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Analysis and cloning of the ethylene-forming enzyme from tomato by functional expression of its mRNA in *Xenopus laevis* oocytes

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The last step in biosynthesis of the plant hormone ethylene, oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC), is catalysed by the elusive ethylene-forming enzyme (EFE). EFE is induced by fungal elicitors in suspension-cultured tomato cells. We demonstrate that *Xenopus laevis* oocytes injected with RNA from elicitor-treated tomato cells gain the ability to convert ACC to ethylene. The enzyme expressed in the oocytes under the direction of plant RNA is indistiguishable from genuine plant EFE with regard to its saturation kinetics, its iron dependency and its stereospecificity to the diastereomeric ethyl derivatives of ACC, allocoronamic acid and coronamic acid. In tomato cells stimulated for different times with elicitor, the level of EFE correlates with the level of RNA directing EFE expression in oocytes. Hybridization and co-injection experiments demonstrate that the tomato RNA species directing EFE expression in oocytes are homologous to clone pTOM13 which has been shown to inhibit ethylene production in plants when expressed in antisense. Using a cDNA library from elicitor-stimulated tomato cells, we have isolated several homologues of pTOM13 and identified one of them, pHTOM5, as a clone of EFE on the basis of its functional expression in the *Xenopus* oocytes.

Key words: ethylene-forming enzyme/fungal elicitor/oocyte expression/stereospecificity/1-aminocyclopropane-1-carboxylate oxidase

Introduction

Ethylene is a plant hormone involved in the regulation of growth and development and in the reaction to environmental stress (Yang and Hoffman, 1984). It is formed in two steps from *S*-adenosylmethionine by way of the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984; Kende, 1989). The first step, conversion of *S*-adenosylmethionine to ACC, is catalysed by ACC synthase, an enzyme discovered by Boller *et al.* (1979), purified from various sources (see Kende, 1989), and recently cloned (Sato and Theologis, 1989; Nakajima *et al.*, 1990; Van Der Straeten *et al.*, 1990).

The second reaction, the oxidation of ACC to ethylene, is catalysed by the so-called EFE, the ethylene-forming enzyme (Yang and Hoffman, 1984; Kende, 1989). This enzyme has remained elusive up to now. Somewhat paradoxically, the main difficulty in identifying genuine plant EFE has been the ease with which plant extracts from various sources as well as model systems convert ACC to ethylene; almost all of these extracts and model systems now appear to represent artefacts since they are not stereoselective (see Yang and Hoffman, 1984; Kende, 1989). Stereoselectivity of genuine EFE was first established by Hoffman *et al.* (1982) using the four stereoisomers of the ethyl derivative of ACC, 1-amino-2-ethylcyclopropane carboxylic acid (AEC), known as (+) and (−)coronamic acid and (+) and (−)allocoronamic acid. They found that genuine EFE *in vivo* converted only one of the four stereoisomers, (+)allocoronamic acid, to the ethyl derivative of ethylene, 1-butene. To date, the only cell-free systems that display the same stereoselectivity as genuine EFE are isolated pea vacuoles (Guy and Kende, 1984) and kiwi fruit membrane vesicles (Mitchell *et al.*, 1988).

Recently a cDNA clone, pTOM13, originally isolated as a tomato ripening related gene (Holdsworth *et al.*, 1987a, b), has been shown to suppress EFE activity when expressed as antisense RNA in transgenic tomatoes (Hamilton *et al.*, 1990), indicating that it might be homologous to a transcript encoding an essential part of tomato EFE.

We have previously shown that EFE activity is induced by a fungal elicitor in tomato cells, and we have used this model system to demonstrate the dependence of EFE on iron (Bouzayen *et al.*, 1991). Here we report that RNA from elicitor-treated tomato cells directs expression of EFE with correct stereoselectivity in *Xenopus laevis* oocytes. Hybridization and co-injection experiments show that the plant RNA species causing EFE expression are homologous to pTOM13. We have isolated several homologues of pTOM13 from a cDNA library of elicitor-treated tomato cells and demonstrate that one of them, clone pHTOM5, encodes genuine EFE on the basis of its functional expression in *Xenopus* oocytes.

