JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

The Implication of Xyloglucan Endotransglucosylase/Hydrolase (XTHs) in Tomato Fruit Infection by *Penicillium expansum* Link. A

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In general, cell wall-degrading enzymes produced by plant pathogenic fungi are considered important pathogenicity factors. In this work, we evaluate the implication of xyloglucan endotransglucosylase/hydrolase (XTHs), a potential hemicellulosic repairing enzyme, in the infection mechanism process by the fungus. This study investigated the *SlXTHs* expresion and xyloglucan endotransglucosylase (XET) activity during infection of two tomato fruit cultivars by *Penicillium expansum* Link. A. In infected fruits, XET specific activity decreased drastically after long infection periods, 24 and 48 h for Canario and Money Maker tomato fruits, respectively. Real Time RT-PCR of eleven *SlXTHs* also showed a decrease in expression as the infection progressed in both tomato fruit cultivars. Results suggest that the reduction in *SlXTHs* expression during infection might be related with the fungus attack mechanism. We suggest a possible transcriptional control of the *SlXTHs* expression by the fungus, causing a decrease in XET activity and, consequently, lower xyloglucan endotransglucosylation, which changes the xyloglucan structure. These changes might increase the fruit softening and wall disassembly, facilitating the fungus colonization and the progress of the infection.

KEYWORDS: Cell wall; fruit infection; hemicelluloses; *Solanum lycopersicum* L; *Penicillium expansum* Link; A. Xyloglucan endotransglucosylase/hydrolase

INTRODUCTION

Plant cell walls provide a physical barrier between pathogens and the internal content of the cells. The high molecular weight polysaccharides, which are the principal components of the cell walls, are cross-linked by both ionic and covalent bonds into a network that resists physical penetration (*1–3*). Many pathogens release enzymes such as polygalacturonase (*4, 5*), and pectin lyases (*6*) which degrade cell wall polysaccharides. The role of these cell wall-degrading enzymes in many aspects of pathogenicity, including penetration, tissue maceration, nutrient acquirement, symptom expression, and plant defense induction has been studied (*2, 3*).

We have previously investigated cell wall metabolism during infection of apple and tomato fruits by *P. expansum* and reported pectin depolymerisation associated with *P. expansum* infection, mainly due to the activation of pectinases (*7*). Hemicelluloses and xyloglucan were also depolymerised by fungal β-glucanases during infection (*8*), which confirms the importance of hemicellulose degradation in the breakdown of plant cell walls.

In addition to the cell wall-degrading enzymes, another type of cell wall enzyme, involved in the cell wall hemicelluloses metabolism, the xyloglucan endotransglucosilase/hydrolase (XTH), has been reported (*9, 10*). This enzyme is encoded by the *XTHs* family, and some members of this family have been shown to act as transglucosylases (XET), catalysing the transfer of a xyloglucan molecule fragment to another xyloglucan molecule, other XTHs act preferentially as hydrolases (XEH), which hydrolyses one xyloglucan molecule, whereas some of XTH proteins present both activities (*11, 12*).

Since xyloglucans comprise the framework of the cell wall, XET action might be significantly important, and its function is considered essential for cell wall remodelling, including cell wall architecture, reconstruction, strength, and extensibility (*13–15*). It has also been reported that *PcXTH* gene induction might be associated with cell wall maintenance during "Rocha" pear development and ripening (*16*), suggesting that XTHs could have a potentially crucial role in fruit texture maintenance.

One preliminary and interesting result in our studies in fruit infected by *Penicillium* was the drastic decrease of the XET activity in infected fruits, and we suggested that this decrease in XET activity during infection could be specifically produced by the fungus by way of an unknown mechanism (*8*).

To further investigate the possible implication of the XTH enzyme and the XET activity with the fungus attack mechanism, we characterized the time course of XET activity and the *SlXTHs* expression, associated with fruit infection, in two different tomato fruit cultivars. The fungus used was *Penicillium expansum* Link. A., which has a broad host range and infects different fruits, such as tomato. We particularly wanted to evaluate if

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10.1021/jf0718244 CCC: $37.00
Published on Web 10/24/2007

the inhibition of the XET, a potential hemicellulosic repairing enzyme, could be a related to the infection mechanism process by the fungus.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Solanum lycopersicum* L. cv. Money Maker) were grown in a greenhouse until ripe. Tomato fruits were harvested at the red stage. Tomato fruits cv. Canario were purchased at the local market. Fruits were selected on the basis of being of a similar size and at a similar stage of ripening at the red stage.

***Penicillium expansum* Link. A. Growth and Fruit Infection.** Fruit infection was carried out with *Penicillium* spores as previously described (*8*) *Penicillium expansum* Link. A. was supplied from CECT (Spanish type Culture Collection; http://www.cect.org/) in the form of a freeze-dried sample. The fungi was reconstituted with sterile water and sown and maintained as a single-spore culture on potato dextrose agar (PDA, Difco, Laboratories) at 20 °C. For fruit inoculation, spores from 7 day old cultures were suspended in sterile distilled water and filtered through glass wool. Ten μL of the conidial suspension, containing 2×10^6 spores mL^{-1}, were used for the infection experiments. Fruits were infected on four opposite sides by injection with a microsyringe. Control fruits were inoculated with 10 μL of distilled water. Fruits were then held at 24 °C and at 60% relative humidity during different periods of time. After the incubation time, cylindrical samples of 1 cm diameter and 1 cm long were obtained from around the injection point of the fruits. Samples were frozen with N_2 liquid at −80 °C until the experiments were carried out.

