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Expression and characterization of three tomato 1-aminocyclopropane-1-carboxylate oxidase cDNAs in yeast

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Heterologous expression in yeast has previously shown that the tomato cDNA *LE-ACO1* encodes a functional 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) protein [Hamilton, A. J., Bouzayen, M. & Grierson, D. (1991) *Proc. Natl Acad. Sci. USA* 88, 7434–7437]. In the present work, full-length cDNAs encoding the two other members of the tomato ACO family (*LE-ACO2* and *LE-ACO3*) were isolated and expressed in *Saccharomyces cerevisiae*. Analysis of the predicted amino acid sequences showed that the ACO1 and ACO3 proteins are highly similar (95%) while ACO2 is more divergent (89%). Yeast strains transformed with each of the three cDNAs were able to convert exogenous ACC to ethylene, the ACO1 strain exhibiting the highest activity *in vivo* and the ACO3 and ACO2 strains reaching 65% and 45% of ACO1 maximum activity, respectively. None of the ACO activities expressed in yeast required addition of ascorbate *in vivo*. ACO activities assayed *in vitro* revealed no significant differences between the three isoforms with regards to optimum temperature (29°C), optimum pH (6.8–7.2), absolute dependence for ascorbate, Fe²⁺ and carbon dioxide, and inhibition by iron-chelating agents (1,10-phenanthroline and EDTA), Co²⁺ and free-radical scavengers (*n*-propyl gallate). However, differences were detected in the apparent *K_m* values for ACC, the *pI* and the specific activity. The biochemical features that might explain the differences between the isoenzyme activities are discussed.

Keywords: ethylene; *Lycopersicon esculentum*; heterologous expression; isoform; *Saccharomyces cerevisiae*.

The gaseous plant hormone ethylene plays an important regulatory role in many aspects of plant growth, development and responses to environmental stresses. 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase (ACO) are the two key enzymes of the ethylene biosynthetic pathway (Fluhr and Mattoo, 1996). Genes encoding these enzymes have been isolated from different plant species, and it is well established that both ACC synthase and ACO (originally identified via the cDNA TOM13) are encoded by multigene families (Holdsworth et al., 1988; Tang et al., 1993; Zarembinsky and Theologis, 1994). The involvement of the TOM13 ripening-related cDNA in ethylene biosynthesis was first demonstrated through an RNA antisense strategy (Hamilton et al., 1990). Thereafter, functional expression of the TOM13 clone in heterologous systems revealed that it encodes ACO (Hamilton et al., 1991; Spanu et al., 1991). During fruit ripening ACC synthase has been assigned the major regulatory role for ethylene biosynthesis, whereas it was assumed that ACO genes were expressed constitutively. The differential expression of ACC synthase and ACO genes during

normal and abnormal plant development have been studied in the last few years. There is increasing evidence demonstrating that ACO genes are under strict regulation in many plant tissues, for example during fruit ripening (Grierson et al., 1992; Tang et al., 1994; Barry et al., 1996; Lasserre et al., 1996; Blume and Grierson, 1997), and regulation of ACO plays a critical role in the control of ethylene synthesis. Although all three tomato ACO genes are expressed at the mRNA level (Barry et al., 1996) a comparative biochemical characterization of the three ACO proteins is lacking.

Until the gene encoding ACO was discovered all attempts to obtain an enzymatically active acellular extract from plant tissues failed. By using the extraction procedure for flavanone-3-hydroxylase, the gene for which TOM13 shows the highest similarity, Ververidis and John (1991) found a reconstituted ACO activity in the soluble fraction of melon extracts. Subsequently, ACO has been extracted and purified from various fruit species and shown to be dependent on Fe²⁺ (Bouzayen et al., 1991; Ververidis and John, 1991) and activated by CO₂ (Dong et al., 1992; Smith and John, 1993; Pirrung et al., 1993). Since these breakthroughs, several studies have contributed to a better characterization of the enzyme activity (Prescott and John, 1996; John, 1997) and subcellular localization (Peck et al., 1992; Rombaldi et al., 1994; Reinhardt et al., 1994). However, many critical characteristics of ACO isoforms remain to be elucidated: do all three ACO genes encode functional proteins?; do individual ACO isoforms share common biochemical features?; is ascorbate a natural substrate for the three isoforms?; and do all three proteins show activation by CO₂?

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Abbreviations. ACC, 1-aminocyclopropane-1-carboxylic acid, ACO, 1-aminocyclopropane-1-carboxylate oxidase; *GraP-K*, phosphoglycerate kinase.

Note. S. Bidonde and M. A. Ferrer contributed equally to this work.

Note. The nucleotide and amino acid sequence data presented here have been submitted to the GenBank database and are available under accession numbers X58273 (ACO1), Y00478 (ACO2) and Z54199 (ACO3).

