J. Plant Biotechnology(2000) *Vol. 2(2). pp.61~71*

Journal of Plant Biotechnology

Isolation and Characterization of ACC Synthase Gene Family in Mung Bean (*Vigna radiata* L.): Differential Expression of Three ACC Synthase Genes in Response to Auxin and Brassinosteroid

Sunjoo Joo, Woo Taek Kim*

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

Key words: 1-aminocyclopropane-1-carboxylate synthase - auxin - brassinosteroid - ethylene - gene expression - hypocotyl - promoter - transgenic tobacco - *Vigna radiata*

Abstract

By screening a cDNA library of auxin-treated mung bean (*Vigna radiata* L.) hypocotyls, we have isolated two full-length cDNA clones, pVR-ACS6 and pVR-ACS7, for 1-aminocyclopropane-1-carboxylate (ACC) synthase, the rate-limiting enzyme in the ethylene biosynthetic pathway. While pVR-ACS6 corresponds to the previously identified PCR fragment pMBA1, pVR-ACS7 is a new cDNA clone. A comparison of deduced amino acid sequences among auxin-induced ACC synthases reveal that these enzymes share a high degree of homology (65-75%) to VR-ACS6 and VR-ACS7 polypeptides, but only about 50% to VR-ACS1 polypeptide. ACS6 and ACS7 are specifically induced by auxin, while *ACS1* is induced by cycloheximide, and to lesser extent by excision and auxin treatment. Results from nuclear run-on transcription assay and RNA gel blot studies revealed that all three genes were transcriptionally active displaying unique patterns of induction by IAA and various hormones in etiolated hypocotyls. Particularly, 24-epibrassinolide (BR), an active brassinosteroid, specifically enhanced the expression of *VR-ACS7* by distinct temporal induction mechanism compared to that of IAA. In addition, BR synergistically increased the IAA-induced *VR-ACS6* and *VR-ACS7* transcript levels, while it effectively abolished both the IAA- and kinetin-induced accumulation of *VR-ACS1* mRNA. In light-grown plants, *VR-ACS1* was induced by IAA in roots, whereas *VR-ACS6* in epicotyls. IAA- and BR-treatments were not able to increase the *VR-ACS7* transcript in the light-grown tissues. These results indicate that the expression of ACC synthase multigene family is regulated by complex hormonal and developmental networks in a gene- and tissue-specific manner in mung bean plants. The *VR-ACS 7* gene was isolated, and chimeric fusion between the 2.4 kb 5'-upstream region and the β-glucuronidase (GUS) reporter gene was constructed and introduced into *Nicotiana tobacum*. Analysis of transgenic tobacco plants revealed the *VR-ACS7* promoter-driven GUS activity at a highly localized region of the hypocotyl-root junction of control seedlings, while a marked induction of GUS activity was detected only in the hypocotyl region of the IAA-treated transgenic seedlings where rapid cell elongation occurs. Although there was a modest synergistic effect of BR on the IAA-induced GUS activity, BR alone failed to increase the GUS activity, suggesting that induction of *VR-ACS7* occurs via separate signaling pathways in response to IAA and BR.

Introduction

The gaseous phytohormone ethylene mediates numerous physiological aspects of plant growth and development (Yang and Hoffman, 1984; Abeles et al., 1992). Ethylene also serves as a signaling molecule to induce specific changes in genetic expression at certain stages of a plant life cycle (Yang and Hoffman, 1984; Abeles et al., 1992). The production of ethylene

* Corresponding author, E-mail; wtkim@bubble.yonsei.ac.kr
Received Mar. 21, 2000; accepted Apr. 4, 2000

in plant tissue is normally low, but can be markedly induced by a wide range of developmental and environmental cues, including seed germination, fruit ripening, leaf and flower senescence and a multitude of biotic and abiotic stresses (Yang and Hoffman, 1984; Abeles et al., 1992). Ethylene synthesis is also enhanced in response to auxin treatment (Yang and Hoffman, 1984; Abeles et al., 1992). In higher plants, ethylene is produced from methionine via S-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylate (Met → AdoMet → ACC → C_2H_4) (Theologis, 1992; Kende, 1993; Kende and Zeevaart, 1997). The last two steps of this biosynthetic pathway are catalyzed by ACC synthase and ACC oxidase, respectively (Theologis, 1992; Kende, 1993; Kende and Zeevaart, 1997). 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; Kende, 1993).

ACC synthase is encoded by a divergent multigene family in numerous plant species and the expression of each members of ACC synthase is modulated differentially by developmental, hormonal and environmental factors in a tissue-specific manner (Theologis, 1992; Kende, 1993; Kende and Zeevaart, 1997). For example, seven ACC synthase genes have been identified in tomato whose individual expression is developmentally and spatially regulated by mechanical wounding, fungal elicitor, flooding stress, auxin treatment, ozone exposure and fruit ripening (Van der Straeten et al., 1990; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Spanu et al., 1993; Olson et al., 1995; Oetiker et al., 1997; Tuomainen et al., 1997; Shiu et al., 1998). There are at least seven and five divergent members of ACC synthase genes in *Arabidopsis* and rice, respectively (Liang et al., 1992; Zarembinski and Theologis, 1993; Vahala et al., 1998; Arteca and Arteca, 1999; Woeste et al., 1999). Five members of the ACC synthase gene family are isolated from potato plants, with the expression of each member of the family being increased differentially during the progression of leaf and petiole senescence, in response to auxin in hypocotyls, in wounded leaves and tubers, and by ozone and Cu^{++} treatment in leaves (Destefano-Beltran et al., 1995; Schlagnhaufer et al., 1997). It has been recently found that three members of the ACC synthase gene family are differentially regulated in various floral organs in response to pollination and different chemical signals in orchid and carnation flowers, respectively (Bui and O'Neill, 1998; Jones and Woodson, 1999).

Auxin stimulates ethylene production in various plant tissues by promoting de novo synthesis of ACC synthase and of ACC (Yang and Hoffman, 1984; Abel and Theologis, 1996). IAA-induced ethylene production in mung bean hypocotyls can be inhibited both by CHI, a protein synthesis inhibitor, and by AVG or AOA, inhibitors of the PLP-mediated reaction catalyzed by ACC synthase (Yoshii and Imaseki, 1982). In our previous investigation, we isolated a cDNA fragment (pMBA1) encoding the region containing the active site center of ACC synthase from auxin-treated mung bean hypocotyls using PCR-based DNA amplification and showed that auxin induced ethylene production transcriptionally by increasing the transcript of pMBA1 (Kim et al., 1992). More recently, Botella et al. (1992a) isolated a full length cDNA clone (pAIM-1) for ACC synthase from mung bean hypocotyls and showed that the expression of this transcript was regulated by auxin. In addition, DNA sequences of five genomic fragments of ACC synthase were reported by the same group, one of which corresponds to pAIM-1 (Botella et al., 1992b; Botella et al., 1993). To gain more insight into the nature of the auxin-induced expression of ACC synthase gene family in mung bean, we have isolated two full length ACC synthase cDNA clones (pVR-ACS6 and pVR-ACS7) and constructed one partial clone (pVR-ACS1); pVR-ACS6 corresponds to the previously reported pMBA1 fragment, while pVR-ACS1 is identical to pAIM-1. In this investigation, we compare and characterize the expression of these three ACC synthase transcripts following auxin treatment in mung bean hypocotyls.

