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Structure and expression of an ethylene-related mRNA from tomato

Michael J.Holdsworth, C.R.Bird[1], J.Ray[1], W.Schuch[1] and Donald Grierson

Department of Physiology and Environmental Science, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough LE12 5RD and [1]ICI Plant Biotechnology Group, PO Box 11, The Heath, Runcorn, Cheshire WA7 4QE, UK

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ABSTRACT.
Messenger RNAs homologous to a cDNA clone (pTOM 13) derived from a ripe-tomato-specific cDNA library are expressed during tomato fruit ripening and after the wounding of leaf and green fruit material. Both responses involve the synthesis of the hormone ethylene. Accumulation of the pTOM 13-homologous RNA during ripening is rapid and sustained, and reaches its maximum level in orange fruit. Following mechanical wounding of tomato leaves a pTOM 13-homologous RNA shows rapid induction within 30 minutes, which occurs before maximal ethylene evolution (2-3h). This RNA also accumulates following the wounding of green tomato fruit. Northern blot analysis of poly(A)$^+$ RNA indicates that the length of the mRNA is about 1400 nucleotides. Nucleotide sequence analysis showed the cDNA insert to contain the complete coding region of the pTOM 13 protein (33.5kD) and an unusual 5' structure of ten dT-nucleotides. Hybridisation of the pTOM 13 cDNA insert to Southern blots of tomato DNA indicates the presence of only a small number of homologous sequences in the tomato genome.

INTRODUCTION.

The phytohormone ethylene is associated with many aspects of plant growth and development, including leaf senescence, fruit ripening, and responses to environmentally induced stress (1). Ethylene evolution is accompanied by a change in the activity of several enzymes (2, 3), and the appearance of new mRNAs (4). The administration of ethylene to plant tissues has been shown to change several facets of cellular biochemistry. These include an increase in respiration and protein synthesis, and increase in the amount of poly(A)$^+$ RNA per μg total RNA, and the transcription of previously quiescent genes (5).

Fruit ripening is a developmentally controlled process which involves coordinated changes in cell physiology and biochemistry. During the early stages of ripening ethylene is produced autocatalytically (3, 6) and triggers many of the changes associated with ripening. As fruit ripen, several mRNAs that are absent from unripe fruit increase in abundance, including those encoding the cell-wall softening enzymes polygalacturonase from tomato (4, 7) and cellulase from avocado (29).

Ethylene synthesis is also associated with the initial response of plant tissue to mechanical wounding (8). This response also involves the accumulation of previously unexpressed mRNAs (9, 10, 11), and an increase in total poly(A)$^+$ RNA (9). mRNAs homologous to a cDNA

clone (pTOM 13) derived from a ripe-tomato-specific cDNA library (7) have been shown to appear following the wounding of leaf material and unripe tomato fruit (10). In all three cases poly(A)$^+$ RNA hybrid-selected by pTOM 13 translated in vitro to give a protein of 35 kD.

Ethylene synthesis during ripening and following wounding occurs by the same pathway. Methionine is converted to S-adenosylmethionine (SAM), and from SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and finally ethylene (8, 12). ACC synthase is the only known enzyme in this pathway that needs to be formed for ethylene synthesis to occur. Its expression may be regulated at the level of RNA accumulation (1, 13) or enzyme activation (14).

In this paper we report the characterisation of a cDNA clone (pTOM 13) whose homologous mRNA is expressed during tomato fruit ripening and after the wounding of leaf and green fruit material, and discuss its possible relevance to ethylene biosynthesis.

MATERIALS AND METHODS.

Plant Material.

Tomato (Lycopersicon esculentum Mill. cv. Ailsa Craig) plants were grown under virus-free conditions as previously described (4). Fruit used in ripening experiments were picked at various stages of development. Their lycopene content (15) and ethylene production were measured, and these fruit were then immediately frozen in liquid nitrogen. Unripe fruit pericarp and young leaves were wounded by cutting into roughly 0.5 cm^2 sections with a sterile scalpel. After suitable time periods ethylene production was measured, and the plant material was frozen in liquid nitrogen.

mRNA isolation and analysis.

