

# Cloning, characterisation and expression analyses of cDNA clones encoding cell wall-modifying enzymes isolated from ripe apples

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## Abstract

Fruit softening is accompanied by modifications of the cell wall pectic and hemicellulosic fractions, as the result of the combined action of several cell wall-modifying enzymes. The objective of this work was to clone specific cDNAs that encode isoforms of cell wall-modifying enzymes, which are expressed during the final stages of apple softening, and to establish a temporal sequence of their accumulation. A cDNA library enriched with mRNA isolated from over-ripe fruit was constructed and screened. A pectin methylesterase (*MdPME1*), a pectate lyase (*MdPL1*), an  $\alpha$ -L-arabinofuranosidase (*MdAF1*), an endo-1,4- $\beta$ -glucanase (*MdEG1*), two xyloglucan endotransglucosylase/hydrolases (*Md-XTH1* and *Md-XTH2*), and an alpha-expansin (*MdEXPA3*) specific cDNAs were identified by homology-based cloning, and their mRNA accumulation was examined during fruit growth and ripening. The expression of an apple  $\beta$ -galactosidase ( $\beta$ -Gal; pABG1) and a polygalacturonase (PG; pGDPG-1) mRNA previously reported was also investigated using the same biological material. Transcripts of all enzymes, except *MdPME1*, could be unambiguously detected by semi-quantitative RT-PCR in fruit during ripening. However, transcripts of *MdEG1* were more abundant at fruit set and *MdPL1* exhibited higher expression before commercial maturity. The strongest RT-PCR signals in over-ripe fruit were observed for PG,  $\beta$ -Gal and *Md-XTH1* clones. Two XTHs were detected in over-ripe fruit. While *Md-XTH1* acts constitutively during fruit development, *Md-XTH2* showed a ripening-related pattern. The *Md-XTH2*-encoded protein was heterologously expressed in *Saccharomyces cerevisiae* and showed both transglycosylase and hydrolase activities. Expression analyses conducted in flowers, peduncles, young and expanded leaves, and petioles of senescent leaves revealed that none of the cloned cDNAs is fruit specific.

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## 1. Introduction

During ripening, fruit undergo many physiological and biochemical modifications that result in the development of colour and flavour and in loss of firmness. Fruit texture has significant commercial importance since excessive softening is the main factor responsible for limitations of shelf-life, transportability and storage, for increased occurrence of physical damage during handling, and for higher susceptibility to diseases. Fruit firmness and texture also affect the integrity of processed fruit and have been considered to be the principal quality attribute for consumer acceptance in the market (Johnson and Ridout, 2000).

Changes in the cell wall composition and dynamics have been considered the most important factor responsible for the textural changes in fruit (Fischer and Bennett, 1991; Hadfield and Bennett, 1998). Recently, it has been shown that, at least in tomato, differences in water loss and turgor pressure derived from alterations in cuticle architecture are also an integral element of softening during ripening (Saladié et al., 2007). Biochemical studies of the modifications that occur in the cell wall during ripening in several fruit indicate that structural changes in pectin, hemicellulose and cellulose together are responsible for the alteration of cell wall structure (Huber, 1983; Seymour et al., 1990). These changes include not only modifications like solubilisation and depolymerisation of the polysaccharides, but also rearrangements of their associations. Studies on the structure of the plant cell wall have disclosed a large number and a large range of distinct biochemical

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linkages between the components. Such linkages represent potential targets for hydrolytic action and draw attention to a putative involvement of several members of enzymes able to act and modify its structure in a developmental and coordinated way.

The analysis of enzymatic activity is often masked by the presence of multiple isoforms that may act with different patterns of expression, sometimes sequentially, overlapping or concomitantly, and under distinct developmental regulation. Therefore, knowledge about individual members of these gene families is needed to understand the overall effect that each gene product has on cell wall metabolism and its consequential effect, if any, on fruit softening.

Furthermore, it is now recognised that, depending on the fruit species, different modifications may occur and to different extents (Brummell, 2006; Goulao and Oliveira, 2008). Since differences exist between different fruit and even between cultivars from the same species, species other than the models tomato and strawberry should be investigated.

Despite the importance of softening in apples, the information available at the molecular level for this species is limited and restricted to the characterisation of only one PG (Atkinson et al., 1998), one  $\beta$ -Gal clone (Ross et al., 1994) and six expansins (Wakasa et al., 2003).

It is important to know the serial expression of the various genes involved in cell wall modifications associated with fruit softening. In fact, the activity of a certain enzyme might improve or be a requirement to the access of others to their specific substrates. Such information is, at the present time, unavailable for most species. A partial picture can only be obtained in tomato, by putting together all the separate data obtained by the different laboratories working with this species as a model. However, this is not totally accurate since the published reports do not use the same exact biological material in their independent research programmes. The objective of this study was to identify, clone and characterise the most abundant isoforms of enzymes from candidate families that are expressed in the later stages of softening and to determine, for the first time in apple, their patterns of mRNA accumulation throughout growth and ripening. The use of the same biological material allowed a direct comparison of the results.

## 2. Materials and methods

### 2.1. Plant materials

Tissue samples from ‘Mondial Gala’ apples (*Malus × domestica* Borkh.) were collected from producing trees growing at the experimental orchard of the Instituto Superior de Agronomia, Lisboa, Portugal, during two growing seasons. The orchard is managed under standard agronomical practices. Fruit were collected at different stages of development, classified and assigned to classes according to their physiological stage, based on their time from anthesis or from harvest, size, skin colour, seed maturation and cortex firmness as: fruit set (stage 1), growing fruit (stage 2), unripe expanded fruit (stage 3), fruit at harvest

(stage 4), softening fruit (stage 5) and over-ripe fruit (stage 6). Stages 1–4 and 6 were previously established (Goulao et al., 2007). Stage 5 was defined in this work. Softening of several apple cultivars, including ‘Gala’, is triphasic at low storage temperatures, consisting of an initial slow softening phase (phase I), a rapid softening phase (phase II) and a final slow softening phase (phase III). Softening fruit (defined here as “Stage 5”) were considered to be those with a measured firmness between 60 and 40 N, which corresponds to phase II of softening (Johnston et al., 2001) while physiologically over-ripe fruit (stage 6 as previously defined by Goulao et al., 2007) were considered to be those in phase III. The characteristics of each class are given in Table 1. In all cases, samples were harvested, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction of nucleic acids. Fruit at fruit set and actively growing were frozen with their skin. For fully expanded fruit, fruit at commercial maturity, softening and over-ripe fruit, the skin was removed and the flesh was quickly diced into small slices immediately before freezing. Young expanded leaves, adult leaves and flowers were collected within two weeks after full bloom. Petioles of senescent leaves were collected in October, just before the vegetative dormancy period.

For the construction of the cDNA library, ‘Royal Gala’ apples were obtained from a local market at commercial maturity, and left to become over-ripe at about  $22^{\circ}\text{C}$  for 2 weeks.

### 2.2. Statistical analyses

The data of the developmental behaviour of apples were analysed by one-way ANOVA using the SPSS 12.0 for Windows software. Statistical significance was judged at the confidence level of  $P < 0.01$ . When the analysis was statistically significant, the Scheffé Multiple Range Test was used for separation of means at a significance level of 0.01.

### 2.3. Nucleic acids extraction

Genomic DNA was extracted from young, fully expanded leaves using the procedure of Ceniz (1992), modified as previously described (Goulao et al., 2001).

Total RNA was extracted from all tissues under study using the protocol of Chang et al. (1993) with some modifications. The starting amount of frozen plant material was optimised empirically for each tissue, since starting with excessive material led to degradation of the extracted RNA. The best starting amounts per extraction were determined to be 8–10 g fruit tissue, 4–6 g leaf, pedicel and peduncle tissues and 2–3 g flower tissue. Total RNA was treated with 2 units of DNase I (Ambion, Austin, TX, USA), according to the manufacturer’s instructions.

Polyadenylated RNA was purified using paramagnetic oligo(dT)<sub>25</sub> Dynabeads of the Dynabead mRNA purification kit (DynaL AS, Oslo, Norway) according to the manufacturer’s instructions. This poly(A)<sup>+</sup> RNA was treated with 2.3 mM methylmercury hydroxide to remove secondary structures and was used to construct a cDNA library.