Results

Expression of the ethylene-forming enzyme in Xenopus oocytes

EFE has a low activity in unstimulated tomato cells and is strongly induced upon treatment with fungal elicitors (Bouzayen *et al.*, 1991). We injected various amounts of total RNA from unstimulated and elicitor-stimulated tomato cells into *Xenopus* oocytes and compared their ability to convert ACC into ethylene. The oocytes were bathed in 5 mM ACC in Barth's saline medium in air-tight vials, and their ethylene production was measured during the second day after injection (Figure 1). Oocytes treated with RNA from elicitor-stimulated cells produced ethylene in a dose-dependent manner and reached a plateau at 500 ng RNA. Similar results were obtained with poly(A)$^+$ RNA from elicitor-treated tomato cells but ethylene production reached

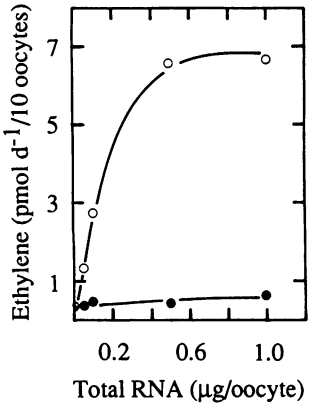

Fig. 1. Ethylene production by *Xenopus laevis* oocytes injected with increasing quantities of total RNA extracted from tomato cells treated with elicitor (10 μg/ml) for 1.5 h (○) or untreated (●). The oocytes were incubated in 5 mM ACC. Ethylene accumulation was measured during the second day after injection of RNA.

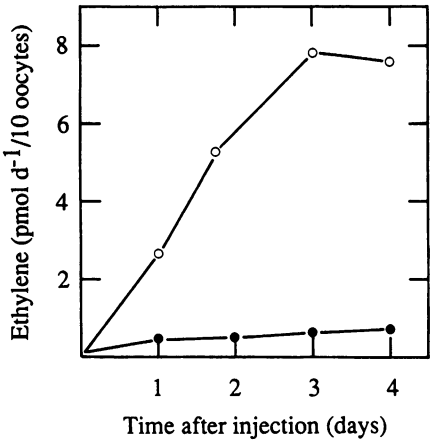

Fig. 2. Dependence of ethylene production rate in *Xenopus* oocytes on the time of incubation after injection of plant RNA. Oocytes were injected each with 500 ng total RNA from untreated (○) or elicitor-treated (●) tomato cells. The rate of ethylene production was determined for 24 h and is plotted at the end of the interval.

a plateau already at 2.5 ng (unpublished data). Oocytes injected with total RNA from non-induced cells produced only little ethylene at all RNA doses (Figure 1).

The time course of expression of the ethylene-forming activity in oocytes was studied, using 500 ng of RNA per oocyte (Figure 2). With RNA from elicitor-treated cells, the rate of ethylene production increased continuously over 3 days and then reached a plateau, indicating that the tomato RNA responsible for the ethylene-forming capacity was maintained and continued to be translated for 3 days. Oocytes injected with RNA from untreated cells produced little ethylene over the whole incubation period.

Untreated oocytes and oocytes injected with water or RNA preparations treated with RNase did not convert ACC to ethylene (unpublished data). Thus, plant RNA directs expression of an ethylene-producing activity in oocytes.

The ethylene-producing activity of oocytes under the direction of RNA from elicitor-treated tomato cells was compared with genuine EFE activity in plants. EFE in plants is saturable *in vivo* at concentrations between 0.01 and 0.5 mM ACC (Yang and Hofmann, 1984; Kende, 1989). Ethylene production in the oocytes increased in a dose-dependent manner and reached a value close to saturation at 0.5 mM ACC in the incubation medium, indicating a K_m of the same order of magnitude as found in plants (Figure 3). In the absence of external ACC, a small but significant amount of ethylene was formed. Control experiments indicated that this was due to a small amount of endogenous ACC in the oocytes (unpublished data).

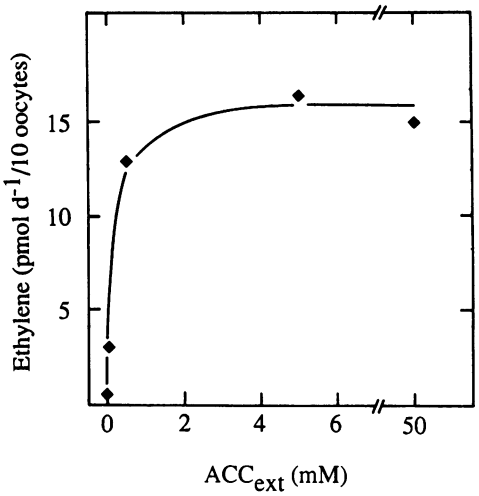

Fig. 3. Dependence of ethylene production in *Xenopus* oocytes on external ACC concentration. The oocytes were injected with 500 ng total RNA from elicitor-stimulated tomato cells. Ethylene accumulation was measured during the second day after injection.

