

## Expression profile of transcripts encoding cell wall remodelling proteins in tomato fruit cv. Micro-Tom subjected to 15°C storage

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**Abstract.** To extend fruit market life, tomatoes are harvested before red ripe and kept at temperatures below optimum (20°C). In this work, Micro-Tom tomatoes stored at 20°C (normal ripening) were compared with those stored at 15°C or 4°C (chilling injury inducer) for 7 days. In contrast to 4°C, storage at 15°C delayed ripening with the benefit of not enhancing oxidative metabolism and of enabling ripening upon being transferred to 20°C. The transcriptional expression profile of enzymes related to cell wall metabolism was compared at the three temperatures. Although *endo-β-1,4-glucanase* (*Cell1*), which is associated with fruit decay, was largely increased after removal from 4°C storage, its expression was not modified in fruits stored at 15°C. Enhanced transcriptional expression of xyloglucan endotransglycosylase/hydrolases (*XTHs*) *XTH1*, *-2*, *-10* and *-11*, and of two *β-xylosidases* (*Xyl1-2*) was detected in fruits stored at 15°C with respect to those at 20°C. Following 2 days at 20°C, these transcripts remained higher in fruits stored at 15°C and *XHT3* and *-9* also increased. Ethylene evolution was similar in fruits kept at 15°C and 20°C; thus, the changes in the transcript profile and fruit properties between these treatments may be under the control of factors other than ethylene.

**Additional keywords:** cell wall metabolism, chilling injury, cold-storage, endotransglycosylase/hydrolase, ripening, xyloglucan *β-xylosidase*.

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### Introduction

The tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop (after potato). It is important in both the fresh vegetable and the food processing industry, with a world production ~100 million tons of fresh fruit (FAOSTAT 2009). Among tomatoes, the cultivar Micro-Tom has been proposed as a convenient model system for research on the regulation of berry fruit development and for genomics. Several resources such as ESTs and mutagenised lines in the Micro-Tom-background are therefore available (Dan *et al.* 2006; Martí *et al.* 2006; Matsukura *et al.* 2008; Aoki *et al.* 2010).

The quality of a fruit is determined by a wide range of desirable characters such as nutrition, flavour, taste, processing quality and shelf-life. Accelerated loss of firmness is considered one of the main factors that limit the shelf-life of fresh fruit. Therefore, firmness is considered an appropriate index for the ripening process and for the associated tomato quality (Lana *et al.* 2005). In this context, temperature regulation is the most effective tool for extending the storage life of fresh commodities so that ripening is delayed. However, tomatoes are

sensitive to chilling injury (CI) damage at temperatures below 10°C if held for longer than 2 weeks or at 5°C for longer than 6–8 days (Jackman *et al.* 1988; López Camelo 2004). The extent of injury depends on the temperature, the length of exposure, the stage of maturity at the moment of cold storage and the post-chilling conditions. The most common CI symptoms observed in tomato include uneven or partial ripening, increased fruit softening, increased susceptibility to postharvest fungal pathogens, reduced flavour and skin pitting (Hobson 1987).

Physiology of the CI involves changes in membrane competence, cell wall metabolism and tissue integrity, and impacts on the production of reactive oxygen species (ROS) (Saltveit and Morris 1990; Malacrida *et al.* 2006; Lim *et al.* 2009). Micro-Tom tomatoes are as prone to CI as other standard tomato varieties, evidenced by the collapse of deep layers of the pericarp, alteration in the kinetics of production of ripening sugars and organic acids and changes in the activities of antioxidants and enzymes that scavenge reactive oxygen species (Gómez *et al.* 2009).

The chilling response is not completely understood. In terms of cell wall metabolism, in cold-stored tomatoes the expression of polygalacturonase (PG) and the activity of PG can be reduced (Watkins *et al.* 1990; Marangoni *et al.* 1995) but in another set of experiments changes in pectin disassembly occurred independently of modifications in the activity of PG (Almeida and Huber 2008). When cold-stored tomatoes were removed from the cold, enhanced softening and mealiness was detected and related to increased pectin methylesterase (PME) activity (Marangoni *et al.* 1995). In contrast, Rugkong *et al.* (2010) reported that chilling had little if any effect on pectin solubilisation and depolymerisation and proposed that the response of other enzymes involved in cell wall metabolism could account for softening and other modifications in chilled tomatoes.

Following commercial packing, tomatoes are routinely palletised and cooled to 20°C for ripening or to 12–15°C for storage. Most commercial storage temperature recommendations have been formulated as a result of CI studies, based on threshold temperatures not inducing the development of visual CI symptoms or major compositional changes (Hobson 1981). However, the effects of currently recommended storage temperatures (12–15°C) on cell wall metabolism have not been addressed. In this context, it is valuable to identify the alterations, if any, that take place in fruits stored at temperatures above those inducers of CI but below ambient conditions which are currently used in the market. Hence, the objective of this work was to analyse the effect of different postharvest storage temperatures on dwarf tomato fruit (*S. lycopersicon* cv. Micro-Tom) focusing on the changes in the expression, at transcript level, of enzymes involved in tomato cell wall remodelling. The study of cell wall enzymes that are modified during storage at temperatures, such as 15°C, that delay ripening but not induce severe CI symptoms will help in the identification of candidate genes whose alteration may be used to improve aspects of shelf life.

## Materials and methods

### *Plant material and storage conditions*

Seeds of *Solanum lycopersicum* L. cv. Micro-Tom (Scott and Harbaugh 1989) were surface-sterilised for 10 min with chlorine solution (5% (v/v) sodium hypochlorite in water) containing 0.05% (w/v) Tween 20, then thoroughly rinsed with tap water for another 3 min before being placed in 500 mL pots with sterile soil to germinate. Germination was conducted under a 16 h fluorescent light at a photosynthetically active photon flux density of 130  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 8 h darkness with day/night temperatures of 22/17°C. Plants were grown in chambers under the same photoperiod and temperature conditions but under a photosynthetically active photon flux density of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and using standard cultural practices. Flowers were tagged after pollination and fruit were allowed to develop until different stages according to the number of days after anthesis (DAA) and harvested for analysis or used for the postharvest treatments.

