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Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (*Lycopersicon esculentum*)

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Abstract

The key enzyme of ethylene biosynthesis, ACC synthase, is encoded by a multigene family. We describe three new DNA sequences encoding members of the ACC synthase family of the tomato. One of these sequences encodes a novel ACC synthase, *LE-ACS6*, which is phylogenetically related to the ACC synthases *LE-ACS1A* and *LE-ACS1B*. Gene-specific probes for seven tomato ACC synthase genes were prepared. They were used for RNase protection assays to study the accumulation of ACC synthase transcripts in suspension-cultured tomato cells after the addition of an elicitor.

The ACC synthase genes *LE-ACS2*, *LE-ACS5* and *LE-ACS6* were strongly induced by the elicitor. In contrast, the genes *LE-ACS1B*, *LE-ACS3* and *LE-ACS4* were constitutively expressed and *LE-ACS1B* was present at all times at a particularly high level. Thus, there are two groups of ACC synthase transcripts expressed in these cells, either elicitor-induced or constitutive. A transcript of *LE-ACS1A* was not detected. Despite the presence of *LE-ACS1B*, *LE-ACS2*, *LE-ACS3*, *LE-ACS4* and *LE-ACS5*, there was only little ethylene produced in the absence of the elicitor. Increased ethylene production is usually correlated with the accumulation of ACC synthase transcripts, indicating that ethylene production is controlled via the transcriptional activation of ACC synthase genes. However, the abundance of several ACC synthase mRNAs studied was not strictly correlated with the rate of elicitor-induced ethylene production. Our data provide evidence that the activity of these ACC synthases may not solely be controlled by the transcriptional activation of ACC synthase genes.

Introduction

The plant hormone ethylene controls many aspects of plant growth and development. Enhanced rates of ethylene production are observed during germination, flower development, pollination, leaf and floral abscission and fruit ripening. Enhanced rates of ethylene production are also observed when plants are subjected to various biotic or abiotic stresses, such as mechanical strain, wounding, hypoxia and flooding, chilling, soil salinity and infection by pathogens [40, 41]. While the phenomenon of enhanced ethylene production as a response to stresses has been observed many times, the function of stress ethylene production is elusive [1, 6]. Ethylene is synthesized from *S*-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC) [2]. The rate limiting step in ethylene production is ACC synthase (ACS), which is encoded by a multigene family [34]. In most cases, enhanced ethylene production rates appear correlated with enhanced ACS transcription [16]. However, transcription of ACS genes may not be the only factor regulating the produc-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U74458 (active-site center from *LE-ACS1A*, U74460 (pPCROR6, active-site center from *LE-ACS1B*); M38822, the (*LE-ACS5* active-site center from plasmid pBTAS3), U74461 (pPCROR2, active-site center from *LE-ACS6*); U74459, (pJO101A), U75692, (pJO101B) and U74462 (pJO105).

tion of ACC; regulatory mechanisms at the posttranscriptional and posttranslational level may be equally important. These could act at the level of mRNA splicing, the control of translation or by modifications of the native ACS protein, including C- or N-terminal processing and covalent modifications such as phosphorylation or alkylation. To various degrees, there is experimental evidence that these posttranscriptional mechanisms of control apply to ACC synthase. For instance, the accumulation of unspliced ACS transcripts was observed under stress conditions, a phenomenon well known from other plant genes that may regulate the availability of translatable ACS mRNA [25]. Indirect evidence for post-transcriptional control of ACC synthase came from two stress ethylene systems, in fruits [38, 39] and in suspension cultures [7, 12]. The basic observation of both lines of research was that inhibitors of translation prevented a stress-induced rise of ACC synthase activity while inhibitors of RNA synthesis did not. Post-translational modification in the form of C-terminal processing of ACC synthase was observed by several groups and modification of its C-terminus can lead to hyperactive forms [19, 23, 28, 36]. ACC synthase has a short half-life [17, 18] and part of this may be due to the mechanism-based inactivation of ACC synthase: an elimination reaction of its substrate, *S*-adenosyl-L-methionine leads to the formation of L-vinylglycine, causing alkylation and inactivation of the enzyme about once in 30 000 turnovers [29]. Apart from this mechanism-based inactivation, the half-life of ACC synthase appears under control of ATP-dependent processes, and evidence for this was provided by work of two groups [18, 32, 33], the consensus being that an ATP-dependent and/or a phosphorylation-event controls the rapid inactivation or degradation of ACC synthase *in vivo*.

In the tomato, six members of the ACC synthase multigene family are known and genomic clones have been characterized of *LE-ACS2*, *LE-ACS3* and *LE-ACS4* [22, 25, 28]. A partial cDNA sequence is known for the ACC synthase *LE-ACS5* [42] and deduced amino acid sequences but no DNA sequences were described of *LE-ACS1A* and *LE-ACS1B* [28]. While nothing is known of the expression of *LE-ACS1A* and *LE-ACS1B*, the genes *LE-ACS2* and *LE-ACS4* are responsible for the burst of ethylene production during the ripening of the tomato fruit [20, 22, 24, 26, 28, 37, 42]. It appears that *LE-ACS2* is the ACS gene mainly responsible for the production of stress-ethylene because its mRNA has been detected at a relatively high level after wounding, pathogen attack, in senescent flowers and flooded tomato roots and in elicited tomato suspension cultures [20, 25, 26, 31, 37]. The transcripts of *LE-ACS3* and *LE-ACS5* are also present in suspension cultures [31, 42].

