

Structure and Ethylene-Induced Expression of the 1-Aminocyclopropane-1-Carboxylate Oxidase Gene in Mung Bean (*Vigna radiata* L.)

Daeseon Park, Jae Hoon Lee, Sunjoo Joo, and Woo Taek Kim*

Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea

Previously we isolated two closely related 1-aminocyclopropane-1-carboxylate (ACC) oxidase cDNAs, pVR-ACO1, and pVR-ACO2, from mung bean seedlings. Ethylene caused a marked increase in the accumulation of VR-ACO1 transcript, whereas the ethylene-induction of VR-ACO2 was much lower in the hypocotyl and root tissues. In the current study, genomic Southern blot analysis confirmed the existence of two ACC oxidase genes in the mung bean genome. To gain information concerning the structure of the ethylene-responsive VR-ACO1 gene as well as to investigate its promoter sequence, we also obtained and sequenced the genomic clone encoding VR-ACO1. The VR-ACO1 gene consists of four exons interrupted by three introns whose junctions are in agreement with the consensus intron/exon borders of plant genes. Individual sizes of the three introns are 100, 413, and 205 bp. The coding region of VR-ACO1 comprises 951 bp encoding a protein of 317 amino acid residues, with a predicted molecular mass of 36 kDa. The major start site of transcription is located 66 nucleotides upstream from the 5'-end of the coding region of the VR-ACO1 gene; the putative TATA box is found at the 27th nucleotide upstream from this start site. The nuclear run-on transcription assay indicated that ethylene induces the expression of VR-ACO1 at the transcriptional level in mung bean hypocotyls. Comparing the sequence of the 5'-flanking region of VR-ACO1 with known ethylene responsive elements (EREs) revealed two copies of sequence (TTCAA) that displays a high degree of identity with the primary ethylene responsive element (PERE) found in the *GST1*, *E4*, and *ERF1* genes. In addition, the promoter contains three octanucleotide sequence motifs reminiscent of the EIN3 binding site that exists in *Arabidopsis* *ERF1* and tobacco *ERF2*. We also discuss the possible molecular mechanism of regulation of ACC oxidase gene expression by ethylene in mung bean seedlings.

Keywords: ACC oxidase, ethylene, gene expression, mung bean hypocotyls, promoter, *Vigna radiata*

The gaseous plant hormone ethylene regulates diverse physiological processes and induces specific changes in gene expression during plant growth and development. The rate of ethylene production is usually low in most plant tissues, but increases markedly at certain developmental stages, including seed germination, leaf senescence and abscission, and fruit ripening (Yang and Hoffman, 1984; Abeles et al., 1992). Ethylene production is also enhanced in response to various biotic and abiotic stresses, such as drought, flooding, anaerobiosis, wounding, pathogen attack, or auxin treatment (Yang and Hoffman, 1984; Theologis, 1992).

In higher plants, ethylene is synthesized from methionine via S-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylate (Met → AdoMet → ACC → C₂H₄) (Yang and Hoffman, 1984; Theologis, 1992;

Kende, 1993). ACC synthase and ACC oxidase catalyze the last two steps of this biosynthetic pathway, respectively. In fruit tissue, these two unique enzymes are induced during ripening, and contribute to the regulatory steps for ethylene production. In vegetative tissues, however, ACC oxidase is constitutively expressed so that ACC synthase is regarded as the rate-limiting step for ethylene biosynthesis (Yang and Hoffman, 1984; Theologis, 1992). ACC synthase is encoded by a divergent multigene family. The expression of each member of that family is modulated differentially by developmental, hormonal, and environmental cues (Theologis, 1992; Kende, 1993; Kende and Zeevaart, 1997).

