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Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast

(1-aminocyclopropane-1-carboxylic acid oxidase/*Lycopersicon esculentum*/*Saccharomyces cerevisiae*)

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ABSTRACT **The ethylene-forming enzyme (EFE), which catalyzes the last step in the biosynthesis of the plant hormone ethylene, has never been purified and no molecular probes are available. Recently, a putative cDNA clone for tomato EFE (pTOM13) has been identified by inhibiting ethylene synthesis with an antisense gene expressed in transgenic plants. A direct test of its function has been made by expression of a pTOM13 gene in *Saccharomyces cerevisiae*. After cloning artefacts were discovered in the 5′ region of the cDNA, a corrected cDNA (pRC13) was created by the fusion of the 5′ end of a genomic clone to the 3′ end of the cDNA and expressed in *S. cerevisiae*. Cultures of transformed yeast converted 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, whereas control cells did not. This EFE activity displays similar characteristics to EFE found in plant tissue: it converts the trans isomer of the ACC analogue 1-amino-2-ethylcyclopropane-1-carboxylic acid to 1-butene in preference to the cis isomer, and it is strongly inhibited by cobaltous ions and 1,10-phenanthroline. Furthermore, information gained from the activity of effectors on yeast EFE activity supports the hypothesis that EFE is one of a group of hydroxylase enzymes.**

Ethylene is a plant growth regulator important for fruit ripening, leaf and flower senescence, and responses to environmental stimuli such as mechanical wounding and invasion by pathogens (1, 2). The biosynthesis of ethylene in all these cases is by the same pathway, involving conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) and conversion of ACC to ethylene by the ethylene-forming enzyme (EFE) (2). Genes encoding ACC synthase were recently isolated and sequenced by two groups (3, 4), but no clones are available for EFE. Many studies related to EFE characterization (5, 6) and subcellular localization (7, 8) failed to provide an efficient method to isolate the enzyme, which has proved recalcitrant to purification. In an alternative strategy to identify genes involved in ethylene biosynthesis, cDNA clones in a ripening-related library from tomato fruit were screened to identify those also expressed in wounded leaves. The cDNA, pTOM13, was identified on this basis (9) and further characterized (10, 11). Subsequent experiments using transgenic plants in which the accumulation of pTOM13 mRNA was inhibited with an antisense gene confirmed its role in ethylene synthesis and suggested it may encode at least part of EFE (12).

In this paper we describe two cloning artefacts in the 5′ region of pTOM13 and reconstruction of this sequence to generate a cDNA with an authentic 5′ coding region, pRC13. We show that this corrected cDNA confers EFE activity when expressed in *Saccharomyces cerevisiae*. The implications of this result for understanding EFE structure and regulation are discussed.

MATERIALS AND METHODS

Materials. Tomato plants (*Lycopersicon esculentum* Mill, variety Ailsa Craig) were grown as described previously (13) from seeds of a homozygous line maintained at Sutton Bonington since 1978. The yeast expression vector pBEJ15 (14) was kindly provided by C. Hadfield and B. Jordan (Leicester Biocentre, U.K.). All chemicals were obtained from Fisons (Loughborough, U.K.), unless otherwise stated. $FeSO_4$ was obtained from Sigma. AEC isomers were generously provided by M. A. Venis (Institute of Horticultural Research, East Malling, U.K.).

Direct Dideoxy Sequencing of RNA. Fruit RNA was extracted as described by Grierson *et al.* (13) and poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose (Pharmacia) chromatography (ref. 15, p. 7.26–7.29). Sequencing of the mRNA homologous to pTOM13 was carried out by using the method of Geliebter (16) with an oligonucleotide primer complementary to nucleotides 277–312 of the pTOM13 cDNA sequence (11).

Molecular Cloning. All steps were carried out by standard methods (15). The plasmid pRC13, containing a full-length coding sequence for the pTOM13 protein, was constructed by ligating the 2.9-kilobase-pair (kbp) *Bst*XI–*Xba* I fragment of pETH1 (17) to the 1.2-kbp *Bst*XI–*Xba* I fragment of pTOM13. The 1.3-kbp *Hin*dIII–*Bam*HI fragment containing this reconstructed cDNA sequence was rendered flush ended with the Klenow fragment of DNA polymerase (Amersham). This was ligated into the *Bgl* II site between the phosphoglycerate kinase (PGK) promoter and terminator of the yeast expression vector pBEJ15 (14), also rendered flush ended with Klenow enzyme. Orientation of the insert with respect to the promoter was determined by restriction enzyme mapping and a clone with the insert in the sense orientation was named pYE13.

