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Expression of xyloglucan endotransglucosylase/hydrolase (*XTH*) genes and XET activity in ethylene treated apple and tomato fruits

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A B S T R A C T

Xyloglucan endotransglucosylase/hydrolase (XTHs: EC 2.4.1.207 and/or EC 3.2.1.151), a xyloglucan modifying enzyme, has been proposed to have a role during tomato and apple fruit ripening by loosening the cell wall. Since the ripening of climacteric fruits is controlled by endogenous ethylene biosynthesis, we wanted to study whether XET activity was ethylene-regulated, and if so, which specific genes encoding ripening-regulated *XTH* genes were indeed ethylene-regulated. XET specific activity in tomato and apple fruits was significantly increased by the ethylene treatment, as compared with the control fruits, suggesting an increase in the *XTH* gene expression induced by ethylene. The 25 *Sl*XTH protein sequences of tomato and the 11 sequences *Md*XTH of apple were phylogenetically analyzed and grouped into three major clades. The *SlXTHs* genes with highest expression during ripening were *SlXTH5* and *SlXTH8* from Group III-B, and in apple *MdXTH2*, from Group II, and *MdXTH10*, and *MdXTH11* from Group III-B. Ethylene was involved in the regulation of the expression of different *SlXTH* and *MdXTH* genes during ripening. In tomato fruit fifteen different *SlXTH* genes showed an increase in expression after ethylene treatment, and the *SlXTHs* that were ripening associated were also ethylene dependent, and belong to Group III-B (*SlXTH5* and *SlXTH8*). In apple fruit, three *MdXTH* showed an increase in expression after the ethylene treatment and the only *MdXTH* that was ripening associated and ethylene dependent was *MdXTH10* from Group III-B. The results indicate that *XTH* may play an important role in fruit ripening and a possible relationship between *XTHs* from Group III-B and fruit ripening, and ethylene regulation is suggested.

Introduction

Fruit ripening and softening are major attributes that contribute to perishability in fleshy or climacteric fruit. Fleshy fruits soften during ripening mainly as a consequence of the disassembly of different cell wall components. The main changes are in pectic and hemicellulosic polysaccharides, two of the major cell wall components that undergo solubilization and depolymerization (Fischer and Bennett, 1991; Wakabayashi, 2004; Vicente et al., 2007; Goulao and Oliveira, 2008; Li et al., 2010). Studies made mainly in climacteric fruits have shown that modification of cell wall polymers seems to be a consequence of the coordinated action of cell wall modifying enzymes and proteins such as polygalacturonase, pectate lyase, pectin methylesterase, β-galactosidase, R-L-arabinofuranosidase, endo-(1,4)-β-D-glucanase, β-xyloxidase, expansin, xyloglucan endotransglucosylase and endomannanase (Brummell and Harpster, 2001; Bennett and Labavitch, 2008; Li et al., 2010).

Depending on the fruit species, different modifications may occur and to different extents, so the role of individual cell wall modifying enzymes in fruit softening and the composition of polymers in the fruit cell wall may differ between fruit species. Tomato fruit has been used as a model system for ripening studies, so ripening and softening have been intensively studied (Saladié et al., 2006, 2007; Bapat et al., 2010; Klee and Giovannoni, 2011), but the underlying molecular mechanisms may not be entirely representative of fruit softening in other species. For instance, apple fruit has limited softening during ripening and the fruit softens without detectable depolymerization of pectins and hemicelluloses, whilst in other species, depolymerization of these compounds appears to occur. In apple fruit, the activity of several cell wall-modifying enzymes has been reported and it seems that ripening is possibly coordinated by members of the same families (Goulao et al., 2007; Goulao

Abbreviations: XEH, xyloglucan endohydrolase; XET, xyloglucan endotransglucosylase; XTH, xyloglucan endotransglucosylase/hydrolase.

and Oliveira, 2008; Johnston et al., 2009; Wei et al., 2010). In addition, Goulao et al. (2008) have cloned and characterized the most abundant isoforms of enzymes from candidate families that are expressed throughout apple growth and ripening.

As we mentioned above, depolymerization of hemicelluloses is also a common feature of ripening fruit, wherein xyloglucan is the predominant hemicellulose of cell walls. Xyloglucan endotransglycosylase/hydrolase (XTH) enzymes are thought to play a key role in fruit ripening by loosening the cell wall in preparation for further modification by other cell wall-associated enzymes and through disassembly of xyloglucan.

XTH can act as a transglucosylase (XET), having a dual role, integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan, restructuring existing cell wall material by catalyzing transglycosylation between previously wall bound xyloglucan molecules, or acting as hydrolase (XEH), hydrolyzing one xyloglucan molecule, depending on the nature of the xyloglucan donor and acceptor substrates (Fry et al., 1992; Nishitani and Tominaga, 1992; Rose et al., 2002).

XTH gene products from GH16 have historically been classified into four major phylogenetic subgroups (Groups I–IV) for *Arabidopsis thaliana* and into three groups for *Solanum lycopersicum* (Campbell and Braam, 1999; Yokoyama and Nishitani, 2001; Rose et al., 2002; Saladié et al., 2006) on the basis of sequence similarity, although bioinformatic analysis of the GH16 subfamily indicated that Group III can be subdivided into two predominant clades designated Group III-A and Group III-B (Baumann et al., 2007).

