throughout the internal coding regions among all six ACC oxidases, C-terminal regions are divergent (Fig. 7).

2. Expression of the ACC oxidase genes

In ripening fruit and senescent flower tissues, both ACC synthase and ACC oxidase are induced during the processes and contribute to the regulation of ethylene biosynthesis (Yang and Hoffman, 1984). Northern blot analysis also similarly demonstrated that expression of ACC synthase and ACC oxidase mRNAs are coordinatedly induced during fruit ripening (Holdsworth *et al.*, 1987; Dong *et al.*, 1992a; Ross et al., 1992; Theologis, 1992) or carnation flower senescence (Woodson et al., 1992), resulting in a surge in ethylene production . With the exception of preclimacteric fruits and flowers, it has been generally believed that ACC oxidase is constitutively present in plant tissues. In order to study the differential expression of pVR-ACO1 and pVR-ACO2 mRNAs in the mung bean

seedling, gene specific probes (probe 1 and 2 for each homology class) were prepared by PCR for our subsequent expression studies. The size of probes 1 and 2 was 350 bp and 260 bp, respectively (Fig. 6A). The expression of the ACC oxidase gene in different parts of mung bean plant was examined by Northern blot analysis. Total RNA, isolated from dark-grown 3-day-old hypocotyls of mung bean seedlings, or from leaves and stems of light-grown plants, were hybridized with 32P-labeled probe 1 or 2. Figure 8 shows that pVR-ACO1 transcript is detectable in all these tissues, while pVR-ACO2 transcript was barely detectable. The size of both transcripts was about 1.35 kb. The higher expression of pVR-ACO1 is not surprising because 10 out of 12 isolated cDNA clones belong to the pVR-ACO1 class, indicating that pVR-ACO1 mRNA is a major transcript expressed in mung bean seedling.

3. Induction and regulation of ACC oxidase mRNA accumulation in hypocotyls by excision and ethylene.

In order to study whether physical wounding of hypocotyl tissues may also cause an increased expression of ACC oxidase transcripts, the accumulation of pVR-ACO1 and pVR-ACO2 mRNAs during the course of incubation following excision was examined by Northern blotting. For wounding, hypocotyls were excised into 1-cm long segments and incubated for various time periods. A marked increase in the pVR-ACO1 mRNA level was observed at 3 h, and the level reached a maximum after 6 h, and then declined slowly (Fig. 8). The induction pattern of pVR-ACO2 mRNA was similar to that of pVR-ACO1 transcript except that its relative abundance was much lower than that of pVR-ACO1 transcript (Fig. 8).

The temporal induction of the wound-induced ACC oxidase gene expression following excision was also compared to that of extractable ACC oxidase enzyme activities. The enzyme activity increased from 13 $nlmg^{-1}h^{-1}$ at time zero to a maximum activity of 34 $nlmg^{-1}h^{-1}$ at 24 h, and then declined (Fig. 8). The change of the *in vivo* enzyme activity was similar to that of the *in vitro* activity (data not shown). Thus, the increase in ACC oxidase enzyme activity during the incubation following excision exhibited a different pattern from that of the mRNA transcript. The level of the transcript reached a maximum at 6 h after excision when the enzyme activity was still rising (Fig. 8). Furthermore, ACC oxidase enzyme activity increases only moderately during the entire 36-h incubation period in spite of a large accumulation of its transcript in a short period (Fig. 8). These results indicate that the low basal level of ACC oxidase transcript in the intact tissue is sufficient to maintain a moderate level of ACC oxidase activity. Furthermore, since the

magnitude and temporal induction of ACC oxidase transcript are not in parallel with those of enzyme activity, we may conclude that ACC oxidase gene expression after wounding is subject not only to transcriptional control but also to other factors such as posttranscriptional/translational control and/or enzyme turnover.

