Characterization of an Auxin-Inducible 1-Aminocyclopropane-1-Carboxylate Synthase Gene, VR-ACS6, of Mungbean (Vigna radiata (L.) Wilczek) and Hormonal Interactions on the Promoter Activity in Transgenic Tobacco

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A genomic clone for VR-ACS6, an isozyme of auxin-inducible ACC synthase of mungbean, was isolated, and its promoter activity was examined in transgenic tobacco. The clone contained 1,612 bp long 5' untranscribed region and its coding sequence consisted of three exons and two introns. Genomic Southern hybridization indicated that VR-ACS6 is a single copy gene. The transcription initiation site was a cytosine present at 231-base upstream the translation start codon. The VR-ACS6 promoter contained DNA sequences homologous to various functionally identified auxin-responsive elements. To demonstrate hormonal response of the promoter region, transgenic tobacco plants carrying the 1,719 bp VR-ACS6 promoter/-glucuronidase (GUS) fusion gene were generated. Strong GUS expression occurred by auxin treatment of leaves of T0 transformants and hypocotyls of T1 etiolated seedlings. Magnitude of the response to auxin was dose-dependent, and the increased GUS activity was detected at 0.1 \( \mu \text{M} \) and higher concentrations of IAA. Other plant hormones did not induce GUS activity, but greatly modified the response to auxin. Cytokinlin enhanced the IAA-induced expression of GUS reporter gene, whereas ABA and ethylene suppressed the expression. These characteristics of VR-ACS6 promoter activity in transgenic tobacco are in good accordance with the expression patterns of the gene in mungbean hypocotyls. Histochemical staining showed that GUS activity was evident in both etiolated and light grown seedlings treated with IAA. Cytokinlin enhanced the intensity of auxin-induced GUS stain and also expanded the stained area, whereas ABA and ethylene reduced both intensity and area of the stain.

Key words: ACC synthase gene — Auxin — Ethylene — Hormonal interaction — Promoter/GUS — Transgenic tobacco.

Ethylene is a gaseous plant hormone which regulates growth and development of a plant throughout its life. It is also closely associated with responses of plants to irregular environmental stresses such as wounding, physical load, disease and water stress (Abeles et al. 1992). Thus, ethylene production must be precisely controlled for plants to survive.

ACC synthase is a key enzyme in the ethylene biosynthetic pathway and catalyzes the conversion of S-adenosylmethionine to ACC (Adams and Yang 1979). The enzyme is encoded by a family of isogenes in most plants so far examined. Many of the isogenes in the gene family are differentially expressed in response to different signals, such as wounding, ripening, senescence, auxin and an aerobiosis as well as in different tissue types (Kende 1993). At least one biochemical evidence indicates that different signals, i.e., auxin and wounding, resulted in the induction of different isozymes (Nakagawa et al. 1988).

Auxin stimulates ethylene production by inducing ACC synthase (Yu and Yang 1979, Yoshii and Imaseki 1981) following increased expression of its gene (Huang et al. 1991, Nakagawa et al. 1991, Yip et al. 1992, Peck and Kende 1995, Yoon et al. 1997). Some of the auxin-responsive ACC synthase isogenes are specifically responsive to the auxin signal, whereas others are also induced by other stimuli such as wounding and mechanical stress (Huang et al. 1991, Lincoln et al. 1993, Botella et al. 1995). The expression of isogenes specific to auxin has been demonstrated for winter squash CM-ACS2 (Nakagawa et al. 1991, Yamagishi et al. unpublished data), tomato LE-ACS3 (Yip et al. 1992, Mori et al. unpublished data), Arabidopsis AT-ACS4 (Abel et al. 1995), and mungbean VR-ACS6 (Yoon et al. 1997).

Many auxin-inducible genes have been reported in a variety of plants, and several auxin-responsive domains were functionally identified in soybean GH3 (Liu et al. 1994, Ulmasov et al. 1995), SAUR (Li et al. 1994), pea IAA4/5 (Ballas et al. 1993, 1995), tobacco parB (Takahashi et al. 1995) and parC (Sakai et al. 1996). Auxin-inducible ACC synthase represents another group of primary auxin-responsive genes (Abel et al. 1996), but information on the function and structure of their promoters is limited. We have previously isolated a cDNA for auxin-inducible ACC synthase, pVR-ACS6, from auxin-treated mungbean hypocotyls (Yoon et al. 1997). Induc-
tion of VR-ACS6 expression was highly specific to the auxin signal and independent of de novo protein synthesis. Furthermore, the auxin-induced expression of the isogene was greatly modified by other plant hormones such as cytokinin, ABA and ethylene. These characteristics of expression of VR-ACS6 make it useful as a molecular probe to study hormonal interactions involved in gene regulation. As a first step toward this end, we isolated and characterized VR-ACS6 gene, and demonstrated that the promoter of this isogene, when introduced into tobacco plant, drove the reporter gene expression specifically in response to auxin and contained all necessary elements for the interaction of auxin with cytokinin, ABA or ethylene.

