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Control of Ethylene Synthesis by Expression of a Bacterial Enzyme in Transgenic Tomato Plants

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Synthesis of the phytohormone ethylene is believed to be essential for many plant developmental processes. The control of ripening in climacteric fruits and vegetables is among the best characterized of these processes. One approach to reduce ethylene synthesis in plants is metabolism of its immediate precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). Soil bacteria containing an enzyme, ACC deaminase, were identified by their ability to grow on ACC as a sole nitrogen source. The gene encoding ACC deaminase was cloned and introduced into tomato plants. Reduction in ethylene synthesis in transgenic plants did not cause any apparent vegetative phenotypic abnormalities. However, fruits from these plants exhibited significant delays in ripening, and the mature fruits remained firm for at least 6 weeks longer than the nontransgenic control fruit. These results indicated that ACC deaminase is useful for examining the role of ethylene in many developmental and stress-related processes in plants as well as for extending the shelf life of fruits and vegetables whose ripening is mediated by ethylene.

INTRODUCTION

Ethylene is a potent plant growth regulator that affects diverse developmental processes, including fruit ripening, senescence, and stress responses (McKeon and Yang, 1987; Reid, 1987). Its role in promoting climacteric fruit ripening is particularly well established. Chemical inhibitors of ethylene synthesis or action completely block ripening in fruits and senescence in flowers of many plant species. At a molecular level, ethylene is known to induce expression of a number of genes involved in ripening (Lincoln and Fischer, 1988) and pathogen response (Ecker and Davis, 1987).

Ethylene is synthesized from *S*-adenosylmethionine by way of the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) (McKeon and Yang, 1987). Recently, the cDNAs encoding ACC synthase (Sato and Theologis, 1989; Van Der Straeten et al., 1990; Huang et al., 1991) and ACC oxidase (Hamilton et al., 1991; Spanu et al., 1991) have been cloned. The cDNA from this latter gene when expressed in an antisense orientation causes a decrease in ethylene synthesis and delayed ripening of excised tomato fruit (Hamilton et al., 1990).

The ability to inhibit synthesis of ethylene in a plant without the necessity for exogenous application and uptake of chemical inhibitors should permit definitive experiments to elucidate the precise role of ethylene in a variety of developmental and stress-related phenomena. Here we present a system for significant reduction of ethylene in vegetative and reproductive tissues and describe the effects on tomato fruit ripening.

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RESULTS

Cloning an ACC-Degrading Enzyme

A generally applicable method for preventing ethylene synthesis would involve irreversible degradation of the precursor of ethylene, ACC, to an inactive compound. Current knowledge of ethylene biosynthesis in plants indicates that ACC is the immediate metabolic precursor of ethylene under physiological conditions. Besides ethylene biosynthesis, only two metabolic reactions of ACC are known. An enzyme that is capable of degrading ACC to α-ketobutyric acid has been purified from a soil bacterium, *Pseudomonas* sp strain ACPC (Honma and Shimomura, 1978), and an enzyme malonylating ACC to malonyl-ACC has been described in plants (McKeon and Yang, 1987). We screened a collection of 600 soil bacteria for ACC-degrading enzymes by selecting for organisms capable of growth on minimal medium containing ACC as a sole nitrogen source. Two organisms were identified from this screening and were further characterized. Both were identified as *Pseudomonas* species. One of them, *Pseudomonas* sp strain 6G5, was chosen for further characterization. A cosmid library was constructed from genomic DNA

and introduced into *Escherichia coli.* Cosmid-containing *E. coli* cells were then screened for their ability to grow on ACC as a sole nitrogen source. Several were identified and 18 were further characterized. Restriction endonuclease digestion patterns indicated that all of the cosmid clones contained an overlapping region of the 6G5 genome. Subsequent subcloning and selection for growth on minimal ACC-containing medium permitted identification of a 2.4-kb DNA fragment containing the ACC-degrading gene. This fragment was subjected to DNA sequence analysis that revealed a single open reading frame (ORF) of 1017 nucleotides encoding a protein with a molecular weight of 36,800, as given in Figure 1.