In this paper we describe the isolation and functional expression of the three tomato ACO cDNAs in *Sacharomyces cerevisiae*. The biochemical characterization of the ACO isoforms undertaken in this work was aimed at determining whether they exhibit similar properties and to understand whether they might play different roles or have distinct regulatory features.

MATERIALS AND METHODS

Materials. *Lycopersicon esculentum* Mill cv. Ailsa Craig plants were grown from seeds in growth rooms (16 h light, 24°C/8 h dark, 18°C) as described previously (Grierson et al., 1985). Mature green fruits were picked and treated with ethylene (20 µl/l) in sealed jars for 15 h. All restriction and modifying enzymes and chemicals were purchased from commercial sources. Radiolabeled nucleotides and amino acids were purchased from Amersham Corp.

Isolation and molecular cloning of ACO isoforms. ACO2 and ACO3 cDNAs were isolated by reverse transcription/PCR methods (Sambrook et al., 1989). Gene-specific primers encompassing the coding region of *LE-ACO2* and *LE-ACO3* genes (Holdsworth et al., 1988) were designed to amplify the corresponding cDNAs. ACO2-5'; AGA TCT ATG GAG AAT TTC CCA ATT ATC; ACO2-3'; AGA TCT TCC AAT TTT TTT AAT TTT TAA TC; ACO3-5'; AGA TCT ATG GAG AAC TTC CCA ATT ATC; ACO3-3'; AGA TCT CTT CCA AGA ACA TAA TAA TTT. *Bg*III restriction sites (underlined) were added to the 5' end of each primer. The amplified fragments were first digested with *Bg*III and then ligated into the *Bg*III site between the phosphoglycerate kinase (*GraP-K*) promoter and terminator of the yeast expression vector pBEJ15 (Hadfield et al., 1990). Sense orientation of the insert with respect to the *GraP-K* promoter was determined by restriction enzyme mapping. Expression of *LE-ACO1* in yeast has been described previously (Hamilton et al., 1991). The clones obtained for *LE-ACO1*, *LE-ACO2* and *LE-ACO3* were named pYACO1, pYACO2 and pYACO3, respectively.

Yeast transformation. *S. cerevisiae* strain JRY188 was transformed with pYACO1, pYACO2 and pYACO3 by using the lithium acetate method described by Carter et al. (1987). Selection of transformants on synthetic complete plates without leucine was based on the complementation of the *leu2* mutation in yeast by a functional gene on the pBEJ15 vector.

Culture conditions. Yeasts were grown on a shaker (300 rpm) at 30°C in a medium containing 0.65% yeast nitrogen base without amino acids (DIFCO), 2% glucose, 10 mg/l adenine, 10 mg/l methionine, 20 mg/l arginine, 20 mg/l histidine, 20 mg/l tryptophan, 10 mg/l uracil, 10 mg/l lysine, 50 mg/l tyrosine, 60 mg/l phenylalanine, 60 mg/l isoleucine and 50 mg/l threonine.

Nucleic acid analysis. After cloning and sequencing of ACO cDNA clones in pBEJ15, the predicted amino acid sequences were aligned using the PC GENE program. Specificity of ACO probes was tested by cross-hybridization in southern blot analysis. The ACO inserts were cut from cloning vectors by *Bg*III and hybridized with either ACO1, ACO2 or ACO3 probes after transfer on Genescreen hybridization membrane (Du Pont) according to the manufacturer's recommendations. Northern blot analysis were performed using yeast total RNA (15 µg) extracted as described previously (Hamilton et al., 1991), separated by agarose gel electrophoresis and transferred to Genescreen hybridization membranes following the supplier's instructions. The membranes were then hybridized with [α -³²P]CTP labeled ACO probes obtained with the Ready to Go system (Pharmacia Biotech Inc.).

Table 1. Comparison of the amino acid composition of the three ACO isoenzymes from tomato. The amino acid contents were determined from the predicted amino acid sequence. The theoretical isoelectric points of ACO1, ACO2 and ACO3 are 4.9, 5.6 and 5.0, respectively.

Amino acid	Number of residues in		
	ACO1	ACO2	ACO3
Ala	21	21	19
Arg	11	12	12
Asp or Asn	35	39	36
Cys	4	4	4
Glu or Gln	40	32	41
Gly	19	19	18
His	7	8	7
Ile	14	14	15
Leu	32	33	33
Lys	27	29	26
Met	12	14	12
Phe	15	15	15
Pro	16	17	17
Ser	15	13	15
Thr	13	10	11
Trp	3	3	3
Tyr	11	11	11
Val	20	20	21
Total	315	316	316
Positively charged	38	41	38
Negatively charged	51	48	50

Measurement of ACO activity in *S. cerevisiae*. Yeast transformed with the native vector pBEJ15 or with pYACO1, pYACO2 and pYACO3 vectors were grown in synthetic complete broth without leucine at 30°C under continuous agitation (300 rpm). Glucose contained in the culture medium induces activation of the *GraP-K* promoter of the pBEJ15 yeast expression vector containing the three ACO cDNAs. After inoculation into a fresh medium (5×10^6 cells/ml), the absorbance of the yeast culture was measured spectrophotometrically (600 nm) at intervals during a complete cycle of cell growth. Samples (1 ml) were incubated for 1 h at 30°C with 1 mM ACC in 7-ml sealed bottles. Gas samples were withdrawn from the headspace and the amount of ethylene produced was analysed by GC (Intersmat IGC 120 FB; 70°C) according to Bouzayen et al. (1990).