Materials and Methods

Plant material—Presoaked seeds of mungbean (Vigna radiata (L.) Wilczek) were placed on agar plates, and grown in darkness at 28°C for 3 d. Hypocotyl segments, 2 cm long, were excised and freeze-dried before isolation of genomic DNA. Tobacco (Nicotiana tabacum L. cv. SR1) seeds were surface sterilized, germinated and grown in a culture bottle which contained agar-solidified Murashige-Skoog medium at 26°C under the continuous light. Leaves of young tobacco plants grown for 4-6 weeks were used for transformation.

Construction and screening of genomic DNA library—Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method, and purified by equilibrium sedimentation in a CsCl gradient. A partial MboI digest of the genomic DNA were fractionated by NaCl density gradient centrifugation. DNA fragments of 10-20 kb in size were treated with the Klenow fragment in the presence of dGTP and dATP, inserted into the partially-filled NotI site of Lambda FixII vector (Stratagene), and packaged with Gigapac II gold (Stratagene). The primary library transfected into Escherichia coli (XL1-Blue MRA) was screened with the 1.8 kb fragment of cDNA for VR-ACS6, pVR-ACS6, as a probe, which had been end-labeled with 32P by thermostable Bca DNA polymerase (TAKARA, Kyoto). Nitrocellulose membranes (Amersham) representing 3.4 × 105 pmol) complementary to nucleotides +169 to +187 of the gene were used for transformation.

Genomic Southern hybridization—Fifteen micrograms of mungbean genomic DNA was digested with an excess amount of restriction enzyme, electrophoresed on 10% agarose gel and transferred to Hybond N+ (Amersham) membrane. The 1.8 kb fragment of pVR-ACS6 was labeled with 32P and used as a probe. Hybridization (with 50% formamide at 42°C and washing (with 2× SSPE and 0.1% SDS at 65°C) was carried out under high stringency conditions.

Primer extension analysis—A specific 3′ primer P2 (ca. 0.5 pmol) complementary to nucleotides +169 to +187 of the gene (see below), which had been 5′ end-labeled, was hybridized with 50 μg of heat-denatured total RNA isolated from IAA-treated mungbean hypocotyls at 50°C for 1 h. The annealed primer was extended with reverse transcriptase (SUPERSCRIPT II, GIBCO BRL) in 20 μl of a reaction mix (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 1 μM each dNTPs, and 10 mM DTT) at 37°C for 30 min. For determination of the nucleotide sequence near the transcription initiation site, the sequencing reaction of pSP6 that contained about 2 kb of the promoter region of VR-ACS6 gene was carried out with the same primer. The primer extension product was analyzed on a 4.5% acrylamide/7 M urea sequencing gel along with the sequencing reaction products.

Construction of fusion plasmid—The promoter region from −1,531 to +187 of the gene was generated by PCR with the plasmid pXC1 as a template. The 5′ primer (P1) was 5′-AAGCTTAAGCATAAGCAATGTAAG-3′ and the 3′ primer (P2) was 5′-TCTAAGCTTCTGGAGATTTTATGG-3′. HindIII and XhoI site were attached to the P1 and P2 primer, respectively. A promoter/GUS chimeric plasmid (pBI-ACS6) was prepared by inserting the HindIII/XhoI fragment of the promoter into the HindIII/XbaI site of the binary vector pBI121 (CLONTECH).