Identification as ACC Deaminase

Because an ACC deaminase activity had been reported in the literature, it was of interest to determine whether the cloned gene also encoded an ACC deaminase. The 6G5 ORF was thus engineered to permit high-level expression in *E. coli.* Figure 2, lane 2, shows expression of the gene under the control of the *recA* promoter in *E. coli.* It can be seen that a protein of the correct size was produced. Bacteria expressing this protein were capable of degrading ACC to equimolar amounts of ammonia and α-ketobutyric acid. To confirm the identity of the 6G5 protein, the previously identified organism, *Pseudomonas* sp strain ACPC, was obtained, and the ACC deaminase from that organism was purified to homogeneity following the published protocol (Honma and Shimomura, 1978). This protein was subjected to amino-terminal amino acid sequencing, and the first 21 amino acids were identified. Seventeen of these 21 amino acids matched the predicted sequence of the 6G5 gene product, whereas 2 of the remaining 4 amino acids were conservative substitutions. Thus, the 6G5 gene appeared to encode an ACC deaminase. Because the native enzyme has been reported to have an apparent molecular weight of 104,000 and the predicted molecular weight of the subunit is 36,800, the enzyme probably exists as a trimer.

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   1  GATATCCCATATCAAGGAGCAGAGTCATGAATCTGAATCGTTTTGAACGTTATCCATTGACC
                                    MetAsnLeuAsnArgPheGluArgTyrProLeuThr

  63  TTCGGTCCTTCTCCCATCACGCCCTTGAAGCGCCTCAGTCAACATCTGGGGGGCAAGGTCGA
      PheGlyProSerProIleThrProLeuLysArgLeuSerGlnHisLeuGlyGlyLysValGl

 125  GCTGTATGCCAAACGTGAAGACTGCAACAGTGGCCTGGCCTTTGGTGGGAACAAGACGCGCA
      uLeuTyrAlaLysArgGluAspCysAsnSerGlyLeuAlaPheGlyGlyAsnLysThrArgL

 187  AGCTCGAATACCTCATTCCCGAAGCGATCGAGCAAGGCTGCGATACGCTGGTTTCCATCGGC
      ysLeuGluTyrLeuIleProGluAlaIleGluGlnGlyCysAspThrLeuValSerIleGly

 249  GGCATCCAGTCGAACCAGACCCGTCAGGTCGCTGCCGTCGCTGCCCACTTGGGCATGAAGTG
      GlyIleGlnSerAsnGlnThrArgGlnValAlaAlaValAlaAlaHisLeuGlyMetLysCy

 311  CGTGTTGGTGCAGGAAAACTGGGTGAACTATTCCGACGCGGTGTATGACCGCGTAGGCAACA
      sValLeuValGlnGluAsnTrpValAsnTyrSerAspAlaValTyrAspArgValGlyAsnI

 373  TCGAGATGTCGCGGATCATGGGCGCTGATGTGCGGCTTGACGCCGCTGGCTTCGATATTGGC
      leGluMetSerArgIleMetGlyAlaAspValArgLeuAspAlaAlaGlyPheAspIleGly

 435  ATTCGGCCAAGTTGGGAAAAGGCCATGAGCGATGTCGTGGAACAGGGTGGCAAACCGTTTCC
      IleArgProSerTrpGluLysAlaMetSerAspValValGluGlnGlyGlyLysProPhePr

 497  GATTCCAGCGGGTTGCTCCGAGCATCCCTATGGCGGCCTCGGTTTCGTCGGCTTTGCCGAAG
      oIleProAlaGlyCysSerGluHisProTyrGlyGlyLeuGlyPheValGlyPheAlaGluG

 559  AGGTGCGGCAGCAGGAAAAGGAACTGGGCTTCAAGTTTGACTACATCGTGGTCTGCTCGGTG
      luValArgGlnGlnGluLysGluLeuGlyPheLysPheAspTyrIleValValCysSerVal

 621  ACCGGCAGTACGCAGGCGGGCATGGTTGTTGGTTTCGCGGCTGACGGTCGTTCGAAGAATGT
      ThrGlySerThrGlnAlaGlyMetValValGlyPheAlaAlaAspGlyArgSerLysAsnVa

 683  GATTGGTATCGATGCTTCGGCCAAGCCGGAACAGACCAAGGCACAGATCCTGCGCATCGCCC
      lIleGlyIleAspAlaSerAlaLysProGluGlnThrLysAlaGlnIleLeuArgIleAlaA