Table 1

Description of the criteria used to classify the fruit into stages 1–6 (according to Goulao et al., 2007 and this work) and the measured characteristics by growing season

Developmental stage	(1) Fruit set	(2) Active growing	(3) Unripe; expanded	(4) Harvest	(5) Softening	(6) Over-ripe
<b>Dpa<sup>a</sup>/dph<sup>b</sup> (days)</b>	<b>35–45<sup>(a)</sup></b>	<b>60–70<sup>(a)</sup></b>	<b>90–100<sup>a</sup></b>	<b>135–140<sup>a</sup></b>	<b>10–20<sup>(b)</sup></b>	<b>21–30<sup>(b)</sup></b>
Season 1	40	65	92	135	16	26
Season 2	41	67	95	137	15	25
<b>Diameter (mm)</b>	<b>15–25</b>	<b>32–40</b>	–	–	–	–
Season 1	18.7 ± 2.7 a	36.6 ± 2.6 b	59.6 ± 2.2 c	62.0 ± 2.2 c	61.8 ± 2.4 c	61.6 ± 2.6 c
Season 2	17.4 ± 2.5 a	37.1 ± 2.4 b	61.6 ± 2.3 c	62.3 ± 2.3 c	61.9 ± 2.6 c	61.6 ± 2.8 c
<b>Mature seeds (%)</b>	–	–	<b>30–70</b>	<b>90–100</b>	<b>100</b>	
Season 1	–	–	52.5 ± 8.3	100	100	
Season 2	–	–	45.7 ± 9.1	100	100	
<b>Skin colour (% red)</b>	–	–	<b>&lt;1/3</b>	<b>About 2/3</b>		
Season 1	–	–	<1/3	About 2/3		
Season 2	–	–	<1/3	About 2/3		
<b>Ground hue<sup>c</sup> (°)</b>	–	–	<b>106–120</b>	<b>100–105</b>	<b>&lt;105</b>	<b>&lt;105</b>
Season 1	–	–	116.4 ± 9.9 a	102.2 ± 7.9 b	97.0 ± 10.1 c	96.8 ± 8.2 c
Season 2	–	–	114.2 ± 9.7 a	103.6 ± 8.3 b	94.9 ± 9.2 c	95.7 ± 7.2 c
<b>Cortex firmness<sup>d</sup> (N)</b>	–	–		<b>65–80</b>	<b>40–60</b>	<b>&lt;40</b>
Season 1	–	–	118.1 ± 9.3 a	74.4 ± 8.1 b	53.5 ± 8.1 c	34.6 ± 7.0 d
Season 2	–	–	121.6 ± 7.9 a	76.2 ± 9.1 b	54.9 ± 6.9 c	32.2 ± 6.3 d

Values are the average ± standard deviation of 30 representative samples and the letters represent statistical significance at 1%.

<sup>a</sup> Days post-anthesis.

<sup>b</sup> Days postharvest.

<sup>c</sup> Transformation of the value of hue angle in the Hunter scale into degrees ( $90 + (90 + \text{hue value} \times 180/\pi)$ ) (McGuire, 1992).

<sup>d</sup> Maximum force (N) necessary for compression of peeled flatten areas of the fruit using a texture analyser (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY) fitted with a 11 mm diameter flat probe. Fruit were compressed 8 mm at speed rate of 1 mm s<sup>−1</sup>.

#### 2.4. Construction of a cDNA library from late softening apples

For the construction of a cDNA library, a mixture of poly(A)<sup>+</sup> mRNA purified from softening fruit (stage 5) and over-ripe fruit (stage 6) was used in a ratio of 3:7. Hence, 1.5 µg of mRNA from “softening fruit” was added to 3.5 µg of mRNA from “over-ripe fruit” and the mixture was used for the construction of the cDNA library. The Lambda ZAP synthesis kit (Stratagene, La Jolla, CA, USA) was used according to the manufacturer’s protocol, except that no radioactive dNTP was used during first-strand cDNA. After fractioning, 100 ng of the selected cDNA was directionally ligated into Uni-ZAP XR vector and packaged using the Gigapack III Gold package extract (Stratagene). The primary library resulting from infection of *Escherichia coli* host strain XL1-Blue MRF<sup>+</sup> was immediately amplified. The primary library had a titer of  $1.6 \times 10^{10}$  pfu mL<sup>−1</sup> and after amplification, resulted in a library with a titer of  $8.6 \times 10^9$  pfu mL<sup>−1</sup> with an estimated average size of the inserts of 1454-bp. The size of the amplification fragments ranged between 775- and 3025-bp in the clones sampled and analysed.

#### 2.5. Cloning of cell wall-modifying enzymes in softening fruit by homology-based cloning

Aliquots of the amplified cDNA library were used in the homology-based cloning experiments. For each gene family, a set of degenerate primers was designed from consensus regions

determined after alignments of amino acid sequences of orthologues available in databases (Supplementary Table S1). These primers were used in standard PCR reactions consisting of 20 µL mixes containing 0.5 µL of the cDNA library as template, 1 unit of *Taq* DNA polymerase (Pharmacia Biotech, Uppsala, Sweden), 0.25 mM each dNTP (Gibco BRL, Eggenstein, Germany) and 0.5 µM each primer, in 1 × reaction buffer (50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris–HCl pH 9.0) and thermal cycled under the following conditions: 4 min at 94 °C for initial denaturation, 35 cycles of 30 s at 94 °C, 60 s at Tann (optimised empirically for each primer pair; see Supplementary Table S1) and 90 s at 72 °C, followed by 10 min at 72 °C. The specific PCR products were gel-purified using S.N.A.P. mini columns (Invitrogen, Karlsruhe, Germany) and cloned into TOPO-TA vector (Invitrogen), according to the manufacturer’s protocol. For each gene family, the plasmid DNA from 10 independent recombinant colonies was purified using the Jet Star Plasmid Mini kit 2.0 (Genomed, Bad Oeynhausen, Germany), and sequenced using commercial services (StabVida, Oeiras, Portugal).

#### 2.6. Determining the full-length sequence of the cloned fragments

##### 2.6.1. Screening of the cDNA library

The cDNA library was screened by four rounds of PCR reactions, according to Amaravidi and King (1994), using specific primers designed from the known partial sequences

(Supplementary Table S1). Screenings were done in 10 90 mm Petri dishes per screening. The appropriate density in each screening was determined experimentally and it was established to be 10 plates with a density of about 3000 pfu (plaque forming units per millilitre) each, 10 plates with a density of about 300 pfu each, from a positive primary plaque, 10 plates with a density of about 30 pfu each, from a positive secondary plate, respectively for primary, secondary and tertiary screenings. Final screening was done using at least 14 single plaques from a positive tertiary plate. Positive clones, confirmed by PCR, were then sub-cloned for single-clone excision. Bluescript SK-plasmids were excised from hybridising phage into *E. coli* SOLR strain with helper phage (ExAssist) according to the *in vivo* excision protocol provided by the manufacturer (Stratagene). Plasmid DNA was then isolated and sequenced.

#### 2.6.2. Rapid amplification of cDNA ends (RACE)

The full-length nucleotide sequences of the *MdEG1* clone and the 5'-region of the *MdAF1* truncated clone obtained after screening the library, were obtained by RACE experiments.

For 3'-RACE, cDNA was synthesised as described below, using a specific adapter (Supplementary Table S1). One microlitre of 3'-adapter tailed cDNA was amplified in nested PCR reactions using 0.8  $\mu$ M of 3'-RACE short primer, 0.2  $\mu$ M 3'-RACE long primer and 0.25  $\mu$ M sequence-specific outer or inner primers (Supplementary Table S1). A polymerase with proofreading activity (Clontech, Palo Alto, CA, USA) and longer (3 min) extension cycles were used in the PCR reactions.

The 5'-end of the cDNA fragments was obtained using the RLM-RACE (RNA Ligase-Mediated Rapid Amplification of cDNA Ends) procedure using the FirstChoice® RLM-RACE kit (Ambion) according to the manufacturer's instructions in nested reactions using outer and inner primers (Supplementary Table S1).

### 2.7. Gene expression studies

#### 2.7.1. Synthesis of cDNA

Complementary DNA was generated in 20  $\mu$ L reactions containing 1  $\mu$ g of spectrophotometrically quantified DNA-free total RNA with 20 units of AMV Reverse Transcriptase (Roche, Mannheim, Germany), 200 units of Protector RNase Inhibitor (Roche), 1 mM each dNTP and either 1.5  $\mu$ g of oligo(dT)<sub>12</sub> or 3  $\mu$ g of random 7-mer primers in 1 $\times$  synthesis buffer (50 mM Tris-HCl, 8 mM MgCl<sub>2</sub>, 30 mM KCl, 1 mM DTT (dithiothreitol) pH 8.5), according to Roche's protocol.

