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A functional tomato ACC synthase expressed in *Escherichia coli* demonstrates suicidal inactivation by its substrate *S*-adenosylmethionine*

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1-Aminocyclopropane-1-carboxylate (ACC) synthase is a key enzyme in the biosynthesis of the plant hormone, ethylene. We have isolated, sequenced and expressed a functional tomato (cv Pik-Red) ACC synthase gene in *Escherichia coli*. ACC synthase expressed in *E. coli* was inactivated by incubation with *S*-adenosylmethionine (SAM), the half-time of which was concentration dependent. Mixing the tomato fruit protein extract with the cell-free extract from transformed *E. coli* did not affect SAM-dependent inactivation of ACC synthase activity. Thus, single isoforms of the ACC synthase enzyme, which demonstrate the biochemical features expected of the tomato fruit enzyme, can be expressed in *E. coli* and their structure–function relationships investigated.

ACC synthase; Gene expression; *Lycopersicon esculentum*; Enzyme inactivation

1. INTRODUCTION

Ethylene is a gaseous plant hormone that influences several aspects of plant growth, development and senescence [1–3]. A major rate-limiting step in the biosynthesis of ethylene is the conversion of *S*-adenosylmethionine (SAM[2]) to 1-aminocyclopropane-1-carboxylic acid (ACC), catalyzed by ACC synthase [3]. ACC synthase is a highly regulated enzyme both in vivo (see [1] and references therein) and in vitro. It is highly labile and undergoes suicidal inactivation by its substrate, SAM, during catalysis [4].

The identification of multiple ACC synthase genes from tomato [5,6] and two from zucchini [7] corroborated previous findings on the existence of various isoforms of ACC synthase [8,9]. Thus, the existence of multiple gene families and expression of multiple ACC synthase isoforms question the validity of earlier results obtained with either crude plant extracts or partially purified enzyme preparations. This is so particularly in regard to the biochemical parameters studied, such as the affinity constants for substrates, K_i's for inhibitors and half-life of the enzyme in vivo and in vitro, properties that must be re-examined for each isoform. At the very best, the earlier data represent average values for a mixture of ACC synthases. The approach of expression of individual genes in a prokaryotic organism provides a unique opportunity to study each isoform independent of one another.

A functional zucchini ACC synthase gene was expressed both in yeast and *E. coli* [10]. However, in the case of tomato ACC synthase gene expression in *E. coli*, one report found the expressed gene product non-functional [11] while a second, very recent report obtained a functional enzyme [5]. We have employed RNA-based polymerase chain reaction (PCR) and isolated full length ACC synthase gene from a different tomato cultivar. This gene is 99.99% homologous to the wound-inducible ACC synthase. The PCR-generated cDNAs were cloned, sequenced and expressed in *E. coli*. The recombinant *E. coli* was found to express ACC synthase activity. Further, this single ACC synthase isoform demonstrated substrate inactivation by SAM.

*The nucleotide sequence data on PikRed tomato ACC synthase can be accessed from the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number, X62536 (PRTOMACCS1).

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid; PCR, polymerase chain reaction; PLP, pyridoxal-5'-phosphate; SAM, *S*-adenosylmethionine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Polymerase chain reaction amplification, cloning and sequencing of the ACC synthase gene*

Two primers, (5'AAAAACC ATG GGA TTT GAG ATT GCA AAG ACC3') and (5'AACAACTATTTCTGAAAATACTCC-GGATCCTAC3'), corresponding to bases 146–177 and 1,739–1,706, respectively, of tomato cDNA clone pcVVA [11] were synthesized using a DNA Synthesizer (Applied Biosystem model 380A). Nucleotides, AA (151–152) in the upstream primer and AG (1,710–1,709) in the downstream primer, were substituted with CC to create *Nco*I and