 745  GACACACCGCTGAGTTGGTGGAGTTGGGGCGCGAGATTACGGAAGAGGACGTGGTGCTCGAT
      rgHisThrAlaGluLeuValGluLeuGlyArgGluIleThrGluGluAspValValLeuAsp

 807  ACGCGTTTTGCCTACCCGGAATATGGCTTGCCCAACGAAGGCACATTGGAAGCCATCCGACT
      ThrArgPheAlaTyrProGluTyrGlyLeuProAsnGluGlyThrLeuGluAlaIleArgLe

 869  GTGCGGCAGCCTTGAAGGCGTGCTGACAGACCCGGTATATGAAGGTAAATCGATGCACGGCA
      uCysGlySerLeuGluGlyValLeuThrAspProValTyrGluGlyLysSerMetHisGlyM

 931  TGATTGAAATGGTCCGTCGTGGTGAATTCCCCGAAGGTTCCAAAGTGCTTTACGCACACTTG
      etIleGluMetValArgArgGlyGluPheProGluGlySerLysValLeuTyrAlaHisLeu

 993  GGTGGGGCGCCGGCGCTGAACGCCTACAGCTTCCTGTTTCGTAACGGCTAAGCGTAGAACTG
      GlyGlyAlaProAlaLeuAsnAlaTyrSerPheLeuPheArgAsnGlyEnd

1055  CTTTTGGAGTCATCTGTGGGAGCTC  1079
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Figure 1. Nucleotide Sequence and Predicted Amino Acid Sequence of an ACC Deaminase Gene from *Pseudomonas* sp Strain 6G5.

The DNA sequence of the ACC deaminase gene has been assigned GenBank Accession No. M80882.

Expression of ACC Deaminase in Plants

The ACC deaminase gene was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and used to transform UC82B tomato plants. Whereas the CaMV 35S promoter is expressed preferentially in certain tissue types (Benfey and Chua, 1989), it has been observed to be expressed at detectable levels in most tissues (M. Hinchee and H. Klee, unpublished data). Reduction in ethylene levels throughout a plant may have significant effects on plant development because ethylene has been implicated in a number of physiological reactions. However, a number of phenotypically normal transgenic plants were obtained from the transformation. Enzymatic and protein gel blot analyses of plant tissue, as shown in Figure 2, indicated that several of these plants expressed the ACC deaminase gene at high levels, up to 0.5% of the total protein.

Young leaf tissue from plants of the two lines expressing the highest levels of ACC deaminase were used in ethylene generation assays. As shown in Table 1, in the line best expressing ACC deaminase, 5673, ethylene was reduced by 90%. In a second experiment, this line exhibited ethylene reductions of greater than 97%. The line with the second-best ACC deaminase expression level, 5854, showed a reduction in ethylene of 78%. These data are consistent with the gene expression data. Line 5673 contained approximately 0.5% of the soluble protein in ACC

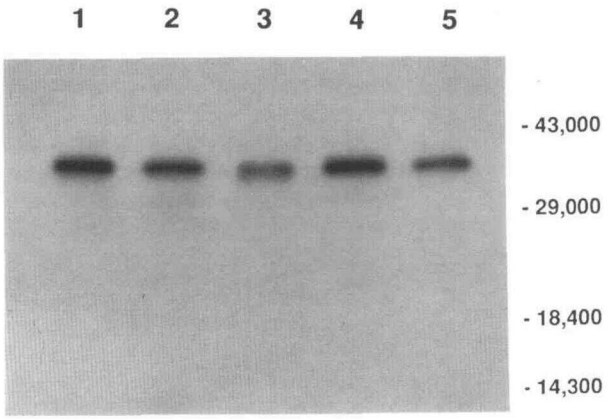

Figure 2. Protein Gel Blot Analysis Showing Expression of ACC Deaminase in Bacteria and Transgenic Tomato Plants.

Lane 1, *Pseudomonas* sp strain 6G5; lane 2, *E. coli* containing pMON10078; lanes 3 to 5, transgenic 5673 tomato leaf, green fruit, and red fruit, respectively. Molecular weights of size standards are indicated.

deaminase, whereas line 5854 contained approximately 0.05% of the soluble protein in ACC deaminase, as measured by protein gel blot analysis (data not shown).