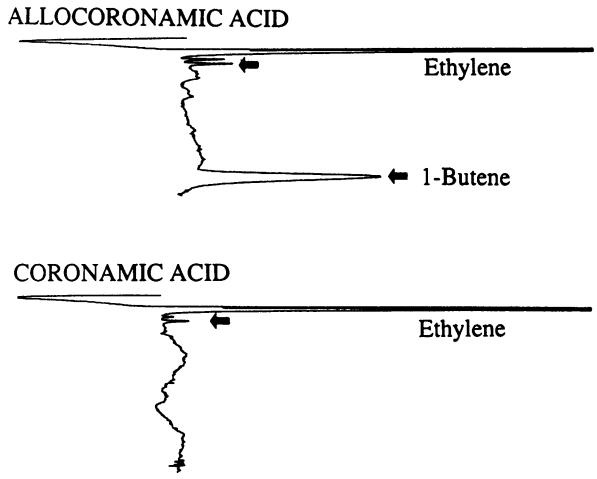

Fig. 4. Stereodiscrimination of the ethylene-forming enzyme expressed in *Xenopus* oocytes. Gas chromatograms of samples of the atmosphere above oocytes injected with 500 ng total RNA from elicitor-treated tomato cells and incubated with 0.1 mM allocoronamic acid or with 0.1 mM coronamic acid for 24 h. The retention times of ethylene and 1-butene are 0.6 min and 3.2 min, respectively.

Activity of plant EFE is inhibited *in vivo* by *o*-phenanthroline (Bouzayen *et al.*, 1991). ACC-dependent ethylene formation in oocytes injected with tomato RNA was blocked by similar concentrations of *o*-phenanthroline (unpublished data). As in plants, Fe^{2+} ions reversed the inhibitory effect of *o*-phenanthroline in the oocytes (unpublished data).

Activity of plant EFE shows stereodiscrimination towards the isomers of AEC, converting only (+)allocoronamic acid into 1-butene (Hoffman *et al.*, 1982). The ability of oocytes injected with plant RNA to distinguish between the two

diastereoisomers of AEC was tested (Figure 4). Oocytes were incubated in 0.1 mM coronamic acid or in 0.1 mM allocoronamic acid. Only allocoronamic acid was converted to 1-butene (3.0 pmol day^{-1} per 10 oocytes).

Taken together, the data presented above demonstrate that plant RNA directs expression of genuine EFE in the oocytes. This implies that the ability of a given sample of plant RNA to cause EFE expression in oocytes can be taken as a measure of its content of EFE mRNA. To test this, RNA was extracted from tomato cells at various times after elicitor treatment and compared with the time course of EFE induction *in planta* (Figure 5). Addition of elicitor to tomato cells resulted in a marked increase of EFE 1−2 h after treatment (Figure 5A), as previously described (Bouzayen *et al.*, 1991). Pretreatment with cordycepin for 1 h completely abolished this response (Figure 5A). The level of EFE mRNA, estimated by functional expression of total RNA in oocytes, increased about two-fold in comparison to controls within 30 min after elicitor addition and reached levels ten-fold higher than controls after 120 min (Figure 5B). Cordycepin totally blocked this increase in EFE mRNA (Figure 5B).

Characterization of tomato RNA coding for EFE by hybridization with pTOM13 and co-injection with pTOM13 transcripts

It was of interest to characterize the tomato RNA species encoding EFE with regard to pTOM13 (Holdsworth *et al.*, 1987a), a ripening-related clone that was recently found to reduce EFE expression in plants when expressed in antisense orientation under a constitutive promoter (Hamilton *et al.*, 1990). Plasmid DNA from clone pTOM13 was denatured and bound to nitrocellulose. For controls, the same amount of an unrelated plasmid was similarly bound to nitrocellulose. Total RNA extracted from elicitor-induced tomato cells was incubated with the nitrocellulose filters. Three pools of RNA were prepared: (i) RNA that hybridized to the bound plasmid DNA (hybrid-selected RNA); (ii) RNA that did not bind to the filter (hybrid-depleted RNA); and (iii) a reconstituted mixture of bound and unbound RNA (total RNA). These were injected into oocytes, and EFE was assayed (Table I). In the experiments with pTOM13, the hybrid-selected RNA contained a high level of EFE mRNA, as assayed by functional expression in the oocytes. Hybrid-depleted RNA contained little EFE mRNA. Mixing hybrid-depleted and hybrid-selected RNA did not result in increased EFE expression, indicating that the hybrid-depleted RNA did not contain RNA species required for optimal EFE expression. In the experiments with the control plasmid, as expected, there was little EFE mRNA in the hybrid-selected RNA preparation eluted from the filter, and most remained in the hybrid-depleted supernatant.

