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Structure and expression of cDNAs encoding 1-aminocyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls

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Abstract. By screening a mung bean (*Vigna radiata* L.) hypocotyl cDNA library using a combination of apple (pAE12) and tomato (pTOM13) 1-aminocyclopropane1-carboxylate (ACC)-oxidase cDNAs as probes, putative ACC-oxidase clones were isolated. Based on restriction-enzyme map and DNA-sequencing analyses, they can be divided into two homology classes, represented by pVR-ACO1 and pVR-ACO2. While pVR-ACO1 and pVR-ACO2 exhibit close homology in their coding regions, their 3'-noncoding regions are divergent. pVR-ACO1 is a 1312-bp full-length clone and contains a single open reading frame encoding 317 amino acids (MW = 35.8 kDa), while pVR-ACO2 is 1172 bp long and is a partial cDNA clone encoding 308 amino acids. These two deduced amino-acid sequences share 83% identity, and display considerable sequence conservation (73–86%) to other ACC oxidases from various plant species. Northern blot analyses of RNAs isolated from hypocotyl, leaf, and stem tissues using gene-specific probes indicate that the pVR-ACO1 transcript is present in all parts of the seedling and that the expression in hypocotyls is further increased following excision. The maximum induction of ACC-oxidase transcripts occurred at about 6 h after excision, while the maximum enzyme activity was observed at 24 h. When excised hypocotyls were treated with ethylene a further enhanced level of transcripts was observed. Aminooxyacetic acid, an inhibitor of ACC-synthase activity, and 2,5-norbornadiene, an inhibitor of ethylene action, suppressed the wound-induced accumulation of ACC-oxidase mRNA, while an addition of ethylene in these tissues restored the accumulation of ACC-oxidase mRNA. These results indicate that the wound-induced expression of ACC-oxidase transcripts is mediated through wound-induced ethylene. Furthermore, when intact mung-bean seedlings were treated with exogenous ethylene, a marked increase in the level of ACC-oxidase mRNA was observed. Together, these results indicate that ethylene plays a key role in activating the expression of the ACC-oxidase gene in both intact and excised mung-bean hypocotyls.

Key words: 1-Aminocyclopropane-1-carboxylate oxidase – Ethylene – Gene expression – Hypocotyl excision – *Vigna* – Wounding

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 tissue is usually low, but is greatly induced at certain stages of plant development, such as seed germination, leaf abscision, and fruit ripening; by auxin treatment; or in response to a wide range of external stimuli including wounding, anaerobiosis, drought, flooding or pathogen invasion (Yang and Hoffman 1984; Theologis 1992; Kende 1993). The biosynthetic pathway of ethylene in higher plants is well established as follows: methionine → S-adenosyl-L-methionine → ACC → C_2H_4 (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).

Based on the observation that transgenic tomato plants expressing antisense copies of pTOM13 exhibit reduced ACC-oxidase activity and ethylene production in ripening fruit, it was first suggested that the pTOM13 gene was related to ACC oxidase (Hamilton et al. 1990). Subsequent work confirmed that pTOM13 or its homolog indeed conferred ACC-oxidase activity when ex-

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Abbreviations: ACC = 1-aminocyclopropane-1-carboxylate; AOA = aminooxyacetic acid; MJ = methyl jasmonate; NBD = 2,5-norbornadiene; PCR = polymerase chain reaction

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pressed 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 seedlings (Peck et al. 1993). These clones share about 85% identity at the amino-acid level to the tomato ACC-oxidase clone, and the levels 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. In this report we describe the isolation and characterization of two putative ACC-oxidase cDNAs from mung-bean hypocotyls and show that the transcripts are constitutively present but can be further induced by wounding. We have also shown that ethylene induces a large increase in ACC-oxidase gene expression not only in wounded (excised) hypocotyls but also in intact mung-bean seedlings.

Materials and methods

Plant material. Dry seeds of mung bean (*Vigna radiata* L. cv. Berken) were germinated and grown in vermiculite for 3 d in darkness at 25°C. For wounding, batches (5 g each) of 1-cm-long hypocotyl segments were cut at 1–2 cm below the hook and incubated in 20 ml of a medium consisting of 2% (w/v) sucrose, 1 mM $CaCl_2$, and 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes) buffer (pH 6.2) in Erlenmeyer flasks for various time periods with continuous shaking. Where indicated, addenda were 50 µl/l ethylene (in the gas phase), 100 µM aminooxyacetic acid (AOA), 5000 µl/l 2,5-norbornadiene (NBD; in the gas phase), or various concentrations of methyl jasmonate (MJ). At the end of incubation, the tissues were immediately frozen in liquid nitrogen and stored at −80°C until use.

