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Cloning and sequence of two different cDNAs encoding 1-aminocyclopropane-1-carboxylate synthase in tomato

(ethylene/fruit ripening/λgt11/*Lycopersicon esculentum* Mill./plant senescence)

DOMINIQUE VAN DER STRAETEN*, LUC VAN WIEMEERSCH*, HOWARD M. GOODMAN†, AND MARC VAN MONTAGU*‡

*Laboratorium voor Genetica, Rijksuniversiteit Ghent, B-9000 Ghent, Belgium; and †Department of Molecular Biology, Massachusetts General Hospital, Boston MA 02114

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ABSTRACT **1-Aminocyclopropane-1-carboxylate synthase (ACC synthase; *S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), the key enzyme in ethylene biosynthesis, was purified 5000-fold from induced tomato pericarp. ACC synthase activity was unambiguously correlated with a 45-kDa protein by two independent methods. Peptide sequences were obtained both from the N terminus after electroblotting and from tryptic peptides separated by reversed-phase chromatography. Mixed oligonucleotide probes were used to screen a λgt11 library prepared from RNA of induced pericarp tissue. Putative ACC synthase clones were isolated with a frequency of 0.01%. One of these contained a 1.9-kilobase insert with a single open reading frame encoding a polypeptide of 55 kDa. A second, partial cDNA clone was found that differed from the first one in 18% of its bases. Genomic Southern blotting suggests possible tandem organization of the two genes in tomato. The entire coding region was expressed in *Escherichia coli* and the denatured recombinant polypeptide was used to raise polyclonal antibodies. The antibody preparation both immunoinhibits and immunoprecipitates ACC synthase activity from an enriched tomato extract, confirming the identity of the clone. Northern blot analysis demonstrates that the ACC synthase messenger accumulation is coordinated with fruit ripening.**

Ethylene plays an important role in virtually every phase of plant development, from germination and seedling growth to flowering, fruit ripening, and organ senescence (1). The precursor of ethylene is L-methionine, which is metabolized in three steps. The first step involves the formation of *S*-adenosyl-L-methionine (AdoMet) by *S*-adenosyl-L-methionine synthetase. The methionyl side chain of AdoMet is converted to the three-membered ring amino acid 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene in higher plants (2). This reaction, catalyzed by ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), is the rate-limiting step of the pathway (1). A variety of factors can lead to an induction of ACC synthase activity and consequently to an increase in ethylene production. These factors include hormones such as auxin and environmental factors such as mechanical wounding, excessive temperatures, drought, flooding, and a number of chemicals (1). Due to the key role of ACC synthase in this pathway, there is considerable interest in understanding its regulation at the molecular level. Efforts from different laboratories have resulted in the purification of ACC synthase from several plant sources [tomato (3–7), winter squash (8), mung bean (9), and zucchini (10)]. In this paper, we present data on the isolation of two different tomato ACC synthase cDNA species by a strategy involving amino acid sequence analysis of the protein followed by oligonucleotide screening of a λgt11 cDNA library. We have sequenced the two cloned cDNA inserts and have characterized the organization and expression of the genes in ripening tomato fruit.[§]

MATERIAL AND METHODS

Plant Material and Induction of ACC Synthase. Pink tomatoes were obtained from a local market (*Lycopersicon esculentum* Mill., cv. Orlando). The induction method has been reported (7).

Assay for ACC Synthase Activity. Protein extraction and assay for ACC synthase activity were as described (7). One unit is defined as the amount of enzyme that converts 1 nmol of AdoMet per hr at 30°C.

Gel Electrophoresis, Transfer, and Detection of Immobilized Proteins. Polyacrylamide gels (10%) were prepared according to Laemmli (11). Electroblotting and amino acid sequencing were performed as described (12).