The cDNA insert of pTOM13 was subcloned into pBLUESCRIPT SK(−). The orientation of the insert, determined by restriction analysis, was such as to provide sense mRNA by transcription with T3 RNA polymerase and antisense RNA by transcription with T7 RNA polymerase. Sense or antisense RNA transcripts (5 ng per oocyte) were injected into oocytes either alone or with total RNA from elicitor-induced tomato cells (500 ng per oocyte). As shown before, total RNA from tomato cells directed expression of EFE in the oocytes. While sense mRNA from pTOM13 did not affect this, antisense mRNA from pTOM13 completely abolished the expression of EFE directed by the RNA from

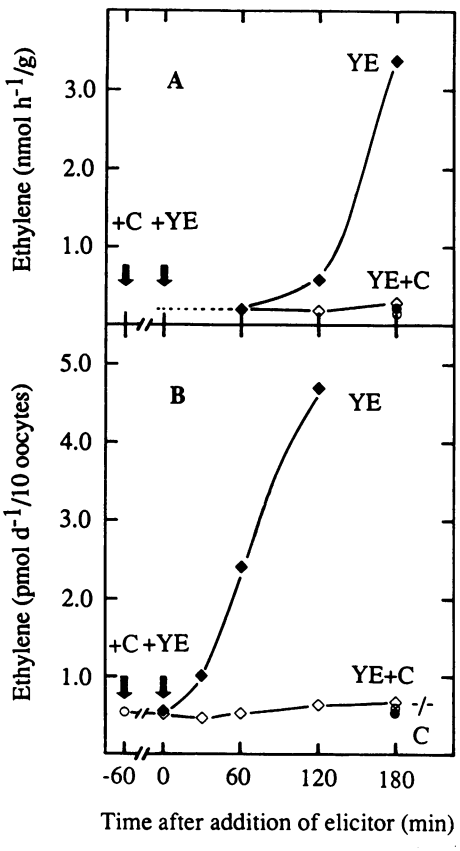

Fig. 5. Induction of the ethylene-forming enzyme (**A**) and of RNA translatable into the ethylene-forming enzyme (**B**) in tomato cells treated with elicitor (YE) or elicitor plus cordycepin (YE + C) or with cordycepin alone (C) Tomato cells were treated with 10 $\mu g\ ml^{-1}$ elicitor (◇, ◆) at time zero (+YE, arrow). Control cells (○, ●) received no elicitor. Part of the cultures received 200 $\mu g\ ml^{-1}$ cordycepin (◇, ○) one h before elicitor addition (+C, arrow). (A) Activity of the ethylene-forming enzyme measured *in vivo* in tomato cells. (B) Production of ethylene in oocytes injected with total RNA (500 ng per oocyte) extracted from cells treated as above. The oocytes were incubated in 5 mM ACC. Ethylene accumulation during the second day after injection of RNA was measured.

tomato cells (Table II). Neither sense nor antisense mRNA obtained from pTOM13 was able to direct functional expression of EFE in the oocytes (Table II).

Taken together, the data of Tables I and II show that the RNA species causing EFE expression in oocytes are homologous to pTOM13 although this clone itself does not direct EFE expression. In order to identify a clone encoding EFE, we therefore initiated a search for pTOM13 homologues in a cDNA library from elicitor-treated tomato cells and screened them for functional expression of EFE in oocytes.

Isolation of a clone directing functional expression of the ethylene-forming enzyme using a cDNA library from elicitor-treated tomato cells

An oriented cDNA library of 5×10^5 independent clones was constructed in λ ZAP II with RNA obtained from elicitor-induced tomato cells. A total of 5×10^4 independent clones was screened with the *Pst*I restriction fragment of pTOM13. Five clones hybridizing to pTOM13 were isolated and designated pHTOM1 to pHTOM5.

Sense RNA transcripts of the five clones were synthesized

Table I. Ethylene production of oocytes injected with tomato RNA after hybridization with pTOM13[a]

RNA injected	DNA used for hybridization	Ethylene produced (pmol day^{-1}/10 oocytes)
Hybrid depleted	pTOM13	0.9
	Control	7.0
Hybrid selected	pTOM13	10.5
	Control	0.9
Total	pTOM13	7.0
	Control	5.3

[a]Total RNA extracted from tomato cells 1.5 h after elicitor treatment was subjected to hybrid-depletion or hybrid-selection with pTOM13 or with an unrelated control plasmid before injection into oocytes. Amounts corresponding to 500 ng of the total RNA preparation were injected into the oocytes. The oocytes were incubated for 24 h in 5 mM ACC before assaying ethylene production.