Extraction and assay of ACC oxidase. Hypocotyl tissues were pulverized in liquid nitrogen and homogenized in 1 ml/g of extraction medium consisting of 200 mM 3-(N-morpholino)propanesulfonic acid (Mops; pH 7.3), 5 mM sodium ascorbate and 30% glycerol. After centrifugation at 28 000·*g* for 20 min, the supernatant (crude extract) was used for enzyme assays. Enzyme activity was assayed at 30°C in a capped 15-ml tube containing 1 ml reaction mixture which consisted of 100 mM Mops (pH 7.3), 50 µM $FeSO_4$, 1 mM sodium ascorbate, 5% CO_2 (in the gas phase), 10% (v/v) glycerol, 1 mM ACC and 50 µl crude extract. Ethylene which accumulated in the head space during 1 h incubation was determined by gas chromatography (Fernandez-Maculet and Yang 1992). For assay of ACC oxidase in vivo, 5 g of mung-bean hypocotyl was incubated in 5 ml of a medium containing 2% (v/v) sucrose, 1 mM $CaCl_2$, 50 mM Mes (pH 6.2) and 2 mM ACC for 1 h, and the ethylene produced was measured (Fernandez-Maculet and Yang 1992).

Isolation of RNA. Total RNAs of mung-bean hypocotyls, leaves and stems were obtained by a method adapted from the established protocols reviewed by Lizzardi (1983) with modifications as described previously (Kim et al. 1992). Polyadenylated RNA was obtained by two cycles of oligo (dT)-cellulose chromatography.

Construction and screening of a cDNA library. The preparation of double-stranded DNA complementary to poly(A)$^+$ RNA from auxin-treated mung-bean hypocotyls (Kim et al. 1992) and its ligation to Lambda Uni-Zap arms were performed according to the protocol of Stratagene (La Jolla, Calif., USA). The cDNA library was screened, using the combination of radiolabeled cDNA inserts encoding apple (Dong et al. 1992b) and tomato (Holdsworth et al. 1987) fruit ACC oxidases as probes, by an established procedure (Maniatis et al. 1983).

Sequencing of DNA. The cDNA inserts containing putative mung-bean ACC oxidase were subcloned into Bluesript SK plasmids by in-vivo excision of pBluescript from Uni-Zap vector as described in the protocol by Stratagene. Sequencing of DNA was performed using the Sequenase DNA sequencing kit according to the manufacturer's manual (US Biochemical, Cleveland, Ohio, USA). Sequence analysis was carried out using PC-GENE computer software.

Polymerase chain reaction (PCR). The DNA sequences corresponding to probes 1 and 2, as shown in Fig. 1, were amplified by PCR from purified plasmids pVR-ACO1 and pVR-ACO2, employing oligonucleotides corresponding to nucleotides 942–961 and 1268–1288 of pVR-ACO1 or nucleotides 883–902 and 1120–1140 of pVR-ACO2 as the upstream and the downstream primers, respectively. The PCR was performed in a total volume of 50 µl containing 1 ng of purified plasmid, 1 µM primers, 10 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM deoxynucleotides, and 2.5 units of Taq polymerase (Perkin-Elmer/Cetus, Norwalk, Conn., USA). Thirty-five thermal cycles were carried out, each consisting of 1 min at 94°C, 2 min at 53°C, and 2 min at 72°C in an automatic thermal cycler (Perkin-Elmer/Cetus). The 350-bp and 260-bp PCR products corresponding to probes 1 and 2, respectively, were separated on a 1.2% agarose gel, eluted, extracted with phenol/chloroform and precipitated with ethanol.