Radiolabeling of protein synthesised in yeast cells. Yeast lines were grown in synthetic complete medium without leucine and at a density of 5×10^6 cells/ml, L-[³⁵S]methionine (5 µCi) was added. At a density of 1.2×10^8 cells/ml, 1 ml cell culture was pelleted, suspended in 100 µl 62.5 mM Tris/HCl, pH 6.8; 10% (mass/vol.) glycerol, 2% SDS and 0.0125% bromophenol blue and boiled for 5 min. After separation on SDS/PAGE (12% acrylamide) proteins were fixed, stained and autoradiography was performed following the manufacturer's recommendations (EN³HANCER, DuPont).

Enzyme extraction. The yeast pellet obtained by centrifugation ($3000 \times g$, 15 min at 4°C) of 300 ml of yeast cell cultures taken at the exponential growth phase ($A_{600} = 1.60$; 1.2×10^8 cells/ml) was washed in cold distilled water, frozen in liquid nitrogen and ground to powder in a ball grinder. The powder was homogenized in 6 ml 50 mM Hepes pH 7.5, 10% (mass/vol.) glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 30 mM sodium ascorbate, 10 µg/ml leupeptin and 10 µg/ml pepstatin A. The slurry was centrifuged at $50000 \times g$ for 30 min at 4°C and the supernatant adjusted to 30% saturation with $(\text{NH}_4)_2\text{SO}_4$. After removal of the precipitated material by centrifugation, the supernatant was brought to 90% saturation