For the ripening analysis on the vine, tomatoes were harvested at the following stages: immature green (IG, 16 DAA), mature green (MG, 36 DAA) and red ripe (RR, 45 DAA). In addition,

fruits harvested at the MG stage were stored for 7 days at different temperatures (4, 15 or 20°C, named 7D4, 7D15 and 7D20) and at a relative humidity (RH) of 90–93%, in ventilated, dark chambers. After this 7-day storage period, half of the fruit from each group were analysed and the other half were kept at 20°C for 2 days and used for analysis (named 7D4+2, 7D15+2 and 9D20).

Immediately after harvest at the different stages (IG, MG and RR) and at the end of each storage period (7D4, 7D15, 7D20, 7D4+2, 7D15+2 and 9D20), ethylene production, fruit firmness, colour, weight and pigment content were measured using fresh fruits. At least 15 fruits, collected at three different harvests, were individually analysed.

For protein and RNA extractions, fruits were cut into pieces; gel and seeds were removed and the remaining pericarp was frozen in liquid nitrogen and stored at –80°C until analysis. At least three different pools, each one composed of five different fruits, were analysed. Fruits were collected at three different harvests.

### *Fruit firmness, coloration and weight*

Whole fruit firmness measurements were evaluated with a firmness testing device Bareiss HPE II FFF (Bareiss, Oberdischingen, Germany) using a 0.25 cm<sup>2</sup> diameter steel cylinder probe. Measurements were carried by pressuring the anvil centred on the middle of a locule during 1 s and expressed in relative units.

Colour development was monitored using the CIE L\*a\*b (lightness/green-to-red scale/blue-to-yellow scale) colour space with a Minolta chromameter CR 300 (Minolta, Ramsey, NJ, USA).

Fruit fresh weight (FW) was also measured at harvest time at MG (FW<sub>MG</sub>) and after storage at different temperatures (FW<sub>X</sub>) to assess the relative loss of weight during storage which was calculated as follows (FW<sub>X</sub> – FW<sub>MG</sub>)/FW<sub>MG</sub>.

### *Ethylene measurement*

Ethylene evolution was measured in individual fruits enclosed in 225 mL sealed jars following the methods by Budde *et al.* (2006). After 1 h at 20°C, 3 mL air samples were withdrawn from the jars and injected through a sampling valve with 1 mL loop into a gas chromatograph (Hewlett Packard 5980 Series II, Wilmington, DE, USA), equipped with a flame ionisation detector and with a 3.048 m Porapack Q column (80/100; Restek, Bellefonte, PA, USA). Nitrogen carrier gas flow rate was 30 mL min<sup>–1</sup>, hydrogen carrier flow was 30 mL min<sup>–1</sup> and air flow was 400 mL s<sup>–1</sup>. Injector, oven and detector temperatures were 100, 100 and 250°C respectively.

### *Electrolyte leakage*

For electrolyte leakage, 20 fruits from each condition (from four different sets of experiments) were used. Peel was carefully removed, and 10-mm disks were cut with a cork borer. Four disks from two fruits were placed in a 200-mL glass bottle, washed twice with deionised water and then incubated in 20 mL of deionised water at 20°C. Conductivity of the incubation medium was measured with a conductivity meter (CDS 5000, LaMotte, Chestertown MD, USA) after 3 h of

incubation under constant shaking. After readings were taken, the flasks were autoclaved at 120°C for 20 min and cooled to 20°C, and the conductivity was measured again for total electrolytes. Data were expressed as (initial conductivity/total conductivity)  $\times$  100 (Perotti *et al.* 2011).

#### *Chlorophyll and carotenoids measurements*

Total chlorophyll and carotenoids were extracted according to Nagata and Yamashita (1992). Five fruits were sliced and ground in a mortar using liquid nitrogen; then 300 mg was transferred to 2 mL tubes and extracted with 500  $\mu$ L of acetone and 1 mL of hexane. After vigorous shaking by 'vortexing' the preparation was centrifuged at 10 000g for 15 min at 4°C. The absorbance was measured at 663, 645, 505 and 453 nm. Pigments concentrations were expressed in micrograms per gram of FW.

#### *Protein extraction and quantitation*

Total soluble protein from five fruits was extracted using a buffer containing 50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.0; 1 mM EDTA; 1 mM ascorbate; 20% (v/v) glycerol; 1 mM phenylmethylsulfonyl fluoride (PMSF); and 33  $\mu\text{g mL}^{-1}$  protease inhibitor cocktail (Sigma, St Louis, MO, USA). The samples (2 mL buffer  $\text{g}^{-1}$  tissue) were ground in a mortar using liquid nitrogen and centrifuged at 10 000g for 10 min at 4°C. After clarification extracts were used for protein quantitation and for measurements of antioxidant activities. For native activity gels, the extract was diluted in 0.25 M Tris-HCl, pH 6.8; 0.05% (v/v) bromophenol blue and 50% (v/v) glycerol.

Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as standard.

#### *Antioxidant activity*

Activities of glutathione reductase (GR, EC 1.8.1.7) and of catalase (CAT, EC 1.11.1.6) were measured spectrophotometrically in a final volume of 1 mL at 30°C and 340 nm using a UNICAM Helios  $\beta$  spectrophotometer (UNICAM Instruments, Cambridge, England). The reaction mixtures used contained: 100 mM  $\text{KH}_2\text{PO}_4$ , pH 7.8, 2 mM EDTA, 1 mM glutathione and 0.2 mM NADPH, for the GR assay and 100 mM  $\text{KH}_2\text{PO}_4$ , pH 7.8, 5 mM  $\text{H}_2\text{O}_2$  for CAT measurement. GR and CAT activities were assayed by measuring the rate of NADPH and  $\text{H}_2\text{O}_2$  oxidation at 340 nm and 240 nm, respectively, and expressed in international units (IU) per mg of protein.

Peroxidase (POD, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) in gel antioxidant enzyme activities were conducted as described by Casati *et al.* (2002). For each enzyme, at least three different gels were conducted using different samples for each condition analysed. Each isoform of the enzymes studied was semi-quantified by densitometric analysis of the bands in the gels. For this, the gels were scanned and the area of the bands was integrated by use of a BioChem System UVP (BioImaging System, Upland, CA, USA). In integrating the peak areas, a rectangular bar covering ~80% of each lane was drawn. Each densitometric analysis was determined in triplicate to minimise experimental errors.