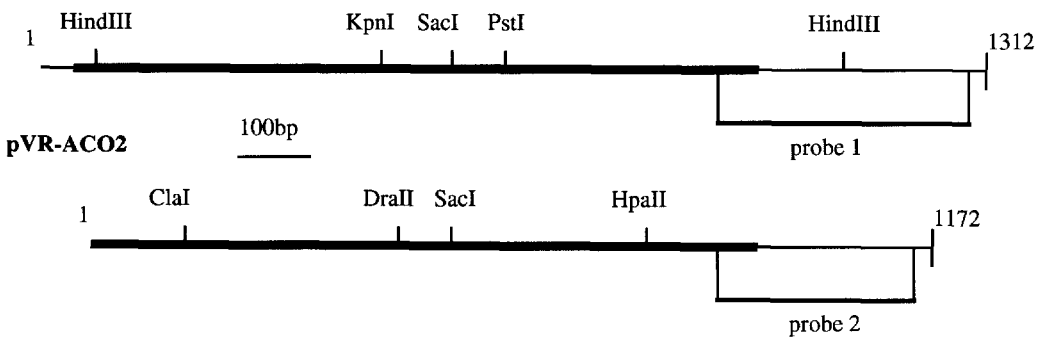
Northern blot hybridization. For Northern blot analysis, total RNA (20 µg) was separated by electrophoresis on a 1.2% formaldehyde-agarose gel, blotted to a Zeta-Probe membrane filter (Bio-Rad, Richmond, Calif., USA), and hybridized to ^{32}P-labeled gene-specific probes as described previously (Kim et al. 1992). The blots were visualized by autoradiography at −80°C using Kodak XAR-5 film and intensifying screen (Crone DuPont).

Results and discussion

Isolation and classification of ACC-oxidase cDNA clones. A mung-bean hypocotyl cDNA library comprising $5 \cdot 10^5$ individual recombinant plaques 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. Restriction-enzyme digests of DNAs isolated from these clones indicated that they contained inserts of about 0.9–1.35 kb. Subsequent restriction-enzyme mapping and analysis by agarose gel electrophoresis revealed that these clones could be divided into two homology classes. Figure 1A 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. While pVR-ACO1 contains restriction sites for *Kpn*I, *Sac*I, *Pst*I and *Hind*III, pVR-ACO2 has a *Sac*I site, but lacks sites for *Kpn*I, *Pst*I and *Hind*III.

Structure and primary sequence of mung-bean ACC-oxidase cDNAs. The pVR-ACO1 clone is 1312 bp long com-

A



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. 1. A Restriction-enzyme map analysis of two mung-bean ACC-oxidase cDNA clones. *Solid bars* indicate the coding regions. The positions of the hybridization probes generated by PCR are presented by *solid lines*. While pVR-ACO1 is a full-length clone, pVR-ACO2 lacks a portion at the 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 primers used for the generation of gene-specific probes 1 are *underlined*. The sequence of pVR-ACO2 has been deposited in the GenBank data base, accession number U06047

prising a 44-bp 5'-uncoding region, a 951-bp-long coding region encoding 317 amino acids and a 317-bp 3'-uncoding region (Fig. 1). The 3'-untranslated region of pVR-ACO1 is enriched for A-T nucleotides and contains two putative polyadenylation signals (AATAAA) (Fig. 1B). 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 sequence was deposited in GenBank, accession number U06047.) The nucleotide sequence homology between pVR-ACO1 and pVR-ACO2 is 81%. Since their 3'-uncoding regions are divergent, we selected these regions to construct gene-

specific probes by PCR (Fig. 1 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 kDa 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 seedlings (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 flowers (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.

Based on the observation that intact protoplasts and vacuoles possess functional ACC-oxidase activity, but the activity is lost upon rupture of plasma and vacuolar membranes, it was previously proposed that ACC oxidase is associated with integral membrane structure (Guy and Kende 1984; Yang and Hoffman 1984). Recent work from various laboratories has, however, shown that ACC oxidase can be isolated in soluble form (Ververidis and John 1991; Kuai and Dilley 1992; Fernandez-Maculet and Yang 1992; McGarvy and Christoffersen 1992). As with other ACC-oxidase cDNAs, the predicted polypeptides encoded by pVR-ACO1 and pVR-ACO2 do not contain any significant stretches of hydrophobic regions, nor membrane-associated helix, suggesting that mung-bean ACC oxidase is likely to be located in the cytoplasmic fraction.

