

# Characterisation of an *endo*-(1,4)- $\beta$ -mannanase (*LeMAN4*) expressed in ripening tomato fruit

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## Abstract

A cDNA, *LeMAN4*, containing an open-reading frame of 1185 bp and putatively encoding an *endo*-(1,4)- $\beta$ -mannanase (EC 3.2.1.78) was isolated from red tomato (*Lycopersicon esculentum* Mill.) fruit. Native PAGE IEF of fruit protein extracts revealed a single mannanase of pI 9, close to the predicted pI of 8.8 for the mature protein ( $M_r$  of 42,400) encoded by *LeMAN4*. In mature green fruit *LeMAN4* mRNA was detectable, increased markedly at the breaker stage and remained at high levels thereafter. *endo*-(1,4)- $\beta$ -Mannanase activity, however, was almost undetectable in the early stages of ripening and did not increase significantly until the pink and red stages of ripeness, possibly reflecting post-transcriptional regulation of the enzyme. Cell wall analysis revealed no net change in non-cellulosic mannosyl residues during ripening, suggesting cell wall mannans may not be the *in vivo* substrate of this enzyme. Southern analysis indicated that *LeMAN4* is a single copy gene although several weakly hybridising bands imply the presence of other related genes. *LeMAN4* is not fruit-specific, being expressed in flowers but not in leaves, and is probably not rate-limiting in the ripening-related softening of tomato fruit. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** *endo*-(1,4)- $\beta$ -Mannanase; Tomato; Fruit ripening; Cell wall

## 1. Introduction

Tomato enjoys the status of being the model fruit for studying the several processes which together constitute ripening. One of these, ripening-related softening, has been more intensely studied in this fruit than any other and yet to date the underlying biochemical changes in the fruit cell wall which achieve this remain elusive [1].

Solubilisation of cell wall pectins certainly accompanies tomato ripening but using polygalacturonase and pectin methyl esterase antisense genes to inhibit or modify expression of these enzymes was shown to have little effect on fruit softening (for review see [1]). Part of the problem with this antisense approach may be the myriad forms of a particular enzyme which may be present, as in the case of  $\beta$ -galactosidase where some

seven genes are expressed in tomato fruit [2]. Other contenders for regulating softening in tomato include *endo*-(1,4)- $\beta$ -glucanases, xyloglucanases, xyloglucan *endo*-transglycosylases and expansins [1]. None of these has yet been identified as the key regulator and it may well be that a complex process such as fruit softening is not controlled by a single rate-limiting step.

*endo*-(1,4)- $\beta$ -Mannanase (EC 3.2.1.78) is one cell wall hydrolase identified some time ago in ripening tomato [3] but which has received less attention. Its presumed substrates, unsubstituted mannans and the heteromannans, galactomannan, glucomannan and galactoglucomannan, become important storage reserves in some seeds, including tomato [4], and certain monocotyledonous bulbs [5] but are present in relatively low amounts in the hemicellulosic fraction of more typical cell walls [1]. Nonetheless, just as a chain is only as strong as its weakest link, it is conceivable that this minor class of cell wall polysaccharides could play a key strengthening role in fruit cell walls and be an important target in ripening-related softening.

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In this paper, we re-investigate the development of *endo*-(1,4)- $\beta$ -mannanase activity in ripening tomato fruit, characterise the gene encoding this activity and analyze its expression, looking for possible changes in its potential cell wall substrate.

## 2. Materials and methods

### 2.1. Plant material

Tomato (*Lycopersicon esculentum* Mill. cv. Castalia) fruit at the green and breaker stages, were obtained from a commercial grower in Almeria, Spain, and were used directly or after storage at 20 °C, at the following ripeness stages: - mature green, breaker, turning, pink, light red, red [6] and over-ripe (red fruit stored 1 week at 20 °C). Fruit firmness was measured with a fruit pressure tester EFFEGI model FT 327 with 0.5 cm<sup>2</sup> tip at three locations along the equatorial diameter of five representative fruit of each firmness stage. Outer pericarp tissue was cut into segments of about 1 cm<sup>3</sup>, frozen in liquid nitrogen and stored at -80 °C prior to use. Seeds of the same cultivar were germinated on moistened filter paper at 25 °C to provide 4-day-old seedlings and mature greenhouse plants from which leaves and flowers were harvested.

### 2.2. Enzyme extraction and assay

Triplicate 200 g samples of pericarp tissue from all seven ripeness stages were each homogenised in a domestic blender in 300 ml of H<sub>2</sub>O and the homogenates, adjusted to pH 3, were centrifuged. The insoluble material was washed with 200 ml of H<sub>2</sub>O at pH 3 and finally stirred with 200 ml H<sub>2</sub>O at pH 1.6 for 1 h [3]. The slurries were centrifuged and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> added to each supernatant to 80% of saturation. The precipitated proteins were collected by centrifugation, dissolved in a minimal volume of 0.15 M NaCl and dialysed against the same overnight. The dialysates, centrifuged to remove insoluble material, were used as enzyme extracts. All steps were carried out at 4 °C with centrifugations at 8000  $\times$  *g* for 20 min. Tomato seedling protein extracts were prepared as described previously [7] but using 0.3 M NaOAc, pH 5, extraction buffer.