#### *RNA isolation and RT-PCR*

Total RNA was isolated from 0.05–0.10 g of tomato pericarp at the IG stage and from 0.2 g in the other conditions. For each extraction, pericarp from five different fruits was used. The Trizol method was used, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), with the following modifications: after isopropanol addition, the pellet was resuspended in 200  $\mu$ L distilled water treated with DEPC and the RNA was precipitated by addition of 2.5 volumes of absolute ethanol for 2 h at  $-20^\circ\text{C}$ . After three washes with 80% (v/v) ethanol, the pellet was dried and resuspended in 40  $\mu$ L distilled water free of RNAses for 20 min at 55°C. The integrity of the RNA was verified by agarose electrophoresis. The quantity and purity of RNA were determined spectrophotometrically. First-strand cDNA was synthesised with MoMLV-reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI, USA) and using 2  $\mu\text{g}$  of RNA and oligo(dT).

#### *Quantitative real-time PCR*

Relative expression was determined by performing quantitative real-time PCR (QRT-PCR) in an iCycler iQ detection system and the Optical System Software ver. 3.0a (Bio-Rad, Hercules, CA, USA), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter, with 2.5 mM  $\text{MgCl}_2$ , 0.5  $\mu\text{M}$  of each primer and 0.04 U  $\mu\text{L}^{-1}$  GoTaq (Promega). PCR primers were designed based on tomato cDNA sequences published in GenBank and *S. lycopersicon* ESTs databases (TIGR Plant Transcript Assemblies; <http://plantta.tigr.org>; Sol genomics network (SGN) <http://solgenomics.net/>, accessed 14 September 2012), with the aid of the web based program 'primer3' ([http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) in a way to produce amplicons of 150 to 300 bp in size (Table 1). A 10-fold dilution of cDNA obtained as described above was used as template. PCR controls were performed in the absence of added reverse transcriptase to ensure RNA samples were free of DNA contamination. Cycling parameters were as follows: initial denaturation at 94°C for 2 min; 40 cycles of 96°C for 10 s, and 58°C for 15 s, 72°C for 1 min; and 72°C for 10 min. The SYBRGreen I fluorescence of the double strand amplified products was measured at 78°C. Melting curves for each PCR reaction were determined by measuring the decrease of fluorescence with increasing temperature (from 65 to 98°C). The specificity of the PCR reactions was confirmed by melting curve analysis using the software as well as by agarose gel electrophoresis of the products. Amplification efficiency (E) for each gene was determined using the relative standard curve method (Čikoš *et al.* 2007). Relative gene expression was calculated using the Comparative  $2^{-\Delta\Delta\text{CT}}$  method (Livak and Schmittgen 2001) and elongation factor 1 $\alpha$  (EF1 $\alpha$ ) as reference gene. Each cDNA sample was run in technical triplicates.

#### *Expression analysis: heat maps and cluster analysis*

Heat maps were created using Multiexperiment Viewer ver. 4.1. (MeV) (Saeed *et al.* 2003; <http://www.tm4.org/mev.html>). Cluster analysis was performed KMC: K-means/K-medians clustering by using Multiexperiment Viewer ver. 4.1 (MeV) which uses the Euclidean default distance metric. For

**Table 1. Sequences of the oligonucleotide primers used for Quantitative Real time RT-PCR**

Gene	Forward primer	Reverse primer
<i>PSY1</i> (AK247060) <sup>A</sup>	5'-GGGTAATTTTGTGTTGAGAGTGG-3'	5'-TGAAACTTGTCCATTGAG-3'
<i>EF1α</i> (X14449) <sup>A</sup>	5'-GCTGGTATCTCCAAAGATGGTC-3'	5'-CATGTTGTCTCCTTCAAACCA-3'
<i>XTH1</i> (SGN-U578547) <sup>B</sup>	5'-ATTTTTGGGGAACAGAACTGG-3'	5'-AACGTCGTCCACAAAAGATCAC-3'
<i>XTH2</i> (SGN-U577260) <sup>B</sup>	5'-AATACTCGAAAACGGGCAACT-3'	5'-CTGACCCACCGAAGATAAAT-3'
<i>XTH3</i> (SGN-U579684) <sup>B</sup>	5'-TCCAATTCTGCAGCTTCAAC-3'	5'-ATCTGCTGGAAATCCCTGTG-3'
<i>XHT5</i> (SGN-U587165) <sup>B</sup>	5'-AATGGACCCATTGGAACAAG-3'	5'-TGGATCAATCTCGCACTCTG-3'
<i>XHT9</i> (SGN-U570013) <sup>B</sup>	5'-TTGTGTGTATCAAGTCGGTGAA-3'	5'-TTAGTGGGCAACAATGGTGA-3'
<i>XHT10</i> (SGN-U213454) <sup>B</sup>	5'-GCAATGAGAGTTTACGCGAGTC-3'	5'-AACCCATCGTATTCTATTCTTC-3'
<i>XHT11</i> (SGN-U213455) <sup>B</sup>	5'-GCAATGAGAGTTTATGCCAGTT-3'	5'-CCCATCGAAGCCTATTTCTG-3'
<i>Exp1</i> (SGN-U578473) <sup>B</sup>	5'-TGAAGGAAGAATCCCTGGTG-3'	5'-TCCAGGAAGACACCATTTC-3'
<i>PG</i> (SGN-312702) <sup>B</sup>	5'-ACCTTTTCAGGTCCATGCAG-3'	5'-AGAAGGTTAAGGCCGTTGGT-3'
<i>PME1</i> (SGN-U12953) <sup>B</sup>	5'-AAAAGCTGGCGTGAAAAAGA-3'	5'-ACCAGTCCGCTTTTCTCTCA-3'
<i>Man4A</i> (AY046588) <sup>A</sup>	5'-GAAATGGCGGATATTGAA-3'	5'-TGGACTTGGATCCATTGTGA-3'
<i>Cell</i> (U13054) <sup>A</sup>	5'-TGCTGGAGCCCAAACCTTAC-3'	5'-CCTATTCCAGCTGCCTCAAG-3'
<i>TBG4</i> (SGN-U577461) <sup>B</sup>	5'-TCTGCTCAGTGTTCCTGTTG-3'	5'-GGCTGCTTTTGAGCCATTAG-3'
<i>Xyl1</i> (SGN-U563237) <sup>B</sup>	5'-CATGAGGCCGAACCTGC-3'	5'-CTGCAGTTTGCCCAACC-3'
<i>Xyl2</i> (SGN-U580888) <sup>B</sup>	5'-TTTGCTGTGCATAATCCTAAAGTC-3'	5'-TTCCAGGGAATCCTGTTTTT-3'
<i>ACO1</i> (HQ322499) <sup>A</sup>	5'-CAAAGTGAGTGGCCTTCAAC-3'	CTGCCTCTTTTCAACCAAA-3'

<sup>A</sup>GenBank Accession number.