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. This view is based on the observation that application of exogenous ACC resulted in a marked increase in ethylene production in organs from a number of plant species (Cameron et al. 1979; Yang and Hoffman 1984). Recent molecular studies by Holdsworth et al. (1987) and Hamilton et al. (1990), however, have shown that the level of pTOM13-hybridizable ACC-oxidase mRNA in tomato leaves was undetectable but increased markedly following wounding. In order to study the differential expression of pVR-ACO1 and pVR-ACO2 mRNAs in the mung-bean seedling, gene-specific probes (probes 1 and 2 for each homology class) were prepared by PCR for our subsequent expression studies. The sizes of probes 1 and 2 were 350 bp and 260 bp, respectively (Fig. 1A). The extent of cross-hybridization of probes was examined by dot-blot analysis. Probe 1 hybridized strongly to its own DNA, pVR-ACO1, but hardly to pVR-ACO2, indicating that probe 1 is highly specific for pVR-ACO1. When probe 2 was used, a weak cross-hybridization to pVR-ACO1 and a strong hybridization to its own DNA, pVR-ACO2, were observed. Results using serially diluted plasmid indicated that hybridization of probe 2 to pVR-ACO1 was about 1/6 of that to its own plasmid DNA (data not shown).

The expression of the ACC-oxidase gene in different parts of mung-bean plants was examined by Northern blot analysis. Total RNA, isolated from dark-grown 3-d-old hypocotyls of mung-bean seedlings, or from leaves and stems of light-grown plants, were hybridized with ^{32}P-labeled probe 1 or 2. Figure 2 shows that the pVR-ACO1 transcript was detectable in all these tissues, while the 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 seedlings. It should be noted that the application of exogenous ACC results in increased ethylene production not only in excised plant tissues (Cameron et al. 1979), but also in intact plants (Grodzinski et al. 1982; Bassi and Spence 1983; Fuhrer 1985; Vangronsveld et al. 1988). Hence, ACC oxidase must also be present in intact plant tissues. Our present results on the expression of ACC oxidase transcripts are, therefore, in agreement with these in-vivo observations, but are at variance with those of Holdsworth et al. (1987) and Hamilton et al. (1990) who observed that ACC-oxidase (pTOM13) mRNA in tomato leaves was not detectable unless they were mechanically wounded. This discrepancy could be reconciled by the interpretation that the level of pTOM13-hydridizable mRNA in intact tomato leaves is too low to be detected by blot-hybridization analyses, or that there are other ACC-oxidase transcripts present in tomato leaves which are not highly homologous to fruit-originated pTOM13, and hence cannot be detected.

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. 2). The induction pattern of pVR-ACO2 mRNA was similar to that of the pVR-ACO1 transcript, except that its relative abundance was much lower (Fig. 2).

The temporal induction of the wound-induced ACC-oxidase gene expression following excision was also compared to that of extractable ACC-oxidase enzyme activi-

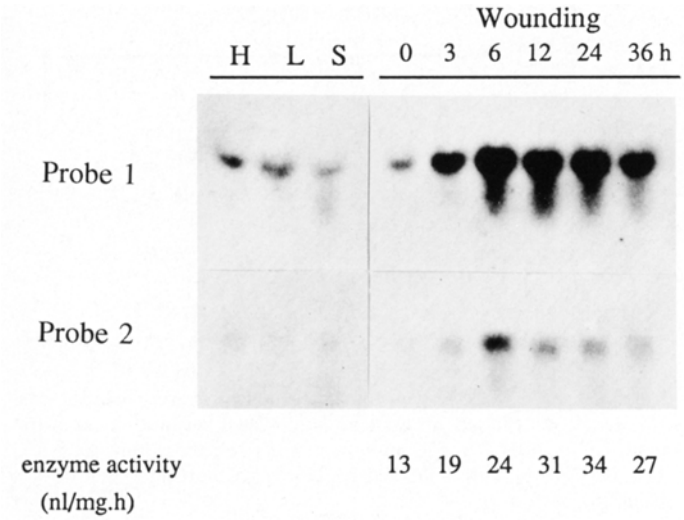

Fig. 2. Northern blot analyses of RNAs (20 μg) isolated from different parts of mung bean seedlings (*left*) or from hypocotyls following excision and incubation for various periods (*right*). *H*, hypocotyls; *L*, leaves; *S*, stems. Blots were hybridized to ^{32}P-labeled probe for pVR-ACO1 (probe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