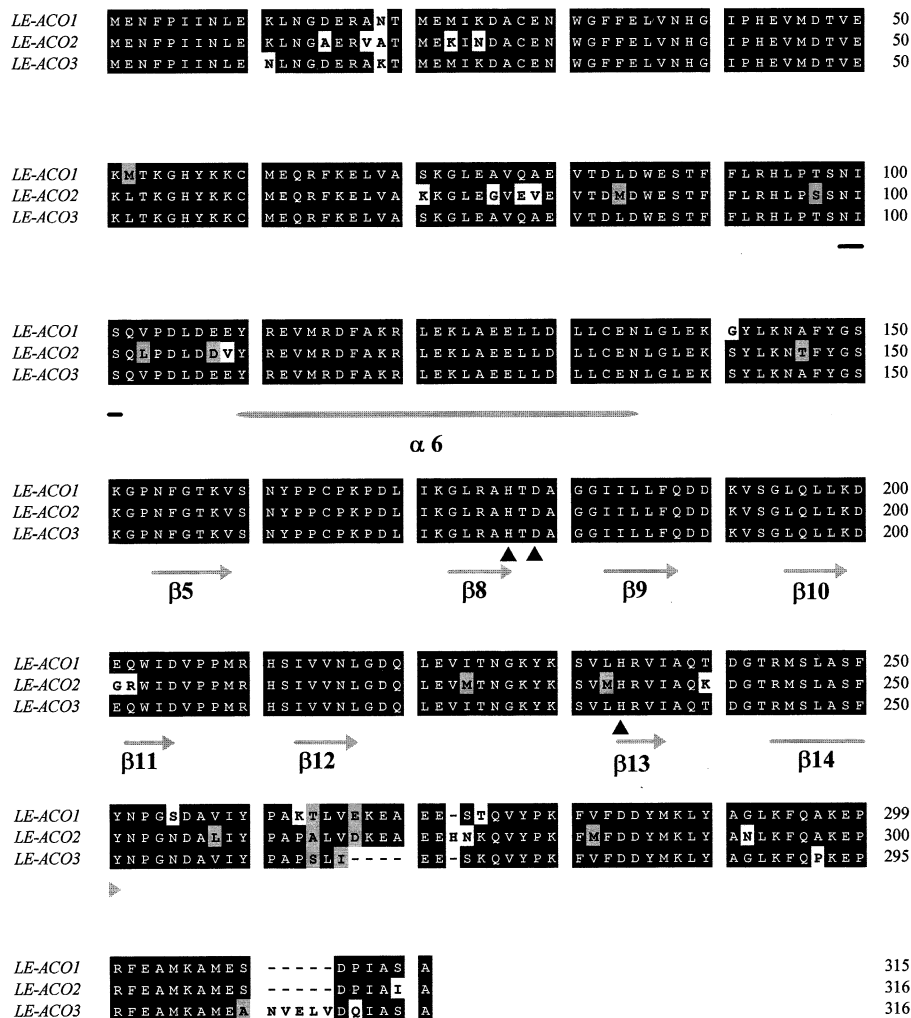


Fig. 1. Alignment of the deduced amino acid sequences of the three ACC oxidase isoforms from tomato. Identical and similar amino acid residues are shown on black and grey backgrounds, respectively. Amino acid deletions are indicated by -. The putative glycosylation site is underlined. Metal ligands are indicated by black triangles. The jelly-roll motif (β -strands 5 and 8–14, grey arrows) and the $\alpha 6$ helix (grey cylinder), which seems to be implicated in its stabilization, are indicated (Roach et al., 1995).

with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged for 20 min at $50000\times g$. The pellet was suspended in 5 ml 25 mM Hepes (pH 7.2), 10% (mass/vol.) glycerol and 1 mM dithiothreitol. ACO activity was measured immediately after desalting of a 2.5-ml aliquot on a PD 10 column (Sephadex G-25 M, Pharmacia).

Protein determination. Total protein content of each sample was determined using the bicinchoninic acid protein assay reagent (Pierce) according to the procedure described by the manufacturer. BSA was used as a standard.

Gel electrophoresis and western immunoblotting analysis. Protein samples were separated on one-dimensional 12% polyacrylamide gels according to the method of Laemmli (1970). Proteins were transferred to nitrocellulose sheets and ACO visualized with a goat anti-rabbit IgG conjugated with peroxidase according to the manufacturer's recommendations (enhanced chemiluminescence; Amersham Corp.).

Denaturing IEF. IEF of recombinant ACO in the pH range 4.5–7.5 was performed in 5% polyacrylamide gels containing 8 M urea and 1% ampholytes (BioRad) as described by Robertson et al. (1987).

Assay for *in vitro* ACO activity. The standard reaction mixture contained 100 μl of enzyme preparation, 10 mM NaHCO_3 , 10 μM Fe SO_4 , 3 mM sodium ascorbate, 1 mM ACC and 0.1 M Hepes, pH 7.2, 10% (mass/vol.) glycerol to 1 ml in a sealed

14-ml test tube. Incubation was carried out at 29°C for 15 min. Ethylene production was measured by GC as described above.

RESULTS

Isolation and structural analysis of three ACO cDNAs from tomato. The tomato gene *LE-ACO1* has been shown previously to encode a functional ACO protein by heterologous expression in yeast (Hamilton et al., 1991). To investigate whether the two remaining members of tomato ACO multigene family also encode functional proteins we have undertaken the isolation and expression of the corresponding cDNAs. Analysis of *LE-ACO2* and *LE-ACO3* genomic clones allowed us to design specific primers directed to the coding region for use in reverse-transcription/PCR amplification. *ACO2* and *ACO3* cDNAs were amplified successfully using total cDNAs from mature green fruit treated for 15 h with exogenous ethylene. In both cases, the 1-kb fragment amplified was consistent with the size of the coding region of the genes. Amplification from contaminating genomic DNA was ruled out since the size of *ACO2* and *ACO3* coding regions including the introns would be 2.5 kb and 1.6 kb, respectively. After cloning, the amplified fragments were sequenced fully and their complete identity with the corresponding genomic

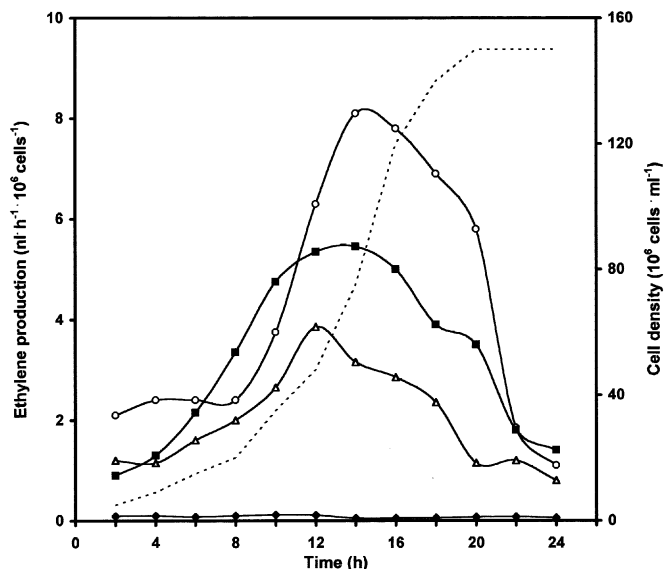


Fig. 2. Ethylene production by transformed *S. cerevisiae*. Yeast strains harboring pYACO1 (○), pYACO2 (△), pYACO3 (■) and pBEJ15 (◆) were grown in selective medium with shaking for 24 h (5×10^6 cells/ml) before determining ethylene production beginning at 0 h. At indicated times, 1 ml yeast culture was taken and its cell density was determined (---). To test the capacity of transformed yeast to convert ACC into ethylene 1 mM ACC was added to 1 ml cell suspension in 5-ml flasks. The amount of ethylene generated during 30 min at 30°C was measured by GC. Each measurement represents the mean of six replicates.