#### 2.7.2. Semi-quantitative PCR

Transcript accumulation in different tissues was estimated through semi-quantitative RT-PCR reactions in multiplex reactions. Co-amplifications with isoform-specific primers (designed from the 3'-UTR region of each sequence) and primers specific for constitutive genes, 18S rDNA (with sequence-specific primers) or GADPH (glyceraldehyde-3-phosphate dehydrogenase; with degenerate primers based on sequences

from other species; Supplementary Table S1) as internal standards, were conducted to assure that equal quantities of cDNA template had been used in each sample. PCR conditions were the ones described for homology-based cloning, except that two primer pairs were used in combination. Clone-specific primers were used at a 0.5  $\mu$ M concentration, while primers from constitutive genes were used at 0.25  $\mu$ M final concentration. PCR amplification of a given RT reaction was carried out in triplicate using samples from different batches as template. One analysis was done starting from cDNA synthesised from RNA extracted during agronomic season 1 and reverse transcribed with oligo(dT)<sub>12</sub>. The second template was cDNA synthesised from RNA from a different extraction and with random primers. Finally, a third sample was used, using cDNA generated with random primers on RNA extracted from samples collected during agronomic season 2. To assure that all PCR reactions started from equal amounts of quantified cDNA, a first aliquot was sampled after 16 cycles (for 18S rRNA) or 18 cycles (for GADPH), which was determined to be the first cycle which generates visible amplification products. The presence of a faint band of similar intensity in all samples is indicative that identical amounts of starting template were used (Figs. 2 and 3). The conditions for all semi-quantitative RT-PCR reactions were identical, except for the number of cycles, which was optimised empirically to determine the linear range of amplification so that the data could be analysed before the amplification reached the plateau (see number of cycles in Figs. 2 and 3).

### 2.8. Southern blots

Aliquots of 10  $\mu$ g of genomic DNA were digested overnight with 10 units of BamHI, EcoRI and HindIII (Roche) separately, size-fractionated by electrophoresis on a 1.0% (w/v) agarose gel, and transferred onto positively charged nylon membranes (Roche) by capillary blotting using 0.4 M NaOH. The membranes were prehybridised for 1 h in prehybridisation solution (DIG EasyHyb, Roche) at 42 °C. Isoform-specific probes were synthesised from the 3'-UTR region of each cDNA and labelled by the incorporation of DIG (digoxigenin) in standard PCR reactions (Supplementary Table S1). Hybridisation conditions were based in the procedure of Engler-Blum et al. (1993) and in the DIG Application Manual for Filter Hybridisation (Roche) using 7.5 ng of DIG-labelled probe per mL of hybridisation solution. The filters were then washed twice for 15 min in 2 $\times$  SSC (3 M NaCl, 300 mM Na<sub>3</sub>-citrate, pH 7.0), 0.1% SDS at room temperature for non-stringent washes. For stringent washes, the membranes were washed twice in a solution of 0.5 $\times$  SSC, 0.1% SDS at 65 °C for 15 min (for *MdAF1*, *MdPL1*, *MdEG1* and *Md-XTH1*), the same solution at 60 °C (for *Md-XTH2*) or 0.1 $\times$  SSC, 0.1% SDS at 65 °C for 15 min (for *MdEXPA3*). These conditions allowed a maximum of 10% mismatches. Colorimetric detection followed standard procedures (Engler-Blum et al., 1993), using CDP (disodium 4-chloro-3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)) as substrate.



## 2.9. Production of *Md-XTH2* recombinant enzyme in *Saccharomyces cerevisiae*

### 2.9.1. Construction of the expression plasmid

The *Md-XTH2* plasmid recovered from the cDNA library of ripening apples was amplified through high-fidelity PCR using 1 unit of *AccuPOL* DNA polymerase (GeneChoice, Frederick, MD, USA), with primers sense: 5'-caggatccatgactaagatattcc-3' and antisense 5'-gtgtactctcacgcagcagagc-3'. The primers were designed to introduce BamHI and XbaI restriction sites (underlined), respectively. The amplification product resulted in the complete double stranded sequence of the coding region, including its native signal peptide, with the necessary restriction overhangs to allow ligation in frame in the expression vector with respect to the GPD (glyceraldehydes-3-phosphate dehydrogenase) promoter. The resultant PCR product was purified, double-digested with BamHI and XbaI (Promega, San Louis Obispo, CA, USA) and ligated in frame with the GPD promoter in the pGAU-KHC expression vector. The expression vector was restricted with BamHI and XbaI (Promega), releasing two fragments, 600 bp and 8.2 Kb. The 8.2 Kb fragment was gel-purified and ligated to the *Md-XTH2* insert generated, using T4 DNA ligase (New England BioLabs, Beverly, MA, USA). The recombinant plasmids were transformed into DH5 $\alpha$  chemically competent *Escherichia coli* cells (Invitrogen) and the plasmid DNA was recovered using the QIAprep Spin Miniprep kit (Qiagen). Finally, the plasmids were sequenced to verify the presence and correct sequence of the insert.

### 2.9.2. Transformation of *S. cerevisiae* and selection of transformants

The *S. cerevisiae* strain W303 (*Mata*, *leu2-3,112*, *his3-11*, *trp1-1*, *ura3-1*, *can1-100*, *ade2-1*) was transformed with the pGAU-KHC:*Md-XTH2* construct by the EZ yeast transformation protocol. Briefly, 100  $\mu$ L of PLATE medium (100 mM LiOAc, 40% PEG (polyethylene glycol) 3350, 1 $\times$  TE (50 mM Tris-HCl, 1 mM EDTA, pH 8)) were added to 3  $\mu$ L of ssDNA previously incubated at 100 °C for 10 min and to 10  $\mu$ L of plasmid construct. The mixtures were vortexed, incubated overnight at room temperature and then centrifuged at 20,000  $\times g$  for 60 s. The pellet was then resuspended in 200  $\mu$ L of YPD (10 mg mL<sup>-1</sup> yeast extract, 20 mg mL<sup>-1</sup> peptone, 20 mg mL<sup>-1</sup> glucose, pH 6.5) and plated on selective plates. Positive transformants were selected for their ability to grow on uracil-deficient 2% glucose YNB (Yeast Synthetic Complete Nitrogen Base with Amino Acids) media containing amino acids. A negative control was included, by transformation of yeast with a vector without insert. A single colony of transformant strains was used to inoculate 100 mL of 2% glucose, YNB containing all amino acids without uracil, and grown at 28 °C for 20 h (until about 2 OD). The clarified supernatants from the cultures were concentrated by filtration through an Ultrafree<sup>®</sup>-15 Centrifugal Filter Device (Millipore, USA). The 100 mL of culture medium was concentrated to about 2.5 mL containing the recombinant expressed XTH enzyme. Samples of total extracellular proteins were quantified using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) and immediately

assayed for endotransglucosylase and hydrolase activities or stored at -80 °C.

### 2.10. Endotransglucosylase activity assay

A solution of 1% (w/v) tamarind xyloglucan (Megazyme, Ireland) was dissolved at 35 °C for 2 h. Five microlitres of this solution were incubated with 40  $\mu$ L of solution containing the recombinant enzyme or negative control samples, and 2  $\mu$ M of 0.5% fluorescent labelled oligosaccharides (XLLG/XXLG/XXXG; for nomenclature see Fry et al., 1993a) in 50 mM MES buffer pH 5.5. After 60 min incubation at 27–28 °C in the dark, the mixtures were resolved by thin-layer chromatography (TLC) on silica gel in butano-1-ol/acetic acid/water (2:1:1, v/v/v) in the dark. The reaction products were visualised under a UV transilluminator and photographed with a digital camera fitted with an orange filter.

### 2.11. Hydrolase activity assay

XTH hydrolytic activity was measured based on the induced changes in viscosity of a solution containing tamarind xyloglucan (Megazyme). One hundred microlitres of enzyme extract were added to 350  $\mu$ L of a 2% (w/v) tamarind xyloglucan solution in 50 mM MES buffer pH 5.5. The initial viscosity was determined by measuring the time taken for the movement of the mixture through the 0 and the 0.05 mL marks of a 0.1 mL glass pipette fixed in a vertical position. Three readings were measured for each replicate using a stopwatch. The mixtures were then incubated for 8 h at RT (22–24 °C) with shaking. After a 15 min equilibration period at RT, the viscosity of the mixture of measured again as described. Activity is reported as the percentage of viscosity reduction after the assay period, with respect to time zero.