Some of the *XTH* genes have been related to fruit growth and ripening in different fruits such as tomato, apple, kiwi fruit, pear, persimmon or cherimoya fruits (Schröder et al., 1998; Maclachlan and Brady, 1994; Arrowsmith and de Silva, 1995; Ishimaru and Kobayashi, 2002; Hiwasa et al., 2004; Fonseca et al., 2005; Lu et al., 2006; Nishiyama et al., 2007; Goulao et al., 2008; Atkinson et al., 2009; Li et al., 2009; Miedes and Lorences, 2009; Nakatsuka et al., 2011). Our previous studies with tomato fruit showed that the ten *SlXTH*s genes examined during tomato fruit development, were detected at all developmental stages (Miedes and Lorences, 2009), and four of the genes (*SlXTH3*, *SlXTH5*, *SlXTH8* and *SlXTH9*) showed mRNA accumulation during fruit growth or maturation. Saladié et al. (2006) also reported a correlation of mRNA accumulation of *SlXTH3*, *SlXTH5* and *SlXTH9* with ripening. A later report with transgenic tomato fruits showing a significant overexpression of the *SlXTH1* showed that the transgenic fruits had much lower xyloglucan depolymerization and lower fruit softening than in the wild type tomatoes, indicating that the xyloglucan structure was related with the softening mechanism and that XET was one of the enzymes involved in the process (Miedes et al., 2010).

Characterization of *XTHs* in apple has focused on changes in enzyme activity and gene expression during fruit maturation and softening (Goulao et al., 2007, 2008) and two *XTH* were detected in overripe apple fruit, *Md*XTH1 acting constitutively during fruit development, and *MdXTH2* showing a ripening-related pattern. A later report showed that eleven *XTH* genes were identified in apple, with two genes (*MdXTH2* and *MdXTH10*) being shown to be abundant in ripe fruit (Atkinson et al., 2009).

Ethylene has been identified as the major hormone that initiates and controls ripening in climateric fruit, and because of that, a substantial amount of the research on fruit softening has been done examining the relationship between ethylene production and perception and the accumulation of cell wall enzymes (Bennett and Labavitch, 2008; Li et al., 2009; Bapat et al., 2010; Klee and Giovannoni, 2011).

Different research groups have studied the effect of 1-methylcyclopropene (1-MCP), an ethylene perception inhibitor, delaying apple fruit ripening (DeEll et al., 2002; Asif et al., 2009) and altering gene expression of cell wall enzymes in ripening apple fruit (Wei et al., 2010). With regard to the possible relationship between *XTH* and ethylene, some research has been made in different fruits, using either 1-MCP in pear fruit (Hiwasa et al., 2004), cherimoya fruits (Li et al., 2009), persimmon fruit (Nakatsuka et al., 2011), or restoring the softening by treatment with exogenous ethylene in Charentais melon (Nishiyama et al., 2007).

Since *XTH* belongs to a multigenic family, it seems that different *XTHs* are subjected to a different expression pattern regulation during fruit growth and ripening. To date, tomato and apple are the two fleshy fruits for which most *XTH* gene sequences have been reported. However, no reports regarding the possible regulation by phytohormones during fruit ripening in those fruits have been published. For this reason, the objective of this work was to determine if ethylene could be involved in the regulation of the expression of the different *XTH* tomato and apple genes during ripening, to further investigate the implication of the cell wall enzyme XET in fruit softening and to determine the relationship between ethylene, *XTH* expression, and XET activity.

Materials and methods

Plant material

Apple fruits (*Malus domestica* var. Golden) and tomato fruits (*Solanum lycopersicum* var. canario) were purchased at the local market. Fruits were selected based on being of a similar size and also at a similar stage of ripening, and fruit flesh firmness. Tomato fruits were selected at the red stage of ripening. Apple fruits stage of ripening was determined by measuring fruit flesh firmness as resistance to puncture using a fruit pressure tester (Model FT 327, Effegi, Alfonsine, Italy) with an 8-mm tip on two areas opposite to each other on the equatorial region of the fruit.

Ethylene treatment

Tomato or apple fruits were placed in 600 mL glass jars, sealed and 5 ppm ethylene was injected, using a syringe, through rubber septa inserted in the jar lids. The fruits were then held at 25 ± 1 °C for 24 or 48 h. Control fruits were left in the jars with the top open and held at 25 ± 1 °C for 24 or 48 h.

Protein extraction

Soluble and ionically bound proteins were extracted together by homogenization of partially thawed tomato fruit pericarp as previously described (Miedes and Lorences, 2004). The protein content of the extracts was assayed by the Coomassie Blue G dye-binding method (Bradford, 1976).

Xyloglucan endotransglucosylase assay

Xyloglucan endotransglucosylase was assayed as described previously (Fry et al., 1992), and [^{3}H]XXXGol was used as the acceptor for the endotransglucosylation reaction. Xyloglucan heptasaccharide XXXG was prepared as described previously (Lorences and Fry, 1993). [^{3}H]XXXGol was prepared by reduction of the reducing terminal glucose moiety of non-radioactive XXXG with NaB_3H_4. The solution of [^{3}H]XXXGol used for the XET assays had a specific activity of 22.5 TBq mol^{-1}. Reaction mixtures (total volume 40 μL) containing 5 mg mL^{-1} of partially purified apple xyloglucan, 0.85 kBq [^{3}H]XXXGol, 50 mM-MES (Na^+) pH 6.0 and 25 μL of enzymatic extract (0.5–0.6 mg mL^{-1}) were incubated for 1 h at 25 °C. The reaction was stopped by the addition of 100 μL of 20% (w/v) formic acid and the solution was then dried on 5 cm × 5 cm Whatman 3MM filter paper, washed for 30 min in running tap water to remove unchanged [^{3}H]XXXGol, re-dried and assayed for ^{3}H by

Table 1
Malus domestica gene-specific oligonucleotides primers pairs used for RT-qPCR. The accession number of each gene was obtained from GenBank.