Tomato fruit RNA was isolated as previously described (4). Total nucleic acids were isolated from young tomato leaves according to Grierson and Covey (16). Poly(A)$^+$ RNA was isolated using one cycle of oligo dT-cellulose chromatography (17) following purification through one cycle of Sigmacell-50 cellulose to remove polysaccharides (18). The polyA-containing RNA was denatured using glyoxal (19) and was electrophoresed through a 1.5% agarose gel and transferred to Genescreen (New England Nuclear) for northern hybridisations, or directly applied to the membrane for RNA dot-blots, as described by the manufacturer. DNA control dots were applied in the same way, having first been denatured by incubation of known amounts of the cDNA in 0.2M NaOH for 15 minutes at room temperature.

RNA dot blots were pre-hybridised in 5 x Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% Bovine Serum Albumin), 0.1% SDS, 250 $\mu g.ml^{-1}$ denatured salmon sperm DNA at 65°C for 4h, and were then hybridised to the nick-translated cDNA insert (20) ($1x10^8$ cpm.μg^{-1} DNA) in 1 x Denhardt's, 0.1% SDS, 250 $\mu g.ml^{-1}$ denatured salmon sperm DNA, 10 x N.T.E. (0.3M NaCl, 0.06M Tris-HCl pH=8.0, 2 mM EDTA) at 65°C for 16h. Membranes were washed for 5 minutes in 10 x N.T.E., 0.1% SDS at room

temperature, followed by 1h at 65°C in 10 x N.T.E., 0.1% SDS, and finally 1 x N.T.E., 0.1% SDS for a further 1h. Membranes were autoradiographed for 1−3 days at −70°C using pre−flashed Kodak X−Omat film. The resulting autoradiograms were quantified by scanning with a Joyce−Loebl densitometer according to the manufacturers recommendations.

cDNA sequence analysis.

Restriction endonuclease (Amersham International PLC) digested pTOM 13 cDNA insert was cloned into suitably digested M13 mp 8 and 9 DNA (21) and grown up in E. coli TG−2. DNA sequence determination by dideoxy−nucleotide chain termination using [^{32}p] dATP (400 ci/mmol, Amersham) was as previously described (22). Sequence data was analysed using the computer programs of Staden (23).

Tomato chromosomal DNA isolation and analysis.

Tomato DNA was extracted from young tomato leaves according to Dellaporta et al. (24). 5 μg samples of DNA were digested overnight with restriction endonucleases, and fractionated on 0.8% agarose gels. Gels were denatured in 0.2M NaOH, 0.6M NaCl for 45 minutes and capillary blotted on to Hybond membranes (Amersham) presoaked in 2 x SSPE (17). Membranes were prehybridised in 5 x SSPE, 5 x Denhardt's, 0.1% SDS, 100 μg.ml^{-1} denatured salmon sperm DNA for 4h at 65°C, and hybridised in the same solution containing the nick−translated cDNA insert at 65°C for 16h. The membranes were washed in 2 x SSPE for 5 minutes at room temperature, followed by 1h at 65°C in 2 x SSPE, 0.1% SDS, and then 30 minutes at 65°C in 0.2 x SSPE, 0.1% SDS. Membranes were autoradiographed at −70°C for 2−7 days.

RESULTS

pTOM 13−homologous RNA expression

The cDNA clone used in this report was derived from a ripe−tomato−specific cDNA library (7). This cDNA hybridises to a poly(A)$^+$ RNA species from red tomato fruit of 1400 nucleotides, that is absent from green fruit (18). Hybridisation of the pTOM 13 cDNA insert to poly(A)$^+$ RNA derived from ripening fruit and wounded leaves has demonstrated that in both cases an homologous RNA accumulated (10, 18). The pTOM 13−complementry RNA was expressed throughout fruit ripening (Fig. 1). Accumulation began at the time of autocatalytic ethylene production exhibiting a pre−maximum peak, increased to a peak in orange fruit, then declined as fruit became red. Ethylene evolution reached its maximum in orange fruit and then declined. Following wounding of tomato leaves a pTOM 13−homologous mRNA rapidly accumulated from an undetectable level in unwounded leaves, to a maximum one hour after wounding (Fig. 1). Three hours after wounding this RNA had declined to a low level. Ethylene evolution in this response began within the first thirty minutes following wounding, reached its peak after 2−3h, and thereafter gradually declined.

mRNA complementary to the pTOM 13 cDNA insert represented about 0.04% of the total