## 3. Results

### 3.1. Identification and cloning of cDNAs encoding cell wall-modifying enzymes

Candidate families to be involved in cell wall-modification during apple softening were selected based on published studies of fruit softening in other species, in which correlative data between activity of these enzyme families and fruit softening is reported, or in which differences exist between wild-type fruit and fruit with modified genetic backgrounds (see review in Goulao and Oliveira, 2008). Amplification with degenerate primers produced a single band of the expected size in all families, but amplification with degenerate primers designed from PME and EGase conserved regions consistently resulted in weaker products in the agarose gels.

To investigate the number of isoforms that are expressed in later stages of ripening, inserts from 10 random independent recombinant colonies from each family were sequenced and pair-wised compared by the BLAST2 algorithm. In all cases, except for XTHs, the 10 sequenced clones displayed more than 98% identity at the nucleotide level, and therefore were con-

sidered to correspond to the same product, which suggests that one isoform is preferentially expressed during latter ripening of apples. Two distinct cDNA encoding two XTHs sequences sharing 55% of amino acid identity in their deduced mature protein sequences were recovered (*Md-XTH1* and *Md-XTH2*). Full-length clones of *MdPL1*, *MdAF1*, *Md-XTH1*, *Md-XTH2* and *MdEXPA3* were recovered from screening about 30,000 pfu of the cDNA library. Screening of the cDNA library was however unsuccessful in the cases of EGase and PME clones, despite exhaustive attempts (screening of about 150,000 pfu in each case). Therefore, the full-length sequence of *MdEG1* was obtained by a combination of 3'-RACE and 5'-RLM RACE, using a mix of RNA from all fruit classes studied, as starting template material. In the case of *MdPME1*, RACE experiments were also unsuccessful in generating the full-length clone and consequently, only the partial sequence is reported.

Alignment of the amino acid sequences with other species using the BLASTp algorithm revealed that all clones were full-length, except for *MdAFase*, which was truncated at the 5'-end. The missing part of the sequence was obtained by 5'-RLM RACE using sequence internal primers (Supplementary Table S1).

### 3.2. In silico sequence analyses of the cloned sequences

Sequence data from this study have been deposited in the EMBL/GenBank databases under the accession numbers AF527800 (*MdEXPA3*), AY144593 (*Md-XTH1*), AY144594 (*Md-XTH2*), AY309436 (*MdAF1*), AY350734 (*MdEG1*), AY376878 (*MdPL1*) and AY530907 (*MdPME1*). The summary of the main characteristics of each full-length clone is given in Table 2. As expected, putative signal peptides are predicted for all sequences, indicating that the nascent polypeptides are likely to be targeted to the cell wall.

Although only a 284-bp partial sequence has been obtained for *MdPME1*, it possesses a pectin methylesterase signature II (GxxDFIFG) (Markovic and Jornvall, 1992), supporting its classification as a member of the PME family. The *MdEG1* cDNA contained a Arg-Gly-Asp (RGD) motif, which has been identified in several endoglucanases and proposed to represent a cell attachment signature (Molhoj et al., 2001). XTH are known to possess four conserved cysteine residues, probably

involved in two disulfide linkages, located at the carboxyl terminal region (Okasawa et al., 1993). The active sites of XTHs are proposed to be DEIDFEFLG or DEIDIEFLG (Okasawa et al., 1993). These features were observed in both isoforms. The *MdEXPA3* sequence contains the highly conserved amino acid residues typical of expansins (Shcherban et al., 1995; Yennawar et al., 2006) and the two characteristic domains (Cosgrove, 1997, 2000). One interesting feature revealed by sequence analysis of *MdEXPA3* is the presence of a putative *N*-glycosylation (Asn-X-Ser/Thr-X; X-any except Pro) motif. However, alpha-expansins are thought not to be glycosylated (Cosgrove et al., 1997). Whether *MdEXPA3* is really glycosylated needs to be demonstrated *in vivo* and the biological significance of this post-translational modification should be investigated. Wakasa et al. (2003) reported a partial sequence for the same gene, isolated from 'Golden Delicious' apples. This clone is identical to the full-length *MdEXPA3*, except for a (GA)<sub>n</sub> microsatellite located in the 3'-UTR which, in 'Golden Delicious', is 10 nucleotides shorter. All genes cloned display a predicted neutral-basic pI, except for *MdAF1* (Table 2). This suggests that this enzyme may be immobilised in specific, positively charged, regions of the cell wall such as the extensin network, acting to bring specific, localised modifications.

### 3.3. Number of copies of the cloned genes in the apple genome

The results obtained from Southern blot analyses carried out under high-stringency conditions suggest that each cDNA cloned corresponds to a single-copy gene (Fig. 1). The presence of two bands with similar intensity in *MdPL1* suggests that two copies of the gene or two closely related sequences in the 3'-UTR region may be present in the apple genome. The absence of signal on the sample restricted with EcoRI, using the EGase probe, was unexpected. The analysis of the *MdEG1* sequence shows a restriction site 322-bp upstream the portion of the sequence used to produce the labelled probe. One can speculate that, if another restriction site exists in the genome a few base pairs downstream this string, the product generated by the digestion with EcoRI that would hybridise with the probe can be too small to be efficiently transferred to the membrane and, therefore, would not be detected.

Table 2  
Main characteristics of the full-length clones under study after *in silico* analysis

Clone	Nucleotide (bp)				Amino acid			
	Sequence length	5'-UTR	3'-UTR	Coding region	Signal peptide (aa)	M <sub>w</sub> (kDa)	pI	N-Glycosylation sites (putative)
<i>MdPL1</i>	1660	73	312	1254	33	42.2	6.70	1
<i>MdAF1</i>	2489	190	253	2025	25	71.3	4.74	8
<i>MdEG1</i>	1942	79	352	1491	20	52.7	8.92	0
<i>Md-XTH1</i>	1268	57	308	882	22	31.9	9.13	1
<i>Md-XTH2</i>	1084	12	206	846	20	29.7	6.77	2
<i>MdEXPA3</i>	1311	65	497	723	20	24.2	9.49	1

Presumed ORFs (open reading frame) were deduced using the NCBI ORF Finder of the National Centre of Biotechnology Information (NCBI) databases. Signal peptides and their cleavage sites were predicted using PSORT (Nakai and Kanehisa, 1992) and SignalP programs (Nielsen et al., 1997). Theoretical isoelectric points and mass values for mature peptides were calculated using the PeptideMass program (Wilkins et al., 1997). UTR, untranslated region; M<sub>w</sub>, molecular weight; aa, amino acids; pI, isoelectric point.

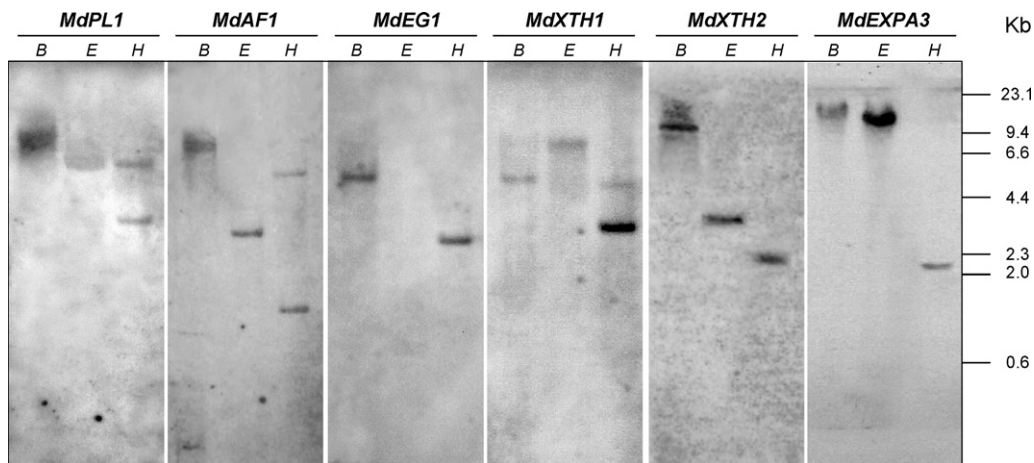


Fig. 1. Southern analysis of genomic organization of the clones studied. Genomic DNA extracted from apple leaves was analysed by Southern blotting using 3'-UTR based specific probes, as described in Section 2. Restriction enzymes used in the analysis are indicated: B, BamHI; E, EcoRI; H, HindIII. Migration positions of DNA size markers are shown on the right.