A prerequisite for a better understanding of the biological significance and the relative contribution of the various control mechanisms of ACC synthase to the overall production of ethylene *in vivo* is to isolate complete sets of the members of the ACC synthase gene family, to investigate their expression pattern and to assign the ethylene-inducing stimuli to individual ACC synthase isoforms. When the accumulation of ACS transcripts is found not to be correlated with the ACC synthase activity or the rate of ethylene production, then these observations may indicate that ethylene synthesis is under post-transcriptional rather than transcriptional control of ACC synthase.

Towards this goal we describe, in this paper, the isolation of further cDNA fragments for tomato ACC synthase genes, the isolation of one corresponding genomic clone and the preparation of gene specific RNase protection assay (RPA) probes for seven ACC synthase genes. We used these to study whether or not the accumulation pattern of ACC synthase transcripts is correlated with the rise of elicitor-induced ethylene production in suspension cultures.

A diverse range of compounds such as monoamines, cell wall fragments and peptides are known to elicit ethylene synthesis in suspension cultures [8, 11, 13, 35]. As an elicitor, we prepared a partially purified fraction from yeast extract which contains yeast elicitor (YE), i.e. glucans with terminal 3-, 6- and 3,6-linked glycosyl residues and small glycopeptides [5, 14]. YE has been shown to effectively stimulate ethylene production of suspension cultures and to induce the ACC synthase genes *LE-ACS2* and *LE-ACS5* [12, 31]. We show that additional tomato ACC synthase transcripts are present in suspension-cultured tomato cells and identify the expression kinetics of *LE-ACS1B*, *LE-ACS3* and *LE-ACS4* as not correlated with the production of elicitor-induced ethylene synthesis.

Materials and methods

Plant material

We utilized a tomato cv. VFNT (cherry tomato) suspension culture that was originally initiated from tomato calyx tissue (from Betty Ishida, Agricultural Research Service, United States Department of Agriculture,

Albany, CA). The suspension was maintained in medium with Murashige and Skoog salts, 3% (w/v) sucrose, 0.5 μM benzyladenine and 9.0 μM 2,4-D, at a pH of 5.8. Before the experiments, the cells were transferred to fresh media with 2,4-D reduced to 0.9 μM and used after 6 days.

Preparation of elicitor

An elicitor-containing crude fraction was prepared from yeast lysate extract, essentially according the initial purification steps of two previously reported procedures [5, 14]. 450 g of yeast extract (Difco, Detroit) were dissolved by heating in 1000 ml water and centrifuged at 7000 $\times$ *g* for 15 min. The supernatant was adjusted to 80% ethanol (v/v) and centrifuged at 7000 $\times$ *g* for 15 min. The supernatant was discarded and the pellet, a gummy precipitate, redissolved in 800 ml H_2O. Ethanol was added to a final concentration of 60% (v/v) and the solution incubated at 4 °C overnight. After centrifugation at 7000 $\times$ *g* for 15 min, the pellet was discarded, the supernatant adjusted to 80% (v/v) ethanol and reprecipitated. After centrifugation at 7000 $\times$ *g* for 15 min, the pellet was redissolved in 100 ml of H_2O and 50 ml of that were dialyzed at 1 °C against 5 $\times$ 5 l of water at 4 °C for 8 days, using a Spectrapor dialysis membrane with a molecular cutoff of 3500 Da. The dialyzed fraction was centrifuged for 10 min at 13 000 $\times$ *g* and the supernatant was lyophilized. This powder was used as the partially purified yeast elicitor (YE) at a concentration of 200 μg/ml.

Measurement of ethylene

For ethylene measurement, 10 ml of the suspension culture, 6 days after subculturing, was transferred to 30 ml Erlenmeyer flasks. The flasks were sealed with rubber caps and after 20 min, 1 ml gas samples were withdrawn and used for ethylene measurement with a gas chromatograph. The suspension culture cells were filtered off and weighed after the C_2H_4 measurements.

RNA extraction

Total RNA was isolated from 5 g of suspension culture cells that were frozen in liquid N_2 and pulverized to a fine powder in a coffee grinder. The powdered tissues were homogenized in an equal volume of a 9:1 phenol/cresol mixture containing 5% triisopropylnaphthalene sulfonic acid and 1% 4-aminosalicylic acid, using a Tissuemizer (Tekmar) for 2 min at top speed. The homogenate was centrifuged at 25 000 $\times$ *g* for 30 min, and the total nucleic acids were precipitated from the aqueous supernatant overnight at -20 °C with 0.3 M sodium acetate (pH 6.0) and 1 volume of 2-propanol. The pellet was resuspended and contaminating carbohydrates were removed by precipitation with LiCl and if necessary, with an additional precipitation in 30% ice-cold ethanol.

Reverse transcriptase PCR and PCR

Five μg of total RNA were annealed with 1 μg of oligo-dT in 0.3 M NaCl, 3 mM EDTA, 10 mM Tris-HCl, pH 7.5, in the presence of 1 μl of RNasin (Promega) for 30 min at 50 °C. The mixture was transferred to 42 °C and adjusted to 1 mM of each nucleotide, 1 mM DTT, 10 mM Tris-HCl pH 8.4, 6 mM $MgCl_2$, 60 μg/ml actinomycin D in a final volume of 40 μl. Two μl of AMV reverse transcriptase were added and the mixture was incubated for 2 h at 42 °C. The nucleic acids were precipitated, resuspended in 250 μl water and 5 μl of this were used for PCR. PCR was performed using *Taq* polymerase according to the manufacturers specifications (Perkin-Elmer Cetus). For reverse transcriptase PCR we used the degenerate primers A and B (Table 1) and an annealing temperature of 42 °C. All the other oligonucleotides used in this study are also listed in Table 1. Primers H through N were chosen based on the published genomic sequences [22, 25, 28].