Total Tryptic Digestion and Separation of the Peptides. One-half of a 5000-fold-enriched ACC synthase preparation (100–200 μg) was subjected to digestion with trypsin (12). Separation was done by reversed-phase chromatography on a Baker C4 column and the major peptide peaks were sequenced (12).

RNA Isolation, Construction, and Screening of a cDNA Library. RNA extraction of tomato pericarp was done essentially as described by Jones *et al.* (13) after 16 hr of 100 mM LiCl incubation and 2.5 hr of wounding. Poly(A)$^+$ RNA was isolated with Hybond messenger affinity paper (Amersham) according to the manufacturer's protocol. Double-stranded cDNA was synthesized (14) and cloned in λgt11 (15). A library of 4×10^6 clones was obtained. Approximately 20,000 plaque-forming units were plated and screened on Hybond N (Amersham) using mixed oligonucleotide probes (100 pmol). The sequences of the oligonucleotides are as follows: 5′ TT (C/T)TC ICC ITG (C/T)TC (C/T)TC ITT IGT IGC 3′ (26-mer, derived from peptide P32) and 5′ GG IA(G/A) ICC (G/A)TG ITA ITC (T/C)TG IAA (G/A)TT IGC 3′ (29-mer, derived from P40, main sequence). Hybridization and filter washes were performed at 35°C in a buffer recommended by Amersham.

Subcloning and DNA Sequence Analysis. The 1846-base-pair (bp)-long pcVV4A clone, consisting of two *Eco*RI fragments, was subcloned into pUC18 after generation of an *Xmn* I site at the 3′ end of the larger fragment and subsequent

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AdoMet, *S*-adenosyl-L-methionine; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside.
‡To whom reprint requests should be addressed.
§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34289).

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insertion of the small fragment (pUC18::pcVV4A). DNA sequence analysis was performed on several subclones (16).

***In Vitro* Mutagenesis by the Polymerase Chain Reaction (PCR).** An *Nco* I site was generated at the start codon of pcVV4A to enable cloning of the complete coding region in an *Escherichia coli* expression vector. PCR conditions were as described by Higuchi *et al.* (17). Fifteen cycles were performed in a Techne Dri-Block Cycler PHC-1 using Cetus *Thermus aquaticus* DNA polymerase (*Taq* polymerase). The product was cloned into pT7-7 (18) (pT7-7::pcVV4A-PCR). To ensure that no mutations had been introduced by PCR, the large 3′ *Eco*RI fragment was exchanged for the original one and the sequence of the small fragment was confirmed.

Expression in *E. coli*, Antibody Production, Immunoblotting, and Immunoprecipitation. Mature ACC synthase polypeptide (55 kDa) was obtained by isopropyl β-D-thiogalactopyranoside (IPTG) induction of BL21(DE3) (19) containing pT7-7::pcVV4A-PCR. A 33-kDa peptide was produced by BL21(DE3) transformed with pT7-7 containing the 890-bp *Eco*RI/*Hin*dIII fragment of pcVV4A. In both cases, bacteria were grown in LB medium containing triacillin (50 μg/ml) at 37°C for 2 hr (A_{600}, 0.5); IPTG was added to 2 mM and incubation continued for 1.5 hr. Bacteria were harvested and then resuspended and sonicated in buffer A (10). Two-month-old female rats were injected twice intraperitoneally with 50 μg of the 55-kDa polypeptide (recovered from 10% SDS gel after separation from the majority of other *E. coli* proteins). Serum was obtained at day 18. In addition, a 5-week-old male New Zealand White rabbit was injected three times with 150 μg of denatured recombinant protein. In this case, the polypeptide was recovered from gel by electroelution and precipitated in acidic acetone to remove SDS (acetone/acetic acid/triethylamine/water, 17:1:1:1). Antisera were able to reveal 10 ng of the recombinant protein on immunological dot blot (1:200 dilution). Immunoblotting was according to Towbin *et al.* (20) with a rabbit anti-rat alkaline phosphatase conjugate used as a secondary antibody (Sigma). Immunoprecipitation of ACC synthase activity was done by incubation of 2 units of 5000-fold-purified ACC synthase in phosphate-buffered saline (PBS)/bovine serum albumin (BSA) (0.5 mg/ml)/20 μM pyridoxal 5′-phosphate (PLP) containing increasing amounts of preimmune or immune serum in a total vol of 20 μl. After 1 hr at 30°C and 7 hr at 4°C, 100 μl of a 10% protein A-Sepharose (Pharmacia-LKB) suspension in PBS/BSA/PLP buffer was added and left at 4°C for 30 min. After centrifugation at 6000 × *g* for 10 min, the supernatant was assayed for ACC synthase activity.