Table 1

Expression of wound-inducible tomato ACC synthase in *E. coli*

Clone	Insert orientation	Insert size (kb)	ACC content		ACC synthase activity (nmol · h^{-1} · mg^{-1})
			Extracellular ACC (nmol · ml^{-1})	Intracellular ACC (nmol · 10^9 $cells^{-1}$)	
pTACC-A2	Sense	1.6	79.25 ± 6.25	9.25 ± 0.25	46.67 ± 3.89
pTACC-B1	Sense	1.6	73.25 ± 1.25	9.25 ± 0.25	63.53 ± 2.28
pTACC-C7	Anti-sense	1.6	0	0	0.329 ± 0.03

*Bam*HI sites, respectively, to facilitate sub-cloning. RNA-based polymerase chain reactions (PCRs) were carried out with 8 μg of total RNA under conditions described in the manufacturer's protocols (Perkin Elmer Cetus, Norwalk, CT), for 30 cycles of 94°C (1 min), 55°C (1 min), and 72°C (2 min). The PCR products were ligated to plasmid vector pCR1000 and transformed in *E. coli* INVαF' (Invitrogen Corp., San Diego, CA). ACC synthase clones were sequenced using the double-stranded DNA template with a sequenase version 2.0 (United States Biochemical Corporation, Cleveland, Ohio) and [^{35}S]dATP (Amersham, Arlington Heights, IL) following the manufacturer's protocols.

2.2. *Preparation of* E. coli *and tomato cell-free extracts and determination of ACC content and ACC synthase activity*

INVαF' *E. coli* (Invitrogen Corp., San Diego, CA) cells, containing the chimeric pCR1000 DNA plasmid harboring the ACC synthase gene, were grown overnight in 5 ml of SOC medium with 50 μg/ml of kanamycin at 37°C on a shaker, then transferred to 200 ml of fresh SOC/kanamycin medium, and the growth allowed to continue until the cell density (OD_{600}) reached 0.9 (close to the end of the exponential growth phase). The cells were then harvested by centrifugation at 4,000 × *g* for 5 min and washed once with buffer A (100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid (EPPS), pH 8.0, 4 mM DTT, 10 μM pyridoxal-5' phosphate (PLP), 20 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). The first supernatant was saved and an aliquot (0.1 ml) assayed for extracellular ACC content [12]. The washed cells were re-centrifuged, the pellets frozen at −70°C for 1 h and then resuspended in 5 ml of buffer A. The cell suspension was sonicated 8 times for 30 s at 1 min intervals. Aliquots (0.1 ml) of this cell homogenate were assayed for intracellular ACC content, and the remaining portion was centrifuged at 4,000 × *g* for 10 min. The supernatants were gel-filtered (G-25 Sephadex; 30-ml column) and then used for ACC synthase activity and [^{14}C]SAM labeling studies. Protein content was quantified using the Rose Bengal method [13].

Tomato (*Lycopersicon esculentum* cv. Pik-Red) extracts were prepared from ripe pericarp tissue as described previously [14] and assayed for ACC synthase activity as described [8].

2.3. *Labeling of ACC Synthase with* S*-adenosylmethionine*

Cell extracts (~15 mg/1.5 ml), prepared as described above, were diluted with 1 vol. of 2 × reaction buffer (100 mM EPPS, pH 8.5; 10 μM PLP) and incubated in the presence of 53 or 96 μM *S*-adenosyl-L-[3,4-^{14}C]methionine (Research Products International Corp., Mount Prospect, IL) at 30°C for 23 h with constant shaking. Following radiolabeling of proteins, the unincorporated [^{14}C]SAM was separated from the proteins by a passage through a 3-kDa cutoff size Centicon-3 filter (Milipore, Bedford, MA).

2.4. *SDS-PAGE and fluorography*

Proteins were solubilized in sample application buffer, fractionated by SDS-PAGE using a 3% stacking and 7.5–15% gradient resolving gel, and then stained with Coomassie brilliant blue, as previously described [15]. The gels were fluorographed with EnHance (DuPont, Boston, MA), dried and exposed to Kodak XAR-5 X-ray film at −70°C for 7–21 days.