<sup>B</sup>Sol Genomics Network (SGN) Accession number.

clustering the means option and 100 maximum iterations were used.

### Statistical analyses

Data were tested using one way analysis of variance (ANOVA). Minimum significance differences were calculated by the Bonferroni, Holm-Sidak, Dunett and Duncan tests ( $\alpha=0.05$ ) using the Sigma Stat Package (R Systat Software Inc. (SSI), San Jose, CA, USA).

## Results

### Comparison of fruit ripening parameters under three different storage temperatures

Under the growing conditions of the present study, *Solanum lycopersicum* cv. Micro-Tom fruit reached the immature green (IG) stage 16 days after anthesis (DAA), the mature green (MG) stage 36 DAA and the red ripe (RR) 45 DAA. From IG to RR, Micro-Tom tomato pericarp showed a great decrease in both chlorophyll *a* and *b* contents, and a 70-fold increase in the levels of lycopene (Fig. 1a). The increase in lycopene matched with the augmentation in the levels of the transcript encoding the ethylene-regulated phytoene synthase 1 (*PSY1*; AK247060) involved in carotenogenesis (Fig. 1b). Changes in fruit pigment composition were correlated with a transition from a green coloration with a yellow component of MG to a red colour with a yellow component of RR (see Table S1, available as Supplementary Material to this paper). In addition, ethylene was not detected at the IG and MG stages and largely increased at the RR stage (Fig. 1c). The levels of the transcript encoding 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO1*) increased with the progression of ripening, reaching maximum levels at the RR stage (Fig. 1b). In parallel with the increase in *ACO1* and ethylene levels, fruit firmness decreased over the ripening process, showing the lowest firmness at the RR stage (Fig. 2).

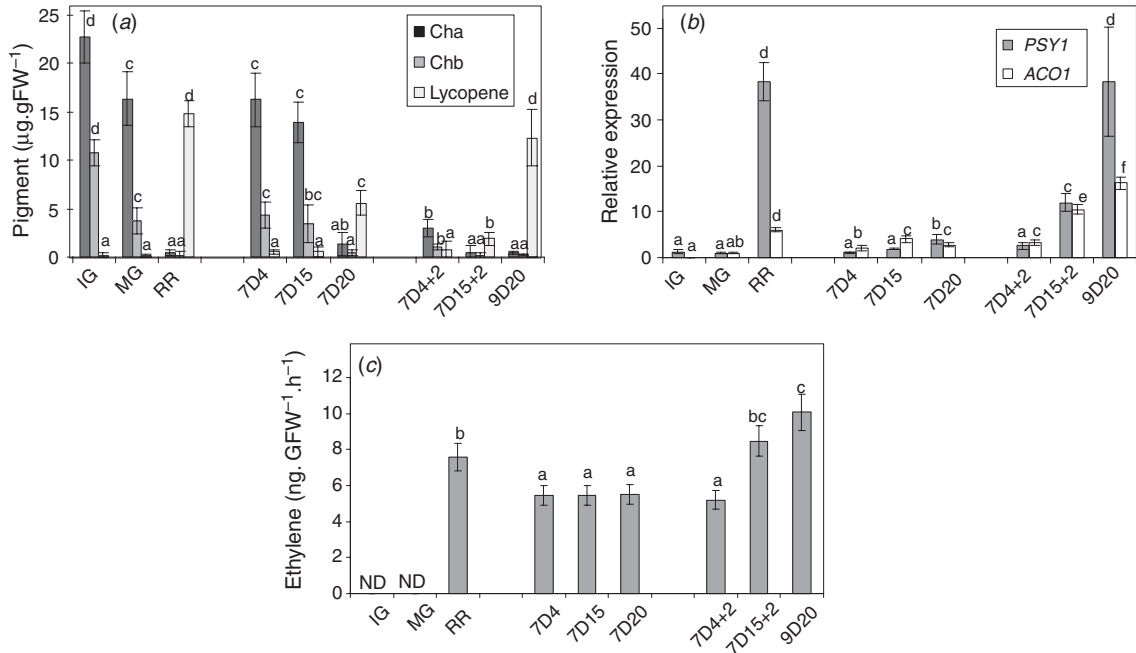
Ripening off the vine from MG stage was analysed until 9 days at 20°C (9D20 fruits) with fruits showing similar characteristic as those ripened on the vine (RR) (Fig. 1) but exhibiting an orange colour rather than dark red (see Fig. S1, available as Supplementary Material to this paper; and Table S1). Ripening was impaired in fruit stored at temperatures below 20°C, with maintenance of firmness, weight, chlorophylls and lycopene values of MG fruits (Figs 1, 2; Tables S1, S2) in either 7D4 (7 days at 4°C) or 7D15 (7 days at 15°C) fruits. Nevertheless, after transferring tomatoes from 15 to 20°C for 2 days (7D15+2) an increase in ethylene and *ACO1* was observed, which was accompanied by the achievement of similar properties as those fruits kept for 9 days at 20°C (loss of weight, firmness, and chlorophyll content, Figs 1, 2; Table S2) except for lower levels of lycopene, which correlated with lower levels of *PSY1* (Fig. 1) and yellow-orange coloration (Table S1). However, storage at 4°C was useful to preserve the firmness, pigment and colour of MG fruit for 7 days (Figs 1, 2; Table S1), after transferring to 20°C for 2 days, 4°C-exposed fruits (7D4+2) failed to complete the ripening process, as shown by lower ethylene evolution, pigment composition and higher firmness than 7D15+2 and 9D20 fruits (Figs 1, 2). In this case, even though these fruits were maintained at 20°C for another 7 days they still failed to fully ripen, with higher firmness and a yellower coloration than RR or 9D20 tomatoes (Fig. S1).