Nucleic Acid Hybridization Analysis. Nuclear DNA was prepared according to Jofuku and Goldberg (21). Total DNA preparation was mainly as described by Dellaporta *et al.* (22), followed by a CsCl gradient. DNA gels were blotted onto Hybond N (Amersham) and the probes were labeled by random priming according to the manufacturer's specifications (Amersham). Hybridization was carried out at 65°C essentially as described by Church and Gilbert (23).

RNA for Northern blotting was purified on Qiagen columns (Diagen) and the poly(A)$^+$ fraction was obtained on oligo(dT)-cellulose (24). Formaldehyde gels (6%) were blotted on Hybond N (Amersham). RNA gel blot hybridization was at 42°C for 48 hr in 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.5% SDS/50% (vol/vol) formamide/5× Denhardt's solution (1× Denhardt's solution = 0.02% BSA/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.1 mg of denatured herring sperm DNA per ml. The first two washes were at room temperature, in 2× SSPE for 10 min each, followed by 30 min at 42°C in 0.5× SSPE and 1% SDS.

RESULTS

Determination of N-Terminal and Internal Amino Acid Sequences. ACC synthase was purified 5000-fold from induced tomato pericarp. Previous studies showed that the ACC synthase activity corresponded to a 45-kDa polypeptide (7). Approximately 10 μg of the highly purified preparation was separated on SDS/PAGE and recovered by electroblotting onto poly(4-vinyl-*N*-methylpyridinium iodide)-coated glass fiber sheets. The 45-kDa and 42-kDa bands (Fig. 1 *Left*) were sequenced. The former resulted in a sequence of 20 residues, which upon comparison with the NBRF data base was found to be 45% homologous with yeast enolase; the