Name gene	Accession No.	Sequence of the 5′–3′ primers, forward/reverse
MdXTH1	EU494960	TACTGCACTGACCGAGCCCGAT
		TCTCTGTCCCTCTGGCACTCTGGT
MdXTH2	EU494961	TCAGGCACTTCCCGTTGTCCTT
		GCCCTGTGGAATCGAGTGACTGA
MdXTH3	EU494962	TCCCCAATGAGAAGCCGATGTACTT
		GCCCATTCGTCTGCGTTCCATA
MdXTH4	EU494963	GTCCCATACCCAAAGCTCCAACCTA
		CCACAACGTCGAAAACACTCCCA
MdXTH5	EU494964	CCACCAGAACCACCAGCTCGTT
		GCGGAATCGGTGCAGTAGTCGTA
MdXTH6	EU494965	CCCTAAAAACCAGCCCATGAGGAT
		CCACCTCTTGTTGCCCAGTCATCT
MdXTH7	EU494966	GACCCCACAAAAACCTTCCACACAT
		AATATAATGCGCTGCGAGTTCCAAA
MdXTH8	EU494967	GCAAAGGCAACAGAGAGCAGCAA
		GGAAGTCGGCAGTTGGGTCAAA
MdXTH9	EU494968	GACCCGACAGCAGTACAGGACCAT
		TCTTGGGGTCCCTGCAATAGTCAT
MdXTH10	EU494969	CAATCCCCAAGAAGCTGAAAGACTG
		TCTGCTCCGGTGGTGATGCTT
MdXTH11	EU494970	GCAGCCACAAACGACAGTCCAA
		CTCGGTTGCGTTTGTGGGACTT
Md actin	CN938023	TGACCGAATGAGCAAGGAAATTACT
		TACTCAGCTTTGGCAATCCACATC

scintillation counting. Inactivated controls were carried out in the same way using enzyme previously boiled for 30 min.

Phylogenetic analysis

A total of 36 full or partial length transcripts of tomato and apple were collected from GenBank, http://www.ncbi.nlm.nih.gov/genbank/, or Solanaceae genomics network, http://solgenomics.net/ (Tables 1 and 2). We have also included the XTH protein sequences from *Annona cherimola*, *Diospyros kaki* and *Cucumis melo* obtained from GenBank of *Ac*XTH1 (ACK36945), *Ac*XTH2 (ACK36946), *Ac*XTH3 (ACK36947), *Dk*XTH1 (BAI66274), *Dk*XTH2 (BAI66275), *Cm*XTH1 (ABI94061) and *Cm*XTH3 (ABI94063). Nucleotide sequences were translated with Jellyfish (v1.5, LabVelocity) and all amino acid sequences were aligned using the ClustalX program (Thompson et al., 1997) version 1.83. The pairwise and multiple alignment parameters were used as described by Saladié et al. (2006). Phylogenetic analyses were performed according to the neighbour-joining method (Saitou and Nei, 1987). Bootstrapping was performed with 2000 replicates to obtain support values for each internal branch and the representation of the calculated consensus tree was drawn using the tree view program (Page, 1996).

mRNA extraction and cDNA preparation

The mesocarp fruit (approximately 130 mg) was harvested from each sample and total mRNA was extracted with an RNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA was quantified by absorbance at 260 nm. After the treatment with RNase-free DNase (Promega Biotech Ibérica. Madrid, Spain), total mRNA (3 μg for tomato and 1 μg for apple) was reverse transcribed using polyT primers and the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Farma, SA, Barcelona, Spain) following the manufacturer's instructions.

Table 2
Solanum lycopersicum gene-specific oligonucleotides primers pairs used for RT-qPCR. The accession number of each gene was obtained from GenBank ([a]) or Solanaceae genomics network ([b]). * Partial protein.

Name gene	Accession No.	Sequence of the 5′–3′ primers, forward/reverse
SlXTH1	D16456[a]/	CTTGAGAAAACCAATTGGGCCAAC
	SGN-U578547[b]	GAACCCAACGAAGTCTCCTATACTGTAATG
SlXTH2	AF176776[a]/	CCTACTCTATTCTTTGGAATCCTCGAAAT
	SGNU577260[b]	GGGATTCTTGGAAGTTTGGGCAT
SlXTH3	AY497476[a]/	CCATCACTTGGAATCCACAACGC
	SGNU579445[b]	GAAGTGGGAATGCAAGCATTAGCAC
SlXTH4	AF186777[a]/	GAGGGTTAGAGAAAACTAATTGGTCTGGG
	SGN-U581597[b]	AACCCTATGAAGTTTTCTATATTCAGGGCC
SlXTH5	AY497475[a]/	CCCTAGTCTTTGTGATGAAAAAGATGTTGA
	SGN-U583026[b]	CCAGTCTCTTTAAAATGTTGTTGCTCAACT
SlXTH6	AY497477[a]/	GGTTGCGCGGCCTACGAGAGT
	SGN-U562982[b]	CTAACACTCAGGTGTGTGAGTATGGTCC
SlXTH7	AY497478[a]/	GACCAGCAAATTGTGCCTCCAAC
	SGN-U565532[b]	CCGGCCCTACATTCTGGTGGG
SlXTH8	AB036338[a]/	TCCCAAGTGTGATATAGTCCTGGATTCTG
	SGN-U575872[b]	CCTCGGAGGCGATTAGCTTCCTTA
SlXTH9	AY497479[a]/	CATGGGAATTCCATTTCCCAAGAG
	SGN-U577928[b]	CGCGTTGTTTGTTGAACCGATAGACTTA
SlXTH10	X82684[a]/	ACCGCTGTGTCATATAGACGCCG
	SGN-U581358[b]	ATCAGCGGAAACAGGGGATAGTAAGAC
SlXTH11	X82685[a]/	AAGTAGACGATGCAGCTGGAATTTCAA
	SGN-U578224[b]	GCCCAAACAGGACATAGCAAGAGAAG
SlXTH12	AK323964[a]/	CTTATCTTCTCAAGGACCCACTCATGATG
	SGN-U580011[b]	CCAAATGATGGAGTAGGTGTGGAAGTTC
SlXTH13	SGN-U221639[b]	GGTCCTCTCTTCCATATTTGGTGT
		CAGGTTGCGACGGGCTGA
SlXTH14	SGN-U219308[b]	CGATCCGGGTATTTTGGGGC
		GGTAGAAAGCGGTGATGACTCCG
SlXTH15	SGN-U577491[b]	CTCCTATAGCTTATTTTCTTGTACTTAGTGC
		CCAGATCTGTCCATCTTCAGAAGTC
SlXTH16	AK322426[a]/	CAAATGTGTATGTCAATGGTGTTGG
	SGNU580834[b]	GGGGTTTCATCTACCAAGAATACAAC
SlXTH17	SGN-U579875[b]	ATTGTTTGGAACGCTAACCGCATA
		GGGTATGCAATTCCAATGCTCTC
SlXTH18	SGN-U578149[b]	TATGTAGTCATTGGGATGACATCAAGTAGC
		AGAACAAGTTGAAGATCATCCCCTA
SlXTH19	SGN-U578397[b]	ATGACCCTTTCCCTCGATAAAGTC
		CTCATCATGACTTGATCCTATTGAAGATAG
SlXTH20	SGN-U577866[b] and	GGATCCTGTGAATGAAGAAAAATTGAG
	SL2.40ch07	CCTTGAGGAAATCTCTTATTATCAGTACAA
	(1722101–1724700)[b]	
*SlXTH21**	SGN-U575876[b]	GGGAATGGTAGCACAAATGTTGGC
		ACTGTATTGATGAAAATCTTCAGAAGG
*SlXTH22**	SGN-U226745[b]	CAGAGTCAAGATATTCCCTATCCGAAC
		GCATTGGTCCAGTCACATTTGACA
SlXTH23	SGN-U563284[b]	CCTTAGACCAAGTATCAGATTGTGGG
		TCTCTCAATGGCCCTGCCTCTACT
SlXTH24	AK323906[a]/	CCCATTATGAGTTACCAAAAGTGTTG
	SGN-U574759[b] and	CCAGAGCCAGAGAATTTGTCCATAGA
	SL2.40ch12	
	(64392001–64394900)[b]	
SlXTH25	SGN-U238267[b] and	TGTCAAACTCAGATGTGTTTCCACAC
	SL2.40ch05	CTCTTCCAGTGTGAACACTTCCATTT
	(506301–508500)[b]	
RPL8	NM_001247186[a]/	ATTCACGATCCAGGGAGAGGTGC
	SGN-U581377[b]	AGGCAACACGTTACCAACCATAAGAGTAG