*endo*- $\beta$ -Mannanase was assayed viscometrically at 20 °C by adding a 0.1 ml aliquot of the enzyme extract to 0.9 ml of 0.5% (w/v) locust bean galactomannan (Sigma) in 50 mM NaOAc, pH 4.8, in a Cannon–Manning 200 semi-micro viscometer (Cannon Instrument Co., State College, PA). Activity was calculated over the initial 20% fall in relative viscosity, with one unit of enzyme activity defined as the amount of enzyme causing a 1% drop in viscosity in 1 h at 20 °C. Aliquots of red stage extracts were also incubated at 40 °C for

0.5 h with 0.1% (w/v) mannan, konjac glucomannan (Megazyme, Bray, Ireland) or locust bean galactomannan in 50 mM NaOAc, pH 4.8, and assayed for increase in reducing groups [8].

### 2.3. Isoelectric focussing

Native IEF-gel electrophoresis was performed using a mini-PROTEAN II (Bio-Rad Laboratories, Richmond, CA). Tomato pericarp extracts (concentrated 10  $\times$ ) together with an extract of 4-day-old tomato seedlings were loaded onto a 6% (w/v) acrylamide, 10% glycerol, 5% ampholyte (pH 3–10; Amersham Pharmacia, Uppsala, Sweden) gel and focused at 4 °C for 1.5 h at 180 V and 0.5 h at 300 V. After electrophoresis, a terminal strip was excised from the IEF gel and cut into 5 mm long segments. Segments were dissolved in 0.5 ml H<sub>2</sub>O and the pH of these solutions measured to verify proper focusing. The gel was then equilibrated for 5 min in 0.1 M NaOAc buffer (pH 4.5) and placed on top of an activity gel consisting of 0.6% (w/v) soluble azo-carob galactomannan (Megazyme, Bray, Ireland) in 0.05 M NaOAc buffer (pH 4.5) solidified with 1.2% (w/v) agarose. The gel sandwich was sealed in a moist chamber, incubated overnight at 37 °C and the activity gel destained [9] and photographed.

### 2.4. Cell wall analyses

Cell wall material (CWM) was prepared from quadruplicate outer pericarp samples (10 g) of each ripeness stage as described previously [10] and 20 mg aliquots were hydrolysed for 1 h at 121 °C with 2 N TFA containing myo-inositol as internal standard. The resulting sugars were converted to alditol acetates [11] and separated isothermally at 240 °C using a gas chromatograph (PU4550; Unicam Chromatography, Cambridge, UK) fitted with FID and a 4 mm  $\times$  1.5 m glass column packed with 3% SP-2340 on 100/120 Supelcoport (Supelco, Bellefonte, PA). Quantification was achieved with an integrator (PU4811; Unicam Chromatography, Cambridge, UK).

### 2.5. DNA and RNA extraction

Tomato genomic DNA was extracted from leaves as described previously [12]. Total RNA from tomato pericarp was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany).

### 2.6. *LeMAN4* cDNA cloning

A partial copy of the fruit *LeMAN4* mannanase cDNA, was obtained by first strand cDNA synthesis from RNA from red tomato pericarp using oligo(dT) as primer and subsequent PCR amplification using the

Table 1  
Primers (5' → 3') used in the cloning of *LeMAN4* and for RT-PCR

Name	Sequence
<i>Cloning of bar5 sequence</i>	
Man1	GCATTACAAATATCACCTGGT
Man2	ACCATAAAATCCCTCCATTCC
<i>Cloning of full length sequence</i>	
Man3	GCAGAAATTAACAAGTGATG
Man4	CATCACTTGTTAATTTCTGC
Man5	CTCCTTTATGCATTATTA
3'RT	GGCCACGCGTCGACTAGTAC(T) <sub>17</sub>
<i>RT-PCR</i>	
Man6	CTCATGGTGG TTCTAGACCTTTAC
Man7	CCAAGAGATGATTTGAATCTATTG
DM155	GTCTCTGATCAGAAATCCTTCTATC
DM156	CATGTCAAATTTCACTGCTTCATCC

primers Man1 and Man2 (see Fig. 4 and Table 1), derived from the *LeMAN1* tomato seed mannanase sequence [13]. First strand cDNA synthesis and amplification were carried out as described previously [14], except that the annealing temperature was 56 °C. The resulting 447-bp fragment (designated *bar5*) and all subsequent PCR products were cloned into the pGEM®-T Easy vector (Promega, Madison, USA), and sequenced as described below. To amplify the 3'-cDNA end of *bar5*, total RNA (2 µg) was reverse-transcribed using the 3'RT primer specific for the poly(A) tail of the mRNA. The cDNA was used as template for PCR amplification employing the same 3'RT primer used previously and the upstream gene-specific primer Man3. The missing 5'-terminal region was amplified with a 5'RACE kit (Gibco-BRL, Rockville, MD) using primer Man4 and the conditions specified by the manufacturer. This amplification yielded an incomplete sequence which had an overlapping and identical sequence with tomato EST, TC94100. The 5'-region of this EST was used to design the Man5 primer which in conjunction with primer Man4 amplified a 508 bp PCR product which was used to reconstruct clone *LeMan4* by *Eco*RI religation of the respective 5'- and 3'-end fragments.

### 2.7. DNA sequencing and sequence analysis

Nucleotide sequences were determined using an automated laser-fluorescent DNA-sequencer system (ABI PRISM® 377 DNA sequencer). Nucleotide and protein sequence comparisons were performed with BLAST searches [15]. Signal sequence predictions were carried out using the interactive web site SignalP [16]. Multiple sequence alignments were performed using the CLUSTALW program [17].