In addition to auxin, brassinosteroid, a growth-promoting natural compound with similar structure to animal steroid hormones, has been shown to induce ethylene production and to act synergistically with auxin to stimulate ethylene synthesis in etiolated mung bean seedlings (Yopp et al., 1979; Arteca et al., 1983). However, the molecular mechanism of brassinosteroid-induced ethylene production has not been addressed. In order to gain more insight into the mechanism of the hormone-regulated ethylene production and differential expression of ACC synthase multigene family, we characterized the gene- and tissue-specific induction pattern of three auxin-inducible genes following various hormone treatments in mung bean plants. In addition, the genomic clone for *VR-ACS7* was isolated and its structural characteristics were analyzed. Here, we report that the *VR-ACS7* gene is tissue-specifically induced by auxin and brassinosteroid in mung bean plants, and 2.4 kb of the 5'-upstream region of *VR-ACS7* contains the putative IAA-responsive cis-acting element

functional in transgenic tobacco seedlings.

Auxin Induces Three Genes Encoding 1-Aminocyclopropane-1-Carboxylate Synthase in Mung Bean Hypocotyls

Isolation and classification of ACC synthase cDNA clones

Previously, we isolated a 328 bp PCR fragment (pMBA1) encoding the active site domain of ACC synthase, using a set of degenerate oligonucleotide primers corresponding to the conserved amino acid sequences of SNPLGTT and MSSFGLV (Kim et al., 1992). We further showed by Northern blot analysis that the expression of the corresponding transcript was regulated by auxin in mung bean hypocotyls. To study auxin regulation of ACC synthase gene expression in more detail, a cDNA library was constructed using poly(A)$^+$ RNA prepared from auxin-treated hypocotyl tissue (Kim and Yang, 1994). The primary cDNA library comprised 5X105 individual recombinant plaques (Kim and Yang, 1994). The library was amplified, and the total recombinant phage DNA was isolated and used as a template for PCR, using the degenerate oligonucleotide primers as described above. Total PCR products of about 330 bp in length were radioactively labeled and used as probes to screen the cDNA library. Three putative ACC synthase clones were isolated containing inserts of about 1.3, 1.4, and 1.6 kb. Subsequent restriction enzyme mapping and DNA sequencing analyses revealed that these clones can be divided into two different homology classes. The 1.3 kb clone (pACS6) corresponds to the previously identified pMBA1 fragment, while the others (pACS7-1 and pACS7-2) belong to a new ACC synthase gene. All were partial clones lacking a portion of the 5'-end of the coding region. A PCR strategy was used to clone the remaining 5'-end of the each coding region from the cDNA library. A sense primer specific for the T_7 promoter in the Uni-Zap arm flanking the 5'-end of the cDNA insert, together with gene specific antisense primers, were used to amplify fragments representing the missing 5'-end of the partial cDNAs. PCR products were cloned and sequenced, yielding complete coding sequences together with 5'-untranslated leader sequences. These full length cDNA sequences were designated as pVR-ACS6 and pVR-ACS7 representing ACS6 and ACS7, respectively .

Structure and primary sequence of mung bean ACC synthase cDNAs

The pVR-ACS6 clone is 1867 bp long comprising a 169 bp 5'-uncoding region, a 1416 bp coding region encoding 472 amino acids and a 282 bp 3'-uncoding region. pVR-ACS7 is 1840 bp long consisting of a 115 bp 5'-untranslated region, a 1404 bp coding region encoding 468 amino acids and 321 bp 3'-untranslated region. The predicted molecular masses of polypeptides encoded by pVR-ACS6 and pVR-ACS7 are 53.6 kDa and 53.1 kDa, respectively. Complete sequences for pVR-ACS6 and pVR-ACS7, and a published sequence for pAIM-1 (renamed as pVR-ACS1) (Botella et al., 1992), allow comparison of the three full length VR-ACS genes and analysis of their structural relationship. The overall nucleotide sequence homology between pVR-ACS1 and pVR-ACS6, pVR-ACS1 and pVR-ACS7, and pVR-ACS6 and pVR-ACS 7 is 57%, 60%, and 74%, respectively. The coding regions of pVR-ACS6 and pVR-ACS7 are 81% identical at the nucleotide level and 88% at the amino acid level. They are both 60% identical to pVR-ACS1 at the nucleotide level, and 51% and 53% identical to pVR-ACS1 at the amino acid level, respectively. Thus, *VR-ACS6* and *VR-ACS7* share high homology with each other, while *VR-ACS1* gene is a more divergent member of this multigene family.

It has been previously reported that the molecular mass for the auxin-induced ACC synthase isolated from etiolated mung bean hypocotyls was 65 kDa on SDS-PAGE (Tsai et al., 1988). This molecular mass, however, does not agree with the predicted molecular masses of polypeptides encoded by these three genes. Subsequently they reported a 26 amino acid sequence from the N-terminal portion of the purified protein (Tsai et al., 1991), and this sequence does not correspond to any of deduced sequences from these genes nor to any sequences in the Genebank database. To examine whether other ACC synthase cDNAs may be present in the auxin-induced cDNA library, degenerate primers corresponding to the conserved regions 2 and 6 (Dong et al., 1991; Kende, 1993) were prepared and used for PCR to amplify ACC synthase sequences in the library. The PCR products of about 680 bp in length were cloned, and the identity of 24 clones were examined by DNA sequencing; 4 corresponded to pVR-ACS1, 4 to pVR-ACS6, and 16 to pVR-ACS7. No other ACC synthase cDNA were found. Thus, *ACS1, ACS6* and *ACS7* appear to be the major ACC synthase genes whose expression is induced by auxin in mung bean hypocotyls.

Genomic southern blot analysis

Southern blot analyses were performed on mung bean genomic DNA digested with *Bam*HI, *Eco*RI, *Hin-*

dIII or *Xba*I. The filters were hybridized to ^{32}P-labeled 330 bp probes derived from pVR-ACS6 and pVR-ACS7 clones containing sequence between the conserved regions 4 and 6 of each cDNA. The genes encoding *ACS6* and *ACS7* have clearly different restriction patterns, and only one DNA band was detected in all restriction digests. These results indicate that *ACS6* and *ACS7* are present in a single copy per haploid mung bean genome. It has been reported that mung bean *ACS1*, *ACS4* and *ACS5* genes are also present as a single copy (Botella et al., 1992a; Botella et al., 1993).