Homozygous 5673 plants were then examined for phenotypic effects. Seed from the transgenic plants germinated normally, and plants were phenotypically indistinguishable from controls. The plants exhibited no delay in the onset of flowering or ripening; however, they did show significant differences in the progression of ripening. Figure 3 shows that both the 5673 and the UC82B fruit exhibited a peak of ethylene at 3 days, but the 5673 fruit synthesized ethylene at a level of only 10% of that of UC28B fruit. Table 2 and Figure 4 illustrate the delay in ripening of fruit detached at the breaker stage. Whereas control fruit passed from the breaker to fully red stages in 7 days and exhibited a marked degree of softening after only 2 weeks, transgenic fruit reached the fully red stage after 24 days and remained firm for an extended period. Figure 4A shows fruit detached at the breaker stage and allowed to ripen at room temperature for 7 days. Figure 4B illustrates the effect on over-ripening that was observed in ACC deaminase-expressing fruit picked at the breaker stage and stored at room temperature for 4 months. Figure 4C illustrates a similar phenomenon when fruit remained on the plants to ripen. The transgenic fruit remained firm for longer than 40 days and did not abscise, whereas the control fruit had abscised after 14 days.

DISCUSSION

Ethylene is known to promote ripening of climacteric fruits and is synthesized from ACC. Because ACC is only known to participate in the ethylene synthesis pathway, it is likely that the only biochemical consequence to the plant of ACC degradation is inhibition of ethylene synthesis. Probably because of an abundance of ACC and its metabolite malonyl-ACC in decaying plant material, microorganisms capable of degrading ACC can be readily isolated from soil. We have independently isolated ACC deaminase-containing bacteria from soil samples obtained from four continents (M. T. Tran and M. B. Hayford, unpublished data). The isolated gene has been introduced into and expressed in tomato plants where it greatly reduces the level of ethylene production. The product of ACC degradation, α-ketobutyric acid, is a naturally occurring plant metabolite. Because it is the substrate for the enzyme acetolactate synthase, all of the α-ketobutyric acid should be converted to branched-chain amino acids in plants.

It is particularly interesting that plants producing greatly reduced levels of ethylene in tissues such as leaves and fruit exhibit no apparent differences from controls except in ripening fruit. These results suggest a minor role for ethylene in plant development until fruit ripening. It is also possible that the remaining ethylene synthesized by the transgenic plants is sufficient for normal tomato growth. The existence of ethylene-insensitive mutants of Arabidopsis that are phenotypically normal under laboratory conditions is also consistent with a minor role for ethylene in plant growth before fruit ripening and/or senescence (Bleecker et al., 1988; Guzman and Ecker, 1990). Rather, ethylene seems to be critical for signaling environmental or physiological alterations such as ripening or stress. Subtle differences in response to environmental influences on the transgenic plants cannot be eliminated at this time.

An alternate approach to reducing ethylene synthesis involves expression of an antisense gene construction to shut off the endogenous biosynthetic pathway (Hamilton et al., 1990). This approach has been demonstrated to delay tomato fruit ripening effectively. Because both ACC synthase and ACC oxidase are members of multigene families, each with its own distinct temporal and spatial

Table 1. Ethylene Synthesis in a Control (UC82B) and Two Transgenic Tomato Lines Expressing ACC Deaminase

Plant	Leaf	Fruit
UC82B	5.15 ± 0.69	11.73 ± 0.86
UC82B-2	5.53 ± 0.37	
5673	0.60 ± 0.09	1.43 ± 0.36
5673-2	0.18 ± 0.02	
5854	1.14 ± 0.21	ND[a]

Measurements were determined as nanoliters per gram per hour (±SE) and were performed as described in Methods.
[a] ND, not determined.

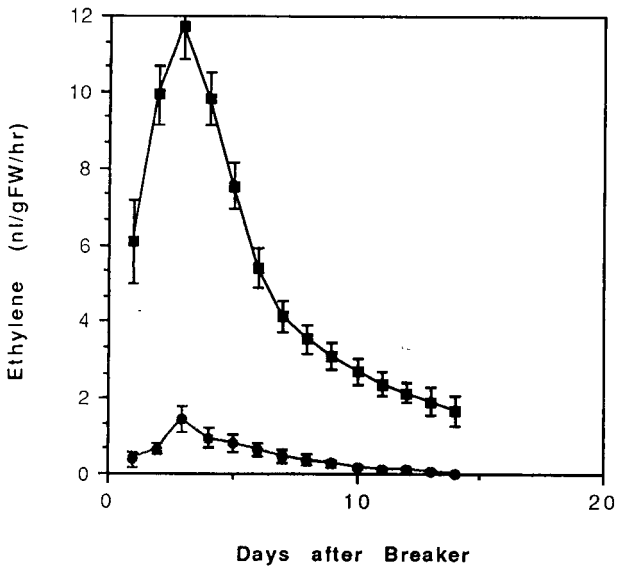

Figure 3. Ethylene Generation by 5673 Transgenic (– ● –) and UC82B Control (– ■ –) Ripening Fruit.