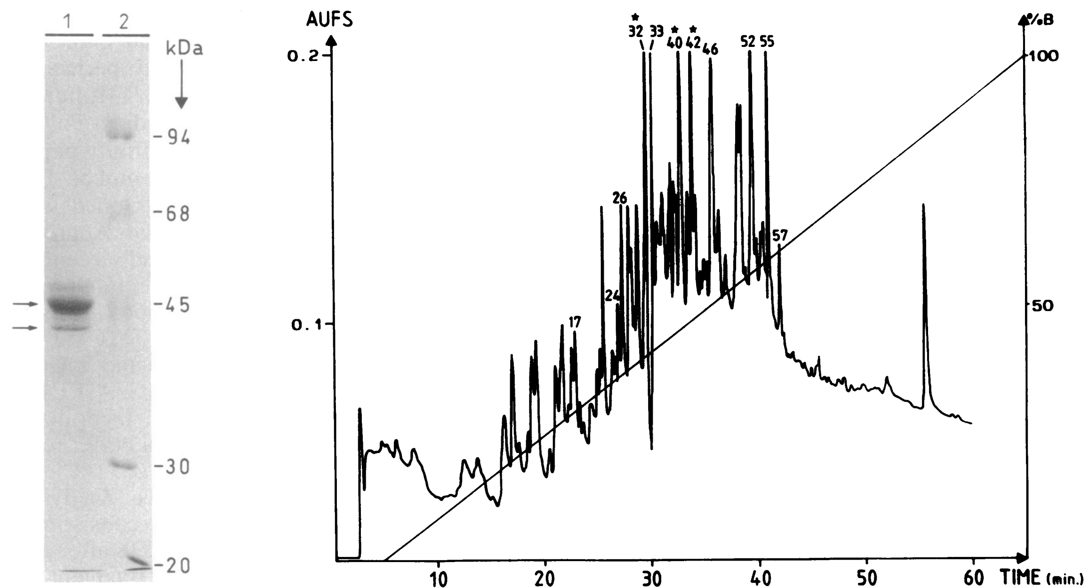

FIG. 1. (*Left*) One-dimensional PAGE analysis of highly purified ACC synthase (10% Laemmli gel, Coomassie blue stain). Lane 1, active fractions after 5000-fold purification; lane 2, size standards. The 45-kDa and 42-kDa bands (arrows) were sequenced after electroblotting on poly(4-vinyl-*N*-methylpyridinium iodide)-coated glass fiber sheets. (*Right*) Reversed-phase chromatography of tryptic peptides. Peptides generated upon trypsin degradation of the highly enriched ACC synthase preparation were separated. The slope of the gradient is given by the solid line and the percentage of solvent B (0.1% trifluoroacetic acid in 70% acetonitrile) is indicated on the right. Detection was at 214 nm. Numbered peaks were sequenced. *, Peaks corresponding to ACC synthase sequences. AUFS, absorbance units full scale.

latter yielded a seven-amino acid sequence that did not correspond to previously characterized proteins. Approximately one-half of the 5000-fold-enriched preparation was subjected to total tryptic digestion. The peptides were separated by reversed-phase chromatography (Fig. 1 *Right*). The peptide pattern was simple, with ≈35 peaks, among which were nine major peptides. Sequence data from 7 well-separated major peaks and 4 others were obtained, yielding a total of ≈210 residues. Of these, eight sequences were found homologous to yeast enolase. However, three major and four secondary sequences were unknown. We reasoned that the 45-kDa band represents a mixture of ACC synthase and enolase and that many or all of these seven unannotated peptides originated from ACC synthase. Therefore, mixed oligonucleotides were synthesized based on the sequences P32 and P40 (underlined in Fig. 2 *Lower*).

Screening of a λgt11 Tomato cDNA Library and Sequencing of the ACC Synthase Clones. By screening 20,000 λgt11 recombinant clones, two candidate ACC synthase clones were isolated (referred to as pcVV4A and pcVV4B). Upon *Eco*RI digestion, pcVV4A was found to contain two fragments, a 442-bp 5′ fragment and a 1404-bp 3′ fragment. Fig. 2 shows a partial restriction map of the pcVV4A clone (*Upper*) and the complete sequence (*Lower*). The cDNA of 1846 bp contains an open reading frame encoding a 54.7-kDa polypeptide (485 amino acids), a 153-bp leader, and a 235-bp trailing sequence. When compared to average protein composition (25), it seems to be rich in phenylalanine (6%), leucine (8.8%), lysine (7.2%), and serine (8.4%). The 11 cysteines and 9 histidines are arranged in four possible Zn^{2+}-binding sites, one of which might alternatively be a copper-binding domain (Intelligenetics Suite, release 5.37). Asparagines in positions 131, 206, and 396 reside in potential glycosylation sites (26). Fig. 2 also indicates the position of the tryptic peptides P32, P40, and P42 and the short N-terminal peptide of the 42-kDa band. pcVV4B is an incomplete cDNA consisting of two *Eco*RI fragments of 250 and 170 bp, respectively. The sequence is aligned above the pcVV4A sequence with an overall similarity of 82% and conserved blocks separated by regions of lower similarity. The deduced amino acid sequences are also 82% homologous.