Generation of transgenic tobacco and determination of GUS activity—Tobacco leaf discs were transformed with Agrobacterium tumefaciens LBA4404 carrying pBI-ACS6 according to Horsch et al. (1988), and transgenic plants were selected in the presence of 100 μg ml−1 kanamycin. Transgenic plants (T0) were transferred to soil and grown in a greenhouse for 4 weeks. Leaf discs (7 mm in diameter) from the second leaves of the T0 transgenic plants were incubated at 28°C in 50 mM sodium phosphate buffer (pH 7.0) in the presence of a combination of plant hormones as indicated. After 6 h, the leaf discs were washed and extracted with cold buffer (50 mM sodium phosphate, 10 mM EDTA, 0.1% sodium lauryl sarcosinate, 0.1% Triton X-100 and 10 mM 2-mercaptoethanol), and GUS activity was fluorometrically determined with 1 mM 4-methylumbelliferyl glucuronide as substrate at 37°C. For histochemical GUS staining, seedlings from T1 seeds germinated in the presence of 100 μg ml−1 kanamycin were used. Transgenic seedlings were grown for 7 d at 27°C under complete darkness, or for 10 d in 16L/8D or under continuous light. The whole seedlings were incubated for 5 h or 18 h at 28°C with a combination of plant hormones, and stained with 1 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid at 37°C.

Results

Isolation and structural characterization of VR-ACS6 gene—Three-round screening of mungbean genomic library by cDNA, pVR-ACS6, as a probe led to isolation of nine positive clones. Southern hybridization indicated that a 7.6 kb NotI/SpeI fragment of the pXC1 encompassed the VR-ACS6 gene. The clone contained 1,612 bp long 5′
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Fig. 1 Genomic structure of VR-ACS6. A, A block diagram of genomic structure. Boxes indicate exons, and the translation start site is shown in exon I. Below the diagram, the promoter region used for fusion with GUS gene (a) and that detailed in B (b) are shown. Arrows indicate restriction sites; B, BamHII; E, EcoRI; H, HindIII; D, DraI. A detailed block diagram of the promoter region. VRA1 and VRA2, domains highly homologous to soybean GH3 D1 and D4 elements; VRA3, a domain homologous to tobacco parB ARE-I domain; G-box and its related elements are shown. M1, M2 and M3, short elements conserved in four different auxin-specific ACC synthase genes. C, Comparison of partial DNA sequences of the upstream side of TATA box of four auxin-specific ACC synthase genes from mungbean (VR-ACS6), winter squash (CM-ACS2, CM-ACS3), and Arabidopsis (AT-ACS4). Four different highly conserved domains (M1 through M4) are shaded.

untranscribed region and 872 bp long 3' nontranslated sequence (the entire nucleotide sequence has been deposited to DDBJ, accession number AB018355). The coding sequence of the gene was 100% identical to that of cDNA (Yoon et al. 1997, accession number DDBJ AB00679) and consisted of two introns and three exons (Fig. 1A). The consensus dinucleotides at the intron/exon junctions were well conserved. ACC synthase genes can be divided into 3 classes based on the number of introns (Rottmann et al. 1991). They have either 2, 3 or 4 introns, but the position of each intron is exactly conserved among ACC synthase genes. VR-ACS6 has intron I and intron III at corresponding positions.

Southern analysis of mungbean genomic DNA with pVR-ACS6 (Fig.3) showed one strong positive band in EcoRI (E), BamHII (B), PstI (P) and XbaI (X) digests and two strong bands in HindIII digest (H). This implies that VR-ACS6 gene is present in a single copy.

The transcription initiation site was determined by primer extension. As shown in Fig. 2, a strong termination signal was obtained at a size of 187 nucleotides, and corresponded to the base cytosine at 231 bp upstream the translation start codon ATG. A sequence located at position -31 to -25, TATAAT (Fig. 1), matched the consensus TATA box, and a potential CAAT box was present at position -96 to -91, GGCAAT.

Structure of VR-ACS6 promoter—In the promoter region of VR-ACS6, several sequences homologous to the functionally identified auxin-responsive elements were present. A 41 bp domain located at position -187 to -147 contained two sequences (VRA1 and VRA2) highly homologous to D1 and D4 domains of soybean GH3 pro-
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Fig. 2 Determination of the transcription initiation site by primer extension. A primer complementary to a segment from +169 to +187 were labeled with $^{32}$P at 5' end, hybridized with 50 µg of total RNA from IAA-treated mungbean hypocotyls and extended by reverse transcriptase. Sequencing reaction for pSP6 plasmid that contained the promoter region was carried out with the same primer. The primer extension product (B) and sequencing reaction products (A) were co-electrophoresed on a 4.5% polyacrylamide gel. Arrow indicates the major extended product.