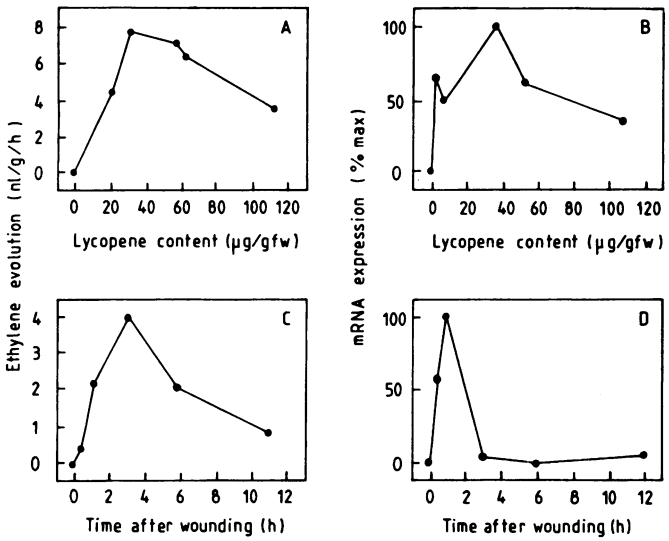

FIGURE 1.
Ethylene synthesis and pTOM 13−homologous RNA expression.
(A) Ethylene synthesis and (B) pTOM 13−homologous RNA accumulation during tomato fruit ripening (C) Ethylene synthesis and (D) pTOM 13−homologous RNA accumulation following leaf wounding. RNA dot blots were quantified using a Joyce−Loebl densitometer to scan X−rays films. Each point represents the average of three experiments.

poly(A)$^+$ RNA population in orange fruit (Fig. 2), and one tenth of this value in leaves 45 minutes after wounding (<0.01%). The level of the pTOM 13−complementary RNA in wounded green tomato fruit, which evolved ethylene to a similar level as leaves (data not shown), was about 0.02% of the poly(A)$^+$ RNA population.

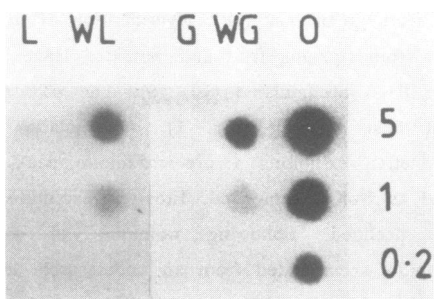

FIGURE 2.
The relative abundance of pTOM 13−homologous RNAs during ripening and in response to wounding. Autoradiograph of dot−blots of 5µg, 1 µg and 0.2 µg samples of poly(A)$^+$ RNA hybridised to the pTOM 13 cDNA insert. G: green fruit, WG: green fruit 45 minutes after wounding, L: leaf, WL: leaf material 45 minutes after wounding O: orange fruit.

