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Scott Charles Peck

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Major professor

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POSITIVE AND NEGATIVE FEEDBACK REGULATION OF ETHYLENE BIOSYNTHESIS INDUCED BY INDOLE-3-ACETIC ACID

By

Scott Charles Peck

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ABSTRACT

POSITIVE AND NEGATIVE FEEDBACK REGULATION
OF ETHYLENE BIOSYNTHESIS INDUCED
BY INDOLE-3-ACETIC ACID

By

Scott Charles Peck

A cDNA clone, pPE8, encoding 1-aminocyclopropane-1-carboxylate (ACC) oxidase from etiolated pea stems was isolated. The insert was expressed in transgenic yeast to confirm that it encoded a functional ACC oxidase. The localization of an ACC oxidase from tomato was studied in transformed yeast. Transgenic ACC oxidase accumulated in the yeast cells but could not be detected in the vacuole as determined by activity assays and by immunoblot analysis using ACC oxidase-specific antibodies. These results indicated that ACC oxidase was not associated with the vacuole as had been previously suggested. In 5- to 6-day-old etiolated pea stems, ethylene induced an increase in ACC oxidase transcript level and enzyme activity. Indole-3-acetic acid (IAA), which stimulates ethylene production, also caused an increase in ACC oxidase mRNA levels and enzyme activity. This increase was blocked by 2,5-norbornadiene (NBD), a competitive inhibitor of ethylene action. An increase in the level of ACC synthase mRNA and enzyme activity preceded the increase in ACC oxidase levels after IAA treatment. These results indicate that the enzymes of ethylene biosynthesis are sequentially induced after treatment of intact pea seedlings with IAA and that ethylene promotes the accumulation of ACC oxidase mRNA and enzyme activity through a

positive feedback loop. Two cDNA clones of IAA-induced ACC synthase, PS-ACS1 and PS-ACS2, were isolated. Ethylene inhibited the IAA-induced accumulation of transcripts of both clones. Results from genomic southern blot analysis, RNA blot analysis using a probe from the 3'-untranslated region, and oligonucleotide-directed RNAse H mapping indicated that a single gene for PS-ACS1 produces two transcripts (1.6 kb and 1.9 kb) with differences in the 5'-ends of the transcripts.

For my parents

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
IAA	indole-3-acetic acid
IPNS	isopenicillin N synthase
NBD	2,5-norbornadiene
PCR	polymerase chain reaction
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SAM	S-adenosyl-L-methionine
UTR	untranslated region

GENERAL INTRODUCTION

Ethylene controls a wide range of physiological and developmental processes including ripening of fruit, abscission, senescence, and responses to wounding and auxin (Abeles et al., 1992). Most of these ethylene effects are associated with changes in ethylene production. Thus, defining the regulation of ethylene biosynthesis is of great importance in understanding the role of ethylene in growth and development.

In higher plants, ethylene is produced from methionine via S-adenosyl-L-methionine (AdoMet) and the cyclic, nonprotein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). The formation of AdoMet from methionine is catalyzed by AdoMet synthase. Besides being an intermediate of ethylene biosynthesis, AdoMet acts as a methyl donor in a number of biosynthetic reactions and as an intermediate in polyamine bioysynthesis. The first committed step of ethylene biosynthesis, the formation of ACC from AdoMet, is catalyzed by ACC synthase (Abeles et al., 1992). The 5'-methylthioadenosine released during this reaction enters a modified methionine cycle which maintains methionine levels even during periods of high ethylene production (Miyazaki and Yang, 1987). The final enzyme, ACC oxidase, converts ACC to ethylene. Besides being converted to ethylene, ACC is metabolized to 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC), permanently removing the ACC from the ethylene biosynthetic pathway under most physiological conditions (Yang and Hoffman, 1984).

Ethylene regulates its own biosynthesis. In ripening fruits and senscing tissues,

ethylene production is autocatalytic. In vegetative tissue, however, ethylene both positively and negatively regulates its own biosynthesis (Abeles et al., 1992). Ethylene stimulates the accumulation of ACC oxidase activity in citrus leaves (Riov and Yang, 1982), tobacco leaves (Chalutz et al., 1984), and in etiolated pea stems (Schierle et al., 1989). Autoinhibition of ethylene seems to be directed primarily at ACC synthase. Ethylene partially suppresses the increase in extractable ACC synthase activity in indole-3-acetic acid (IAA)-treated mung bean hypocotyl sections (Yoshii and Imaseki, 1982). In etiolated pea stems, propylene (an ethylene analog) completely inhibits wound-induced ethylene accumulation (Saltveit and Dilley, 1979).

The goal of this dissertation was to examine the role of negative and positive feedback on IAA-induced ethylene production in etiolated pea stems. It is now known that multiple ACC synthase genes responsive to either IAA or wounding would have been induced under the conditions used in the previous IAA-induction experiments. Gene specific probes can determine if expression of some or all of the genes expressed after auxin treatment are negatively regulated by ethylene. Molecular probes also allow greater sensitivity in detecting rapid and subtle changes in ACC synthase and ACC oxidase transcript levels after IAA treatment. This sensitivity is especially important in examining the role of ethylene-induced ACC oxidase activity in the regulation of ethylene production.

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CHAPTER 1

CLONING OF A cDNA ENCODING 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE FROM PEA

Abstract

A clone for 1-aminocyclopropane-1-carboxylate (ACC) oxidase was isolated from a cDNA library made from poly(A)$^+$ RNA of apical hooks of etiolated peas treated for 4 h with 10^{-4} M IAA and was sequenced. The 1122-bp insert was subcloned into a yeast expression vector which was then used to transform yeast strain F808. When induced with galactose, transgenic yeast with the insert in the coding orientation was able to convert ACC to ethylene, and the conversion was inhibited by $CoCl_2$ and aminoisobutyrate, two inhibitors of ACC oxidase activity. By immunoblot analysis, it was shown that polyclonal antibodies raised against a tomato ACC oxidase recognized a protein of the expected size in transgenic yeast if the insert was in the coding orientation but not if it was in a non-coding orientation. These results confirm that the cDNA clone encodes a functional ACC oxidase from peas.

Introduction

The final step of ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by ACC oxidase (also referred to as the "ethylene-forming enzyme," EFE). Knowledge about ACC oxidase remained limited long after the pathway of ethylene biosynthesis had been elucidated (see Kende, 1993). Attempts to purify and characterize the enzyme failed because enzymatic activity disappeared when the tissue was homogenized. While a number of cell-free systems were capable of converting ACC to ethylene, they all lacked the stereospecificity exhibited by the *in vivo* ethylene-forming system with respect to the conversion of 2-ethyl-ACC stereoisomers to 1-butene (Yang and Hoffman, 1984).

The enzyme was finally identified by the characterization of a ripening-related cDNA clone from tomato fruits. Slater et al. (1985) isolated a number of cDNA clones corresponding to mRNAs that were present in ripe but not in mature green tomato fruits. One of these clones, pTOM13, coded for a 35-kDa protein whose expression is correlated with increased ethylene synthesis (Smith et al., 1986). Tomato plants transformed with an antisense construct of pTOM13 had greatly reduced ethylene biosynthesis in wounded leaves and ripening fruits (Hamilton et al., 1990). The level of reduction in ACC oxidase activity was related to the number of copies of antisense pTOM13 genes (Hamilton et al., 1990). Based on these results, Hamilton et al. (1990) suggested that pTOM13 encoded ACC oxidase. Final proof came when a corrected version of pTOM13 containing the full 5' end was expressed in yeast and was shown to

possess stereospecific ACC oxidase activity (Hamilton et al., 1991). Independent confirmation came from the injection of mRNA for pHTOM5, another cDNA from tomato with 88% nucleotide identity with pTOM13, into oocytes of *Xenopus laevis*. Expression of pHTOM5 conferred ACC oxidase activity that also met the stereospecificity requirements (Spanu et al., 1991).

The identification of the cDNA for ACC oxidase in tomato led to the isolation of a number of cDNA clones for ACC oxidase from other species (see Zarembinski and Theologis, 1994). These new cDNA clones were identified solely by their sequence similarity to the two tomato clones. While these clones did have a high degree of identity to pTOM13, they also shared significant identity with other dioxygenase-related cDNAs, such as E8 (Deikman et al., 1988).

Because the deduced amino acid sequence of pTOM13 showed 58% similarity to the amino acid sequence of flavanone 3-hydroxylase, a 2-oxoglutarate-dependent dioxygenase (Hamilton et al., 1990), Ververidis and John (1991) extracted and assayed ACC oxidase activity from melon fruits using conditions that had been shown to preserve flavanone 3-hydroxylase activity from *Petunia hybrida*. By extracting the enzyme under N_2 gas and including Fe^{2+} and ascorbate in the assay medium, they completely reconstituted ACC oxidase activity that displayed the proper stereospecificity *in vitro*. Previous failures to isolate ACC oxidase activity can be attributed to the dilution of the essential cofactors Fe^{2+} and ascorbate during homogenization. Despite the sequence similarity to oxoglutarate-dependent dioxygenases, ACC oxidase does not require 2-oxoglutarate as a cofactor (Smith et al., 1992).

A unique feature of ACC oxidase is its requirement of CO_2 for enzyme activity (Dong et al., 1992). In a number of plant systems, ACC oxidase activity is enhanced by high levels of CO_2 (Yang and Hoffman, 1984). Working with purified enzyme, Dong et al. (1992) found that *in vitro* activity is completely abolished if CO_2 is trapped with KOH. They also determined that half-maximum activity is reached at 0.5% CO_2 and maximum activity at 2-4% CO_2. By analogy to the activation of ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco; Lorimer and Miziorko, 1980) and urease (Park and Hausinger, 1995), the CO_2 is predicted to form a carbamate with the ϵ-amino group of a lysine residue. This carbamate would interact with a cation, Fe^{2+} in the case of ACC oxidase, to bind the metal and facilitate its incorporation into the catalytic site. Although an aspartate or glutamate residue should, theoretically, be able to perform a similar role, crystal structure data of urease (Jabri et al., 1995) suggests that the added length of a carbamate on a lysine residue is necessary to span the distance to the active site.

Work with ACC oxidase reported below has been separated into two parts: (i) cloning of a cDNA encoding an ACC oxidase that is expressed in the stem of etiolated pea seedlings; and (ii) confirming that the insert of the clone encoded a functional ACC oxidase by expression of the cDNA in transgenic yeast.

Materials and Methods

Cloning and sequencing of ACC oxidase cDNA

From a comparison of nucleotide sequences of pTOM13 from tomato (Holdsworth et al., 1987) and an ACC oxidase-related cDNA from avocado (McGarvey et al., 1990), two conserved regions of FGTKVSN and PKEPRFE were selected for the synthesis of non-degenerate oligonucleotide primers HK-21, 5'-TTTGGTACTAAAGTTAGCAAC-3', and HK-20, 5'-G(GAATTC)AAATCTTGGCTCTTTGGC-3' (nucleotides in parenthesis encode an EcoRI site), corresponding to the respective pTOM13 sequences. These primers were used for a polymerase chain reaction (PCR)-based amplification from a Lambda Zap II cDNA library (Stratagene, La Jolla, CA, USA) made from poly(A)$^+$ RNA from apical hooks of etiolated peas treated for 4 h with 10^{-4} M IAA. Thermocycling was performed at 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min, for 30 cycles. The PCR product (PE1) was sequenced to confirm its similarity to pTOM13 and then used to screen the pea cDNA library. Hybridization was done in 6X SSC, 0.1% SDS, and 5X Denhardt's Reagent at 62°C. The final wash was performed at 0.5X SSC, 0.1% SDS at 62°C. Both strands of the insert of the longest full-length cDNA, pPE8, were sequenced.

Production of transgenic yeast

The full-length cDNA insert was excised from pBluescript SK$^-$ (Stratagene, La Jolla, CA) by EcoRI digestion, recovered by electrophoresis onto DE-81 paper

(Sambrook et al., 1989), and ligated into a yeast expression vector with a galactose-inducible promoter, pYES2.0 (Invitrogen, San Diego, CA), to yield pYPE8. Yeast strain F808 (*MATa/α GAL1$^+$ leu2-3 leu2-112 his4-519 ade1-100 ura3-52*) was transformed by electroporation (Bio-Rad Technical Bulletin) with pYPE8 to yield TYPE8-F808. Transformants were selected by their ability to grow on a medium that lacked uracil.

Culture conditions for transgenic yeast

Yeast was grown on a shaker at 30°C to mid-log phase in a medium containing 0.65% yeast nitrogen base without ammonium sulfate or amino acids (DIFCO, Detroit, MI), 2% galactose, 0.5% ammonium sulfate, 0.002% adenine sulfate, and 470 mg/L of an amino acid mixture consisting of 20 mg arginine, 100 mg aspartate, 60 mg histidine, 60 mg leucine, 30 mg lysine, 60 mg methionine, 50 mg phenylalanine, 60 mg threonine, 40 mg tryptophan, and 30 mg tyrosine.

Measuring ACC oxidase activity in transgenic yeast

To measure ACC oxidase activity, 690 μL of mid-log cells, 100 μL 1 M Tris-HCl, pH 7.2, 100 μL 100 mM ACC (Sigma Chemical Co., St. Louis, MO), 100 μL 300 mM Na-ascorbate, and 10 μL 0.1 mM $FeSO_4$ were mixed in 4-mL vials which were capped and incubated on a shaker at 30°C for 1 h. Ethylene concentrations were determined by analyzing 1 mL of the headspace in a gas chromatograph (GC). The number of cells per reaction was determined by counting on a hemocytometer. In the inhibitor studies, the inhibitors were resuspended in the above Tris buffer and added to

the mixture such that the other volumes were not affected.

Gel electrophoresis and immunoblotting

To isolate protein, yeast cells were collected by centrifugation at 600 x *g* for 15 min in microcentrifuge tubes. The pelleted cells were washed in ice-cold extraction buffer [50 mM Tris-2-N-morpholinoethanesulfonic acid (MES), pH 7.8, 2 mM dithiothreitol (DTT), 1 mM PMSF, 250 mM sucrose]. The pellet was then suspended in an equal volume of ice-cold extraction buffer and an equal volume of glass beads (diameter: 0.45 mm). The tubes were shaken vigorously on a Vortex mixer for 10 min at 4°C. The tubes were punctured at the bottom, placed inside fresh tubes, and centrifuged at 100 x *g* to separate the extract from the glass beads. The supernatant from the lower tube was transferred to a fresh tube and centrifuged again at 100 x *g* for 15 min at 4°C to remove remaining cellular debris. The supernatant was combined with 0.25 volume of 4X sample buffer (1X sample buffer is 0.03 M Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, 0.0125% bromophenol blue) and boiled for 10 min.

Samples were subjected to electrophoresis at 50 V in 10% SDS-polyacrylamide gels (Laemmli, 1970) with 1-mm spacers using the Bio-Rad Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). Transfer to 0.45 μm nitrocellulose (Schleicher and Schuell, Keene, NH) was performed at 40 V for 3 h. For immunoblot detection of the protein, a secondary antibody conjugated to alkaline phosphatase was used as described by Burnette (1981).

Results

Cloning of ACC oxidase

To isolate a cDNA clone for ACC oxidase, a PCR product was obtained from a pea cDNA library made from poly(A)$^+$RNA isolated from etiolated pea hooks treated for 4 h with 10^{-4} M IAA using non-degenerate oligonucleotide primers based on the nucleotide sequence of a tomato ACC oxidase, pTOM13. The PCR product, PE1, was used to screen the same cDNA library for a full-length clone. From the 300,000 plaques screened, 26 independent clones were isolated. Of these, eight clones were sequenced and found to be identical except for different lengths at the 5' and 3' ends. The longest clone, pPE8, contained an insert of 1122 bp encoding an open reading frame of 317 amino acids with a predicted mass of 36 kDa (Figure 1.1). The deduced amino acid sequence shared 71% and 69% identity with those of two tomato ACC oxidase cDNAs (Hamilton et al., 1991; Spanu et al., 1991, respectively).

```
   1  AAAATGGAAAACTTTCCAATTGTTGACATGGGGAAGCTTAACACAGAAGACAGAAAATCA
         M  E  N  F  P  I  V  D  M  G  K  L  N  T  E  D  R  K  S
  61  ACCATGGAGTTGATCAAAGATGCCTGTGAGAACTGGGGTTTCTTTGAGTGTGTGAATCAT
      T  M  E  L  I  K  D  A  C  E  N  W  G  F  F  E  C  V  N  H
 121  GGTATATCTATTGAGATGATGGATACGGTTGAGAAGCTGACAAAGGAACACTACAAGAAG
      G  I  S  I  E  M  M  D  T  V  E  K  L  T  K  E  H  Y  K  K
 181  TGTATGGAACAAAGGTTCAAAGAAATGGTTGCAACCAAAGGTTTGGAGTGTGTTCAGTCA
      C  M  E  Q  R  F  K  E  M  V  A  T  K  G  L  E  C  V  Q  S
 241  GAGATAGATGATTTGGATTGGGAAAGCACTTTCTTTTTGCGCCATCTTCCTGTTTCCAGT
      E  I  D  D  L  D  W  E  S  T  F  F  L  R  H  L  P  V  S  S
 301  ATCTCAGAGATCCCAGATCTTGATGATGATTACAGGAAGGTGATGAAGGAATTTGCGTTG
      I  S  E  I  P  D  L  D  D  D  Y  R  K  V  M  K  E  F  A  L
 361  AAATTGGAAGAACTTGCGGAGGAACTTCTTGACTTGTTGTGTGAGAATCTTGGGCTTGAG
      K  L  E  E  L  A  E  E  L  L  D  L  L  C  E  N  L  G  L  E
 421  AAAGGGTATTTGAAGAAGGCTTTTTATGGATCAAAGGGTCCAAATTTTGGGACGAAAGTT
      K  G  Y  L  K  K  A  F  Y  G  S  K  G  P  N  F  G  T  K  V
 481  AGTAACTACCCTCCTTGTCCTAAACCGGAACTCATCAAGGGACTTAGAGCTCACACAGAT
      S  N  Y  P  P  C  P  K  P  E  L  I  K  G  L  R  A  H  T  D
 541  GCCGGCGGAATCATTCTTCTCTTCCAAGATGACAAAGTCAGTGGACTTCAGCTTCTCAAA
      A  G  G  I  I  L  L  F  Q  D  D  K  V  S  G  L  Q  L  L  K
 601  GATGACCAATGGATTGATGTCCCTCCAATGCGTCACTCTATTGTCATCAATCTTGGTGAT
      D  D  Q  W  I  D  V  P  P  M  R  H  S  I  V  I  N  L  G  D
 661  CAACTCGAGGTGATAACCAATGGAAAGTACAAGAGTGTGATGCATAGAGTAATAGCACAA
      Q  L  E  V  I  T  N  G  K  Y  K  S  V  M  H  R  V  I  A  Q
 721  ACAGATGGTGCTAGAATGTCCATAGCATCCTTCTACAATCCAGGGGATGATGCTGTGATT
      T  D  G  A  R  M  S  I  A  S  F  Y  N  P  G  D  D  A  V  I
 781  TCACCAGCTTCAACCTTATTGAAGGAAAATGAAACAAGTGAAGTTTATCCAAAGTTTGTG
      S  P  A  S  T  L  L  K  E  N  E  T  S  E  V  Y  P  K  F  V
 841  TTTGATGATTACATGAAACTCTACATGGGACTCAAGTTCCAAGCTAAAGAGCCTAGATTT
      F  D  D  Y  M  K  L  Y  M  G  L  K  F  Q  A  K  E  P  R  F
 901  GAAGCTATGATGAAAGCCATGTCAAGTGTTAAAGTGGGACCAGTAGTAAGCATATAGTTA
      E  A  M  M  K  A  M  S  S  V  K  V  G  P  V  V  S  I  *
 961  TGTGTGTGGTTTATATTACTAAGGTATTATTAATGTTTGGTTGGTGTTTTAAGATTGTGC
1021  AGTGTCTAAAGTGTTATATTAAAAAAAATAAAGTATGATCTTAGATTGTGTGTGACAATT
1081  AATCAGTTATGATTAAGTGATTAAGTTTATGTAATGCGATGC
```

Figure 1.1. Nucleotide and deduced amino acid sequence of a pea ACC oxidase cDNA clone PE8 (Peck et al., 1993). The primer sites used to amplify PE1 are underlined.

Expression of pPE8 in transgenic yeast

To determine whether the pPE8 insert encoded a functional ACC oxidase, the insert was cloned into yeast expression vector pYES2.0 (Invitrogen, San Diego, CA, USA) and transformed into yeast strain F808 to create TYPE8-F808. Expression of the insert was controlled by a galactose-inducible promoter. When grown under inductive conditions, transgenic cells contained ACC oxidase activity as determined by their ability to convert ACC to ethylene (Table 1.1). Yeast cells containing a pYES2.0 plasmid with the PE8 insert in the opposite orientation (TY8EP-F808) did not produce ethylene above background level (medium only; Table 1.1). ACC oxidase activity in the transgenic cells was reduced by $CoCl_2$, a potent inhibitor of ACC oxidase activity, and aminoisobuterate (AIB), a weak inhibitor (Table 1.2). A transgenic protein with the expected mass of 36 kDa was recognized by immunoblot detection (Figure 1.2) using antibodies raised to tomato ACC oxidase (Peck et al., 1992). TYPE8-F808 cells grown under non-inductive conditions did not produce ethylene from ACC above background levels and did not accumulate antigenic protein (data not shown). These results confirm that the clone PE8 encodes a functional ACC oxidase.

Table 1.1: ACC oxidase activity in transgenic yeast. Yeast containing plasmids with inserts in the coding orientation (TYPE8-F808) and in the non-coding orientation (TY8EP-F808) were grown under inductive conditions. Values are the average of duplicate samples. Experiments were performed twice with similar results.

	Ethylene Production (pmol h^{-1} 10^{-6} cells)
TYPE8-F808	1371.2
TY8EP-F808	19.1
Medium Only	12.8

Table 1.2: Effect of inhibitors on ACC oxidase activity in transgenic yeast. Values are averages of duplicate samples. Experiments were performed twice with similar results.

ACC (mM)	$CoCl_2$ (mM)	AIB (mM)	Ethylene Production (pmol h^{-1} 10^{-6} cells)
10	-	-	1658.3
10	1	-	118.8
10	5	-	12.2
10	10	-	5.4
1	-	-	515.7
1	-	10	365.9
1	-	50	279.5
1	-	100	229.1

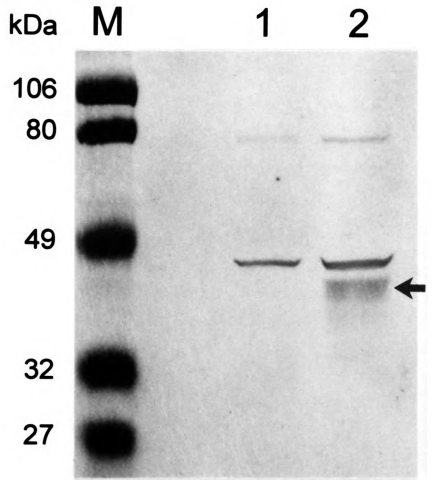

Figure 1.2. Immunoblot analysis of protein extracts from transgenic yeast. Yeast containing the cDNA insert PE8 in the non-coding orientation (lane 1) and in the coding orientation (lane 2) were induced by galactose for 24 h. Bio-Rad molecular weight markers are in lane M. The arrow marks the expected size of ACC oxidase.