Table II. Ethylene production of oocytes injected with pTOM13 RNA transcripts and tomato RNA[a]

RNA injected	Ethylene produced (pmol day^{-1}/10 oocytes)
Total RNA	5.3
pTOM13 sense RNA	0.2
pTOM13 sense RNA + total RNA	4.6
pTOM13 antisense RNA	0.2
pTOM13 antisense RNA + total RNA	0.4

[a]Sense or antisense RNA transcripts of pTOM13 (5 ng per oocyte) were injected with or without total RNA (500 ng per oocyte) extracted from tomato cells 1.5 h after elicitor treatment. The oocytes were incubated for 24 h in 5 mM ACC before assaying ethylene production.

Table III. Functional expression of pHTOM5 in oocytes[a]

Substrate	Product (pmol day^{-1}/10 oocytes)	
	Ethylene	1-Butene
ACC	5.3	<0.2
Allocoronamic acid	0.3	3.7
Coronamic acid	0.3	<0.2

[a]RNA transcripts (5 ng per oocyte) were injected into *Xenopus* oocytes. The oocytes were incubated for 24 h before analysing the products.

using T3 RNA polymerase and injected individually into oocytes. RNA transcripts derived from one clone, designated pHTOM5, was capable of directing expression of EFE in the oocytes as shown by their capacity to convert the substrate ACC into ethylene (Table III). Oocytes injected with RNA transcripts from the four other clones (pHTOM1–pHTOM4) did not convert ACC into ethylene (unpublished data). The EFE activity expressed in oocytes under the direction of pHTOM5 displayed the same stereoselectivity as genuine plant EFE: only allocoronamic acid but not coronamic acid was converted to 1-butene (Table III).

Clone pHTOM5 has a cDNA insert of 1035 bp (Figure 6). It contains one long open reading frame of 316 amino acids, starting with an ATG codon at position 39. From the derived amino acid sequence, the molecular mass of the polypeptide encoded by pHTOM5 is 35.9 kd. The 3′ end has 46 bp of nontranslated sequence but does not end with poly(A), indicating that the 3′ end is incomplete.

Comparison of the sequence of pHTOM5 with pTOM13 reveals a high homology (88% identity at the nucleotide level). For clarity, only positions differing in the aligned sequence of pTOM13 are given. pTOM13 has an ATG at the same position as the start codon of pHTOM5 but two single bp deletions in the 30 bp downstream, with the first ATG in the reading frame at the position corresponding to 99 in pHTOM5. Further differences include a region towards the 3′ end where pHTOM5, with respect to pTOM13, contains a 12 bp deletion at position 837 of the aligned sequences and a 15 bp insertion at position 965 (Figure 6).

Discussion

RNA from elicitor-treated tomato cells directs the expression of EFE when injected into *Xenopus* oocytes: the oocytes convert ACC to ethylene. The fact that expression of EFE in the oocytes requires intact RNA and depends on the dose of RNA injected, supports the notion that the ability to oxidize ACC is the result of RNA translation and not of the activation of some unspecific mechanism of ethylene production. The ability to synthesize EFE is saturable with respect to quantity of RNA injected (Figure 1). This could be the result of saturation of the translation machinery or of some co-factors required for EFE expression. The increase of EFE activity for up to 3 days (Figure 2) indicates a high stability of EFE mRNA in the oocytes.

We have used the following criteria to determine whether the EFE activity in oocytes has the same properties as the EFE in plants:

(i) Kinetics and sensitivity to inhibitors: The EFE in oocytes displays typical saturation kinetics with respect to the concentration of its substrate ACC, resembling those found both in cell cultures and in adult plant tissue *in vivo*. It differs markedly from inorganic model systems (Yang and Hoffman, 1984) as well as from the lipoxygenase mediated conversion of ACC (Wang and Yang, 1987) which are not saturated even at 10 mM ACC. Like plant EFE, the EFE in oocytes is inhibited by *o*-phenanthroline and appears to require iron as an essential co-factor.

(ii) Stereospecificity: plant EFE converts 1-amino-2-ethyl-cyclopropane-1-carboxylic acid (AEC) to 1-butene and shows stereospecificity with regard to the four isomers of AEC. Only (+)allocoronamic acid is a substrate for the reaction, while (−)allocoronamic acid and both optical isomers of coronamic acid are not (Hoffman *et al.*, 1982). Stereoselectivity has ever since been considered the criterion for 'genuine' EFE activity (McKeon and Yang, 1984; Guy and Kende, 1984). We did not have access to all four isomers in pure form but we were able to test stereoselectivity between the racemic diastereoisomers allocoronamic acid and coronamic acid. Oocytes, like plants, converted only allocoronamic acid but not coronamic acid to 1-butene. Thus the EFE expressed in oocytes displays the stereoselectivity of genuine plant EFE.