Genomic cloning

A library constructed from VFN8 tomato genomic DNA cloned into λ-EMBL3 was from Clontech (Palo Alto, CA). Plating, plaque lifting, and filter hybridization methods were essentially as described [3]. The probes used in screening were the ^{32}P-labelled inserts of plasmids pPCROR6 and pPCROR2, containing the reaction centers of *LE-ACS1B* and *LE-ACS6*, respectively.

Cloning and DNA sequencing

All DNA manipulations were performed using standard methods [3]. DNA fragments were ligated either in vector pKSII+ (Stratagene) or TA-cloned in pCRII (Invitrogen). Dideoxy sequencing of double-stranded DNA was performed with universal and synthetic primers using ^{35}S-dATP and the modified T7 DNA polymerase, Sequenase, according to the manufacturer's instructions (US Biochemicals). DNA

Table 1. Oligonucleotide primers.

	Name	DNA sequence	Gene
A	OLSACS1F	aycewtswaatccaytrggnac	degenerate
B	OLSACS1R	acwarnccraarctngacat	degenerate
C	XLEACS1A3F	gc(ctcgag)actcaccagctcactctccg	*LE-ACS1A*
D	XLEACS1A3R	gc(tctaga)ttatttttctttcatcagtttgcaca	*LE-ACS1A*
E	XLEACS1B3F	gc(ctcgag)gcctttggttaggacataaagatg	*LE-ACS1B*
F	XLEACS1B3R	gc(tctaga)ccaaaatggaatgaattgtataatca	*LE-ACS1B*
G	OLS1BR1	gtatccgaaatggttgag	*LE-ACS1B*
H	XLEACS23F	gc(ctcgag)gcaacaatggaagaagaataatttg	*LE-ACS2*
I	XLEACS23R	gc(tctaga)tatgatgtctaagtacatagaccagttgtc	*LE-ACS2*
K	XLEACS33F	gc(ctcgag)ggatcatcatgccattgtac	*LE-ACS3*
L	XLEACS33R	gc(tctaga)atggacagagtgcaccctct	*LE-ACS3*
M	XLEACS43F	gc(ctcgag)gagatcgcacttgcaaggat	*LE-ACS4*
N	XLEACS43R	gc(tctaga)caagctttataactttatttgattgtac	*LE-ACS4*
O	XLEACS53F	gc(ctcgag)agacctcgcgatgcaaag	*LE-ACS5*
P	XLEACS53R	gc(tctaga)tctcaacaactccctctgattg	*LE-ACS5*
Q	XLEACS63F	gc(ctcgag)aagattcatcaatgacaaaaacataca	*LE-ACS6*
R	XLEACS63R	gc(tctaga)gcatccaacaacttcactcttg	*LE-ACS6*

sequences were analyzed with the sequence analysis software packages of the Genetics Computer Group (University of Wisconsin) and PcGene.

RNase protection assay

For RPA assay we used plasmids pJO101A, pJO101B, pJO102, pJO103, pJO104, pJO105, and pJO106, corresponding to the tomato ACC synthases *LE-ACS1A*, *LE-ACS1B*, *LE-ACS2*, *LE-ACS3*, *LE-ACS4*, *LE-ACS5* and *LE-ACS6*, respectively. The plasmids were linearized with appropriate restriction enzymes that produce 5′-protruding ends and purified by agarose gel electrophoresis. The templates pJO105 and pJO106 were transcribed by SP6 RNA polymerase in the presence of 1 mM spermidine at 1 °C overnight, the remaining templates by T7 RNA polymerase at 22 °C for 2 h. To assure the RPA probes produce equal signal intensities despite their different uracil contents, the [α-^{32}P]-UTP (3000 Ci/mmol) used for radiolabelling was diluted with unlabeled UTP to specific activities inversely proportional to the uracil content of each transcript. The antisense transcripts were purified by 8 M urea/5% polyacrylamide gel electrophoresis before they were used for the assays, each in at least a 4-fold molar excess. Hybridization was performed for 10 min at 67 °C in Hyb-speed RPA assay buffer (Ambion, Austin, TX). The hybridization products were digested with a mixture of ribonucleases A and T1. To gain a high resolution of the products, they were separated on a 8 M urea/5% polyacrylamide 0.4 mm thick and 40 cm long DNA-sequencing gel which was run for 2 h and 40 min at 90 W. Prior to autoradiography, the dried polyacrylamide gels were analyzed on a Fuji BAS1000 phosphorimager (Fuji Medical Systems, Stamford, CT) and for quantification the integ-

rated optical density of the radioactive RNA bands was determined by the Sigmascan image analysis program.