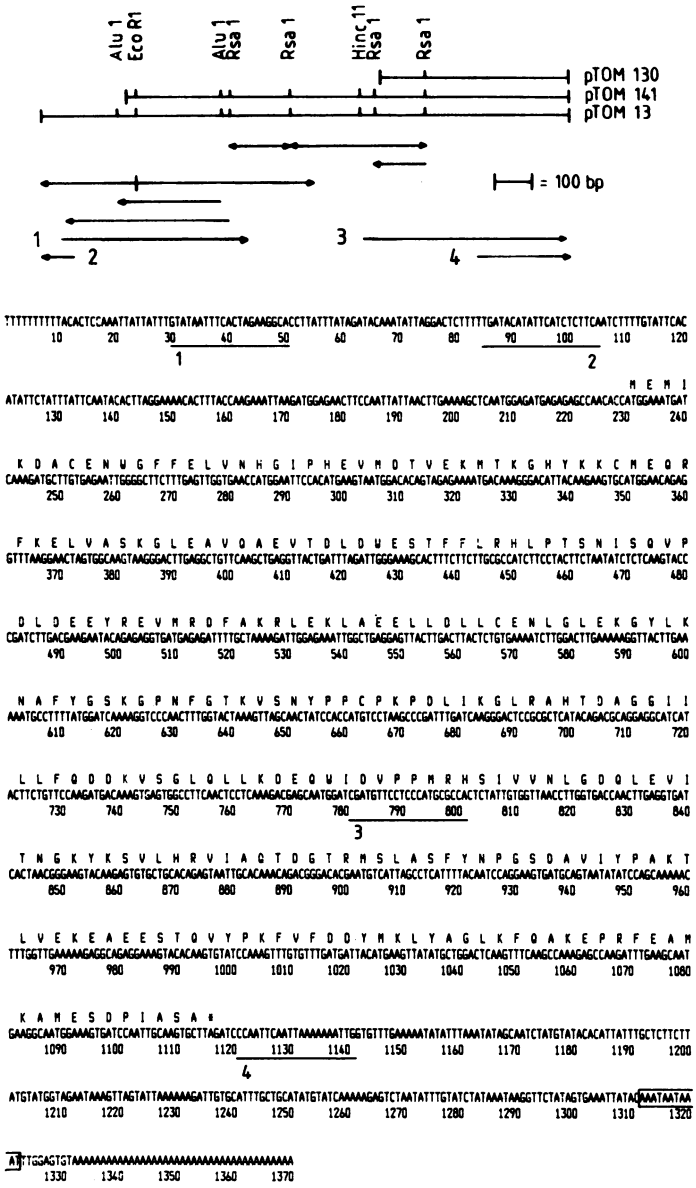

FIGURE 3.
Structure of the pTOM 13 cDNA insert. Restriction map of the pTOM 13, 141 and 130 cDNA inserts and sequencing strategy (upper). Numbers indicate the positions of synthetic oligonucleotides used for sequencing. Nucleotide sequence and derived amino−acid sequence of pTOM 13 cDNA insert (lower). Boxed nucleotides indicate the position of a putative polyadenylation signal. Underlined nucleotides indicate the position of synthetic oligonucleotides used in the sequencing strategy.

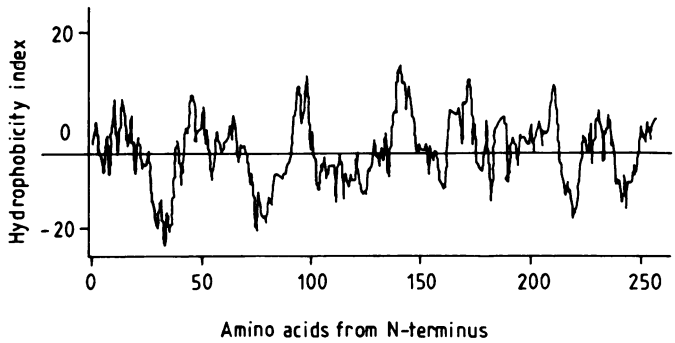

FIGURE 4.
Hydropathy profile for the pTOM 13-derived protein sequence. Hydrophilic domains are below the line, hydrophobic domains above (30).

Structure of the pTOM 13 cDNA.

Two cDNAs from the ripe-tomato-specific cDNA library (7) cross-hybridise with the pTOM 13 cDNA insert (pTOM 130 and pTOM 141). pTOM 13 contains the largest insert of 1400 bases, approximately the same size as the hybridising RNA species from red fruit (18). The restriction enzyme cleavage sites of the pTOM 13 cDNA insert were determined (Fig. 3). These were used to subclone fragments of the insert into the M13 vectors mp8 and 9 (21). The sequencing strategy utilized both the universal primer (Amersham) and several synthetic oligonucleotides made to the pTOM 13 insert sequence.

The sequence is 1370 nucleotides long, and contains a poly(A) tail of about 30 bases. The 5' end of the cDNA insert contains an oligo dT-head of 10 nucleotides. Between 10-21 nucleotides from the 3' end are several putative polyadenylation signals, including the animal consensus sequence (AATAAA) (25), and the most conserved plant polyadenylation signal (AATAAT) (26).

The longest open reading frame within the cDNA insert codes for a protein of 295 amino-acids, with a molecular weight of 33,512. The hydropathy plot of the pTOM 13-derived protein (Fig. 4) shows it to contain an even distribution of hydrophobic and hydrophilic regions, and not to contain an N-terminal hydrophobic region, which is usually associated with transit peptides.

Genomic organisation of pTOM 13-related sequences.