Discussion

Analysis of deduced amino acid sequences is often useful in providing insights into the structure and reaction mechanism of enzymes. A number of reviewers have presented sequence alignments between ACC oxidases and related dioxygenases (Prescott, 1993; Zarembinski and Theologis, 1994). These alignments were structured to highlight identical residues. While this type of analysis is helpful in determining common domains, such as binding sites for Fe^{2+} and ascorbate, it will not identify domains unique to ACC oxidase. The analysis presented below was designed to supplement these previous alignments by focusing on regions conserved in ACC oxidases but not in other dioxygenases.

As seen in Figure 1.3, all known ACC oxidases share 46% identity and 64% similarity at the amino acid level. The consensus sequence derived from this comparison facilitates comparison with other dioxygenases. As pointed out by Prescott (1993), dioxygenases from animals, plants, and microorganisms share suprisingly little similarity. It may be more meaningful, therefore, to compare the consensus sequence of ACC oxidase with a consensus sequence obtained only from plant dioxygenases (Figure 1.4).

Legend for Figure 1.3. Sequences are from pea (Peck et al., 1993), tomato (Kock et al., 1991; Spanu et al., 1991), avocado (McGarvey et al., 1990), carnation (Wang and Woodson, 1991), apple (Dong et al., 1992b), petunia (Tang et al., 1993), peach (Callahan et al., 1992), Arabidopsis (Gomez-Lim et al., 1993), and orchid (Nadeau et al., 1993). Identical residues aligned below the pea sequence are shown by an asterisks (*). Gaps in the alignment are shown by periods (.).

```
PEA          MEN...FPIV  DMGKLN...T  EDRKSTMELI  KDACENWGFF  ECVNHGISIE
TOMATO1      ***...***I  NLE***...G  DE*AN***M*  **********  *L*****PH*
TOMATO2      ***...***I  NLEN**...G  DE*AK***M*  **********  *L*****PH*
AVOCADO      *DS...**VI  N*E**E...G  QE*AA**K**  N*********  *L***S*PV*
CARNATION    *A*IVN***I  **E***NYNG  VE*SLVLDQ*  ****H*****  QV***SL*H*
APPLE        *AT...**VV  *LSLV*...G  *E*AA*L*K*  N*********  *L****M*T*
PETUNIA      ***...***I  SLD*V*...G  VE*AA***M*  **********  *L*****PR*
PEACH        ***...G***  N*EG**...G  *G**A***K*  **********  *L*S***PT*
ARAB.        **S...****  NLE*D*...G  *E*AI***K*  **********  ********L*
ORCHID       **S.GS**VI  N*EL*Q...G  SQ*PAA*A*L  R*******LY  *LL*****H*

PEA          MMDTVEKLTK  EHYKKCMEQR  FKEMVATKGL  ECVQSEIDDL  DWESTFFLRH
TOMATO       V******M**  G*********  ***L**S***  *A**A*VT**  **********
TOMATO2      V**R******  G*********  ***L**S***  *A**A*VT**  **********
AVOCADO      L**E**R***  **********  ***LM*S*.V  *GAVVDAN*M  *******I**
CARNATION    L**K**RM**  *****FR**K  **D**Q****  VSAE*QVN*I  ******Y***
APPLE        LL*****M**  D****T****  ******A***  DD*****H**  **********
PETUNIA      V******M**  G*********  ***L**S*A*  *G**A*VT*M  ********K*
PEACH        FL****R***  ***RQ*L***  ***L**S***  *A*KT*VN*M  ******Y***
ARAB.        LL*K***M**  ********E*  ***SIKNR**  DSLR*SVN*V  ******Y*K*
ORCHID       L*NR**TVE*  ***RRFR***  ***FASKTLD  TVENV*PEN*  **********

PEA          LPVSSISEIP  DLDDDYRKVM  KEFALKLEEL  AEELLDLLCE  NLGLEKGYLK
TOMATO       **T*N**QV*  ***EE**E**  RD**KR**K*  **********  **********
TOMATO2      **T*N**QV*  ***EE**E**  RD**KR**K*  **********  **********
AVOCADO      ****NL****  **T*EH****  ****E***K*  **QV******  **********
CARNATION    R*T*N***V*  ****Q***L*  ****AQI*R*  S*Q*******  ******A***
APPLE        **S*N*****  **EEE***T*  ****VE**K*  **K*******  **********
PETUNIA      **I*N***V*  ***EE**E**  RD**KR**K*  **********  **********
PEACH        **K*N***V*  **E*Q**N**  **********  **********  **********
ARAB.        **V*N**DV*  *******TL*  *D**G*I*K*  S*********  **********
ORCHID       **T*N**Q**  *****C*ST*  *S***E**N*  **R*******  D*********

PEA          KAFYGSK.G.  PNFGTKVSNY  PPCPKPELIK  GLRAHTDAGG  IILLFQDDKV
TOMATO       N******.*.  **********  ******D***  **********  **********
TOMATO2      N******.*.  **********  ******D***  **********  **********
AVOCADO      M**A*TTT*L  *T********  ****R*E*F*  **********  L*******R*
CARNATION    N****AN.*.  *T********  ******D***  **********  **********
APPLE        *V*****.*.  **********  ******D***  *****S****  **********
PETUNIA      N******.*.  **********  ******D***  **********  **********
PEACH        *****TN.*.  *T********  **********  **********  **********
ARAB.        *V*****.R.  *T********  ****N*D*V*  **********  **********
ORCHID       *V*C*GSD*L  *T********  ******D**N  *****S****  **********

PEA          SGLQLLKDDQ  WIDVPPMRHS  IVINLGDQLE  VITNGKYKSV  MHRVIAQTDG
TOMATO       ********E*  **********  **V*******  **********  L*********
TOMATO2      ********E*  **********  **V*******  **********  **********
AVOCADO      A*******GE  *V*****N**  ********V*  **********  ****V*****
CARNATION    ********GH  *V*****K**  **V*******  **********  **********
APPLE        ********GE  *V*****H**  ********I*  **********  *******S**
PETUNIA      ********G*  **********  **V*******  **********  *******K**
PEACH        ********GW  Q*L*******  **********  **********  E*********
ARAB.        ********GE  *V****VK**  **V*******  **********  E***LS****
ORCHID       ********GE  ******V***  **V*I*****  **********  L***V*****
```

Figure 1.3. Amino acid sequence alignment of ACC oxidases.

Figure 1.3 (continued)

```
PEA          .ARMSIASFY  NPGDDAVISP  ASTLL.KENE  T.....SEVY  PKFVFDDYMK
TOMATO       .T***L****  ***S****Y*  *K**VE**A*  ES....TQ**  **********
TOMATO2      .T***L****  ***N****Y*  *PS*I....*  ES....KQ**  **********
AVOCADO      .N***L****  ***S****F*  *PA*VE**A*  EK....K***  *****E***N
CARNATION    .N********  ***S****Y*  *P**VE**.*  EK....CRA*  *****E***N
APPLE        .T********  ***N*SF***  *PAV*....*  KKTED.APT*  **********
PETUNIA      .****L****  ***S****Y*  *PA*VE**A*  EN....KQ**  **********
PEACH        .T********  ***S****Y*  *P**VE**A*  EK....NQ**  *****E****
ARAB.        EG********  ***S*S**F*  VPE*IG**A*  .K.EK.K*N*  *R***E****
ORCHID       .N****SA**  ***S****F*  *PA*VE**A*  EKEEKKK*I*  *****Q***N

PEA          LYMGLKFQAK  EPRFEAM.MK  AMSSV..KVG  ..PVVSI
TOMATO       **A*******  *******..*  **E*D.....  ..*IA*A
TOMATO2      **A*****P*  *******..*  **EANVEL*D  ..QIA*A
AVOCADO      **A*******  *****V*KM*  *VETA..NLS  ..*ITT*
CARNATION    **LK****ED  *******..*  **ETT....*  ..**PTA
APPLE        **S*******  *******..*  *KE*T.....  ..**ATA
PETUNIA      **A*******  *******..*  **ETD.V*MD  ..*IATV
PEACH        **M*****P*  *******..*  *VETN.ISL*  ..*IATA
ARAB.        **SAV*****  *******..*  **ETTVANNV  G.*LATA
ORCHID       **IRK**E*D  *******..*  S*EI*MSSQ.  ..*IPTA
```

```
CONSENSUS      1 M------Pii  -l--------  --R---m--I  kDAC-NWGff  e-vNHgi--E
DIOXY.         1 M--//-vP-I  -L--------  ----------  --AC---GiF  -vv-Hgv--e

CONSENSUS     51 vm--VE-m-K  -HYkk--Eqr  FKe-------  ----------l  DWESTFflrH
DIOXY.        51 li--------  -FF-lP---K  -rv-------  --//------  -W-e------

CONSENSUS    101 -P-S-iS-iP  DL-ee-R--M  k-FA--lE-L  aE-lLDLLCE  -LGLEKgYLK
DIOXY.       101 ----//----  --------v-  --y---l--l  s--im-ll--  slGv-//y-k

CONSENSUS    151 --F-G-----  P-FGTKVSNY  PPCPkPdLik  GLRAHtDAGG  iILLFQDDkV
DIOXY.       151 --f----//-  ----v-lNfY  P-C--P---l  G---H-D--s  itll-QD---

CONSENSUS    201 sGLQLLKD--  wi-VPPm-HS  IViN-GDQlE  VITNGKYKSV  -HRViaQ-DG
DIOXY.       201 .GLq------  W-sv-P---A  FVvNlG----  -lsN--y---  -HraV-----

CONSENSUS    251 --RMSlasFY  NPG-DavI-P  ---lv----E  ----------Y  PkFVFdDYMk
DIOXY.       251 --R-SiA-f-  -P-------P  ----------  ----------  ----------

CONSENSUS    301 LY---KFq--  EPRFE-M--K  a---------  ---i-t-
DIOXY.       301 ----------  ----------  ----------  ---------
```

Figure 1.4. Amino acid alignment of consensus sequences of ACC oxidases (from Figure 1.3) and dioxygenases from plants: GA-20 oxidases (Lang et al., 1994; Xu et al., 1995), flavanone 3-hydroxylases (Britsch et al., 1992; Meldgaard, 1992), hyoscyamine 6-hydroxylase (Matsuda et al., 1991). Identical residues are in upper case. Functionally conserved residues are in lower case. Shaded residues are absolutely conserved in all ACC oxidases and dioxygenases from this comparison. Double backslashes (//) indicate regions of variable length.

Similarity with other dioxygenases ends at Pro270. In fact, similarity between all dioxygenases ends at that same proline. However, at various distances after that residue, enzymes compared by substrate specificity have extremely well-conserved domains (Figure 1.5). The high degree of identity suggests that these residues may be involved in the binding of the respective substrates of these enzymes. The crystal structure data of isopenicillin N synthase (IPNS) indicate that the C-terminal tail will fold to enter the active site region, supporting this hypothesis.

Another unique feature of ACC oxidase activity is its absolute requirement for CO_2 (Dong et al., 1992a). By analogy to Rubisco (Lorimer and Miziorko, 1980) and urease (Park and Hausinger, 1995), the only other known CO_2-activated enzymes, it is believed that CO_2 forms a carbamate with the ϵ-amino group of a lysine residue. This carbamate binds the cation and brings it into the catalytic site. Sequence comparison between ACC oxidase and Rubisco suggests a candidate lysine for this activation step. Lys202 is the residue involved in the formation of the carbamate in Rubisco (Lorimer, 1981). The consensus sequence around this residue agrees well with the consensus sequence around Lys208 from ACC oxidase (Figure 1.6). Future experiments will be directed at determining which amino acid residue interacts with CO_2 to activate ACC oxidase.

ACC Oxidases:	^{290}YPkFVFdDYMkLY---KFq--EPRFE-M--Ka
GA-20 Oxidases:	^{345}Df-W-mlLEf-QKhYR-D-nTL-aF--Wl
Flavanone-3-hydroxylase:	^{319}eEPITFaEMYRRKM-rDLeLAr-KKQAKeQ-MQ

Figure 1.5. Amino acid consensus sequences from the carboxy-terminal portions of plant dioxygenases. Alignments were derived from sequences cited in Figure 1.4. Identical residues are shown in upper case while functionally conserved residues are shown in lower case. Numbering of residues is based on consensus alignments.

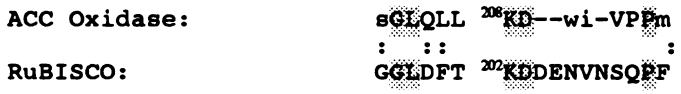

Figure 1.6. Amino acid alignment of sequence surrounding the lysine residue which binds CO_2 in Rubisco (Lorimer, 1981) with consensus sequence of ACC oxidase. Identical residues are shown in upper case while functionally conserved residues are shown in lower case. Shaded residues are identical in both enzymes. Residues that are similar are connected by two periods (:).

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CHAPTER 2

LOCALIZATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE FROM TOMATO IN TRANSGENIC YEAST

Abstract

The localization of the ethylene-forming enzyme, ACC oxidase, was studied in yeast that had been transformed to express ACC oxidase from tomato fruits when induced with galactose. Since at least part of the ethylene-forming activity in plants was found in association with vacuoles, we were particularly interested to know whether ACC oxidase would be targeted to the yeast vacuole. Transgenic ACC oxidase began to accumulate ca. 2 h after transfer of yeast cells to medium containing galactose, as was determined by measuring ACC oxidase activity and by immunoblot analysis using ACC oxidase-specific antibodies. Protoplasts prepared from transgenic yeast retained 84% of the ACC oxidase activity of intact cells. Vacuoles isolated from such protoplasts did not possess ACC oxidase activity. Immunoblot analysis confirmed that intact cells and protoplasts contained approximately equal amounts of ACC oxidase whereas vacuoles did not contain any detectable amounts. Differential centrifugation indicated that ACC oxidase from transgenic yeast was associated with the particulate fraction of the homogenate. It is not known whether this represents specific binding of the enzyme or nonspecific attachment that may have occurred during homogenization.

Introduction

The identity of 1-aminocyclopropane-1-carboxylate (ACC) oxidase, the enzyme that converts ACC to ethylene, remained elusive until recently. The breakthrough in identifying ACC oxidase came from experiments where the antisense mRNA of a ripening-related cDNA clone, pTOM13, was expressed in tomato plants (Hamilton et al., 1990). These transgenic tomato plants exhibited greatly reduced capacity to form ethylene from added ACC. Functional expression of the putative ACC oxidase cDNA in yeast (Hamilton et al., 1991) and *Xenopus* oocytes (Spanu et al., 1991) provided conclusive evidence that it indeed encoded the ethylene-forming enzyme. Results of earlier work indicated that ACC oxidase was associated, at least in part, with the central vacuole (Erdmann et al., 1989; Guy and Kende, 1984) and that the activity of the enzyme required an intact membrane (Mayne and Kende, 1986). However, the deduced amino acid sequence of ACC oxidase does not show a vacuolar targeting signal or membrane association site. Also, *in vitro*, ACC oxidase activity was found in the soluble fraction of tissue homogenates (Ververidis and John, 1991). In the work below, a tomato cDNA with sequence identical to the insert of pTOM13 was expressed in yeast. ACC oxidase activity was localized by cell fractionation with the aim of determining whether the enzyme was targeted to the vacuole.

Materials and Methods

Cloning and sequencing of cDNA

Non-degenerate oligonucleotide primers described in Chapter 1 were used to amplify by the polymerase chain reaction a 442-bp fragment from a Lambda Zap II cDNA library (Stratagene, La Jolla, CA, USA) which had been constructed from poly(A)$^+$ RNA of ripe, unwounded tomato fruits (*Lycopersicon esculentum* Mill., cv. UC82B). This fragment was used to isolate from the above library a full-length cDNA clone encoding ACC oxidase, which was subcloned into pUC19 for sequencing.

Production and culture conditions of transgenic yeast

Transgenic yeast containing a tomato ACC oxidase was produced as described in Chapter 1. For the isolation of vacuoles, yeast was grown as described in Chapter 1. For the time course of induction and differential centrifugation experiments, the yeast was grown to mid-log phase in medium containing 2% glucose instead of galactose, collected by centrifugation at 1000x*g*, and washed twice with sorbitol medium [0.6 M sorbitol, 5 mM Tris-piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH 6.8]. The yeast was then resuspended in medium containing 2% (+)-galactose.

Assays of ACC oxidase activity

ACC oxidase activity was measured as in Chapter 1.

Isolation of protoplasts and vacuoles

Protoplasts and vacuoles were isolated as described by Boller et al. (1989) with minor modifications. Yeast cells were incubated in cell wall-digesting solution for 60 min instead of 90 min. The concentration of diethylaminoethyl-dextran (DEAE-dextran) was lowered to 5 mg/mL to achieve better mixing with the protoplasts. Dextran sulfate was not found to be necessary to protect the vacuoles from lysis. The vacuoles were collected from the interface above the 2.5% Ficoll layer.

Assays of marker enzymes

α-Mannosidase and carboxypeptidase Y assays were performed following the procedures of Opheim (1978) and of Hemmings et al. (1981), respectively.

Protein extraction

To analyze the time course of induction, protein was extracted as in Chapter 1. For differential centrifugation, lysed cells were centrifuged at 100x*g* to remove cellular debris (P1). A portion of the supernatant (150 μL) was centrifuged at 18,500x*g* for 15 min at 4°C, yielding a pellet (P2) and supernatant. This supernatant was removed to an airfuge cylinder (Beckman, Palo Alto, CA, USA) and centrifuged at 100,000x*g* for 30 min in a 18° A-100 rotor. This pellet (P3) as well as P2 were resuspended in 150 μL of extraction buffer. The final supernatant was also brought to 150 μL. Ninety μL of each sample were combined with 30 μL of 4x sample buffer and boiled for 10 min.

Isolated protoplasts and vacuoles were resuspended in an equal volume of 2x sample buffer and boiled.

Gel electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting were performed as described in Chapter 1 except for development of the blot which was done with horseradish peroxidase conjugated to the secondary antibody (Hawkes, 1982).

Digestion with endo-β-N-acetylglucosaminidase H (Endo-H)

Digestion of proteins with Endo-H followed a modified procedure of Trimble and Maley (1984). Fifty μL of total protein extract from yeast induced for 5 h was precipitated with acetone and resuspended in 20 μL of Endo-H reaction buffer (50 mM Na-citrate, pH 5.5, 100 mM NaCl, 1 mM PMSF). Samples were digested with 5 mUnits Endo-H (Sigma Chemical Co., St. Louis, MO, USA) for 15 h at 37°C before addition of 8 μL 4x sample buffer and boiling for 10 min.

Results

From a comparison of nucleotide sequences of pTOM13 from tomato (Holdsworth et al., 1987) and a ripening-related gene from avocado (McGarvey et al., 1990), two conserved regions were selected to synthesize two non-degenerate oligonucleotide primers common to both. These primers were used to amplify by PCR a 442-bp fragment from a tomato cDNA library prepared from poly(A)$^+$ RNA of ripe, unwounded tomato fruit. This fragment was subcloned into pUC19 to yield pTE1. Partial sequencing of pTE1 confirmed 100% identity with the corresponding region of pTOM13. The pTE1 insert was used to screen at moderately high stringency (final wash = 0.5x SSC, 65°C) approximately 50,000 primary plaques of the same cDNA library for a full-length clone. Five strongly hybridizing plaques were selected. One of these, pTE2, was chosen for sequencing based on its insert size. Sequencing of the ends of the clone confirmed that it contained the correct 5' and 3' coding sequence and, therefore, was assumed to be full-length. With the exception of two unreadable base pairs, the sequence of pTE2 was identitical to the corrected sequence of pTOM13 (Hamilton et al., 1991).

To determine whether the insert encoded a functional ACC-oxidase, we subcloned the pTE2 insert into pYES2.0, a yeast expression vector with a galactose-inducible promoter, to yield pYTE2. We then transformed yeast strain F808 with pYTE2 to create TF808. Of the three transformants tested, all were able to convert ACC to ethylene. One of the transformants, TF808-1, was selected for further investigation.

When transferred to inductive conditions, ACC-oxidase activity began to increase in TF808-1 cells after about 2 h. Activity continued to increase for the duration of the experiment (Figure 2.1 A). Immunoblot detection showed that the appearance and accumulation of the transgenic ACC oxidase (M_r 36 kDa) followed the pattern seen in the activity assay during the first 4 h (Figure 2.1 B). TF808-1 grown in noninducing glucose medium did not produce ethylene from ACC above background levels and did not accumulate antigenic protein (data not shown). Yeast transformed with the vector alone was also unable to produce ACC oxidase, even under inductive conditions (data not shown). The antibody also recognized a protein of 36 kDa in extracts from elicitor-treated tomato cell cultures (Figure 2.1 B, lane L). It had been shown previously that elicitor-treated tomato cells expressed high levels of ACC oxidase mRNA and displayed a high activity of the enzyme (Spanu et al., 1991).

Legend for Figure 2.1. (A) Immunoblot analysis of protein extracts from yeast cells induced with galactose for 0 to 5 h (lanes 0-5; each lane represents the extract from an equal number of cells) and from tomato cell cultures treated with yeast-derived elicitor for 4 h (lane L). (B) Ethylene synthesis in transformed yeast cells incubated with 1 mM ACC, 30 mM ascorbate, and 0.1 mM $FeSO_4$.

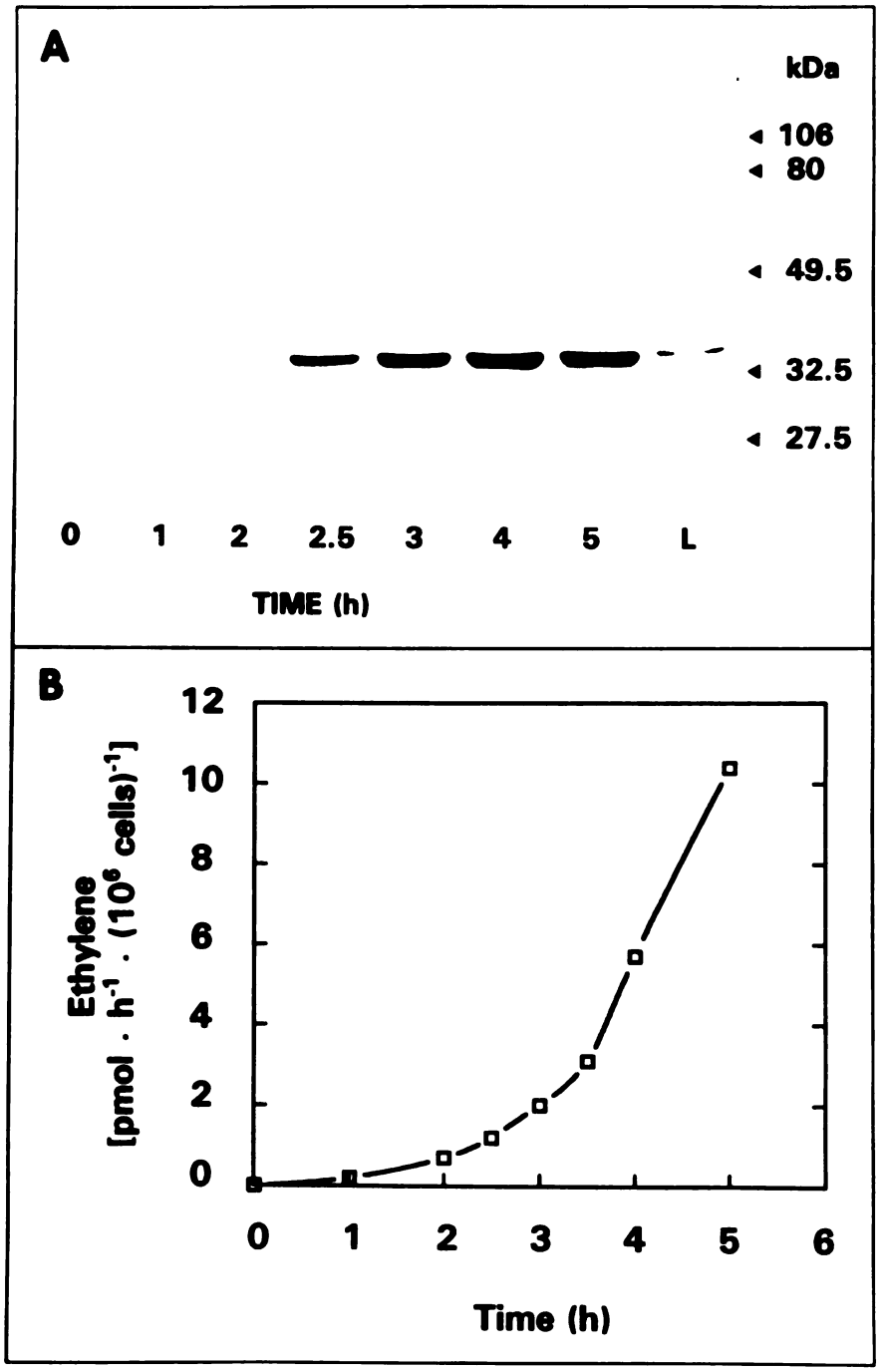

Figure 2.1. Time course of induction of ACC oxidase in transgenic yeast (TF-808-1) following transfer to galactose medium.