Expression of ACS6 and ACS7 genes

To confirm that expression of *ACS6* and *ACS7* are indeed regulated by auxin, Northern blot analyses were performed using pVR-ACS6 and pVR-ACS7 as probes. To minimize cross-hybridization among different ACC synthase genes, Northern blot analyses were carried out under high stringency conditions, and the filters were extensively washed. A strong signal of about 2 kb mRNA was detected in mung bean hypocotyls after 5 h incubation with 500 mM IAA + 100 mM BA. In contrast, no hybridization signal was detected without auxin. These results indicate that the level of ACC synthase mRNAs, as probed by pVR-ACS6 and pVR-ACS7, is indeed closely regulated by auxin. To examine the role of protein synthesis in auxin-regulated ACC synthase gene expression, the translation inhibitor CHI was used. Total RNA was isolated from mung bean hypocotyls which had been incubated for 4 h with 100 mM IAA , 100 mM IAA + 25 mM CHI, or 25 mM CHI, and Northern blottings were carried out. CHI effectively inhibited the auxin-induced accumulation of both *ACS6* and *ACS7* mRNAs, and CHI alone was not capable of inducing the ACC synthase message in mung bean hypocotyls. In contrast, CHI greatly induced the mRNA for *ACS1*. These results suggest that CHI exerts its effect differentially on the ACC synthase multigenes and that protein synthesis may be necessary for the accumulation of *ACS6* and *ACS7* transcripts in the mung bean hypocotyl system. In green and pink tomato fruit, CHI superinduces the wound-induced ACC synthase mRNA accumulation (Linclon et al., 1993). Similarly, in etiolated rice seedling, CHI is capable of inducing the expression of IAA-inducible *OS-ACS1* gene and does not prevent its induction by IAA (Zarembinski and Theologis, 1993).

Differential induction of ACC synthase genes by auxin

To examine the temporal pattern of expression for each of the 3 ACC synthase genes in response to auxin, the time courses of ACC synthase mRNA levels were compared by Northern blot analyses. The *Kpn*I/*Xba*I fragment of pVR-ACS6 and *Sal*I/*Xba*I fragment of pVR-ACS7 both about 1 kb in length were used as probes. A probe of similar size for *VR-ACS1* was cloned by PCR using gene specific primers for the conserved region 1 and 7 (Botella et al., 1992). No cross-hybridization was detected among these probes under our hybridization and washing conditions (data not shown). Northern blot analyses showed that mRNAs for *ACS6* and *ACS7* were not expressed at a detectable level before auxin treatment, while marked induction of both transcripts was observed during the incubation with 100 mM IAA. Although the overall magnitudes of both transcripts during auxin treatment were similar, their induction kinetics appeared to be somewhat different. The level of transcript for *ACS6* reached a maximum at 2 h incubation, and subsequently decreased. In contrast, the maximum level of *ACS7* transcript was observed at 8 h incubation. The level of both transcripts rapidly declined after 8 h auxin treatment. As demonstrated by Botella et al. (1992), *ACS1* transcript was also induced during auxin treatment, but the level of this mRNA accumulation was variable and always lower than those of *ACS6* and *ACS7* mRNAs during the entire incubation period. Thus, auxin exerts its inductive effect on these genes differentially. No detectable transcripts for *ACS6* and *ACS7* were observed during the incubation without auxin, indicating that excision itself is not capable of inducing the expression of these genes. On the other hand, a low level of transcript for *ACS1* was detectable. These results suggest that *ACS1* gene may be also inducible by wounding and may not be an auxin-specific gene. This notion is consistant with the observation that CHI induces the expression of *ACS1* but not those of *ACS6* and *ACS7*.

Comparison of amino acid sequences of auxin-inducible ACC synthases

A comparison of deduced amino acid sequences among auxin-induced ACC synthases from *Arabidopsis* (*AT-ACS4* and *AT-ACS5*; Liang et al., 1992), winter squash (*CM-ACS4*; Nakagawa et al., 1991), tomato (*LE-ACS3*; Yip et al., 1992; Lincoln et al., 1993) and rice (*OS-ACS1*; Zarembinski and Theologis, 1993) reveals that these enzymes share a high degree of homology, about 65-75% sequence identity, to VR-ACS6 and VR-ACS7 polypeptides, but only about 50% to VR-ACS1 polypeptide. On the other hand, VR-ACS1 polypeptide shares 90% homology with GM-ACS1 enzyme whose synthesis is induced by

wounding in soybean (Liu et al., 1993). In addition to the seven highly conserved regions found in all ACC synthases studied (Dong et al., 1991; Kende, 1993), all auxin-induced ACC synthases including VR-ACS6 and VR-ACS7 polypeptides contain four more regions of high homology (indicated by stars) which are at least ten amino acid residues and show greater than 90% identity. The VR-ACS1 polypeptide, however, is more heterologous in these regions. Although the carboxyl termini are widely divergent among these polypeptides, C-terminal amino acid residue is always R except for VR-ACS1 enzyme. Phylogenetic analysis of amino acid sequences shows that *VR-ACS6* and *VR-ACS7* along with other auxin inducible genes in other species fall into the same lineage, but *VR-ACS1* belongs to a different lineage (Zarembinski and Theologis, 1993), indicating that *VR-ACS6* and *VR-ACS7* are closely related but are divergent from *VR-ACS1*. Our results support the notion that a striking correlation exists between the phylogenetic relationship and the pattern of expression among the ACC synthase family from various plant species (Lincoln et al., 1993).

Auxin and brassinosteroid differentially regulate the expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in mung bean (*Vigna radiata* L.)

Transcriptional induction of ACC synthase multigene family in response to IAA

We have previously shown that three ACC synthase genes, *VR-ACS1, VR-ACS6* and *VR-ACS7*, are induced by auxin in mung bean hypocotyls (Kim et al., 1997). In order to evaluate the effect of auxin on the transcriptional activities of these ACC synthase gene family, nuclei were obtained from mock- and 100 mM IAA-treated hypocotyls and subjected to nuclear run-on transcription (Walling et al., 1986). cDNAs representing the ACC synthase gene family were immobilized onto membrane filter and probed with equal amounts of radioactivity from the nuclear run-on reactions under high stringency. No detectable run-on transcriptional activities for all three genes were observed during the incubation without IAA. At 0.5 h IAA treatment, however, the transcriptional activities of *VR-ACS6* and *VR-ACS7* were relatively high, with the activity of *VR-ACS6* being 2 times higher than that of *VR-ACS7*. The transcription rates for both genes rapidly increased at 1 h and reached a maximum level at 2 h IAA treatment. The transcription of *VR-ACS1* was also induced by IAA, but the activity was significantly lower than those of *VR-ACS6* and *VR-ACS7* during the 2 h incubation period. As a negative control, plasmid DNA containing no cDNA insert showed undetectable level of hybridization. Thus, IAA rapidly induces (within 30 min) the expression of *VR-ACS6, VR-ACS7* and to a lesser extent *VR-ACS1* at the transcriptional level. In addition, these results are consistent with the view that IAA exerts its inductive effect differentially on these genes.