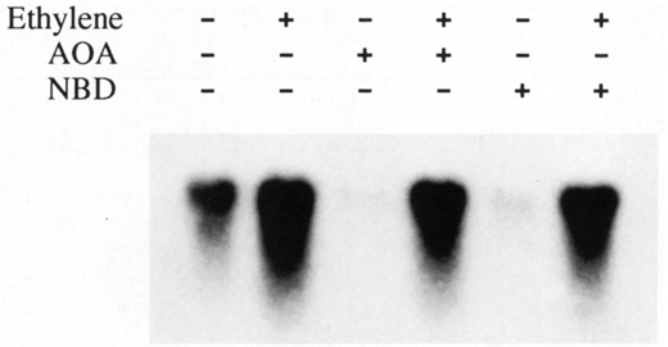

Fig. 3. Effect of ethylene, AOA and NBD on the level of wound-induced ACC-oxidase mRNA. Total RNAs (20 μg), isolated from excised hypocotyls which had been incubated for 7 h without or with C_2H_4 (50 μl/l), AOA (100 μM), NBD (5000 μl/l), or combinations as indicated, were analyzed by Northern blotting using ^{32}P-labeled probe 1

ties. The enzyme activity increased from 13 nl·mg^{-1}·h^{-1} at time zero to a maximum activity of 34 nl·mg^{-1}·h^{-1} at 24 h, and then declined (Fig. 2). The change of enzyme activity in vivo was similar to that in vitro (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 transcripts. Transcript levels reached a maximum at 6 h after excision when the enzyme activity was still rising (Fig. 2). Furthermore, ACC-oxidase enzyme activity increased only moderately during the entire 36-h incubation period in spite of a large accumulation of its transcripts in a short period (Fig. 2). These results indicate that the low basal level of ACC-oxidase transcripts 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 transcripts 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.

The ability of exogenous 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 exogenous 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 transcripts was compared by Northern blot analysis. As shown in Fig. 3, ethylene treatment further enhanced the transcript level, while AOA or NBD treatment blocked the accumulation of wound-induced pVR-ACO1 mRNA. Furthermore, the addition of ethylene to the AOA- or NBD-treated hypocotyls restored the accumulation of pVR-ACO1 mRNA (Fig. 3). The induction pattern of the pVR-ACO2 mRNA was similar to that of the pVR-ACO1 transcript except that its 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 rates of ethylene production by seedlings of various species are low and that excision causes only a 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.

It has been well documented that auxin markedly stimulates ethylene production by promoting ACC-synthase activity and its mRNA level (Yang and Hoffman 1984; Kende 1993). When excised mung-bean hypocotyls were incubated with 500 μM indole-3-acetic acid + 100 μM N^6-benzyladenine for 5 h, a higher level of pVR-ACO1 transcript was observed as compared to the basal level caused by excision alone (data not shown). Since wounding + auxin had a similar effect on the level of ACC-oxidase mRNA as wounding + C_2H_4, we conclude that auxin exerted its promotive effect via increased ethylene production.

When intact preclimacteric apple and tomato fruits, or presenescent carnation petals which produce very little ethylene, were treated with exogenous 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 expression of the ACC-oxidase gene not only in wounded but also in intact fruit tissues. In order to study if ethylene is also able to