The localization of ACC oxidase activity in transgenic yeast was examined to determine if it was present in the vacuole. While protoplasts retained 84% of the ACC oxidase activity found in intact cells, isolated vacuoles were unable to convert ACC to ethylene (Table 2.1). Microscopic inspection was used after each assay to verify that the loss of activity did not result from lysis of vacuoles or protoplasts. The results of the ACC oxidase activity assays were confirmed by immunoblot detection (Figure 2.2). An aliquot from each of the samples used in the activity assays was loaded on an SDS-polyacrylamide gel such that each lane represented an equal number of cells, protoplasts, and vacuoles, as determined both by measuring α-mannosidase activity and by counting under a microscope. Whole cells and protoplasts contained similar amounts of ACC oxidase protein, but none could be detected in vacuoles. Carboxypeptidase Y assays confirmed that the contents of the vacuolar lumen were still present. Thus, the lack of immunologically reactive protein in the vacuolar fraction could not have resulted from loss of vacuolar components during the isolation procedure. Also, comparison of freshly isolated protoplasts and protoplasts kept on ice for 6 h showed that the protein had not been degraded, even after periods well in excess of the time required for isolation of the vacuoles (Figure 2.2, lane 3).

Table 2.1. Ethylene-forming activities of cells, protoplasts, and vacuoles. Values represent the means of 4 assays from 2 separate isolations.

Cells	Protoplasts	Vacuoles
Ethylene [pmol h^{-1} (10^6 cells or vacuoles)$^{-1}$]		
0.68 ± 0.06	0.57 ± 0.08	<0.01

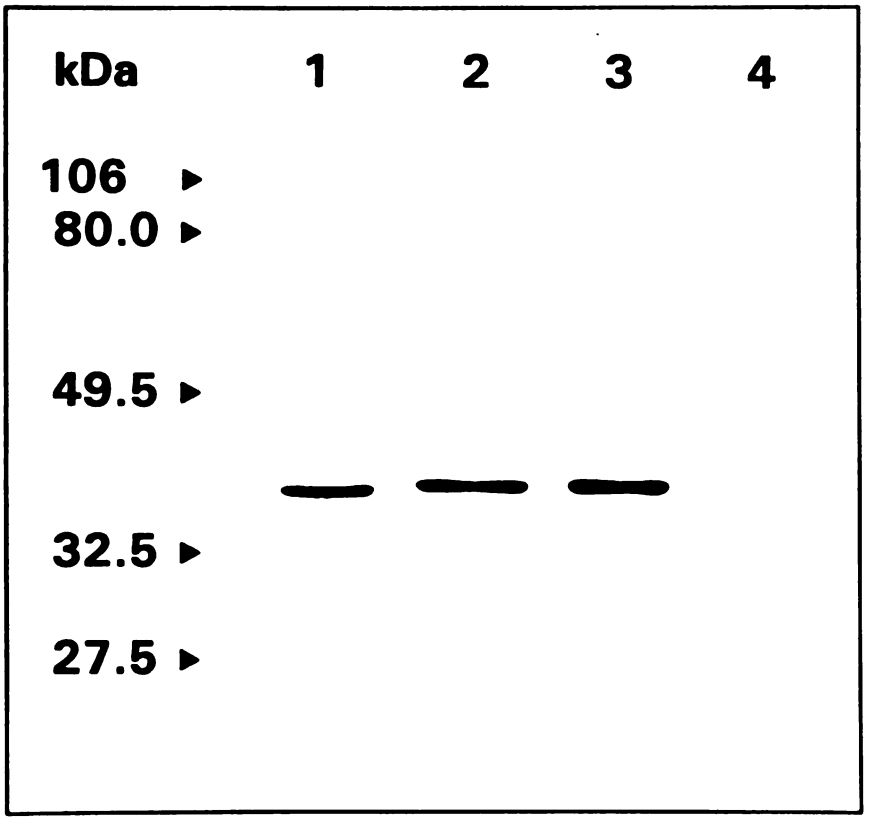

Figure 2.2. Immunoblot analysis of protein extracts from yeast cells, protoplasts, and vacuoles. Lanes represent equal number of cells (lane 1), protoplasts (lane 2), protoplasts stored on ice for 6 h (lane 3), and vacuoles (lane 4) from cells that had been induced with galactose for 24 h.

Upon differential centrifugation, the transgenic ACC oxidase was recovered in the particulate (P2) fraction (Figure 2.3, lane 6). The microsomal fraction (P3) and the soluble fraction contained little or no ACC oxidase (Figure 2.3, lanes 7 and 8, respectively). The higher molecular weight bands seen on the blot were present in the uninduced yeast (lanes 1-3), suggesting cross-reactivity with yeast proteins. Samples treated with Endo-H (Figure 2.3, lane 9) and the respective control not treated with Endo-H (lane 10) had the same molecular mass, indicating that ACC oxidase is not glycosylated.

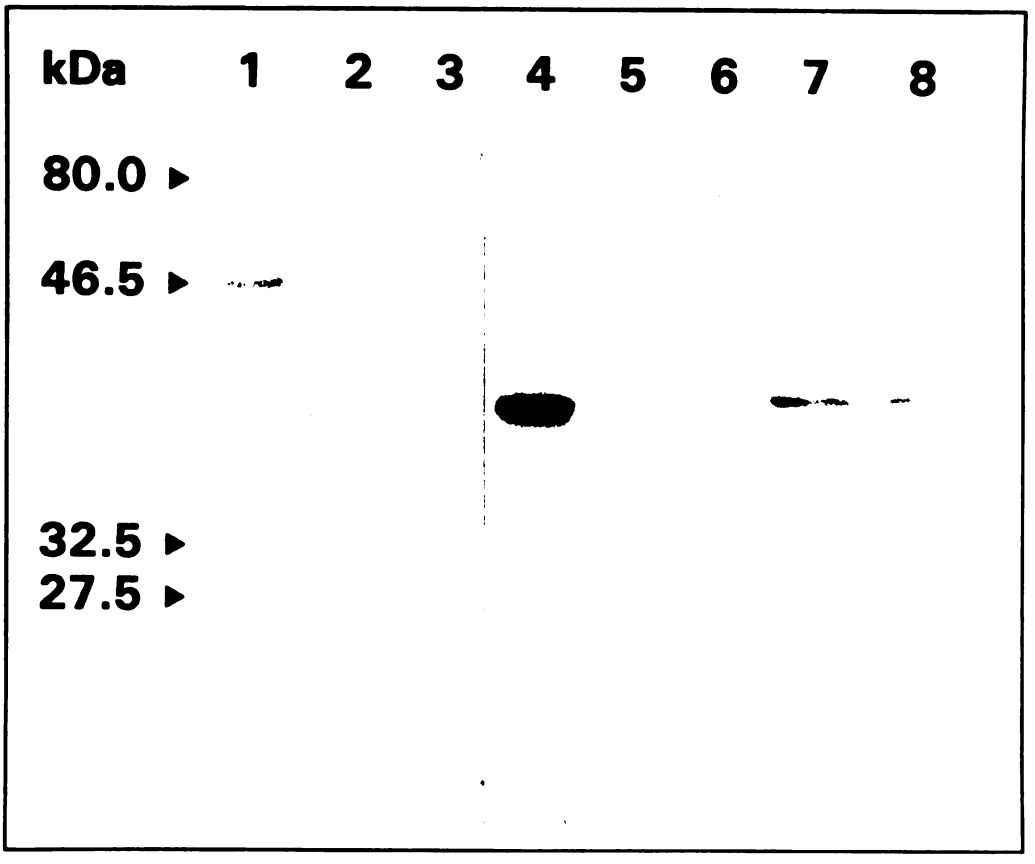

Figure 2.3. Immunoblot analysis of proteins from differential centrifugation and from Endo-H treatment. Lanes 1 to 3, 0-h induction; lanes 4 to 6, 5-h induction with galactose; lanes 1 and 4, 18,500 x *g* pellet (P2); lanes 2 and 5, 100,000 x *g* pellet (P3); lanes 3 and 6, soluble fraction. Each fraction was brought to the same volume (150 μL) of which aliquots were used for analysis (see Materials and Methods). Lanes 7 and 8, immunoblot analysis of total protein extracted from cells induced for 5 h and incubated with (lane 7) and without (lane 8) Endo-H.

Discussion

For many years, ACC oxidase remained the elusive enzyme in the ethylene biosynthetic pathway. A large body of evidence supported the notion that the conversion of ACC to ethylene required membrane integrity. Osmotic and cold shock and treatment with detergents inhibited oxidation of ACC to ethylene (Apelbaum et al., 1981a) as did ionophores (Apelbaum et al., 1981b; Mayne and Kende, 1986; Yu et al., 1980). Vacuoles isolated from pea leaves contained 80% of the ethylene-evolving capacity of protoplasts from which the vacuoles had been prepared (Guy and Kende, 1984). The ACC-oxidase activity in the vacuole displayed the same stereospecificity as exhibited by the enzyme *in vivo*. The activity of the enzyme was lost upon lysis of the vacuole, leading to the conclusion that membrane integrity was required for the functioning of the ethylene-forming enzyme (Mayne and Kende, 1986). However, recent results based on analysis of cDNA clones encoding ACC oxidase do not support vacuolar localization of ACC oxidase or requirement for membrane integrity. The cDNA-derived amino acid sequence of ACC oxidase from tomato (Hamilton et al., 1991; Spanu et al., 1991) and that of putative ACC oxidase from avocado fruits (McGarvey et al., 1990) and carnation flowers (Wang and Woodson, 1991) do not indicate targeting sequences or domains for association with a membrane. Also, the reconstituted *in vitro* activity of ACC oxidase from melon fruits proved to be soluble (Ververidis and John, 1991).

The localization of ACC oxidase in transgenic yeast was examined mainly to determine whether at least part of the ethylene-forming activity was associated with the

vacuole. Previous work has shown that certain plant proteins targeted to the vacuole by way of the endomembrane system are also properly transported to the vacuole when expressed in yeast cells (Chrispeels, 1991). In addition, there is a possibility that cytoplasmic proteins lacking a conventional signal sequence may be imported directly into the yeast vacuole (Chiang and Schekman, 1991). The results shown in Table 2.1 and Figure 2.2 demonstrate conclusively that ACC oxidase is not present in the yeast vacuole. Because the vacuole can act as a lysosome-like compartment, the possibility exists that a portion of the protein may have been targeted to the vacuole and rapidly degraded. High levels of ACC oxidase activity, however, were measured in cells and protoplasts without detecting the protein in the vacuole. Thus, the tomato fruit ACC oxidase does not require a vacuolar localization in order to be functional in yeast.

While the tomato protein does not contain a recognizable targeting signal for the endoplasmic reticulum, there is a potential glycosylation site (Asn-X-Ser) at amino acids 335-337. To determine whether the protein was glycosylated, the protein extact from induced cells was incubated with Endo-H, an enzyme that hydrolyzes N-linked high mannose oligosaccharides. As shown in Figure 2.3 (lanes 9 and 10) there is no difference in molecular size between the Endo-H-treated and untreated proteins.

The results from differential centrifugation show that ACC-oxidase is associated with a particulate fraction. We obtained identical results regardless of whether the fractionation was performed 2 or 24 h after the induction of expression. Thus, association with a membrane fraction does not appear to be the result of overexpression of the protein. It is not yet known whether it represents specific binding of the enzyme

to a particular cell component or nonspecific attachment that may have occurred during homogenization.

The consistent association of ACC oxidase with the particulate fraction raised the possibility of protein-protein interactions between ACC oxidase and an integral membrane protein. A predicted secondary structure for ACC oxidase from tomato fruits was obtained by a modified Chou-Fossman analysis from sequence analysis programs MCF and Amphi (available from A.R. Crofts, University of Illinois, Urbana, IL, USA). A predicted amphipathic helix extends from Met120 to Glu140 and possibly extending to Lys150 (see consensus ACC oxidase sequences, Figure 1.3). Beginning with Phe123, which is known to be able to substitute for leucine residues in leucine zippers without the loss of function (Gentz et al., 1989), a leucine residue repeats every seven amino acids, a characteristic of a leucine zipper (Landschultz et al., 1988). While an amphipathic helix/leucine zipper motif is present in all ACC oxidases, it is also present in a similar region of 2-oxoglutarate-dependent dioxygenases from plants (Figure 1.4). Crystal structure data from a related fungal enzyme, isopenicillin N synthase, indicate that the amphipathic helix is a structural feature involved in stabilizing the ß-sheets that form the active site region (Roach et al., 1995). Thus, the helix is more likely to fill a structural role in ACC oxidases than to be involved in protein-protein interactions. The association of ACC oxidase with the particulate fraction appears to be from nonspecific attachment that occurred during homogenization.

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CHAPTER 3

SEQUENTIAL INDUCTION OF THE ETHYLENE BIOSYNTHETIC ENZYMES BY INDOLE-3-ACETIC ACID IN ETIOLATED PEAS

Abstract

Ethylene induced an increase in the accumulation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase transcript level and enzyme activity in the first internode of 5- to 6-day-old etiolated pea (*Pisum sativum* L.) seedlings. Indole-3-acetic acid (IAA), which stimulates ethylene production by enhancing ACC synthase activity, also caused an increase in ACC oxidase transcript and activity levels. The IAA-induced increase in ACC oxidase mRNA level and enzyme activity was blocked by 2,5-norbornadiene (NBD), a competitive inhibitor of ethylene action. This indicates that IAA induces ACC oxidase through the action of ethylene. The level of ACC synthase mRNA and enzyme activity started to increase less than 1 h after the start of IAA treatment, whereas ACC oxidase activity and transcript levels began to rise after 2 h of IAA treatment. These results indicate that the enzymes of ethylene biosynthesis are sequentially induced after treatment of intact pea seedlings with IAA. The increase in ACC synthase activity leads to the production of ACC, which is converted by the low constitutive level of ACC oxidase activity to ethylene. Ethylene promotes the accumulation of ACC oxidase mRNA and the increase in ACC oxidase activity through a positive feedback loop.

Introduction

Ethylene is a gaseous hormone that regulates a wide range of physiological responses in vegetative tissues (Abeles et al., 1992). In etiolated pea seedlings, an asymmetric distribution of ethylene biosynthesis may be involved in the asymmetry of growth that leads to the formation of the apical hook (Schierle and Schwark, 1988). This asymmetry in ethylene production may result from a gradient in auxin concentration between the outer and inner portions of the apical hook. Ethylene production in vegetative tissues is thought to be regulated by the level of free IAA (Yang and Hoffman, 1984). While it is difficult to establish the exact auxin concentration at its site of action, experiments with ^{3}H-IAA show that the IAA level within the interior portion of the hook is approximately 4-fold greater than in the outer portion of the hook (Schwark and Schierle, 1992). Although auxin is normally associated with cell elongation, higher concentrations (3 μM to 1 mM) of IAA inhibit growth by stimulating ethylene production (Burg and Burg, 1966). Thus, a high local concentration of IAA in the inner portion of the hook may be responsible for the increased ethylene production in this region (Schierle and Schwark, 1988) and this may, ultimately, lead to inhibition of growth. Studying the effects of IAA on the enzymes of ethylene biosynthesis will help to understand how these hormones interact in this response.

Ethylene production is normally low in vegetative tissues but can be increased by a variety of stimuli, including wounding and IAA (Abeles et al., 1992). In most cases, the increase in ethylene production appears to result from an increase in the transcript

and activity levels of ACC synthase, the first committed enzyme of ethylene biosynthesis (Kende, 1993). ACC synthase catalyzes the formation of ACC from *S*-adenosyl-L-methionine (AdoMet). ACC is converted to ethylene by the second enzyme, ACC oxidase, which is thought to be constitutively present in most vegetative tissues (Yang and Hoffmann, 1984). Because a stimulus that promotes ethylene production usually causes ACC synthase activity to increase rapidly from extremely low or undetectable levels, and because ACC oxidase is usually constitutively present at least at low levels, ACC synthase has been widely regarded as the rate-limiting step in ethylene biosynthesis (Yang and Hoffman, 1984). There is evidence, however, that changes in ACC oxidase levels may also contribute to the overall increase in rates of ethylene production. Using *in vivo* assays, it has been shown that ethylene pretreatment increases ACC oxidase activity in citrus leaves (Riov and Yang, 1982), etiolated pea seedlings (Schierle et al., 1989), and carnation petals (Drory et al., 1993), and wounding of ripening tomato fruit induces the accumulation of ACC oxidase transcript levels (Holdsworth et al., 1987). Wounding also causes an increase in ACC oxidase activity levels in winter squash mesocarp (Hyodo et al., 1993) and in ACC oxidase transcript and activity levels in etiolated mung bean hypocotyls (Kim and Yang, 1994). In these cases, wound-induced accumulation of ACC oxidase could be blocked by treatment with NBD, a competitive inhibitor of ethylene action (Sisler and Yang, 1984). In orchid flowers, pollination caused an increase in ACC oxidase transcript levels, and this increase could be inhibited by NBD (O'Neill et al., 1993). These results show that a stimulus causing an increase in ethylene production can also cause an increase in ACC oxidase activity. It has also

been suggested that the enzymes of ethylene biosynthesis are sequentially induced by a stimulus promoting ethylene production (Hyodo et al., 1993). It is currently not known whether, in these instances, ACC oxidase is a rate-limiting enzyme in the production of ethylene.

The work below reports on the effects of IAA on ACC oxidase transcript and activity levels. The timing of the induction of both ACC synthase and ACC oxidase is also compared. These studies use the first internode of 5- to 6-day-old etiolated peas because this tissue has a very low basal level of ethylene production (Schierle et al., 1989), making it easy to detect changes in ACC synthase and ACC oxidase mRNA and enzyme activity levels.

Materials and methods

Plant material

Pea seeds (*Pisum sativum* L., cv. Alaska; Cliston Seed Co., Faison, NC) were imbibed overnight in aerated tap water. Seedlings were grown in vermiculite at 25°C in darkness. In all experiments, treatments were performed on intact, 5- to 6-day-old seedlings that had formed a third node, and the start of the treatments was designated as 0 h. At the times indicated, 2-cm sections were isolated from the seedlings and immediately frozen in liquid nitrogen. Tissue from the same experiment was used for both the enzyme assays and RNA isolations. All manipulations were performed under safe green light (530-590 nm).

Hormone and inhibitor treatment of plant material

For ethylene treatments, seedlings were enclosed in 9-L desiccators fitted with injector ports that were sealed with rubber serum vial caps. Ethylene was injected into the desiccators, and the proper internal ethylene concentration was confirmed by gas chromatography. Purafil (Purafil, Inc., Norcross, GA), which absorbs ethylene, was added to control treatments in a dish at the bottom of the desiccator to prevent accumulation of ethylene produced by the seedlings. IAA solutions contained 100 μM IAA, 0.05% ethanol, and 0.05% Tween-20, adjusted to pH 6.0 with dilute NaOH. Control solutions of the same pH were identical except that IAA was omitted. To disturb the seedlings as little as possible, the solutions were applied to the intact plants as a fine

mist using a spray bottle. Treatments with NBD were performed in 9-L desiccators. Immediately after being treated with IAA or control solutions, the seedlings were placed in the chamber and NBD was injected to yield a concentration of 3000 μL/L in the gas phase.

Measuring the rate of ethylene production

At the indicated times after treatments, 7 to 10 sections were isolated from the first internode and enclosed in 4-mL tubes fitted with serum vial caps. After 30 min, 1 mL of headspace was withdrawn for determination of ethylene concentration by gas chromotography. Because the lag time for wound-ethylene production in etiolated pea stems is approximately 26 min (Saltveit and Dilley, 1978), taking the measurements after 30 min minimizes any contributions of wound effects to the ethylene production rates.

ACC oxidase enzyme assays

The ACC oxidase assay was modified from that of Ververidis and John (1991). For the experiments with IAA treatment, weighed, frozen stem sections were ground in liquid nitrogen. An extraction buffer (1 mL/g FW) consisting of 100 mM Tris-HCl, pH 7.2, 30 mM Na-ascorbate, and 10% glycerol was added to the frozen powder which was allowed to thaw to a slurry. The total extract was transferred to a cold, 1.5-mL microcentrifuge tube on ice and centrifuged at 15,000 *g* for 10 min at 4°C. The supernatant was used directly in the assay. Neither polyvinylpolypyrrolidone nor Triton X-100 were necessary in the extraction buffer to recover maximum activity from the stem

sections, as was reported for apple fruits (Dong et al., 1992). For the time course of induction by ethylene, the addition of 1 mM ACC to the extraction buffer was necessary to recover maximum enzyme activity. The addition of ACC to the extraction buffer for the IAA-treated tissue did not increase the recoverable ACC oxidase activity. A likely explanation for this observation is that the ACC stabilized the enzyme during extraction and that IAA-treated tissue produced sufficient amounts of ACC to stabilize ACC oxidase without the addition of ACC to the extraction buffer.

The activity assays consisted of 200 μL extraction buffer, 50 μL of 40 mM ACC, 50 μL of 2 mM $FeSO_4$, and 1.7 mL of the enzyme extract in a total volume of 2 mL. The reaction mixtures were shaken at 30°C for 1 h in 9-mL screwcap tubes, each fitted with a teflon-coated septum (Fischer Scientific, Pittsburgh, PA). At the end of this time period, 1 mL of the headspace was removed and analyzed by gas chromatography. The *in vitro* activity was saturated at 1 mM ACC, required ascorbate and $FeSO_4$ for maximal activity, was inhibited by Co^{2+}, and was completely abolished by 5-min treatment at 90°C. These results agree with previously established parameters for specific ACC oxidase activity.

ACC synthase enzyme assays

Because of the low ACC synthase activity recovered from pea-stem tissue, it was necessary to modify the extraction procedure from that described by Jones and Kende (1979). Approximately 10 g FW of stem tissue was ground in liquid nitrogen and extracted with 0.5 mL/g FW of buffer (200 mM Na-phosphate, pH 8.0, 5 mM DTT, 10

μM pyridoxal 5-phosphate). The slurry was centrifuged at 25,000 *g* for 20 min at 4°C. The supernatant was dialyzed overnight against two changes of buffer (10 mM Na-phosphate, pH 8.0, 5 μM pyridoxal 5-phosphate) at 4°C. The volume of the dialyzed extract was reduced by placing the dialysis bag onto dry polyethylene glycol until 1 mL of extract represented approximately 1 g FW of tissue. In the assay, 2 mL of concentrated extract was combined with 200 μL of 1 M Na-phosphate, pH 8.0, and either 50 μl of 0.2 M AdoMet or H_2O for a total volume of 2.25 mL in a 9-mL tube, and the reaction mixture was shaken at 30°C for 1 h. The ACC produced was determined by the method of Lizada and Yang (1979).