### 2.8. RT-PCR analysis

Reverse transcription-PCR analysis was performed in a single tube. First strand cDNA was synthesised from total RNA in a 20 µl reaction volume containing 125 ng of total RNA, 100 units of M-MLV reverse transcriptase (Promega), 100 ng oligo-(dT)<sub>15</sub> primer (Promega), 20 U RNase inhibitor, 0.2 mM of each dNTP in the buffer supplied with the enzyme. The reaction was carried at 37 °C for 60 min. As an internal control, the RNA template GeneAmplicer® pAW109 (Perkin Elmer, Boston, MA) was added to each reaction tube. PCR amplification was performed by adding 80 µl of a common PCR mixture containing the *LeMAN4*-specific primers Man6 and Man7, together with the RNA control specific primers GeneAmplicer® DM155 and DM156 (Perkin Elmer, Boston, MA) to the reverse transcribed RNA. PCR conditions comprised an initial denaturing step at 94 °C for 3 min, followed by 35 cycles of incubation at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min and a final elongation step of 10 min at 72 °C.

### 2.9. DNA gel-blot analysis

Tomato genomic DNA (10 µg) was digested with restriction enzymes, fractionated on a 0.8% agarose gel and transferred onto a nylon membrane (Hybond-N, Amersham). The DNA probe corresponding to the coding region from positions +508 to +869 of the *LeMAN4* gene, was radiolabelled using a random-primed DNA labelling kit (Boehringer, Mannheim, Germany). Hybridisation was carried out as described previously [18]. Hybridisation temperature was 42 °C, and filters were washed with 3 × SSC, 0.5% SDS at 65 °C.

## 3. Results and discussion

### 3.1. β-Mannanase activity during fruit ripening

Very low levels of *endo*-(1,4)-β-mannanase activity were measured in fruit in the early stages of ripening of Castalia tomato. Enzyme activity then increased significantly at the pink stage and finally peaked at the red stage, declining by the over-ripe stage (Fig. 1A). A broadly similar trend was reported in previous investigations of tomato fruit mannanase whether using low pH [3,19] or high salt [20] extractants. Corresponding to the delayed rise in mannanase activity, there was also a lag before pericarp firmness declined (Fig. 1B) but the largest increase in mannanase activity was seen as fruit turned from light red to red while the sharpest fall in firmness occurred at the preceding pink to light red transition. This anomaly suggests that mannanase is not

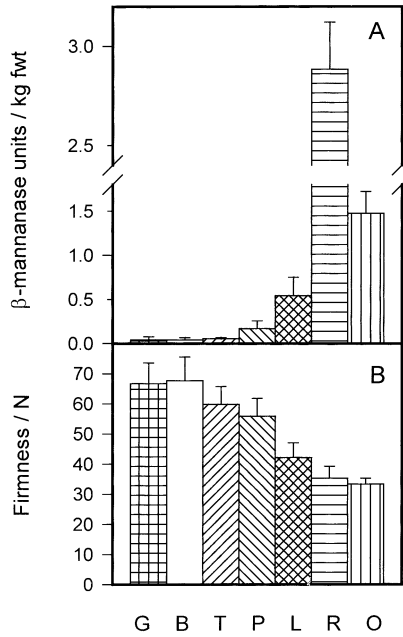


Fig. 1. *endo*-(1,4)- $\beta$ -Mannanase activity (A), as measured viscometrically (mean of 3 replicates  $\pm$  S.D.), and firmness (B), as measured by penetrometer (mean of 5 fruit  $\pm$  S.D.), of pericarp of ripening tomato fruit. G, mature green; B, breaker; T, turning; P, pink; L, light red; R, red; O, overripe.

the rate-limiting enzyme in ripening-related softening of tomato.

When tomato protein extracts were subjected to native PAGE IEF, and *endo*-(1,4)- $\beta$ -mannanase activity was revealed through clearing in an azo-galactomannan overlay, a similar pattern of activity emerged (Fig. 2). Extracts from green and breaker fruit showed no such activity but clearing became just detectable at the turning stage, increasing to a maximum in red fruit and declining again in the over-ripe fruit (Fig. 2). Only a single isoform seemed to be present in the fruit extracts with a *pI* of approximately 9, which is reasonably close to the *pI* of 9.3 determined for this enzyme in another tomato cultivar [3]. Using a single high salt extraction it has recently been claimed that the skin of tomato fruit also contains a minor acidic isoform of mannanase [21].



Fig. 2. Native PAGE IEF of tomato *endo*- $\beta$ -mannanases resolved on a broad range pH 3–10 gel and visualised using an azo-galactomannan overlay. Equal amounts of crude protein extract were loaded in each lane from ripening fruit (G, mature green; B, breaker; T, turning; P, pink; L, light red; R, red; O, overripe) and 4-day-old seedlings (S).

That study did not show whether this acidic form was also present in skin-free pericarp extracts but we were certainly unable to detect any such acidic isoform in our pericarp extracts (Fig. 2) even though we had no difficulty in detecting similar acidic isoforms [7] in our tomato seedling extract (Fig. 2).