(iii) Inducibility: EFE activity in tomato cells can be induced by fungal elicitors (Bouzayen *et al.*, 1991). The

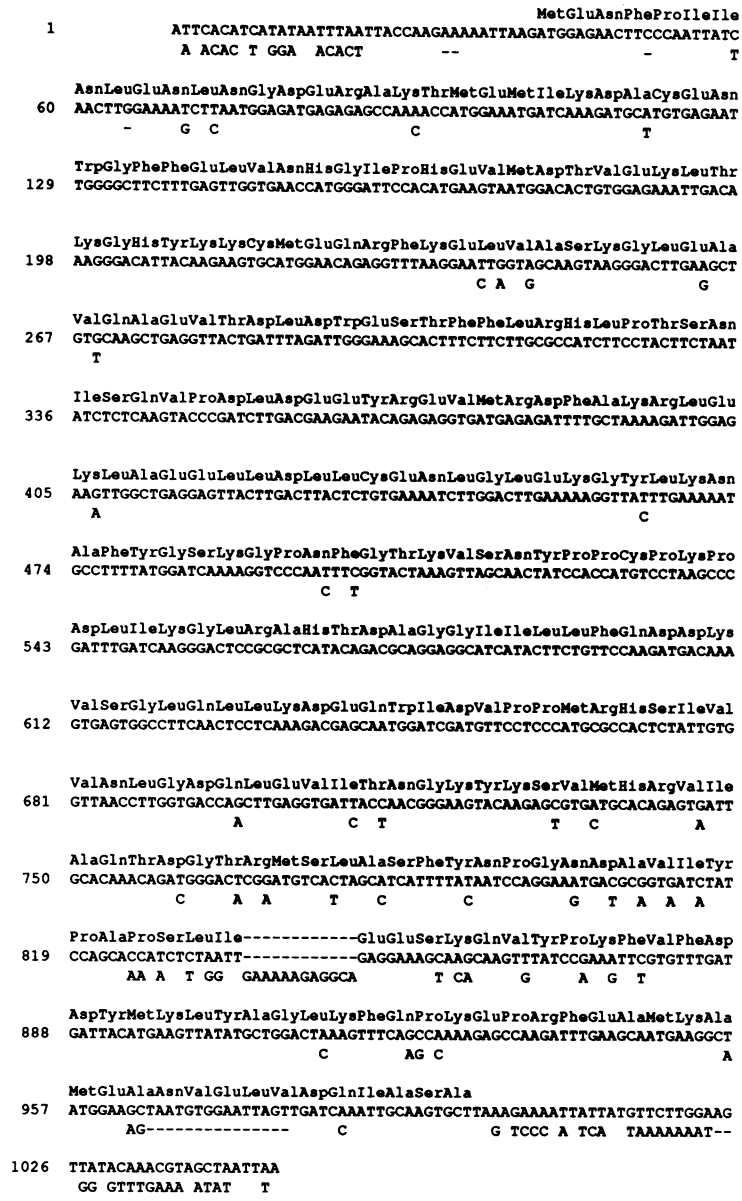

Fig. 6. DNA sequence and derived amino acid sequence of the cDNA clone pHTOM5 yielding functional expression of the ethylene-forming enzyme in oocytes. The sequence is compared with the cDNA clone pTOM13 (Holdsworth *et al.*, 1987a). Bases differing from pHTOM5 are shown below the DNA sequence. Gaps introduced for optimal alignment are indicated (−).

induction is probably due to increased transcription as it is sensitive to inhibitors of RNA synthesis, e.g. cordycepin (Figure 5A). RNA extracted from tomato cells at various times after elicitor addition directed synthesis of EFE activity in oocytes in a manner consistent with the time course of enzyme induction in cell cultures. No EFE RNA accumulated in cordycepin treated cells. This confirms that the elicitor-dependent induction of EFE in tomato cells is the result of increased steady state levels of mRNA for EFE, possibly because of a transcriptional activation of its gene.

These observations provide strong evidence that the activity expressed in oocytes is genuine plant EFE. In view of the difficulties of isolating plant EFE by biochemical techniques (see Kende, 1989, for a review), we have used functional expression in the oocytes as an approach to clone a cDNA encoding EFE.

We have first further characterized the EFE mRNA from tomato cells in relation to pTOM13, a cDNA clone isolated from ripening tomatoes (Holdsworth *et al.*, 1987a). It has been suggested that pTOM13 codes for an essential part of the EFE because, when expressed as antisense in transgenic tomatoes, it inhibits ethylene formation and EFE activity (Hamilton *et al.*, 1990). We have found that RNA which directs EFE expression in oocytes specifically hybridizes to pTOM13. In addition, antisense RNA from pTOM13, when injected together with EFE RNA from tomato cells, inhibits expression of EFE. These findings support the hypothesis that EFE RNA is at least partially homologous to pTOM13. However, sense mRNA from pTOM13 is not able to direct EFE activity in the oocytes. Therefore pTOM13 itself does not appear to code for a polypeptide with EFE activity.