moter (Fig. 1B). GH3 promoter contains three independent auxin-responsive elements, D1, D4 and E1 (Ulmasov et al. 1995). The D1 and D4, respectively, have a composite structure which contains a constitutive element adjacent to a conserved TGTCTC element that confers auxin inducibility. Although the same constitutive element of D1 or D4 was not found in the 41 bp domain of VR-ACS6 promoter, both VRA1 and VRA2 elements contained the TGTCTC(nnn)AATAAG core-like sequence. The VR-ACS6 promoter also contained a DNA domain (VRA3) located at position $-206$ to $-237$ which is homologous to the ARE I of parB promoter. The ARE I and ARE II of parB promoter have conserved sequences different from the TGTCTC motif, but have conferred auxin responsiveness in transgenic tobacco (Takahashi et al. 1995).

Furthermore, three G-box related motifs were found proximal to the VRA1 domain, though one of them was in a reverse orientation (Fig. 1B). Among them, G-box 1, CACGTGGC, located at $-104$ to $-110$ was identical to the functionally identified ABA responsive element found in rice Rab-16A gene (Mundy et al. 1990) and wheat Em gene (Marcott et al. 1989).

We compared the 5' flanking regions among four auxin-inducible ACC synthase genes, VR-ACS6, CM-ACS2, CM-ACS3 and AT-ACS4. The expression of these genes are highly auxin-specific (Yoon et al. 1997, Abel et al. 1995, Nakagawa et al. 1991, Imaseki et al. unpublished data). Interestingly, there are four short sequences conserved in the promoter region proximal to TATA box of these genes (M1 to M4). Although CM-ACS2 and CM-ACS3 contained all of four M sequences, VR-ACS6 and AT-ACS4 lacked M4 and M2, respectively (Fig. 1C).

Hormonal regulation of VR-ACS6 promoter in transgenic tobacco—To demonstrate functionality of the promoter region of VR-ACS6, we generated transgenic tobacco carrying a promoter/GUS fusion transgene. When leaf discs of T₀ transgenic tobacco were incubated with IAA at different concentrations, the magnitude of GUS activity increased in a dose-dependent manner (Fig. 4A). A similar auxin dose-response was also found in etiolated seedlings of T₁ generation (Fig. 4B). In both cases, a significant increase in GUS activity over the control was detected at a concentration as low as 0.1 µM IAA. Thus, the response to auxin of the VR-ACS6 promoter was not regarded as a stress response. Treatment of leaf discs of T₀ transformants with synthetic auxins, 1-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid also greatly increased GUS activity, but other plant hormones (benzylaminopurine, ABA and GA₃) did not (Fig. 5). Sucrose which has been known to modify plant gene expression neither induced GUS activity nor modified the auxin action. These results indicate that response of the VR-ACS6 promoter is highly specific to the auxin signal.

We found that IAA-inducibility of the 1.7 kb promoter segment of VR-ACS6 gene was greatly modified by
other plant hormones, as observed in auxin-induced ethylene production in hypocotyl sections (Imaseki et al. 1995, Kondo et al. 1975). In etiolated seedlings of T₁ generation, kinetin (Fig. 6A) greatly enhanced auxin-induced expression of GUS reporter gene, whereas ABA (Fig. 6B) and ethylene (Fig. 6C) significantly suppressed the expression. The enhancement by kinetin and the suppression by ABA were detected at 1 μM of each plant hormone. Magnitude of the cytokinin response appeared to be dose-dependent. These characteristics of VR-ACS6 promoter activity observed in transgenic tobacco were in good agreement with the expression patterns of the gene in etiolated mungbean hypocotyls (Yoon et al. 1997).

**Fig. 4** IAA dose response of GUS expression in transgenic tobacco carrying VR-ACS6 promoter/GUS chimeric construct. A, 30 leaf discs (7 mm in diameter) excised from T₂ transgenic tobacco grown for 4 weeks in a greenhouse were incubated with IAA at indicated concentrations in 50 mM sodium phosphate, pH 7.0 at 28°C for 6 h. B, 7-day-old whole etiolated T₁ seedlings were incubated with IAA as above for 8 h. Tissues were extracted with an extraction buffer (see Materials and Methods), and GUS activity was fluorometrically determined. GUS activities in controls are 17 pmol MU (mg protein)⁻¹ min⁻¹ for A and 25 pmol MU (mg protein)⁻¹ min⁻¹ for B.