Ion leakage measurements were used to evaluate membrane integrity and thus putative damage due to cold storage. As expected, tomatoes stored for 7 days at 4°C presented significant higher ion leakage ( $81.0 \pm 1.0\%$ ) than those kept at 15°C ( $76.5 \pm 1.5\%$ ).

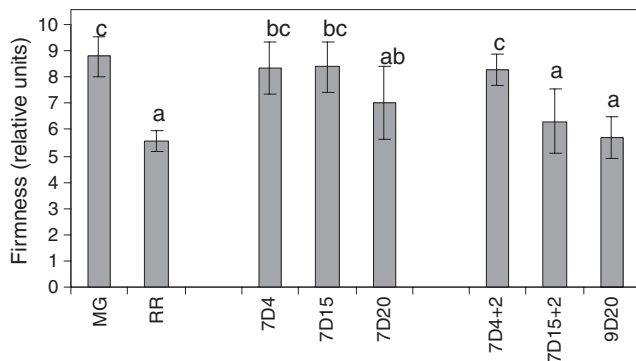
### Enzymes involved in the antioxidant metabolism under different storage temperatures

The enzymatic antioxidant activity of four enzymes was investigated by activity staining in native PAGE (POD and



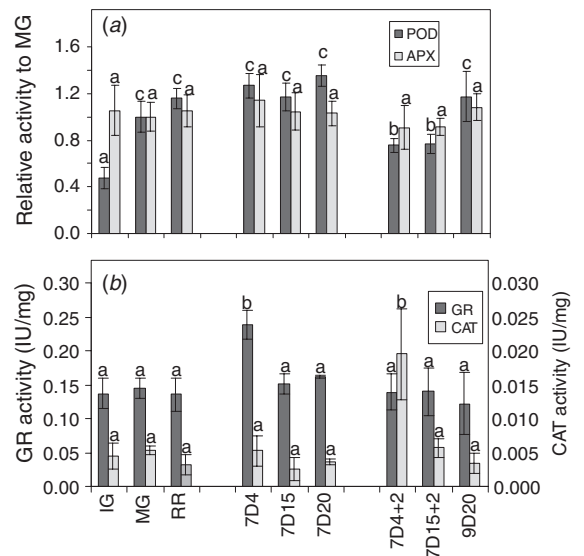


**Fig. 1.** Maturation analysis of tomato fruit cv. Micro-Tom. Fruits were harvested at the immature green (IG), mature green (MG) and red ripe (RR) stages. A group of MG fruit was kept for 7 days at 4°C (7D4), 15°C (7D15) or 20°C (7D20), followed by 2 days at 20°C (7D4+2; 7D15+2 and 9D20 respectively). Fruits were collected at least at three different harvests. (a) Chlorophyll *a* and *b* and lycopene; (b) expression analysis of *PSY1* and *ACO1* of determined by qRT-PCR; y-axis refers to the fold difference in a particular transcript level relative to its amount in MG; (c) ethylene production. ND: not detected. Values represent the mean  $\pm$  s.d. For each variable analysed, bars with the same letters are not significantly different ( $P < 0.05$ ). (a, c) At least 15 fruits were individually analysed; (b) at least three different pools, each one composed of five different fruits, were analysed.



**Fig. 2.** Changes in fruit firmness during ripening on and off the vine under different postharvest conditions. Values represent the mean  $\pm$  s.d. Bars with the same letters are not significant different ( $P < 0.05$ );  $n = 15$ . Samples analysed are described in Fig. 1.

APX, Fig. 3a) or by in tube activity assay (GR and CAT, Fig. 3b) as a means of oxidative stress evaluation. Although APX activity was almost constant in all the samples analysed, POD activity increased during the IG to MG transition (Fig. 3a). With respect to storage under different conditions, significant increases in activity were observed for GR in 7D4 tomatoes (~2-fold with respect to MG) and for CAT activity in 7D4+2 tomatoes (~4-fold increase with respect to MG) (Fig. 3b). In contrast, a decrease in POD activity was measured after 2 days at 20°C in tomatoes that had



**Fig. 3.** Antioxidant activity. (a) Densitometric analyses of peroxidase (POD) and ascorbate peroxidase (APX) activities on native PAGEs. Representative gels are shown in Fig. S2. (b) Catalase (CAT) and glutathione reductase (GR) specific activities are expressed in International Unit (IU) per mg of total soluble protein. For each enzyme analysed, bars with the same letters are not statistically different ( $P < 0.05$ ). Samples were collected as in Fig. 1. At least three different pools, each one composed of five different fruits, were analysed.

been stored at either 4 or 15°C (7D4+2 and 7D15+2 respectively) (Fig. 3a).

*Expression analysis of enzymes involved in cell wall metabolism during storage at three different temperatures*