clones confirmed. Nucleotide sequence comparisons showed that *ACO1* and *ACO3* cDNAs share high similarity (95% identity) with each other and relatively less compared with *ACO2* (89% identity). Analysis of the predicted amino acid sequences revealed that all the cDNAs encode 36-kDa protein of 316 amino acids for *ACO2* and *ACO3* and 315 amino acids for *ACO1* (Table 1). Amino acid residue analysis revealed that the *ACO2* protein contains more positively charged groups, resulting in a higher predicted isoelectric point (Table 1). Optimal alignment showed that *ACO3* has a five-amino-acid deletion between positions 266 and 271 of *ACO1* and a five-amino-acid insertion at position 310. None of the three *ACO* isoforms has an obvious transit peptide or hydrophobic membrane-spanning domain, whereas all three proteins contain a potential glycosylation site (Fig. 1). Structural analysis indicated that all three *ACO* isoforms showed sequence conservation for the eight β -strands involved in the jelly-roll motif of isopenicillin N-synthase as deduced from the crystal structure of this protein (Roach et al., 1995). However, the *ACO2* protein presented amino acid variation within β -strand 11, where the glutamine at position 202 was replaced by an arginine residue (Fig. 1). Furthermore, the aspartate residue at position 201, in the direct vicinity of this β -strand, was changed to glycine. On the other hand, the longest α -helix (α_6) of isopenicillin N-synthase was conserved in the three *ACO* proteins (Fig. 1). His177, Asp179 and His234, which are assumed to be involved in binding Fe^{2+} (Shaw et al., 1996; Lay et al., 1996), were conserved in the three tomato *ACO* proteins.

Expression in *S. cerevisiae*. The three *ACO* coding sequences were cloned into the pBEJ15 yeast expression vector between the *GraP*-K promoter and terminator to generate pYACO1, pYACO2 and pYACO3 recombinant vectors, which were used subsequently for transformation of *S. cerevisiae*. Transformed yeast colonies were screened by PCR using specific primers for

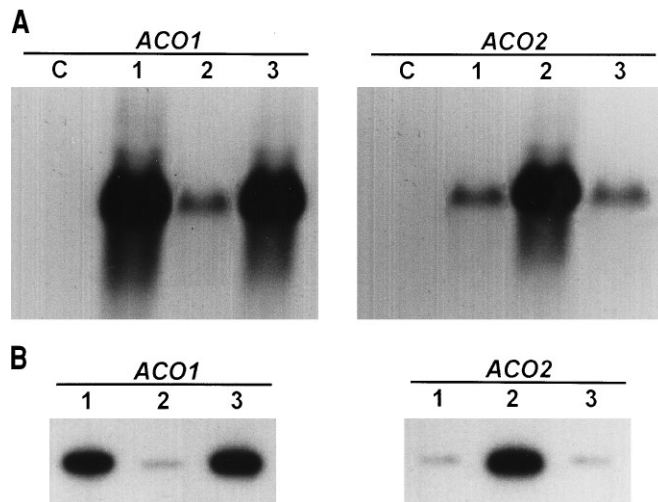


Fig. 3. RNA and DNA analysis. (A) *ACO* mRNA expression. Total RNA (15 μ g) from yeast lines transformed with pYACO1 (lane 1), pYACO2 (lane 2), pYACO3 (lane 3) or pBEJ15 vector (lane C) was separated on an agarose gel, transferred to nylon membrane and hybridized to *ACO1* or *ACO2* probes. (B) Specificity of the *ACO* probes. Cross-hybridization between *ACO* probes was analysed by Southern blotting. *ACO* cDNA inserts were hybridized either to *ACO1* or *ACO2* probes as described in Materials and Methods.