### 3.4. Analysis of mRNA expression patterns in apple tissues

Semi-quantitative RT-PCR analyses were performed with cDNA generated from RNA extracted from a variety of apple organs and tissues in order to quantify the relative amount of gene transcription of the cDNAs cloned during apple growth and ripening, and to examine if the transcripts are fruit specific. In addition, the  $\beta$ -Gal (pABG1) and PG (pGDPG-1) cDNAs previously reported to be expressed in ripe apples (Ross et al., 1994; Atkinson et al., 1998) were also included in the analyses.

Each cDNA displays a unique expression profile during fruit development and differs in its expression in other tissues. This suggests a coordinate but not concomitant action of these cell wall-modifying enzymes in the putative cell wall breakdown that accompanies softening.

Expression of pGDPG-1 correlated precisely with the onset of ripening (harvest) and remained high until the over-ripe stage (Fig. 2). A slightly weaker signal for the pGDPG-1 transcript was also detected in the cDNA obtained from petioles of senescent leaves but it was undetectable in the other tissues studied (Fig. 3).

Surprisingly, despite a faint amplification product corresponding to PME transcripts was occasionally present when the cDNA library is used as template in the PCR reactions, no amplification was consistently obtained in PCR reactions using fruit cDNA as template, neither using degenerate primers for PMEs nor specific primers designed from the partial sequence obtained. Nevertheless, the results show a very strong transcription of *MdPME1* in flowers and a faint signal in young leaves and petioles from senescent flowers (Fig. 3). The analysis was also conducted in fruit from 'Golden Delicious' apples, since a PME protein was purified from fruit of this cultivar (Denès et al., 2000). The results were similar to the ones obtained with 'Mondial Gala' apples (data not shown) which suggests that the absence of mRNA accumulation is not specific to the 'Mondial Gala' cultivar.

*MdPL1* was expressed most highly from fruit set to the fully expanded stage. Thereafter, its expression declined markedly to

lower levels (Fig. 2). Only a fainter band was seen in fruit at commercial maturity and no signal detected in softening and over-ripe fruit material. On the other hand, *MdPL1* was also expressed in flowers, flower peduncles and petioles of senescent leaves (Fig. 3).

By contrast, the  $\beta$ -Gal transcripts accumulated at very high levels in ripening fruit, with a similar pattern to the one observed for PG (Fig. 2). The pABG1 expression was also significant in all of the non-fruit tissues investigated, particularly in the petioles of senescent leaves (Fig. 3). In developing fruit, a slightly, reproducible, stronger signal was observed at fruit set.

*MdAF1* expression was most abundant in fruit at harvest and petioles of senescent leaves, although it was clearly present in flowers, petioles and leaves, and at a slightly lower level, in fruit from the other developmental stages (Figs. 2 and 3).

The role of EGases in the ripening of apples seems to be de-emphasised based on the transcription pattern of *MdEG1* (Fig. 2). Transcripts of this clone were preferentially expressed at fruit set (Fig. 2) and in the flower peduncles and petioles of senescent leaves (Fig. 3). In the remaining tissues and in fruit after fruit set, the *MdEG1* transcripts were present but barely detectable. It should be noted that the relative expression reported for *MdEG1* was much lower, when compared to the other cDNAs since the results reported for RT-PCR are based on 35 amplification cycles, in contrast with the 30 cycles suitable for the other clones.

The two XTH clones displayed different patterns of expression. The expression of *Md-XTH1* was nearly constitutive in all tissues employed, although with lower intensity in flowers (Fig. 3) and higher expression in fruit at harvest (Fig. 2). As stated for *MdEG1*, it should be noted that fewer amplification cycles were used in this analysis, which is indicative of higher levels of transcription for *Md-XTH1*. During fruit development, *Md-XTH2* transcripts started to accumulate in unripe fully expanded fruit, reached a maximum expression in fruit at harvest and declined to lower, but easily detectable, levels in the postharvest period (Fig. 2). Lower levels of transcription

were detected in all non-fruit tissues, in particular in petioles of senescent leaves (Fig. 3).

The *MdEXPA3* transcription was initiated at fruit set, increased progressively as the fruit grows and the highest level of signal was detected in fruit at harvest (Fig. 2). After that the expression was reduced to lower, but detectable levels.

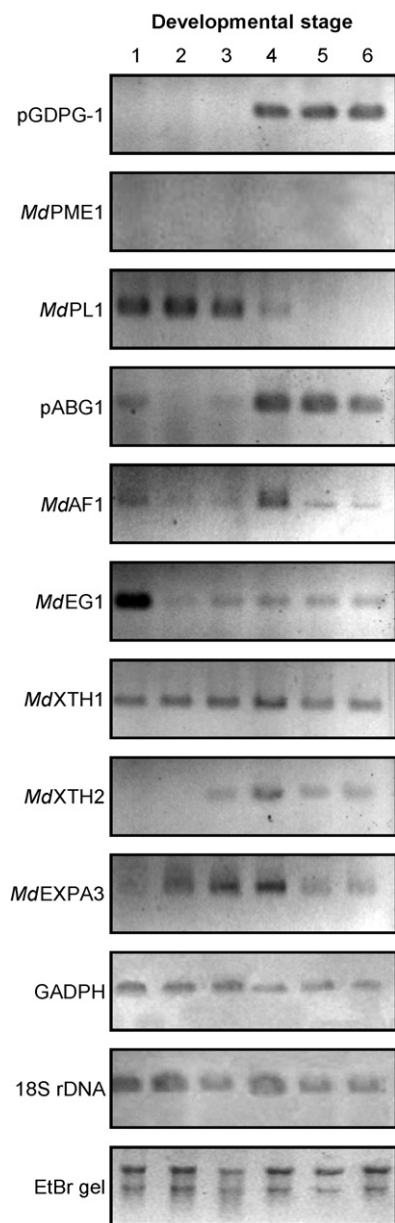


Fig. 2. Patterns of mRNA accumulation of the clones studied by semi-quantitative RT-PCR analyses in developing fruit. PCR and electrophoresis conditions are described in Section 2. GADPH and 18S rDNA were used and internal controls to normalise the amount of starting template. A representative ethidium bromide staining gel of RNA samples confirming equal loadings based on spectrophotometric quantification and showing RNA integrity is shown at the bottom of the figure. The colour was inverted to allow an easier visualization of the faint fragments. Each gel is representative of at least three replications. Legend: 1, fruit set; 2, growing fruit; 3, unripe fully expanded fruit; 4, fruits at commercial maturity; 5, softening fruit; 6, over-ripe fruit. The number of cycles was 30 in each case, except for *MdPME1* (40 cycles), *MdEG1* (35 cycles) and *Md-XTH1* (28 cycles). For constitutive genes, 18 and 16 cycles were used for GADPH and 18S rDNA, respectively.

*MdEXPA3* transcripts were also present in developing flowers, petioles and young expanding leaves, although the amplification is weaker than the one obtained in fruit (Fig. 3). This was the only clone studied without detectable transcription in petioles of senescent leaves.

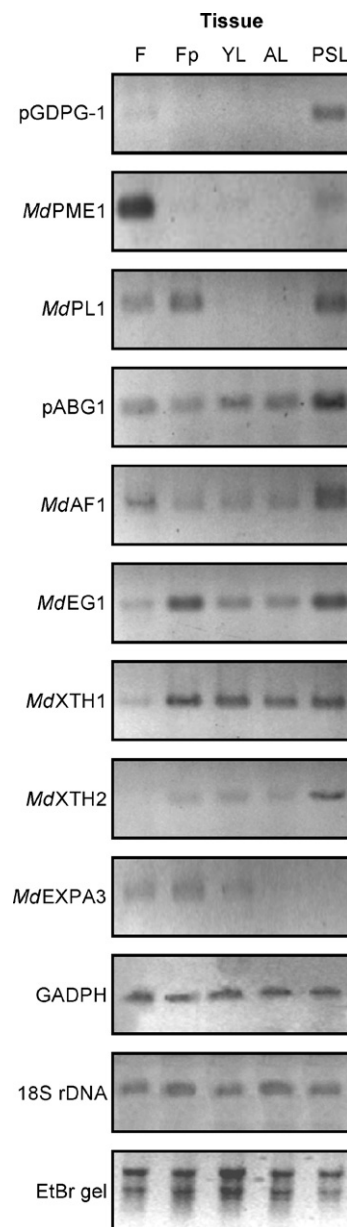


Fig. 3. Patterns of mRNA accumulation of the clones studied by semi-quantitative RT-PCR analyses in several apple tissues. PCR and electrophoresis conditions are described in Section 2. GADPH and 18S rDNA were used and internal controls to normalise the amount of starting template. A representative ethidium bromide staining gel of RNA samples confirming equal loadings based on spectrophotometric quantification and showing RNA integrity is shown at the bottom of the figure. The colour was inverted to allow an easier visualisation of the faint fragments. Each gel is representative of at least three replications. Legend: F, flower; Fp, flower peduncle; YL, young leaves; AL, adult leaves; PSL, petioles of senescent leaves. The number of cycles was 30 in each case, except for *MdPME1* (40 cycles), *MdEG1* (35 cycles) and *Md-XTH1* (28 cycles). For constitutive genes, 18 and 16 cycles were used for GADPH and 18S rDNA, respectively.