Specificity of ACC synthase mRNA accumulation

Abel et al. (1995) have demonstrated that among five *Arabidopsis* ACC synthase genes *ACS4* is rapidly (within 25 min) and specifically induced by IAA, and other plant hormones do not increase *ACS4* mRNA in etiolated *Arabidopsis* seedlings. We wanted to investigate the effect of various plant hormones on steady-state levels of mung bean ACC synthase mRNAs. Total RNAs were isolated from mung bean hypocotyls which had been incubated for various time periods with 10 mM IAA, 100 mM kinetin, 100 mM ABA, or 1 mM 24-epibrassinolide (BR), an active brassinosteroid (Zurek et al., 1994), and analyzed by Northern hybridization using pVR-ACS1, pVR-ACS6 or pVR-ACS7 as probes. *VR-ACS1* is highly inducible by 100 mM kinetin and 100 mM ABA in hypocotyls; the inductive effect of these two hormones is more evident than that of 10 mM IAA, indicating that *VR-ACS1* is not an auxin-specific gene. By contrast, the expression of *VR-ACS6* was specifically enhanced by IAA, and other growth regulators failed to induce the accumulation of the *VR-ACS6* mRNA. The expression of *VR-ACS7* was not affected by kinetin and ABA, but markedly induced by 1 mM BR. The time course studies showed that the temporal induction of *VR-ACS7* mRNA by BR exhibited a different pattern from that by IAA. With auxin treatment, the level of transcript for *VR-ACS7* reached a maximum at 4 h incubation, and subsequently declined. In contrast, the accumulation of mRNA continuously increased in response to BR until 24 h BR treatment. Brassinosteroid, an endogenous plant growth regulator, causes the elongation and division of the cell in a number of plant species (Clouse, 1997; Creelman and Mullet, 1997). From soybean epicotyls, Zurek and Clouse (1994) isolated the first BR-induced gene, *BRU1*, encoding a wall modifying enzyme possibly involved in BR-promoted stem elongation. Subsequently, Xu et al. (1995) found that the *TCH4* gene for xyloglucan endotransglycosylase was induced not only by various environmental stimuli, such as touch, cold stress and

heat shock, but also by auxin and BR in *Arabidopsis*. The time course studies revealed that 1 mM IAA rapidly induced the accumulation of *TCH4* transcript within 10 min, while the induction kinetics by 1 mM BR were slower than those by auxin, with increase in mRNA level being observed at 30 min BR treatment (Xu et al., 1995; Xu et al., 1996). In addition, the *SAUR* or *GH1* genes whose expressions are rapidly up-regulated by auxin, could be induced by BR only after 18-24 h treatment in soybean epicotyls (Clouse et al., 1992; Zurek et al., 1994). Consistent with those previous findings, our present results show that the temporal induction patterns of *VR-ACS7* by auxin and BR are different; the level of auxin-induced *VR-ACS7* mRNA peaks at 4 h incubation, whereas the accumulation of the transcript increases continuously until 24 h BR treatment, indicating that BR has a longer lag time and a greater prolonged effect on the induction of the *VR-ACS7* gene compared to auxin. Taken together, all these results give strong support for the suggestion that in addition to the interdependent cross-talk between auxin and BR there exist parallel signaling pathways of these two growth-promoting hormones for the regulation of *VR-ACS7* gene expression. Promoter analysis of *VR-ACS7* using transgenic tobacco plants provides additional evidence for this view (see below).

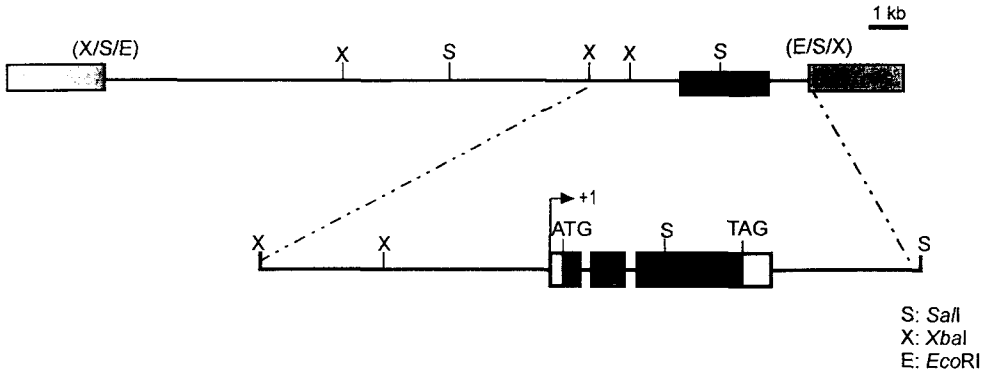
Differential effect of brassinosteriod on the expression of ACC synthase genes

BR was previously reported to stimulate ethylene production in mung bean hypocotyls (Yopp et al., 1979). Therefore, our present results are in agreement with the suggestion that the BR-induced ethylene production in hypocotyls is due to the expression of *VR-ACS7* mRNA. Furthermore, 1 mM BR has been known to promote the auxin-induced ethylene production in a synergistic manner in etiolated mung bean segments by stimulating the ACC synthase activity (Arteca et al., 1983; Schlagnhaufer et al., 1984). To assess the effect of BR (1 mM) in conjunction with IAA (10 mM) or kinetin (100 mM) on ethylene production and ACC synthase gene expression, we incubated mung bean hypocotyls for 6 h in the presence of various combinations of these hormones. A marked synergistic effect of BR on the IAA- and kinetin-induced ethylene synthesis was observed at 6 h hormone treatment, and the greatest amount of ethylene was produced when all three hormones were present together. BR enhanced the inductive effect of IAA on the level of *VR-ACS6* mRNA. While BR or kinetin alone has very little effect on the expression of *VR-ACS6* mRNA, a clear synergistic induction of *VR-ACS6* was observed when these two hormones worked together. A modest synergistic effect of BR on the accumulation of *VR-ACS7* mRNA was also detected when BR was present along with IAA or kinetin As was the case in ethylene production, there was a great induction of both the *VR-ACS6* and *VR-ACS7* genes when all three growth regulators were present. These results indicate that BR in conjunction with IAA and kinetin positively mediates the expression of the *VR-ACS6* and *VR-ACS7* genes in mung bean hypocotyls. By contrast, BR effectively abolished both the IAA- and kinetin-induced accumulation of *VR-ACS1* mRNA. Only a near background level of *VR-ACS1* transcript was detected when hypocotyls were incubated with 10 mM IAA + 100 mM kinetin + 1 mM BR. Thus, BR acts negatively on the expression of *VR-ACS1*. These results suggest that BR exerts its effect differentially on these IAA-inducible genes by the opposite regulatory mechanisms in mung bean hypocotyls. Thus, it appears that there are two groups of IAA-responsive ACC synthase transcripts, either BR-induced or BR-suppressed. Overall, these results indicate that regulation of ACC synthase genes occurs via multiple mechanisms by which IAA and BR act by both the parallel and interdependent pathways in hypocotyls.

Tissue specific expression of ACC synthase mRNAs

To examine whether the *VR-ACS1*, *VR-ACS6* and *VR-ACS7* genes are differentially induced by auxin and BR in a tissue specific manner, Northern analysis was carried out with RNAs isolated from mock- or hormone-treated roots, leaves and epicotyls of four-week-old light-grown mung bean seedlings. The substantial level of *VR-ACS1* transcript was found in control roots but not in leaves and epicotyls. The *VR-ACS1* was slightly induced in the roots after 6 h of 10 mM IAA incubation, but its induction was not detected in other tissues. As found in dark-grown hypocotyls, 1 mM BR suppressed the IAA-induced *VR-ACS1* gene expression in roots. The abundance of *VR-ACS6* mRNA increased in response to IAA only in the epicotyls, and the IAA-induction was synergistically enhanced by BR treatment in this tissue. On the other hand, the expression of *VR-ACS7* was not observed in all light-grown tissues examined except for in the IAA + BR treated epicotyls, where the *VR-ACS7* mRNA was barely detectable. Thus, the *VR-ACS7* gene is expressed mainly in the etiolated hypocotyls, suggesting that *VR-ACS7* may play a role in the early stage of development of seedling. Taken together, these results indicate that the expression of ACC synthase gene family is spatially regulated by auxin and BR in a gene specific manner

A.



