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Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants

(plant senescence/hormone action/gene expression/λgt11/*Cucurbita pepo*)

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ABSTRACT **Ethylene is the plant hormone that controls several features of plant growth and development. The rate-limiting step in its synthesis is the formation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) from *S*-adenosylmethionine (AdoMet), catalyzed by ACC synthase. We have isolated a complementary DNA sequence encoding ACC synthase from zucchini (*Cucurbita*) fruits. The biological activity of the clone was confirmed by the ability of the cloned sequence to direct ACC synthase activity in *Escherichia coli* and yeast. *In vivo* studies using the ACC cDNA as probe showed that the ACC synthase gene is induced by a diverse group of inducers, including wounding, Li^+ ions, and the plant hormone auxin.**

Ethylene, one of the simplest organic molecules with biological activity, is a plant hormone that influences many aspects of plant growth and development (1). The production of ethylene is strictly regulated. It is induced during several stages of plant growth, including fruit ripening, seed germination, leaf and flower senescence, and abscission (1). It is also induced by a variety of external factors, including the application of auxins, wounding, anaerobiosis, viral infection, elicitor treatment, chilling injury, drought, Cd^+ and Li^+ ions (1).

It has been established that methionine is the biological precursor of ethylene in all higher plants (2–5), and it is converted to ethylene via the following biosynthetic route:

methionine → *S*-adenosylmethionine (AdoMet) →
1-aminocyclopropane-1-carboxylic acid (ACC) → C_2H_4

The rate-limiting step is the conversion of AdoMet to ACC, catalyzed by the enzyme ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), the only enzyme in the pathway that has been studied in detail (6–12). Induction of ethylene production by a variety of agents is due to *de novo* synthesis of this enzyme (2, 13). It has been partially purified from several sources, with wide discrepancies in its molecular mass reported (9–12). Isolation of the gene encoding ACC synthase has been difficult, in part because the enzyme is unstable and present in low abundance (7). Here we report the isolation of a complementary DNA sequence to ACC synthase mRNA from zucchini (*Cucurbita*) fruit by a strategy that requires only partially purified enzyme.

MATERIALS AND METHODS

Plant Material. Slices 1 mm thick were prepared from zucchini fruits (*Cucurbita pepo*) and incubated for 24 hr in induction medium [50 mM KPO_4 buffer, pH 6.8/0.5 mM indoleacetic acid (IAA)/0.1 mM benzyladenine (BA)/50 mM LiCl/0.6 mM aminooxyacetic acid (AOA)/chloramphenicol (50 μg/ml)].

ACC Synthase Assay. During the course of the protein purification, ACC synthase activity was monitored by incubating enzyme samples with 200 μM AdoMet/10 μM pyridoxal phosphate/40 μg of bovine serum albumin in 200 mM Hepes buffer (pH 8.5) in a total vol of 600 μl at 30°C for 30 min. The amount of ACC formed was assayed according to ref. 14. The protein was determined according to Bradford (15).

Purification of ACC Synthase. The purification of the enzyme will be described in detail elsewhere (unpublished data) and is available upon request. Briefly, the proteins in tissue homogenates were sequentially bound to and eluted from (in batch) Butyl-toyopearl, (Toyo Soda, Tokyo), SP-Sephadex, and QAE-Sephadex. The eluate was then chromatographed sequentially through columns containing Butyl-toyopearl, Sephacryl S-300, Bio-Gel HT, and finally FPLC Mono Q. After the final step, the specific activity was 35,000 nmol per hr per mg of protein, representing 6000-fold purification.

Antibody Production and Immunoinhibition of ACC Synthase. A New Zealand White rabbit was immunized four times (16) at 3-week intervals with 5000 nmol of ACC synthase activity per hr (specific activity, 1500 nmol of ACC per hr per mg of protein; Bio-Gel HT column). Crude antiserum (2 ml) was purified by incubation with 10 ml of Sepharose 4B coupled with soluble proteins from intact *Cucurbita* fruit. Immunoinhibition of ACC synthase was carried out by incubating 2 nmol of enzyme activity per hr (specific activity, 1500 nmol of ACC per hr per mg of protein) in 50 μl of 500 mM Hepes, pH 8.5/40 μM pyridoxal phosphate/bovine serum albumin at 400 μg/ml with 50 μl of TBS (50 mM Tris·HCl, pH 8.0/0.14 M NaCl) containing various amounts of preimmune or immune antiserum. The mixture was incubated for 12 hr at 4°C. The enzyme–antibody complex was precipitated by centrifugation at 16,000 × *g* and the remaining enzyme activity in the supernatant was assayed.