Results

Isolation of ACC synthases

In a search for novel transcripts of tomato ACC synthase genes we reverse-transcribed RNA from tomato roots [25]. We used the degenerate primers A and B which correspond to the conserved Blocks 4 and 6 of ACC synthase (Figure 2A) [10]. These blocks are flanking Block 5 which encodes the active center of ACC synthase [43]. The DNA sequences of the resultant PCR products predominantely corresponded to the genes *LE-ACS2* and *LE-ACS3* which were already known and are expressed in tomato roots [25]. However, three cDNA sequences were novel, two PCR products represented the same gene, a novel ACC synthase which we named *LE-ACS6*; the third product corresponded to the gene *LE-ACS1B*, which was previously described, but only as a deduced amino acid sequence [28]. Between Blocks 4 and 6 the amino acid sequence of *LE-ACS1B* is highly similar to the tomato ACC synthase *LE-ACS1A*, with the exception of only three amino acids [28]. The genes encoding them are closely linked on chromosome 8, convergently oriented and separated by a 2 kb intergenic region [28]. To obtain a DNA probe for *LE-ACS1A* and to isolate genomic clones for *LE-ACS6*, *LE-ACS1A* and *LE-ACS1B* we screened 500 000 plaques from a λ-EMBL3 tomato genomic library (Clontech) with the inserts of plasmids pPCROR6 and pPCROR2 as the DNA probes for *LE-ACS1B* and *LE-ACS6*. We were unable to isolate genomic clones for *LE-ACS6*, but Southern analysis of 2 of the 6 positively hybridizing phage λ clones with the pPCROR6 probe revealed 2 distinct bands, indicating the presence of two highly similar sequences at molecular sizes of 4.8 kb and 10 kb. To investigate more closely these hybridizing sequences, DNA of this phage λ-clone, designated λ-LEACS1A/B, was amplified with primers A and B. Sequence analysis of the PCR products confirmed the Southern analysis and showed that it contained the active site centers of *LE-ACS1A* and of *LE-ACS1B*. Their DNA sequences are 95% identical and 73% identical to *LE-ACS6* (Figure 1A).

With members as similar as *LE-ACS1A* and *LE-ACS1B*, it appeared logical to construct gene-specific probes from the 3′-ends to study gene expression, a strategy that previously proved useful to distinguish highly similar tomato ACC synthase and oxidase transcripts [4, 26, 28]. To isolate the 3′-ends of *LE-ACS1A* and *LE-ACS1B* we amplified λ-LEACS1A/B DNA with primer G which, apart from two nucleotides, consists of a DNA sequence common to the active-site centers of both *LE-ACS1A* and *LE-ACS1B*. If successful, this should amplify a stretch of ca. 3.4 kb containing parts of the active-site centers of *LE-ACS1A* and *LE-ACS1B*, the 3′ ends of their coding regions, their 3′-UTRs and the intergenic region. However, the PCR produced two bands of 1.3 and 0.9 kb (data not shown) which were partially sequenced. In both cases, the primer annealed at the expected 5′ positions in the active-site centers, but at two different positions in the intergenic region, 3′ to the presumptive polyadenylation signals of either gene, artefactously, yet producing the required PCR products. The deduced amino acid sequences of the coding regions of both plasmids were identical to the previously reported ones [28].

Preparation of RPA probes

3′ to the region encoding amino acid Block 7 we detected stretches of DNA sequences of *LE-ACS1A* and *LE-ACS1B* that were not highly homologous. In this area, we selected the amplimers C/D and E/F to obtain gene-specific RPA assay probes for *LE-ACS1A* and *LE-ACS1B*, resulting in plasmids pJO101A and pJO101B, respectively. Similarly, we selected regions for the preparation of probes for the other tomato ACS genes (Figure 2A). For *LE-ACS2* and *LE-ACS4*, we amplified genomic tomato DNA with primers H/I and M/N, respectively. A probe for *LE-ACS3* was obtained by amplification of pGTAS2R2 [25] with amplimer K/L, and the probe for *LE-ACS5*, pJO105, was constructed by PCR amplification of a genomic *LE-ACS5* clone, with the primers O and P. Since we did not know the 3′-end of *LE-ACS6*, we chose to amplify 113 nt flanking the active-site center which were the most divergent from *LE-ACS1A* and *LE-ACS1B* with amplimer Q/R, resulting in pJO106. To provide the later option of combining several RPA probes in a single tube assay [27], the amplimers were chosen to yield products of different molecular sizes (Figure 2B). *In vitro* transcription of these RPA assay probes should yield RNA molecules of the following native (n) and expected protected (p) nucleotide sizes: pJO101A (*LE-ACS1A*, 249n, 200p); pJO101B (*LE-ACS1B*, 207n, 158p); pJO102 (*LE-ACS2*, 331n, 282p); pJO103 (*LE-ACS3*, 427n, 378p); pJO104 (*LE-*

Figure 1. A. DNA sequence alignment of the ACC synthases *LE-ACS1A*, *LE-ACS1B* and *LE-ACS6* flanking the active-site center. ∗ indicates sequence identity and • indicates sequence similarity. B. Amino acid alignment of the known tomato ACC synthases in the region between the conserved blocks 4 to 6. The conserved amino acid blocks are shaded. Sequences that correspond to the minor active-site peptide isolated from the tomato fruit [43] are underlined. The sequences are from *LE-ACS1A* and *LE-ACS1B* (this work), *LE-ACS2* [37], *LE-ACS3* [25], *LE-ACS4* [26], and *LE-ACS5* [42] (deduced from GenBank accession number M38844) and *LE-ACS6*.

ACS4, 288n, 239p); pJO105 (*LE-ACS5*, 560n, 422p) and pJO106 (*LE-ACS6*, 223n, 113p). The authenticity of all probes was confirmed by DNA sequencing in both orientations.