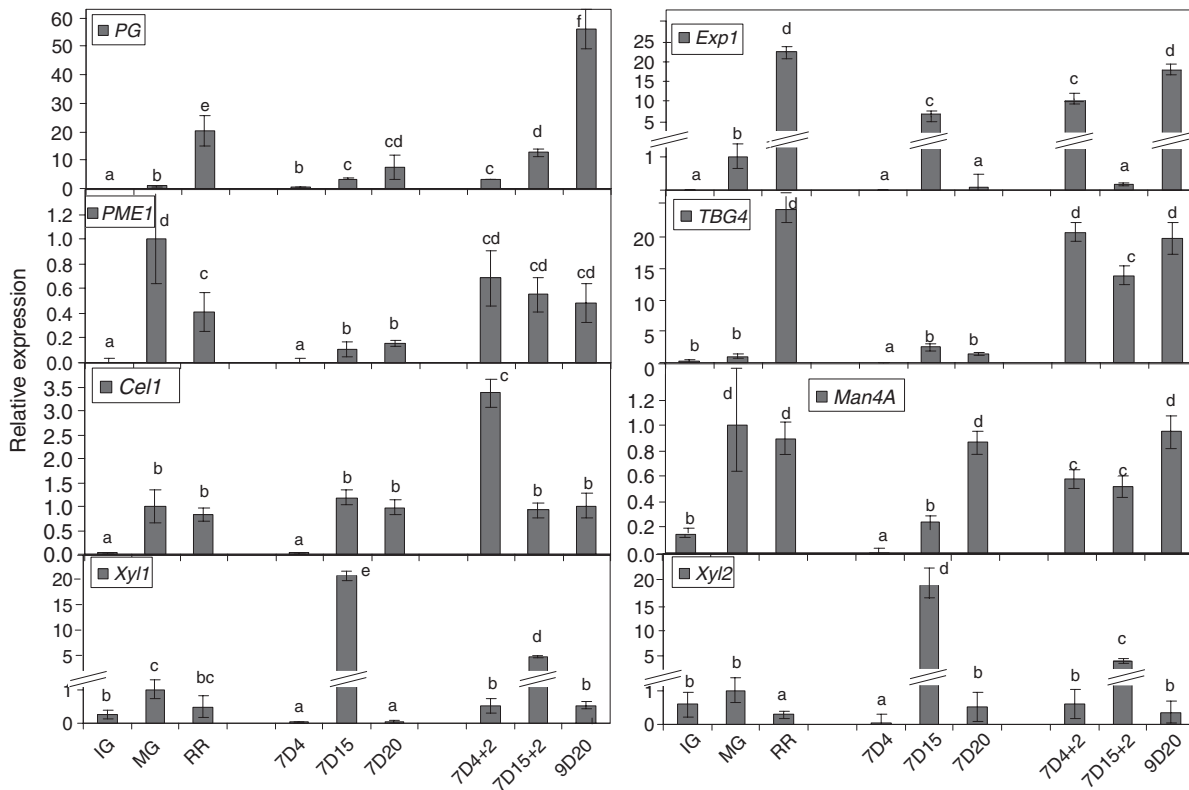
The levels of 15 transcripts encoding cell wall-related proteins were investigated by quantitative real-time PCR (qRT-PCR) during storage at different temperatures. The genes evaluated were the following: two β-xylosidases (EC 3.2.1.37) (*Xyl1* and *Xyl2*), a β-galactosidase (EC 3.2.1.23) (*TBG4*), an endo-β-1,4-glucanase (*EGase*, EC 3.2.1.4) (*Cell1*), a pectin methylesterase (EC 3.1.1.11) (*PME1*), an expansin (*Exp1*), a polygalacturonase (EC 3.2.1.15) (*PG*); an endo-β-1,4-mannanase (EC 3.2.1.78) (*Man4A*), and seven xyloglucan endotransglycosylase/hydrolases (EC 2.4.1.207 and/or 3.2.1.151) (*XTHs*).

A great number of the transcripts analysed increased from IG to MG (Figs 4, 5), with *PME1* showing the greatest enlargement (274-fold), followed by *XTH5* (83-fold) and *Cell1* (57-fold) (Figs 4, 5). In the transition from MG to RR, *PG*, *TBG4*, *Exp1* and *XTH3*, -9 and -10 increased, whereas *Xyl2*, *PME1* and *XTH1*, -2 and -5 decreased. No significant changes were detected in *Cell1*, *Man4A*, *Xyl1* and *XTH11* in the transition from MG to RR (Figs 4, 5). In ripened fruits which had matured off the vine for 9 days at 20°C (9D20), the transcripts analysed showed basically the same levels as those ripening on the vine (RR), as visualised in the heat map shown in Fig. S3. Although after

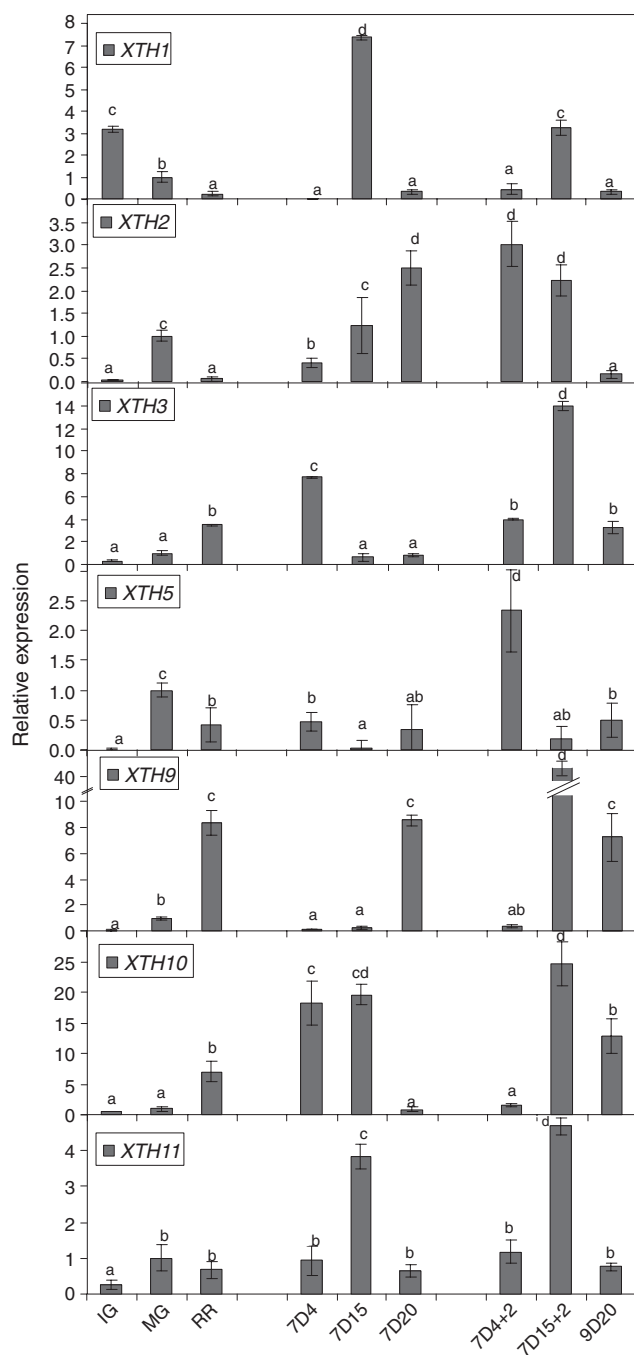
7 days at 20°C (7D20 fruits) many transcripts showed intermediate levels between MG and 9D20 fruits, *XTH2* showed a peak at 7D20 and then decreased in 9D20. In contrast, *Exp1* slightly decreased at 7D20 with respect to MG and then greatly increased at 9D20 (Figs 4, 5).