Bars represent means ± SE at specific time points. Fruits were detached at breaker stage, and ethylene generation was measured daily as described by Ward et al. (1978).

pattern of expression, it is difficult to assess the degree of ethylene inhibition in different organs. The approach described in this paper leads to a reduction of ethylene throughout the plant. Furthermore, ACC deaminase should function well in any plant species in which it is introduced, offering the possibility to reduce ethylene synthesis in species in which mutants are not currently available. Thus, plants expressing ACC deaminase should be generally useful for study of the role of ethylene in many aspects of disease resistance, environmental stress, and development.

In transgenic tomato fruit expressing the gene encoding ACC deaminase, there is a correlation between the degree of ethylene inhibition and delay in the progression of ripening. Visual observation of transgenic fruit stored at room temperature also indicates a significant reduction in softening, with fruit remaining firm for longer than 5 months. In this same period, control fruit is almost completely dessicated, as shown in Figure 4B. Degradation of ACC inhibits ethylene synthesis but does not interfere with the fruit's ability to perceive ethylene because transgenic fruit exposed to exogenous ethylene ripens normally (data not shown). All of these factors indicate that expression of ACC deaminase should result in a greatly extended shelf life for tomato and other climacteric fruits and vegetables.

METHODS

Microbial Screen

A collection of 600 bacterial strains was screened for organisms capable of degrading ACC. The majority of the microorganisms were fluorescent *Pseudomonas* species with the remainder being microbes typically found in soil. The screen was designed to select for organisms that would grow in a minimal medium containing 3.0 mM ACC as the sole source of nitrogen. The samples were grown in 96-well microtiter dishes at 30°C for 4 days. Each well contained 0.2 mL of medium consisting of DF salts (Dworkin and Foster, 1958) minus nitrogen supplemented with ACC and 0.2% each glucose, gluconic acid, and citric acid. Three organisms were identified as growing on ACC-containing medium. Their ability to grow on ACC-containing minimal medium was confirmed by regrowth in 300 mL of liquid cultures of the same medium. Two isolates that grew best on ACC, designated 3F2 and 6G5, were chosen for further characterization.

To prepare chromosomal DNA from the *Pseudomonas* sp strain 6G5 strain, a 200-mL culture was grown in Luria broth. The cells were collected and resuspended in 10 mL of 25 mM Tris-HCl, pH 8, 10 mM EDTA. SDS was added to a final concentration of 1%, and the suspension was subjected to three freeze-thaw cycles, each consisting of immersion in dry ice for 15 min and in water at 70°C for 10 min. The lysate was then extracted four times with equal volumes of phenol:chloroform, and was ethanol precipitated. The pellet was resuspended in 5 mL of 10 mM Tris, pH 7.5, 1.0 mM EDTA and dialyzed for 16 hr at 4°C against 2 L of the same buffer. The 6G5 DNA was partially digested with EcoRI and size fractionated on a 10% to 40% sucrose gradient. Fractions containing DNA fragments greater than 20 kb were pooled. A cosmid bank was constructed in EcoRI-cut pMON17016, a derivative of cosmid vector pHC79 (Hohn and Collins, 1980). The pMON17016 plasmid contains the gene 10 promoter from phage T7 (Tabor and Richardson, 1985) adjacent to the cloning sites (M. Weldon and G. Barry, unpublished data). The cosmid library was packaged into λ phage particles (Stratagene; Gigapack Plus) using the manufacturer's procedure and introduced into *Escherichia coli* MM294. To select the cosmid clones containing the ACC deaminase gene, a portion of the

Table 2. Ripening of Control and Transgenic Tomatoes

	Stage			
Plant	3	4	5	6
Transgenic	2.8 ± 0.53	5.3 ± 0.98	11.3 ± 3.1	23.5 ± 3.8
Control	1.4 ± 0.19	2.8 ± 0.26	5.1 ± 0.45	7.0 ± 0.53

Control and transgenic (5673) tomatoes were detached at the breaker stage. Fruits were the same as used to generate the data in Figure 3. Data are expressed as the number of days to reach color stage after being detached (±SE). Ripening stages were defined as follows: breaker, first sign of color change; 3, fully orange; 4, orange to red; 5, greater than 50% red; 6, fully red. Fruits in this experiment were the same fruits used for ethylene generation in Figure 3.