Yeast Transformation. *S. cerevisiae* JRY188 was transformed with pYE13 by using the lithium acetate method, with transformants being selected on synthetic complete plates without leucine (18).

Nucleic Acid Analysis. Total DNA was extracted from *S. cerevisiae* as described (18). To extract total RNA from *S. cerevisiae*, cell walls were digested for 5 min at 37°C with Lyticase (Sigma) at 5 mg·ml^{-1} in 1 M sorbitol/50 mM EDTA. An equal volume of buffer used for extracting total plant

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene-forming enzyme; AEC, 1-amino-2-ethylcyclopropane-1-carboxylic acid; PA, 1,10-phenanthroline; PGK, phosphoglycerate kinase.

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RNA was added and RNA was purified as described (12). DNA and RNA were separated by agarose gel electrophoresis (ref. 15, p. 7.40–7.42) and blotted on to Hybond N hybridization membranes (Amersham) or blotted directly on to these by using a slot blot manifold (BRL). The immobilized nucleic acids were hybridized with a sense-specific RNA probe labeled with ^{32}P transcribed from pTOM13 sequences cloned in the transcription vectors pGEM3 and pGEM4 (Promega) as described (12).

Measurement of *S. cerevisiae* Cell Number. Routinely, the optical density of cultures at 600 nm was measured and related to cell density by using a calibration curve. Yeast cells were counted in a hemocytometer under ×40 magnification.

Measurement of EFE Activity in *S. cerevisiae*. Untransformed yeast or yeast transformed with pYE13 were grown in synthetic complete broth (18), with or without leucine, respectively, at 27°C with continuous agitation (200 rpm). The glucose in this broth induces activation of the PGK promoter as well as providing an energy and carbon source for yeast growth. Samples (1 ml) were taken at intervals and their cell density was determined spectrophotometrically. The capacity of cells to convert ACC to ethylene was determined at different stages in the growth cycle, beginning about 30 h after inoculation in the fresh medium (4.5×10^6 cells per ml). The samples were incubated in the same medium with 1 mM ACC (Sigma) in 5-ml glass bottles sealed with Subaseal vaccine caps for 1 h at 27°C with agitation (200 rpm). Ethylene in the headspace was measured by gas chromatography using an alumina column as described (12).

Analysis of Stereospecificity of EFE Activity. *S. cerevisiae* culture (1 ml, 7×10^7 cells per ml) transformed by pYE13, with or without ascorbate (50 mM), was incubated at 27°C in the presence of 1 mM racemic mixture of *trans*- (1*R*,2*S*- and 1*S*,2*R*)- or *cis* (1*R*,2*R*- and 1*S*,2*S*)-1-amino-2-ethylcyclopropane-1-carboxylic acid (AEC). After 2 h of incubation the 1-butene evolved in the headspace was measured by gas chromatography using the same conditions as for ethylene. Identities of these olefins were verified by cochromatography with standard samples.

Factors Affecting EFE Activity in *S. cerevisiae*. *S. cerevisiae* culture (1 ml, 8×10^7 cells per ml) transformed by pYE13 was preincubated for 15 min with 1 mM ACC. Effectors were added as indicated in Table 2 and incubation was continued for a further 15 min. Cultures were then sealed for ethylene measurement as described above. 1,10-Phenanthroline (PA; Sigma) was added from stock solution prepared in 50% (vol/vol) ethanol. The ethanol concentration did not exceed 0.5% in the cell suspension.

RESULTS

The 5′ transcribed sequence of pTOM13 was determined by direct RNA sequencing (Fig. 1). The sequence was identical to the cDNA between nucleotides 172 and 274 in all but two positions (underlined in Fig. 1), where an additional C and G were present. When included in the cDNA sequence, these two nucleotides render the first ATG in frame with the previously reported longest open reading frame started by the fourth ATG, 60 nucleotides downstream (11). A clone with the correct coding sequence (pRC13) was constructed from the 5′ sequence of the cloned pTOM13-homologous gene pETH1 and the pTOM13 cDNA as described in *Materials and Methods* and ligated between the PGK promoter and terminator of the yeast expression vector pBEJ15 to create pYE13. This plasmid was used to transform *S. cerevisiae*. Complementation of the *leu2* mutation in *S. cerevisiae* JRY188 by a functional gene on pYE13 allowed selection of transformants on medium without leucine. The presence of pYE13 was confirmed by Southern blotting (data not shown) and the production of pRC13 transcripts in the transformed cells was measured by Northern blotting or slot blotting of RNA and hybridization with a sense-specific probe. Transcripts of the expected size (1.2 kb) were detected in cells transformed with pYE13 but not in control cells (Fig. 2). The steady-state level of pRC13 RNA during the culture period increased approximately 2-fold between 2 and 8 h, reached a maximum between 8 and 14 h, and then declined to an undetectable level late in stationary phase (Fig. 3). Yeast transformed with pYE13, but not untransformed cells, were able to convert ACC to ethylene (Fig. 3). EFE activity increased 2- to 3-fold during the exponential phase of growth and declined sharply as the cells entered stationary phase. In the absence of exogenous ACC, both untransformed and transformed cells failed to produce ethylene (data not shown).