3. RESULTS AND DISCUSSION

3.1. *ACC synthase cDNA clone identity*

ACC synthase cDNAs were identified by hybridization to an oligonucleotide probe specific to ACC synthase [16]. The complete nucleotide sequence of the Pik-Red tomato ACC synthase showed only two changes when compared to the cDNA sequence of ACC synthase from the Mill cultivar [11]: a T for a C at position 972 and a C for a T at position 1,203. These two nucleotide changes in the cDNA sequence result in changes in the deduced amino acid sequence: Leu^{322} and Pro^{399}

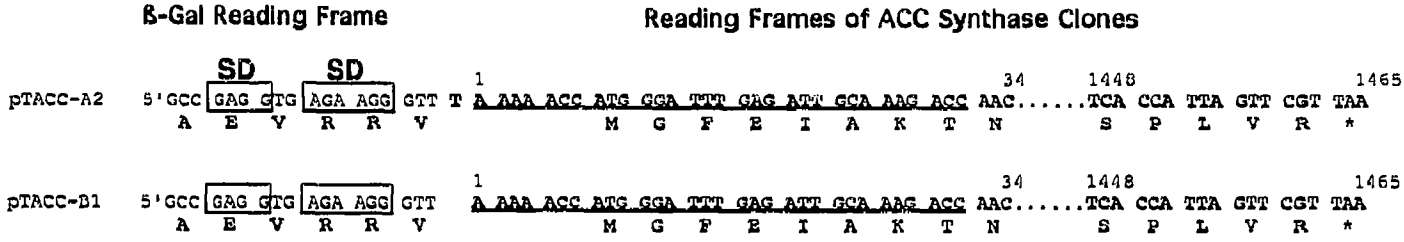

Fig. 1. Nucleotide sequences at the 5' end of the vector/insert junction in different cDNA clones. The reading frame of β-galactosidase encoded by the vector and that of the ACC synthase cDNA clones are shown as single letters below the nucleotide sequences. Shine–Dalgarno (SD) sequences in the vector are bordered. Double digestion of pTACC-B1 plasmid DNA was carried out in the presence of *Nur*I and *Bam*HI restriction enzymes. The digests were treated with Mungbean nuclease and then re-ligated. Asterisks represent the stop codon. In pTACC-A2 clone, an extra T was introduced between the insert and the vector due to double adenylation of PCR-amplified DNA fragment by Amplitaq DNA polymerase.

in the Pik-Red ACC synthase are Pro^{322} and Leu^{399} of Mill ACC synthase, confirming identical alterations found in Rutgers ACC synthase [5].

3.2. *Expression of functional ACC synthase in* Escherichia coli

We cloned PCR-amplified ACC synthase cDNAs, in sense as well as antisense orientations, into a pCR1000 cloning vector (Invitrogen Corp., San Diego, CA) in which constitutive transcription of the introduced gene fragment was driven by a LacZ promoter in a host cell (INVαF') with an inactivated LacI repressor. Successful expression was quantified by assaying for extracellular and intracellular contents of ACC, a product of ACC synthase activity.

Results (Table I) indicated that when the ACC synthase open reading frame is in the same direction as the LacZ transcription (correct orientation = sense direction), ACC was produced in *E. coli* cultures both intracellularly and extracellularly (pTACC-A2 and pTACC-B1). On the other hand, when the ACC synthase open reading frame was in the opposite direction (antisense) to LacZ transcription (pTACC-C7), ACC was not detected either in the cells or in the medium (Table I). The accumulation of ACC in pTACC-A2 and pTACC-B1 correlated with high specific activity of ACC synthase in their cell extracts, while in the control pTACC-C7 ACC synthase was barely detectable (Table 1).

To determine if the enzymatically active ACC synthase expressed in *E. coli* resulted from a fusion with β-galactosidase, the nucleotide sequence at the vicinity of the 5′ junction site of the insert with the vector was determined for both pTACC-A2 and pTACC-B1. In both cases, β-galactosidase and ACC synthase were found not to be in the same reading frame (Fig. 1). These sequence data suggested that the enzymatically active ACC synthase was not a fusion protein, implying that a full-length polypeptide of 53.4 kDa (deduced from the open reading frame) should be found in the transformed *E. coli* cells.