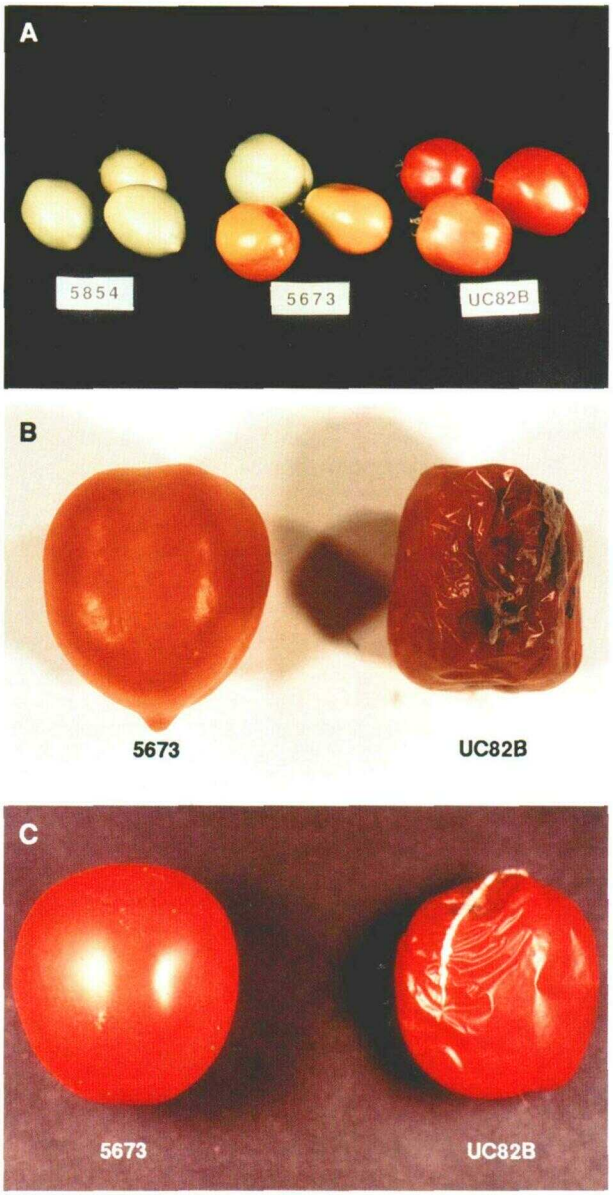

Figure 4. Effect of ACC Deaminase Expression on Ripening Tomatoes.

(A) Ripening tomatoes from control (UC82B) and transgenic (5673 and 5854) plants were removed from the plants at the breaker stage and stored at room temperature for 7 days.
(B) Tomatoes from 5673 (left) and UC82B (right) plants were picked at the breaker stage and stored at room temperature on a lab bench. This photograph was taken 121 days after harvest.
(C) Overripe 5673 (left) and UC82B (right) tomatoes. Tomatoes were left to ripen on the plants and were photographed 40 days after the breaker stage.

library was plated on M9 medium containing 3 mM ACC as a sole nitrogen source. The plates were incubated for 3 days at 37° C. Approximately 300 of the 56,000 cosmids plated (1 per 200) grew on the minimal plates.

ACC deaminase activity was measured by following production of α-ketobutyric acid and ammonia. The α-ketobutyric acid was measured with 2,4-dinitrophenylhydrazine derivitization as described in Honma and Shimomura (1978). Ammonia was measured with a glutamate dehydrogenase assay kit as described by the manufacturer (Sigma). The identity of α-ketobutyric acid was verified by coelution of derivatized standard by ion-pairing reverse phase HPLC. The assay mixture was extracted twice with ethyl acetate, discarding the aqueous fraction. The ethyl acetate fraction was then extracted once with 10% sodium carbonate, and the aqueous phase was applied directly onto a C-18 column. Samples were eluted under isocratic conditions using 60% methanol, 1.5% acetic acid with 1.0 mM tetrabutylammonium phosphate as the ion-pairing agent. Eluates were monitored at 374 nM.