B.

```
                                                                    tctagtttaacaaaaacatgtatgaatttatcttctcttaatgttggaaacataaa
taagagaatcaagattaaatgtggagtgtgagaatttgttaaaaatatgttataattaagaaatggtgaaagaatataaaatatagggttggttttatag
ttaaaaaagagaaaggaaatcagtgagcggagggggcaatgaaagtgagtagagtagtaaaagtaagtgtgagagtgtgatgagttgaatggtt
ttagcaacgacctaataacagagatatatactaaaaagagatgaattgttgttttggcttatgtgaccctatcacttcacctaatgcatctcatttatcca -2001
ttttaatacatcaccaaatcagctacctcaataaaactaagggcggatcacacctttccaactctctcctcttattattgcttcctttcccattctctttt
ttttttttttttaattcttcccatcccaccaacttcccatcttctttttttttccctttttactaattatatacacacatgcctcttgtttatacaa
aaacctaggggcgaaactgcattcaggacttttctctgtttcattaaatttaacttactctcaaaccaacctactttcaatattttccaaaacagaaaag
tattcccaagaatacagtcattgatatttaagaaaatctatatgcacacccaaagaaaaacaaatagttactacatttaaattaatttttctttttctc
actttaaaaaagacaaatatcctctcaagtgaagcatgattaatatgagattatattagaaataagtaagcgtatttttttttaacaaaatataattatt -1501
gttattgaaaaaatggtaaaatacaaaagtatgttttgagtgacatggaaacagacaagtaataaccaccagagatttgtgattatttgtgtttgaattt
gttattcttttatcttgagttgtatatttatattatagctccaacactgaacagattcaccaatcttcaacctttttagatatctagaaagtattaca
ttgtttcatcatcatctccttttagagtgggcctcacctgattgagaatctttgttattgtgattttataatagtagaaaacaaactattattatcttgg
ttacagaagaaaatcaaacccaattatcttatttgttttctcatgtatgattatttattggtagaaggaacataatttaactgagcagtaaggaaaattg
acgtatatgaagagtgttgaaccccagaaaatgtttctccaatggtaagagggaagagaaaaatatcgtgtatgagtacattcgaataaaatttaaatgt -1001
gggtatctgtttatttagatttattttcataaatttttaaataaatattcaataaataatagaaaaagtaaggaataaaaaatttgtttgatatgaatag
gtaatattggacctaacatgttgtcatccgaagacacagatgtcacatgacaagttgcagttatataggattttacttgtatagttttttgtggttgaa
tagaaatttatgatggcgataatcatatgtgtgttaatatagaaattggaaagtttgaccaatgaaattgatatgcttacgtgatgcaatctctaact
ctgggtgagagagtataattaaaaggtatgagtgatattgtgttgaaattgaattctatagtggagaatccaaatcaataaatttgttagtttggta
ggcagagccaaaggcgagacattggacatgtttggggaggttaaaatgaacaaaataccgacatcaacttggctggttaggctaaaaggaagcagatggac -501
catccccaagggtccatatataaccttttgatggacctcctgacccacttttttccatgagatttctctcccaaacaaaaaatacccctatcaactttc
tcttaacttacccctcctccaattaacattaccttctaacaaacctagacatcccaataaaaacaacaaacacaacaaaaacattcattatttcttga
ctcttataaaattcaatatcccacgctagattatacccgctgggagacactttccacaaatactgtcaataacattttagtccctgctacttattcagcga
tactagggagatttggacttttctcgtgacatgcttcggacggtggacaaaagcgctttaaattaaacccatttattcactcactctctctcttctcgagaa
accctacatttttttattctcaaactgtttcaccattgccacttggctctctacccttttggacttccactataaatatataaccaacctactcctcttc   -1
atacacacacaaaatttctcttcaacattccactccgaatattcttctcttcttcttcctttccgccatcacctcttcattcctttcctaccatcccaaacac
acactaacatacaaaATGAGGCTCTTGTCCACAATAGCAACGTGCAATAGTCACGGCCAAGACTCTTCATACTTTTTAGGATGGCAGGAATACGAGAAAA
                  M  R  L  L  S  T  I  A  T  C  N  S  H  G  Q  D  S  S  Y  F  L  G  W  Q  E  Y  E  K
ACCCTTACGATGAACTCCATAACCCCAAAGGAATTATCCAGATGGGTTTAGCGGAGAATCAGgttagacattcatttttctttcttcaaaataataacca
N  P  Y  D  E  L  H  N  P  K  G  I  I  Q  M  G  L  A  E  N  Q
ctgtctttattctattacttacttcaaaacttgtttcacttacaaaatttttctttttcaattgcagCTCTCTTTCGACCTCCTAGAGTCATGGCTGGCTA
                                                                  L  S  F  D  L  L  E  S  W  L  A
AGAACCCTGACGTCGCCGGCTTCAAACGAGACGGCAAATCCATATTTAGAGAACTGGCTCTTTTCCAGGACTACCACGGCCTCCCTTCCTTCAAAAAGGC  500
K  N  P  D  V  A  G  F  K  R  D  G  K  S  I  F  R  E  L  A  L  F  Q  D  Y  H  G  L  P  S  F  K  K  A
ATTGGTAGATTTCATGTCTGAAATCAGAGGAAACAAAGTCACTTTCGACCCCAACCACATCGTTCTCACTGCCGGTTCCACTTCCGCAAACGAAACCTTA
  L  V  D  F  M  S  E  I  R  G  N  K  V  T  F  D  P  N  H  I  V  L  T  A  G  S  T  S  A  N  E  T  L
ATGTTCTGTCTTGCCGAACAAGGAGACGCATTCCTCCTTCCGACTCCTTATTACCCAGGgtgcgtatatttttttatcttaatcttatcgcttatattcca
 M  F  C  L  A  E  Q  G  D  A  F  L  L  P  T  P  Y  Y  P  G
cttcatgctatgatatttttacgtgacttttatttttataatttttttatgcattcccctgaccttgctttattctatacagATTTGACAGAGACCTCAAGTG
                                                                                    F  D  R  D  L  K  W
GAGAACCGGTGCTGAGATTGTTCCAATACAATGCACAAGTTCTAACAACTTCCAAATTACGGAGCCAGCTCTGAAACAGGCTTACGAGGAGGCCCGCAAG
  R  T  G  A  E  I  V  P  I  Q  C  T  S  S  N  N  F  Q  I  T  E  P  A  L  K  Q  A  Y  E  E  A  R  K
CGCAACCTCAGAGTCAAAGGCGTTCTGGTGACAAACCCGTCAAACCCGCTAGGCACCACCATGTCTCGGAGTGAATTGAACCTTCTCGTCGACTTCATCA  1000
 R  N  L  R  V  K  G  L  L  V  T  N  P  S  N  P  L  G  T  T  M  S  R  S  E  L  N  L  L  V  D  F  I
AAGAGAAAAAAGACACGCATTTGATAAGCGACGAGATATACTCCGGGACGGTTTTTAGCTCTCCAGGTTTCGTCAGTGTCATGGAAATCCTCAAGGAGCG
K  E  K  K  T  H  L  I  S  D  E  I  Y  S  G  T  V  F  S  S  P  G  F  V  S  V  M  E  I  L  K  E  R
AAACGATACTGAGGAAATCTGGAACAGAGTTCACGTTGTCTACAGTCTATCCAAAGACTTGGGTTTGCCGGGTTTCCGCGTCGGCGCTATCTACTCGGAA
  N  D  T  E  E  I  W  N  R  V  H  V  V  Y  S  L  S  K  D  L  G  L  P  G  F  R  V  G  A  I  Y  S  E
AATGACACCGTGGTTGCCGCCGCAACGAAGATGTCGAGCTTTGGGTTGGTCTCTTCGCAGACGCAGTACCTCCTCTCTGCCATGCTGGGCGACAAGAAGT
  N  D  T  V  V  A  A  A  T  K  M  S  S  F  G  L  V  S  S  Q  T  Q  Y  L  L  S  A  M  L  G  D  K  K
TCGCCCGAAACTACATCGTTGAGAACCAGAAGAGGCTTAAGCGACGACAGAGAATGATGGTTTCGGGGTTACAAAAAGCTGGTATTTCTTGCCTGGAGAG
F  A  R  N  Y  I  V  E  N  Q  K  R  L  K  R  R  Q  R  M  M  V  S  G  L  Q  K  A  G  I  S  C  L  E  S
CAATGCCGGGTTGTTTTGCTGGGTTGACATGAGACACCTTTTGCATTCGAACACGTTCGAAGCTGAGATGGAGCTGTGGAAGAAAATAGTGTACCAAGTT  1500
  N  A  G  L  F  C  W  V  D  M  R  H  L  L  H  S  N  T  F  E  A  E  M  E  L  W  K  K  I  V  Y  Q  V
GGGTTAAACATTTCCCCTGGGTCGTCGTGCCATTGCACCGAGCCAGGGTGGTTCCGTGTGTGTTTCGCTAACATGTCCGAGGAGACTCTCACCTTGGCCA
 G  L  N  I  S  P  G  S  S  C  H  C  T  E  P  G  W  F  R  V  C  F  A  N  M  S  E  E  T  L  T  L  A
TGAAACGCTTGAAGAACTTCGTCGTTGAGTCCACCTGCACTCAGAGAAGTGCTCTGTTCAACGGAACCTCCAAAAGAAAGTCTCTCACCAAGTGGGTTTT
M  K  R  L  E  N  F  V  V  E  S  T  C  T  Q  R  S  A  L  F  N  G  T  S  K  R  K  S  L  T  K  W  V  F
CCGGTTATCGTCTTGCGACCATCGTGAACAGGAAGAACGGTAGcctggtcttagtctatgcgtgttcgctcatgcataaattcatttcaatttcttttttc
 R  L  S  S  R  D  H  R  E  Q  E  E  R  *
ttttccccttttttgcctccccaatattttttggtggagtagttcaaaaaattccctttattttttcttcatttttttttttttgtgataaaattaatgtagaga
gagagaaacaaaaacaaagcactcggtccatgtggatgtgtagtggtttgggcttctagtcagccttgtttgtatgtaagttgttccatatctcagtcattaa  2000
ttccctacaaaatgtccttatatcattaatgaaatgtttattgatacta
```