Gene expression analysis by real time quantitative PCR (qPCR)

The real time qPCR amplification was performed with gene-specific primers (Tables 1 and 2). The apple and several tomato primers were designed as previously described (Miedes and Lorences, 2009; Atkinson et al., 2009). RPL8 (a housekeeping gene, ribosomal protein L2) was used as internal control for all the tomato genes, and the apple actin gene was used for all apple genes. Each reaction was performed in triplicate for each sample in 25 μL final volume containing 1 μL cDNA, 25 pmol specific primers, and 12.5 μL of *Power*SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. PCRs were

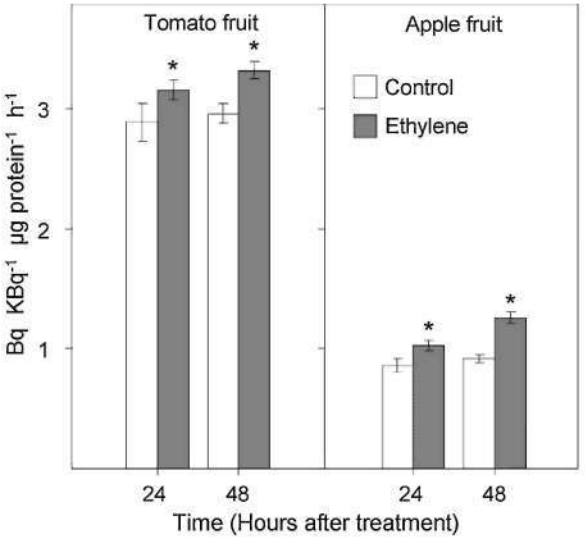

Fig. 1. Specific activity of xyloglucan endotransglucosylase (XET) of tomato and apple fruits in control (□) and ethylene treated fruits (■). The XET assay was performed by measuring [^{3}H]XXXG incorporation into XG by the protein extract. Dashed bars indicate control fruits. Filled bars indicate ethylene treated fruits. Values shown are the means of 3 independent experiments, using 20 fruits in each experiment ± SE. The symbol * indicate significant differences between control and ethylene treated fruits (*t*-test, *p*-value <0.05).

carried out using the ABI7000 (Applied Biosystems, Foster City, CA, USA) for 10 min at 95 °C (initial denaturation) and then for 40 cycles as follows: 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. The specificity of the PCR amplification was confirmed with a melt curve analysis consisting of a 0.1 °C temperature gradient from 55 to 90 °C. Primer efficiencies were determined for each oligonucleotide pair. To account for different primer efficiencies, relative quantification of transcript levels to the reference tomato gene RPL8 or apple actin was calculated using the method described in Pfaffl (2001).

Fruit firmness

Fruit flesh firmness, after the different treatments, was measured as resistance to puncture using a fruit pressure tester (Model FT 327, Effegi, Alfonsine, Italy) with an 8 mm tip on four areas opposite each other on the equatorial region of the fruit, after removal of a 2 mm thick slice.

Results

Effect of exogenously added ethylene on the XET activity

To investigate the possible regulation of the XET enzyme by ethylene, soluble endotransglucosylase activity was determined in the tomato and apple fruits after 24 and 48 h of ethylene treatment, using [^{3}H]XXXGol as the acceptor for the endotransglucosylation reaction (Fig. 1). The results showed that in both tomato and apple fruits, there was an increase in XET activity in the fruits incubated with ethylene as compared with the respective controls. The increase in XET activity was statistically significant in tomato and in apple fruit after 24 and 48 h of ethylene treatment.