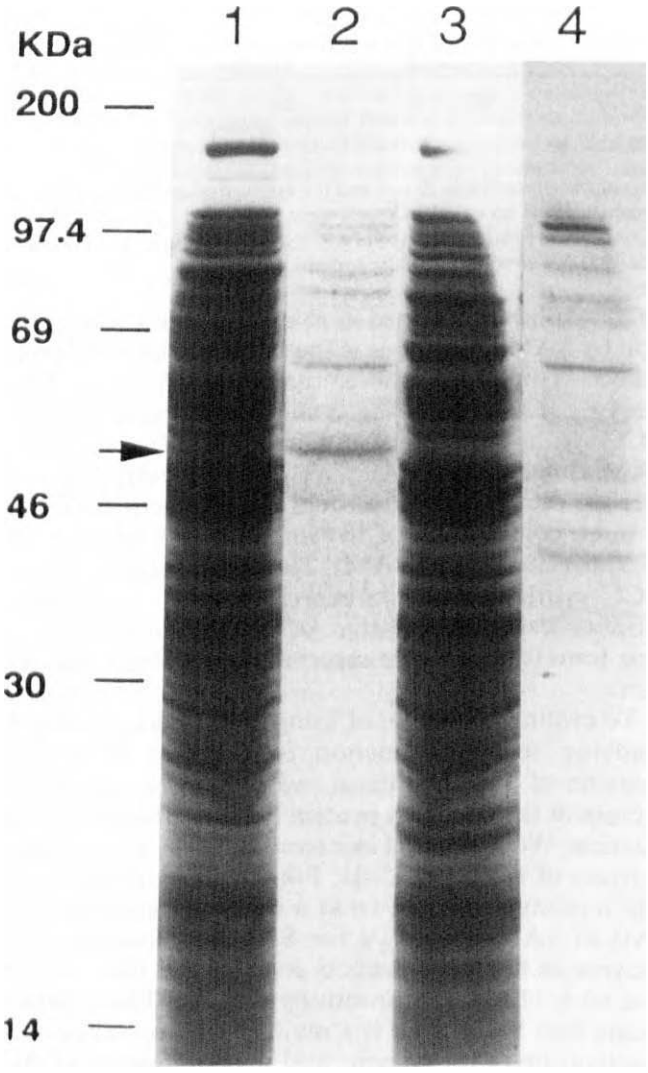

Fig. 2. *S*-Adenosyl-[3,4^{14}C]methionine labeling of ACC synthase expressed in *E. coli*. Cell extracts from pTACC-A2 clone (lanes 1 and 2) or from pTACC-7C (lanes 3 and 4) were fractionated on 7.5–15% gradient SDS polyacrylamide gels, stained with Coomassie brilliant blue and fluorographed. Lanes 2 and 4 are the respective fluorographs of lanes 1 and 3 which show the Coomassie-stained protein patterns. Samples were loaded on the gels on an equal protein basis (200 μg). The [^{14}C]SAM concentrations used for labeling proteins in extracts prepared from pTACC-A2 and pTACC-C7 clones were 53 and 96 μM, respectively. Arrow indicates the radiolabeled ACC synthase protein band.