A 10.6-kb BamHI-XbaI fragment that retained ACC deaminase activity was subcloned from one of the cosmids into pUC118 (Vieira and Messing, 1987). Subsequent HindIII and SmaI deletions narrowed down the ACC deaminase activity to a 2.4-kb fragment. Both strands of the 2.4-kb region were sequenced using a U.S. Biochemical Corporation Sequenase DNA sequencing kit following the manufacturer's directions. A 1017-bp ORF was identified as the ACC deaminase gene.

Protein Sequencing

Purified ACC deaminase was sequenced using automated Edman degradation on an Applied Biosystems model 470A protein sequencer as described in Hunkapillar (1983).

Bacterial Gene Expression and Antibody Preparation

An NdeI site was introduced at the ATG start site of the ACC deaminase ORF by site-directed mutagenesis (Kunkel, 1985). The gene was subcloned on a 1127-bp NdeI-HindIII fragment into an *E. coli* expression vector containing the *recA* promoter and the gene 10 leader sequence (Olins and Rangwala, 1989). This plasmid, pMON10078, was introduced into *E. coli* W3110, and gene expression was induced with 50 μg/mL nalidixic acid for 20 hr at 37°C. The cell pellet was resuspended and sonicated for 1 min, and the soluble and insoluble fractions were run on a 10% to 20% denaturing gel. A major band with a molecular weight of ~37,000, corresponding to the predicted molecular weight of 36,800 for ACC deaminase, was present in the insoluble fraction. Its identity was verified by amino-terminal amino acid sequencing of the sample. Antibodies were obtained from a goat injected with 1.5 mg of protein.

Expression of ACC Deaminase in Tomato

A 1071-bp EcoRV-SacI fragment from pMON10027 containing the ACC deaminase ORF was cloned into a plant transformation vector, pMON893. This fuses the ORF to a duplicated CaMV 35S promoter described in Kay et al. (1987) and a pea *rbcS-E9* gene

3′ end (Coruzzi et al., 1984; Morelli et al., 1985). The plasmid vector was mobilized into the ABI strain of Agrobacterium by the triparental conjugation system using the helper plasmid pRK2013 (Ditta et al., 1980). The ABI strain is C58 Agrobacterium carrying the disarmed pTiC58 plasmid pMP90RK (Koncz and Schell, 1986). Transconjugants were selected on Luria plates containing 50 μg/mL kanamycin, 25 μg/mL chloramphenicol, 100 μg/mL spectinomycin. Tomato UC82B leaf tissue was transformed using the procedure described by McCormick et al. (1986). Fruits were visually graded for stages of maturity as described by Kader and Morris (1976). Each experimental point represents a minimum of 10 fruits. Fruit softening was based on observation and has not been measured quantitatively at this point.

Ethylene Measurements

Ethylene generation was measured by enclosing whole leaves or fruit in sealed containers and withdrawing 1.0-mL gas samples after 1 hr. Ethylene was quantified on a gas chromatograph (Ward et al., 1978) equipped with an alumina column and flame ionization detector.

Protein Gel Blotting

Protein samples were boiled for 3 min in the gel-loading buffer (50 mM Tris, pH 7, 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and run on a 4% to 20% polyacrylamide Mini-PROTEAN II Ready Gels (Bio-Rad). The protein was transferred to nitrocellulose membrane using a MilliBlot-SDE Electroblotting Apparatus (Millipore, Bedford, MA) following the manufacturer's directions. The membrane was incubated overnight at 4°C in 1% BSA and 10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20 (TBST). To hybridize the membrane, the incubations were done at room temperature with gentle agitation. The primary ACC deaminase antibody was bound by incubating the membrane in a 1:1000 dilution of the goat serum in TBST for 1 hr. This was followed by three 10-min washes in TBST. The secondary reagent was bound by incubating the membrane with 5 μC of ^{125}I-labeled protein G in 20 mL of TBST for 30 min. The membrane was washed four times for 10 min with 0.1% Triton X-100 and exposed to film.

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