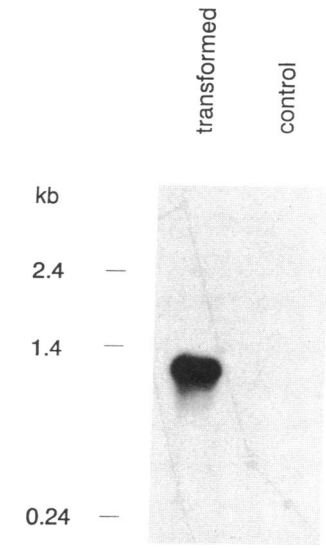

FIG. 2. Northern blot analysis of RNA from transformed and untransformed *S. cerevisiae* (8×10^7 cells per ml). Total RNA was hybridized to a sense-specific RNA probe transcribed from pTOM13 sequences in the pGEM3 transcription vector.

The ability of EFE activity in yeast transformed with pYE13 to discriminate between stereoisomers of AEC was examined. Table 1 shows that conversion of the *trans*-AEC

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          172                                                    232
           ▼                                                      ▼
cDNA  ATGGAGAACTTC CAATTATTAACTTG AAAAGCTCAATGGAGATGAGAGAGCCAACACCATG

RNA:  AUGGAGAACUUCCCAAUUAUUAACUUGGAAAAGCUCAAUGGAGAUGAGAGAGCCAACACCAUG

amino M E N F P I I N L E K L N G D E R A N T M
acid
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FIG. 1. Comparison of pTOM13 cDNA sequence, between nucleotides 172 and 232, with the sequence of the same region determined directly from pTOM13 homologous mRNA. ATG trinucleotides are doubly underlined. Nucleosides present in the RNA but absent from the cDNA are singly underlined. The previously reported longest open reading frame in the cDNA starts from the ATG at positions 230–232. However, in the RNA sequence, this AUG is in frame with the upstream AUG trinucleotide between positions 172 and 174 and thus the coding region is extended by 60 nucleotides. The additional N-terminal peptide sequence is shown underneath.