### 3.2. Cell wall compositional changes

Analysis of the non-cellulosic neutral sugar composition of cell walls of ripening tomato revealed the consistent presence of 6 monosaccharides. Of these, only Ara and Gal showed any change with ripening, both declining significantly during ripening (Fig. 3B,E) as reported previously [22,10]. Neither Rha, Xyl nor Man showed any significant change during ripening (Fig. 3A,C,D) while Glc showed 2 peaks (Fig. 3F), probably reflecting transient starch accumulation. The absence of any net decline of cell wall Man contrasts with the massive upsurge in mannanase activity measured (Fig. 1A) but is consistent with results of previous analyses of walls of ripening tomato fruit [22,10].

### 3.3. Identification and cloning of a $\beta$ -mannanase cDNA in tomato fruit

To isolate the gene for fruit mannanase, a 446 bp fragment was amplified by RT-PCR from total RNA of red tomato fruit using primers derived from the tomato seed *LeMAN1* sequence [13]. This PCR product, termed *bar5*, was cloned, its sequence confirmed as a likely  $\beta$ -mannanase and its full-length sequence obtained by a combination of 5'-RACE, 3'-RACE and further PCR. The final full-length clone obtained, *LeMAN4* (acces-

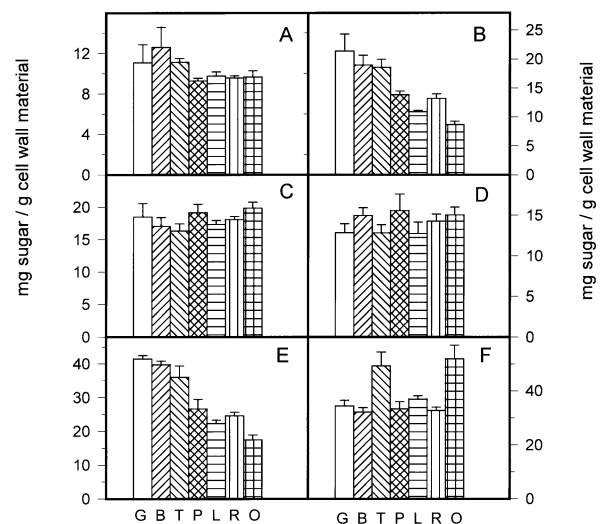


Fig. 3. Non-cellulosic neutral sugar composition (A = Rha, B = Ara, C = Xyl, D = Man, E = Gal, F = Glc) of cell wall material purified from ripening tomato fruit (G, mature green; B, breaker; T, turning; P, pink; L, light red; R, red; O, overripe). Mean of 4 fruit  $\pm$  S.D.

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CTCCTTTATGCATTATTAATAAAAAATATTGATGAATAACTCAATCATCTTAATTTTTGTGCTATTTTAATAATATTTC 80
      M N N S I I L I F V A I L I I F P
AAATGAATTTAGCAAGCCTACTAGAGCTTTTCAAATAATAATTTTGTTTATACAGATGGAACCTATTTTGCATTAATG 160
      N E F S ▲ K P T R A F S N N N F V Y T D G T H F A L N
GAAAGTCACTTTATATAAATGGTTTTAATGCATATTGGTTAATGTATATAGCTTATGATCCATCAACAAGAATAAAGTT 240
      G K S L Y I N G F N A Y W L M Y I A Y D P S T R I K V
ACAAATACATTCACAAGCTTCTAAATATAAAATGAATGTTGCTAGAACTTGGGCTTTTTCTCATGGTGGTTCTAGACC 320
      T N T F Q Q A S K Y K M N V A R T W A F S H G G S R P
TTTACAATCTGCACCTGGTGTTTACACGAACAATGTTTCAGGGATTGGATTTTGTGATATCAGAAGCTAAAAAATATG 400
      L Q S A P G V Y N E Q M F Q G L D F V I S E A K K Y
GAATTCACCTTAATATGTCATTAGTCAATAATTGGGATGCTTTTGGAGGAAAGAAACAATATGTAGAATGGGCAGTCAA 480
      G I H L I M S L V N N W D A F G G K K Q Y V E W A V Q
AGAGGGCAGAAATTAACAAGTGTATGATTTCTTCACTAATCCTATGGTCAAAGGATCTACAAAAATAATGTCAGGT 560
      R G Q K L T S D D D F F T N P M V K G F Y K N N V K V
TGTGCTTACAAGAGTGAATACAATAACCAAGTAGCATATAAAGATGATCCAACAATCTTTTCATGGGAATTAATAATG 640
      V L T R V N T I T K V A Y K D D P T I L S W E L I N
AGCCTAGATGCCCTCTGACCTCTCTGGGAAAACATTTCAAGACTGGGTTTTAGAAATGGCGGGATATTTGAAATCAATA 720
      E P R C P S D L S G K T F Q N W V L E M A G Y L K S I
GATTCAAATCATCTCTTGGAGATTGGACTTGAAGGATTTTATGGGAATGACATGAGACAATACAATCCTAATCTTACAT 800
      D S N H L L E I G L E G F Y G N D M R Q Y N P N S Y I
TTTTGGGACTAATTTTATCTCCAACAATCAAGTTCAAGGAATTGATTTTGCACAATTCATATGTACCCATAATCAATGGT 880
      F G T N F I S N N Q V Q G I D F A T I H M Y P N Q W
TGCCAGGTTTAACTCAAGAGGCCCAAGACAAATGGGCTTCACAATGGATCCAAGTCCATATAGATGACTCCAAAATGTTG 960
      L P G L T Q E A Q D K W A S Q W I Q V H I D D S K M L
AAAAAGCCCTTATTAATTGCAGAATTTGGCAAGTCTACAAAACCCAGGGTACTGTTGCAAAGAGGGGATAATATTT 1040
      K K P L L I A E F G K S T K T P G Y T V A K R D N Y F
TGAAAAAATATATGGAACCATTTTTAATTTGCCCCAAAGTGGAGGCCCATGTGGTGGTGGGCTTTTTTGGCAAGTATTGG 1120
      E K I Y G T I F N C A K S G G P C G G G L F W Q V L
GCCAAGGAATGTCAGTTTTGTATGATGGTTATCAAGTGGCTTTCGAAGAGAGCCCATCAACTTCTAGAGTTTACTCTTA 1200
      G Q G M S S F D D G Y Q V V L Q E S P S T S R V I L L
CAATCTCTGGCTCTCTAAGCTATCATAGAAACAAAAATAGATTGAAGGCTATAGTAGTATTTTTAAGATCCATGAAAAGT 1280
      Q S L A L *
TGTTTGGGTTGGCATATACTATTAAATCATCTGGTTGAGTTATAGTATTGATTATAAATATGTATAAATACTTTGTATACA 1360
TGTTATTTATTAATAATAAATAAAGTATTTTGTGTTGAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1426