***In vitro* Translation and Immunoprecipitation.** $Poly(A)^+$ RNA was isolated from uninduced and induced tissue and *in vitro* translated in a wheat germ lysate (17) in the presence of [^{35}S]methionine (specific activity >1000 Ci/mmol; 1 Ci = 37 GBq). Immunoprecipitation with preimmune and immune purified antiserum was carried out according to ref. 18. The translation products were analyzed on a 10% SDS/polyacrylamide gel (19).

cDNA Library Construction and Immunoscreening. $Poly(A)^+$ RNA from 18-hr induced tissue was used for constructing a cDNA library in λgt11 (20) with insert sizes

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indoleacetic acid; BA, benzyladenine; AOA, aminooxyacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

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between 200 and 500 base pairs. The basis for the size fractionation was our previous experience that chimeric β-galactosidase proteins made from small cDNAs are more stable than those derived from large cDNAs. The library was screened with purified ACC synthase antiserum at 1:200 dilution essentially as described (20).

Antibody Selection by λgt11 Recombinants. The appropriate λgt11 clones were plated on *E. coli* strain Y1090 (20) to yield 10^5 plaque-forming units per 90-mm plate. Dry nitrocellulose filters presoaked in 10 mM isopropyl β-D-thiogalactopyranoside (IPTG) were laid on the lawn after incubation for 2 hr at 42°C and then incubated for an additional 4 hr at 37°C. The filters were then soaked for 30 min in TBST (50 mM Tris·HCl, pH 8.0/0.14 M NaCl/0.05% Tween 20)/2% milk protein and then in 5 ml of diluted (1:500) purified antiserum per filter for 2 hr. After washing five times (10 min each) with TBST, bound antibody was eluted by shaking for 3 min at room temperature with 0.2 M glycine hydrochloride buffer (pH 2.3) containing 1% milk protein (21). The antibody solution was neutralized and used for immunoblotting (22).

Expression of ACC Synthase in *E. coli*. *λgt11 lysogens. E. coli* strain Y1089 was lysogenized with appropriate λgt11 recombinant phages (20). Lysogens were grown in LB medium containing ampicillin (50 μg/ml) at 30°C for 2.5 hr (A_{600} = 0.5), the temperature was shifted to 42°C for 20 min, and IPTG was added (final concentration, 1 mM), and the culture was grown for an additional 1.5 hr at 37°C. The ACC secreted in the medium was determined (14) by assaying 50 μl of the culture supernatant. Bacterial pellets of 100 ml of culture were frozen in liquid N_2 and kept at −80°C for 2 hr. The cells were resuspended in 5 ml of buffer A (100 mM Tris·HCl, pH 8.0/20 mM EDTA/10 μM pyridoxal phosphate/0.5 mM phenylmethylsulfonyl fluoride/20 mM 2-mercaptoethanol), sonicated five times (3 sec each), centrifuged at 16,000 × *g* for 25 min at 4°C, and ACC synthase activity was assayed.

pKK223-3 recombinants. ACC synthase cDNA clone ACC1 was subcloned into the *Eco*RI site of the expression vector pKK223-3 (23) and introduced into *E. coli* strain JM107. Transformants were grown in LB medium in the presence of ampicillin (50 μg/ml) at 37°C for 4 hr, IPTG was added to 1 mM, the cultures were incubated for 2 hr at 37°C, and ACC synthase activity and ACC formation were assayed.

Expression of ACC Synthase in *Saccharomyces cerevisiae*. The cDNA clone ACC1 was subcloned into the *Eco*RI site of the yeast expression vector pBM258 (24) and introduced into yeast strain YM2061. The yeast cells grown on YP medium (25) at 37°C for 24 hr containing either 2% galactose or 2% glucose were harvested and the supernatant was assayed for ACC released into the medium. The pellet was resuspended in buffer A containing 5 g of glass beads, Vortex mixed 10 times for 30 sec (21), and centrifuged at 2000 × *g* for 3 min, and the supernatant was collected. Solid $(NH_4)_2SO_4$ was added to achieve 80% saturation, and the precipitate was collected and dissolved in 2 ml of buffer B (20 mM Tris·HCl, pH 8.0/10 μM pyridoxal phosphate/10 mM EDTA/0.5 mM dithiothreitol) and dialyzed against buffer B.