Expression pattern of ACC synthases

To study the expression pattern of these ACC synthase genes in a stress-ethylene model system, we treated 6-day old suspension cultures with the yeast elicitor (YE) and observed a strong stimulation of ethylene biosynthesis (Figure 3). Increased rate of ethylene production was observed after 20 min and the ethylene continued to increase over 15 nl g^{-1} h^{-1}, 2 h after the treatment with elicitor. Ethylene production of non-treated cells remained at the basal level of around 0.2 nl g^{-1} h^{-1}. To determine whether the induction of ethylene biosynthesis is correlated with the induction of specific ACC synthase transcripts, RNA was analyzed from cells treated for various times with the elicitor.

While *LE-ACS1A* was not detectable at any time (Figure 4A), *LE-ACS1B* was strongly expressed (Figure 4B). *LE-ACS1B* was enhanced by addition of the elicitor and remained high throughout the experiment. The transcript of *LE-ACS2* was near to the detection limit without the addition of the elicitor (Figure 4C). However, it was strongly inducible and reached a maximum after 2 h. There was a second protected fragment, ca. 20 nt shorter than the expected fragment at 282 nt and less intense. At all times, the abundance of this second fragment was proportional to the band at 282 nt. This second band is likely to reflect heterogeneity in termination of the mRNA, ie. the same transcript but with a 3′-untranslated region 20 nt shorter than expected. Six different cDNA clones of *LE-ACS2* transcripts have been isolated with 3′ ends and poly(A) tails beginning at six different positions, dispersed over a range of 75 nt [28]. Our probe ends 18 nt before the 3′ end of the shortest known *LE-ACS2* transcript. The banding pattern in our RPA assays indicates that in suspension-cultured tomato cells there are even shorter forms of this transcript. Further, the intensities of these two bands suggest that the two forms of the *LE-ACS2* transcript occur in a ratio of ca. 2:1. *LE-ACS3* was constitutively expressed at a low level (Figure 4D). We detected five smaller than expected bands and estimated the larger two at sizes of 330 nt and 280 nt. While these could be due to heterogeneity of termination, we discount that possibility for the remaining three bands, all estimated smaller than 230 nt, because this is the

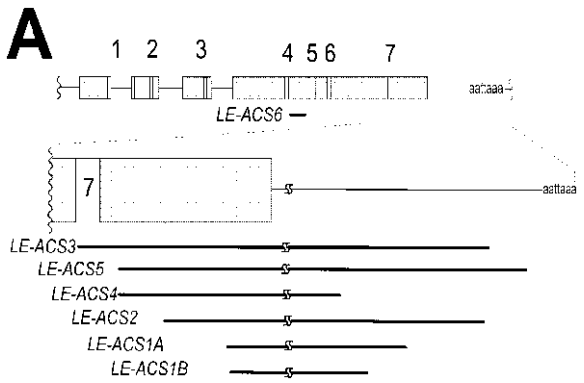

Figure 2. Seven gene-specific antisense-RNA probes for tomato ACC-synthase genes. A. General organization of an ACC synthase gene with 4 exons. Exons are depicted as shaded boxes, introns and untranslated regions as lines. The regions encoding the seven conserved amino acid boxes are shown as white boxes at their relative positions on the exons and numbered accordingly [10]. A putative polyadenylation signal is shown. The sizes and number of the introns are based on the *LE-ACS3* gene [25]. The extent of the probes with respect to block 7 and the putative polyadenylation sign are shown as fat lines under the enlargement. Breaks were inserted to represent the various lengths of the 3′-UTR of the ACC synthase genes. B. *In vitro* transcription products obtained from pJO101A (*LE-ACS1A*), pJO101B (*LE-ACS1B*), pJO102 (*LE-ACS2*), pJO103 (*LE-ACS3*), pJO104 (*LE-ACS4*), pJO105 (*LE-ACS5*) and pJO106 (*LE-ACS6*). The molecular sizes in nts from the native (n) and expected protected fragments (p) are shown. The antisense RNA molecules were labeled with ^{32}P-UTP, gel-purified after synthesis and 10^4 dpm each were separated on a 5% DNA sequencing gel. To compensate for different signal intensities due to the different length of these probes, the specific activity of the [α-^{32}P]-UTP used for radio labelling was adjusted to be inversely proportional to the uracil content of the probes.

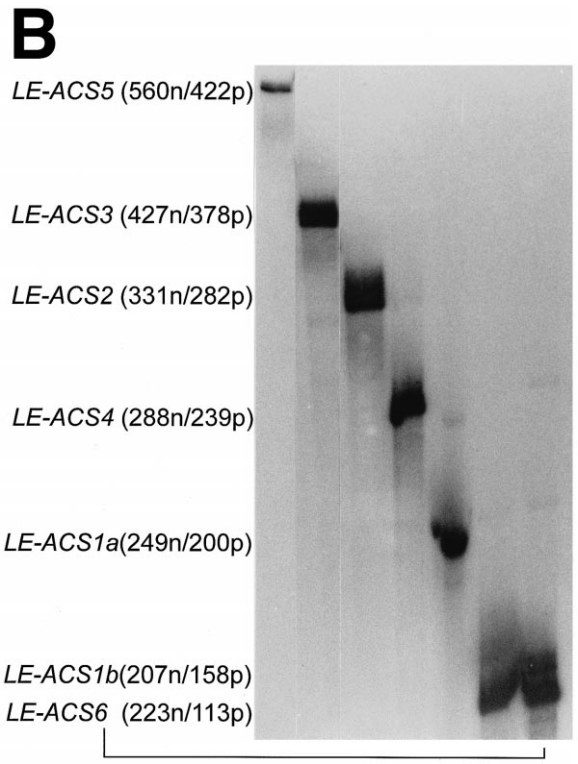

Figure 2. Continued.