**Fig. 5** Specificity to auxin of GUS expression in transgenic tobacco carrying VR-ACS6 promoter/GUS chimeric construct. Incubation of leaf discs and determination of GUS activity were the same as in Fig. 4A. IAA, 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA (1-naphthaleneacetic acid) at 100 μM; sucrose, 1%; BA (benzylaminopurine), 20 μM; ABA, 50 μM; GA (GA₃), 20 μM. GUS activity in control is 25 pmol MU (mg protein)⁻¹ min⁻¹.

**Histochemical GUS staining**—Histochemical GUS staining showed that GUS activity was evident in both etiolated and light grown seedlings treated with IAA. The intensity of auxin-induced GUS stain was enhanced by cytokinin and decreased by ABA (Fig. 7A, B) or ethylene (Fig. 7C). In green seedlings, auxin-induced GUS stain appeared in shoot tip extending to petioles and basal part of cotyledons, whereas in etiolated seedlings, hypocotyls were a major part of GUS staining. Unexpectedly, in hook region of etiolated seedlings where endogenous auxin is considered high, GUS stain was not detected in the control seedlings. The effect of cytokinin was to enhance the stain intensity as well as to expand the stained area, and reverse situation was true for ABA and ethylene. Microscopic observation of sections of the stained part of green leaves and shoot tips indicated that all mesophyll cells and vascular bundles were stained (data not shown).

**Fig. 6** Hormonal interactions of GUS expression in transgenic tobacco carrying VR-ACS6 promoter/GUS chimeric construct. Etiolated T₁ seedlings were treated with a combination of plant hormones. A, Enhancement of IAA-induced GUS expression by cytokinin. Seedlings were incubated with 1 μM IAA in the presence of kinetin (Kin) at an indicated concentration in μM at 28°C for 8 h. For kinetin alone, at 100 μM. B, Suppression of IAA-induced GUS expression by ABA. Seedlings were incubated with 100 μM IAA in the presence of ABA at an indicated concentration in μM of ABA. For ABA alone, at 100 μM. C, Suppression of IAA-induced GUS expression by ethylene treatment. Seedlings were pretreated with ethylene (300 μl liter⁻¹) or air for 6 h, then incubated with buffer or 100 μM IAA for another 12 h. GUS activity of control (None) is 27 pmol MU (mg protein)⁻¹ min⁻¹.
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Fig. 7  Histochemical GUS staining of hormonal interactions of GUS expression in transgenic tobacco carrying \( VR-ACS6 \) promoter/GUS chimeric construct as observed by. A, Etiolated \( T_1 \) seedlings of two independent transgenic tobacco lines (18-1 and 18-2) were treated with buffer (a), 100 \( \mu \text{M} \) IAA (b), 100 \( \mu \text{M} \) IAA + 20 \( \mu \text{M} \) benzylaminopurine (c), 500 \( \mu \text{M} \) IAA (d), and 500 \( \mu \text{M} \) IAA + 50 \( \mu \text{M} \) ABA (e) at 26°C for 5.5 h. B, Green seedlings (line 18-2) were treated as in A. C, Suppression by ethylene. Green seedlings at the 4-leaf stage were incubated with 200 \( \mu \text{M} \) IAA in air (upper panel) and 200 \( \mu \text{M} \) IAA in 20 \( \mu \text{l} \) liter\(^{-1} \) ethylene at 26°C for 5.5 h (lower panel).
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**Discussion**

Plant hormones usually interact with one another to control the growth and development of plants. The balance of auxin and cytokinin controls the organogenesis from callus, lateral root formation and outgrowth of lateral buds. Auxin and ethylene together control leaf abscission and senescence. The interaction can be synergistic, antagonistic, or additive depending on the tissue type, developmental stage and plant species. However, molecular mechanisms of these hormonal interactions are not well understood. Auxin-induced ethylene production provides a good model system, by which the hormonal interaction can be studied at the molecular level, because cytokinin synergistically enhances auxin-induced ethylene production (Fuchs and Lieberman 1968, Imaseki et al. 1975), and ABA suppresses it (Kondo et al. 1975). These hormonal interactions are also found in the endogenous level of ACC synthase activity (Yoshii et al. 1981), and in the steady state level of mRNA for the enzyme (Yoon et al. 1997).