Regarding fruits stored for 7 days at 4°C, a decrease in the level of most transcripts encoding enzymes related to cell wall metabolism was observed with respect to the levels in MG tomatoes (Figs 4, 5, S3). The exceptions were *PG* and *XTH11*, which remained unchanged, and *XTH3* and -10 that increased 7.7 and 18 times respectively (Fig. 5), reaching higher levels than those found in RR and 9D20 fruits. After transferring these fruits to 20°C for 2 days the analysed transcripts exhibited similar levels to ripe fruits, either RR or 9D20, with the exception of *Cell1*, *XTH2* and -5 which were greatly increased with respect to ripe tomatoes (RR and 9D20), and *PG*, *XTH9* and -10, which were lower (Figs 4, 5).

In contrast, for the majority of the cell wall related transcripts analysed, the effect of storage at 15°C was clearly different from that observed at 20°C and 4°C (Fig. S3). Further, *Xyl1* and -2, and *XTH1*, -10 and -11 showed significant increases with respect to either MG, RR, 7D20 or 9D20 tomatoes, and *Exp1* showed an increase with respect to MG and 7D20 fruits. Two days after transferring the fruits to 20°C (7D15+2), the levels of *PME1* and *Cell1* reached those of ripe fruits (RR, Figs 4, 5). In the case of *Man4A* and *TBG4*, although an increase was observed, the levels were smaller than those of ripe fruits. In the case of *Xyl1* and -2,



**Fig. 4.** Expression analysis by qRT-PCR of transcripts encoding proteins involved in cell wall metabolism. Values represent the mean ± s.d.: y-axis refers to the fold difference relative to mature green (MG). Bars with the same letters are not significantly different ( $P < 0.05$ ). Fruit sampling is described in Fig. 1. At least three different pools, each one composed of five different fruits, were analysed.



**Fig. 5.** Expression analysis by qRT-PCR of transcripts encoding xyloglucan xyloglucosyltransferase/endohydrolases (XTHs). Values represent the mean  $\pm$  s.d. y-axis refers to the fold difference relative to mature green (MG). Bars with the same letters are not significantly different ( $P < 0.05$ ). Treatments are described in Fig. 1. At least three different pools, each one composed of five different fruits, were analysed.

*XTH1*, *-2*, *-3*, *-9*, *-10* and *-11* the level in 7D15+2 was greater than those of RR or 9D20 tomatoes whereas *Exp1* was remarkably lower (Figs 4, 5).

Based on the pattern of expression of all the tomato samples analysed, the transcripts encoding enzymes involved in cell wall

metabolism were clustered in six different groups (Fig. 6), with *ACO1* and *PSY1* grouping with *Man4A* and *PG* (Fig. 6).

## Discussion

### *Below ambient temperature is effective for delaying ripening*

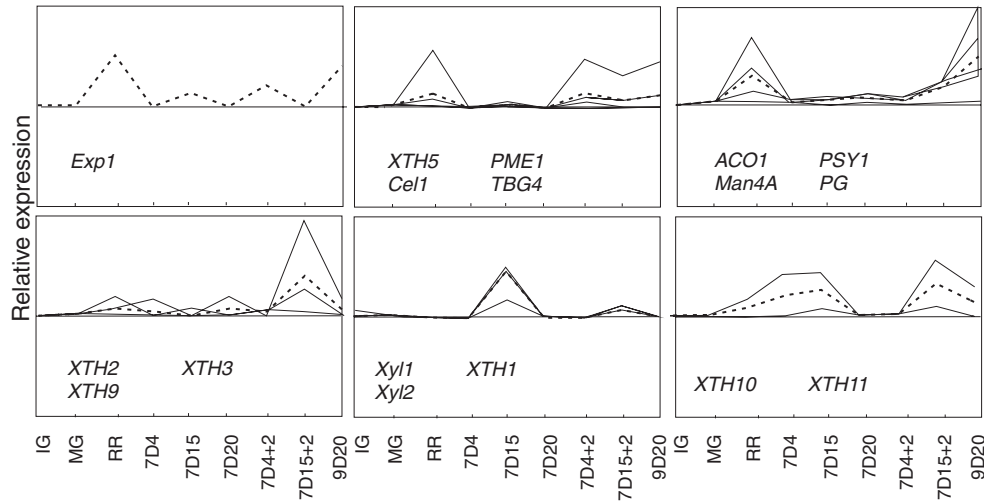
Storage at either 4 or 15°C for a week was effective in slowing down pigment changes that occur during fruit ripening at 20°C, although similar ethylene levels were detected (Fig. 1). After transferring to 20°C, fruits stored at 4°C failed to ripen whereas those kept at 15°C showed similar properties of those stored at 20°C, except for lycopene and *PSY1* levels that were lower (Figs 1, 2, S1; Table S1).

The increase in POD activity when ripening on the vine (Fig. 3a) correlates with the reported increase of ROS (Malacrida *et al.* 2006; Gómez *et al.* 2009). Tomatoes stored at 4°C (7D4) showed a rise in GR (Fig. 3b) and an increase in CAT activity after transferring to 20°C (7D4+2, Fig. 3b), in relation to the increase of H<sub>2</sub>O<sub>2</sub> when chilled fruits are transferred to non-chilling temperatures (Sala 1998; Malacrida *et al.* 2006). Modifications in the antioxidant enzymes were not detected in fruits stored at 15°C and only a decrease in POD was observed after transferring to 20°C (Fig. 3a). Even though ROS species were not measured, the results suggest that ROS production would not be enhanced at 15°C; and thus support 15°C as an adequate temperature to preserve fruit quality and to extend postharvest shelf life without oxidative damage.