Tomato genomic DNA was cut with several restriction enzymes and fractionated on a 0.8% agarose gel, before being transferred to Hybond (Amersham). The resulting filter was hybridised to the nick-translated pTOM 13 cDNA insert. The filter was washed under the same high stringency conditions as RNA dot blots to reduce hybridisation to partially related sequences. One hybridising band of 20 kbp was visible for tomato DNA cut with Bgl II (Fig. 5), whereas Eco R1 produced four bands of which two hybridised strongly, and three were

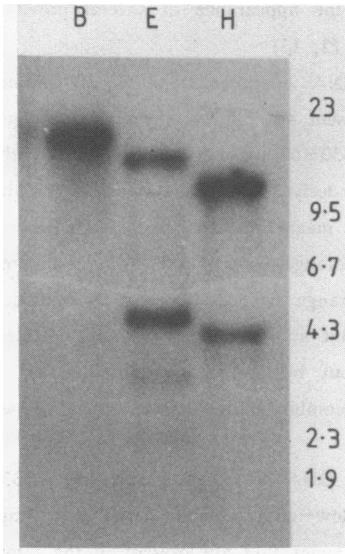

FIGURE 5.
Southern analysis of tomato genomic DNA. DNA (5 μg) was cut with Bgl II (B), Eco RI (E) and Hind III (H). The nick-translated pTOM 13-cDNA insert was used to probe the blot. Numbers refer to the positions of labelled λDNA fragments cut with Hind III and used as size markers (kbp).

generated by Hind III, one of which hybridised weakly. The cDNA insert itself contains one Eco R1 site. These data imply that the corresponding sequence is represented in the tomato genome as a low copy number gene.

DISCUSSION.

The phytohormone ethylene is involved in several plant responses that result in a change in gene expression (27,18). Fruit ripening represents a developmental process during which several new mRNAs accumulate, following an autocatalytic burst of ethylene production (4, 18). pTOM 13 represents one fruit-specific mRNA (18) which is absent from unripe green fruit and rapidly accumulates in the initial stages of ripening, in concert with ethylene and lycopene synthesis.

Wounding of ripening tomatoes, leaves, and storage (tuber) tissue induces several changes in aspects of cellular biochemistry/physiology (9, 13). These can be separated into those responses which occur shortly after wounding, and those which occur after a longer lag period. Shirras and Northcote (9) have demonstrated that the mechanical wounding of potato tuber tissue causes a slow accumulation of several mRNAs which reach maximum expression 16-24h after wounding. Ethylene evolution represents a much more rapidly induced response

(28) which is correlated with the appearance of several previously unexpressed mRNAs (10) and the enzyme ACC synthase (2, 13).

The pTOM 13−homologous RNA is expressed within 30 minutes following the wounding of tomato leaves, and is also present 45 minutes after wounding green fruit material. Maximum expression is reached between 30−60 minutes after wounding, the mRNA then declines. This suggests that the pTOM 13−derived protein is present in the cell during the stages of greatest ethylene evolution. At maximum expression this RNA represents about 0.04% of the poly(A)$^+$ RNA population in orange fruit. There is approximately ten times as much pTOM 13−complementary RNA in orange fruit as there is in wounded leaves. Even so, dot−blot analysis of RNA from ripe fruit and wounded material indicates that it is a low abundance species. This is also borne out by the observation that the original ripe−tomato−specific cDNA library (7) of 2000 recombinants contained only two which cross−hybridised to the pTOM 13 cDNA insert.

Genomic Southerns of tomato DNA demonstrate that the pTOM 13 sequence is represented in the tomato genome as a low−copy number gene. It is important to measure the exact number of pTOM 13−related genes that are present in the tomato genome in order to know which genes are expressed in specific situations. The presence of one functional gene would imply promoter sequences that control expression of the gene during both ripening and in response to wounding. Alternatively there could be two similar genes with different promoter sequences. We are currently investigating these possibilities. Preliminary sequencing of genomic clones indicates the presence of more than one pTOM 13−related gene (data not shown).

Sequence analysis of the pTOM 13 cDNA shows it to contain the usual poly(A) addition signals (25, 26) and an unusual 5' poly dT−head which may affect the stability or translation of the RNA. The largest open reading frame encodes a protein of 33.5 kD, which is similar to the 35 kD size already estimated by hybrid−release translation of RNA from red tomato fruit, wounded leaves and wounded green fruit (7, 10). As it contains no large hydrophobic moieties it is probable that the protein is located in the cytosol and is not associated with or transported across membranes.

The observation that this RNA is expressed during ripening and following wounding implies that it may encode a protein involved in the synthesis and/or regulation of ethylene. Those enzymes which have been studied in greatest detail include ACC synthase, the ethylene forming enzyme, and malonyl ACC synthase (8). The pTOM 13−derived protein may represent one of these enzymes, or others involved in the recycling of sulphur in the ethylene biosynthetic pathway (8). Further work on the characterisation of these enzymes will be necessary to determine whether the pTOM 13−complementary RNA encoded protein has a function in ethylene synthesis and metabolism.

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