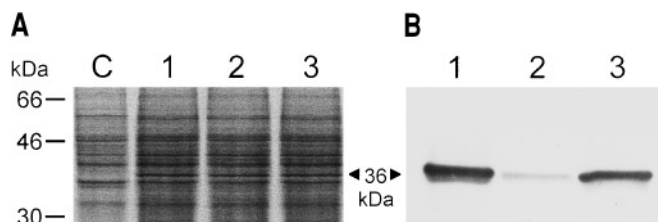


Fig. 4. Radiolabeling and immunoblotting of *ACO* proteins expressed in yeast. (A) SDS/PAGE analysis of total [^{35}S]Met proteins extracted by SDS and visualized by autoradiography. (B) Western blot of soluble proteins after ammonium sulphate precipitation (10 μ g/lane) immunodetected with antibodies to recombinant tomato *ACO1*. Positions of the markers are shown on the left. Lane C, proteins from yeast transformed with the pBEJ15 vector without insert; lanes 1–3, proteins from *ACO1*, *ACO2* and *ACO3* yeast clones. The arrowhead indicates the location of the *ACO* protein.

each *ACO* cDNA. Selected colonies harboring pYACO1, pYACO2 and pYACO3 clones were grown on liquid selective medium lacking leucine, and their capacity to convert ACC to ethylene was tested. The three strains of transformed yeast, *ACO1*, *ACO2* and *ACO3*, were able to convert exogenous ACC to ethylene without addition of cofactors to the culture medium while untransformed cells and yeast transformed with the native pBEJ15 vector were not. Moreover, when transformed and untransformed yeast cultures were not supplemented with exogenous ACC no ethylene production could be detected (data not shown). Ethylene production of all three *ACO* yeast increased during the exponential phase of cell growth and then declined rapidly during the stationary phase (Fig. 2). The *ACO1* line showed the highest activity and *ACO3* activity was slightly lower (65%) while *ACO2* reached less than 45% of the ethylene-producing capacity of the *ACO1* strain. To assess whether these differences in *ACO* activity were related to different levels of transcription or translation of *ACO* cDNAs in individual yeast strains, we performed northern blot analysis and L-[^{35}S]methionine-protein labeling experiments.

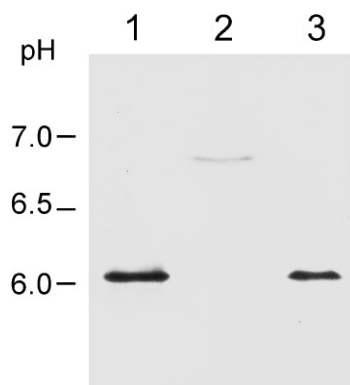


Fig. 5. Western blot of recombinant ACO isoforms separated by IEF. Protein extracts for ACO1 (lane 1, 10 µg), ACO2 (lane 2, 28 µg) and ACO3 (lane 3, 10 µg) clones were immunostained with antibodies raised against recombinant tomato ACO1.

Transcripts of the expected size (1.2 kb) were detected in all yeast lines transformed with pYACO1, pYACO2 and pYACO3, while no hybridization to an *ACO* probe could be detected in untransformed lines or in lines transformed with the pBEJ15 native vector (Fig. 3A). Moreover, *ACO* transcript accumulation was similar in the three transformed lines. The *ACO2* probe gave weak cross-hybridization with *ACO1* in northern and Southern analysis (Fig. 3B). By contrast, *ACO3* exhibited good recognition of *ACO1* but poor hybridization with *ACO2* (data not shown). To check whether the three ACO yeast strains accumulated the same levels of ACO proteins we have performed *in vivo* labeling experiments of total protein with L-[³⁵S]methionine. Autoradiography (Fig. 4A) showed that a protein with the expected size for ACO (36 kDa) accumulated in all three transformed yeast lines but not in yeast transformed with the pBEJ15 native vector. These data revealed no detectable differences in the amount of ACO protein accumulated in the three transformed yeast lines.

Immunoblotting of recombinant ACO isoforms. Western blotting (Fig. 4B) of total proteins precipitated by ammonium sulfate challenged with polyclonal antibodies raised against recombinant tomato ACO1 oxidase (Rombaldi et al., 1994) revealed a single band of 36 kDa. However, the immunostaining signal generated by the ACO2 protein was significantly weaker than those of ACO1 and ACO3, indicating poor recognition of the ACO2 isoform by the antibodies used in this experiment. Using either SDS or phenol for total protein extraction did not improve recognition of ACO2 protein by our antibodies (data not shown). The experimental determination of the isoelectric point (pI) performed by combining an IEF protein-separation step to subsequent western analysis (Fig. 5) indicated that the pI

Table 3. Apparent K_m values of ACO isoforms towards ACC in the presence and in the absence of saturating concentration of bicarbonate.

Isoforms	Apparent K_m	
	no added bicarbonate	10 mM bicarbonate
	μM	
ACO1	30 ^a	67
ACO2	12 ^b	29 ^a
ACO3	13 ^b	30 ^a

^a Not significantly different, $P = 0.05$.

^b Not significantly different, $P = 0.05$.

value for the ACO2 isoform (6.8) diverged significantly from those of ACO1 and ACO3 (6.1).