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Fig. 4. Nucleotide sequence and deduced amino acid sequence of *LeMAN4* cDNA (AY034075). The deduced amino acid sequence is shown below the nucleotide sequence and the location of PCR primers used for the transcript expression analysis are overlined. ▲, Putative cleavage site of the N-terminal signal sequence.

sion no. AY034075), has a size of 1426 bp and contains an open reading frame of 1185 bp that predicts a 395 amino acid polypeptide, *LeMAN4* (Fig. 4), with a molecular mass of 44.8 kDa and a theoretical *pI* of 8.67. This protein has a potential signal peptide cleavage site at residues 21–22, suggesting that the preceding 21 residues constitute a transit peptide for targeting the protein to the cell wall. The mature protein has a predicted  $M_r$  of 42,400 and a *pI* of 8.82, the latter being similar to that determined experimentally by IEF analysis (Fig. 2).

Sequence comparison against gene databases showed that the *LeMAN4* amino acid sequence is highly homologous to *endo*-(1,4)- $\beta$ -mannanases from various plant species. The deduced tomato fruit protein shares the highest homology to coffee (AJ278996) and *Arabidopsis* (ABO13389) GenBank sequences with a 62 and

54% identity, respectively (Fig. 5). The protein also shares extensive although lower identity to the post-germinative and germination-specific tomato seed  $\beta$ -mannanases, *LeMAN1* and *LeMAN2* [13,4]. *LeMAN4* shows overall less homology to other members of glycoside hydrolase family 5 such as mannanases from fungi [23], except in one regard. *LeMAN4* contains a stretch of 28 amino acids (residues 124–151) which is present in many fungal mannanases but absent from some plant mannanases (e.g. *LeMAN1*). In contrast to several microbial mannanases [24], neither *LeMAN4* nor any of the plant mannanases so far characterized have a recognisable carbohydrate-binding domain.

Eight amino acid residues are strictly conserved in the glycoside hydrolase family 5 to which this mannanase belongs. *LeMan4* shares seven of these conserved residues, the catalytic residues Asn203, Glu204 and