Cloning of ACC synthase

To clone the gene for IAA-induced ACC synthase, reverse transcription (RT)-polymerase chain reaction (PCR) was performed using total RNA isolated from tissue treated for 2 h with IAA. In the RT reaction, 2 μL of 0.2 μg/μL oligo$(dT)_{15}$ (Pharmacia, Piscataway, NJ), 10.6 μL of 2.5 mM dNTP mixture, 0.4 μL of 40 U/μL RNAsin RNAse inhibitor (Promega, Madison, WI), 1 μL of 200 U/μL M-MLV reverse transcriptase with 4 μL of the supplied 5X RT buffer (Promega), and 2 μL of 1 μg/μL total RNA were combined for a total volume of 20 μL. This mixture was incubated at 37°C for 1 h, and the reaction stopped by incubation at 95°C for 10 min. Two μL of the RT reaction was used for PCR. Two degenerate oliogonucleotide primers, 5'-CTC(GAATTC) ACCAAYCCNTCNAAYCCNYTRGG-3'and5'-CTC(AAGCTT)ACNARNCCRAARC-TYGACAT-3' based on conserved amino acid sequences (TNPSNPLG and MSSFGLV,

respectively) were synthesized containing *Eco*RI and *Hind*III restriction sites (shown in parentheses) at the 5'-ends, respectively, and used for PCR. Thermocycling was performed at 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min, for 35 cycles. The products were digested with *Eco*RI and *Hind*III, separated by agarose gel electrophoresis, recovered by electrophoresis onto DE-81 paper (Sambrook et al., 1989), ligated into pBluescript SK- (Stratagene, La Jolla, CA), and transformed into INVαF' cells (Invitrogen, San Diego, CA). Five independent clones were selected for sequencing. The sequences of all five were identical in both strands except for the primer regions. One of these clones (317 bp) was selected for further study. Since a different ACC synthase cDNA clone (PS-ACS1, see Chapter 4) had been previously isolated from the hook region of etiolated pea seedlings, the PCR-generated clone was designated PS-ACS2 (see Figure 3.1).

RNA blot analysis

All RNA isolations were performed as described by Puissant and Houdeline (1990). For ACC oxidase RNA blots, 20 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel. Ethidium bromide staining of the ribosomal bands was used to confirm equal loading of lanes. Gels were transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). The filters were baked at 80°C for 1-2 h and prehybridized at 62 °C with 5X SSPE, 10X Denhardt's, 0.1% SDS, 0.25 mg/mL salmon sperm DNA, and 50% formamide, for at least 4 h. A full-length (1122 bp) pea ACC oxidase clone (pPE8) (Peck et al., 1993) in pBluescript SK- (Stratagene) was used to

synthesize a ^{32}P-labeled antisense strand RNA probe by using T7 RNA polymerase. Hybridization was performed at 62°C in the prehybridization buffer. The filters were washed 3 times for 15 min each in 0.2X SSC, 0.2% SDS, at 62°C. Autoradiography was performed using Hyperfilm-MP (Amersham) at -80°C with two amplification screens. The signals were quantified with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

For ACC synthase RNA blots, conditions were as described except that 5 μg of poly(A)$^+$ RNA was used. The poly(A)$^+$ RNA was isolated using the Mini-Oligo(dT) Cellulose Spin Column kit (5 Prime → 3 Prime, Inc., Boulder, CO) according to the manufacturer's instructions. The riboprobe was prepared from PS-ACS2, a 317-bp PCR product.

```
  1         ACTGTTATGGACAGAAACACACTAAGAACTGTCATCACTTTCATCAACGAAAAGCGTATC
            T  V  M  D  R  N  T  L  R  T  V  I  T  F  I  N  E  K  R  I

 61         CAATCTAATAAGCGACGAAATCTACGCTGCAACGGTTTTTAGCCACCAAGTTTCATAAGC
            H  L  I  S  D  E  I  Y  A  A  T  V  F  S  H  P  S  F  I  S

121         ATAGCGGAGATAATCGAACATGACACAGACATTGAATGCGACCGTAACCTCGTTCACATA
            I  A  E  I  I  E  H  D  T  D  I  E  C  D  R  N  L  V  H  I

181         GTTTATAGTCTTTCAAAAGTCATGGGATTCCCGGGGTTTAGAGTTGGTATAATATACTCA
            V  Y  S  L  S  K  D  M  G  F  P  G  F  R  V  G  I  I  Y  S

241         TACAACGACACCGTTGTTGATTGCACGCGCAAA
            Y  N  D  T  V  V  D  C  T  R  K
```

Figure 3.1. Nucleotide and deduced amino acid sequence of ACC synthase cDNA clone PS-ACS2. The sequence does not contain the primers used for PCR (described in Materials and Methods). The conserved active site region is underlined.

Results

Cloning of an IAA-inducible ACC synthase

To obtain a probe for measuring ACC synthase transcript levels, RT-PCR was used to clone an ACC synthase cDNA fragment (PS-ACS2) from the first internode of etiolated pea seedlings treated for 2 h with IAA. This cDNA contained the nucleotide sequence encoding the active site of ACC synthase. PS-ACS2 (Figure 3.1) has 79% and 67% nucleotide identity with the corresponding regions of two IAA-induced ACC synthase cDNA clones from etiolated mung bean hypocotyl sections (Botella et al., 1992; Kim et al., 1992).

Ethylene increases ACC oxidase activity and transcript levels

Schierle et al. (1989) found that ethylene pretreatment of stem sections from etiolated pea seedlings stimulated *in vivo* ACC oxidase activity. The present work expanded upon the previous experiments by using intact seedlings for treatments and an *in vitro* enzyme assay. ACC oxidase transcript levels and enzyme activity increased with the duration of exposure to 40 μL/L ethylene (Figure 3.2). ACC oxidase transcript and enzyme activity were present at low but detectable levels in the untreated tissues. After 12 h of exposure to ethylene, the transcript level had increased almost 100-fold, and the enzyme activity had increased about 10-fold. The changes measured in *in vitro* enzyme assays were consistent with those seen *in vivo* when the conversion of exogenous ACC (1 mM)

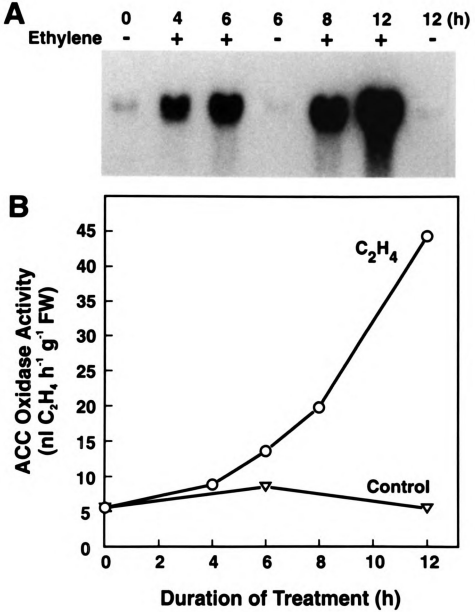


Figure 3.2. Effect of ethylene on ACC oxidase transcript abundance (A) and enzyme activity (B). At 0 h, intact seedlings were placed in desiccators with (○) or without (▽) 40 μL/L ethylene. At the times indicated, the seedlings were removed from the desiccators, and 2-cm sections from the first internode were isolated and used for RNA isolation and enzyme assays. For RNA blot analysis (A), 20 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with a ^{32}P-labelled antisense RNA strand prepared from pPE8 (Peck et al., 1993). The experiment was performed four times with similar results.

to ethylene was measured in stem sections (data not shown). The increase in ACC oxidase activity was not a result of handling during treatment or of enclosing seedlings in desiccators because neither the transcript level nor the activity of the enzyme increased in the control treatments (Figure 3.2). Since ethylene can cause the accumulation of ACC synthase activity in some tissues (Yang and Hoffman, 1984), it could be argued that ethylene induced ACC oxidase activity by increasing the availability of ACC. To address this possibility, the levels of ACC synthase transcript, ACC synthase enzyme activity, and ACC were measured after ethylene treatment. No changes in any of these parameters were detected during the treatment of the seedlings with ethylene (data not shown). Therefore, ACC oxidase activity is induced by ethylene and not by ACC.

IAA increases ACC oxidase activity and transcript levels via ethylene

Because ethylene induced the accumulation of ACC oxidase activity, a stimulus that promotes ethylene production may also stimulate ACC oxidase activity. In the first internode of etiolated pea seedlings, 100 μM IAA stimulated ethylene production via an increase in extractable ACC synthase activity (Figure 3.3). A control solution of the same pH did not cause this increase, indicating that the response is specific to auxin and that the application method does not cause an increase in ACC synthase activity or ethylene production. ACC oxidase transcript levels and enzyme activity increased following treatment with IAA but not in control tissue (Figure 3.4). NBD, an inhibitor of ethylene action, prevented the increase in ACC oxidase transcript and enzyme activity levels after treatment with IAA (Figure 3.4). NBD did not prevent the IAA-induced

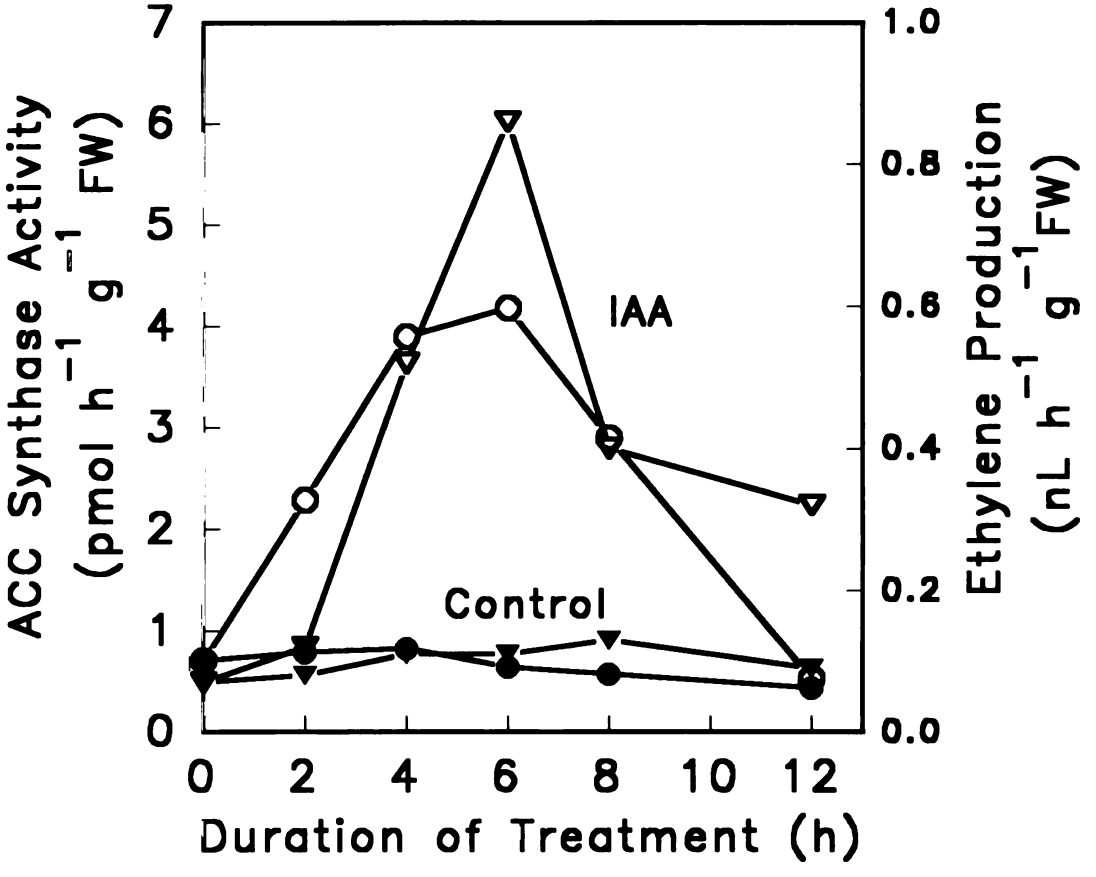
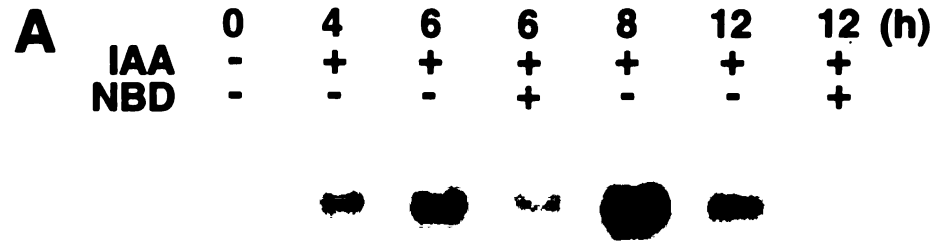
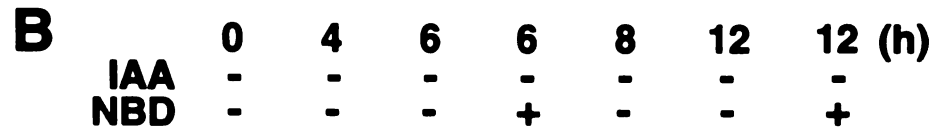


Figure 3.3. Effect of IAA on ACC synthase activity and rate of ethylene production in the first internode of etiolated pea seedlings. At 0 h, seedlings were sprayed with solutions containing 100 μM IAA (○, ▽) or no IAA (○, ▼). At the times indicated, 2-cm sections were isolated from the first internode and used for ACC synthase enzyme assays (○, ○) or for measuring ethylene production (▽, ▼). The experiment was performed twice with similar results.

Legend for Figure 3.4. Treatments were as described in Figure 3.3. Plants were treated with auxin (○,●) or control solutions (▽,▼) in the presence (○,▽) or absence (●,▼) of NBD. RNA blot analysis was performed as described in Figure 3.2. The experiment was performed four times with similar results.





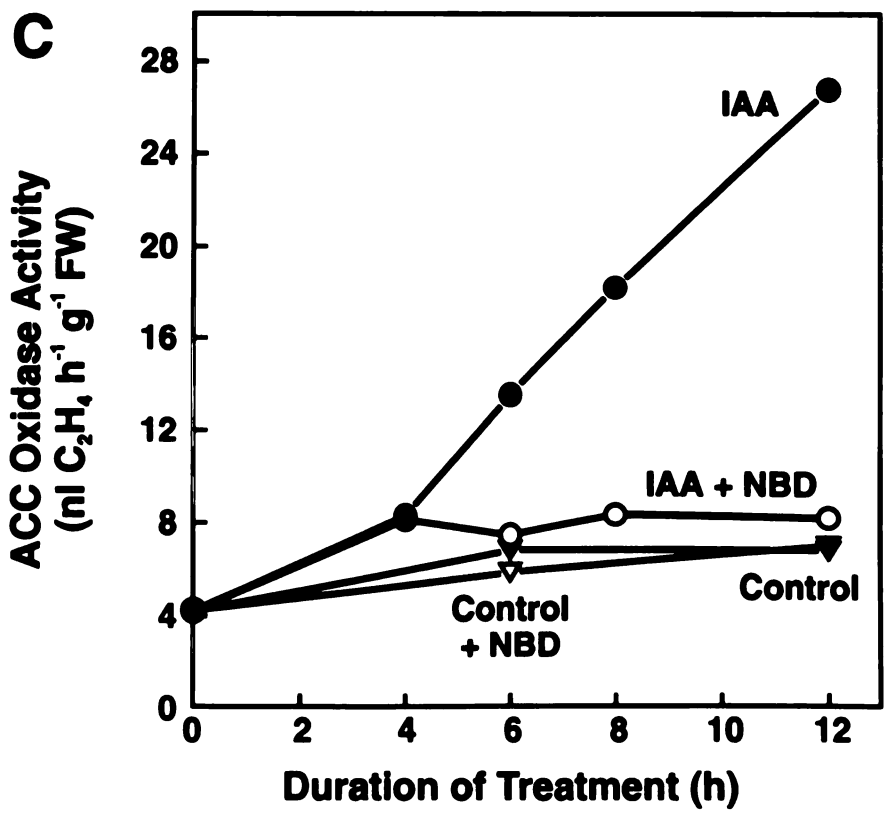


Figure 3.4. Effect of IAA and NBD on ACC oxidase transcript abundance (A,B) and enzyme activity (C).

accumulation of ACC synthase activity (data not shown), showing that NBD did not prevent the response to IAA. To determine if NBD prevented the increase in ACC oxidase activity non-specifically, saturating levels of ethylene (300 μL/L) were added to the IAA+NBD treatment. Ethylene restored the ACC oxidase activity and transcript levels in the presence of NBD (Figure 3.5), supporting earlier evidence that NBD acts specifically as a competitive inhibitor of ethylene action. The difference in transcript abundance between the IAA and the IAA+NBD+ethylene treatments (Figure 3.5) was not surprising. In the IAA treatment, the amount of transcript reached a maximum at 8 h and decreased by 12 h (Figure 3.4A), presumably because IAA-induced ethylene production had ceased (note the pattern of ethylene production in Figure 3.3). In the presence of ethylene, the ACC oxidase transcript level continued to increase during entire period of treatment (Figure 3.2). These results show that IAA increases the levels of ACC oxidase transcript and activity via ethylene.

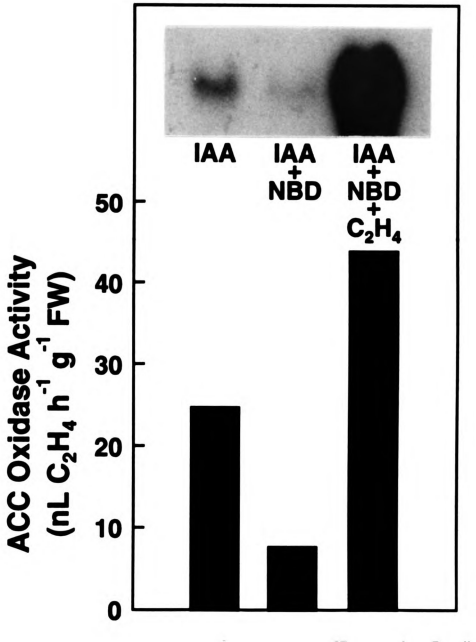


Figure 3.5. Competitive effects of NBD and ethylene on ACC oxidase transcript abundance and enzyme activity during IAA treatment. Seedlings were treated with auxin as described in Figure 3.3 for 12 h with or without NBD or ethylene . RNA blot analysis was as described in Figure 3.2. The experiment was performed twice with similar results.

The evidence that IAA treatment resulted in an increase in ACC synthase activity and that the ethylene produced caused an increase in ACC oxidase activity indicated that the ethylene biosynthetic enzymes are sequentially induced. If so, the increase in ACC synthase activity should be detectable before the increase in ACC oxidase activity. Figure 3.6 shows the changes in ACC synthase and ACC oxidase transcript levels and activities during the first 2 h of IAA treatment. The levels of ACC synthase transcript and enzyme activity increased within the first hour (Figure 3.6, A and C). ACC oxidase transcript, however, did not begin to increase until 2 h after the start of IAA treatment (Figure 3.6B). ACC oxidase activity did not increase during the first 2 h (Figure 3.6C), although it did increase about 2-fold by 4 h (Figure 3.4C).

Legend for Figure 3.6. Treatment conditions were as described in Figure 3.3, and RNA blot analysis as in Figure 3.2, except that 5 μg poly(A)$^+$ RNA was used with an ACC synthase antisense RNA strand made from pPS-ACS2 as a probe. The experiment was performed three times with similar results.

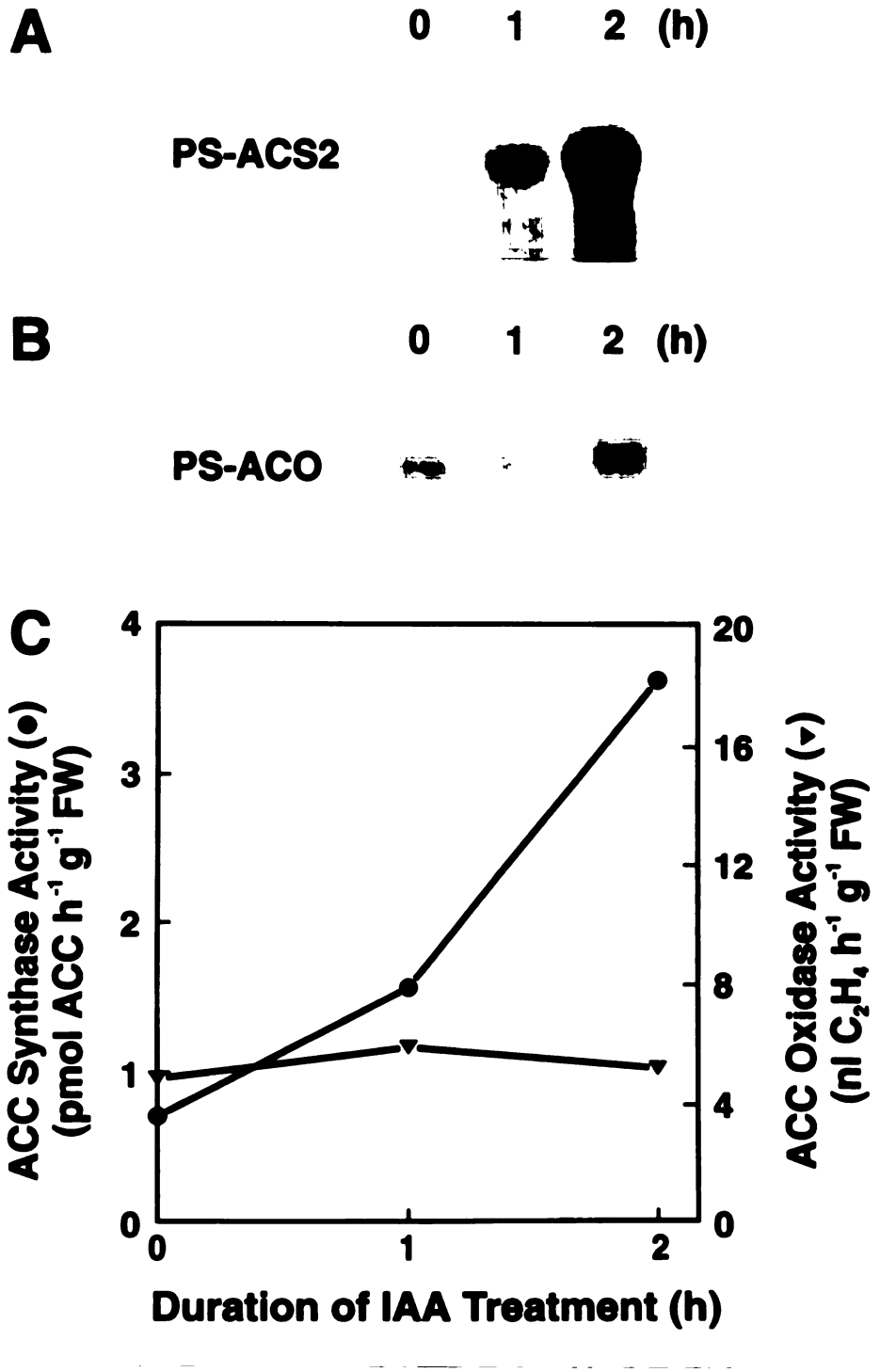


Figure 3.6. Comparison of rates of accumulation of ACC synthase (●) and ACC oxidase (▾) transcript and enzyme activity after auxin treatment.