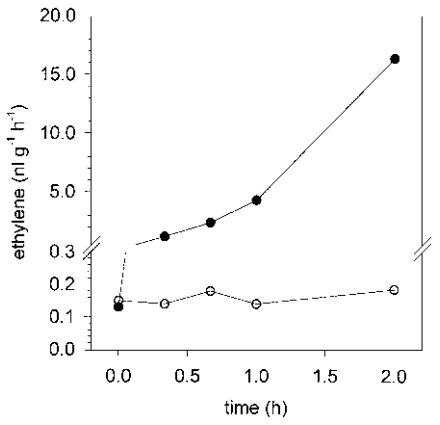

Figure 3. Elicitor-induced ethylene production by tomato suspension cultures. A VFTN cherry tomato culture was treated with 200 μg/ml of yeast elicitor. The rate of ethylene production was determined at various times after addition of the elicitor (●) or in untreated controls (○).

distance from the 5′ end of our probe to the stop codon of *LE-ACS3* (Figure 2A). Therefore, it appears more likely that they are due to the presence of similar RNAs such as unknown ACC synthase transcripts which can crossprotect small fragments of this *LE-ACS3* probe. *LE-ACS4* was at the limit of detection at time 0, slightly induced by the elicitor after 20 min and leveled off after 1 h (Figure 4E). Again, a smaller band was detectable that could be explained by an alternative 3′ end of this transcript, three of which have been cloned and sequenced [22]. *LE-ACS5* was present in the absence of elicitor at an amount similar to *LE-ACS2* and *LE-ACS3* in unelicited tomato cells (Figure 4F). *LE-ACS5* was strongly inducible and continued to increase over 2 h (Figure 4F). Very little *LE-ACS6* transcript was detectable at time 0, but it was induced by the elicitor, peaked at 1 h and then strongly decreased (Figure 4G).

Quantification of the expected protected radioactive RNA bands by the phosphorimager revealed two groups of expressed ACC synthases (Figure 5). The first group contains the genes *LE-ACS2*, *LE-ACS5* and *LE-ACS6* whose transcripts were stimulated tenfold or more by the elicitor (Figure 5A). Their change of abundance paralleled the increase of the rate of ethyl-

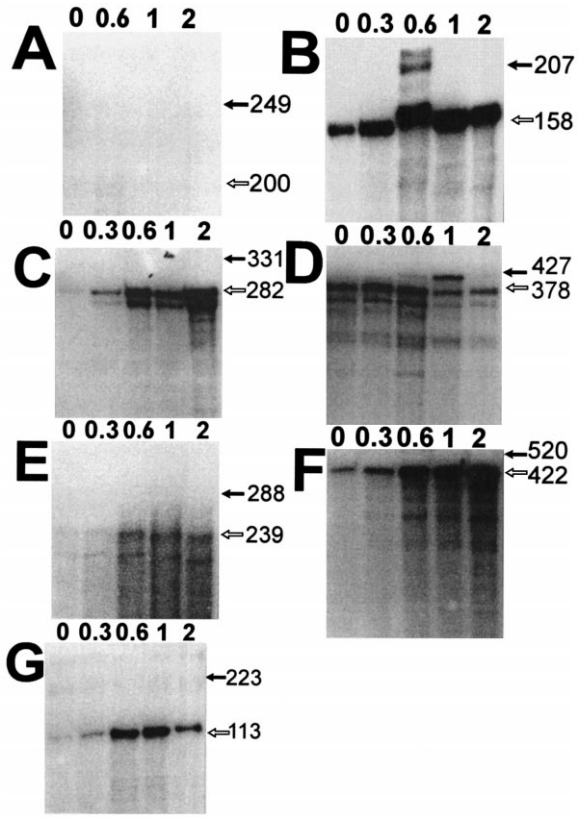

Figure 4. Expression pattern of seven tomato ACC synthase genes as a response to yeast elicitor. The panels are: A, *LE-ACS1A*; B, *LE-ACS1B*; C, *LE-ACS2*; D, *LE-ACS3*; E, *LE-ACS4*; F, *LE-ACS5*; G, *LE-ACS6*. 30 μg of total RNA were analyzed by means RPA assay, using the seven probes shown above. ←, indicates the size/position of the native RNA probe; ⇐, indicates the size/position expected for the protected fragment. Sizes were estimated either using the known sequences of the undigested RNA probes or an end-labelled single-stranded DNA marker. The times (hours) after the addition of the elicitor are shown above the panels. In several lanes there are also bands visible at the size of the undigested probe. They are artefacts, either due to the presence of low levels of residual template fragments that co-purified with and protect the undigested probe or due to low levels of promoter-independent transcription from the end of the probe template [30]. The band at 0.6 h in panel B migrated at a higher molecular weight, most likely due to the presence of residual RNase inactivation buffer.

ene production. The second group contains *LE-ACS1B*, *LE-ACS3* and *LE-ACS4*, whose transcripts were stimulated less than threefold after addition of the elicitor, i.e. they were only weakly induced by the elicitor and nearly constitutive (Figure 5B). Their change of abundance was not correlated with the increase of ethylene synthesis. The sum of all expressed ACC synthase transcripts increased ca. 3-fold during the experiment (Figure 5C).