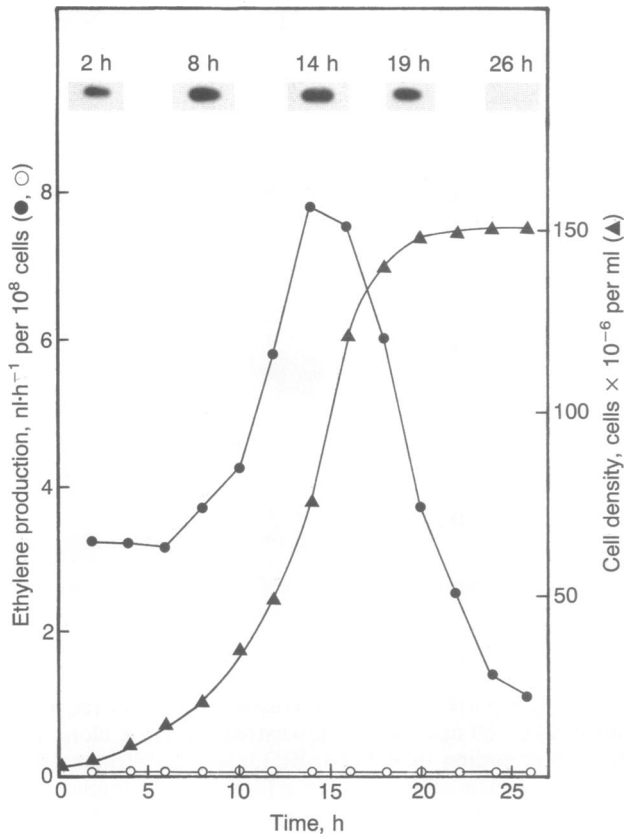

FIG. 3. Ethylene production by transformed and untransformed *S. cerevisiae*. Yeast were grown with shaking for 33 h (4.5×10^6 cells per ml) before ethylene determination was started, indicated as 0 h. At indicated times 1 ml of yeast culture was withdrawn and its cell density was determined (▲), and then the sample was supplemented with 1 mM ACC and incubated at 30°C for 1 h in a 5-ml sealed bottle. At the end of the incubation time the ethylene produced was measured by gas chromatography. Each value represents the mean of two replicates. ●, Yeast transformed with pYE13; ○, untransformed yeast. (*Inset*) Samples (20 μg of total RNA) from cells taken from culture between 2 and 26 h were blotted onto a nylon membrane. pRC13-homologous RNA was measured by hybridization to a sense-specific RNA probe transcribed from pTOM13 sequences in the pGEM3 transcription vector. Hybridization intensities are shown. No hybridization to the probe was detected with RNA taken from an untransformed yeast culture at 14 h.

mixture was approximately 80 times greater than that of the *cis*-AEC mixture. The sensitivity of ethylene production by transformed yeast to various effectors known to influence EFE activity in plants was analyzed (Table 2). $CoCl_2$ (25 μM) and PA (200 μM) drastically reduced ethylene production. Addition of $FeSO_4$ (200 μM) slightly enhanced conversion of ACC to ethylene, whereas conversion was stimulated more than 4-fold by ascorbate (50 mM). EFE activity with and without ascorbate displayed identical stereodiscrimination between isomers of AEC, and activities under both conditions were inhibited to the same extent by $CoCl_2$ and PA and weakly stimulated by iron (Table 2).

Table 1. Conversion of AEC isomers into 1-butene by *S. cerevisiae* transformed by pYE13 carrying the pRC13 cDNA

Substrate	1-Butene produced, $pl \cdot h^{-1}$	
	Without ascorbate	With ascorbate
trans-AEC mixture	1060 ± 190	4907 ± 270
cis-AEC mixture	14 ± 5	59 ± 11

One-milliliter cultures (7×10^7 cells per ml) of transformed *S. cerevisiae*, with or without ascorbate (50 mM), were incubated in the presence of 1 mM *trans*- or *cis*- racemic mixtures of AEC. After 2 h of incubation at 30°C the 1-butene produced was determined by gas chromatography. Data are means (±SEM) of three replicates.

DISCUSSION

The accumulation of mRNA homologous to the cDNA pTOM13 has previously been correlated with ethylene synthesis (9, 11). Recently, it has been shown that inhibiting accumulation of the corresponding mRNA by the expression of an antisense gene in transgenic plants greatly reduced ethylene synthesis and EFE activity (12). This suggested that pTOM13 might encode at least part of EFE, although direct evidence was lacking. Before this proposal was tested directly by heterologous expression of this clone in yeast, the structure of the cDNA was examined for any anomalous features that might affect translation. The presence of three out-of-frame ATG trinucleotides upstream of the fourth ATG, which begins the longest open reading frame (11), gave rise to concern. Such a structure is inconsistent with the ribosome scanning model of eukaryotic translation initiation (19), which proposes that ribosomes bind to the 5′ end of the mRNA and initiate translation predominantly at the most proximal ATG. Initiation of translation at any of the first three ATG trinucleotides in mRNA transcribed from the pTOM13 cDNA would produce only very short peptides. According to the model, translation initiation from the fourth ATG would occur very rarely. Direct dideoxy sequencing of pTOM13 homologous mRNA from tomato fruit showed that two nucleotides were present between the first and fourth ATG that were not present in the cDNA. This has been confirmed by sequencing of a genomic clone, pETH1 (17). The absence of the two "new" nucleotides from the cDNA is assumed to be a cloning artefact. Inclusion of these nucleotides in the cDNA sequence renders the first ATG in frame with the fourth and adds 60 nucleotides to the 5′ end of the longest open reading frame. Attempts at heterologous expression of pTOM13 to study EFE activity would thus be beset by two problems: first, only very low levels of protein would be generated, and second, this protein would lack its first 20 amino acids.