Nucleic Acid Hybridization Analysis. Total nucleic acids or poly(A)$^+$ RNA were separated, blotted, and hybridized as described (17). Nuclear DNA was prepared from 4-day-old etiolated *Cucurbita* seedlings by a modification of the method of Dellaporta *et al.* (26), and DNA fragments were separated, transferred to Genetran 45 nylon membrane, and probed as described (27).

RESULTS

Cloning Strategy. The cloning of ACC synthase mRNA has been hindered because traditional cloning approaches require that the protein be purified and at least partially sequenced. Unfortunately, ACC synthase has been difficult to purify to homogeneity because of its lability and low abundance [e.g., 0.0001% of the total protein in ripe tomatoes (7, 8)]. Our cloning strategy does not demand that the protein be pure, but only inducible. (Its mRNA need not be inducible.) The strategy has five main steps: (*i*) partial purification of the ACC synthase (10–20%) from a highly induced tissue by conventional protein purification procedures; (*ii*) use of the partially purified enzyme to produce an antiserum to ACC synthase that inhibits enzyme activity; (*iii*) purification of the crude antiserum by affinity chromatography with total proteins from uninduced tissue (antibodies against uninduced proteins are removed in this step, resulting in purification of the ACC synthase antibody); (*iv*) immunoscreening λgt11 cDNA libraries with the purified antiserum and isolation of putative cDNA clones to ACC synthase; (*v*) identification of the cDNA clones by immunoblotting analysis with antiserum released from putative λgt11 cDNA clones and, finally, by expressing and recovering ACC synthase activity in *E. coli* and yeast using putative ACC synthase cDNA clones.

The Inducible System. Thin slices of immature zucchini fruit tissue treated for 24 hr with IAA plus BA plus LiCl plus AOA were found to develop higher levels of enzyme activity (15–20 nmol of ACC per hr per g fresh weight) than most of the systems previously used (6–12). Whereas tissue treatment results in a 100-fold stimulation in enzyme activity, ethylene evolution is the same in treated and untreated tissue. This is due to the inhibition of the ethylene forming enzyme by Li^+ (unpublished data).

Antibody Production and Purification. Polyclonal antibodies were made to 1500-fold purified ACC synthase preparations. The antiserum inhibits the enzyme activity (Fig. 1*IE*, curve b) and recognizes numerous polypeptides in highly impure enzyme preparations (Fig. 1*IC*, lane 1) but only two polypeptides [46 and 67 kDa (Fig. 1*IC*, lane 2) in highly purified enzyme preparations. The crude antiserum was purified by passing it through a Sepharose 4B column containing total proteins from uninduced tissue to remove all antibodies to uninduced proteins present in the initial enzyme preparations. The column eluate is able to immunoinhibit ACC synthase activity (Fig. 1*IE*, curve c) with the same intensity as the nonpurified antiserum. The purified antiserum recognizes only two polypeptides, predominantly a 46-kDa and weakly a 67-kDa species in both crude and highly purified enzyme preparations (Fig. 1*ID*, lanes 1 and 2, respectively).

The specificity of the antibody was tested by its ability to recognize an inducible polypeptide in (*i*) total protein extracts and (*ii*) *in vitro* translation products. Immunoblotting analysis reveals that the 46-kDa protein is inducible, whereas the 67-kDa protein is constitutively expressed (Fig. 1*IIC*, lanes 1 and 2, respectively). The 46-kDa polypeptide has been identified during the protein purification process to be the ACC synthase subunit by disk gel electrophoresis followed by SDS/PAGE. The molecular mass of the native enzyme is ≈86 kDa, suggesting that it is a dimer of two identical subunits, 46 kDa each (unpublished data). Furthermore, the purified antiserum immunoprecipitates an inducible *in vitro* translation product encoding a 53-kDa polypeptide (Fig. 1*III*, lane 6), suggesting that the ACC synthase is synthesized as a larger precursor (53 kDa).

Immunoscreening λgt11 cDNA Libraries. Sixty-six immunoreactive clones were isolated by screening 1.4×10^5 λgt11 recombinant clones with the purified antiserum. Upon rescreening only 30 were truly positive. Southern analysis revealed that 19 clones represented the ACC synthase mRNA. Fig. 2 shows a partial restriction map of two representative ACC synthase cDNA clones, pACC1 and pACC7. Clone pACC1 has an open reading frame encoding a 55.8-kDa polypeptide (unpublished data). Clone pACC7 is a small cDNA clone (220 base pairs) and gave the strongest signal during immunoscreening.