### 3.5. Probing XET and XEH activities of *Md-XTH2*

XTH enzymes can be active on xyloglucan molecules acting as strict endotransglucosylases (XET), as hydrolases (XEH), or may display both activities. In order to examine these two possible activities of *Md-XTH2*, the entire ORF, including the putative N-terminal signal sequence, was cloned into the cloning site of a yeast expression vector, containing a GPD constitutive promoter and terminator. The GPD promoter is active in the presence of glucose, allowing the recombinant proteins to be constitutively expressed. Since the signal peptide was included, the proteins were expected to be targeted to the secretory pathway. Therefore, the culture medium of transformed yeast was desalted, concentrated and directly examined for the presence of XET and XEH activities.

Fig. 4A illustrates the results of the XET activity assay. XET activity was assayed based on the ability to generate a fluorescent high- $M_r$  xyloglucan after transglycosylation between tamarind seed xyloglucan and sulphorhodamine-labelled oligosaccharides of xyloglucan (XLLG, XXLG and XXXG). The analysis of the TLC image reveals that unequivocal XET activity was easily detected using the *Md-XTH2* recombinant enzyme, after a 60 min incubation period with the xyloglucan source and fluorescent-labelled oligosaccharides used. The product of XET activity remained at the origin of the TLC, indicating incorporation of supplied labelled oligosaccharides in high molecular weight material.

The XEH activity of *Md-XTH2* was tested using a viscosimetric assay. Xyloglucan preparations are viscous and the cleavage of the polysaccharides is expected to significantly reduce the viscosity of the preparation. Fig. 4B shows the decrease of the viscosity of xyloglucan solutions in the absence of oligosaccharides. Again, *Md-XTH2* displayed XEH activity under the

conditions employed in the analysis. In fact, after 8 h incubation, the medium containing *Md-XTH2* enzymes caused more than a 50% reduction of the viscosity of the xyloglucan preparations.

### 3.6. Phylogenetic analyses

The deduced amino acid sequence of each clone identified, after removal of the signal peptide, was aligned with additional plant sequences and phylogenetic trees were constructed (Fig. 5). In each case, mature protein sequences were aligned with representative members of *Arabidopsis thaliana*, with sequences reported to be expressed in fruit and with other representative sequences cited in the literature. For construction of the PME dendrogram, the amino acid alignment considered only the region corresponding to the region available for *MdPME1*. The results suggest that *MdPME1* belongs to a phylogenetic cluster that does not include fruit isoforms. It clusters with two *A. thaliana* sequences, *AtPME4* and *AtPME6*. Interestingly, *AtPME4* is flower specific, which is in agreement with the pattern of gene expression observed for *MdPME1*. Putative fruit-specific sequences like *LePME1* or *LePME2* are also grouped in different clusters, which suggest that fruit-specific PMEs are not closely related.

The phylogenetic tree obtained for AFases and PLs suggests that the clones identified in this work in ripening apples may cluster together with other fruit-related homologues. In fact, *MdAF1* displays high identity to an AFase clone from pear that is up-regulated during ripening. With regard to PLs, *MdPL1* clusters with genes known to accumulate during ripening of grapes (Nunan et al., 2001) and banana (*MaPL1*), but it belongs to a different cluster than a ripening-induced PL from strawberry.

*MdEG1* shows higher similarity with *PsEG1*, a pea EGase expressing in etiolated seedlings, and with *LIEG1*, which is asso-

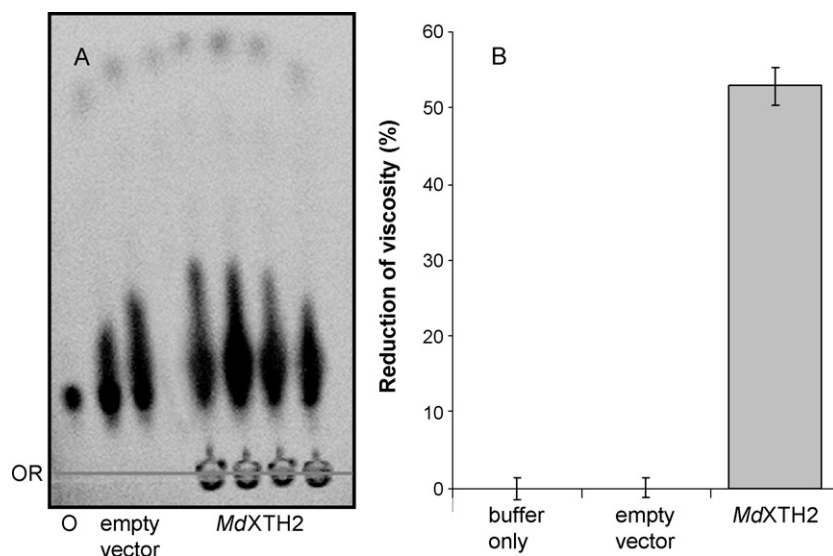


Fig. 4. Enzymatic activity assays for *Md-XTH2*. (A) Thin-layer chromatography (TLC) of products formed by incubation of xyloglucan polysaccharides, fluorescent-labelled oligosaccharides and medium containing recombinant *Md-XTH2* and medium from yeast transformed with an empty vector, under the conditions described in the Section 2. O, oligosaccharide only and OR, origin. Lanes with the same label correspond to replicate experiments. (B) Reduction of the viscosity of concentrated tamarind xyloglucan in the presence of recombinant *Md-XTH2* and negative control samples. The assays were conducted in the absence of oligosaccharides and are the mean  $\pm$  S.E. of six replicates.