Hybridization Analyses. Genomic Southern blot analysis revealed hybridization to a 4.2-kilobase (kb) *Bgl* II and a 3-kb *Eco*RI fragment, both with the intensity of a single copy

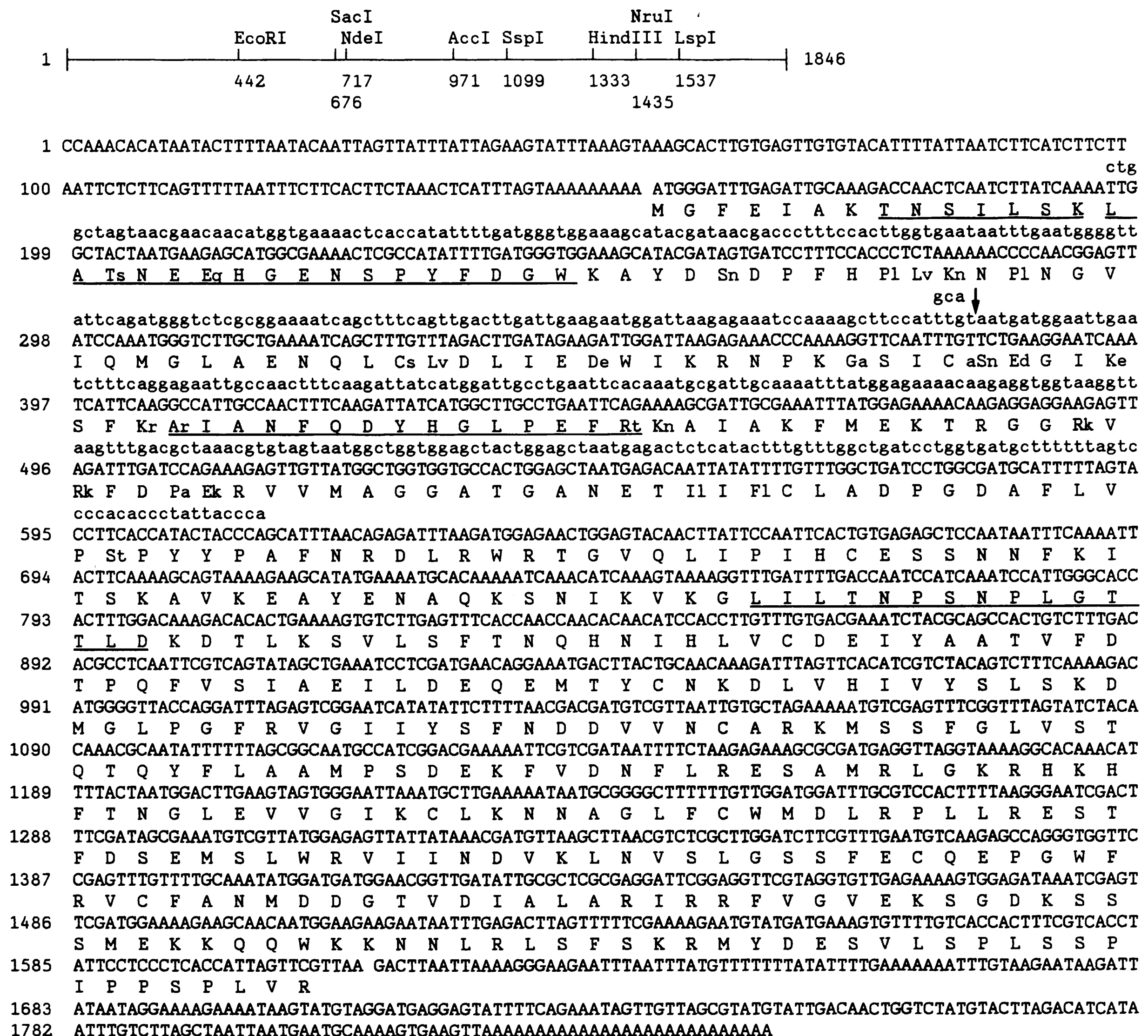

FIG. 2. (*Upper*) Partial restriction map of the tomato ACC synthase clone. (*Lower*) DNA sequences of the tomato ACC synthase cDNA clones. The pcVV4B DNA sequence is shown above pcVV4A. The derived pcVV4A amino acid sequence is shown in the one-letter code under the DNA sequence and is numbered separately. Nonidentical amino acids in pcVV4B are in lowercase letters. Peptides recovered upon tryptic digestion as well as the N-terminal sequence of the 42-kDa band are underlined (42-kDa N terminus, P32, P40, and P42, respectively).

equivalent (Fig. 3 *Left*). Under high-stringency conditions (65°C) weak hybridization was observed in tobacco, and no signals were obtained in *Arabidopsis* or rice. When the same blot was rehybridized under low-stringency conditions (53°C) faint bands were detected, suggesting that the homology between *Arabidopsis* or rice and tomato genes is at the most 70% (data not shown).

Expression of the pcVV4A gene was examined in ripening tomato fruit. As shown in Fig. 3 (*Right*), a 1.9-kb mRNA was detected at low levels in pink tomato fruit. By contrast, no signal was present in mature green fruit. Moreover, the ACC synthase mRNA is at least 100-fold higher in concentration in tomatoes treated by wounding and LiCl/indoleacetic acid/benzyladenine/aminooxyacetic acid (10). Since ACC synthase clones were isolated with a frequency of 0.01% from the cDNA library, the level of its messenger is estimated as low as 0.0001% of total mRNA in ripening tomato fruit.

Expression in *E. coli*, Antibody Production, Immunoblotting, Immunoinhibition, and Precipitation of ACC Synthase Activity. The complete coding region of the pcVV4A clone was expressed under control of the T7-7 promoter in *E. coli* (Fig. 4 *Left*). The 55-kDa polypeptide was recovered from preparative Laemmli gels and injected into rats and rabbits. Western