Since the first ACC oxidase cDNA pTOM13 was identified in tomato (Hamilton et al., 1990), numerous ACC oxidase cDNA clones have been isolated from a variety of plant species (see Kende, 1993 and Kende and Zeevaart, 1997). As with ACC synthase, ACC oxidase is encoded by a gene family (Kende, 1993; Kende and Zeevaart, 1997). Recent molecular studies have shown that the ACC oxidase gene family

*Corresponding author; fax +82-2-312-5657

e-mail wtkim@yonsei.ac.kr

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is differentially expressed in different organs, and at distinct developmental and physical conditions (Kende and Zeevaert, 1997). In tomato and melon plants, three ACC oxidase genes have been identified (Barry et al., 1996; Lasserre et al., 1996). The expression of each gene family member is spatially and developmentally regulated in wounded and ethylene-treated leaves, during flower and leaf senescence, in the fruit-ripening process, and in response to pathogen attack (Barry et al., 1996; Lasserre et al., 1996, 1997; Blume and Grierson, 1997; Bouquin et al., 1997). In the process of flower development and senescence in petunia, the *ACO1* gene was expressed specifically in the senescing corollas, whereas the *ACO3* and *ACO4* genes were specifically expressed in developing pistil tissue (Tang et al., 1994). Liu et al. (1997) reported that three ACC oxidase homologs, *ACCO1*, *ACCO2*, and *ACCO3*, were differentially regulated in sunflower organs, and were induced by wounding and silver ion treatment in seedlings. In *Nicotiana glutinosa* plants, diverse biotic and abiotic stresses, such as TMV infection, wounding, or treatments with CuSO_4 , salicylic acid, methyl jasmonate (MJ) or ethylene, induced three members of the ACC oxidase genes in a gene- and developmental-specific fashion (Kim et al., 1998). During leaf ontogeny of white clover, the *TRACO1* gene was expressed specifically in the apex; *TRACO2* in the apex and in developing and mature green leaves; with the third gene, *TRACO3*, being active in senescent leaf tissue (Hunter et al., 1999). Finally, Chae et al. (2000) have demonstrated that expression of two *OS-ACO* genes is differentially regulated by auxin and ethylene in rice plants. All these results could be interpreted as evidence that ACC oxidase genes are not constitutive, but that specific expression of each member of the gene family is crucial for controlling ethylene biosynthesis in vegetative tissues and in ripening flowers and fruits.

Exogenous and wound-induced ethylene stimulated in-vivo conversion of ACC to ethylene in wounded preclimacteric cantaloupe and excised winter squash fruits (Hoffman and Yang, 1982; Hyodo et al., 1993). When intact preclimacteric tomato and apple fruits were treated with ethylene, ACC oxidase activity as well as its mRNA level greatly increased (Liu et al., 1985; Dong et al., 1992). These results indicate that ethylene is closely associated with the induction of the ACC oxidase gene in fruit tissues. We previously showed that ethylene markedly stimulated the accumulation of ACC oxidase mRNA in mung bean hypocotyls, and suggested that ethylene plays an important role in regulating ACC oxidase gene expression in

vegetative mung bean hypocotyl tissue, as it does in fruit tissues (Kim and Yang, 1994; Kim et al., 1997a; Jin et al., 1999). From these results, we might infer that the low, basal level of ACC oxidase transcript constitutively expressed in mung bean hypocotyls is regulated by the endogenous ethylene present in those tissues. To gain more insight into the mechanism of ethylene-regulated ACC oxidase gene expression in vegetative tissues, we obtained a genomic clone encoding mung bean ACC oxidase (*VR-ACO1*). In this paper, we report the structure, expression pattern, and putative ethylene-responsive cis-acting elements present in the promoter of *VR-ACO1*.