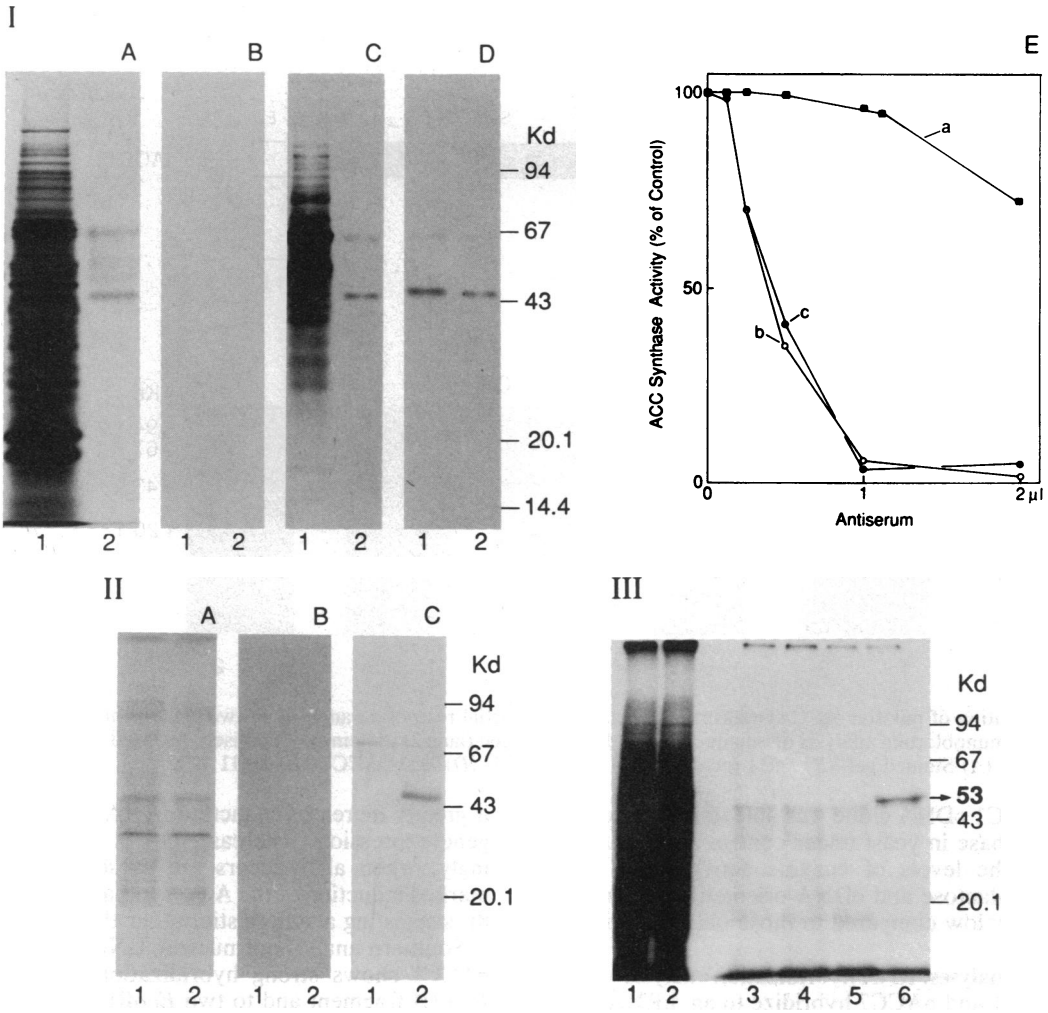

FIG. 1. Induction of ACC synthase polypeptide and mRNA. (*I*) Purification of the ACC synthase antiserum. (*A*) Silver-stained SDS/polyacrylamide gel. Lanes: 1, highly impure enzyme preparation (150-fold; 30 μg of protein with 4 nmol of enzyme activity per hr); 2, highly purified enzyme preparation (6000-fold; 30 ng of protein with 1 nmol of enzyme activity per hr). Three replicas of a gel as shown in *A* were immunoblotted with the following antisera: preimmune (*B*), unpurified immune (*C*), and purified immune (*D*) antiserum. (*E*) Immunoinhibition of ACC synthase activity by preimmune (curve a), unpurified immune (curve b), and purified immune antiserum (curve c). (*II*) Induction of ACC synthase polypeptide. (*A*) Coomassie blue-stained SDS/polyacrylamide gel (10%). Lanes: 1, uninduced protein preparation; 2, induced protein preparation. Two replicas of an identical gel as shown in *A* were immunoblotted with preimmune (*B*) and purified immune (*C*) antiserum to ACC synthase. Tissue treatment was for 24 hr. Immunoblotting was done as described (22). (*III*) *In vitro* translation and immunoprecipitation of ACC synthase mRNA. Poly(A)$^+$ RNAs from uninduced and induced *Cucurbita* fruit tissue were *in vitro* translated and translation products were immunoprecipitated with purified antiserum to ACC synthase. Lanes: 1 and 2, total translation products from uninduced and induced tissue, respectively; 3 and 4, immunoprecipitations with preimmune serum; 5 and 6, immunoprecipitations with purified immune antiserum with the translation mixtures in lanes 1 and 2, respectively. The arrow at 53 kDa indicates the size of the primary translation product of *Cucurbita* ACC synthase.

Antiserum released from filter-bound λgt11 cDNA ACC1 and ACC7 fused proteins recognizes the 46-kDa polypeptide, which is induced in treated tissue (Fig. 2*II C* and *D*). However, antiserum released from a nondifferential clone λgt11 W7 recognizes the same polypeptide weakly as the λgt11 control. This is attributed to the nonspecific binding of the antiserum to the filters with λgt11 or λgt11 W7 plaques.