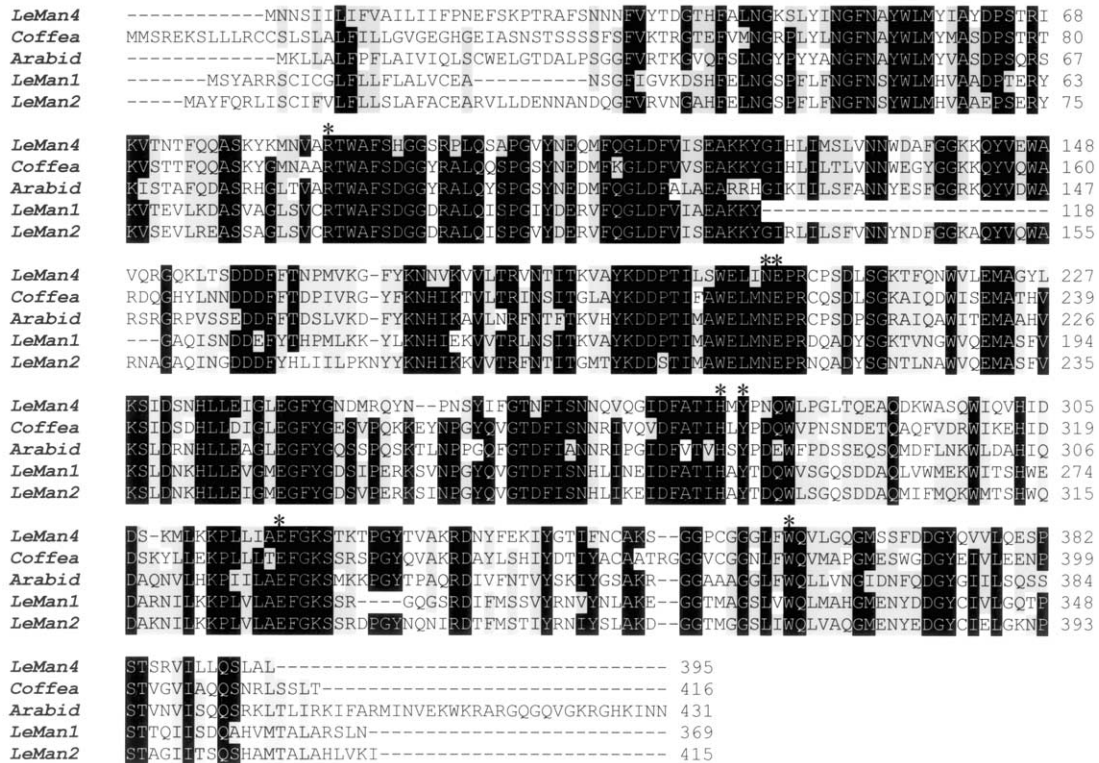


Fig. 5. Alignment of the deduced amino acid sequence of *LeMAN4* and other putative mannanases. Asterisks indicate the seven conserved catalytic residues. *LeMAN4*, from tomato fruit (accession no. AY034075); coffee, from coffee seeds (accession no. AJ278996); *Arabid*, genomic sequence from *Arabidopsis* (accession no. AB013389); *LeMAN1*, *LeMAN2*, from tomato seeds (accession nos. AF017144 and AF184238, respectively). Identical amino acid sequences and conservative amino acid changes are represented by black and grey boxes, respectively. Numerals refer to amino acid position beginning at the first ATG codon of the open reading frame. Dashes were introduced to maximize the alignment.

Glu319, and active site residues, Arg86, His277, Tyr279 and Trp360 [23]. The absence in plant mannanases of the eighth conserved residue, a His approximately midway between Arg86 and Asn203, is consistent with the observation that this residue lies on the opposite side of the molecule to the other conserved residues with no obvious functional role in catalysis or substrate binding [23].

### 3.4. Expression pattern of *LeMAN4* gene

The expression pattern of the *LeMAN4* gene was studied by RT-PCR analysis, using total RNA isolated from tomato leaf, flowers and fruit at different ripening stages. As an internal control, the RNA template pAW109 was added to each sample and coamplified along with *LeMAN4*. The primers used for RT-PCR amplified a 437-bp fragment corresponding to the *LeMAN4* cDNA and a 308-bp fragment corresponding to the control pAW109 transcript. It should be noted that the *LeMAN4* gene contains three introns in this region (data not shown) so that it would be amplified as a 1.5-kb fragment if genomic DNA rather than mRNA was the source of template.

The results (Fig. 6) show that the *LeMAN4* transcript was present in the fruit at all stages studied but at very low levels in the mature green fruit. *LeMAN4* mRNA increased markedly at the breaker stage and remained at constant elevated levels until the red and overripe stages in which a slight decrease of expression was apparent. The transcript was not detected in leaf, but a band of the appropriate size was present in flowers, which when cloned and sequenced was shown to be identical to *LeMAN4*. Two recent studies have reported a low unchanging level of mannanase mRNA in ripening tomato fruit [20,25]. However, these Northern analyses employed a seed mannanase (*LeMAN1*) probe which has only 52% sequence homology with the fruit man-



Fig. 6. *LeMAN4* mRNA levels in tomato leaf, flower and fruit detected by RT-PCR. For all samples, 125 ng of total RNA was used for RT-PCR. For fruit, RNA was extracted from outer pericarp of seen ripeness stages G, mature green; B, breaker; T, turning; P, pink; L, light red; R, red; O, overripe. Inclusion of the pAW109 transcript PerkinElmer served as a control for the RT-PCR reaction.

nanase gene, *LeMAN4*, and so must be interpreted with caution.

The upregulation of *LeMAN4* at the breaker stage is reminiscent of other tomato ripening-related genes such as *LeCEL1* and *LeEXPI* [1] which are ethylene-regulated and which apparently encode cell wall modifying activities. In view of the dramatic increase in mannanase activity during the latter stages of ripening (Figs. 1A and 2), we anticipated a parallel rise in the level of *LeMAN4* mRNA but this remained constant (Fig. 6). This suggests that either this gene is subject to post-transcriptional regulation or that the *LeMAN4* protein remains inactive or sequestered until the later stages of ripening. The latter regulatory mechanism is supported by the detection of mannanase immunologically on western blots in extracts of unripe tomatoes which showed no mannanase activity [20].

### 3.5. Southern analysis of *LeMAN4*

The number of genes encoding *LeMAN4* in the tomato genome was estimated by Southern blot analysis. Tomato genomic DNA obtained from tomato leaves was digested with the restriction enzymes *EcoRI*, *EcoRV* and *HindIII*. As shown in Fig. 7, the *LeMAN4* probe hybridized strongly to single *EcoRI* and *EcoRV* genome fragments, implying that *LeMAN4* is a single copy gene. Additional faint bands were also observed which suggest the presence of additional related sequences within the tomato genome. The pattern of