MATERIALS AND METHODS

Plant Material

Dry seeds of mung bean (*Vigna radiata* L.) were germinated and grown in the dark on a 0.5% agar plate (15 cm × 15 cm) for 3 d at 25°C. One-gram batches of 1-cm-long hypocotyl segments were cut at 1 to 2 cm below the hook and incubated for various time periods without or with ethylene (20 $\mu\text{L}\cdot\text{L}^{-1}$) in 3 mL of a medium consisting of 2% (w/v) sucrose, 1 mM CaCl_2 , 100 μM aminooxyacetic acid (AOA), and 50 mM MES buffer (pH 6.2). AOA, an inhibitor of ACC synthase, was always included in the incubation medium to eliminate the wound (excision)-induced ethylene production inherent with this system (Kim and Yang, 1994). All tissues either were immediately used for in-vivo ACC oxidase enzyme assays or were frozen in liquid nitrogen and stored at -80°C until they were used for the in-vitro enzyme assay and RNA extraction.

Determination of Ethylene Level

A 1-mL gas sample was withdrawn from the flask with a hypodermic syringe, and ethylene was assayed in a gas chromatograph equipped with an aluminum column and flame ionization detector.

Isolation of Genomic DNA and Southern Blot Analysis

The mung bean leaf genomic DNA was isolated as described by Kim et al. (1999), with modifications. Leaf tissue was pulverized under liquid nitrogen, then 1-g samples were suspended in 2.5 mL of extraction buffer (8.0 M urea, 50 mM Tris-Cl [pH 7.5], 20 mM

EDTA, 350 mM NaCl, 2% [w/v] sarkosyl, 5% [v/v] phenol, and 20 mM 2-mercaptoethanol). After successive extractions with phenol/chloroform (1:1, v/v), the aqueous phase was concentrated by ethanol precipitation. The pellet was resuspended in 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA, and adjusted to a density of 1.5 g·mL⁻¹ by the addition of saturated CsCl. Afterward, the DNA was banded overnight in a vertical rotor at 200,000g. The DNA band was collected, extracted with 1-butanol, and dialyzed extensively against 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA. Genomic DNA (10 µg per lane) was digested with the appropriate enzymes, separated by electrophoresis on a 0.7% agarose gel, and blotted to a nylon membrane filter (Bio-Rad, Richmond, CA, USA). The filter was hybridized to ³²P-labeled pVR-ACO1 under low hybridization, with washing conditions as described previously (Kim et al., 1999).

RNA Isolation and Northern Blot Hybridization

Total RNAs of mung bean plants were obtained by adapting a method from the established protocols reviewed by Lizzard (1983). Our modifications have been described previously (Kim et al., 1997b). The total RNA was precipitated overnight at 4°C by the addition of 0.3 volumes of 10 M LiCl, then precipitated in ethanol. Total RNA (30 µg) was separated by electrophoresis on a 1% formaldehyde-agarose gel and blotted to a nylon membrane (Bio-Rad). To ensure equal loading of RNA, the gel was stained with ethidium bromide after electrophoresis. Complete transfer of RNA to the membrane filter was confirmed by viewing both the gel and the membrane under UV light. The filter was hybridized to ³²P-labeled pVR-ACO1 (Kim and Yang, 1994). Blots were washed as described previously (Kim et al., 1997b) and visualized by autoradiography at -80°C using Kodak XAR-5 film and an intensifying screen.

Construction and Screening of Genomic Library

Procedures for constructing the genomic library of mung bean hypocotyls were described by Yi et al. (1999). Our library was screened using the pVR-ACO1 as a probe, according to an established procedure (Sambrook et al., 1989).

Sequencing of DNA

The DNA inserts containing putative mung bean VR-ACO1 were subcloned into Bluescript SK plasmids

(Stratagene, La Jolla, CA, USA). Sequencing of DNA was performed using the Sequenase DNA sequencing kit, according to the manufacturer's manual (US Biochemical, Cleveland, OH, USA). Sequence analysis was carried out with PC-GENE computer software.