Expression of ACC Synthase in *E. coli* and Yeast. Expression experiments carried out in *E. coli* and yeast prove the authenticity of the ACC synthase cDNA clones. ACC synthase activity is present in *E. coli* lysogenized with λgt11 ACC1 (Table 1). The expression of enzyme activity depends on the presence of IPTG and on the correct orientation of the cDNA insert (Table 1). [The low levels of expression in the absence of IPTG are presumably due to insufficient amounts of *lac* repressor in strain Y1089 (28).] Formation of ACC is also detected in the lysates of λgt11 ACC1 lysogens (Table 1). Neither enzymatic activity nor ACC formation is detected in lysogens of the smaller cDNA clone λgt11 ACC7 or the non-ACC synthase cDNA clone λgt11 W7.

Immunoblotting analysis of crude lysogen lysates with purified antiserum reveals that the λgt11 ACC1 lysogen accumulates a hybrid protein (170 kDa) (data not shown). Since low molecular mass degradation products were never detected, the enzymatic activity is most likely associated with the 170-kDa chimeric protein, as has been previously observed for other hybrid proteins (29, 30). The immunoblotting analysis is consistent with the expression data presented in Table 1.

High levels of ACC synthase activity and ACC are recovered in crude extracts after induction of the pKK-ACC1 transformant containing the cDNA insert in the correct orientation (Table 1). Recovery of ACC synthase activity is observed in the absence of IPTG, presumably because of an insufficient amount of *lac* repressor (28) (Table 1). The pKKACC1 transformant accumulates four polypeptides of 53, 47, 44, and 35 kDa upon induction with IPTG (data not shown). The size of the largest polypeptide coincides with that of ACC synthase translated *in vitro* (Fig. 1*III*). This agrees well with the 55.8-kDa size predicted by DNA sequencing of the ACC1 coding region (unpublished data).