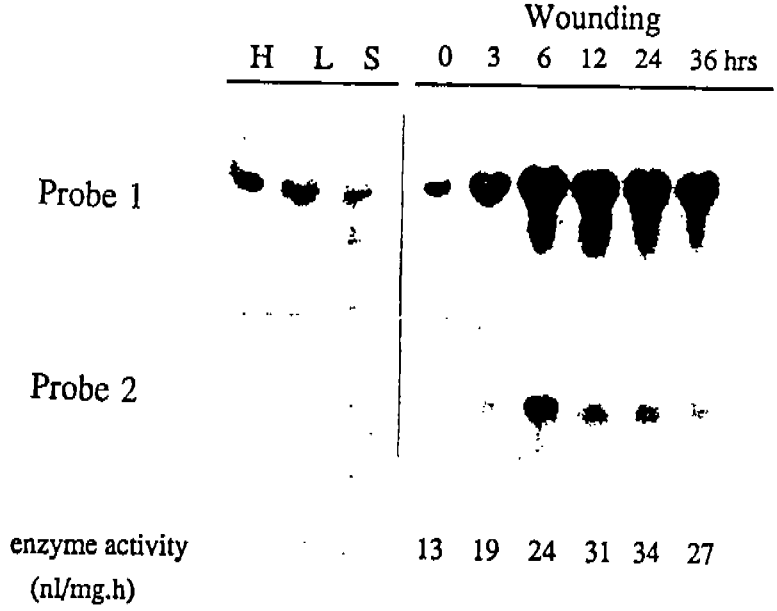

Fig. 8 Northern blot analyses of RNAs (20 μg) isolated from differents parts of mung bean seedlings (left) or from pypocotyls following excision and incubation for various periods (right). H, hyplcotyls; L, leaves; S, stems. Blots were hybridized to ^{32}P-labeled probe for pVR-ACO1 (probe 1) or pVR-ACO2 (probe 2); X-ray films were exposed overnight. ACC oxidase enzyme activities estracted from the same excised hypocotyl samplese were assayed (bottom).

The ability of exogeneous ethylene or wound-induced ethylene to stimulate the *in vivo* conversion of ACC to ethylene has been observed in wounded preclimacteric cantaloupe fruits (Hoffman and Yang, 1982), and citrus leaf discs (Riov and Yang, 1982). Most recently, Hyodo et al. (1993) have shown that the wound-induced ethylene acts to promote the induction of *in vitro* ACC oxidase activity in excised winter squash fruits. In order to assess the possible role of ethylene on wound-induced ACC oxidase mRNA in vegetative tissues, excised hypocotyls were incubated with exogeneous ethylene or in the presence of AOA, an inhibitor of ACC synthase activity (Yu *et al.*, 1979), or NBD, an ethylene action inhibitor (Sisler and Yang, 1984). Total RNAs were then extracted from these tissues and the relative

abundance of ACC oxidase transcript was compared by Northern blot analysis. As shown in Fig. 9, ethylene treatment further enhances the transcript level, while AOA or NBD treatment blocks the accumulation of wound-induced pVR-ACO1 mRNA. Furthermore, the addition of ethylene to the AOA- or NBD-treated hypocotyls restores the pVR-ACO1 mRNA accumulation (Fig. 9). The induction pattern of pVR-ACO2 mRNA was similar to that of pVR-ACO1 transcript except that relative abundance was lower (data not shown). These results indicate that the expression of the ACC oxidase gene in response to wounding is mediated by ethylene. It should be noted that the basal ethylene production rates of seedlings from various species are low and that excision causes only several-fold increase in ethylene synthesis (Saltveit and Dilley, 1978). Thus, the wound-induced accumulation of ACC oxidase mRNA must be responsive to a low level of ethylene.

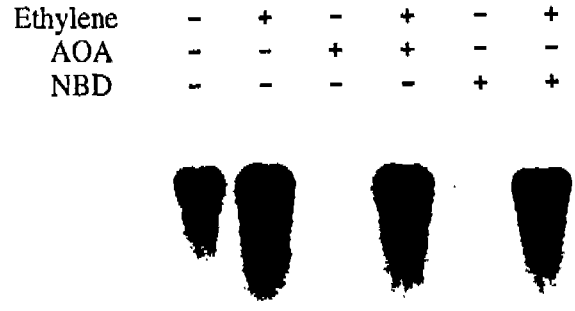

Fig. 9 Effect of ethylene, AOA and NBD on the wound-induced ACC oxidase mRNA level. Total RNAs (20 μg), isolated from excised hypocotyls which had veen incubated for 7 h without or with c_2H_4 (50 μl/l), AoA (100 μM), NBD (5000 μl/l), or combinations as indicated, were analyzed by Nothern blotting using ^{32}p-labeled probe 1.

When intact preclimacteric apple and tomato fruits, or presenescent carnation petals which produce very little ethylene were treated with exogeneous ethylene, ACC oxidase enzyme activity as well as its mRNA level markedly increased depending on the duration of the ethylene treatment (Liu *et al.*, 1985; Dong *et al.*, 1992b; Ross *et al.*, 1992; Woodson *et al.*, 1992; Drory *et al.*, 1993), indicating that ethylene stimulates the ACC oxidase gene expression not only in wounded but also in intact fruit tissues. In order to study if ethylene is also able to induce the accumulation of ACC oxidase mRNA in intact vegetative tissues, 3-day-old, dark-grown mung bean seedling were incubated with air or air containing 50 ml/l ethylene for 7 h. Total RNAs were then extracted from the hypocotyls and similarly analyzed. As shown in Fig. 10, applied C2H4 caused a marked increase in ACC

oxidase mRNA level, while *in vivo* enzyme activity increased only moderately. These results indicate that ethylene activates the expression of ACC oxidase genes not only in wounded but also in intact mung bean hypocotyls. Since a low level of ethylene is capable of inducing the expression of ACC oxidase transcript, it is most likely that the low, basal level of ACC oxidase transcript present in intact mung bean hypocotyls is under the control of their endogeneous ethylene.