We have isolated five cDNA clones which hybridize to pTOM13 and designated them pHTOM (homologous to pTOM13). One clone, pHTOM5, is transcribed into mRNA which directs expression of EFE activity in the oocytes. This activity also displays stereoselectivity. Analysis of the

sequence reveals a high level of homology with pTOM13. Aligning pTOM13 with pHTOM5 shows that two single base pairs are missing at the 5′ end of pTOM13 (Figure 6), which leads to a frameshift and therefore to a short open reading frame terminating after 12 amino acids in the sequence of pTOM13. These deletions are not present in a genomic sequence related to pTOM13 (Holdsworth *et al.*, 1987b), and the two bases have recently been shown to be present in tomato RNA as well as in a genomic sequence representing pTOM13 (Köck *et al.*, 1991). The main parts of the open reading frames of pHTOM5 and pTOM13 show differences leading only to silent or conservative mutations at the amino acid level. A divergence between pHTOM5 and pTOM13 is a 12 bp deletion in pHTOM5 at position 837 and a 15 bp insertion at position 965. We cannot establish at this stage which of these differences is responsible for the inability of pTOM13 to direct EFE activity in oocytes. pHTOM5 codes for a 35.9 kd polypeptide, a value in close agreement with the 35 kd measured for the hybrid select translation product from tomato fruits (Holdsworth *et al.*, 1987a).

pHTOM5 has no obvious hydrophobic membrane-spanning domain, as is also true for pTOM13. The fact that pHTOM5 is sufficient for functional expression of EFE activity in oocytes is somewhat surprising in view of the fact that EFE has long been considered to be associated with membranes, in particular with the vacuolar membrane (Kende, 1989). EFE localization will have to be reconsidered.

Although clone pHTOM5 directs functional expression of EFE in oocytes, this is no proof that the ethylene-forming enzyme activity consists of a single polypeptide *in vivo*. The polypeptide encoded by clone pHTOM5 might only be part of an ethylene-forming enzyme complex. Additional polypeptides might belong to the enzyme complex but might be present endogenously in the oocytes, thus obviating the need to express all participating polypeptides in the oocyte system.

Nevertheless, our work provides strong support for the hypothesis that a single gene product, encoded by pHTOM5 (and homologous to pTOM13), is sufficient for EFE activity, at least for its high substrate affinity and for its stereospecificity with regard to AEC. It further illustrates that functional expression of mRNA in oocytes is a useful approach to study and eventually clone the much sought after but often elusive enzymes in plant hormone pathways.

Materials and methods

Plant materials

Suspension-cultured tomato cells derived from callus line Msk8 (Koornneef *et al.*, 1987) were used 2–3 days after sub-culture. EFE was induced by addition of 10 μg ml^{-1} purified yeast extract elicitor as described (Spanu *et al.*, 1990).

RNA extraction

After removal of the culture medium by vacuum filtration, the cells were frozen in liquid air and stored at −80°C. Frozen cell clumps were broken up and homogenized in 50 mM Tris buffer containing 150 mM LiCl, 5 mM EDTA and 5% SDS, adjusted to pH 9.0 with HCl (2 ml of buffer/g fresh weight of cells) with a 'Polytron' homogenizer for 30 s on ice. The homogenate was cleared by centrifugation at 3000 *g* for 15 min. The supernatant was extracted twice with one vol of acid phenol and once with one vol of chloroform. Each time the suspension was shaken and the two phases separated by centrifugation at 3000 *g* for 15 min. The upper (aqueous) phase was removed, taking care to avoid the thick interphase precipitate. RNA was precipitated by addition of 8 M LiCl to a final concentration of 2 M, incubation for at least 2 h at −20°C and centrifugation at 3000 *g* for 20 min. The pellet was resuspended in DEPC-treated water and reprecipitated with LiCl (Prescott and Martin, 1987). The RNA was finally dissolved in sterile DEPC-treated water and precipitated with 2.6 vol of ethanol plus 0.1 vol 3 M, DEPC-treated, sodium acetate. Where stated, poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Boehringer) as indicated by the manufacturer. RNA was dissolved in sterile DEPC-treated water, quantified by measuring absorption at 260 nm and stored at −20°C.

Oocyte preparation and microinjection

Oocytes were obtained from mature *Xenopus laevis* toads. The ovary sacs were dissected manually and the intact ones selected individually and incubated overnight in Barth's saline medium at 18°C (Colman, 1987). Each oocyte was injected with 50 nl of RNA solution. The oocytes were then incubated in 100 μl medium supplemented with 5 mM ACC (unless otherwise stated) in sterile, air-tight 2 ml GC vials (Varian) at 18°C. One ml of the gas in the headspace was removed, simultaneously substituted with 1 ml medium, and analysed by gas chromatography (Spanu *et al.*, 1990).