Figure 1. The organization, nucleotide sequence and translation product of *VR-ACS7*. A. The organization graphic depicts the overall structure of *VR-ACS7*. Exons are shown as filled in boxes, introns are connecting lines, and 5'- and 3'-untranslated regions open boxes. B. The complete nucleotide sequence of the *VR-ACS7* gene including three exons, two introns, and 5' and 3'-flanking regions. The coding sequence is capitalized, while the introns and untranslated regions are illustrated in lowercase. Numbering is relative to the transcriptional start site. Putative cis-acting elements, TATA box and two direct repeated sequences (5'-TGAAATTGA-3' and 5'-TGGACTTC-3') in the 5'-upstream region are underlined in bold. The predicted amino acid sequence is presented in the one letter code under the DNA sequence. The translational initiation and termination signals are shown as bold letters. The primer sequence for the primer extension analysis is underlined. The invariant amino acid residues conserved between ACC synthase and various aminotransferases are shaded. The underlined dodecapeptide indicates the active site center of ACC synthase.

in mung bean plants.

Isolation and characterization of the VR-ACS7 gene

Although many auxin-inducible ACC synthase genes have been studied extensively in various species, information about their promoter and the elements responsible for the auxin-mediated gene expression have been limited. In order to gain information concerning the structure of the IAA- and BR-responsive *VR-ACS7* gene as well as to investigate its promoter sequence, we proceeded to isolate and characterize the genomic clone. Three clones were obtained from a library of genomic DNA fragments prepared from leaves of mung bean plants. Restriction enzyme digests of DNAs isolated from these clones showed that they contained inserts of approximately 10-15 kb. Subsequent restriction enzyme map and partial DNA sequencing analyses of each of these clones revealed that the genomic clones represent a single group of overlapping sequences. Thus, one clone, lVR-ACS7-1, was selected for further analysis (Figure 1A). The coding region, as well as the 5'- and 3'-flanking regions of lVR-ACS7-1 were sequenced for a total 4.4 kb (GenBank accession number AF151961). This 4.4 kb DNA fragment contained the entire coding sequence and about 2.4 kb of the 5'-upstream region (Figure 1B). The *VR-ACS7* gene consists of three exons interrupted by two introns whose junctions are in agreement with the consensus intron/exon borders of plant genes (Brown, 1986). The sizes of each of the two introns are 104, and 120 bp, respectively. The coding region of *VR-ACS7* comprises 1404 bp encoding a protein of 468 amino acid residues with a predicted molecular mass of 53.1 kDa. This coding sequence completely matched to the previously reported sequence of pVR-ACS7. The start site of transcription for *VR-ACS7* was determined by primer extension analysis using reverse transcriptase and the primer that is complementary to the 5'-untranslated region of *VR-ACS7* (Figure 1B). One major primer extension product was obtained with RNA prepared from 100 mM IAA-treated hypocotyls but not from control tissue, confirming that *VR-ACS7* was highly inducible by IAA (Figure 2). The start site is located 115 nucleotides upstream from the 5'-end of the coding region of the *VR-ACS7* gene, defining the size of the 5'-untranslated region of the *VR-ACS7* mRNA to be 115 nucleotides long (Figure 1B). A putative TATA box was found at the 25 nucleotide upstream from the transcriptional start site.

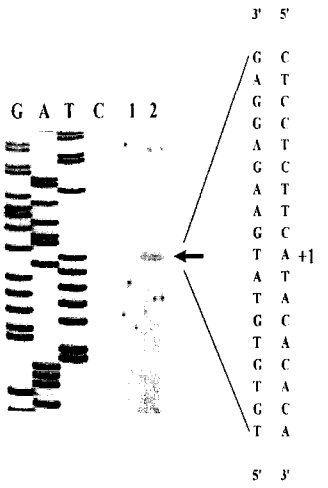


Figure 2. Primer extension analysis to identify the transcriptional start site of VR-ACS7. ^{32}P-labeled primer (Figure 1B) was hybridized with 30 mg total RNA obtained from mock-and IAA-treated hypocotyls, and extended with reverse transcriptase. Single primer extension product is indicated by arrow. The transcriptional start site is shown as + 1 in the DNA sequence. Lane 1, control (without IAA); lane 2, 100 mM IAA.