Effect of ethylene pretreatment on IAA-induced ethylene biosynthesis

ACC oxidase enzyme activity increases following IAA treatment. It is possible that the change in ACC oxidase contributes to the overall increase in the rate of ethylene production in the tissue. If so, ACC synthase cannot be considered the sole, rate-limiting enzyme in ethylene biosynthesis. To address this possibility, seedlings were pretreated for 4 h with 40 μL/L ethylene to raise the levels of ACC oxidase before treating the seedlings with IAA. If the level of ACC oxidase is initially limiting, the rate of ethylene production should increase more rapidly in seedlings pretreated with ethylene before IAA treatment than in seedlings treated with IAA alone. Figure 3.7 shows that ethylene pretreatment results in increased ethylene production. As a control, ACC synthase activity was measured to determine if the change in ethylene production could be attributed solely to the increased levels of ACC oxidase. Unexpectedly, the ethylene pretreatment before the application of IAA resulted in elevated ACC synthase activity and transcript abundance (Figure 3.8).

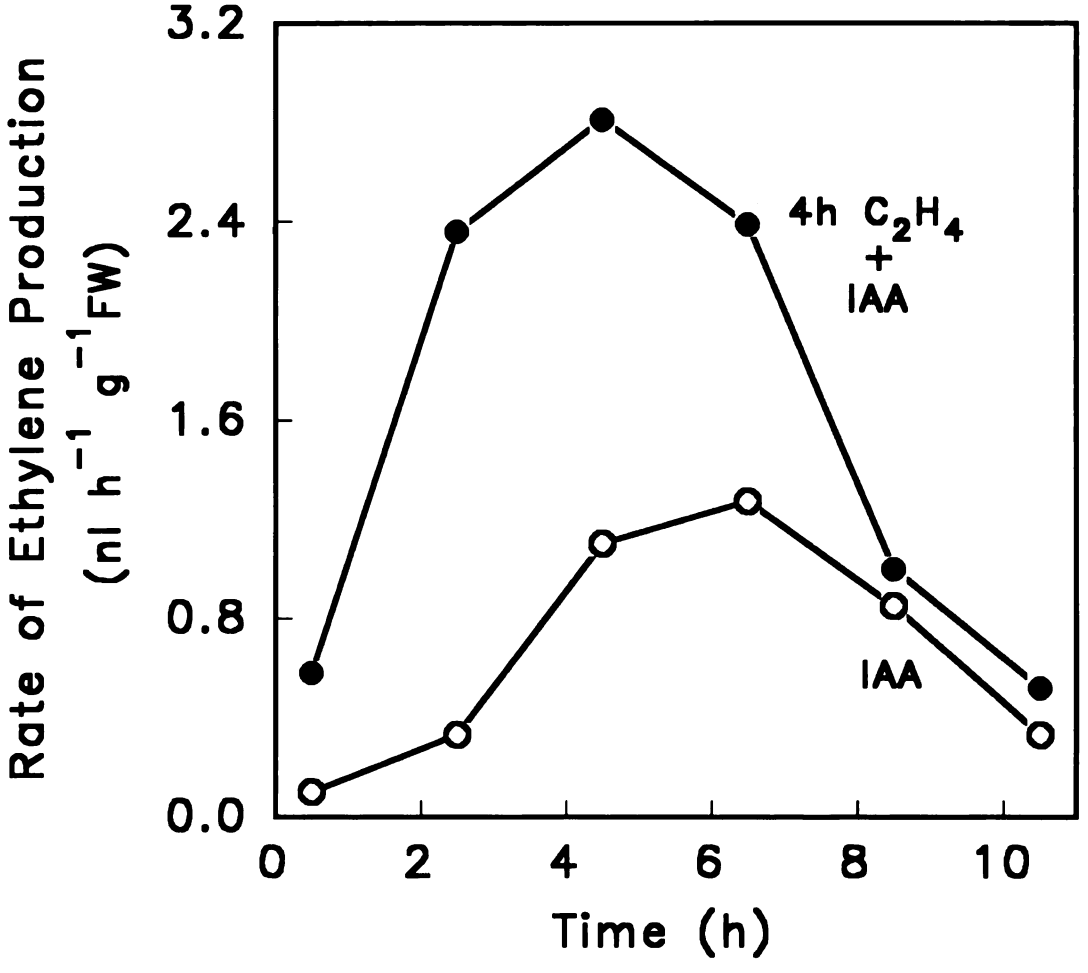


Figure 3.7. Effect of ethylene pretreatment on rate of auxin-induced ethylene production. Intact seedlings were placed in desicators with (●) or without (○) 40 μL/L ethylene for 4 h. The seedlings were then treated with auxin as described in Figure 3.3. At the times indicated, 1-cm sections were isolated from the first internode and used for measuring ethylene production. The experiment was performed three times with similar results.

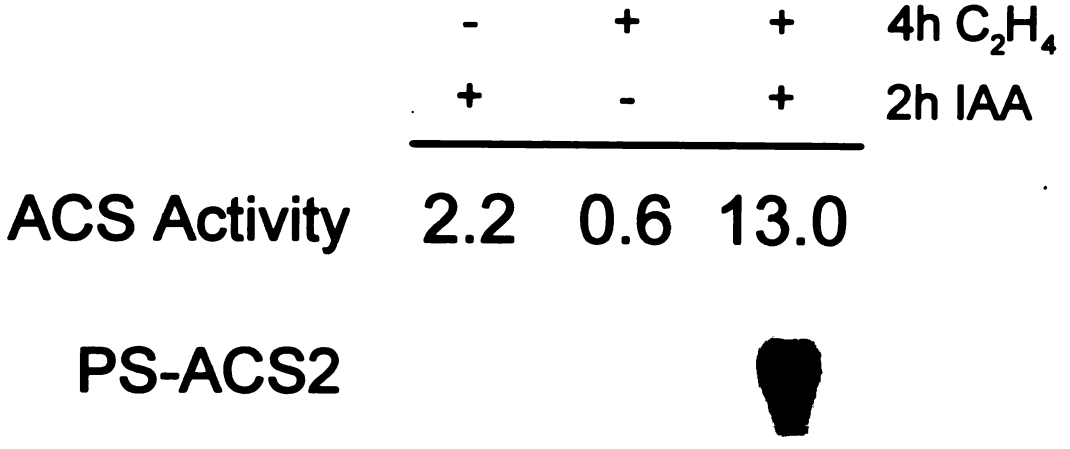


Figure 3.8. Effect of ethylene pretreatment on auxin-induced ACC synthase activity and transcript abundance. A portion of the sections used in Figure 3.7 were used for ACC synthase activity assays and RNA isolation. RNA blot analysis was done as described in Figure 6.6, except that 30 μg of total RNA was loaded in each lane. The experiment was performed twice with similar results.

Discussion

Most of the studies on the control of ethylene production have focused on the regulation of ACC synthase, primarily because it has been considered the rate-limiting enzyme of the pathway. Recent evidence indicates that ethylene-inducing stimuli also cause an increase in ACC oxidase activity (Kende, 1993). Therefore, the effect of IAA and ethylene on ACC oxidase in the first internodes of etiolated peas was examined.

Before Ververidis and John (1991) determined how to recover *in vitro* ACC oxidase enzyme activity, the only recourse was to determine ACC oxidase activity *in vivo* by incubating tissue with high concentrations of ACC for 1 to 3 h and measuring the amount of ethylene produced. This *in vivo* method has two disadvantages. First, isolating stem sections induces, over time, a wound response which has been shown to increase ACC oxidase activity in winter squash mesocarp (Hyodo et al., 1993) and etiolated mung bean hypocotyls Kim and Yang (1994). Second, ACC oxidase activity continues to increase during the incubation period of the *in vivo* assay. Because the enzyme activity is changing, any value measured is an average between two time points and cannot be attributed to any one time point on a curve. This complicates comparisons between activity levels and transcript abundance because the RNA is isolated at specific time points.

To exclude the effects of wounding, it was important to treat intact seedlings with the auxin solutions. Previously, IAA-induction studies involved incubating tissue sections on IAA solutions that often contained a cytokinin to maximize changes in the normally

low ACC synthase activity (Kim et al., 1992; Yoshi and Imaseki, 1982). It is now apparent, however, that this type of treatment can cause both wound-induced and IAA-induced transcription of different ACC synthase genes, with unknown effects resulting from the benzyladenine treatment. The multiple gene products could contribute to changes in ACC synthase activity and ethylene production. The obvious complexity of these types of experiments complicates the interpretation of results and demonstrates the necessity of using intact seedlings with gentle application of test solutions to specifically study the effects of auxin on ethylene biosynthesis.

A ten-fold difference between the ethylene-induced ACC oxidase transcript abundance and enzyme activity was observed. The difference is most likely a result of the underestimation of the enzyme activity. As reported previously by Dong et al. (1992), the addition of CO_2 to the reaction mixture greatly stimulates ACC oxidase activity (data not shown). However, since the level of CO_2 within the tissue was not known, and since ACC oxidase activity could be assayed without the addition of CO_2, all experiments were performed at ambient CO_2 concentrations. Moreover, Smith et al. (1994) have shown that the enzyme undergoes oxidative damage in the presence of ACC, Fe^{2+}, and ascorbate, with a half-life of less than twenty minutes. Thus, the enzyme activity would have been substantially lowered by the end of the 1 h incubation period. As discussed by Smith et al. (1994), questions still remain about the conditions that should be used for *in vitro* ACC oxidase enzyme assays.

From the data presented in this work, a model for the sequential induction of the ethylene biosynthetic enzymes after IAA treatment is proposed. IAA causes an increase

in ACC synthase transcript abundance leading to an increase in ACC synthase activity. The newly formed ACC is converted to ethylene by a low, constitutive level of ACC oxidase. The ethylene produced then causes an increase in the levels of ACC oxidase transcript and activity via a positive feedback loop (Figure 3.9).

The question remains as to whether the increase in ACC oxidase activity contributes to the higher level of ethylene production. The rate of ethylene production increases steeply between 2 and 4 h after IAA treatment (Figure 3.3), which is approximately the same time when ACC oxidase activity begins to increase. While this result indicates that the increase in ACC oxidase activity and the increase in ethylene production rate are related, the evidence is correlative.

While attempting to determine if ACC oxidase contibutes to changes in the rate of ethylene production, an unexpected result was observed. If the tissue was pretreated with ethylene to increase the ACC oxidase activity, the ACC synthase transcript abundance and activity was super-induced by IAA treatment. Because ethylene treatment alone does not affect ACC synthase activity or transcript level (Figure 3.8), the ethylene pretreatment must alter the response to the IAA treatment. One explanation is that the increased levels of ACC oxidase would convert more of the ACC to ethylene, possibly relieving product inhibition on ACC synthase enzyme activity. This hypothesis, however, would not explain the increased levels of ACC synthase transcript that is also observed.

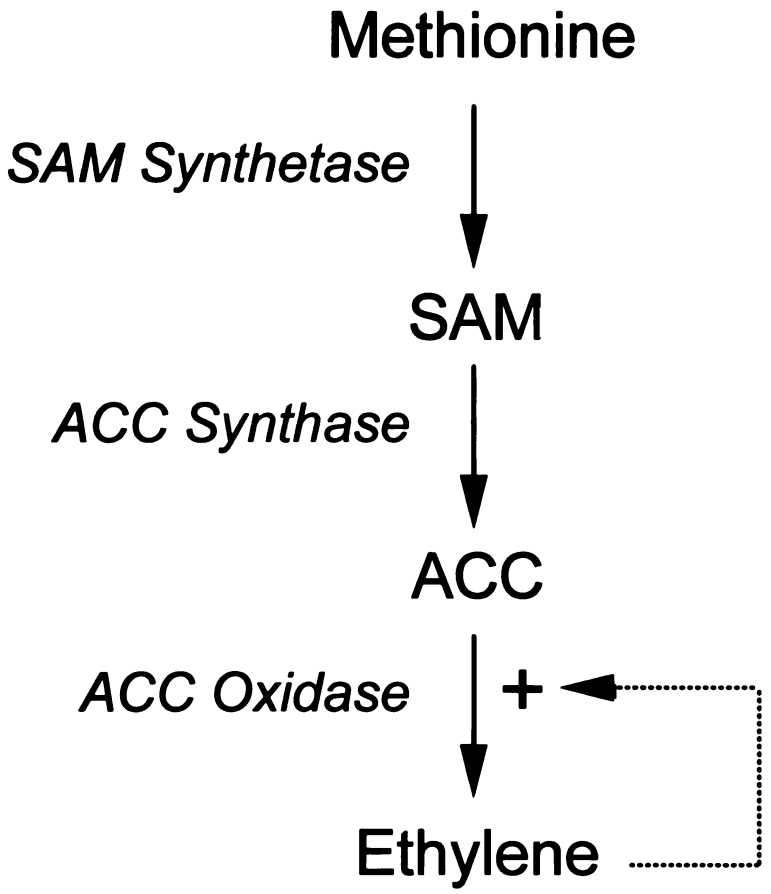


Figure 3.9. Positive feedback of ethylene on ACC oxidase after auxin treatment.

Another possibility is that the increase in ACC synthase activity is a result of the step-down in the ethylene concentration from 40 μL/L ethylene during the pretreatment to ambient concentrations (0.001 μL/L) during the IAA treatment, suggesting an adaptive response in ethylene perception. This possibility is intriguing because ETR, the ethylene receptor, resembles proteins of the prokaryotic two-component system of signal transduction (Chang et al., 1993). Some of these bacterial regulators, such as those in the chemotaxis pathway utilizing CheA and CheY in *E. coli*, utilize dynamic protein phosphorylation cascades in adaptive responses (Borkovich and Simon, 1990). If ethylene responses also involve adaptation, the increase in ACC synthase activity following the ethylene pretreatment might involve a decrease in the level of a functional inhibitor as the tissue undergoes deadaptation. It would be interesting to investigate whether other IAA-inducible genes are similarly affected or if the response is specific for ACC synthase.

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CHAPTER 4

CLONING OF TWO cDNAs ENCODING 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE INDUCED BY INDOLE-3-ACETIC ACID IN ETIOLATED PEAS

Abstract

Two cDNA clones of IAA-induced ACC synthase, PS-ACS1 and PS-ACS2, were isolated from a cDNA library made from the apical hooks of etiolated pea seedlings treated for 4 h with 10^{-4} M IAA. The transcript level of PS-ACS1 increased 75-fold in the third internode of IAA-treated etiolated pea stems. Simultaneous treatment with ethylene inhibited this increase by almost 90%. Similarly, the transcript of PS-ACS2 increased 15-fold after IAA treatment, and ethylene caused a 70% inhibition of transcript accumulation. The inhibition of IAA-induced transcript accumulation by ethylene is consistent with the 70% decrease in the ACC synthase enzyme activity. These results indicate that ethylene inhibits its own biosynthesis by decreasing ACC synthase levels via a negative feedback loop. It was observed that PS-ACS1 hybridized to two transcripts on RNA blot analysis, one of 1.6 kb and one of 1.9 kb. Results of genomic DNA blot analysis and RNA blot analysis using the 3'-untranslated region as a probe indicate that both transcripts are derived from a single gene. Sequencing the ends of eight independent clones of PS-ACS1 showed what appears to be three, unique polyadenylation sites. Moreover, oligonucleotide-directed RNase H mapping of the PS-ACS1 transcripts indicates that the larger transcript contains an additional sequence in the 5'-untranslated region.

Introduction

ACC synthase catalyzes the first committed step of ethylene biosynthesis, the formation of ACC from AdoMet. The enzyme was first identified in homogenates of ripening tomato pericarp tissue by Boller et al. (1979) and Yu et al. (1979). Its activity was shown to be enhanced by factors which stimulate ethylene production, such as wounding, ripening, and auxin (Yang and Hoffman, 1984). Soon after Sato and Theologis (1989) isolated the first cDNA clone for ACC synthase by screening an expression library with antibodies, ACC synthase cDNAs were isolated from a number of different plants (for a review, see Kende, 1993). It was shown that ACC synthase was encoded by a multigene family whose members were differentially regulated, primarily at the transcriptional level, by the various factors which stimulate ethylene production (Kende, 1993). While the amino acid sequence identities vary from 48-97%, seven regions are highly conserved among all ACC synthases (Kende, 1993). These regions provide target sequences for designing oligonucleotide primers to be used in PCR, facilitating the cloning of new ACC synthase cDNAs.

In etiolated pea seedlings, an asymmetric distribution of ethylene biosynthesis may be involved in the asymmetry of growth that leads to the formation of the apical hook (Schierle and Schwark, 1988). Because ethylene production in vegetative tissues is thought to be regulated by the level of free IAA (Yang and Hoffman, 1984), the asymmetry in ethylene production might result from a gradient of auxin concentration between the outer and inner portions of the apical hook. To understand how these two

hormones may interact in this response, the effects of IAA on the enzymes of ethylene biosynthesis are being studied. Rather than attempting to catalogue the factors influencing the expression of a particular gene in all parts of the plant, the present work concentrated on studying the effects of auxin only in etiolated pea seedlings, a well-characterized system for studying ethylene production (Abeles et al., 1992). The rapid response time (changes occur within 0.5 h and are completed by about 8 h) negates concerns about developmental changes in the tissue that might arise if the response continued over several days. The use of etiolated tissue eliminates the complications light-induced changes in development and sensitivity to various growth regulators. Finally, as discussed in Chapter 3, the application method of gently spraying the auxin onto the seedlings reduces the contribution of wound-induced changes.

Materials and Methods

Plant material and hormone treatments

Growth of pea seedlings and treatment with hormones were as described in Chapter 3. In all experiments, sections were isolated from the third (highest) internode.

Cloning and sequencing of cDNA

PCR-based amplification was carried out using the primers described in Chapter 3. A cDNA library (Stratagene, La Jolla, CA) made from poly(A)$^+$ RNA from apical hooks of etiolated peas treated for 4 h with 10^{-4} M IAA and cDNA obtained via reverse transcription of RNA from the third internode of etiolated peas treated for 2 h with 10^{-4} M IAA were used as templates. Reaction conditions and procedures were as described in the Materials and Methods section of Chapter 3. The two PCR products obtained, PACC1 and PACC2, were used to screen the unamplified pea cDNA library. Inserts of the longest full-length cDNAs hybridizing to each PCR product, PS-ACS1 and PS-ACS2 were sequenced completely on both strands.

Cross-hybridization of PS-ACS1 and PS-ACS2

To determine the degree to which the two ACC synthase probes would cross-hybridize, control experiments using sense-strand RNA from each of the clones were performed. For PS-ACS1, the plasmid was digested with Eco RI to linearize the vector, and the sense strand was produced by *in vitro* transcription using T3 RNA polymerase.

For PS-ACS2, Bam HI was used to linearize the plasmid, and T7 RNA polymerase was used to produce the sense strand. Visualization of the sense-strand products on ethidium bromide-stained agarose gels was used to confirm that both templates produced approximately equivalent amounts of transcript. Serial dilutions of each reaction were separated on 1.2% formaldehyde-agarose gels, blotted, and probed with a ^{32}P-labeled anti-sense strand of either PS-ACS1 or PS-ACS2. The signals were quantified with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

ACC synthase assays

Enzyme assays were performed as described in Chapter 3 except that the concentration step with PEG was not needed.

RNA blot analysis

RNA isolation and RNA blot analysis was done as described in Chapter 3. Unless otherwise noted, probes were the full anti-sense RNA strand of either PS-ACS1 or PS-ACS2 labeled with ^{32}P-UTP. For the 3'-untranslated region (UTR) probe of PS-ACS1, a 340-bp Sca I fragment of PS-ACS1 containing only the 3'-UTR was subcloned into pBSK$^-$ which was used to produced the anti-sense RNA probe.

Genomic DNA blot analysis

Genomic DNA was isolated from pea leaves as described by Sambrook et al. (1989). Ten μg of genomic DNA was digested with Bam HI, Eco RI, or Hind III,

separated on a 0.7% agarose gel, and blotted to Hybond-N membrane (Amersham). Prehybridization and hybridization were performed at 65°C in 5X SSC, 5X Denhardt's solution, and 0.5% SDS. The probe was generated by random prime labeling with ^{32}P-αCTP using the full insert of pPS-ACS1 as template. Final wash conditions were 0.2X SSC, 0.5% SDS at 65°C.

Oligonucleotide-directed RNase H mapping of PS-ACS1 transcript

Twenty μg of total RNA was mixed with either 2 μg oligo d(T), 250 ng of oligonucleotides designated SEQB (5'-CAAGATTCGTCCTATTTCCT-3') and SEQC (5'-GTGGATTTGATGGATTTGTG-3'), or water. The total volume was brought to 25 μL with water. The mixture was heated to 65°C for 5 min before slow cooling to room temperature. Ten μL 5X RNase buffer (1X = 20 mM KCl and 4 mM Tris-HCl, pH 8.0), 6.5 μL sterile DEPC-treated H_2O, 5 μL 100 mM $MgCl_2$, 2.5 μL 20 mM DTT, and 1 uL 1.0 unit/μL RNase H were added to a total of 50 μL. This mixture was incubated at 37°C for 45 min. The entire reaction was then precipitated with 5 μL 3 M NaOAc, pH 4.8, and 50 μL isopropanol at -20°C for 1 h. The pellet was resuspended in RNA loading buffer (Sambrook et al., 1989) and used for RNA blot analysis as described above.

Results

Isolation of cDNAs for two auxin-induced ACC synthases

To isolate cDNA clones for ACC synthase transcripts expressed in etiolated seedlings after auxin treatment, PCR was used to amplify fragments containing the active site region. Degenerate oligonucleotide primers based on conserved regions surrounding the active site (TNPSNPLGT and MSSFGLVS; Kende, 1993) were used in PCR with two templates, a cDNA library from etiolated pea apical hooks treated for 4 h with 0.1 mM IAA and cDNA obtained by reverse transcription of mRNA from first internodes of etiolated seedlings treated for 2 h with 0.1 mM IAA. By this method, two unique PCR fragments, PACC1 and PACC2, were isolated. Further attempts to isolate cDNAs for other ACC synthases yielded only these two fragments, indicating that PACC1 and PACC2 represented the two predominant transcripts expressed in etiolated seedlings following IAA treatment.

These fragments were used to screen 600,000 plaques from the unamplified, cDNA library from IAA-treated apical hooks. Thirty-two independent clones which hybridized with PACC1 were isolated. The sequence of PS-ACS1, the longest cDNA clone hybridizing with PACC1, is given in Figure 4.1. It contains a predicted open reading frame of 1443 bp (481 amino acids), a 375-bp 3'-untranslated region (UTR), and a 245-bp 5'-UTR. Of the eight cDNAs sequenced, the three longest clones shared 133 bp of identical 5'-UTR.