Nuclear Run-on Transcription Assay

Nuclear run-on synthesis was performed at 30°C for 40 min in a 600-µL reaction volume (Yi et al., 1999). The reaction mixture contained 100 mM (NH₄)₂SO₄, 4 mM MgCl₂, 0.3 mM phosphocreatine, 25 mg·mL⁻¹ phosphocreatine kinase, 0.5 mM ATP, CTP, GTP, 3.3 µM UTP, 0.5 mCi ³²P-α-UTP, and the nuclei extract (100 µg of DNA). After 40 min of incubation, the DNA was removed by incubating the mixture with 100 units of RQ1 DNase (Promega Biotech) for 20 min at 30°C. Then, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% SDS were added, and the mixture was treated with Proteinase K (200 µg·mL⁻¹) at 42°C for 1 h. RNA was extracted with an equal volume of phenol/chloroform and ethanol precipitated along with yeast tRNA (10 µg·mL⁻¹). After centrifugation, the RNA pellet was washed with 95% ethanol, resuspended in H₂O, and used for blot hybridization.

RESULTS AND DISCUSSION

Genomic Southern Blot Analysis

Previously, we isolated two closely related ACC oxidase cDNAs, pVR-ACO1, and pVR-ACO2, from a mung bean hypocotyl cDNA library (Kim and Yang, 1994). That expression study demonstrated that VR-ACO1 was a major gene that was constitutively expressed in all parts of the seedlings, whereas the relative abundance of the VR-ACO2 gene was much lower. Applying 50 µL·L⁻¹ ethylene has been shown to markedly increase the accumulation of VR-ACO1 transcript, while ethylene-induction of VR-ACO2 is much lower (<5%) in mung bean hypocotyls (Kim and Yang, 1994; Jin et al., 1999).

To assess the exact gene copy number of the ACC oxidase gene in the mung bean genome, genomic Southern blot analysis was carried out using pVR-ACO1 as a probe. The genomic DNA isolated from mature leaves was digested with EcoRI, HindIII, or XbaI, and hybridized with ³²P-labeled pVR-ACO1 under low stringent hybridization and washing conditions. This hybridization detected one major band and an additional weaker band (Fig. 1). The weaker

band possibly corresponds to *VR-ACO2* (Kim and Yang, 1994). Nonetheless, we could not detect any additional band after longer exposure of the blot. Thus, the results indicate the existence of two ACC oxidase genes in the mung bean genome.

Isolation and Characterization of the *VR-ACO1* Gene

To help determine the structure of the ethylene-responsive *VR-ACO1* gene as well as to investigate its promoter sequence, we proceeded to isolate and characterize the genomic clone. The DNA was fully digested with EcoRI and size-fractionated on a 10 to 40% sucrose gradient (data not shown). Restriction fragments of about 12- to 20 kb in size were collected, ligated into EcoRI-digested lambda EMBL4 arms, and packaged in-vitro. The existence of *VR-ACO1* in these fragments was demonstrated in our genomic Southern blot analysis (Fig. 1) and further confirmed by PCR (data not shown). Using ^{32}P -

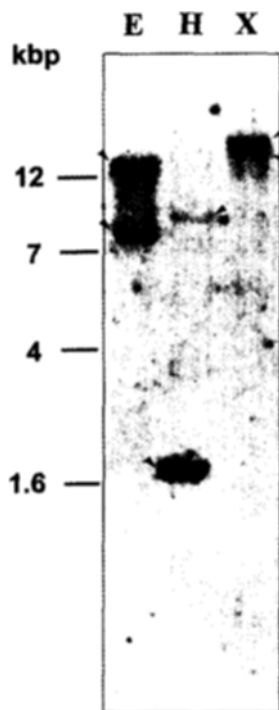


Figure 1. Genomic Southern blot analysis of the mung bean ACC oxidase gene. Genomic DNA (10 μg per lane) was isolated from leaf tissue, digested with either EcoRI (E), HindIII (H), or XbaI (X), and resolved on a 0.7% agarose gel. DNA on the gel was transferred to a nylon membrane filter. The filter was hybridized to the ^{32}P -labeled pVR-ACO1 under low stringent hybridization and washing conditions. The blot was visualized by autoradiography.