blot analysis showed that the antibody recognizes the 33-kDa peptide (data not shown), the complete 55-kDa protein, as well as a 45-kDa polypeptide at different stages of purification (1:200 dilution) (7) (Fig. 4 *Upper Right*). The amounts of protein were calculated according to the ACC synthase purification factor, so that approximately the same intensity of signal could be expected. Fig. 4 (*Lower Right*) represents the immunoinhibition curve obtained with the rabbit antiserum. One microliter of serum is enough to precipitate 95% of the activity present. In addition, this antibody directly inhibits ACC synthase activity. After a 2-hr incubation of 5 units of ACC synthase in the presence of 10 μl of antiserum only 20% of the activity is recovered. These experiments provide strong evidence for the nature of the clones isolated.

DISCUSSION

In this paper we have reported the cloning of tomato ACC synthase. By total tryptic digestion of a partially purified ACC synthase preparation and microsequencing, enough sequence data were obtained to design corresponding oligonucleotides. The strategy proposed here could also be useful for cloning proteins involved in hormone biosynthesis or secondary metabolite pathways, often present in the same low concentration as ACC synthase. We have isolated two different ACC synthase cDNAs, which are 82% similar at both the DNA and the protein level. The authenticity of the clones was demonstrated by immunoinhibition and precipitation of ACC synthase activity with an antibody raised against the recombinant protein expressed in *E. coli*. Recently, Sato and Theologis (10) reported cloning one of the zucchini ACC synthase mRNAs, proving its identity by recovering an active recombinant protein in *E. coli* and yeast. However, we were unable to demonstrate activity when expressing the pcVV4A tomato clone. Therefore, we hypothesize either that the tomato enzyme is quite different from the zucchini gene product or that the isozyme we have cloned is not functional (pseudogene product) or is not functional in a heterologous system.