### *Despite similar ethylene evolution, the response of transcripts encoding enzymes related to cell wall metabolism is different at 4, 15 and 20°C*

Transcript profiling of cell wall related enzymes during ripening of tomato cv. Micro-Tom agrees with proposed mechanisms for fruit softening. *PG*, *Exp1*, *PME1* and *TBG4* followed the pattern of colour change and ethylene evolution (Figs 1, 4; Brummell *et al.* 1999a, 1999b; Smith and Gross 2000; Brummell and Harpster 2001; Smith *et al.* 2002). *Man4A* followed the same trend of *PSY1* and *ACO1* (Fig. 6), in correlation with the increase of endo- $\beta$ mannanase activity during ripening (Bewley *et al.* 2000; Wang *et al.* 2009). Regarding enzymes involved in xyloglucan depolymerisation, *Cell1* (EGase) and *XTH5*, which clustered with the ripening related *TBG4* and *PME1*, also increased during fruit softening (Fig. 5; Real *et al.* 2004; Miedes and Lorences 2009). In contrast, *Xyl1* and *-2* remained similar, in accord with constant  $\beta$ -D-xylosidase activity during ripening (Fig. 4; Itai *et al.* 2003).

Although ethylene evolution was similar (Fig. 1c), the response of transcripts encoding enzymes related to cell wall metabolism was very different after 7 days of storage at 4, 15 or 20°C (Fig. S3), suggesting there might be other signals regulating the expression cell wall related enzymes (Page *et al.* 2010; Rugkong *et al.* 2010, 2011). Specifically, *Exp1*, *Xyl1* and *-2*, *XTH1*, *-10* and *-11* levels were higher in 7D15 than in 7D20 fruits (Figs 4, 5). After transferring to 20°C, these transcripts remained higher in fruits removed from 15°C (7D15+2) than in those kept at 20°C (9D20), with the exception of *Exp1* which largely decreased (Fig. 4). Again, despite both groups of fruits exhibited the same



**Fig. 6.** Cluster analysis expression profiles of transcripts shown in Figs 4 and 5. The mean expression levels of genes in the clusters are shown as a centroid graph (dashed line) overlaid on top of the individual expression graphs. Each line represents the graph of an individual gene, which level of expression is expressed relative to the amount in MG. Genes within each cluster are mentioned at the bottom of each graph.

ethylene evolution and firmness (Figs 1c, 2), their transcriptional profiles were different.

*Xyl1* and *-2* clustered together with *XTH1* (Fig. 6) and separated from *ACO1*, supporting the suggestion that their expression is independent of ethylene (Itai *et al.* 2003). Increases in *Xyl2* protein were detected upon storage below 20°C in two other tomato genotypes (Page *et al.* 2010). Even though their pattern of softening was similar, differences in the levels of *Xyl1-2* and a remarkable decrease in *Exp1*, may translate to differences in the quality of 15°C stored tomatoes at 20°C (7D15+2), in comparison with 9D20 fruits. These potential differences should be tested using other structural measurements. This could be similar to the case of PG and PME1, whose reduction had little effect on fruit firmness but did impact on quality traits such as decreased cracking and improved paste quality (Schuch *et al.* 1991; Kramer *et al.* 1992; Thakur *et al.* 1996). Hence, further antisense studies are needed to elucidate the function(s) of the members of the *Xyl* family and to evaluate the impact of changes in their expression under regular storage conditions around 15°C.

Regarding *XTHs*, many of them showed greater levels in 7D4 (*XTH3* and *-10*), 7D4+2 (*XTH2* and *-5*) or 7D15 (*XTH1*, *-10* and *-11*) fruits than in 9D20 or RR fruits, with 7D4, 7D4+2 and 7D15 being as firm as MG fruits and firmer than ripe tomatoes (Figs 2, 4). In another study, *XTH5* was increased in 3°C stored fruits harvested at the breaker stage (Rugkong *et al.* 2011); differences in the stage of harvesting – MG in the present work – may account for differences in the response of the *XTHs*. Together our data provides evidence that *XTHs* do not represent primary cell wall-loosening agents but could contribute to maintenance of xyloglucan and cell wall structure through the cleavage and reformation of bonds between xyloglucan chains. In this respect, decrease in XET activity is correlated with fruit softening, and overexpression of *XTH1* delays softening during ripening (Fonseca *et al.* 2005; Miedes *et al.* 2010).

The seven fruit-expressed *XTHs* analysed here align in three different phylogenetic groups (Saladié *et al.* 2006). In agreement with the classification of *XTH1* in the phylogenetic group 1, this transcript appeared in a separate cluster from the other *XTHs* (Fig. 6). Members of the phylogenetic group 2 (*XTH2*, *-3*, *-9*, *-10* and *-11*, Saladié *et al.* 2006) clustered in two different groups based on their expression (Fig. 6). Thus, it seems that regulatory features of gene expression are conserved within the phylogenetic groups and that *XTHs* are regulated in an ethylene independent manner.

Plant cell wall remodelling proteins influence pathogen susceptibility (Cantu *et al.* 2008). *Cell1* has been related to the response to the fungal necrotroph *Botrytis cinerea* (Flors *et al.* 2007). Therefore, the increase in the expression of *Cell1* in 7D4+2 fruits may be related to the increase in the disease susceptibility of chilled tomatoes that usually occurs after removal from cold storage. In contrast, 15°C-stored tomatoes did not show enhanced levels of *Cell1*, supporting the use of 15°C as a storage temperature.

Fruit softening is a multi-gene trait with each enzyme activity having its own role to play in softening and textural changes. Taken together, storage at 15°C delayed ripening with the benefit of not enhancing oxidative metabolism and enabling ripening upon transferring to ambient conditions, with an expression profile of cell wall enzymes at 15°C remarkably different to those at 20 and 4°C. Data obtained point out that enzymes involved in the hemicellulose fraction metabolism, such as *Xyl* and *XTHs*, could be important determinants of either fruit softening or quality; and thus, the physiological significance of these changes should be further explored. Moreover, the expression of these transcripts is under the control of factors other than ethylene. A study of the response to cold storage of cultivars varying in *Xyls* and *XTHs* contents may also help in elucidating the contribution of these enzymes to fruit quality and in the process of variety selection.



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