Biochemical properties of ACO activity *in vitro*. To establish the optimal assay conditions, the linearity of the ACO reaction with the incubation time was studied. The reaction rate decreased markedly for incubation times over 20 min (data not shown). All biochemical parameters were therefore determined by incubating the enzymes for no longer than 15 min. The involvement of an enzymatic mechanism for the conversion of ACC to ethylene in our yeast extract was demonstrated by the complete loss of activity upon heat treatment of the extracts (100°C, 5 min). Extracting and assaying the activities *in vitro* indicated an eight-fold and two-fold higher activity for ACO1 compared with ACO2 and ACO3, respectively (Table 2). The half-life for ACO2 activity *in vitro* (24 min) was slightly higher than for ACO1 and ACO3 (18 min).

Maximum activity for the three isoforms was found between 28°C and 30°C and half-maximal activity was reached at 40°C. ACO2 was the most sensitive to high-temperature inactivation since at 45°C the activity of this isoform underwent a 35-fold decrease, whereas ACO3 and ACO1 fell 4-fold and 7-fold, respectively. The pH dependence of ACO activity, determined within the pH range 5.8–8.0, indicated that the maximum activity for the three isoforms was pH 6.8–7.2.

The three ACO isoforms expressed in yeast showed an absolute requirement for ascorbate *in vitro*. The optimal concentration of ascorbate was 3 mM for the three isoforms and the enzyme activity declined about twofold at supraoptimal concentrations of ascorbate (20 mM). ACO isoforms also required Fe²⁺ for activity (Bouzayen et al., 1991), with maximum activity at 10 µM FeSO₄. Furthermore, the enzyme activity was almost completely abolished in the presence of iron-chelating agents such as 1,10-phenanthroline (10 µM) or EDTA (100 µM) (Table 2). The free-radical scavenger, *n*-propyl gallate (0.1 M), and

Table 2. Effect of different inhibitors on the *in vitro* activity of the three ACO isoforms of tomato. Enzyme activity was determined under standard conditions (see Materials and Methods) in the presence or absence of inhibitors. Data are means \pm SD of three replicates.

Inhibitor	ACO1 activity		ACO2 activity		ACO3 activity	
	nmol · h ⁻¹ · mg protein ⁻¹	%	nmol · h ⁻¹ · mg protein ⁻¹	%	nmol · h ⁻¹ · mg protein ⁻¹	%
None	25.1 \pm 2.1	100	2.9 \pm 0.6	100	11.5 \pm 2.4	100
1,10-Phenanthroline (10 µM)	0.05 \pm 0.00	0.2	0.03 \pm 0.02	1.0	0.12 \pm 0.02	1.0
EDTA (0.1 M)	0.80 \pm 0.02	3.2	0.06 \pm 0.00	2.0	0.30 \pm 0.01	2.6
<i>n</i> -Propyl gallate (0.1 M)	0.29 \pm 0.01	1.2	0.03 \pm 0.01	1.0	0.05 \pm 0.00	0.4
Co ²⁺ (10 µM)	0.75 \pm 0.06	3.0	0.11 \pm 0.03	3.8	0.12 \pm 0.09	1.0

CoCl₂ (10 μM) almost completely inhibited the *in vitro* activity of the three isoforms (Table 2).

ACO activity of the three isoforms was stimulated by addition of NaHCO₃ to the reaction medium and the maximum activity was attained at 10 mM. A high concentration of bicarbonate (100 mM) resulted in strong inhibition of the enzyme activity. The dependence of enzyme activity on its substrate, ACC, in the presence and in the absence of bicarbonate exhibited Michaelis-Menten kinetics. Table 3 shows the apparent K_m values calculated using the Sigma Plot Scientific Graphing Software (Jandel Scientific) for a hyperbolic curve. The apparent K_m for ACC in the absence of bicarbonate was twofold higher for ACO1 compared with ACO2 and ACO3. The addition of bicarbonate increased the apparent K_m approximately twofold with respect to ACC for all three isoforms (Table 3). Bicarbonate also stimulated the V_{max} for the three isoforms (data not shown).

DISCUSSION

We report here on the functional expression of the three tomato ACO cDNAs in *S. cerevisiae* and the biochemical analysis of the three isoforms. Full-length cDNAs corresponding to *LE-ACO2* and *LE-ACO3* genes were obtained by reverse-transcription/PCR of total RNA isolated from mature green fruit treated 15 h with exogenous ethylene. Analysis of the amino acid sequences indicated that none of the three ACOs has an obvious signal peptide that might allow the protein to cross the plasma membrane (Bouzayen et al., 1993). However, while their structure predicts a cytosolic location of the enzyme, previous data obtained by immunocytological techniques showed an apoplasmic localization of ACOs in tomato fruit (Rombaldi et al., 1994). Nucleotide sequence alignments indicated that all three cDNAs are closely related, although *ACO1* and *ACO3* share higher similarity between them than with *ACO2*. Surprisingly considering the high level of sequence identity, *ACO1* and *ACO3* probes showed only weak cross-hybridization with *ACO2* on Southern blots. Similarly, in northern blot analysis the *ACO2* probe only weakly detected *ACO1* and *ACO3* transcripts. Polyclonal antibodies raised against the ACO1 protein readily recognized the ACO3 protein but exhibited a very weak signal when challenged with the ACO2 isoform. These data suggest a distinctive structure for the ACO2 protein and indicate that information obtained from subcellular-localization studies with antibodies raised against ACO1 might be incomplete. The significantly divergent pI value revealed by IEF for ACO2 may not account for different subcellular localization. Nevertheless, the yeast strains expressing individual ACO isoforms generated in this study should be an interesting model for addressing the question of whether the three isoforms are targeted to the same cell compartment.