labeled pVR-ACO1 as a probe, we were able to obtain one clone, $\lambda\text{VR-ACO1}$ from the resulting partial genomic library. Restriction enzyme digests of DNA isolated from this clone showed that it contained inserts of approximately 15 kb (Fig. 2). Subsequent restriction enzyme mapping and partial DNA sequencing analysis revealed that the genomic clone encodes *VR-ACO1*. The coding region, as well as the 5'- and 3'-flanking regions of $\lambda\text{VR-ACO1}$, were sequenced for a total of 4.5 kb (GenBank accession number AF315316). This 4.5-kb DNA fragment contained the entire coding sequence and about 2.0 kb of the 5'-upstream region. The *VR-ACO1* gene consists of four exons interrupted by three introns whose junctions are in agreement with the consensus intron/exon borders of plant genes (Brown, 1986). Individual sizes for the three introns are 100, 413, and 205 bp. The coding region of *VR-ACO1* comprises 951 bp encoding a protein of 317 amino acid residues, with a predicted molecular mass of 36 kDa.

The start site of transcription for *VR-ACO1* was determined by primer extension analysis using reverse transcriptase and the primer that is complementary to the 5'-end of the *VR-ACO1* coding sequence. This site was decided upon by sizing a fragment produced by an end-labeled primer hybridized to RNA that had been isolated from ethylene-treated hypocotyls and extended by reverse transcriptase. Single primer extension product was obtained with RNA from mung bean hypocotyls (Fig. 3). The major start site is located 66 nucleotides upstream from the 5'-end of the coding region of the *VR-ACO1* gene. The putative TATA box is found at the 27th nucleotide upstream from the start site. Thus, our primer extension data define the size of the 5'-untranslated region of the *VR-ACO1* mRNA to be 66 nucleotides long (Fig. 2B). In addition, two putative polyadenylation signals (AATAAA) were found in the 3'-untranslated region. These results indicate that *VR-ACO1* is indeed actively transcribed in mung bean hypocotyls.

Ethylene-Induced Expression of *VR-ACO1*

To assess whether the expression of *VR-ACO1* is induced by ethylene treatment, two experiments were performed. Intact, three-day-old mung bean hypocotyls were treated in jars with or without 20 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 8 h. Total RNAs were then isolated and analyzed by RNA gel blot analysis using pVR-ACO1 as a probe under high stringent hybridization and washing conditions. The intact mung bean hypocotyl tissue contained a considerable amount of *VR-ACO1*

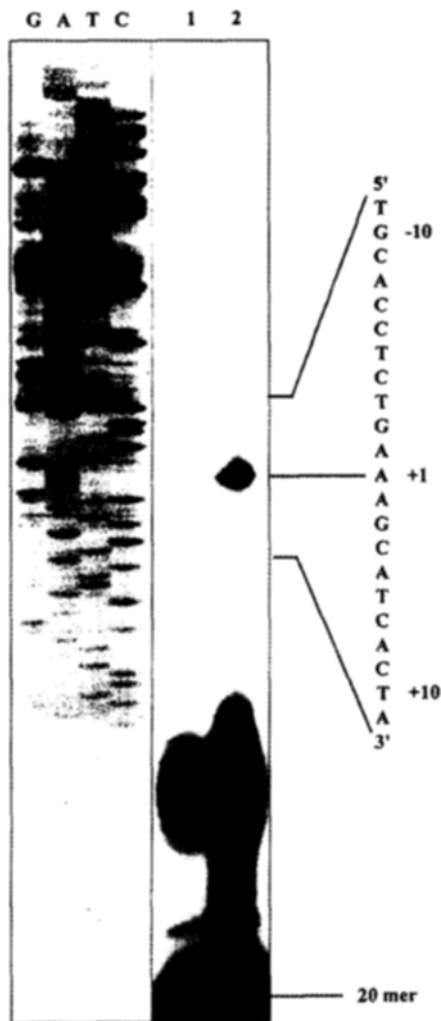


Figure 3. Primer extension analysis to identify the transcriptional start site of *VR-ACO1*. ^{32}P -labeled primer (Fig. 2B) was hybridized with 10 μg of total RNA obtained from ethylene-treated mung bean hypocotyls (Lane 2), and extended with reverse transcriptase. Single primer extension product is indicated. The transcriptional start site is shown as +1 in the DNA sequence. Lane 1 is the control without RNA.