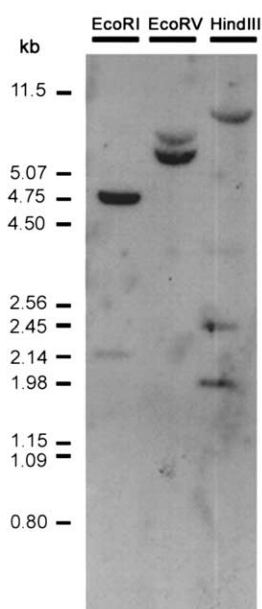


Fig. 7. Genomic DNA gel-blot analysis of *LeMAN4*. Tomato genomic DNA (10  $\mu$ g) was digested with *EcoRI* (lane 1), *EcoRV* (lane 2) or *HindIII* (lane 3) and resolved on a 0.8% agarose gel. A 363bp (nt 508–869) *LeMAN4* fragment was used as a probe.  $M_r$  markers are indicated at the left in kilobases.

hybridisation shown for the *HindIII* digest would indicate the presence of an internal *HindIII* site in the *LeMAN4* gene. As *HindIII* sites are not present in the cDNA clone, this site appears to be located within an intron. Previous Southern analyses of the related *LeMAN1* gene suggested the presence of at least three mannanase genes in the tomato genome [13,25]. That result is compatible with the fact that two tomato seed mannanase genes have been reported [4] and that several EST *endo*-(1,4)- $\beta$ -mannanase sequences from different plant tissues have been described for tomato.

### 3.6. In vivo functions of fruit mannanase

We have isolated a cDNA clone, *LeMAN4*, putatively encoding an *endo*-(1,4)- $\beta$ -mannanase from tomato fruit and characterised expression of this gene during ripening. If the increase in mannanase activity observed during ripening is due to expression of *LeMAN4* the lack of concomitant increase in transcript level would suggest post-transcriptional regulation of this enzyme. The potential substrate mannans present in the tomato pericarp walls are not yet characterised although it has been suggested that these are glucomannans [22]. This may be so, but our enzyme extracts showed no preference for glucomannan over galactomannan or an artificial homomannan (Table 2) leaving this issue unresolved. If mannanase serves to solubilise fruit cell wall mannans, one might expect to see a loss of Man from the wall as ripening ensued but this was not the case and is puzzling. One could argue that a particular class of mannan is specifically hydrolysed by *LeMAN4* which would not be detectable in our global wall analysis but there is no evidence of such specificity of the enzyme (Table 2) nor do more in-depth analyses of the tomato fruit cell wall support this [22]. It is also conceivable that mannanase action lead to some decrease in the size of cell wall mannans, reducing the cooperative weak interactions between wall components, but that these shortened polymers remained part of the framework of an albeit weakened wall. Some support for this comes from the observation that tomato fruit mannanase failed to cleave the artificial substrate locust bean galactomannan below a  $M_r$  of 2500 even though other mannanases hydrolyse this to oligomers [3]. On the other hand, tomato fruit mannans

Table 2  
Tomato fruit mannanase activity with different substrates as measured by generation of reducing groups (mean of 3 extracts  $\pm$  S.D.).

Substrate	Substrate $M_r$	% Man	Activity (nmol min <sup>-1</sup> g f wt <sup>-1</sup> )
Galactomannan	310,000	78	2.45 $\pm$ 0.09
Glucomannan	300,000	60	2.49 $\pm$ 0.12
Mannan	2700	97	2.53 $\pm$ 0.48

have been analysed before and failed to show any size shift during ripening [26]. Similarly, in kiwifruit pericarp where the mannan has actually been purified and shown to be a galactoglucomannan neither size nor composition changed during ripening [27].

Alternatively, marked mannan synthesis occurs throughout tomato ripening [28] and if this keeps pace with hydrolysis this turnover could explain the apparent lack of cell wall Man decline. It is also plausible that the natural substrate of tomato fruit mannanase is not a cell wall mannan. One possible alternative target might be the Man- $\beta$ -(1,4)-GlcNAc linkage of the *N*-glycans of plant glycoproteins although quite a distinct class of *endo*- $\beta$ -mannosidase seems to cleave this [29].

Much more needs to be known about the substrate specificity of *LeMAN4* as well as the types of mannans found in tomato and their functional role in the fruit cell wall. Until such time, this enzyme will remain something of an enigma.