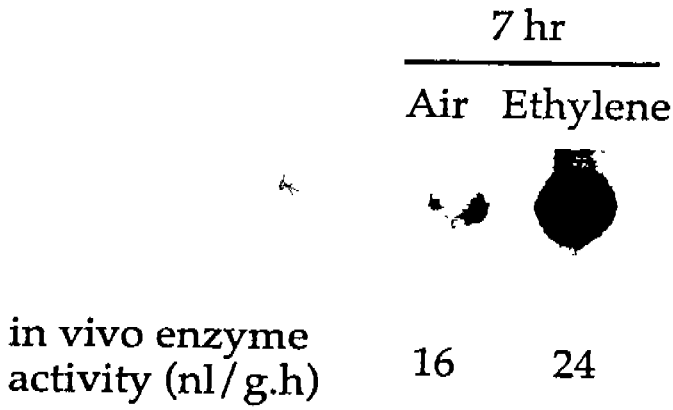

Fig. 10 Effect of exogeneors ethtylene on the accumulation of ACC oxidase transcript in the intact mung bean seedling. Three-day-old seedlings were enclosed in 4-liter jars containing air or air with 50 μl/l ethylene. After 7 h treatment pypocotyls were excised, and used for total RNA extratment hypocotyls blot analysis as described in Fig. 9. For *in vivo* ACC oxidase assay, 5 g hypocotyls described above were incubated in a medium containing 2 mM ACC, and the ethylene produced during the 1-h incubation perion period was taken as the *in vivo* ACC oxidase activity.

4.Effect of methyljasmonate (MJ) on the wound-induced ACC oxidase mRNA expression.

Recently, it has been proposed that jasmonic acid (JA) or its methyl ester may serve as a signal transducer in wound-induced plant responses. This hypothesis is based on the observation that an exogeneous application of JA stimulates several wound-inducible genes (Farmer and Ryan, 1992; Hildman *et al.*, 1992), and that a rapid increase in endogeneous JA level results from wounding (Creelman *et al.*, 1992). While Chou and Kao (1992) reported that MJ (0.45-450 mM) is capable of stimulating the ACC-dependent ethylene synthesis in detached rice leaves, Bailly *et al.* (1992) observed that MJ (10 mM-1 mM) inhibits the ACC-dependent ethylene production in sunflower seedlings. To study the possible involvement of MJ in wound-induced ACC oxidase gene expression, we incubated excised hypocotyls for 6

h in the presence of various concentrations of MJ, and isolated the total RNAs for Northern blot analysis. While 5 mM MJ significantly decreases the wound-induced pVR-ACO1 transcript, 125 mM MJ abolishes totally the accumulation of the transcript (Fig. 11). The mode of action of MJ in inhibiting the expression of the ACC oxidase transcript is not known.

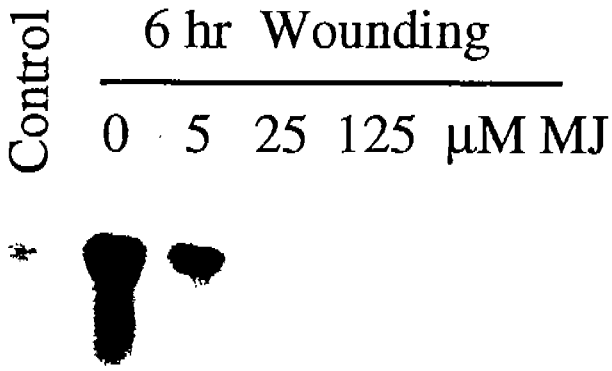

Fig. 11 Effect of MJ on the ACC oxidase gene expression. Total RNAs isolated from excised mung bean hypocotyls without incubation (control) or incubated for 6 h with various concentrations (0, 5, 25, 125 μM) of MJ were analyzed by bortherm blotting using ^{32}p-labeled probe 1.

Our present results indicate that the ACC oxidase transcript is constitutively present in all parts of mung bean seedlings, but its level can be further increased by wounding (excision). We also demonstrate that the accumulation of wound-induced ACC oxidase mRNAs in excised mung bean hypocotyls is regulated by ethylene. Likewise, ethylene also caused a marked enhancement of the ACC oxidase mRNA level in intact seedling. Thus, ethylene plays a key role in regulating the ACC oxidase gene expression in mung bean hypocotyls as in fruit tissues. These results also indicate that the low, basal level of ACC oxidase transcript constitutively expressed in intact hypocotyls is regulated by the endogeneous ethylene present in these tissues. However, in preclimacteric fruit tissues, both ACC oxidase transcript and enzyme activity are undetectable, but are markely induced to a comparable extent following an application of exogeneous ethylene (Dong *et al.* 1992a; Ross et al. 1992), indicating that ethylene-dependent expression of ACC oxidase gene is regulated at the transcriptional level. In contrast, in mung bean hypocotyls both ACC oxidase transcript and enzyme activity are constitutively present. Although the level of ACC oxidase transcript increased greatly following

excision or an application of exogeneous ethylene, the increase in enzyme activity was relatively small, indicating that the regulatory mechanism of the expression of ACC oxidase in mung bean hypocotyls is complex. In addition to transcriptional control, other factors such as posttranscriptional/translational control and/or the stability of the enzyme protein may also be involved.

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