mRNA, and the level of transcript markedly increased with ethylene treatment (Fig. 4). In a parallel experiment, hypocotyl segments were excised and incubated in a medium containing 100 μM AOA, an inhibitor of ACC synthase enzyme activity. Here, the basal level of *VR-ACO1* transcript was significantly reduced. However, when the excised tissue was treated with ethylene, the transcript level of *VR-ACO1* was greatly enhanced (Fig. 4). Taken together, these results indicate that *VR-ACO1* is indeed the ethylene-inducible gene in mung bean hypocotyls.

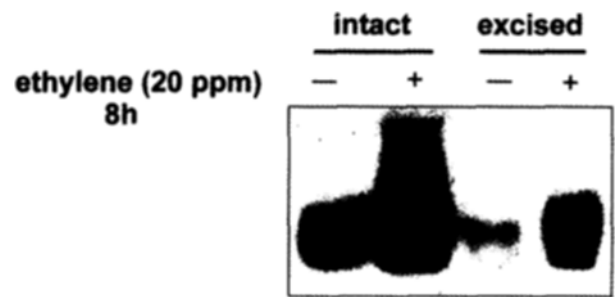


Figure 4. Effect of ethylene on *VR-ACO1* mRNA expression. Intact three-day-old etiolated mung bean seedlings were enclosed in 3-L jars containing air or air plus 20 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene. After the 8-h treatment, hypocotyl tissue was excised and used for RNA extraction. In a parallel experiment, the hypocotyl segments were excised and incubated in a medium containing 100 μM AOA, an inhibitor of ACC synthase enzyme activity, with or without 20 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene. Total RNAs (30 μg per lane) were resolved on a 1.0% agarose-formaldehyde gel, then transferred to a nylon membrane filter. The filter was hybridized to the ^{32}P -labeled pVR-ACO1 under high stringent hybridization and washing conditions. The blot was visualized by autoradiography. + and - indicate with and without ethylene, respectively.

Nuclear Run-on Transcription Assay

The results described in Figure 4, as well as those from previous studies (Kim and Yang, 1994; Jin et al., 1999) clearly demonstrate that the level of *VR-ACO1* transcript markedly increases in response to ethylene in mung bean hypocotyl tissue. However, we still could not determine whether ethylene induced gene expression at either the transcriptional or the post-transcriptional level. To evaluate this ethylene effect, nuclei were obtained from the hypocotyls that were treated with 20 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for different time periods. They were then subjected to a nuclear run-on transcription assay (Yi et al., 1999). ACC oxidase cDNA (pVR-ACO1) was immobilized onto a membrane filter and probed with equal amounts of radioactivity from the nuclear run-on reactions under high stringency. The relative transcription rates were estimated by quantifying the autoradiographic signals with a Fuji PhosphorImager.

Figure 5 shows that the transcriptional activity of *VR-ACO1* was slightly enhanced at 1-h ethylene treatment. In the 8-h ethylene treatment, the transcription rate was further stimulated (Fig. 5). As a positive control, we estimated the transcriptional activity of *ERS1* that is known to be induced by ethylene in mung bean seedlings (Kim et al., 1999). Here, the *ERS1* gene was also induced transcriptionally by ethylene.