Promoter analysis in transgenic tobacco plants

To link the expression properties of the *VR-ACS7* gene with its promoter region, the transcriptional fusion of the β-glucuronidase (GUS) coding region with *VR-ACS7* upstream sequence comprising the 2356 bp promoter and 82 bp 5'-untranslated regions was constructed in the binary plant transformation vector. The expression of the *VR-ACS7-GUS* fusion was then analyzed by qualitative and quantitative measurements of GUS activity in transgenic tobacco plants. Before auxin treatment, transgenic etiolated tobacco seedlings contained low levels of GUS specific activity. Interestingly, histochemical assay showed weak but detectable *VR-ACS7* promoter-driven GUS blue staining at a highly localized region of the hypocotyl-root junction of control plants (Figure 3A). After treatment with 100 mM IAA for 24 h, transgenic seedlings harboring the *VR-ACS7-GUS* fusion revealed an 8 to 11-fold (an average 9-fold) induction of GUS specific activity depending on the transgenic lines. The auxin induction of GUS activity was also verified by the histochemical analysis which showed a marked increase in the *VR-ACS7* promoter blue staining in the hypocotyl region of transgenic seedlings (Figure 3B). These results indicate that the auxin-responsive sequences are present in the 2.4 kb *VR-ACS7* promoter region and that the auxin-inducible expression of *VR-ACS7* is primarily controlled at the transcriptional level. The induction of GUS staining was not detected in transgenic cotyledons and roots,

suggesting that the promoter contains the regulatory sequence for the tissue specific expression of *VR-ACS7*. GUS expression was also investigated in etiolated transgenic seedlings after incubation with 1 mM BR for 24 h. In contrast to auxin, BR did not result in the induction of GUS activity. These results are at variance with those obtained by RNA gel-blot analysis which shows the marked BR induction of *VR-ACS7* in mung bean hypocotyls. Thus, it could be possible to suggest that the induction of *VR-ACS7* by BR is largely controlled by posttranscriptional processes. In deed, Zurek and Clouse (1994) have shown that the BR-specific induction of *BRU1* is regulated at the posttranscriptional level in soybean epicotyls. Alternately, it could be argued that there might be the negative regulatory sequences in the promoter region for the BR-responsive expression of *VR-ACS7*. Finally, there may be the specific *cis*- and *trans*-acting factors functional only in the mung bean hypocotyl cells for the BR induction of *VR-ACS7*. Consistent with Northern data, there was a modest synergistic effect of BR on the auxin-induced GUS activity (an average 12-fold induction) in transgenic tobacco seedlings. The synergistic effect of BR was more evident when the transgenic seedlings were incubated with lower concentration (1 mM) of IAA.

In conclusion, our present results are consistent with the hypothesis that the expression of ACC synthase, the rate-limiting step in ethylene biosynthesis, is controlled by multiple regulatory pathways of auxin and BR in mung bean seedlings. The characterization of the molecular mechanism of auxin- and BR-regulated ACC synthase gene expression, by means of the identification of regulatory *cis*-acting elements and *trans*-acting factors interacting with these elements as well as cellular components involved in the signaling of these growth-promoting hormones, would bring us one step closer to understanding the mode of interactions between auxin, BR and ethylene in higher plant cells.

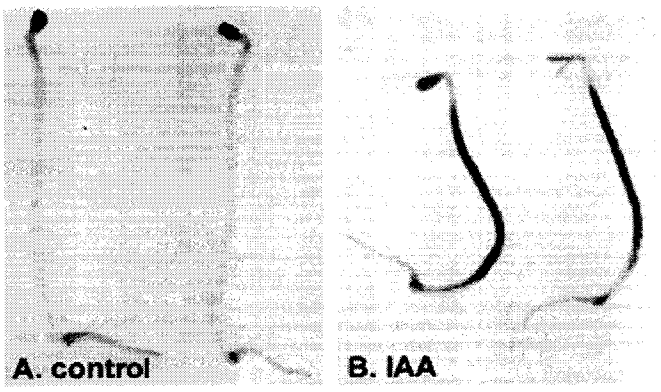


Figure 3. Analysis of the *VR-ACS7* promoter in transgenic tobacco seedlings. Histochemical localization of GUS activity in 5-day-old etiolated transgenic tobacco seedlings. Transgenic seedlings harboring the *VR-ACS7-GUS* fusion were incubated with (A) or without (B) 100 mM IAA for 24 hours.

References

Abel S, Nguyen MD, Chow W, Theologis A (1995) *ACS4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J Biol Chem 270: 19093-19099.

Abel S, Theologis A (1996) Early genes and auxin action. Plant Physiol 111: 9-17.

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in Plant Biology. Academic Press, San Diego, CA.

Arteca JM, Arteca RN (1999) A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (ACS 6) in mature *Arabidopsis* leaves. Plant Mol Biol 39: 209-219.

Arteca RN, Tsai D-S, Schlagnhaufer C, Mandava NB (1983) The effect of brassinosteroid on auxin-induced ethylene production by etiolated mung bean segments. Physiol Plant 59: 539-544.

Botella JR, Arteca JM, Schlagnhaufer CD, Arteca RN, Phillips AT (1992) Identification and characterization of a full-length cDNA encoding for an auxin-induced 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyl segments and expression of its mRNA in response to indole-3-acetic acid. Plant Mol Biol 20: 425-436.

Botella JR, Schlagnhaufer CD, Arteca JM, Arteca RN, Phillips AT (1993) Identification of two new members of the 1-aminocyclopropane-1-carboxylate synthase-encoding multigene family in mung bean. Gene 123: 249-253.

Botella JR, Schlagnhaufer CD, Arteca RN, Phillips AT (1992) Identification and characterization of three putative genes for 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyl segments. Plant Mol Biol 18: 793-797.

Brown JWS (1986) A catalogue of splice junctions and putative branch point sequences from plant introns. Nucleic Acids Res 14: 9549-9559.

Bui AQ, O'Neill SD (1998) Three 1-aminocyclopropane-1-carboxylate synthase genes regulated by primary and secondary pollination signals in orchid flowers. Plant Physiol 116: 419-428.

Clouse SD (1997) Molecular genetic analysis of brassinosteroid action. Physiol Plant 100: 702-709.

Clouse SD, Zurek DM, McMorris TC, Baker ME (1992) Effect of brassinolide on gene expression in elongating soybean epicotyls. Plant Physiol 100: 1377-1383.

Creelman RA, Mullet JE (1997) Oligosaccharins, brassinolides, and jasmonates: Nontraditional regulators of plant growth, development, and gene expression. Plant Cell 9: 1211-1223.

Destefano-Beltran LJC, Van Caeneghem W, Gielen J, Richard L, Van Montagu M, Van Der Straeten D (1995) Characterization of three members of the ACC synthase gene family in *Solanum tuberosum* L. Mol Gen Genet 246: 496-508.

Dong JG, Kim WT, Yip WK, Thompson GA, Li L, Bennett AB, Yang SF (1991) Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. Planta 185: 38-45.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227: 1229-1231.

Jin ES, Lee J-H, Park JA, Kim WT (1999) Temporal and spatial regulation of the expression of 1-aminocyclopropane-1-carboxylate oxidase by ethylene in mung bean (*Vigna ra-*

diata). Physiol Plant 105: 132-140.