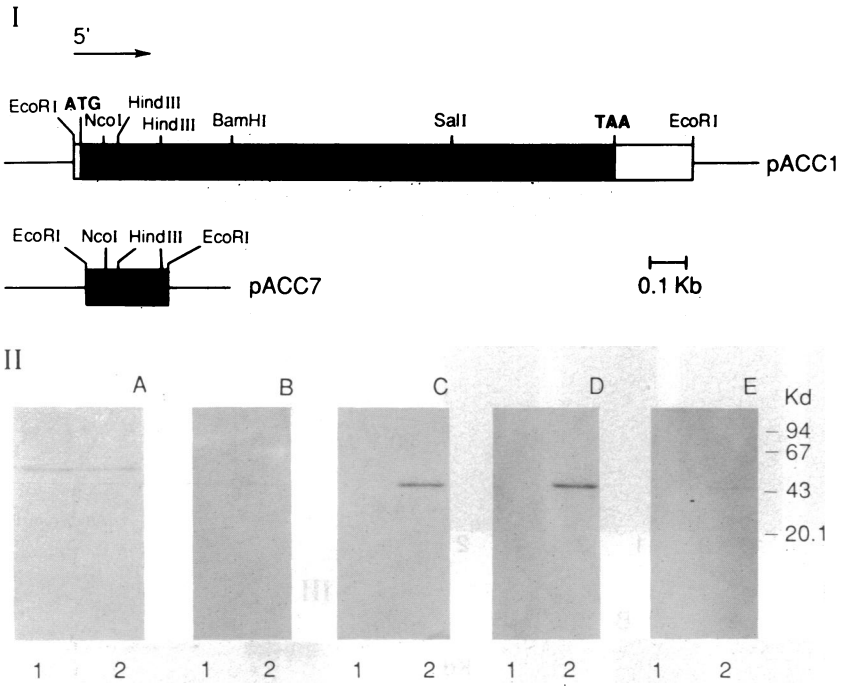

FIG. 2. Identification of putative ACC synthase cDNA clones. (*I*) Partial restriction analysis of two ACC synthase cDNA clones pACC1 and pACC7. (*II*) Immunoblotting analysis of uninduced (lane 1) and induced (lane 2) proteins with antisera selected by various immunopositive λgt11 cDNA clones. (*A*) Stained gel. (*B*) λgt11 (control). (*C*) λgt11 ACC1. (*D*) λgt11 ACC7. (*E*) λgt11 W7.

Finally, the ACC1 cDNA clone was able to direct expression of ACC synthase in yeast under control of the GAL10 promoter (24). The levels of enzyme activity and ACC formation were galactose and cDNA orientation dependent but were relatively low compared to those obtained with *E. coli* (Table 1).

Hybridization Analyses. RNA hybridization analysis shows that clones pACC1 and pACC7 hybridize to an mRNA 1900 nucleotides long (Fig. 3*I A* and *B*) that is greatly induced in tissue treated with IAA plus BA plus LiCl plus AOA. Wounding intact fruit results in small induction of ACC synthase mRNA. The nondifferential clone pW7 hybridizes to a slightly larger RNA, which is constitutively expressed (Fig. 3*IC*). Similar analysis with RNAs isolated from tissue slices treated with the individual inducers is shown in Fig. 3*II*. IAA modestly induces the ACC synthase mRNA (Fig. 3). While BA alone has no effect on the induction of the mRNA, it greatly increases induction by IAA. Li^+ ions also induce gene expression, whereas AOA is without effect. Surprisingly, when all inducers are present together there is a marked induction of the ACC synthase mRNA (Fig. 3*II*, lane 8), suggesting a synergistic rather than an additive effect.

Southern analysis of nuclear DNA with the cDNA clone pACC1 shows strong hybridization to a 14-kilobase (kb) *Bam*HI fragment and to two *Eco*RI fragments (9 and 7 kb), suggesting the presence of one or two gene copies (Fig. 3*III*).

DISCUSSION

We have described the isolation of a cDNA encoding an enzyme in a plant hormone biosynthetic pathway. Isolation of the cDNA did not require the isolation and purification of ACC synthase to homogeneity, which has been difficult because of its low abundance. We believe this approach to cloning a cDNA by immunoscreening should be applicable

Table 1. Expression of ACC synthase in *E. coli* and *S. cerevisiae*

Construct	Orientation	Size of cDNA, kb	ACC synthase activity, nmol per hr per mg of protein		ACC formation, nmol per 100 ml of culture	
			− IPTG	+ IPTG	− IPTG	+ IPTG
E. coli λgt11 lysogen						
λgt11	—	—	0	0	0	0
λgt11 ACC1	Correct	1.7	6.6	83.7	421	3310
λgt11 ACC1	Opposite	1.7	0	0	0	0
λgt11 ACC7	Correct	0.2	0	0	0	0
λgt11 W7	Correct	1.7	0	0	0	0
E. coli pKK223-3						
pKK-223-3	—	—	0	0	0	0
pKK-ACC1	Correct	1.7	20	42	2280	4070
pKK-ACC1	Opposite	0	0	0	0	0
S. cerevisiae			+ glucose	+ galactose	+ glucose	+ galactose
pBM-258	—	—	0	0	0	0
pBM-ACC1	Correct	1.7	0	2.6	0	354
pBM-ACC1	Opposite	1.7	0	0	0	0