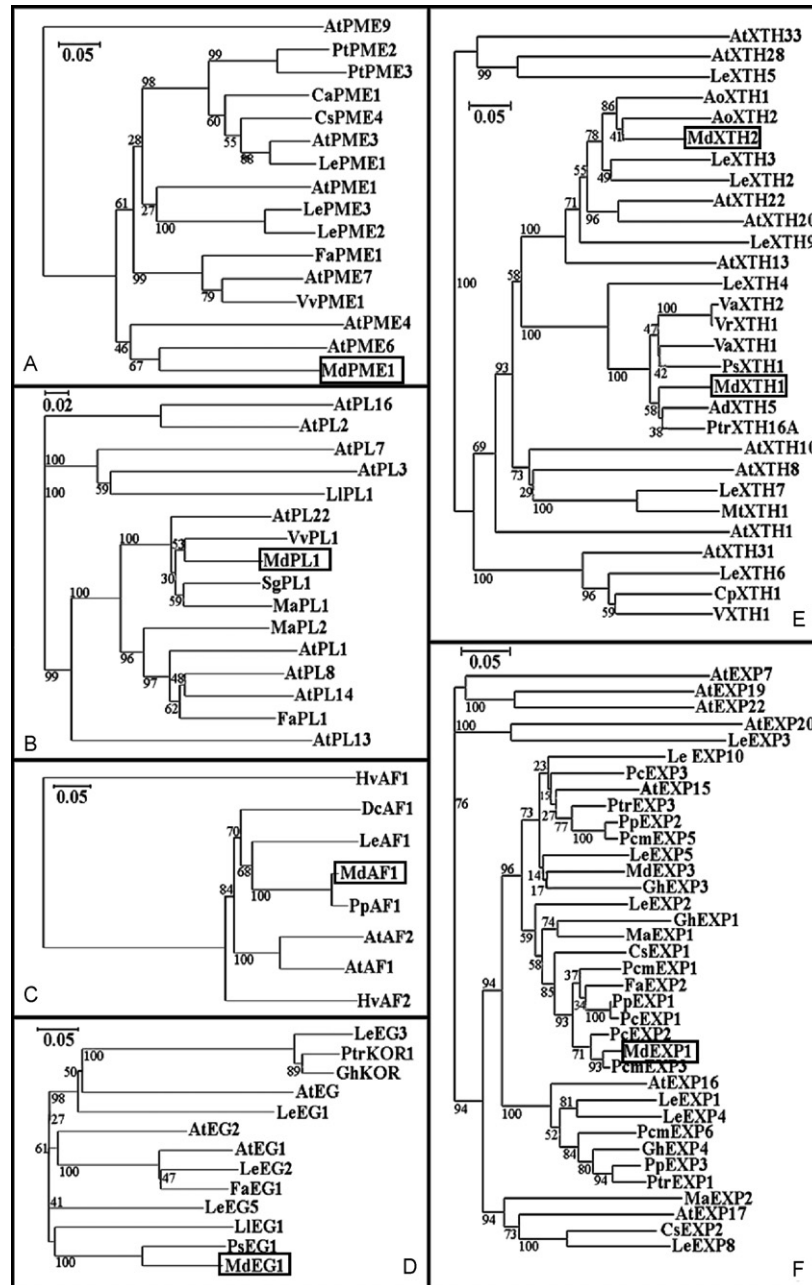


Fig. 5. Phylogenetic analysis of each of the proteins identified in this work (A, PME; B, PL; C, AFase; D, EGase; E, XTH and F, alpha-expansins) with other sequences with published expression patterns. Signal peptides were removed and alignment of the mature protein sequences, bootstrap analysis and phylogenetic trees were obtained using the ClustalX software. Accession numbers are listed in [Supplementary Table S2](#). Numbers on the branches of the dendrograms show bootstrap probability values. Boxes represent the apple sequences identified in this work.

ciated with pollen germination in *Iolium*. The sequence isolated from apple is not closely related to other sequences reported in fruit.

A phylogenetic tree generated from the alignment of the amino acid sequences of the two apple XTHs and other published sequences suggests five major clusters. The two apple XTHs are present in distinct phylogenetic groups. Interestingly, both *Md*-XTH1 and *Md*-XTH2 cluster preferentially with isoforms that are not related with rapid growth or expansion of organs. *Md*-XTH1 groups together with *Ad*XH5 and *Ptr*-XTH16A, which are thought to have a role in fruit ripening and secondary cell wall

formation respectively, while *Md*-XTH2 displays higher similarity with *Ao*-XTH1 and *Ao*-XTH2, which are known to express after growth cessation. In XTHs, although some reports state that members from some phylogenetic groups seem to catalyze only hydrolase activity rather than transglucosylase activity (Farkas et al., 1992; Fanutti et al., 1993; Tabuchi et al., 2001), others indicate that the current grouping of XTHs on the basis of their complete primary sequences does not predict their enzymatic activity or tissue specificity (Schroder et al., 1998; Saladié et al., 2006). The clustering of *Md*-XTH2 that proved to have both XET and XEH activities agrees with the latter hypothesis.

The *MdEXPA3* clone aligned with some sequences isolated from members of the *Rosaceae* family but does not belong to the same subgroup as other ripening-related expansins, namely *LeExp1*.

#### 4. Discussion

##### 4.1. Number of isoforms that express during late softening

In this work, a strategy to identify the isoforms most abundant during apple softening was employed by amplification of cDNA generated from total RNA extracted from over-ripe fruit with degenerate primers designed from conserved regions. Theoretically, this approach allows the amplification of virtually all the isoforms present in the template sample, but due to competition, it is expected that the most abundant transcripts would be favourably amplified. Except for the XTH family, in which two distinct isoforms were identified with a similar representation in the cDNA library, for all the other families only one isoform was identified. Despite the fact that cell wall-modifying enzymes belong to multigenic families, the low number of isoforms per family expressing in fruit after commercial maturity observed here is in accordance with previous works (e.g. Wakasa et al., 2003; Mwaniki et al., 2005).

Recently, EST sequences from developing and ripening 'Royal Gala' apple fruit (Newcomb et al., 2006; Park et al., 2006) were deposited in the GenBank databases. BLAST searches of UniGene sequences against ESTs from 'Royal Gala' fruit show that, except *MdEG1*, all genes cloned are represented in the 150 DAFB (days after full bloom) libraries. Furthermore, in general, the genes investigated in this work are the only ones expressing during late softening (detected 150 DAFB). An additional XTH isoform is represented at 150 DAFB, but only in tree-ripened fruit. Surprisingly, *Md-XTH1* ESTs were represented only in libraries constructed from fruit 10 DAFB. From the six expansins identified in apple fruit, *MdEXPA3* is well represented in the 150 DAFB libraries. However, Wakasa et al. (2003) reported that only *EXPA2* (referred to as *EXPA3* in their paper) showed increased accumulation after harvest in 'Golden Delicious'. Our results together with EST analysis suggest less transcription rates of *EXPA2* in 'Gala' apples. In fact, recently, Wakasa et al. (2006) analysed the mRNA accumulation patterns of the referred clone in a set of apple cultivars and the results show that this isoform is transcribed in ripening apples at relative high levels in some cultivars, including 'Golden Delicious' or 'Kitarou', in other cultivars it was below the experimental detection level, like 'Fuji' and 'Ralls Janet', and in others it increases until commercial maturity and decreases thereafter, during ripening (e.g. 'Kotaro'). That work did not include 'Gala' apples and it should be interesting to investigate the pattern of mRNA accumulation of this clone also in this cultivar. In contrast with our results and with Wakasa et al. (2003), *EXPA5* was also represented in ESTs from apples 150 DAFB in 'Royal Gala'. Despite the fact that this isoform has not been identified in this work, this gene should also be considered.

##### 4.2. Expression patterns of the cloned genes during ripening

###### 4.2.1. Pectolytic enzymes

Biochemically, the major changes related to apple softening are an increased content of water-soluble pectin (Knee, 1978b; Yoshioka et al., 1992) with residual or no depolymerisation (Yoshioka et al., 1992), and a massive loss of galactosyl residues from side-chains of rhamnogalacturonans (Knee, 1973; Bartley, 1974, 1976; Gross and Sams, 1984). Such events have been attributed to the action of exo-PG and  $\beta$ -Gal, which, releasing essentially monosaccharides, might act to increase the porosity of the gel structure of the wall enough to allow the dissolution of entangled polysaccharides, or to act as oligosaccharins (Fry et al., 1993b).

A PG was purified from apples (Wu et al., 1993), and the corresponding cDNA clone was isolated and characterised (Atkinson et al., 1998). The expression pattern of this clone was investigated in this work and it agrees with the results of Atkinson et al. (1998). According to the authors, PG mRNA expression is cultivar-dependent, occurring first and with significantly higher abundance in 'Royal Gala' and later in 'Braeburn' and 'Granny Smith' cultivars. In the present report, high levels of mRNA could be detected in fruit at harvest, supporting this observation.

Similarly, a  $\beta$ -Gal cDNA clone had been identified in apple (Ross et al., 1994) and its pattern of mRNA accumulation was examined in this work. The expression is very high in fruit at harvest, and remains high in softening and in over-ripe fruit. The transcription reported here using 'Mondial Gala' apples started earlier than with fruit from 'Granny Smith' in which the mRNA only accumulates after harvest (Ross et al., 1994), but its pattern of accumulation during softening was similar. As discussed for PG, that aspect probably reflects the later softening behaviour of 'Granny Smith', when compared with 'Gala' cultivars.

Supported by known biochemical modifications, these two enzymes were extensively studied in apples and, for a long time, were the only enzymes studied at the molecular level in this species. More recently, six expansins were cloned in apple and their pattern of gene expression was investigated during apple ontogenie (Wakasa et al., 2003).

However, it has been suggested that small localised changes in the polysaccharide structure may have significant implications in the overall cell wall metabolism, and the disassembly of the cell wall structural network probably involves the concerted and synergistic action of several different enzymatic activities, where one family of cell wall-modifying enzymes may mediate the activity of another, resulting in ordered cell wall re-organisation (Rose and Bennett, 1999).