Legend for Figure 4.1. The shaded region shows the variable sequence. The alternative sequences (U102 and U11) for the 5'-end are given at the top of the figure.

```
U102 (GCCATGTTCATGTTTCAATTCCAAACCAAACCAAGACATGGACCTAACATTCATCATACACGATT
      ATTCAAAACCACACTCAAATTCAAAACTATAAACTTT)
 U11 (TATAAAGCATAGTTATATTTATAGTAATAATACCTCAAACTTT)

   1 TTTTTTTTTTTTAAAAAAAAGACACATCCATTGCACCAACAAGGTAACAATAACATTCCATCATAA
  67 CAAAAATTAAATTGTAAGTAAGAAAAAAAAACAACAAAAGTAGCAACAATCCATCTTCAAACATTT
 133 TTCACTCATAACATCAACTATCTTTCTATAACCTTCTCTCTCTCTCTCTCTCCCTATAGTCCCTCT
 199 CATACATTGCTTTTCATTGTTTCATTTCTTACTCCTACAAAAGAAAATGAAGTTGCTATCTACAAA
                                                     M  K  L  L  S  T  K
 265 AGCCACATGCAACTCTCATGGCCAAGATTCGTCCTATTTCCTAGGATGGCAAGAATATGAAAAAGA
      A  T  C  N  S  H  G  Q  D  S  S  Y  F  L  G  W  Q  E  Y  E  K  E
 331 AAACCCTTATTATCATGTTCAAAATCCCAAAGGAATTATTCAGATGGGTCTCGCCGAAAATCAGCT
      N  P  Y  Y  H  V  Q  N  P  K  G  I  I  Q  M  G  L  A  E  N  Q  L
 397 GTCTTTTGATCTATTAGAATCATGGCTTGCTAAGAATCAAGATGTAGGAGGATTCAAACGTGATGG
      S  F  D  L  L  E  S  W  L  A  K  N  Q  D  V  G  G  F  K  R  D  G
 463 AAAATCAATATTTAGAGAACTTGCTCTCTTCCAAGACTACCATGGTCTACCATCTTTCAAGAAAGC
      K  S  I  F  R  E  L  A  L  F  Q  D  Y  H  G  L  P  S  F  K  K  A
 529 ATTGGTTGATTTCATGGCTGAGATTAGAGGAAACAAAGTCACTTTTGATCCAAACCACATTGTTCT
      L  V  D  F  M  A  E  I  R  G  N  K  V  T  F  D  P  N  H  I  V  L
 595 AACAGCTGGTGCCACTTCTGCTAATGAGACACTCATGTTTTGTCTTGCTGAAAAAGGAGAAGCCTT
      T  A  G  A  T  S  A  N  E  T  L  M  F  C  L  A  E  K  G  E  A  F
 661 TCTTCTTCCCACTCCCTATTATCCAGGATTTGATAGAGACTTGAAGTGGAGAACTGGGGTGGAGAT
      L  L  P  T  P  Y  Y  P  G  F  D  R  D  L  K  W  R  T  G  V  E  I
 727 AGTTCCAATACAATGCACAAGTTCAAACAATTTCCAAATGACTGAATCTGCTTTGCAACAAGCTCA
      V  P  I  Q  C  T  S  S  N  N  F  Q  M  T  E  S  A  L  Q  Q  A  H
 793 TGAAGATGCAAAGAAGAAGAACCTAAAAGTCAAAGGTGTCTTAGTCACAAATCCATCAAATCCACT
      E  D  A  K  K  K  N  L  K  V  K  G  V  L  V  T  N  P  S  N  P  L
 859 AGGCACTACAATGTCAAAGAATGAATTAAACCTTCTCATTGACTTCATCAAAGACAAAAACATGCA
      G  T  T  M  S  K  N  E  L  N  L  L  I  D  F  I  K  D  K  N  M  H
 925 TTTAATAAGCGACGAGATTTACTCCGGTACAGTTTTCACTTCTCCAAACTTCGTCAGCGTCATGGA
      L  I  S  D  E  I  Y  S  G  T  V  F  T  S  P  N  F  V  S  V  M  E
 991 AATCCTTAACGAAAGAACCGATAAGGATTTCCTCGACGCTAACGTCTCAGAAAGAGTTCACATCGT
      I  L  N  E  R  T  D  K  D  F  L  D  A  N  V  S  E  R  V  H  I  V
1057 CTATAGTCTCTCCAAAGATCTCGGTCTACCAGGCTTCCGTGTCGGCGCTATCTATTCCGACAACGA
      Y  S  L  S  K  D  L  G  L  P  G  F  R  V  G  A  I  Y  S  D  N  E
1123 AACCGTCGTTGCAGCCGCGACGAAAATGTCTAGCTTTGGTTTGGTTTCATCACAAACTCAATACCT
      T  V  V  A  A  A  T  K  M  S  S  F  G  L  V  S  S  Q  T  Q  Y  L
1189 TCTCTCAGCTATGCTAGGTGACAAAAAATTCACAAGAAACTACTTATCCGAGAATCAAAAAAGACT
      L  S  A  M  L  G  D  K  K  F  T  R  N  Y  L  S  E  N  Q  K  R  L
1255 CAAAAAACGACAGAAAATGCTTGTTAACGGATTGCAAAAAGCCGGTATTAGCTGTCTGAAAACAAA
      K  K  R  Q  K  M  L  V  N  G  L  Q  K  A  G  I  S  C  L  K  T  N
1321 CAACGCTGGTTTGTTTTGTTGGGTTGATATGAGAAATCTTCTAACATCAGACACATTCGAAGCTGA
      N  A  G  L  F  C  W  V  D  M  R  N  L  L  T  S  D  T  F  E  A  E
1387 AATGGATTTATGGAAGAAGATATTATACGAAGTTGGGTTGAACATTTCACCAGGTTCATCATGTCA
      M  D  L  W  K  K  I  L  Y  E  V  G  L  N  I  S  P  G  S  S  C  H
1453 TTGCACTGAACCAGGTTGGTTTCGTGTTTGTTTTGCTAACATGTCGGAAGATACATTAAACCTAGC
      C  T  E  P  G  W  F  R  V  C  F  A  N  M  S  E  D  T  L  N  L  A
1519 TATGAAAAGGTTAAAGGACTTTGTCTCAAACTCCAACGGTGAAGAGGGTAGTAATAGTGATAACAA
      M  K  R  L  K  D  F  V  S  N  S  N  G  E  E  G  S  N  S  D  N  K
1585 GAGAACTAGAAGTTCCCAATCTTCTAGAAGTTTTACAAGAAAATCGATTTCGAATTGGGTTTTTAG
      R  T  R  S  S  Q  S  S  R  S  F  T  R  K  S  I  S  N  W  V  F  R
1651 GTTATCTTCTCGTGATCATCATGAACAAGAAGAACGATAACCTGGTTAACTATGGTGTGAATGTGA
      L  S  S  R  D  H  H  E  Q  E  E  R  *
1717 AGTACTACGCATGCATGCACAATAGTATCTATTACTATTAACTTTTTATTTTTATTGTTTTTTTAC
1783 ACTCCTTGGCCAAATATTTTTTGGGGGGATTTTAGGATCTTACTTTTGGAAAAATATATATTGTAA
1849 TGGTAAATTGTAGAGGGAAGGAAATTAAGAAAAACTCACTCTGTCCATTTGGAATTGAAGTGTGAG
1915 GTGTGTAGGATTATGACAAACTTTAAAGTGTGGTTGAAAAATGTTGTTGGTGTGAATGTGATGGAT
1981 GGAAAGTTTGAGGTATTATTACTATAAATATAACTATTGCTTTATATTTCCGTCCTCATTTTTTAT
2047 AAAAAAAAAAAAAAAAAA
```

Figure 4.1. Nucleotide and deduced amino acid sequence of PS-ACS1.

The remaining 39 bp (clone ACS1-U11), 92 bp (clone ACS1-U102), and 113 bp (ACS1-14; shown as the shaded region of PS-ACS1 in Figure 1) of the 5'-UTRs were unrelated. Isolation of a genomic clone for PS-ACS1 will be necessary to determine which of these 5'-untranslated sequences are correct. The 3'-UTRs of the eight cDNAs sequenced differed only in length, falling into three, distinct groups (Figure 4.2). Each of these groups had one cDNA which contained a poly(A)-tail, suggesting that these groups may represent true differences in polyadenylation sites. The sequence of PS-ACS1 predicts a hairpin structure formed by basepairs 978 to 1035 (Figure 4.3). This structure would contain a 26-bp stem and 7-bp loop, with a predicted $_{\Delta}G$ of -38 kcal (Sasker, 1977).

Five independent clones hybridizing with PACC2 were isolated. The sequence of the longest cDNA hybridizing with PACC2, PS-ACS2, is given in Figure 4.4. The sequence contains a predicted open reading frame of 1461 bp (487 amino acids), a 137-bp 5'-UTR, and a 251-bp 3'-UTR.

Using *in vitro* transcribed sense-strand RNAs as controls, it was determined that the antisense RNA strand probes for the two clones show less than 0.01% cross-hybridization with each other (Figure 5). Thus, the expression patterns of the two genes coding for PS-ACS1 and PS-ACS2 can be distinguished by RNA blot analysis.

Group I

```
          1950       1960       1970
ACS1-U21  TTGAAAA
ACS1-15   TTGAAAAATGTTGTT
ACS1-U102 TTGAAAAATGTTGTTGGTGTG(A)10
ACS1-8    TTGAAAAATGTTGTTGGTGTGAATGTGAT
```

Group II

```
          1970       1980       1990       2000       2010       2020
ACS1-U72  GAATGTGATGGATGGAAAGTTTGAGGTATTATTACTATAAATATAACTATGC
ACS1-A122 GAATGTGATGGATGGAAAGTTTGAGGTATTATTACTATAAATATAACTATGCTT
ACS1-U11  GAATGTGATGGATGGAAAGTTTGAGGTATTATTACTATAAATATAACTATGCTTT(A)21
```

Group III

```
          2020       2030       2040
ACS1-14   GCTTTATATTTCCGTCCTCATTTTTTAT(A)18
```

Figure 4.2. 3′ ends of eight PS-ACS1 clones. The sequences of all the shorter clones in Groups I and II were identical to the internal sequence of the longest clone PS-ACS1-14 (Figure 4.1) from which the numbering of base pairs was taken. A poly(A) tail was present in each of the three groups.

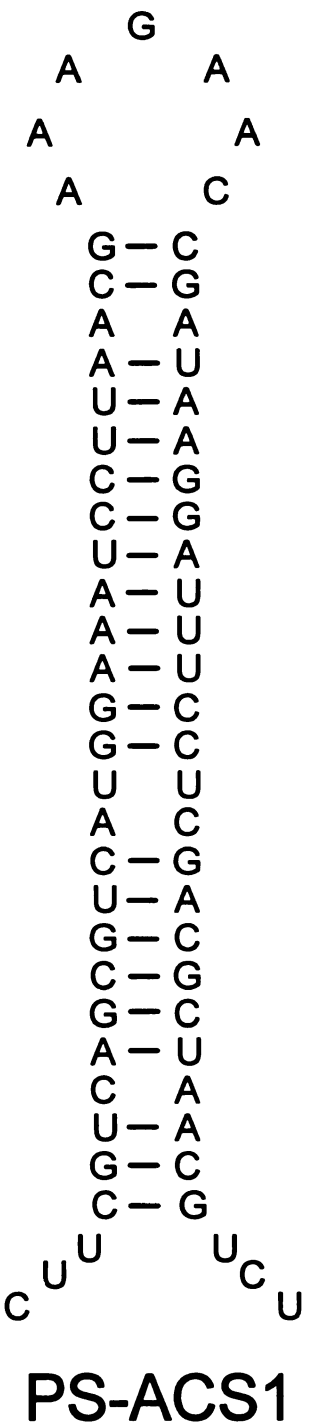

Figure 4.3. Predicted hairpin structure formed by base pairs 978 to 1035 of PS-ACS1.

```
   1  CTTCAAGCTATAAATAACCACCCATACTTCATTCCTAGATATCATTGCAATCAATCTCTC
  61  AACTACAAACACAACAATTTTACTTTCTTTGCTAATATCACACTACATTAATCATTTTTA
 121  TACTACTATAATTAACAATGGGAGTGATGAACTTGGATCAACCACAATTGTTGTCTAAGA
                       M  G  V  M  N  L  D  Q  P  Q  L  L  S  K  I
 181  TAGCCATGGGTGATGGCCATGGTGAAGCATCATCTTACTTTGATGGATGGAAAGCCTATG
        A  M  G  D  G  H  G  E  A  S  S  Y  F  D  G  W  K  A  Y  D
 241  ACAAAGATCCTTTCCATCCATCCAAAAATCCCCACGGAGTCATCCAAATGGGTCTTGCAG
        K  D  P  F  H  P  S  K  N  P  H  G  V  I  Q  M  G  L  A  E
 301  AGAATCAGCTTACTGCTGATATGGTTCAAAATTGGATTATGAGTAATCCAGAAGCTTCGA
        N  Q  L  T  A  D  M  V  Q  N  W  I  M  S  N  P  E  A  S  I
 361  TTTGTACGCTAGAAGGAGTTCATAATTTCAAACAGATGGCGAATTTTCAGGATTATCATG
        C  T  L  E  G  V  H  N  F  K  Q  M  A  N  F  Q  D  Y  H  G
 421  GTTTACCAGAGTTCAGAAATGCTGTGGCGAAATTCATGTCTAGAACCAGAGGAAACAGAG
        L  P  E  F  R  N  A  V  A  K  F  M  S  R  T  R  G  N  R  V
 481  TGACGTTCGATCCCGAACGGATCGTAATGAGCGGCGGAGCCACCGGAGCTCATGAGGCTA
        T  F  D  P  E  R  I  V  M  S  G  G  A  T  G  A  H  E  A  T
 541  CGGCGTTTTGTTTGGCGGATCGTGGTGAAGCTCTTTTGGTGCCTACTCCTTATTATCCAG
        A  F  C  L  A  D  R  G  E  A  L  L  V  P  T  P  Y  Y  P  G
 601  GTTTTGATAGAGATTTGAGGTGGAGAACAGGAGTTAAACTTGTACCAGTTATCTGTGAAA
        F  D  R  D  L  R  W  R  T  G  V  K  L  V  P  V  I  C  E  S
 661  GCTCAAACAATTTCAAATTAACCAAACAAGCCTTAGAAGAAGCTTATGAAAAAGCCAAAG
        S  N  N  F  K  L  T  K  Q  A  L  E  E  A  Y  E  K  A  K  E
 721  AAGATAACATCAGATTCAAAGGTTTACTCATCACAAATCCTTCAAATCCTTTAGGCACTG
        D  N  I  R  F  K  G  L  L  I  T  N  P  S  N  P  L  G  T  V
 781  TTATGGACAGAAACACACTAAGAACTGTCATCACTTTCATCAACGAAAAGCGTATCCATC
        M  D  R  N  T  L  R  T  V  I  T  F  I  N  E  K  R  I  H  L
 841  TAATAAGCGACGAAATCTACGCTGCAACGGTTTTTAGCCACCCAAGTTTCATAAGCATAG
        I  S  D  E  I  Y  A  A  T  V  F  S  H  P  S  F  I  S  I  A
 901  CGGAGATAATCGAACATGACACAGACATTGAATGCGACCGTAACCTCGTTCACATAGTTT
        E  I  I  E  H  D  T  D  I  E  C  D  R  N  L  V  H  I  V  Y
 961  ATAGTCTTTCAAAAGACATGGGATTCCCGGGGTTTAGAGTTGGTATAATATACTCATACA
        S  L  S  K  D  M  G  F  P  G  F  R  V  G  I  I  Y  S  Y  N
1021  ACGACACCGTTGTTGATTGCACGCGCAAAATGTCGAGTTTCGGACTAGTTTCAACACAGA
        D  T  V  V  D  C  T  R  K  M  S  S  F  G  L  V  S  T  Q  T
1081  CACAGTATTTGATCGCGAAAATGCTATCCGATGATGACTTCGTCGAGAAATTTCTTCCCG
        Q  Y  L  I  A  K  M  L  S  D  D  D  F  V  E  K  F  L  P  E
1241  AGAGTGCGAAGAGGTTAGCACAAAGGTACAGAGTTTTCACGGGCGGATTAATCAAAGTCG
        S  A  K  R  L  A  Q  R  Y  R  V  F  T  G  G  L  I  K  V  G
1301  GAATCAAGTGTTTGCAAAGTAACGGTGGACTTTTCGTGTGGATGGATTTAAGAGGACTTC
        I  K  C  L  Q  S  N  G  G  L  F  V  W  M  D  L  R  G  L  L
1361  TTAAGAATGCAACATTCGAATCAGAAATCGAACTATGGAGAGTGATTATTCATGAAGTTA
        K  N  A  T  F  E  S  E  I  E  L  W  R  V  I  I  H  E  V  K
1421  AGATCAATGTTTCACCTGGTGTTTCATTTCATTGCTCTGAACCAGGTTGGTTTAGAGTGT
        I  N  V  S  P  G  V  S  F  H  C  S  E  P  G  W  F  R  V  C
1481  GTTATGCTAACATGGATGATAGAGATGTGCAAATTGCATTACAAAGGATTAGATCATTTG
        Y  A  N  M  D  D  R  D  V  Q  I  A  L  Q  R  I  R  S  F  V
1541  TGACTCAGAATAACAAAGAGGCTATGGGTTCTGATAAGAACTCTAAACCTTACTGGCATA
        T  Q  N  N  K  E  A  M  G  S  D  K  N  S  K  P  Y  W  H  S
1601  GTAATTTGAGGTTGAGCCTTAAACCAAGAAGGTTTGATGATATTATGATGTCACCTCATT
        N  L  R  L  S  L  K  P  R  R  F  D  D  I  M  M  S  P  H  S
1661  CTCCAATTCCTCAATCACCTCTTGTGAAAGCTACTACTTGAATTGATTACATGGTTTTTA
        P  I  P  Q  S  P  L  V  K  A  T  T  *
1721  GTATCATATAGATTATGAAGAAATAACTGATAGAAGATTCTTTGGTTTGTTGATTTATTA
1781  TTTTAGGAGCCTAGATAATAGGTCCTTTAAATGTTGGGGCATTTTTTTTCTTTTTTCACT
1841  TGTTGAATCACATTGTGGTGAAAGAAGTTTATAGAATTTGTAAGGCTATTGTTTGTAATT
1901  TGTTTGAAGGGTTCAGAAAATATCTGTAATCTGTTTTATGAAATTGTTA
```

Figure 4.4. Nucleotide and deduced amino acid sequence of PS-ACS2.

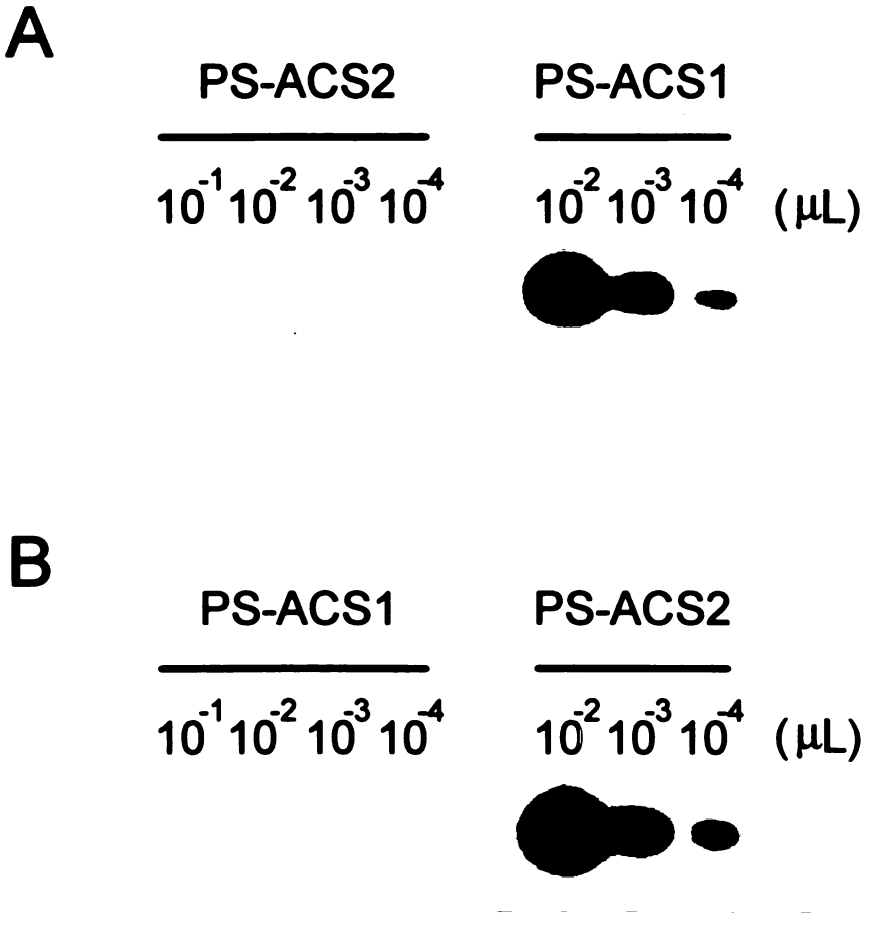

Figure 4.5. Cross-hybridization of PS-ACS1 and PS-ACS2. The cDNA clones were used to produce sense strand RNA by *in vitro* transcription. The transcription reaction yielded approximately equal amounts of the sense- strand product as determined by visualization on an ethidium bromide stained agarose gel. Serial dilutions of the sense-strand transcription reactions were separated on a 1.2% agarose-formaldehyde gel, blotted, and probed with ^{32}P-labeled antisense RNA prepared from either (A) pPS-ACS1 or (B) pPS-ACS2.

Effect of IAA and C_2H_4 on ACC synthase expression and activity.

Extractable ACC synthase activity (Figure 4.6) and transcript levels of both PS-ACS1 and PS-ACS2 (Figure 4.7A) increased in the third internode of 6 to 7 d-old intact etiolated seedlings after treatment with 0.1 mM IAA. While the transcript level of PS-ACS1 reached a maximum at 4 h and then declined to almost undetectable levels by 6 h, the level of PS-ACS2 remained elevated over the course of the experiment (Figure 4.7A). Transcript levels of PS-ACS1 increased 75-fold after IAA-treatment (Figure 7A) but increased only 9-fold when the IAA-treated seedlings were treated simultaneously with ethylene (Figure 4.7B). Similarly, PS-ACS2 transcript increased 15-fold after IAA treatment (Figure 4.7A) but only 5-fold during simultaneous ethylene treatment (Figure 4.7B). The repression of ACC synthase transcript accumulation was consistent with the reduced levels of ACC synthase activity measured in the IAA-treated tissue co-treated with ethylene (Figure 4.6). Treatment with a water control of the same pH did not cause a detectable increase in ACC synthase activity (Figure 4.6) or transcript levels of PS-ACS1 (Figure 4.7C). Levels of PS-ACS2 transcript did increase after the water treatment (Figure 4.7C), but not to the same extent as with IAA-treatment (Figure 4.7A). It has been proposed that VR-ACS1, an ACC synthase clone from mung bean with 81% amino acid identity with PS-ACS2, is induced by touch (Botella et al., 1995), which may explain why the level PS-ACS2 transcript increased following the water treatment.