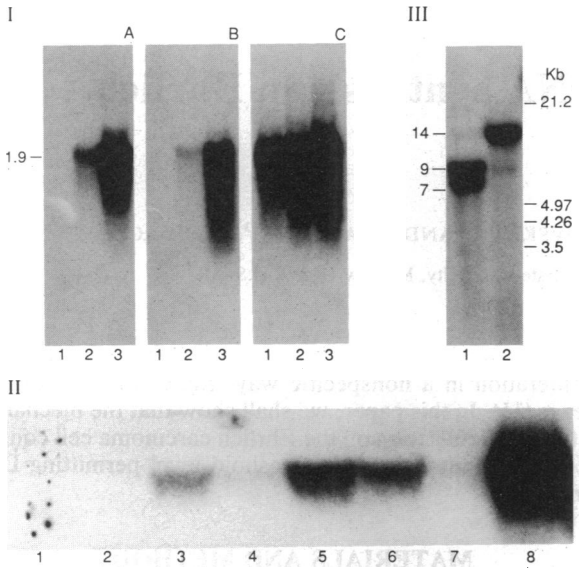

FIG. 3. RNA and DNA hybridization analyses. (*I*) Induction of ACC synthase mRNA by wounding and IAA (0.5 mM) plus BA (0.1 mM) plus LiCl (50 mM) plus AOA (0.6 mM). Autoradiograms of RNA filter papers hybridized with ^{32}P-labeled ACC1 (*A*), ACC7 (*B*), and W7 (*C*) cDNA inserts. Lanes: 1, intact fruit; 2, uninduced; 3, induced tissue (18 hr). Each lane contains 10 μg of poly(A)$^+$ RNA. (*II*) Induction of ACC synthase mRNA by various inducers. Autoradiogram of RNA filter paper hybridized with ^{32}P-labeled ACC1 cDNA insert. Lanes: 1, intact fruit; 2, uninduced tissue (9 hr); 3, 0.5 mM IAA; 4, 0.1 mM BA; 5, IAA plus BA; 6, 50 mM LiCl; 7, 0.5 mM AOA; 8, IAA plus BA plus LiCl plus AOA. Twenty-five micrograms of total nucleic acids was loaded on each lane. (*III*) Southern analysis of *Cucurbita* nuclear DNA. Lanes: 1, *Eco*RI; 2, *Bam*HI. Each lane contains 10 μg of DNA.

for isolating cDNAs encoding inducible enzymatic activities that have not yet been purified to homogeneity.

The *Cucurbita* ACC synthase activity is associated with a 46-kDa polypeptide similar in size to that isolated from tomato (7, 8) and winter squash (11). The protein constitutes 0.01% of the total protein in the induced state. The polypeptide is encoded by an mRNA 1900 nucleotides long that is greatly induced by IAA plus BA plus LiCl plus AOA in a synergistic fashion (Fig. 3*II*). The abundance of the mRNA is ≈20-fold higher than that of the ACC synthase protein (13). The induction of ACC synthase mRNA by IAA, wounding, and Li^+ ions may be due to transcriptional activation, since IAA and wounding have been shown to transcriptionally activate other plant genes (31, 32). However, posttranscriptional induction is also possible. How a single-copy gene can be transcriptionally activated by a diverse group of inducers is currently poorly understood. We propose that each inducer modifies directly or indirectly the properties of a specific transcriptional factor(s), which interacts positively or negatively with a specific DNA domain in the regulatory region of the ACC synthase gene.

The cloning of the ACC synthase mRNA provides the potential to manipulate endogenous ethylene production and consequently to prevent senescence. This is of paramount importance in the agricultural industry, where billions of dollars each year are lost worldwide due to overripening of fruits and vegetables during transportation. It may be possible to reduce ethylene production by reducing expression of the ACC synthase gene. This could potentially be accomplished by a variety of approaches, including gene disruption (33), antisense RNA technology (34), dominant negative mutations (35), expression of antibody genes (36), or with anti-gene ribozyme (37). All approaches will require the use of strong inducible and tissue-specific promoters. Finally, the prospect arises that the ACC synthase cDNA can be introduced and expressed in a photosynthetic bacterium or alga (38) for industrial scale production of ACC, followed by chemical conversion to ethylene (14). This approach substitutes the use of petroleum products for ethylene production (39, 40), and ethylene regenerated from ACC can be catalytically hydrated for production of ethanol (41) for use as an alternative energy source.