Phylogenetic analysis of XTH proteins

We constructed a neighbour-joining phylogenetic tree using the aligned protein sequences of tomato (*Sl*XTHs) and apple (*Md*XTHs) (Tables 1 and 2). We also included the XTH protein sequences of *Ac*XTH1, *Ac*XTH2, *Ac*XTH3, *Dk*XTH1 *Dk*XTH2, *Cm*XTH1 and *Cm*XTH3, since their expression have been reported to be ethylene dependent (Nishiyama et al., 2007; Li et al., 2009; Nakatsuka et al., 2011).

To confirm that the tomato XTH proteins were complete we searched for homology in other species using the BLAST algorithm (NCBI) and in a first approximation, we concluded that 20 *Sl*XTH proteins were complete and proteins *Sl*XTH20, *Sl*XTH21, *Sl*XTH22, *Sl*XTH24 and *Sl*XTH25 were incomplete. In the case of *Sl*XTH20, *Sl*XTH24 and *Sl*XTH25, the missing DNA fragments required to complete the proteins were obtained from genomic fragments. *Sl*XTH21 and *Sl*XTH22 could not be completed, so incomplete sequences were used instead.

The results are shown in Fig. 2. The *Sl*XTHs sequences of tomato were aligned in three groups. Six tomato *Sl*XTHs were aligned within Group I (*Sl*XTH1, *Sl*XTH4, *Sl*XTH7, *Sl*XTH15, *Sl*XTH16, and *Sl*XTH23), and thirteen *Sl*XTHs were associated with Group II (*Sl*XTH2, *Sl*XTH3, *Sl*XTH9, *Sl*XTH10, *Sl*XTH11, *Sl*XTH12, *Sl*XTH13, *Sl*XTH17, *Sl*XTH18, *Sl*XTH19, *Sl*XTH20, *Sl*XTH22 and *Sl*XTH24). Group III was subdivided in two clades designated as Group III-A and Group III-B, where two tomato *Sl*XTHs were associated with Group III-A (*Sl*XTH6 and *Sl*XTH14) and four were aligned within Group III-B (*Sl*XTH5, *Sl*XTH8, *Sl*XTH21 and *Sl*XTH25).

The *Md*XTHs apple sequences were also grouped, as previously reported by Atkinson et al. (2009), within three phylogenetic subgroups. *Md*XTH1, *Md*XTH3, *Md*XTH4 and *Md*XTH5 were aligned within Group I; *Md*XTH2, *Md*XTH6, *Md*XTH7 and *Md*XTH8 were associated with Group II; *Md*XTH9 was aligned with Group III-A, and *Md*XTH10 and *Md*XTH11 were aligned with Group III-B. The other *XTHs* sequences included for further comparisons, *Ac*XTH1, *Dk*XTH1, *Dk*XTH2, *Cm*XTH1 and *Cm*XTH3, were all aligned with Group II, and *Ac*XTH2 and *Ac*XTH3 which were associated with Group I.

Effect of exogenously added ethylene on XTH expression genes

The results of the expression analysis of the *SlXTHs* tomato genes by real-time qPCR, grouped into the three phylogenetic groups described above, are shown in Fig. 3. The expression of *SlXTH10*, *SlXTH13*, *SlXTH18*, *SlXTH20*, *SlXTH22* and *SlXTH24* was almost negligible, so it was not plotted in the figure. The *SlXTHs* genes with highest expression during ripening were *SlXTH5* and *SlXTH8* from Group III-B (Fig. 2). All the *SlXTHs* with measurable expression aligned in Group II and Group III, showed similar behaviour, since all of them increased their expression after 24 and 48 h of ethylene treatment as compared with the control fruits, with the only exception of *SlXTH14*, belonging to Group III-B where an decrease of expression was observed. In Group I, *SlXTH4*, *SlXTH7*, and *SlXTH16* also showed an increase in expression induced by ethylene. However, *SlXTH1*, *SlXTH15* and *SlXTH23* showed a changeable expression pattern.

In apple fruit, all *MdXTHs* studied were expressed in ripe fruits, with the highest expression found in *MdXTH2*, *MdXTH10*, and *MdXTH11*, followed by *MdXTH4*, *MdXTH3* and *MdXTH1*, the expression of the rest of the *MdXTHs* being much lower (Fig. 4). The effect of ethylene on the *MdXTHs* expression was not as uniform as in tomato fruit after the ethylene treatment. *MdXTH3*, *MdXTH5* and *MdXTH10* showed an increase in expression with the incubation with ethylene, being the *MdXTH5* the one that showed the highest increase in expression. On the other hand, *MdXTH2*, and *MdXTH11* showed a decrease in expression, meanwhile the rest of the *MdXTHs* studied, showed a changeable expression pattern.

Changes in fruit firmness during ethylene treatment

We evaluated the firmness of fruits using a fruit pressure tester (Fig. 5) after the incubation time in ethylene. In both tomato and