PME enzymes have been considered to play an important role in cell wall disassembly during fruit ripening by increasing the *in vivo* susceptibility of pectins to hydrolases (Pressey and Avants, 1982; Seymour et al., 1987; Koch and Nevins, 1989). Although a cDNA clone with homology with PME could be identified in a cDNA library of over-ripe fruit, only spurious amplification was detected in the subsequent RT-PCR analyses, even after 40 amplification cycles. Even though it has been reported that

no gross change of esterification of the whole pectin fraction occurs during ripening of apples (Knee, 1978a; Irwing et al., 1984; Yoshioka et al., 1992), the water-soluble polyuronide has a lower degree of methoxylation than those lost in the EDTA and HCl soluble fractions (Yoshioka et al., 1992; Klein et al., 1995). Moreover, PME enzymes have already been purified from apples (MacDonald and Evans, 1996; Denès et al., 2000). Three explanations can be hypothesised to explain the result obtained in this work. One is that very little transcription is required to produce enough protein to carry out the required action. PMEs have been suggested to be present in distinct and defined micro-domains within the cell wall, acting to bring co-localised changes in pectin structure (Morvan et al., 1998). Another possibility is that the transcription may occur during a relatively short period of time and the RNA may have not been extracted during the peak of PME transcription. Alternatively, the degenerate primers used were designed from regions not conserved in the isoforms present in apple fruit. Interestingly, also in 'Rocha' European pear, no PME clone could be identified in ripening fruit (Sandra Tavares, personal communication). Also, transcripts of *FaPE2*, a PME clone isolated from a strawberry cDNA library of ripe fruit, are not detected in fruit during development and ripening (Castillejo et al., 2004), similar to the results obtained in this work.

A possible contribution of PL to pectic depolymerisation and loss of mesocarp firmness during fruit ripening started to be addressed after a PL gene has been isolated from strawberry (Medina-Escobar et al., 1997; Benitez-Burraco et al., 2003), and banana (Dominguez-Puigjaner et al., 1997), with expression restricted to ripening fruit. In the present work, a cDNA clone encoding a putative PL was identified. Although detected at harvest, the expression of *MdPL1* is considerably lower than the one observed during fruit growth. In strawberry and banana, PL mRNA accumulation increases with ripening but declines in over-ripe fruit and, in peach, two PLs transcripts were well represented in libraries from mature fruit but absent in the over-ripe stages (Trainotti et al., 2003). Therefore, one can suggest that the role of *MdPL1* in apple softening, if any, should be in the very early stages of ripening.

Together with galactose, arabinose-rich side-chains of pectins are degraded in the cell wall of 'Red Delicious' apple during ripening. Although loss of arabinose residues during ripening has been reported in several other fruit such as European pears (Gross and Sams, 1984), few reports have been focused in enzymatic activity or mRNA expression of AFases during ripening. In this work, a cDNA encoding an AFase was identified. In contrast with pABG1 ( $\beta$ -Gal) mRNA accumulation, the transcription of *MdAF1* decreases after harvest, but it is still easily detected in the postharvest period. This pattern of transcription is surprising since, while galactose is mostly lost before fruit maturity, loss of arabinose residues was reported to be associated especially with the onset of the over-ripened state (Peña and Carpita, 2004).

#### 4.2.2. Non-pectolytic enzymes

In addition to degradation and solubilisation of pectic polysaccharides, depolymerisation of high molecular mass matrix glycans has been reported in several species including

tomato (Maclachlan and Brady, 1994; Brummell et al., 1999a), strawberry (Huber, 1984), melon (Rose et al., 1998) and avocado (O'Donoghue and Huber, 1992). In these fruit, EGases ripening up-regulated cDNAs have been reported (e.g. Tucker et al., 1987; Cass et al., 1990; Brummell et al., 1997; Harpster et al., 1998; Llop-Tous et al., 1999; Spolaore et al., 2003). However, in apples, cellulose and other non-cellulosic polysaccharides show little change in amount during ripening of fruit (Bartley, 1976; Percy et al., 1997).

Transcripts of *MdEG1* could be detected at fruit set and, at very low levels, in fruit during the whole development. However, a higher number of PCR cycles was necessary to obtain a similar signal, which evidences lower transcription rate compared with the other cell wall enzymes under study. In tomato, some clones like *LeCel1*, *LeCel2* and *LeCel4* accumulate at the earliest stages of fruit cell expansion (Lashbrook et al., 1994; Gonzalez-Bosch et al., 1996; Brummell et al., 1997) in a pattern similar to the one obtained in the current work. However, both *LeCel1* and *LeCel2* accumulate again in fruit at ripening, suggesting different regulation and a role in fruit softening.

XTHs may act as xyloglucan specific endohydrolases or transglucosylases. Due to their role in xyloglucan modification, a role in fruit ripening has been suggested and ripening-related XTH gene expression has been reported in several species like tomato (Arrowsmith and de Silva, 1995), kiwifruit (Schroder et al., 1998), or European, Japanese and Chinese pear (Fonseca et al., 2004; Hiwasa et al., 2003a). Since XTH acting as transglucosylases can cleave and re-attach the xyloglucan polysaccharides without changes in the overall molecular weight of xyloglucans, this gene family should be investigated even in species like apple in which degradation of the hemicelluloses does not seem to occur. In fact, XTH gene expression was detected in grapes during ripening (Nunan et al., 2001) although no significant loss or degradation of xyloglucans occurs in this fruit after the 'veraison' (Nunan et al., 1998; Yakushiji et al., 2001). In the present work, two XTH cDNA clones were identified in ripening apples exhibiting a different pattern of mRNA expression. Although *Md-XTH1* mRNA accumulation reaches its maximum at harvest, it is nearly constitutive during the whole fruit developmental phases. On the other hand, *Md-XTH2* expresses at low levels in fully expanded unripe fruit, reaches its highest expression at harvest and slightly declines in the over-ripe stage. The activity of *Md-XTH2* was investigated using recombinant enzymes and displayed both endotransglucosylase and hydrolase activities. Since the overall molecular weight of xyloglucans does not change during apple ripening, the hydrolase activity, if present *in muro*, should be acting in specific and localised linkages, introducing subtle modifications to the polysaccharides.

Since the first fruit-related expansin identified in tomato (Rose et al., 1997), a large number of expansins have been cloned from fruit tissues and some clones show positive correlation between mRNA accumulation and fruit ripening (e.g. Civello et al., 1999; Catalá et al., 2000; Hayama et al., 2003; Hiwasa et al., 2003b; Dotto et al., 2006). In apple, *MdEXPA3* is expressed throughout fruit development and reaches its highest abundance at harvest, declining during softening and in the over-ripe stages. As stated previously, accumulation of mRNA



of a clone identical to the one cloned in this work was initiated when cell enlargement had started until commercial maturity (Wakasa et al., 2003), in a pattern similar to the one observed in this work.

#### 4.3. Tissue specificity of ripening-associated genes

None of the cDNAs studied in this work is fruit specific. This seems to be a common feature of members of these gene families. For example, out of seven  $\beta$ -Gal identified in tomato fruit, only TBG2 was found to be potentially fruit specific (Smith and Gross, 2000). The fact that all cDNAs clones, except for *MdEXPA3*, express at relatively high abundance in petioles of senescent leaves is particularly interesting since this tissue contains abscission zones; non-growing tissues where cell wall disassembly plays an important role. A major site of degradation during abscission is the middle lamella (Sexton and Roberts, 1982) and the mechanisms of abscission have been proposed to display analogy with fruit softening. In the literature, ripening-related EGases from avocado (Tonutti et al., 1995) and tomato (Lashbrook et al., 1994; Gonzalez-Bosch et al., 1996) have also been reported to express in abundance in tissues containing abscission zones. Furthermore, suppression of *Cel2* to lower than 5% of control values did not alter fruit firmness but did enhance the break strength of abscission zone (Brummell et al., 1999b).

#### 4.4. Softening in non-model fruit

The mRNA levels of each cDNA cloned were examined during fruit development and the results indicate that all clones, except *MdPME1*, *MdEG1* and *MdPL1* accumulate at their highest levels in fruit at commercial maturity. In the case of PG and  $\beta$ -Gal (identified in previous reports), the expression persist at high levels throughout ripening, until the over-ripe stage. Based only on the molecular results reported, the pattern of transcription of PMEs, EGases, expansins (known to be well correlated with tomato softening) and PL (important in banana and strawberry softening) presents some differences with other species and seems to de-emphasise the role of these enzymes in ripening of apples. On the other hand, the role of AFase, which has been less studied in tomato softening, may be important in fruit like apple or pear. Differences between model plants and other fruit were also observed in other species (recently reviewed by Brummell, 2006 and by Goulao and Oliveira, 2008).

Differences in softening of apples compared with model species, can be explained by the fact that limited or no depolymerisation of polysaccharides seems to occur. However, based on the results of this work, hydrolytic action by XTH and PL may exist as an early event of softening. These two cDNA clones and the corresponding encoded proteins should be studied with further detail to help clarification of the occurrence of depolymerisation in polysaccharides during apple softening. These two families of enzymes have been less studied than PGs and EGases in fruit ripening and the information available so far has been mainly correlative.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.postharvbio.2007.09.022.

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