To generate a clone with an authentic 5′ coding region, the first 60 base pairs of the coding region from the genomic clone pETH1 (equivalent to the RNA sequence given in Fig. 1) were fused to the last 1.1 kbp of the cDNA. Expression of this corrected sequence (pRC13) in *S. cerevisiae* using the general yeast expression vector pBEJ15 resulted in the production of EFE activity with properties similar to those determined from studies with plant systems (2). The dependence of EFE activity on growth phase (Fig. 3) may be influenced by a

Table 2. Compounds influencing the conversion of ACC to ethylene by *S. cerevisiae* transformed with pYE13

Effectors	Ethylene produced, $nl \cdot h^{-1}$
None	5.79 ± 0.40
$CoCl_2$	0.92 ± 0.09
PA	0.42 ± 0.07
$FeSO_4$	7.15 ± 0.62
Ascorbate	24.47 ± 1.39
Ascorbate + $FeSO_4$	26.49 ± 2.85
Ascorbate + $CoCl_2$	1.06 ± 0.17
Ascorbate + PA	0.76 ± 0.01

One-milliliter cultures of transformed *S. cerevisiae* (8×10^7 cells per ml) were incubated with 1 mM ACC and 25 μM $CoCl_2$, 200 μM $FeSO_4$, 50 mM ascorbate, or 200 μM PA. Transformed cells used as control were incubated in the presence of 1 mM ACC only. After 1 h at 30°C the ethylene evolved was measured by gas chromatography. Each value represents the mean (±SEM) of three replicates.

number of factors. The activity of the PGK promoter, and hence pRC13 RNA transcription, is expected to follow changes in glycolytic flux. Our results show a doubling of RNA and a 2- to 3-fold rise in EFE activity per cell during the exponential growth phase. This may be due to increased transcription or increased stability of the mRNA. The rapid decline in both RNA and EFE activity with the onset of stationary phase may be a result of specific down-regulation of the PGK promoter as the glycolytic flux decreases or of a general redirection of RNA and protein synthesis during this phase.

EFE has proved intractable to purification and ideas about its properties have been obtained from indirect observations. Because of the deleterious effect of lipophilic compounds and osmotic shock on EFE activity, it was thought that the enzyme system requires membrane integrity (20, 21), and several workers have suggested that the enzyme is membrane bound (8, 22–24). *In vivo* systems are characterized by high stereoselectivity for the *trans* isomer of the ACC analogue AEC (25). The results described here show that only one polypeptide, that encoded by pRC13, is necessary for EFE activity with the same stereodiscrimination as found *in vivo*. Analysis of the amino acid sequence derived from pRC13 indicates that it is not an integral membrane protein and shows no obvious signal peptide, suggesting that the protein may be located in the cytosol. However, failure to purify EFE has previously been explained by the requirement of the protein for membrane integrity. These two observations could be reconciled if the protein encoded by pRC13 interacts with, or depends for its activity on, other proteins located in membranes. If such factors are necessary, they can be assumed to be of a general nature, since the present results indicate their counterparts exist in yeast cells. Alternatively, the enzyme may be soluble but have unexpected requirements, as suggested recently for EFE from melon (26).

Study of pRC13 expression in yeast shows that, as in plant cells, ethylene synthesis is almost abolished in the presence of cobaltous ions (27, 28). The inhibition of ethylene formation in yeast by the metal chelating agent PA, which has a strong affinity for iron, is in agreement with recent data demonstrating that iron is an essential cofactor in the conversion of ACC to ethylene in higher plants (26, 29). It is possible that methionine-rich regions in the protein sequence (11) represent part of iron-binding domains. The poor stimulation by iron reported here may indicate that this cofactor is not limiting in the culture medium.

The deduced amino acid sequence of pTOM13 shows substantial homology to flavarone-3-hydroxylase (A. Prescott and C. Martin, personal communication, cited in ref. 12). It is shown here that, like this and related enzymes (30), EFE activity in yeast is greatly enhanced by ascorbate and probably requires iron, whereas α-ketoglutarate, which is required by some hydroxylases (30), has no effect (data not shown). These observations suggest that the reaction mechanism for ACC oxidation to ethylene by EFE may proceed by a hydroxylation as proposed by Yang (31). This deduced information about the requirements of the enzyme may prove useful in devising procedures for the purification of EFE from plants.

Previous studies on EFE activity have often depended on assays employing excised plant tissue (27, 32). The observation that the level of pTOM13-homologous RNA rises extremely rapidly following wounding of plant tissue (11) suggests that this assay procedure may measure wound-induced EFE activity, and previous conclusions about endogenous enzyme activity and factors regulating ethylene synthesis in plants may need to be reevaluated.

The ability to express plant EFE in yeast with the easy manipulation and variety of genetic backgrounds available should prove very useful in the biochemical analysis of this enzyme and the characterization of the polypeptides encoded by the three different pTOM13 genes (10). Site-directed mutagenesis of the nucleic acid sequence in conjunction with this heterologous expression system should allow detailed analysis of these polypeptides.

After completion of this work we learned that pTOM13 had been used to isolate an elicitor-induced EFE clone, which was identified by functional expression in *Xenopus* oocytes (33).

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