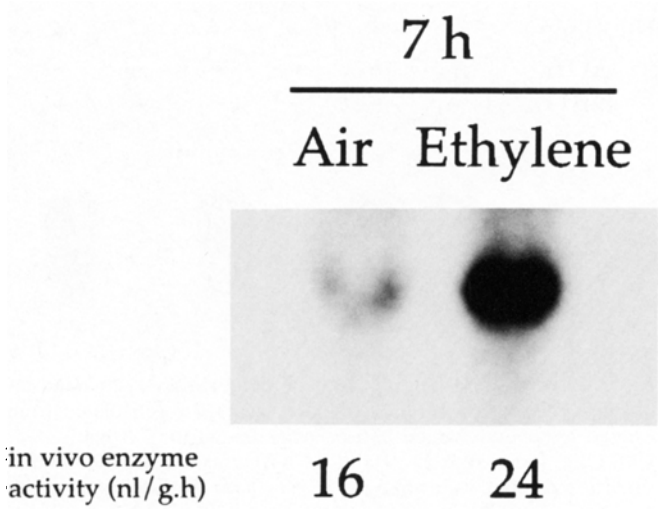

Fig. 4. Effect of exogenous ethylene on the accumulation of ACC-oxidase transcripts in the intact mung-bean seedling. Three-day-old seedlings were enclosed in 4-l jars containing air or air with 50 µl/l ethylene. After 7 h treatment, hypocotyls were excised, and used for total RNA extraction and Northern blot analysis as described in Fig. 3. For assay of in-vivo ACC oxidase, 5 g of the hypocotyls described above were incubated in a medium containing 2 mM ACC, and the ethylene produced during the 1-h incubation period was taken as the in-vivo ACC-oxidase activity

induce the accumulation of ACC-oxidase mRNA in intact vegetative tissues, 3-d-old, dark-grown mung-bean seedlings were incubated in air or in air containing 50 µl/l ethylene for 7 h. Total RNAs were then extracted from the hypocotyls and similarly analyzed. As shown in Fig. 4, applied C_2H_4 caused a marked increase in the level of ACC-oxidase mRNA, 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 transcripts, 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 endogenous ethylene.

Effect of MJ on the wound-induced expression of ACC-oxidase mRNA. Recently, it has been proposed that jasmonic acid (JA) or its methyl ester (MJ) may serve as a signal transducer in wound-induced plant responses. This hypothesis is based on the observation that an exogenous application of JA stimulates several wound-inducible genes (Farmer and Ryan 1992; Hildman et al. 1992), and that a rapid increase in the level of endogenous JA results from wounding (Creelman et al. 1992). While Chou and Kao (1992) reported that MJ (0.45–450 µM) is capable of stimulating the ACC-dependent synthesis of ethylene in detached rice leaves, Bailly et al. (1992) observed that MJ (10 µM–1 mM) inhibits the ACC-dependent production of ethylene 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 µM MJ substantially decreased the wound-induced pVR-ACO1 transcript,

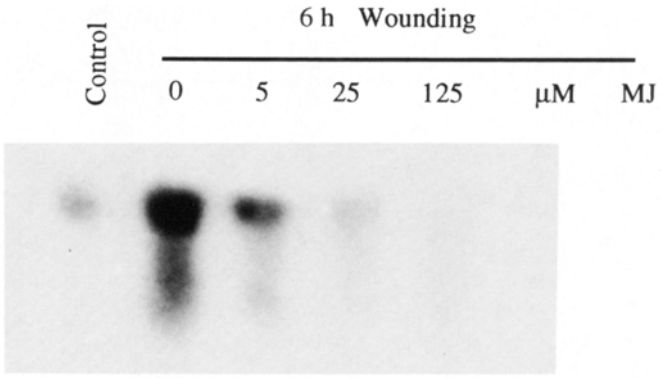

Fig. 5. Effect of MJ on ACC-oxidase gene expression. Total RNAs isolated from excised mung-bean hypocotyls without incubation (control) or incubated for 6 h with various concentrations (0, 5, 25, 125 µM) of MJ were analyzed by Northern blotting using ^{32}P-labeled probe 1

125 µM MJ totally abolished its accumulation (Fig. 5). The mode of action of MJ in inhibiting the expression of the ACC-oxidase transcript is not known.

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 level of ACC-oxidase mRNA in intact seedlings. Thus, ethylene plays a key role in regulating ACC-oxidase gene expression in mung-bean hypocotyls, as it does in fruit tissues. These results also indicate that the low, basal level of the ACC-oxidase transcript constitutively expressed in intact hypocotyls is regulated by the endogenous ethylene present in these tissues. However, in preclimacteric fruit tissues, both the ACC-oxidase transcript and enzyme activity are undetectable, but are markely induced to a comparable extent following an application of exogenous ethylene (Dong et al. 1992a; Ross et al. 1992), indicating that ethylene-dependent expression of the ACC-oxidase gene is regulated at the transcriptional level. In contrast, in mung-bean hypocotyls, both the ACC-oxidase transcript and enzyme activity are constitutively present. Although the level of the ACC-oxidase transcript increased greatly following excision or an application of exogenous 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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