In tomato extracts, ACC synthase activity is associated with a 45-kDa protein after 5000-fold enrichment (7). This is probably the same as the 50-kDa polypeptide assigned to ACC synthase (3, 6). Our data are also in good agreement with the 46-kDa molecular mass published for zucchini (10). However, we found that the single open reading frame in the tomato ACC synthase clone pcVV4A encodes a 55-kDa protein. Thus, the question that arises is whether this apparent processing from a larger precursor is significant *in vivo* or is an artifact that occurs during purification. Although no sequence data from zucchini ACC synthase are currently available, it was demonstrated that a 53-kDa precursor was present after *in vitro* translation and immunoprecipitation (10). Moreover, it was proposed (8) that the winter squash enzyme is processed both *in vivo* and *in vitro* from a 58-kDa precursor to a polypeptide ≈8 kDa smaller. Our data suggest that this processing of ≈85 amino acids occurs at the C terminus of tomato ACC synthase as well, since all of the ACC synthase peptides recovered from the tryptic digestion are located in the N-terminal half of the protein (Fig. 2 *Lower*). This can be the result of either inaccessibility of the C-terminal part for trypsin or C-terminal processing of the

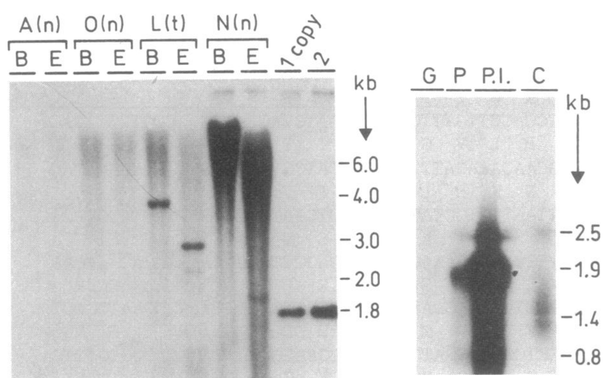

FIG. 3 (*Left*) Genomic Southern blot analysis of *Arabidopsis thaliana* (ecotype C24), *Oryza sativa* (cv. Taipei), *L. esculentum* (cv. Orlando), and *Nicotiana tabacum*. A(n), *A. thaliana* (1 μg of nuclear DNA); O(n), rice (3 μg of nuclear DNA); L(t), tomato (5 μg of total DNA); N(n), tobacco (20 μg of nuclear DNA). B, *Bgl* II; E, *Eco*RI. One- and two-copy reconstructions are on the right. Filters were hybridized with a ^{32}P-labeled 1.8-kb *Dra* I/*Xmn* I fragment of the pUC18::pcVV4A subclone. Exposure was for 6.5 hr on flash-sensitized film. (*Right*) RNA blot analysis. Each lane contains 10 μg of poly(A)$^+$ RNA. G, green; P, pink; P.I., pink-superinduced tissue; C, positive control; pcVV4A *Dra* I/*Nru* I fragment, also used as a probe. Exposure was for 24 hr on flash-sensitized film.