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## References

- [1] D.A. Brummell, M.H. Harpster, Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants, *Plant Mol. Biol.* 47 (2001) 311–340.
- [2] D.L. Smith, K.C. Gross, A family of at least seven  $\beta$ -galactosidase genes is expressed during tomato fruit development, *Plant Physiol.* 123 (2000) 1173–1183.
- [3] R. Pressey, *endo*- $\beta$ -Mannanase in tomato fruit, *Phytochemistry* 28 (1989) 3277–3280.
- [4] H. Nonogaki, O.H. Gee, K.J. Bradford, A germination-specific *endo*- $\beta$ -mannanase gene is expressed in the micropylar endosperm cap of tomato seeds, *Plant Physiol.* 123 (2000) 1235–1245.
- [5] T. Wozniowski, W. Blaschek, G. Franz, Isolation and characterization of an *endo*- $\beta$ -mannanase of *Lilium testaceum* bulbs, *Phytochem* 31 (1992) 3365–3370.
- [6] US Department of Agriculture, United States standards for grades of fresh tomatoes. Agr. Mkt. Serv., US Department of Agriculture, Washington, DC, 1976.
- [7] L.M.A. Dirk, A.M. Griffen, B. Downie, J.D. Bewley, Multiple isozymes of *endo*- $\beta$ -mannanase in dry and imbibed seeds, *Phytochemistry* 40 (1995) 1045–1056.
- [8] K.C. Gross, A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide, *HortScience* 17 (1982) 933–934.
- [9] P. Biely, O. Markovic, D. Mislovicová, Sensitive detection of *endo*-1,4- $\beta$ -glucanases and *endo*-1,4- $\beta$ -xylanases in gels, *Anal. Biochem.* 144 (1985) 147–151.
- [10] C.M.S. Carrington, L.C. Greve, J.M. Labavitch, Cell wall metabolism in ripening fruit. VI. Effect of the antisense polygalacturonase gene on cell wall changes accompanying ripening in transgenic tomatoes, *Plant Physiol.* 103 (1993) 429–434.
- [11] A.B. Blakeney, P.J. Harris, R.J. Henry, B.A. Stone, A simple and rapid preparation of alditol acetates for monosaccharide analysis, *Carbohydr. Res.* 113 (1983) 201–299.
- [12] S.L. Dellaporta, J. Wood, J.B. Hicks, A plant DNA miniprep: version II, *Plant Mol. Biol. Rep.* 1 (1983) 19–21.
- [13] J.D. Bewley, R.A. Burton, Y. Morohashi, G.B. Finche, Molecular cloning of a cDNA encoding a (1 $\rightarrow$ 4)- $\beta$ -mannan endohydrolase from the seeds of germinated tomato (*Lycopersicon esculentum*), *Planta* 203 (1997) 454–459.
- [14] I. Llop-Tous, E. Domínguez-Puigjaner, X. Palomer, M. Vendrell, Characterisation of two divergent *endo*- $\beta$ -1,4-glucanase cDNA clones highly expressed in the nonclimacteric strawberry fruit, *Plant Physiol.* 119 (1999) 1415–1421.
- [15] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zheng, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 25 (1997) 3389–3402.
- [16] H. Nielsen, J. Engelbrecht, S. Brunak, G. von Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, *Protein Eng.* 10 (1997) 1–6.
- [17] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* 22 (1994) 4673–4680.
- [18] R.M. Amasino, Acceleration of nucleic acid hybridisation rate by polyethyleneglycol, *Anal. Biochem.* 152 (1986) 304–307.
- [19] G.O. Sozzi, O. Cascone, A.A. Fraschina, Effect of high temperature stress on *endo*- $\beta$ -mannanase and  $\alpha$ - and  $\beta$ -galactosidase activities during tomato fruit ripening, *Postharvest Biol. Technol.* 9 (1996) 49–63.
- [20] J.D. Bewley, M. Banik, R. Bourgault, J.A. Feurtado, P. Toorop, H.W.M. Hilhorst, *endo*- $\beta$ -Mannanase activity increases in the skin and outer pericarp of tomato fruits during ripening, *J. Exp. Bot.* 51 (2000) 529–538.
- [21] R. Bourgault, J.D. Bewley, A. Alberici, D. Decker, *endo*-1,4- $\beta$ -Mannanase activity in tomato and other ripening fruits, *HortScience* 36 (2001) 72–75.
- [22] G.B. Seymour, I.J. Colquhoun, M.S. DuPont, K.R. Parsley, R.R. Selvendran, Composition and structural features of cell wall polysaccharides from tomato fruits, *Phytochemistry* 29 (1990) 725–731.
- [23] M. Hilge, S.M. Gloor, W. Rypniewski, O. Sauer, T.D. Heightman, W. Zimmerman, K. Winterhalter, K. Piontek, High-resolution native and complex structures of thermostable  $\beta$ -mannanase from *Thermomonospora fusca*—substrate specificity in glycosyl hydrolase family 5, *Structure* 6 (1998) 1433–1444.
- [24] E. Sabini, H. Schubert, G. Murshudov, G.S. Wilson, M. Siika-Aho, M. Penttilä, The three-dimensional structure of a *Trichoderma reesei*  $\beta$ -mannanase from glycosyl family 5, *Acta Crystallogr. D Biol. Crystallogr.* 56 (2000) 3–13.
- [25] M. Banik, R. Borgault, J.D. Bewley, *endo*- $\beta$ -Mannanase is present in an inactive form in ripening tomato fruits of the cultivar Walter, *J. Exp. Bot.* 52 (2001) 105–111.
- [26] N. Sakurai, D.J. Nevins, Changes in physical properties and cell wall polysaccharides of tomato (*Lycopersicon esculentum*) pericarp tissues, *Physiol. Plant.* 89 (1993) 681–686.
- [27] R. Schroder, P. Nicolas, S.J.F. Vincent, M. Fischer, S. Reymond, R.J. Redgwell, Purification and characterisation of a galactoglucomannan from kiwifruit (*Actinidia deliciosa*), *Carbohydr. Res.* 331 (2001) 291–306.
- [28] C.B.S. Tong, K.C. Gross, Glycosyl-linkage composition of tomato fruit cell wall hemicellulosic fractions during ripening, *Physiol. Plant.* 74 (1988) 365–370.
- [29] A. Sasaki, M. Yamagishi, T. Mega, S. Norioka, S. Natsuka, S. Hase, Partial purification and characterization of a novel *endo*- $\beta$ -mannosidase acting on N-linked sugar chains from *Lilium longiflorum* Thumb, *J. Biochem.* 125 (1999) 363–367.