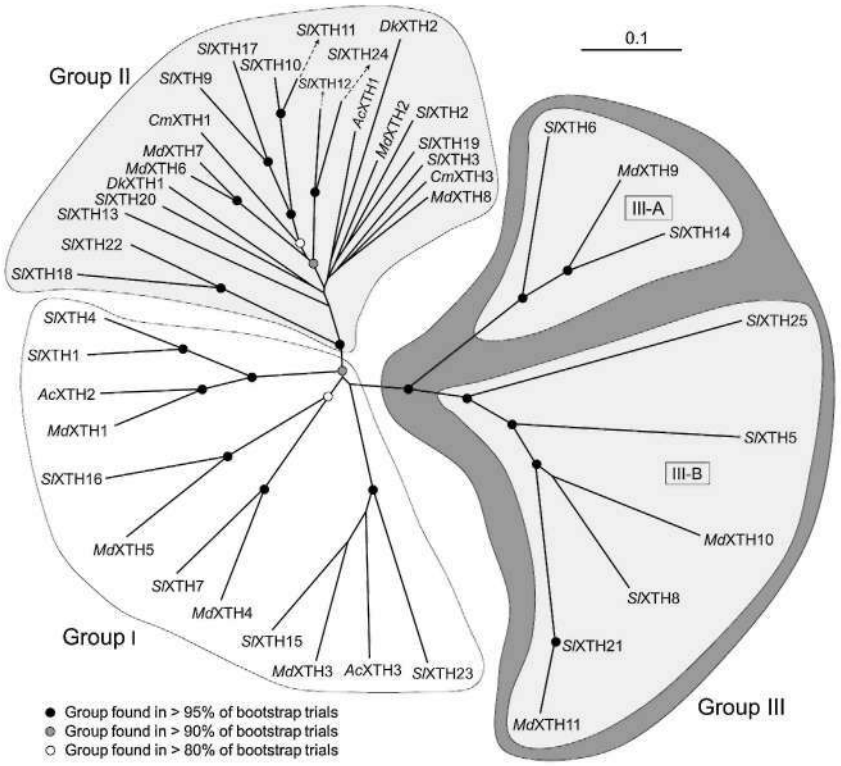

Fig. 2. Unrooted phylogram of xyloglucan endotransglucosylase/hydrolase (XTH) protein sequences from *Solanum lycopersicum* (*Sl*XTH1–25), *Malus domestica* (*Md*XTH1–11), *Diospyros kaki* (*Dk*XTH1 and 2), *Cucumis melo* (*Cm*XTH1 and 3) and *Annona cherimola* (*Ac*XTH1–3). Confidence values for grouping in the tree were obtained using Bootstrap N-J Tree using 2000 bootstrap trials. Trees were visualized in TreeView and the sequences grouped into three distinct clades (Groups I–III). Tree nodes with bootstrap values >95%, >90% and >80% are indicated with black, grey and white dots, respectively. Details and GenBank accession numbers are described in Materials and methods and Tables 1 and 2.

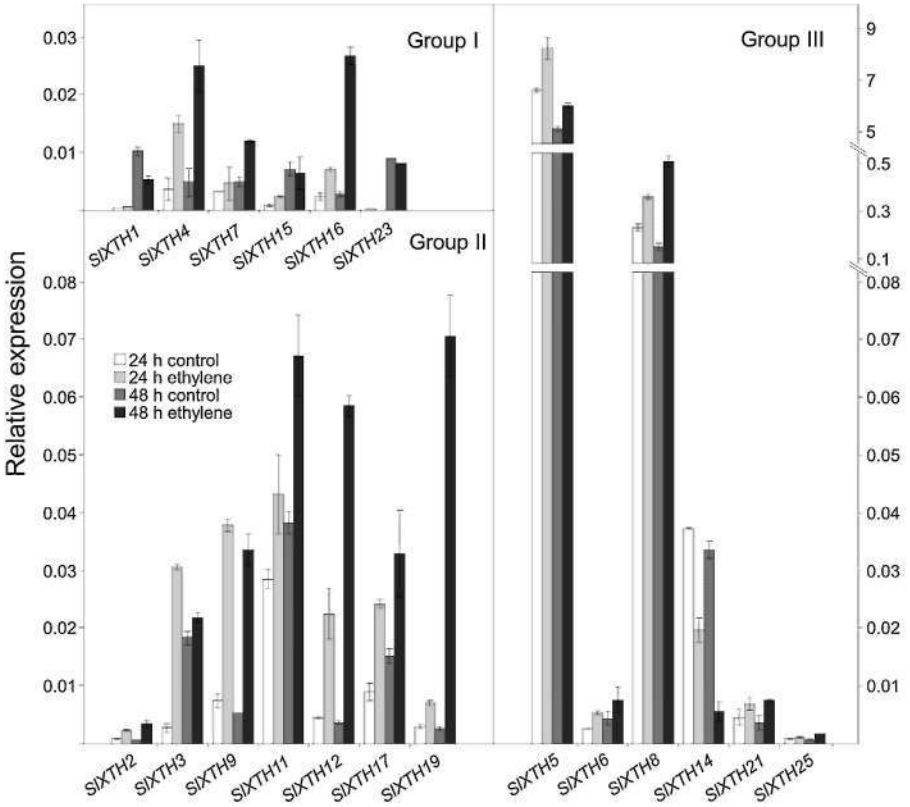

Fig. 3. Expression analysis of *SlXTHs* tomato genes in treated or untreated fruits with ethylene by real-time qPCR. Expression of *RPL8* gene was used as an internal control of gene expression to normalize the expression of *SlXTHs*. The genes are grouped into the three groups obtained from the unrooted phylogram in Fig. 2. Values shown are means of three independent experiments, measuring in triplicate, using 12 fruits in each experiment ± SE.