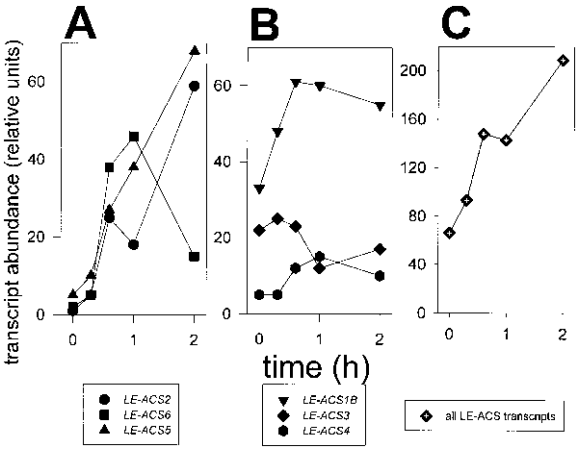

Figure 5. Quantification of ACC synthase transcript abundance. The signal intensities were determined by a phosphor imager and for quantification, the integrated optical densities of the radioactive RNA bands was determined by the Sigmascan image analysis program. The relative values for optical density obtained were divided by 10^6 and plotted. A, induced transcripts; B, nearly constitutive transcripts; C, sum of all ACC synthase transcripts.

The seven tomato ACC synthase genes investigated here are phylogenetically subdivided into three classes (Figure 6). The evolutionary trifurcation of the ACC synthase genes, prior to the evolution of dicots and monocots, was described earlier and it has been observed that in various plants the ACC synthase genes belonging to class III, but not those of class I and class II, are inducible by auxin [21, 22]. This led to the proposal that the ACS genes of a class are coordinately regulated and that the regulatory networks controlling their expression coevolved with the development of this gene class. However, the inducibility by elicitor of the seven tomato ACS genes studied here is not correlated with their phylogenetic relationship because each class contains one member that is strongly inducible and at least one member that is not induced or constitutively expressed (Figure 6). This indicates that the signalling mechanisms of elicitation controlling the expression of these genes evolved independently of the genes themselves, which is evidence against the general validity of the theory of Liang *et al*. [21].

Discussion

We described three novel tomato ACC synthase DNA sequences, the isolation of a genomic clone of *LE-ACS1A* and *LE-ACS1B* and the preparation of seven gene specific probes to distinguish these homologous

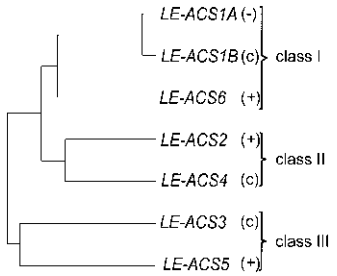

Figure 6. The phylogenetic relationship of seven tomato ACC synthase genes. The phylogenetic tree was constructed using the program Clustal and DNA sequences encoding the polypeptide between the conserved amino acid blocks 4 and 6, including the active-site center, Block 5 (see [10] for nomenclature). Symbols following the ACC synthase genes are: +, inducible by yeast elicitor; −, not inducible by yeast elicitor; c, constitutively expressed.

ACC synthase transcripts. We used the probes to investigate the expression pattern of the ACC synthase genes as a response to a yeast-derived elicitor in suspension-cultured tomato cells. Six of the seven ACC synthase genes investigated were expressed, three were strongly inducible and three were nearly constitutive. The most abundant transcripts were the constitutive *LE-ACS1B* and the inducible *LE-ACS5*. The induction pattern of the ACC synthase transcripts was not related to their phylogenetic relationship.

To analyze the expression pattern of a large multigene family, the ability to specifically distinguish its members is essential. One good test for this ability is if highly similar transcripts can be distinguished in complex RNA preparations. The most similar transcripts of the tomato ACC synthase gene family are *LE-ACS1A* and *LE-ASC1B* (Figure 6). The data of Figure 4A and B show that the probes for these genes do not crossprotect and are specific. Similarly, this appears also true for the probe of *LE-ACS6* which does not crossprotect *LE-ACS1B* (Figure 4B and 4G) and for the probes corresponding to *LE-ACS2* and *LE-ACS4* (Figure 4C and 4E). We conclude that we can specifically distinguish these tomato ACC synthase transcripts. These probes may be of further use to investigate the regulation of *LE-ACS1A* and *LE-ACS1B*, which like the zucchini ACC synthases *CP-ACS1A* and *CP-ACS1B* are convergently oriented twin genes, each pair with virtual sequence identity, all four belonging to the same phylogenetic class and none of them with the structural characteristics of a pseudogene [15, 28]. In contrast to *CP-ACS1A*, expression of its twin, *CP-ACS1B*, was never detected [15]. While here we report the first instance of expression of *LE-ACS1B*, we could not detect the transcript of its twin, *LE-ACS1A*. Should future studies reveal the existence of such twins in many plant species and that only one of them can be expressed, then comparison of their regulatory regions might become instructive of structural features that prevent the expression of an ACC synthase gene and this might have wider implications for genetic means to control ethylene synthesis. Twin ACC synthase genes were also discovered in the potato but these genes, namely *ST-ACS1A* and *ST-ACS1B*, belong to the phylogenetic class of *LE-ACS3* and are, unlike the twins of zucchini and tomato, divergently oriented and both expressed [9].