Jones ML, Woodson WR (1999) Differential expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in carnation. Plant Physiol 119: 755-764.

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44: 283-307.

Kende H, Zeevaart JAD (1997) The five "classical" plant hormones. Plant Cell 9: 1197-1210.

Kim WT, Campbell A, Moriguchi T, Yi HC, Yang SF (1997) Auxin induces three genes encoding 1-aminocyclopropane-1-carboxylate synthase in mung bean hypocotyls. J Plant Physiol 150: 77-84.

Kim WT, Silverstone A, Yip WK, Dong JG, Yang SF (1992) Induction of 1-aminocyclopropane-1-carboxylate synthase mRNA by auxin in mung bean hypocotyls and cultured apple shoots. Plant Physiol 98: 465-471.

Kim WT, Yang SF (1994) Structure and expression of cDNAs encoding 1-aminocyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. Planta 194: 223-229.

Liang XL, Abel S, Keller JA, Shen NF, Theologis A (1992) The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 89: 11046-1105.

Lincoln JE, Campbell AD, Oetiker J, Rottmann WH, Oeller PW, Shen NF, Theologis A (1993) LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene in tomato (*Lycopersicon esculentum*): Expression in *E. coli*, structural characterization, expression characteristics, and phylogenic analysis. J Biol Chem 268: 19422-19430.

Liu D, Li N, Dube S, Kalinski A, Herman E, Mattoo AK (1993) Molecular characterization of a rapidly and transiently wound-induced soybean gene encoding 1-aminocyclopropane-1-carboxylate synthase. Plant Cell Physiol 34: 1151-1157.

Lizzardi PM (1983) Methods for the preparation of messenger RNA. Methods Enzymol 96: 24-38.

Martin T, Wohner R-V, Hummel S, Willmitzer L, Frommer WB (1992) The GUS reporter system as a tool to study plant gene expression. In: S.R. Gallagher (Ed.), GUS protocol: using the GUS gene as a reporter of gene expression. Academic Press, Inc., San Diego, CA.

Oetiker JH, Olson DC, Shiu OY, Yang SF (1997) Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*). Plant Mol Biol 34: 275-286.

Olson DC, Oetiker JH, Yang SF (1995) Analysis of *LE-ACS3*, a 1-aminocyclopropane-1-carboxylic acid synthase gene expressed during flooding in the roots of tomato plants. J Biol Chem 270: 14056-14061.

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. Proc Natl Acad Sci USA 88: 5340-5344.

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. Proc Natl Acad Sci USA 88: 5340-5344.

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy, BP, Taylor LP, Campbell AD, Theologis A (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol 222: 937-961.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schlagnhaufer C, Arteca RN, Yopp JH (1984) Evidence that brassinosteroid stimulates auxin-induced ethylene synthesis in mung bean hypocotyls between S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid. Physiol Plant 61: 555-558.

Schlagnhaufer CD, Arteca RN, Pell EJ (1997) Sequential expression of two 1-aminocyclopropane-1-carboxylate synthase genes in response to biotic and abiotic stresses in potato (*Solanum tuberosum* L.) leaves. Plant Mol Biol 35: 683-688.

Shen WJ, Forde BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucleic Acids Res 17: 8385.

Shiu OY, Oetiker JH, Yip WK, Yang SF (1998) The promoter of *LE-ACS7*, an early flooding-induced 1-aminocyclopropane-1-carboxylate synthase gene of the tomato, is tagged by a *Sol3* transposon. Proc Natl Acad Sci USA 95: 10334-10339.

Spanu P, Boller T, Kende H (1993) Differential accumulation of transcripts of 1-aminocyclopropane-1-carboxylate synthase genes in tomato plants infected with *Phytophthora infestans* and in elicitor-treated tomato cell suspensions. J Plant Physiol 141: 557-562.

Theologis A (1992) One rotten apple spoils the whole bushel: The role of ethylene in fruit ripening. Cell 70: 181-184.

Tsai DS, Arteca RN, Arteca JM, Phillips AT (1991) Characterization of 1-aminocyclopropane-1-carboxylate synthase purified from mung bean hypocotyls. J Plant Physiol 137: 301-306

Tsai DS, Arteca RN, Bachman JM, Phillips AT (1988) Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyls. Arch Biochem Biophys 264: 632-640.

Tuomainen J, Betz C, Kangasjarvi J, Ernst D, Yin Z-H, Langebartels C, Sandermann Jr H (1997) Ozone induction of ethylene emission in tomato plants: regulation by differential accumulation of transcripts for biosynthetic enzymes. Plant J 12: 1151-1162.

Vahala J, Schlagnhaufer CD, Pell EJ (1998) Induction of an ACC synthase cDNA by ozone in light-grown *Arabidopsis thaliana* leaves. Physiol Plant 103: 45-50.

Van der Straeten D, Van Wiemeersch L, Goodman HM, Van Montagu M (1990) Cloning and sequence of two different cDNAs encoding 1-aminocyclopropane-1-carboxylate synthase in tomato. Proc Natl Acad Sci USA 87: 4859-4863.

Walling L, Drews GN, Goldberg RB (1986) Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc Natl Acad Sci USA 83: 2123-2127.

Woeste KE, Ye C, Kieber JJ (1999) Two *Arabidopsis* mutants that overproduce ethylene are affected in the post-transcriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. Plant Physiol 119: 521-529.

Xu W, Campbell P, Vargheese AK, Braam J (1996) The *Arabidopsis XET*-related gene family: Environmental and hormonal regulation of expression. Plant J 9: 879-889.

Xu W, Purugganan MM, Polisensky DH, Antosiewicz DM, Fry SC, Braam J (1995) *Arabidopsis* TCH4, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. Plant Cell 7: 1555-1567.

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155-189.

Yip W-K, Moore T, Yang SF (1992) Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. Proc Natl Acad Sci USA 89: 2475-2479.

Yoon IS, Mori H, Kim JH, Kang BG, Imaseki H (1997) *VR-ACS6* is an auxin-inducible 1-aminocyclopropane-1-carboxylate synthase gene in mung bean (*Vigna radiata*). Plant Cell Physiol 38: 217-224.

Yopp JH, Colclasure GC, Mandava N (1979) Effects of bras-

sin complex on auxin and gibberellin mediated events in the morphogenesis of the etiolated bean hypocotyl. Physiol Plant 46: 247-254.

Yoshii H, Imaseki H (1982) Regulation of auxin-induced ethylene biosynthesis. Repression of inductive formation of 1-aminocyclopropane-1-carboxylate synthase by ethylene. Plant Cell Physiol 23: 639-649.

Zarembinski TI, Theologis A (1993) Anaerobiosis and plant growth hormones induce two genes encoding 1-aminocyclopropane-1-carboxylate synthase in rice (*Oriza sativa* L.). Mol Biol Cell 4: 363-373.

Zurek DM, Clouse SD (1994) Molecular cloning and characterization of a brassinosteroid-regulated gene from elongating soybean (*Glycine max* L.) epicotyls. Plant Physiol 104: 161-170.

Zurek DM, Rayle DL, McMorris TC, Clouse SD (1994) Investigation of gene expression, growth kinetics, and wall extensibility during brassinosteroid-regulated stem elongation. Plant Physiol 104: 505-513.