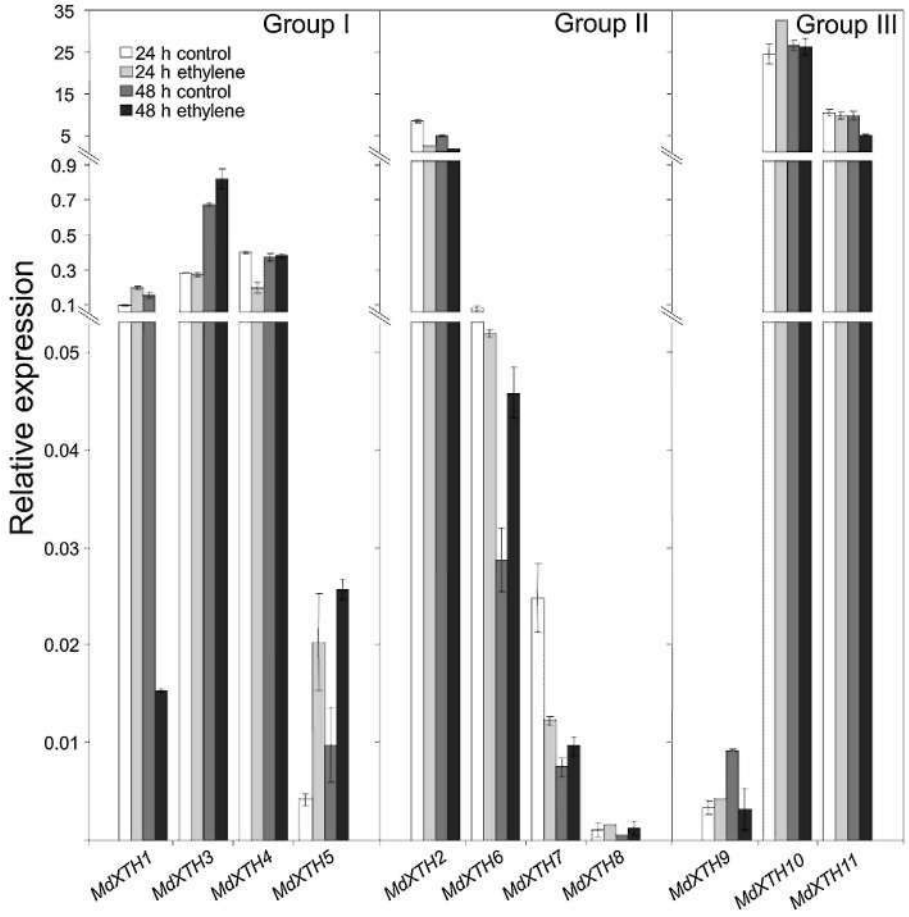

Fig. 4. Expression analysis of *MdXTHs* apple genes in control and ethylene treated fruits by real-time qPCR. Expression of *actin* gene was used as an internal control of gene expression to normalize the expression of *MdXTHs*. The genes are grouped into the three groups obtained from the unrooted phylogram in Fig. 2. Values shown are means of three independent experiments, measuring in triplicate, using 12 fruits in each experiment ± SE.

apple fruits, a decrease in texture was observed after 24 and 48 h of ethylene treatment, although the differences were not statistically significant.

Discussion

Fruit undergoes extensive cell wall disassembly and this is considered to play a major role in ripening-associated fruit softening. The changes in hemicellulosic polysaccharides have been described as an important feature in the whole softening process. For this reason, xyloglucan endotransglycosylase/hydrolase enzymes are thought to play a key role in fruit ripening by loosening the cell wall through disassembly of xyloglucan (Vicente et al., 2007; Goulao and Oliveira, 2008; Li et al., 2010; Klee and Giovannoni, 2011).

Since the ripening of climacteric fruits, such as tomato and apple, is controlled by endogenous ethylene biosynthesis (Bapat et al., 2010), we wanted to study whether XET activity was ethylene-regulated and if so, which specific genes encoding ripening-regulated *XTH* genes were indeed ethylene-regulated.

When we measured the specific XET activity (Fig. 1), we found a significant increase in activity in the ethylene treated fruits compared with the control fruits, in both tomato and apple fruits, suggesting an increase in the *XTH* gene expression induced by ethylene. It is important to mention that when we determined the XET activity, we were measuring the contribution of all the *SlXTHs* or *MdXTH* genes in each case.

XTHs genes belong to a multi-genetic family and their sequences can be aligned within distinct phylogenetic subgroups, depending on the parameters used for the alignment and the species used for these analyses (Baumann et al., 2007; Atkinson et al., 2009). Our phylogenetic analysis of XTHs sequences (Fig. 2) supported previous divisions of XTH sequences into three large clades and

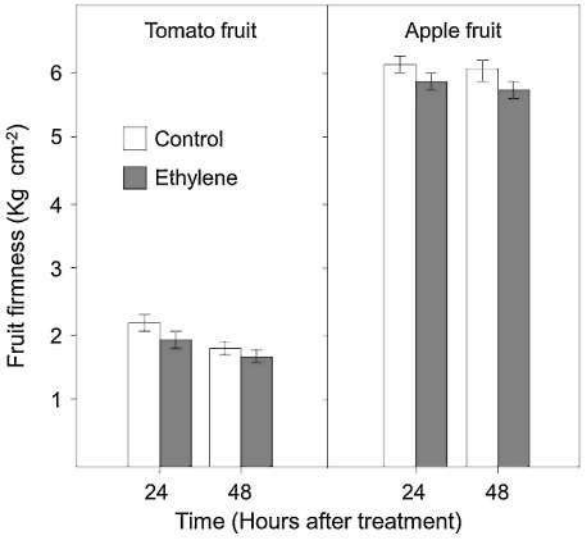

Fig. 5. Changes in firmness of tomato and apple fruits in control (□) and ethylene treated fruits (■). Firmness was determined in the pericarp tissue using a fruit pressure tester. Values shown are means of 3 independent experiments using 20 fruits for each determination ± SE.

also revealed further well supported branches within these three clades (http://labs.plantbio.cornell.edu/XTH/tomato.htm; Rose et al., 2002; Baumann et al., 2007; Atkinson et al., 2009). We have included the eleven *MdXTHs* sequences identified in apple (Atkinson et al., 2009) as well as twenty-five *SlXTHs* sequences of tomato (http://labs.plantbio.cornell.edu/XTH/tomato.htm). This is the first time that the all *SlXTH13* to *SlXTH25* tomato XTH have been phylogenetically analyzed. We have also included the protein sequences of *Ac*XTH1, *Ac*XTH2, *Ac*XTH3, *Dk*XTH1 *Dk*XTH2, *Cm*XTH1 and *Cm*XTH3, since their expressions have been reported to be ethylene dependent (Nishiyama et al., 2007; Li et al., 2009; Nakatsuka et al., 2011).

It has been reported that heterologously expressed *XTH* genes from Groups I and II have exclusively XET activity. In Group III-A, *TmNXG1* from nasturtium and *VaXGH* from azuki bean, are thus far the only *XTH* gene products with demonstrable hydrolytic (XEH) activity. In contrast, *XTH* genes in Group III-B, show predominantly or exclusively XET activity, thus validating a functional distinction between the A and B clades (see review of Eklöf and Brumer, 2010).