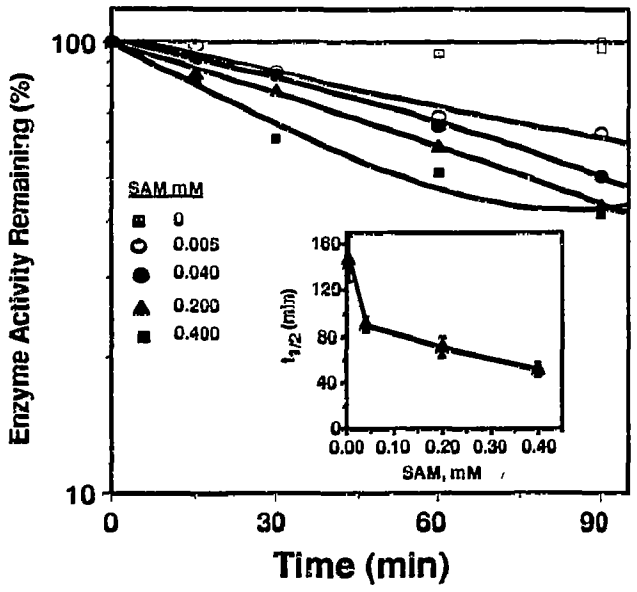

Fig. 3. Concentration dependence of SAM-mediated inactivation of the ACC synthase expressed in *E. coli*. Cell extracts of the pTACC-B1 clone were incubated with 0, 5, 40, 200 or 400 μM SAM for 15, 30, 60 and 90 min as indicated. Aliquots were then gel-filtered and assayed for the remaining ACC synthase activity. From the semi-log plot, half times ($t_{1/2}$) were calculated and plotted against the SAM concentrations (inset). ACC synthase activity in the untreated controls was 2.3 μmol·ACC·h^{-1}.

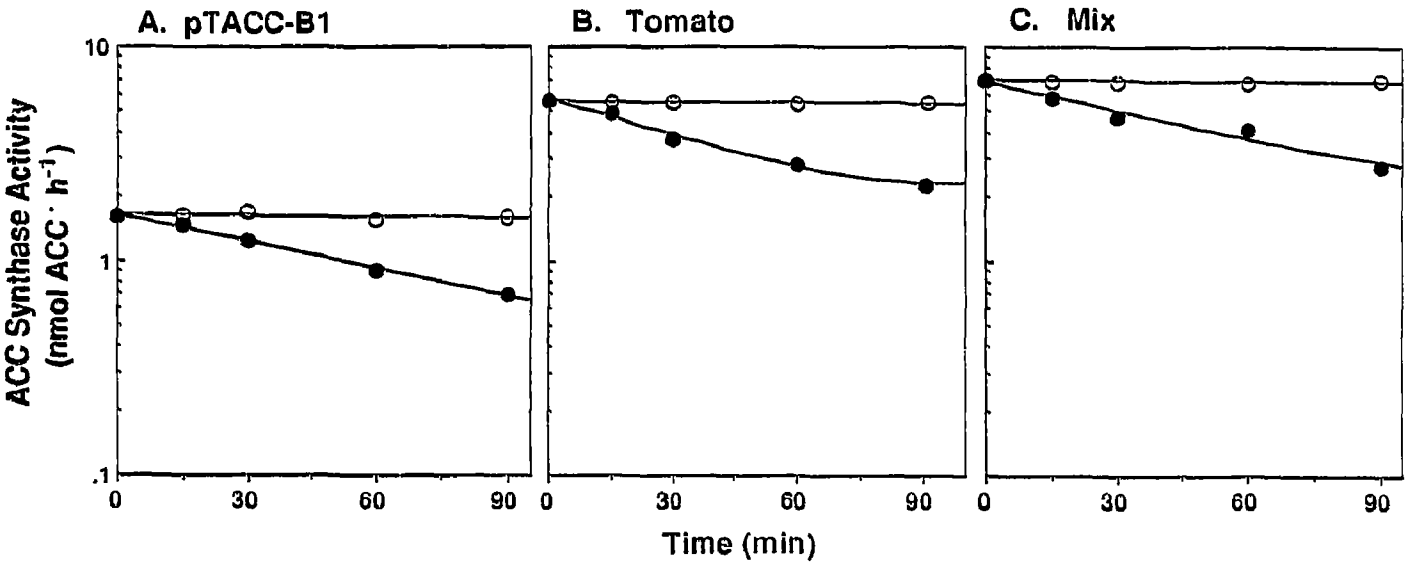

Fig. 4. Comparative inactivation kinetics of native tomato ACC synthase, ACC synthase expressed in *E. coli* and the mixture of the two. Cell extracts of the pTACC-B1 clone (pTACC-B1) and red tomato fruit (Tomato) by themselves, or as an equal mixture, were incubated with 200 μM SAM for the indicated times. Aliquots were removed, gel-filtered and assayed for remaining ACC synthase activity. ACC synthase activity in the untreated controls was 1.6 μmol·ACC·h^{-1} for the pTACC-B1 clone and 5.6 μmol·h^{-1} for the tomato extract.