Hybrid-selection and -depletion of RNA

Plasmid DNA was denatured in 0.2 M NaOH at 100°C for 1 min and then transferred to ice. DNA (2 μg per sample) was spotted onto an autoclaved nitrocellulose membrane, dried and baked at 80°C for 2 h under vacuum. The filters were incubated in hybridization buffer [50% (v/v) formamide, 0.9 M NaCl, 0.2% SDS, 1 mM EDTA, 20 mM PIPES (Na$^+$) buffer, pH 6.4]. 500 μg total RNA was dissolved in 200 μl hybridization buffer and incubated with the filters for 6 h at 37°C. Unbound RNA was removed, precipitated with ethanol and resuspended in 50 μl DEPC-treated water. The filters were washed five times with 500 μl 50% formamide, 20 mM NaCl, 8 mM sodium citrate, 1 mM EDTA, 0.5% SDS; then twice with 1 ml DEPC-reated water. Bound RNA was removed by incubating the filters in 1 mM EDTA, 0.1% SDS for 1 min at 100°C, then freezing them in liquid air, thawing and collecting the fluid. RNA was precipitated as above with 10 μg tRNA as carrier and resuspended in 50 μl DEPC-treated water (Mason and Williams, 1988).

Expression of strand-specific RNA transcripts of pTOM13

The cDNA insert of pTOM13 was isolated by restriction digestion with *Pst*I and electrophoresis in 1% low melting point agarose. The insert fragment was ligated into a *Pst*I-digested dephosphorylated pBLUESCRIPT SK(−) (Stratagene). The resulting construct was transformed into competent XL-BLUE (Stratagene) *Escherichia coli*. Transformed clones were analysed by restriction analysis to determine the orientation of the inserts. One clone was chosen in which T3 RNA polymerase would promote synthesis of sense RNA transcripts and T7 RNA polymerase antisense transcripts. A large scale plasmid preparation was carried out and purified by ultracentrifugation over CsCl gradients. Templates for transcription were prepared by digestion with *Apa*I or with *Not*I restriction nucleases for sense or antisense transcripts respectively, followed by 'filling in' with T4 DNA polymerase (Sambrook *et al.*, 1989). Capped RNA transcripts were synthesized using the mCAP mRNA capping kit (Stratagene) as directed by the manufacturer. The DNA template was removed by DNase treatment prior to quantification. Each oocyte was treated with 5 ng RNA.

Construction and screening of a cDNA library from tomato cells

poly(A)$^+$ RNA was isolated from suspension-cultured tomato cells 1.5 h after being treated with elicitor (10 μg ml^{-1}). A directional cDNA library was constructed using the ZAP-cDNA synthesis kit (Stratagene) and inserted into a UNI-ZAPII λ phage derived vector (Stratagene) following the instructions of the manufacturer. 5×10^5 clones were obtained. These were amplified and maintained as separate sub-libraries each from an original base of 5×10^3 clones. Duplicate nitrocellulose replicas of 10 sub-libraries, containing phage DNA of ~8000 plaques, were denatured with alkali, fixed by baking at 80°C for 2 h under vacuum, washed, prehybridized and hybridized according to standard procedures (Sambrook *et al.*, 1989), using the isolated *Pst*I restriction fragment of pTOM13 as a probe. The probe was labeled by random nucleotide priming with [α-^{32}P]dATP (Stratagene) following the instructions of the manufacturer.

Five independent plaques hybridizing to pTOM13 were identified and purified. Sense RNA transcripts were prepared as described above and injected into *Xenopus laevis* oocytes.

DNA sequencing

The pHTOM5 insert was sequenced using the single-stranded dideoxynucleotide chain termination procedure using Sequenase (USB) following the manufacturer's instructions. Both strands were sequenced

completely. The (−) strand was sequenced directly from a template obtained from pHTOM5. The (+) strand was sequenced after subcloning the insert into pTZ18U.

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The sequence of PHTOM5 has been deposited in the EMBL Data Library under accession number X58885.

After completion of this manuscript, we became aware of recent related work of the group of Professor D.Grierson (Nottingham University). They report functional expression of the ethylene-forming enzyme in yeast, using a derivative of pTOM13 in which the two apparent single base pair deletions in the 5′ region of the coding strand have been corrected (Hamilton,A.J., Bouzayen,M. and Grierson,D. *Proc. Natl. Acad. Sci. USA*, in press).