LE-ACS6 is a novel ACC synthase and its sequence is similar to *LE-ACS1A* and *LE-ACS1B*. The deduced amino acid sequence of *LE-ACS6* in the region of the active-site center (Figure 1B) is identical to the minor of two tryptic active-site peptides previously isolated from wounded ripe tomato fruit [43]. The major peptide corresponded to the transcript *LE-ACS2*, but the minor one did not correspond to the transcript of *LE-ACS4* which is also expressed in the tomato fruit. It was therefore suggested that there are at least three isoforms of ACC synthase in this tissue [26]. Subsequently, it appeared that *LE-ACS3* encodes that minor peptide because its transcript was detected in the fruit and its DNA sequence could account for that minor peptide [42]. However, *LE-ACS6* also could encode that minor peptide and, consequently, the expression pattern of the ACC synthase transcripts in the ripening tomato will need further examination. *LE-ACS6* and *LE-ACS1B* were cloned using RNA isolated from tomato roots, a tissue where *LE-ACS2* and *LE-ACS3* are also expressed [25]. These four genes are dispersed over all three phylogenetic classes of ACC synthase (Figure 6) and this corroborates our view that the mechanism of ACC synthase gene induction did not necessarily coevolve with any particular class of ACC synthase genes.

The suspension-cultured cells produced only low, basal levels of ethylene, but ethylene production was strongly induced by the elicitor. In YE-treated MSK8 tomato suspension cultures the induction of ACC synthase activity preceeds the induction of ACC oxidase and appears to be a limiting factor of ethylene synthesis [12]. If the VFNT suspension used here behaves similar and elevated ethylene synthesis is due to ACC synthase activity and increased transcription of ACC synthase genes, one would expect that the level of all ACS transcripts paralleled the induction of ethylene synthesis. While this is generally true for the genes in Figure 5A, it is not for *LE-ACS1B*, *LE-ACS3* and *LE-ACS4*. The sum of transcript abundance of all the expressed ACC

synthase transcripts revealed a net increase of only 3-fold during the course of the experiment while ethylene production increased more than 100-fold. Also, there were considerable amounts of several transcripts, particularly *LE-ACS1B* and *LE-ACS3*, in the absence of elicitor when the suspension culture produced only basal levels of ethylene. This may not surprise and explain the previous observation that transcriptional inhibitors do not inhibit elicitor induction of ACC synthase in tomato cells [12]. One possible explanation is that these transcripts encode ACC synthase isoforms of low catalytic efficiencies. Alternatively, the elicitor signal could activate these isoforms via increasing availability of translatable mRNA or via translational control mechanisms. It is also possible that the stability of these ACC synthases is altered by the addition the elicitor. In that context it is interesting that the addition of protein kinase inhibitors rapidly and completely inactivates the ACC synthase activity induced by the elicitor [33]. The next step towards understanding the relative contribution of these ACC synthase transcripts and possible post-transcriptional control mechanisms will require to study these isoforms individually.

The elicitor clearly induces several of the ACC transcripts investigated, but it is also true that their overall abundance is not strictly correlated with the production of elicitor-induced stress ethylene. Spanu *et al.* [33] have reported that levels of ACC synthase mRNA poorly correlate with rates of ACC synthase production in suspension-cultured tomato cells. However, their study of elicitor-induced accumulation of the transcripts *LE-ACS2* and *LE-ACS5* [31] revealed a good correlation with elicitor-induced induction of ACC synthase activity (Figures 2, 3A and 4A) and the work presented here supports that. While they did not investigate *LE-ACS1A*, *LE-ACS1B*, *LE-ACS3* and *LE-ACS6*, our expression data of the remaining ACC synthase genes differ from theirs in that they did not detect *LE-ACS4* nor the basal level of *LE-ACS2* and *LE-ACS5* in unelicited suspension cultures. This may reflect intrinsic properties of the MSK8 and the VFNT suspension cultures used. Alternatively, it might be due to the different techniques used: they employed northern blot analysis which is 10 to 100 times less sensitive than the RPA assay. The basal level of *LE-ACS2*, *LE-ACS3* and *LE-ACS5* transcripts was previously observed in the VFNT suspension culture used here [42]. In that study, the RPA assay probes harbored the active-site centers of the ACC synthases, between the conserved amino acid Blocks 4 and 6. Thus, they would not detect heterogeneity of the 3′-untranslated regions. Despite that and like the *LE-ACS3* probe used here, the probe harboring the active-site center of *LE-ACS3* produced multiple and shorter than expected protected bands (Figure 3B, lane 5 in [42]). In principle, such bands could also be due to differential processing of the primary transcript. However, the regions of both probes employed are not interrupted by any introns of the *LE-ACS3* gene [25]. Therefore, the smaller protected bands of both studies point towards the existence of further ACC synthases, similar in their sequence to *LE-ACS3* and expressed in suspension-cultured tomato cells.

In summary, we described novel tomato ACC synthase sequences and investigated the expression characteristics of seven tomato ACC synthase genes using elicited tomato cells as a model system for the production of stress ethylene. The expression kinetics of several ACC synthase mRNAs appears unrelated to the biosynthetic rate of ethylene and this provides further evidence that the activity of the key enzyme of ethylene synthesis, ACC synthase, is not solely controlled by the transcriptional activation of its genes.

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