ACC synthase has been previously radiolabeled with [^{14}C-Met] SAM [4] via 'trans-Schiffization' [17]. Fig. 2 is a composite of SDS-PAGE gels showing that indeed a radiolabeled and Coomassie-stained 53-kDa polypeptide is present in the pTACC-A2 clone but not in the pTACC-C7 clone. These results are consistent with the data in Table I showing high expression of ACC synthase activity in the pTACC-A2, but not in pTACC-C7 clone. Many other polypeptides (ranging from 40 to 130 kDa) were also labeled by [^{14}C]SAM; however, these were labeled in both cell extracts independent of whether or not they produced ACC synthase. These bands represent either non-specific labeling or labeling of other SAM-utilizing/binding enzymes, such as SAM decarboxylase, SAM synthetase and others. The radiolabeled 53 kDa ACC synthase protein agrees well with the 53.4 kDa size predicted from the deduced amino acid sequence. Together, these data demonstrated that the ACC synthase expressed in *E. coli* is not a fusion product.

3.3. *ACC synthase expressed in* E. coli *is inactivated by SAM*

The successful expression of an enzymatically active tomato fruit ACC synthase in *E. coli* enabled us to further characterize this isolated gene product. First, we determined its saturation kinetics with respect to the primary substrate, SAM. Clear hyperbolic kinetics were observed that yielded a K_m (for SAM) of 42 μM at pH 8.3 by regression analysis of the Lineweaver–Burk plot (data not shown). This value is within the range of affinity constants reported for the partially purified preparations of tomato ACC synthase [3].

We also examined if the isolated tomato isozyme of ACC synthase expressed in *E. coli* is subject to inactivation by SAM (Fig. 3), as is the case with enzyme preparations containing more than one ACC synthase isozyme. The inset in Fig. 3 shows a biphasic nature of ACC synthase inactivation kinetics as a function of the SAM concentration. As the SAM concentration is increased the enzyme undergoes faster inactivation (Fig. 3, inset; compare $t_{1/2}$ of 147 min at 5 μM SAM to that of 52 min at 400 μM SAM). These data suggest that the ACC synthase isozyme expressed in *E. coli* behaves more or less like the native ACC synthase enzyme(s) of ripe tomato fruit in its response to substrate inactivation.

To evaluate the merit of using the bacterial system for studying structure–function relationship of a single isozyme of ACC synthase, we sought to see if other factors in the bacterial protein extract affected its inactivation. We compared inactivation of ACC synthase in extracts of the pTACC-B1, Pik-Red tomato fruit tissue and a mixture of the two at a fixed concentration (200 μM) of SAM. The $t_{1/2}$'s for SAM inactivation of the enzyme in the three extracts were 74.3 ± 18.8, 58 ± 8.6 and 60 ± 11.0 min, respectively (Fig. 4). These data indicate that SAM itself is a major factor involved in the inactivation of the enzyme and that expression of ACC synthase in *E. coli* does not impair or enhance that property.

Indirect evidence has suggested that normal turnover of ACC synthase in vivo is more complex than the in vitro inactivation of the enzyme by SAM [18]. However, no direct in vivo studies have been conducted to test if SAM inactivation of ACC synthase occurs in vivo. The deduced amino acid sequence of the Pik-Red ACC synthase when compared to ACC synthases sequenced thus

far using the program Key bank 7.1 (Intelligenetic Suite 5.4) revealed conservation of casein kinase II phosphorylation site (S(F)ND; aa 292–295). Interestingly, phosphorylation of ACC synthase has recently been observed (Boller, T., personal communication). Phosphorylation of proteins can result in charge heterogeneity and mobility differences on gel eletrophoresis [19] and references therein). Possibly, phosphorylation of ACC synthase could contribute to the existence of its different pI forms. Alternatively, phosphorylation–dephosphorylation may be involved in the inactivation/ activation of the enzyme in vivo. These possibilities await further experimentation.

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