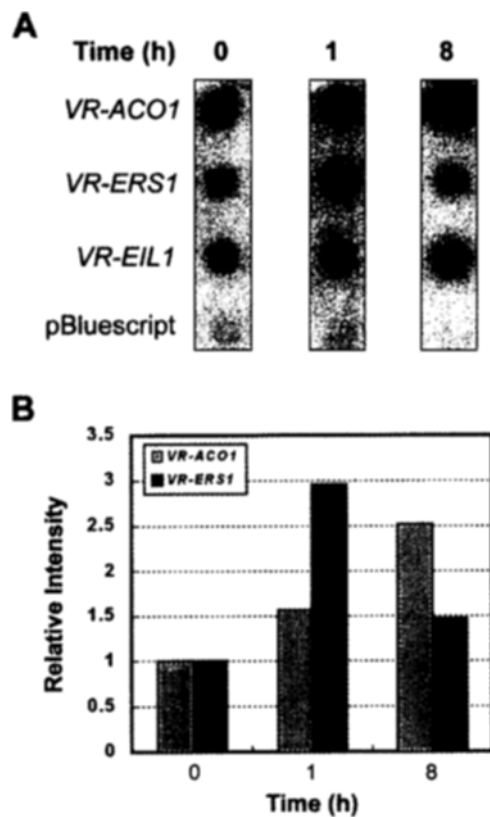


Figure 5. Nuclear run-on transcriptional analysis. **A.** Nuclei were isolated from mock- and $20\text{-}\mu\text{L}\cdot\text{L}^{-1}$ ethylene-treated hypocotyls and used for in-vitro transcription reactions. The in-vitro synthesized ^{32}P -labeled RNA was then hybridized to DNA dot blots containing $10\text{ }\mu\text{g}$ each of cDNA plasmids for pVR-ACO1, pVR-ERS1, pVR-EIL1, or Bluescript plasmid. **B.** The relative transcriptional activities of VR-ACO1 and VR-ERS1 in response to ethylene. Hybridization signals obtained from the dot blot analyses of panel A were quantified with a PhosphorImager.

By contrast, the transcriptional activity of VR-EIL1, whose expression is not induced by ethylene (our unpublished data), was unchanged. As a negative control, plasmid DNA containing no cDNA insert showed an undetectable level of hybridization. Thus, these results indicate that ethylene induces the expression of VR-ACO1 at the transcriptional level in mung bean hypocotyls.

Putative Ethylene-Responsive Elements in the Promoter Region of VR-ACO1

Ethylene-responsive *cis* regions and proteins that specifically interact with these *cis* regulatory regions have been found in the tomato fruit *E4* and *E8* genes (Cordes et al., 1989; Montgomery et al., 1993;

Coupe and Deikman, 1997; Deikman et al., 1998), the carnation glutathione-S-transferase (*GST1*) gene (Itzhaki et al., 1994; Maxson and Woodson, 1996), and the tobacco *PRB-1b* and osmotin-like *PR-5* genes (Meller et al., 1993; Sato et al., 1996). These studies have identified two different types of ethylene response elements (EREs). Deletion analysis of promoters from the ethylene-inducible tobacco chitinase and *PRB-1b* genes has defined the GCC box as an ethylene-responsive *cis*-acting element that is an 11-bp sequence (TAAGAGCCGCC) conserved in the 5'-upstream region of these ethylene-inducible PR protein genes (Sessa et al., 1995; Shinshi et al., 1995). Ohme-Takagi and Shinshi (1995) have isolated four different cDNAs that encode DNA binding proteins specific for the GCC box sequence. They have also shown that the accumulation of mRNAs for these putative transcription factors is induced by ethylene in tobacco. These ethylene-responsive factors (ERFs) have been identified as transcriptional factors that respond in various ways to extracellular signals to modulate GCC box-mediated gene expression, either as transcriptional activators or repressors in tobacco and *Arabidopsis* plants (Fujimoto et al., 2000; Ohta et al., 2000).