In vivo and *in vitro* studies indicated that ACO2 activity was significantly lower than those of ACO1 and ACO3. This feature raises the question of whether the differences in the protein sequences reflected differences in enzyme properties. We have demonstrated that the marked differences exhibited by the three isoforms *in vivo* cannot be ascribed to differences in transcription or translation levels. Extracting and assaying the enzyme activities *in vitro* confirmed the higher capacity of ACO1 to convert ACC to ethylene. The differential activity was even greater *in vitro* since ACO2 showed eight-fold lower activity than ACO1. The differential sensitivity of the isoforms to oxidative damage or proteolytic digestion, which could account for the differences in activity, is not supported by a number of arguments and observations. The same differential activity is found *in vivo*, where the three isoforms face the same background conditions. ACO2, which shows the lowest activity, presents the

highest half-life, suggesting that it is less prone to oxidative degradation. The potential for proteolytic digestion of ACO2 was minimized through the addition of three protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin and pepstatin A) in the extraction medium, and no shift in the molecular mass of the protein could be detected on SDS/PAGE. It is unlikely that differences in ACO activities are due to variable amounts of bicarbonate in the reaction medium since all enzyme activities were assayed in the presence of a saturating concentration of bicarbonate. In addition, the lower activity observed for ACO2 *in vitro* matches the differential activity *in vivo*, where no differences in bicarbonate concentrations are expected. The idea that the differential activity *in vivo* might be due to incorrect folding or lower stability in the yeast expression system, however, cannot be ruled out.

The biochemical properties of the three isoforms revealed little differences with regard to substrate and cofactor requirements and optimum pH and temperature (John, 1997). The only significant difference was found in the apparent K_m towards ACC. The lower apparent K_m value found for ACO2, however, cannot explain its lower activity. The absence of different biochemical properties is not surprising given the very weak divergence of amino acid sequence in the putative active site of the enzyme. Roach et al. (1995) have described the crystal structure of isopenicillin N-synthase and made sequence comparisons with ACO and other 2-oxo-acid-dependent oxygenases. These proteins present a conserved jelly-roll motif composed of β -strands harboring the active site of the enzyme. No amino acid divergence has been observed in the corresponding putative β -strands of the three tomato ACO (Fig. 1), except for ACO2 where a glutamine is replaced by an arginine residue on β -strand 11. The sequence is also very well conserved within the amino acids of the $\alpha 6$ helix, which seems to be involved in stabilization of the distorted jelly-roll motif. The putative metal ligand amino acids, His177 and His234 and Asp179 (Roach et al., 1995) are also present within the putative active site of the three tomato ACOs. Arg300, which has been suggested as a putative site for CO₂-dependent activation of ACO activity (Lay et al., 1996), is conserved in all three isoforms. This residue could be involved in the activation of the enzyme by CO₂ through a carbamylation process.

The biochemical properties of the three tomato ACO isoenzymes were similar to those described for ACOs either extracted from various plant sources (Smith et al., 1992; Dong et al., 1992; Kuai and Dilley, 1992; Dupille et al., 1993; Pirrung et al., 1993; Nijenhuis-De Vries et al., 1994; Moya-León and John, 1995) or recombinant proteins (McGarvey and Christoffersen, 1992; Zhang et al., 1995). One feature common to the three ACO isoforms is that none of them required the addition of ascorbate for *in vivo* activity, although the addition of ascorbate to the yeast incubation medium resulted in a twofold increase of ethylene production. By contrast, all three isoforms exhibit an absolute requirement for ascorbate *in vitro*. These data are consistent with the hypothesis of John (1995), who considered the possibility that another reductant might replace ascorbate *in vivo*. On the other hand, it cannot be excluded that yeast cells produce limited amounts of ascorbate sufficient for sustaining some ACO activity (Leung and Loewus, 1985; John, 1997).

While the present work allowed the functional expression of the three tomato ACO and their structural and biochemical characterization, some important questions remain to be elucidated: what is the structural basis for the differences in activity of the three isoforms?; if ascorbate is not absolutely required for *in vitro* activity what is the nature of the endogenous reductant?; and do the isoforms have different subcellular locations?

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