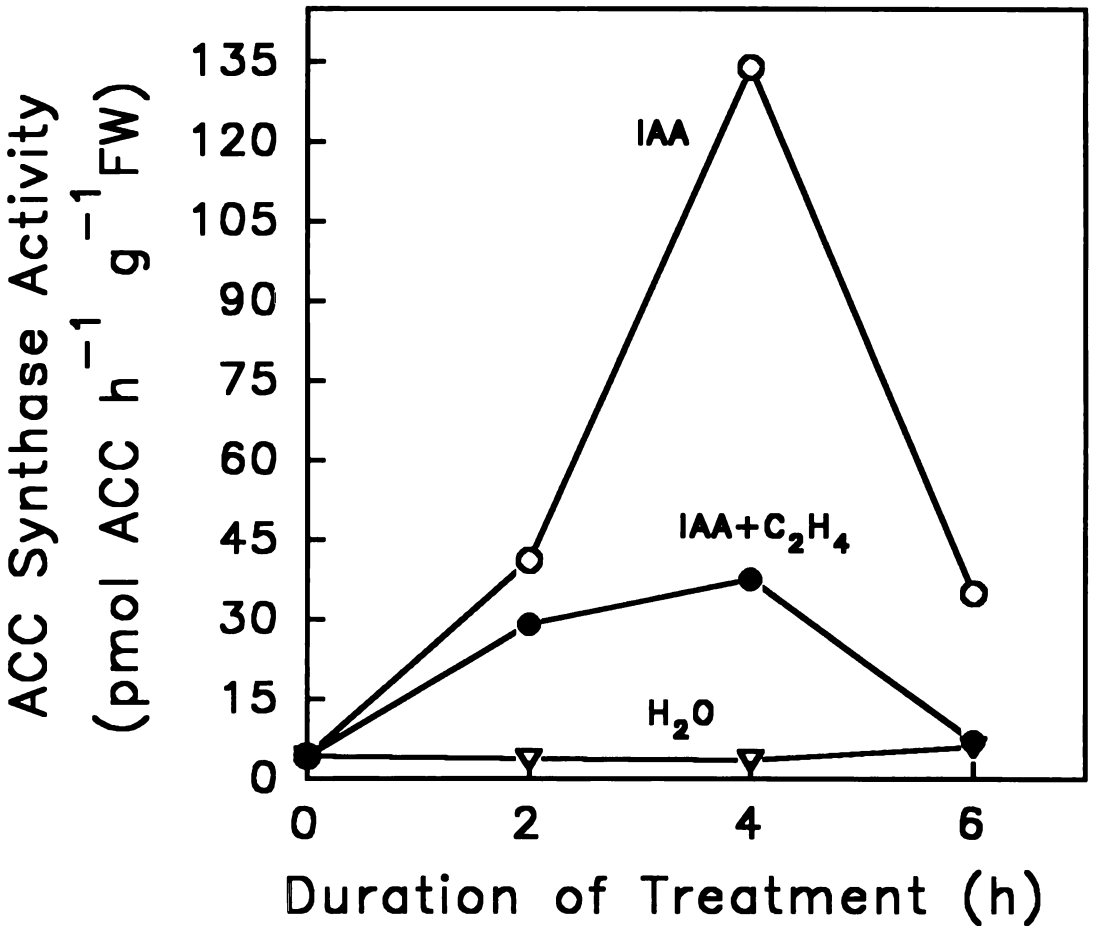

Figure 4.6. Effect of auxin and ethylene on ACC synthase activity. At 0 h, intact seedlings were sprayed with solutions containing 100 μM IAA (●, ○) or no IAA (▽). The IAA-treated seedlings were placed in desiccators with (●) or without (○) 40 μL/L ethylene. At the times indicated, 1-cm sections were isolated from the third (highest) internode and assayed for ACC synthase enzyme activity. The experiment was performed twice with similar results.

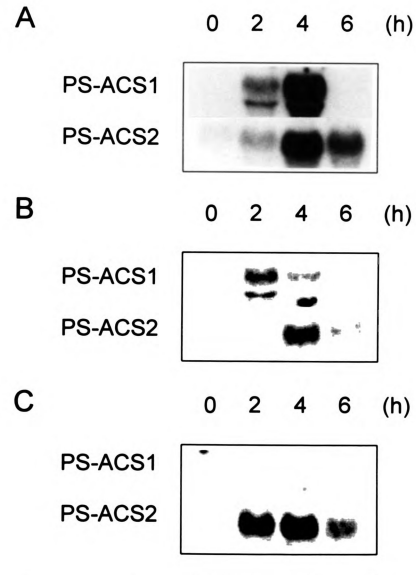

Figure 4.7. Effect of auxin and ethylene of ACC synthase transcript levels. Seedlings were treated with (A) 100 μM IAA, (B) 100 μM IAA + 40 μL/L ethylene, or (C) water. Treatments were as described in Figure 4.6. RNA blot analysis was performed as described in Figure 4.5.

PS-ACS1 hybridizes to two transcripts.

As seen in Figure 4.7, the probe for PS-ACS1 detected two distinct transcripts of 1.6 and 1.9 kb on RNA blots. A time course of the changes after IAA treatment show that the 1.6-kb transcript accumulated first (Figure 4.8A). Moreover, the two transcripts accumulated with different patterns after IAA treatment (Figure 4.8B). These results indicate that the smaller transcript is not a degradation product of the larger.

Because of the predicted hairpin structure in PS-ACS1, the secondary structure could lead to the appearance of two bands. In the cross-hybridization experiment (Figure 4.5), however, the sense-strand RNA product of an *in vitro* transcription reaction from pPS-ACS1 produced only a single band. Because the *in vitro* transcription product had approximately the same size as the larger transcript (data not shown), the appearance of two transcripts is probably not an artifact of secondary structure formation.

To determine if the two transcripts are encoded by one gene or two highly similar genes, the probe used in the RNA blot analysis was used on a genomic DNA blot. Under stringent conditions, the full-length PS-ACS1 probe hybridized with a single band on a genomic DNA blot using three different restriction enzymes (Figure 4.9). In addition, a 300-bp probe containing only the 3′-UTR of the PS-ACS1 cDNA hybridized with both bands on an RNA blot (Figure 4.10). These results indicate that both transcripts are encoded by a single gene but do not exclude a tightly linked gene duplication.

Legend for Figure 4.8. Seedlings were treated with 100 μM IAA as described in Figure 4.6. (A) RNA blot analysis was performed as described in Figure 4.5. (B) Quantitation of abundance of 1.6 kb transcript (○) and 1.9 kb transcript (●) was done on a densitometer. The experiment was performed four times with similar results.

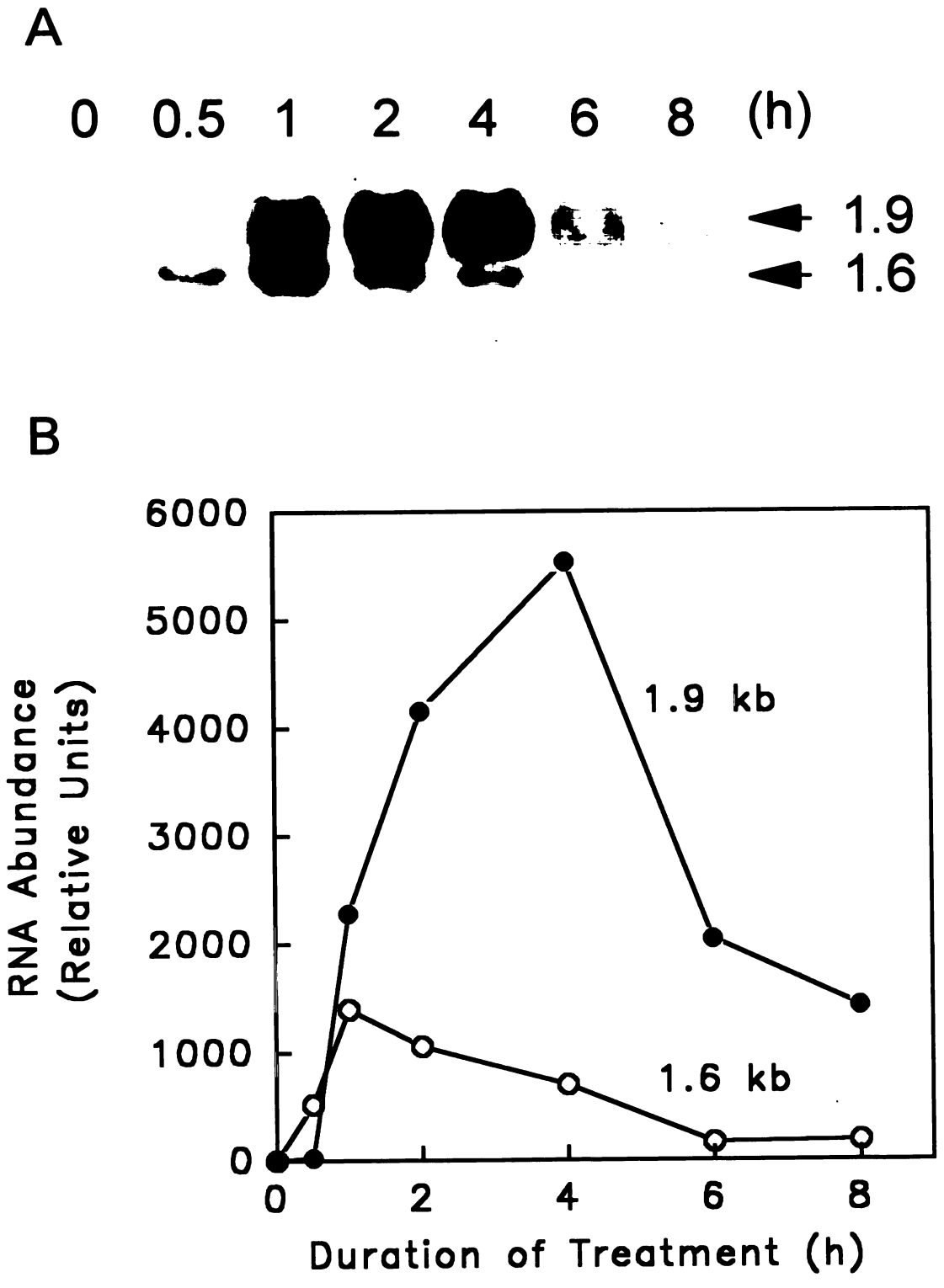

Figure 4.8. Expression pattern of two transcripts hybridizing to PS-ACS1.

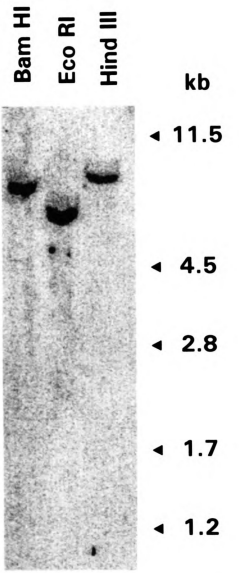

Figure 4.9. Genomic DNA blot probed with PS-ACS1. Ten μg of pea genomic DNA was digested with either Bam HI, Eco RI, or Hind III, and separated on a 0.7% agarose gel. The entire insert of pPS-ACS1 was random prime labelled with ^{32}P-αCTP and used as a probe. Final wash conditions were 0.2X SSC, 0.5% SDS at 65°C.

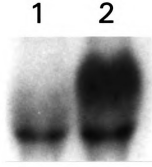

Figure 4.10. RNA blot analysis using 3'-UTR of PS-ACS1. RNA was isolated from the third internode of seedlings treated with 100 μM IAA for 0.5 h (lane 1) or 2 h (lane 2). The RNA was separated on a 1.2% formaldehyde agarose gel, blotted, and probed with a 300-bp ^{32}P-labeled antisense RNA strand from only the 3'-UTR of PS-ACS1. The experiment was performed twice with similar results.

If the two transcripts are produced from the same gene, the four most plausable explanations for the size difference are (1) a difference in poly(A)-tail length, (2) different polyadenylation sites, (3) different transcription start sites, and (4) alternative splice sites.

RNase H mapping was used to remove the poly(A)-tail from the transcripts. RNase H will specifically degrade RNA in a RNA-DNA hybrid. Oligonucleotides can be used to specifically cleave RNA at the complementary region, thus producing a shorter transcript that can be detected by RNA blot analysis (Figure 4.11). Oligo $d(T)_{15}$ will randomly anneal to most of the poly(A)-tail, effectively removing the majority of the sequence. With the poly(A)-tail removed (Figure 4.12), the transcripts shifted in size very slightly but remained distinct, indicating that poly(A)-length is not responsible for the two transcripts. A large shift would not be expected because removal of 40-65 bp, the average steady-state poly(A)-tail length (Jacobson, 1987), would be a minor change in 1.6 to 1.9-bp transcripts. As was shown in Figure 4.2, cDNA clones for PS-ACS1 containing different polyadenylation sites were isolated. The difference in length among the three groupings was only 100-bp, which does not appear to be enough to account for the difference in size between the two transcripts (about 300-bp). Different polyadenylation sites, however, may explain the appearance of the larger transcript as a broad band on RNA blots (Figures 4.7 and 4.8).

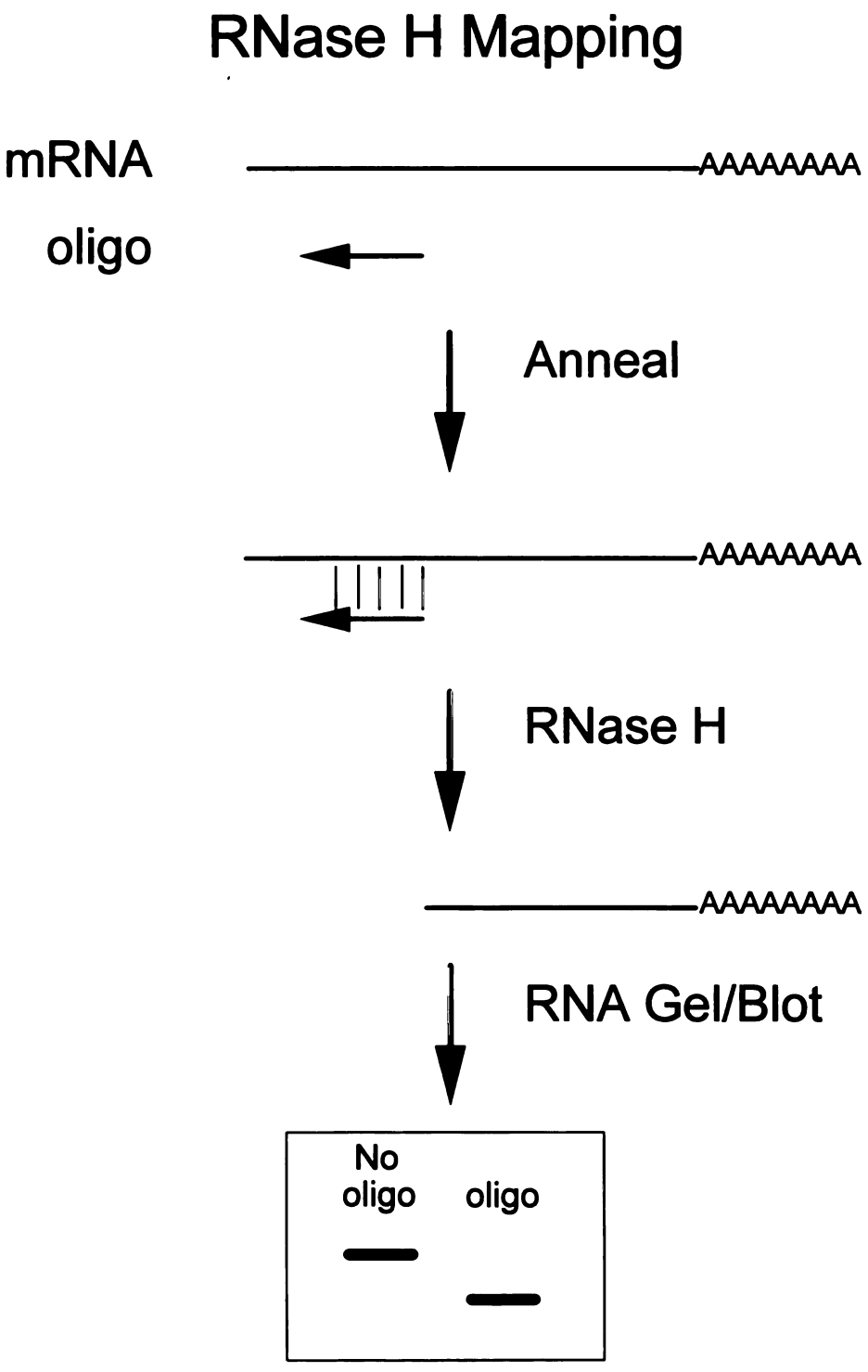

Figure 4.11. Diagram of the procedure for RNase H mapping.

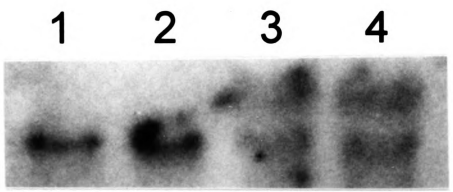

Figure 4.12. RNA blot analysis after removal of poly(A)-tail. Twenty μg of total RNA from 0.5 h (lanes 1 and 2) or 2.0 h (lanes 3 and 4) IAA-treated seedlings were heated to 65°C and allowed to cool slowly to room temperature in the absence of oligonucleotides (lanes 1 and 3) or in the presence of oligonucleotide $d(T)_{15}$ (lanes 2 and 4). The mix was incubated with RNase H for 45 min at 37°C. RNA blot analysis was performed as described in Figure 4.6. The experiment was performed twice with similar results.

Two oligonucleotides, one 40-bp downstream of the predicted translational start site (SEQB) and one about 600-bp downstream (SEQC), were used in RNase H mapping of the 5′ end of PS-ACS1. The primer closest to the translational start site removed a portion of the larger band (Figure 4.13, lane 6) but did not visibly affect the size of the smaller band (Figure 4.13, lane 2). The oligonucleotide directed to a sequence towards the middle of the transcript caused both transcripts to be truncated (Figure 4.13, lanes 3 and 7). In fact, with this oligonucleotide, both transcripts yielded truncated products of similar sizes, suggesting that the difference in size between these two transcripts is upstream of this second primer. Sequence information obtained from the isolation of a genomic clone for PS-ACS1 will be necessary to determine if the difference in the 5′ ends is the result of different transcriptional start sites, alternative splicing, or both.

Legend for Figure 4.13. Twenty μg of total RNA from 0.5 h or 2.0 h IAA-treated seedlings was heated to 65°C for 5 min and allowed to cool slowly to room temperature in the absence of oligonucleotides (lanes 1,4,5, and 8), in the presence of oligonucleotide SEQB (lanes 2 and 6), or in the presence of oligonucleotide SEQC (lanes 3 and 7). The mix was incubated with RNase H for 45 min at 37°C. RNA blot analysis was performed as described in Figure 4.6. The experiment was performed twice with similar results.

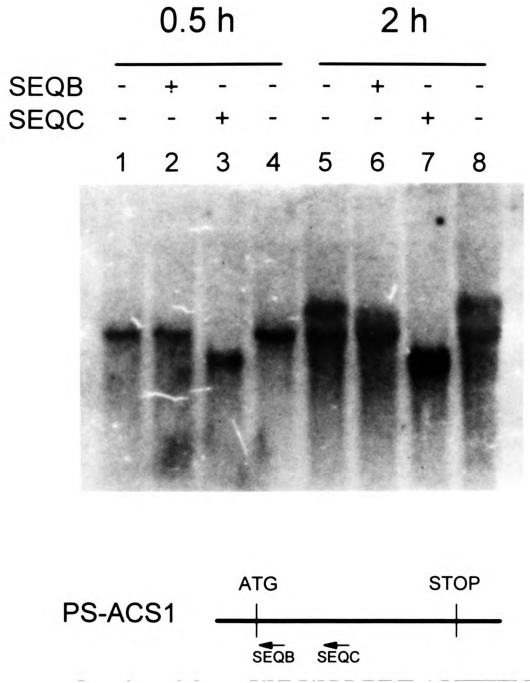

Figure 4.13. Oligonucleotide-directed RNase H mapping of the PS-ACS1 transcript.

Discussion

As was found in other plants (tomato: Yip et al., 1992; mung bean: Botella et al., 1992, Kim et al., 1992), more than one gene encoding ACC synthase is expressed in IAA-treated etiolated pea seedlings. It has been suggested that the different members of the ACC synthase gene family can be placed into groups that are highly related in both amino acid sequence identity as well as pattern of expression (Liang et al., 1992). While both PS-ACS1 and PS-ACS2 are regulated by IAA in etiolated pea seedlings, they share only 53% amino acid identity. PS-ACS1, however, shares 74% amino acid identity with LE-ACS3, an IAA-induced clone from tomato (Olson et al., 1995). As shown in Figure 4.14, 55% of the amino acids are absolutely conserved in all members of the LE-ACS3-type group, which includes both monocots and dicots. Similarly, PS-ACS2 more closely resembles the group associating with LE-ACS2 with which it shares 64% amino acid identity (Figure 4.15). Within this group, 44% of the amino acids are absolutely conserved. Between the groups of LE-ACS3 and LE-ACS2 types, only 29% of the amino acids are conserved. The high level of conservation of amino acids within but not between these groups indicates that, even though the enzymes are catalyzing the same reaction in response to auxin, there may be unique forms of regulation or constraints at the protein level that favor one form over the other. Currently, nothing is known about post-transcriptional regulation of ACC synthase.

All members of the LE-ACS3 group are expressed in auxin treated vegetative tissue, except for apple which was not examined (see references in legend of Figure 4.14); and all members have an Arg as their C-terminal residue. Expression seems to be primarily in the vegetative tissue, as it was either not present (Nakagawa et al., 1991) or expressed at a low level in ripening fruit (Dong et al., 1991; Yip et al., 1992). In tomato roots (Olson et al., 1995) and in rice stems (Zarembinski and Theologis, 1993), expression was increased by flooding or low oxygen. Anaerobiosis, however, did not increase expression in etiolated Arabisopsis seedlings (Liang et al., 1992). As in the present work, multiple bands were detected on RNA blots analysis in tomato (Olson et al., 1995), winter squash (Nakagawa et al., 1991), Arabidopsis (Abel et al., 1995), and possibly in apple (Dong et al., 1991).

The appearance of multiple bands on RNA blot analysis in other plants is intriguing. In tomato (Olson et al., 1995), the larger transcript (2.1 kb) is a result of introns not being spliced during flooding. Unspliced transcripts, however, are not uncommon under stress conditions (Czarnecka et al., 1988; Winter et al., 1988) and may be unrelated to what was observed in this work. The results of the present work indicate that a single gene PS-ACS1 has different transcriptional start sites, alternative splicing at the 5'-end, or both. Because the transcripts produced in some of the other plants are doublets of similar size as in pea, the production of two transcripts may be a conserved response.