The other type of ERE was found to be responsible for ethylene-regulated expression of tomato *E4* and carnation *GST1* genes that are induced during fruit ripening and flower senescence, respectively (Montgomery et al., 1993; Itzhaki et al., 1994). Interestingly, a similar *E4*- and *GST1*-like *cis*-acting element has also been found in the promoter of *Arabidopsis* ERF1 that binds to the GCC box (Solano et al., 1998). Therefore, Solano et al. (1998) has renamed the *E4*- and *GST1*-like element as a primary ethylene responsive element (PERE), and the GCC box as a secondary ethylene responsive element (SERE). Furthermore, they have shown that the *Arabidopsis* EIN3/EIL family of nuclear proteins, essentially involved in the ethylene signal transduction pathway, directly binds to a PERE to mediate ethylene-inducible gene expression. Using a random binding site selection analysis, Kosugi and Ohashi (2000) have found that the consensus binding sequence for tobacco EIL homolog (TEIL) is A(T/C)G(A/T)A(C/T)CT, and that this sequence is well conserved in the promoter of *ERF1*, *E4*, and *GST1*.

To link the ethylene-inducible expression properties of the VR-ACO1 gene with its promoter region, we compared the sequence of the 5'-flanking region with known EREs. One 23-bp region, located between -192 and -169 (Fig. 2), contains two copies of

A. PERE (primary ethylene responsive element, ATTCAA)

VR-ACO1	-192	AAA <u>ATTCAA</u> TTTTATTTATG	-169	(in this paper)
GST1	-497	TATTT <u>ATTCAA</u> AGCCTAGCATGTAGCATCTCAA	-467	(Itzhaki et al., 1994)
ERF1	-1205	GG <u>ATTCAA</u> AGGGGCATGTATCTTGAATCC	-1178	(Solano et al., 1998)
E4	-118	AA <u>ATTCAA</u> ATGGCAAATGTATAACGCATCT	-89	(Montgomery et al., 1993)

B. EIN3 binding site (AY(T/C)GW(T/A)AY(T/C)CT)

VR-ACO1	-417	<u>ATGATAT</u>	-410	(in this paper)
	-399	<u>ATGTATTA</u>	-392	(in this paper)
	-368	<u>ATGTATTA</u>	-361	(in this paper)
ERF1	-1192	<u>ATGTATCT</u>	-1185	(Solano et al., 1998)
NsERF2	-132	<u>ATGTA</u> CTC	-125	(Kitajima et al., 2000)

Figure 6. Putative ethylene-responsive *cis*-acting regulatory elements (EREs) in the promoter of *VR-ACO1*. **A.** Sequence motifs of the *VR-ACO1* promoter (underlined in bold in Fig. 2B) were compared with functionally identified primary ethylene-responsive *cis*-acting elements (PERE) in carnation *GST1* gene (Itzhaki et al., 1994), *Arabidopsis ERF1* (Solano et al., 1998), and tomato *E4* gene (Montgomery et al., 1993). **B.** Sequence motifs of the *VR-ACO1* promoter (underlined in bold in Fig. 2B) are compared with EIN3 binding site identified in *Arabidopsis ERF1* (Solano et al., 1998) and tobacco *NsERF2* (Kitajima et al., 2000). The nucleotide position is relative to the transcriptional start site of each gene.

sequence (TTCAA) that displays a high degree of identity with the PERE of the *GST1*, *E4*, and *ERF1* genes (Montgomery et al., 1993; Itzhaki et al., 1994; Solano et al., 1998) (Fig. 6). In addition, the promoter contains three octanucleotide sequence motifs (-417 to -410, -399 to -392, and -368 to -361), which are reminiscent of the EIN3 binding site that exists in *Arabidopsis ERF1* (Solano et al., 1998) and tobacco *ERF2* (Kitajima et al., 2000). Thus, the presence of putative EREs in *VR-ACO1*, with their similarities to functionally defined *cis*-acting elements in other ethylene-regulated genes, suggests, at least in part, the utilization of analogous *trans*-acting factors for signaling ethylene-mediated *VR-ACO1* gene activation. It would be of great interest to investigate whether the mung bean EIN3 homolog binds to the putative ethylene-responsive *cis*-acting element in *VR-ACO1* to mediate ethylene-responsive gene activation.

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