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1. Abeles, F. B. (1973) *Ethylene in Plant Biology* (Academic, New York).
2. Yang, S. F. & Hoffman, N. E. (1984) *Annu. Rev. Plant Physiol.* **35**, 155–189.
3. Adams, D. O. & Yang, S. F. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 170–174.
4. Yu, Y. B., Adams, D. O. & Yang, S. F. (1979) *Arch. Biochem. Biophys.* **198**, 280–286.
5. Yung, K. H. & Yang, S. F. (1982) *Biochem. Biophys. Res. Comm.* **104**, 771–777.
6. Acaster, M. A. & Kende, H. (1983) *Plant Physiol.* **72**, 139–145.
7. Bleecker, A. B., Kenyon, W. H., Somerville, S. C. & Kende, H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7755–7759.
8. Privalle, L. S. & Graham, J. S. (1987) *Arch. Biochem. Biophys.* **253**, 333–340.
9. Nakajima, N. & Imaseki, H. (1986) *Plant Cell Physiol.* **27**, 969–980.
10. Mehta, A. M., Jordan, R. L., Anderson, J. D. & Mattoo, A. K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8810–8814.
11. Nakajima, N., Nakagawa, N. & Imaseki, H. (1988) *Plant Cell Physiol.* **29**, 989–998.
12. Tsai, D. S., Arteca, R. N., Bachman, J. M. & Phillips, A. T. (1988) *Arch. Biochem. Biophys.* **264**, 632–640.
13. Bleecker, A. B., Robinson, G. & Kende, H. (1988) *Planta* **173**, 385–390.
14. Lisada, C. C. & Yang, S. F. (1979) *Anal. Biochem.* **100**, 140–145.
15. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–253.
16. Shiigi, S. M. & Slomich, M. (1980) in *Selected Methods in Cellular Immunology*, eds. Mishell, B. B. & Shiigi, M. J. (Freeman, San Francisco), pp. 245–277.
17. Theologis, A., Huynh, T. V. & Davis, R. W. (1985) *J. Mol. Biol.* **183**, 53–68.
18. Giudice, L. C., Waxdall, M. J. & Weintraub, B. D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4798–4802.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
20. Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning Techniques*, ed. Glover, E. E. (IRL Press, Oxford), Vol. 1, pp. 49–88.
21. Johnson, L. M., Snyder, M., Chang, L. M. S., Davis, R. W. & Campbell, S. L. (1985) *Cell* **43**, 369–377.
22. Burnette, N. W. (1981) *Anal. Biochem.* **112**, 195–203.
23. DeBoeh, H. A., Comstock, L. S. & Vasser, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 21–25.
24. Johnston, M. & Davis, R. W. (1984) *Mol. Cell. Biol.* **4**, 1440–1448.
25. Sherman, F., Fink, G. R. & Hicks, J. (1979) *Methods in Yeast Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
26. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1**, 19–21.
27. Davis, R. W., Botstein, D. & Roth, J. R. (1980) *Advanced Bacterial Genetics*, (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
28. Brosius, J. (1988) in *Vectors—A Survey of Molecular Cloning Vectors and Their Uses*, eds. Rodriquez, R. L. & Denhardts, D. T. (Butterworths, Stoneham, MA), pp. 205–225.
29. Kaufman, D. L., McGinnis, J. F., Krieger, N. R. & Tobin, A. L. (1986) *Science* **232**, 1138–1140.
30. Marullo, S., Klutchko, C. D., Eshdat, Y., Strosberg, A. D. & Emorine, L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7551–7555.
31. Theologis, A. (1986) *Annu. Rev. Plant Physiol.* **37**, 407–438.
32. Thornburg, R. W., An, G., Cleveland, T. E., Johnson, R. & Ryan, C. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 744–748.
33. Scherer, S. & Davis, R. W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4951–4955.
34. Ecker, J. R. & Davis, R. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5372–5376.
35. Herskowitz, I. (1987) *Nature (London)* **329**, 219–222.
36. Carlson, J. R. (1988) *Mol. Cell. Biol.* **8**, 2638–2646.
37. Haselhoff, J. & Gerlach, W. L. (1988) *Nature (London)* **334**, 585–591.
38. Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Anderson, B. L. R., Robertson, D., Klein, T. M., Shark, K. B. & Sanford, J. C. (1988) *Science* **240**, 1534–1538.
39. Greek, B. F. (1983) *Chem. Eng. News* **61**, 43.
40. Heylin, M. (1988) *Chem. Eng. News* **66**, 25.
41. Cram, D. J. & Hammond, G. S. (1964) *Organic Chemistry* (McGraw-Hill, New York), 2nd Ed.