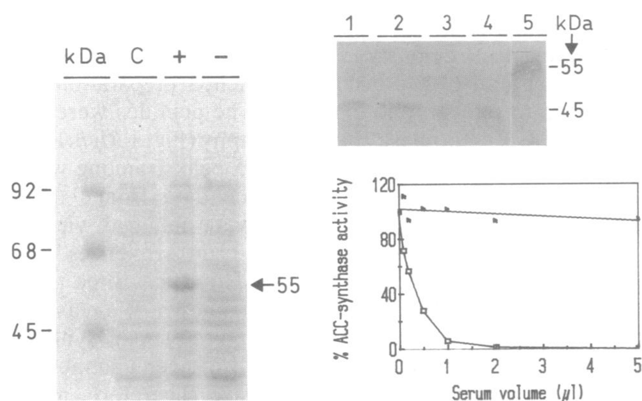

FIG. 4. (*Left*) Expression of recombinant ACC synthase in BL21(DE3) cells. Lanes: 1, size standards; 2, negative control—nontransformed host cells, IPTG treated; 3 and 4, BL21(DE3) (pT7-7::pcVV4A-PCR) with and without IPTG, respectively. (*Upper Right*), Immunoblot analysis of ACC synthase at different purification stages. Lanes: 1, 60 μg after phenyl-Sepharose; 2, 25 μg after hydroxyapatite; 3, 3 μg after Affi-Gel Blue; 4, 1 μg after high-pressure hydroxyapatite purification (each lane contains ≈200 ng of ACC synthase); 5, positive control [200 ng of recombinant 55-kDa polypeptide gel purified from a BL21(DE3) (pT7-7::pcVV4A-PCR) extract]. (*Lower Right*) Immunoinhibition curve of ACC synthase activity in the presence of various amounts of preimmune (●) and immune (□) antiserum.

protein. The latter explanation is more likely for the following reasons: (*i*) the majority of ACC synthase (45 kDa) in the 5000-fold-enriched preparation is N terminally blocked; (*ii*) the 42-kDa band yielded an N-terminal sequence only seven amino acids downstream from the initiator methionine, suggesting that the C-terminal end was degraded; (*iii*) the calculated pI of the mature 55-kDa protein is 7.7, whereas elimination of 85 amino acids at the C terminus results in a predicted pI of 6.5, which is close to that found in tomato extracts (7).

Several conclusions can be drawn from the predicted polypeptide sequence. We postulate that ACC synthase is a cytoplasmic protein, since no homology with the consensus chloroplast, mitochondrial, endoplasmic reticulum, or peroxisomal target sequences was found (27–30). Immunolocalization or cell fractionation experiments will have to confirm this hypothesis. Three putative N-glycosylation sites were found. The presence of 11 cysteines evenly dispersed over the pcVV4A polypeptide chain might suggest the formation of several disulfide bridges within the protein, which may not be forming correctly in *E. coli*, leading to negligible activity. More interestingly, they seem to be arranged in four putative Zn-binding sites as predicted by the Intelligenetics Suite (release 5.37). Although two of these regions are at the same time extremely rich in aromatic amino acids and leucines, they do not show the spacing required for a zinc finger structure in DNA-binding proteins (31).

Genomic Southern blot data revealed the presence of at least one gene in tomato and a low degree of homology with the *Arabidopsis* and rice genes. However, since we have isolated two different cDNA species, it appears that there are two genes that either have the same restriction pattern for the enzymes used or are arranged in tandem. Upon rescreening the cDNA library with the 1.9-kb clone, we found pcVV4A and pcVV4B inserts in a 5:1 ratio. Therefore, both genes are either coregulated but expressed at different levels or are differentially regulated. It is conceivable that one of them would respond to internal signals (during development—e.g., ripening), whereas the other one would be triggered by external stimuli (stress factors). In that case, pcVV4A could be corresponding to the latter, since peptides recovered from an ACC synthase preparation from LiCl-treated wounded tomatoes are 100% homologous to its deduced amino acid sequence. RNA gel blot analysis indicates the existence of a 1.9-kb messenger, the same size as was found for the zucchini mRNA (10). The message was undetectable in green tomato, weakly represented in pink tissue, and highly prevalent in pink superinduced tissue. In normal ripening tomato fruit, the mRNA is estimated as low as 0.0001%.

The induction of the ACC synthase genes at various stages in plant development and by diverse external factors remains an intriguing question not only from the point of view of ethylene physiology but also as a model system for the study of transcriptional activation of a gene by various inducers. The cloning of the ACC synthase cDNAs will enable us to isolate the corresponding genes and resolve this question in detail. At the same time it will allow a better understanding of regulation of endogenous ethylene production on the molecular level and open the possibility of an improved control over ripening and senescence of fruits, vegetables, and cut flowers.

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