The *SlXTHs* genes with highest expression during ripening were *SlXTH5* and *SlXTH8* (Fig. 3), in agreement with our previous reports (Miedes and Lorences, 2009). In tomato fruit *SlXTH5* have been previously published to be the *SlXTH* with highest expression during ripening (Saladié et al., 2006).

In apple, the highest expression was found in *MdXTH2*, from Group II, and *MdXTH10*, and *MdXTH11* from Group III-B (Fig. 4). In agreement with our results, previous reports for apple fruit described that *MdXTH2* showed a ripening-related pattern (Goulao et al., 2008) and that *MdXTH2* and *MdXTH10* were the *MdXTH* with the highest expression during fruit ripening (Atkinson et al., 2009).

Although, to date, the phylogenetic classification of XTH sequences into three clades has not revealed any temporal or tissue specificity within clades, our results as well as the reports mentioned above showed that in tomato fruit, the *XTH* genes that are ripening associated belong to Group III-B, and in apple fruit, the *XTHs* that are ripening associated belong to Group II and Group III-B.

Since the ethylene treatment increased XET activity in both tomato and apple fruit (Fig. 1), and since the XTH protein belongs to a multigenic family, we wanted to determine which members of the family had altered expression by exogenous ethylene treatment. In tomato fruit, the results indicate that the increase en XET activity during ethylene incubation could be due to the increase in the expression of fifteen different *SlXTH* genes that showed an increase in expression after ethylene treatment (Fig. 3). Moreover, we suggest that the increase in XET activity in tomato fruit was mainly to the increase in expression of *SlXTH5* and *SlXTH8* since those genes were the *XTH* genes with highest expression levels during ripening. It is also remarkable that with the only exception of *SlXTH14*, all the *SlXTHs* from Groups II and III-B increased expression after ethylene treatment.

In apple fruit, however, only *MdXTH3* and *MdXTH5* from Group I and *MdXTH10* from Group III-B showed a clear increase in expression after the ethylene treatment (Fig. 4).

The *MdXTH5* was the *MdXTH* that showed the highest increase in expression induced by ethylene, although the expression level was quite low in comparison with the rest of the *MdXTHs* during apple fruit ripening, so the contribution of this gene to the total XET specific activity could be also reduced. Bearing in mind that the ethylene treatment increased the XET specific activity (Fig. 1), and that the *MdXTHs* that showed higher expression values during ripening were *MdXTH2*, *MdXTH10* and *MdXTH11* (Fig. 4), we could speculate that the increase in XET activity measured in apple fruit after the ethylene treatment might be attributed to the increase in expression of *MdXTH10*.

Previous reports on the effect on ethylene on *XTH* expression showed an increase of different *XTHs* after the ethylene treatment, or a decrease in the expression when the ethylene synthesis was inhibited by 1-MCP treatment. For instance, the effect of 1-MCP on the *XTH* expression in harvest cherimoya fruits, Li et al. (2009) reported that 1-MCP delayed softening and affected expression of the *XTH* genes *AcXTH1*, *AcXTH2*, *AcXTH3*. Another report showed that pretreatment with 1-MCP during the deastringency treatment of persimmon fruit delayed accumulation of *DkXTH1* mRNA but intensified the expression of *DkXTH2* (Nakatsuka et al., 2011). On the other hand, application of exogenous ethylene to Charentais melon, induced a substantial increase in both *CmXTH1* and *CmXTH3* mRNA, but their levels declined between 1 d and 4 d of ethylene exposure, and it seems that both *XTH* genes are likely to be ripening-associated but only partially ethylene-dependent (Nishiyama et al., 2007). All the above mentioned *XTHs* genes belong to Group II, with the exception of *AcXTH2* and *AcXTH3*, that belong to the Group I (Fig. 2).

As we have mentioned before, in both tomato and in apple fruit, the *XTHs* that are ripening associated belong to Group II and Group III-B. On the other hand, a large number of the *XTH* genes that are ripening associated seem to be also ethylene dependent, in particular in tomato fruit. In both fruits, the increase in expression could be contributing to the increase in XET specific activity measured after the ethylene treatment (Fig. 1), and hence to the incipient fruit softening (Fig. 5). The differences observed between both fruits in the magnitude of the response to the ethylene treatment, wider in tomato than in apple fruit, could be explained based on the fact that both fruits have a different pattern of fruit ripening and softening, and may proceed via different mechanisms. Apple fruit has limited softening during ripening, without extensive depolymerization of pectins and hemicelluloses, whilst in tomato fruit, depolymerization and solubilization of polyuronides and depolymerization of hemicelluloses, including xyloglucan, appears to occur (Klee and Giovannoni, 2011). In apple fruit, increased XET activity was detected in apple fruits after the growth had stopped and throughout softening (Goulao et al., 2007), but in this species, the molecular weight profile of xyloglucan presents a high molecular weight peak which does not change during fruit development and ripening (Percy et al., 1997). Whatever the precise mechanism it seems that xyloglucan structure is related to the softening process and that XET is one of the enzymes involved in its modification.

In summary, our results showed that some *XTH* genes were associated with fruit ripening, *SlXTH5* and *SlXTH8* in tomato fruit and *MdXTH2*, *MdXTH10*, and *MdXTH11* in apple fruit. In addition, ethylene was involved in the regulation of the expression of different *SlXTH* and *MdXTH* genes during ripening. In tomato fruit, the *XTHs* that are ripening associated are also ethylene dependent, and belong to Group III-B (*SlXTH5* and *SlXTH8*). In apple fruit, only the expression of *MdXTH10* (from Group III-B) was increased after the ethylene treatment. The results indicate that *XTH* may play an important role in fruit ripening and a possible relationship between *XTHs* from Group III-B and fruit ripening, and ethylene regulation.

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