The expression of genes of ACC synthase isozymes is regulated by different stimuli, and we have identified \textit{CM-ACS2} of winter squash (Nakagawa et al. 1991) and \textit{VR-ACS6} of mungbean (Yoon et al. 1997) as auxin specific isogenes by northern blot analysis. To further analyze the specificity of promoter function, we cloned \textit{VR-ACS6} gene and the responsiveness of its promoter to auxin was characterized. To date, auxin-responsive elements have been functionally identified in the promoters of several auxin-regulated genes. The TGTTCTC motif was conserved in the auxin responsive domain of \textit{PS-IAA4/5} (Ballas et al. 1995), \textit{GH3} (Liu et al. 1994) and \textit{SAUR} (Li et al. 1994), which were isolated from elongating tissues. On the other hand, the same sequence does not seem to be related to the expression of another group of auxin-regulated genes isolated from dividing tobacco cells such as \textit{parA} (Takahashi et al. 1990), \textit{parB} (Takahashi et al. 1995) and \textit{arcA} (Ishida et al. 1996). It was also demonstrated that the \textit{as-1} sequence found in the promoter of some auxin-responsive genes was a multi-stimulus-responsive element (Ulmasov et al. 1994).

A 1.7 kb segment of the promoter region of \textit{VR-ACS6} gene contained multiple auxin-responsive elements; two sequences, VRA1 and VRA2 that were highly homologous to D1 and D4 domain, respectively, of soybean \textit{GH3} promoter and VRA3 homologous to the ARE I of \textit{parB} promoter (Fig. 1B). The promoter of \textit{GH3} has, in addition to D1 and D4, another auxin-responsive element E1, which contains multiple TGA box or G-box related sequences (Liu et al. 1994). As \textit{VR-ACS6} promoter also contains G-box related sequences, structure of the promoter is similar to that of soybean \textit{GH3} promoter. These elements were found in genes which are expressed by auxin in elongating cells. In addition to these elements, the presence of \textit{parB} I-like element (VRA3) which was found in auxin-inducible genes of dividing cells (Takahashi et al. 1995) is interesting. Since auxin-induced ethylene production occurs at a wide range of auxin concentrations (0.1 \textmu M to 1 mM IAA) and since the elongation-stimulating activity of various synthetic and natural auxins is not correlated with their ethylene-producing activity (Imaseki, unpublished data), auxin action to induce ethylene production is unlikely to be related to the auxin action to stimulate either cell elongation or cell division. VRA1, VRA2 and VRA3 together and lack of as-1 element may contribute to high specificity to auxin of this particular isogene.

We found 4 different short sequences (M1 to M4) which were conserved in auxin-inducible ACC synthase isogenes from different sources (\textit{VR-ACS6}, \textit{CM-ACS2}, \textit{CM-ACS3} and \textit{AT-ACS4}), and \textit{VR-ACS6} contains M1, M2 and M3 (Fig. 1C). Among them, M1 sequence, (T/G)(T/G)ATTTT, may act as another auxin-responsive element in this isogene, since the sequence was also found in several auxin inducible promoters (Zaal et al. 1991, Ainley et al. 1988), but their functionality has not been demonstrated yet.

The presence of a typical G-box (G-box 1, Fig. 1B) is also interesting, because its sequence, ACGTG, was identified as a core element of genes expressed in response to ABA (Marcott et al. 1989, Mundy et al. 1990). Nevertheless, ABA alone did not induce the expression of \textit{VR-ACS6}, but rather suppressed the auxin-induced expression. Together with two other G-box-related sequences present within 80 bp, G-box 1 may be involved in the interaction of ABA with auxin.

Although the functionality of such elements present in \textit{VR-ACS6} must be experimentally determined, the present results indicate that the 1.7 kb promoter of \textit{VR-ACS6} contained all the elements necessary to show hormonal interactions and functioned in transgenic tobacco as in mungbean. The promoter introduced into a heterologous plant not only directed specific auxin-responsive expression, but also responded to cytokinin leading to enhancement of the expression, and to ABA and ethylene resulting in suppression. These plant hormones are to modify the expression by auxin, probably affecting the pathway of signal transduction for auxin. This is in part because the hormonal interactions observed in ethylene production and ACC synthase activity results from regulation of a particular ACC synthase isogene at the transcriptional level and because cytokinin, ABA, or ethylene by itself does not induce the expression of this isogene.

The results obtained in this study also indicate that tobacco cells contain and/or induce necessary components in response to plant hormones that interact with \textit{VR-ACS6} promoter, and transgenic tobacco plant can be used for a more detailed analysis of the promoter's functional domains for the hormonal interaction.
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References


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