Legend to Figure 4.14. The clones are from tomato (LE-ACS3; Olson et al., 1995), pea (PS-ACS1; this work), potato (ST-ACS1; Destafano-Beltran, 1995), winter squash (CM-ACS2; Nakagawa et al., 1991), Arabidopsis (AT-ACS4; 1995), apple (MS-ACS1; Dong et al., 1991, modified for this work), and rice (Zarembinski and Theologis, 1993). Residues identical to those of LE-ACS3 are shown by "*". Gaps in the sequence are shown by "-". The original sequence for MS-ACS1 differs from the current in that the sequence terminates prematurely, apparently a result of a frameshift caused by a single nucleotide addition within the shaded backslash region. Without this nucleotide,the open reading frame presented here continues the reading frame that aligns with the other sequences.

```
LE-ACS3  MKLLSEKATC NSHGQDSSYF LGWWEYEK-N PYDEIQNPKG IIQMGLAENQ LSFDLLESWL
PS-ACS1  *****T**** ********** ***Q****E* **YHV***** ********** **********
ST-ACS1  *****K**M* ********** ***E****-* **D*TR**** ********** **********
CM-ACS2  **M**T**** ********** ***EA**N-* *FHHTS**N* ********** **********
AT-ACS4  *VQ**R**** *****V**** ***E****-* **DVTK**Q* ********** *C********
MS-ACS1                   * ***Q****-* **H*VL*TN* ********** *C********
OS-ACS1  //***K**G* ********** ***Q****-* *FDPVS**S* ********** *******E**

LE-ACS3  AQNPDAAGFK RNGE--SIFR ELALFQDYHG LPAFKNAMTK FMSEIRGNRV SFDSNNLVLT
PS-ACS1  *K*Q*VG*** *D*K--**** ********** **S**K*LVD **A*****K* T**P*HI***
ST-ACS1  T******A** ***N--**** ********** *****D*LVQ ********K* *****K****
CM-ACS2  SK*****S** *D*K--**** ********** *****K*LVE **A*****K* **EA**I***
AT-ACS4  ***T***C** *D*Q--*V** ********** *SS****FAD ****N***** **********
MS-ACS1  *K**E**A** K***--***A ********** *****K**VD **AK****K* T**P*H****
OS-ACS1  EK**H*L*LR *E*GGA*V** ********** *******LAR ****Q**YK* V**PS*I***

LE-ACS3  AGATSANETL MFCLANQGDA FLLPTPYYPG FDRDLKWRTG AEIVPIHCSS SNGFRITESA
PS-ACS1  ********** *****EK*E* ********** ********** V*****Q*T* **N*QM****
ST-ACS1  ********** *G***DP*** ********** ********** ******Q*T* **********
CM-ACS2  ********** *****EA*** ********** ********** V*******T* ****Q**Q**
AT-ACS4  ********** *****DP*** ********** ********** V*****QS** T******KL*
MS-ACS1  *********F I****DP*E* V*I******* ********** V*******T* ****Q***T*
OS-ACS1  ********A* *****DH*** **I******* ********** *****V**A* A****V*RA*

LE-ACS3  LEEAYLDAKK RNLKVKGVLV TNPSNPLGTT LNRNELELLL TFIDEKG-IH LISDEIYSGT
PS-ACS1  *QQ*HE**** K********* ********** MSK***N**I D**KD*-NM* **********
ST-ACS1  *****KE*ER ***R****** ********S* *TKK**Q*** **VST*-Q** **********
CM-ACS2  **Q**K**QT ***R****** ********** M**D**N*VF D**TS*G-** **********
AT-ACS4  *****EQ*** LD*N***I*I ********** TTQT**NI*F D**TKNK*** *V********
MS-ACS1  *****QE*E* ***R****** ********** MT****Y*** S*VED*G-** **********
OS-ACS1  *DD**RR*Q* *R*R*****I *********A SP*AD**TIV D*VAAKG-** *******A**

LE-ACS3  VFN-SP-GFV SVMEVLI--- EKNYMKTRVW ERVHIVYSLS KDLGLPGFRI GAIYSNDEMV
PS-ACS1  **T-**-N** ****I*NERT D*DFLDAN*S ********** *********V *****DN*T*
ST-ACS1  ***-**-K** ********--- *N***Y*E** D********* *********V *******D*I
CM-ACS2  **G-**-*** *A****K--- *RSSEDEE** K********* *********V *******D**
AT-ACS4  ***-*S-E*I ****I*K--- NNQLEN*D*L N*****C*** *********V *******KD*
MS-ACS1  A*S-**-S*I ******K--D RNCDENSE** Q***V***** *********V *******D**
OS-ACS1  A*AEP*A*** *AL**VA--- GRDGGGAD*S D***V***** *********V *****ANAA*

LE-ACS3  VSAATKMSSF GLVSSQTQYL LSCMLSDKKF TKKYISENQK RLKKRHAMLV KGLKSAGINC
PS-ACS1  *A******** ********** **A**G**** *RN*L***** *****QK*** N**QK***S*
ST-ACS1  ********** **I******* **AL***Q** M*N*V***** ******E*** G***QI**R*
CM-ACS2  *A******** ********** **A******* *IS******* ***Q*QK*** S**QK*****
AT-ACS4  I********* ********** **SL****** **N*LR**** ***N*QRK** L**EAI**K*
MS-ACS1  *A******** ********H* **A******L **N**A**H* ***Q*QKK** S**QKS**S*
OS-ACSA  ********** ********** *AAL*A*RD* *RS*VA**KR *I*E**DQ** D**REI**G*

LE-ACS3  LESN-AGLFC WVDMRHLLSS NNFDAEMDLW KKIVYDVGLN ISPGSSCHCT EPGWFRVCFA
PS-ACS1  *KT*N***** *****N**T* DT*E****** ***L*E**** ********** **********
ST-ACS1  ****-***** ********** *T**G**E** *****E**** ********** ******A***
CM-ACS2  ****-***** ********E* DK*ES*LE** *****E**** ********** **********
AT-ACS4  ****-***** *****P**R* KT*E****** *****E*K** *********E **********
MS-ACS1  *NG*-***** ********R* *T*E***E** *****E*H** ********** **********
OS-ACS1  *P**-***** ****S**MR* RS*AG**E** **V*FE**** *********R **********
```

Figure 4.14. Amino acid sequence comparison of ACC synthase clones similar to LE-ACS3.

Figure 4.14 (continued)

```
LE-ACS3  NMSEDTLDLA MRRIKDFVES TAPNATNHQN QQQSN-ANS- K---KKSFSK WVFRLSFNDR
PS-ACS1  *******N** *K*L****SN SNGEEGSNSD NKRTRSSQ*S RSFTR**I*N ******SRDH
ST-ACS1  *******NI* IQ*L*A**D* RDNKDDI-** *KH******** *---****** ********E*
CM-ACS2  ****S**K** V**L*S**TE LRSTT*S-NH RNHD*--KIC *NIK*NI*T* ****Q*AQEA
AT-ACS4  **IDE**K** LK*L*ML*DD ENSSRRC-*K SKSERLNG*R *KTMSN-V*N *******H**
MS-ACS1  *LP*R***** *Q*L*A**GE YYNVPEVN// /////HLSHS R---RQ*LT* **S****DDR
OS-ACS1  ***AK***V* *Q*LRS**D* ATGGGD*AAL RRAAVPV--- RSVSCPLAI* *AL**TPSIA

LE-ACS3   ----RER
PS-ACS1   --HEER*
ST-ACS1   ----RE*
CM-ACS2   NRKMAE*
AT-ACS4   --EAEE*
MS-ACS1   -GPIPG*
OS-ACS1   -DRKAE*
```

Legend for Figure 4.15. The clones are from tomato (LE-ACS2; Van Der Straeten et al., 1990); pea (PS-ACS2; this work); mung bean (VR-ACS1; Botella et al., 1992); zucchini (CP-ACS1; Huang et al., 1991); winter squash (CM-ACS1; Nakajima et al., 1990), carnation (DC-ACS1; Park et al., 1992, modified in this work), Arabidopsis (AT-ACS1; Liang et al., 1992), and tobacco (NT-ACS1; Bailey et al, 1992). Residues identical to those of LE-ACS3 are shown by "*". Gaps in the sequence are shown by "-". The sequence of DS-ACS1 in this work differs from the original. The original amino acid sequence was deduced from a genomic clone. In the original sequence, a putative splice junction that would not affect the reading frame was ignored leading to the addition of 35 amino acid residues including an unlikely string of 18 threonine residues. In this work, the intervening sequence was interpreted as an intron and deleted (original position is indicated by the shaded backslashes) yielding the sequence that matches well with this alignment.

```
LE-ACS2  MGFEIAKT-N S-ILSKLATN EEHGENSPYF DGWKAYDSDP FHPLKNPHGV IQMGLAENQL
PS-ACS2  **VMNLD--Q PQL***I*MG DG***A*S** *******K** ***S****** **********
VR-ACS1  ***KAMD--Q TPL***MAIG DG***S**** *******QN* ***TD**N** M*********
CP-ACS1  ***HQIDER* QAL***I*LD DG******** *******N** ***EN**L** **********
CM-ACS1  *E*HQIDER* QAL***I*VD DG******** *******N** ***ED**L** **********
DC-ACS1  **SYKGVYDR -E****I*** DG****LE** *******R** Y*ST**SN** **********
AT-ACS1  **LPGKNKGA --V***I*** NQ*****E** *******K** **LSR****I **********
NT-ACS1  ****NE*NSS --******** **L******* *******N** *******N** **********

LE-ACS2  CLDLIEDWIK RNPKGSICS- EGIKSFKAIA NFQDYHGLPE FRKAIAKFME KTRGGRVRFD
PS-ACS2  TA*MVQN**M S**EA***TL **VHN**QM* ********** **N*V****S R***N**T**
VR-ACS1  TS**VE***L N**EA***TP ***ND*RA** ********A* **N*V****A R***N*IT**
CP-ACS1  SF*M*V***R KH*EA***TP **LER**S** ********** **N***N**G *V*****K**
CM-ACS1  SF*M*V***R KH*EA***TP K*LER**S** ********** **NG**S**G *V*****Q**
DS-ACS1  *F***TE*LL K**QA***TN **VNK*MD** I********* **S*V****G *A*DEK*I*N
AT-ACS1  *****K**V* E**EA***T* ***HQ*SD** ********KK **Q***HG*G *A*****T**
NT-ACS1  *F***EE*** ***NA***TT ******R*** ********** **S******* *******T**

LE-ACS2  PERVVMAGGA TGANETIIFC LADPGDAFLV PSPYYPAFNR DLRWRTGVQL IPIHCESSNN
PS-ACS2  ***I**S*** ***H*ATA** ***R*E*L** *T****G*D* ********K* V*VI******
VR-ACS1  *D*I**S*** ***H*VTA** *****E**** *I****G*D* ********K* V*VM*D****
CP-ACS1  *S*I**G*** ***S**V*** ********** *****AG*D* **K***RAQI *RV**NG***
CM-ACS1  *S*I**G*** ***S**V*** ********** *****A**D* **K***RAQI *RV**N*****
DS-ACS1  *D*I**S*** SAS-**LL** **N******I ********N* ********N* **FT*S****
AT-ACS1  ******S*** *******M** ******V**I *****A**D* ********EI **VP*S**D*
NT-ACS1  ********** ********** ***T****** ********** ********Q* ***P*D****

LE-ACS2  FKITSKAVKE AYENAQKSNI KVKGLILTNP SNPLGTTLDK DTLKSVLSFT NQHNIHLVCD
PS-ACS2  **L*KQ*LE* ***K*KED** RF***LI*** ******VM*R N**RT*IT*I *EKR***IS*
VR-ACS1  *VL*KE*LED ***K*RED** R****LI*** ******IM*R K**RT*V**I *EKR******
CP-ACS1  *QV*KA*LEI **KK**EA*M ****V*I*** *******Y*R ****TLVT*V ***D***I**
CM-ACS1  *QV*KA*LEI **KK**EA** ****V*I*** *******Y*R ****TLVT*V ***D***I**
DS-ACS1  ****KETLQS ***EL*-K** ****I*V*** ******V*** ****ML*T*V *AK*******
AT-ACS1  **L*VD*AEW **KK*****K ********** ******M*** ***TNLVR*V TRK*****V*
NT-ACS1  *Q**TK*VR* ********** ********** ********** ****NL*T** **********

LE-ACS2  EIYAATVFDT PQFVSIAEIL DEQEMTYCNK DLVHIVYSLS KDMGLPGFRV GIIYSFNDDV
PS-ACS2  *********SH *S*I*****I EHDTDIE*DR N********* ****F***** *****Y**T*
VR-ACS1  *********SQ *G*I****** EDETDIE*DR N********* ****F***** *****Y**A*
CP-ACS1  ***S****KA *T*T*****V EQM*H--*K* E*I**L**** ********** *****Y**V*
CM-ACS1  ***S****KA *T*I***Q*V E*M*H--*K* E*I**L**** ********** *****Y**V*
DS-ACS1  **F*T***NS *S*I*V**VI KDMPHV--*Q *****L**** ****M***** *****Y**R*
AT-ACS1  ********AG GD***V**VV NDVDISEV*V **I******* ********** **V*****S*
NT-ACS1  *********N* ********** *DE*SH-*** ********** ********** **V*****A*

LE-ACS2  VNCARKMSSF GLVSTQTQYF LAAMPSDEKF VDNFLRESAM RLGKRHKHFT NGLEVVGIKC
PS-ACS2  *D*T****** *********L I*K*L**DD* *EK**A***K **AQ*YRV** G**IK*****
VR-ACS1  ********** *********L **S*LN*DE* *ER**A***K **AQ*FRV** G**AK*****
CP-ACS1  *RR**R**** ****S***HL ****L***D* **K**A*NSK *V*E**AR** KE*DKM**T*
CM-ACS1  *RR**Q**** ****S***HL ****L***D* **K**A*NSK **AE**AR** KE*DKM**T*
DS-ACS1  *ST**R**** ****S***FM I**LL**DD* *RR**V**RD **FR**Q*** SE*AKI**G*
AT-ACS1  *S******** ****S***LM L*S*L**DQ* *****M**SR ***I***V** T*IKKAD*A*
NT-ACS1  ********** *********L **E*L***R* *S***T**SK **A******* ****E*****
```

Figure 4.15. Amino acid sequence comparison of ACC synthase clones similar to LE-ACS2.

Figure 4.15 (continued)

```
LE-ACS2  LKNNAGLFCW MDLRPLLRE- STFDSEMSLW RVIINDVKLN VSLGSSFECQ EPGWFRVCFA
PS-ACS2  *QS*G***V* ****G**KN- A**E**IE** ****HE**I* **P*V**H*S ********Y*
VR-ACS1  *QS*****V* ****Q**KK- P*****TE** ****HE**I* **P*Y**H*T ********Y*
CP-ACS1  *NS***V*V* ****R**KD- Q**KA**E** *****E**** **P****HVT **********
CM-ACS1  *NS***V*V* ****R**KD- Q**KA**E** *****E**** **P****HVT **********
DS-ACS1  *QG**A**V* ****H**D*- A*VER*LK** *****E**I* **P****L*S **********
AT-ACS1  *TS*****A* ****H***DR NS*E**IE** HI**DR**** **P****R*T ******I***
NT-ACS1  *RS******* *******K*- ********** ********** **P****D** ***F******

LE-ACS2  NMDDGTVDIA LARIRRFV-- GVEKSGDKSS SME------- -KKQQWKKNN LRLSFS---K
PS-ACS2  ****RD*Q** *Q***S**-- TQNNKEAMG* DK-------- NS*PY*-HS* ****LKP--R
VR-ACS1  ****MA*Q** *Q***N**-- LQN*EVVVS- ---------- N**HC*-HS* ****LKT--R
CP-ACS1  ****N***V* *N**HS**-- ENIDKKEDNT VAMPS----- --*TRHRD*K ******FSGR
CM-ACS1  ****N***V* *N**HS**-- ENIDKKEDNT VAMPS----- --*TRRRE*K ******FSGR
DS-ACS1  ***NA*L*V* *N***S**-- TRGR-///// ---------- --*K-RGQME *****NN--R
AT-ACS1  ****D*LHV* *G**QD**SK NKN*IVE*A* ENDQVIQNKS AK*LK*TQT* *********---R
NT-ACS1  ****E***** *****S**-- **K****E*T PILME----- -K******** *********---*

LE-ACS2  RMYVLDES-- ----SPLS-S PIPPSPLVR
PS-ACS2  *FDDIMM--- -----**H--* ***Q****KA TT
VR-ACS1  *FDDITM--- -----**H--* *L*Q**M*KA TN
CP-ACS1  *YDEGNVLN- -----**HIM* *--H****IA KN
CM-ACS1  *YDEGNVLN- -----**HTM* *--H****IA KN
DS-ACS1  *FEDGLM*PH SILL**H--* *M*Q****KA RT
AT-ACS1  *L*EDGL*-- -----**GIM* *--H***L*A
NT-ACS1  ***DESVNL- ----*****-* ***H*****A RT
```

Of all the ACC synthases, the LE-ACS2 group appears to be induced by the widest range of stimuli. Although other groups such as those related to LE-ACS4 (Olson et al., 1991; Rottman et al., 1991) show significant similarity to the LE-ACS2-like group, members of the LE-ACS2 group can be distinguished from the rest by the residues Ser-Pro repeated three times at the C-terminus. This group seems to represent the primary transcript in ripening fruits of tomatoes (Olson et al., 1991; Rottman et al., 1991) and zucchini (Huang et al., 1991). In Arabidopsis, it is expressed in all light-grown tissues examined and in etiolated seedlings (Liang et al., 1992). It is present at low levels and increases following auxin treatment in Arabidopsis (Liang et al., 1992), zucchini (Huang et al., 1991), mung bean (Botella et al., 1992), apple (Kim et al., 1992), and tomato (Yip et al., 1992). Its expression is increased by wounding in Arabidopsis seedlings (Liang et al., 1992), tomato fruit (Olson et al., 1991; Rottman et al., 1991; Yip et al., 1992), and zucchini hypocotyls (Huang et al., 1991). Flooding in tomato roots (Olson et al., 1995) and pathogen infection in tomato leaves or suspension cells (Spanu et al., 1993) also cause the transcript to accumulate. It also has been proposed that VR-ACS1 is induced by touch (Botella et al., 1995).

The diversity of stimuli that induce expression of the PS-ACS2 family suggests surprisingly complex regulation. Six to nine genes for ACC synthase are thought to be expressed in tomato (Zarembinski and Theologis, 1994). If ACC synthase is encoded by a multi-gene family whose members are induced

under different conditions in different tissues, why is there a member that appears to be induced under so many conditions? One possibility is that this group represents the earliest ACC synthase which was originally responsible for all ethylene production. The rest of the gene family may have evolved from gene duplication and diverged to fill more specific roles. In support of this hypothesis, a PCR fragment for an ACC synthase from *Marsilea quadrifolia*, a semi-aquatic fern, has been isolated (J. Chernys, personal communication), and it shows the greatest similarity to the PS-ACS2-type group. The C-terminal sequence will have to be determined to verify whether it contains the Ser-Pro repeat characteristic of this group.

The IAA-induced accumulation of transcripts for PS-ACS1 and PS-ACS2 was repressed in the presence of ethylene. The repression by ethylene is consistent with previous studies which showed that ethylene negatively regulates its own biosynthesis in vegetative tissues (Kende, 1993).

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CONCLUSIONS

From the data presented in this work, a model of how positive and negative feedback are involved the regulation of ethylene biosynthesis is proposed (Figure 5.1). A stimulus, IAA in the present work, causes an increase in ACC synthase transcript abundance leading to an increase in ACC synthase activity. The newly formed ACC is converted to ethylene by a low, constitutive level of ACC oxidase. The ethylene produced then causes an increase in the levels of ACC oxidase transcript and activity via a positive feedback loop. Ethylene also causes a decrease in ACC synthase transcript and activity via a negative feedback loop. Although the sequential induction of the ethylene biosynthetic enzymes has not been shown in other plants, results from studies on wound-induced ethylene production in mung bean hypocotyls (Kim and Yang, 1994) and in winter squash mesocarp (Hyodo et al., 1993) indicate that the model could apply to the regulation of ethylene formation by other stimuli.

At first, the positive and negative feedback loops would appear contradictory. The increase in ACC oxidase would presumably enhance ethylene production while the repression of ACC synthase would decrease ethylene production. However, this apparent contradiction arises from considering these two events to be simultaneous. The increase in ACC oxidase may precede the negative feedback on ACC synthase. This temporal separation could arise from the kinetics of different signal transduction pathways. Alternatively, the positive feedback on ACC oxidase may be initiated by a lower ethylene

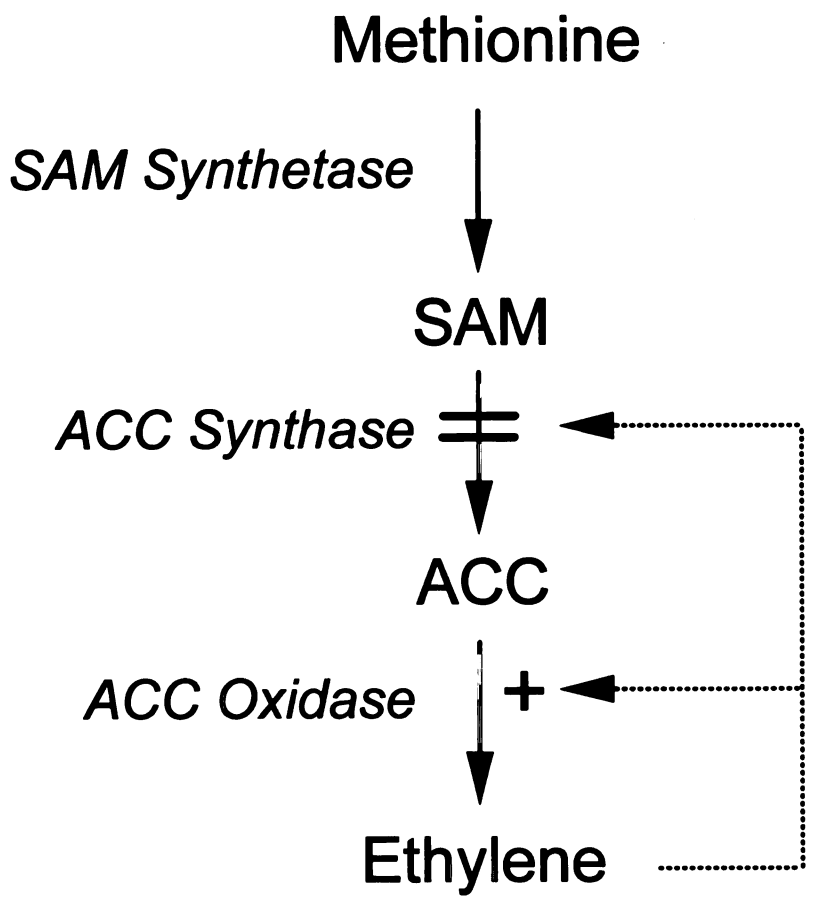

Figure 5.1. Positive and negative feedback of ethylene on the biosynthetic pathway after auxin treatment.

concentration than that required by the negative feedback on ACC synthase. These possibilities need to be examined to understand the roles of the two feedback loops.

Like ACC synthase, ACC oxidase is also encoded by a multigene family (for a review, see Zarembinski and Theologis, 1994). Because the coding region of ACC oxidase is very highly conserved, it is likely that the probe (PE8) used in the current work would hybridize to transcripts of any ACC oxidase genes that were expressed. Only clones related to PE8 were isolated during the library screening. However, the library was made from tissue that would be enriched for an ethylene-inducible ACC oxidase. A sufficient number of clones were not isolated to ensure that a lower abundance constitutive ACC oxdidase would be isolated. Thus, it is not clear whether PE8 is both constitutively expressed and ethylene-inducible or whether different genes are responsible for the two types of expression. Analysis using 3'-UTRs should be able to answer this question.

The role of ACC oxidase in ethylene-insensitive mutants of Arabidopsis, such as *etr1*, is an interesting problem. The level of ACC oxidase seems to correspond to the level of endogenous ethylene (Schierle et al., 1989; Hyodo et al., 1993; Kim and Yang, 1994; Peck and Kende, 1995). Thus, the level of ACC oxidase should be quite low in insensitive mutants. Although ACC oxidase activity has never been measured directly, it has been shown that the rate of ethylene production in even the most severe mutants is actually higher than in wild type (Bleecker et al., 1988; J. Ecker, personal communication). Is a low level of constitutive ACC oxidase sufficient to maintain normal ethylene production? The fact that ethylene production is reduced by about 70%

when the ACC oxidase activity is decreased by 70% in leaves of transgenic tomato plants expressing antisense ACC oxidase would argue that this is not the case (Hamilton et al., 1990). These results indicate that the positive feedback of ethylene on ACC oxidase may utilize a signal transduction pathway different from the one involved in the formation of the apical hook.

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