Protein Extraction and Xyloglucan Endotransglucosylase Assay. Soluble and ionically bound proteins were extracted together by homogenization of partially thawed tomato fruit pericarp as previously described (*8*). The protein content of the extracts was assayed by the Coomassie Blue G dye-binding method (*17*).

Xyloglucan endotransglucosylase was assayed as described previously (*9*), and [^{3}H]XXXGol was used as the acceptor for the endotransglucosylation reaction. Xyloglucan heptasaccharide XXXG was prepared as described previously (*18*). [^{3}H]XXXGol was prepared by reduction of the reducing terminal glucose moiety of nonradioactive XXXG with NaB_3H_4. The solution of [^{3}H]XXXGol used for the XTH 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 one hour 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, redried and assayed for ^{3}H by scintillation counting. Inactivated controls were carried out in the same way using enzyme previously boiled for 30 min.

Analysis "In Silico" of *SlXTH1-SlXTH12* Genes. Corrected sequences of the full length transcripts were submitted to Genbank and are listed at http://labs.plantbio.cornell.edu/XTH. The phylogenetic tree was based on multiple alignments done with CLUSTAL W and a neighbour-joining tree was constructed based on the alignment. The consensus tree was drawn using the TREE VIEW program (*19*).

mRNA Extraction and cDNA Preparation. The mesocarp fruit (approximately 130 mg) was harvested 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 was reverse transcribed using the First Strand cDNA Synthesis Kit (Roche Farma, SA. Barcelona, Spain) following the manufacturer's instructions.

Real Time RT-PCR. The PCR amplification was performed with gene-specific primers. Primer sequences for all the studied genes are presented in **Table 1**. In the primers design we avoided the highly conserved regions of the *XTHs* gene of tomato. The primers were designed using couples of similar genes and the primers which discriminate between comparing sequence genes were used. The size of these primers was chosen to have PCR product ranges between 120 and 220 bp, CG 50% and melting point of approx. 59 °C. *RPL2* (a housekeeping gene, ribosomal protein large. subunit 2) was used as internal control for all the studied genes. Three replicates were performed for each sample in 25 μL final volume containing 1 μL cDNA, 25 pmol *SlXTHs* or *RPL2* (18S) specific primers, and 12.5 μL of *Power*SYBR Green PCR Master Mix according to the manufacturer's instructions. PCRs were carried out using the ABI7000 (Applied Biosystems, Foster City, CA) 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. Real-time quantification is based on Ct values and were interpolated in a quantification standard curve (*20*). The Ct for each sample was normalized to *RPL2*, 18S rRNA.

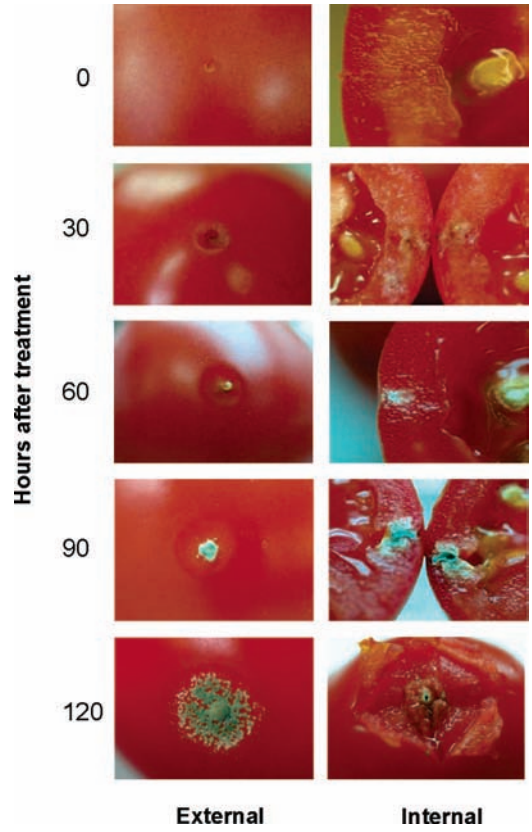

Figure 1. Infection progress in the internal tissues of Money Maker tomato fruits infected with *Penicillium expansum Link A.* Fruits were then held at 24 °C and at 60% relative humidity during different periods of time.

Infection Experiments and Analysis. Three independent fruit infection experiments were performed using 24 fruits in each experiment. All enzyme assays and the gene expression assays were performed from the tree experiments in duplicate.

RESULTS

Fruit Infection with *Penicillium expansum* Link. A. Tomato fruits were infected with *P. expansum* (control fruits were inoculated with distilled water) the fruits were then held at 24 °C and at 60% relative humidity during different periods of time. We found no differences between noninoculated fruits and water-inoculated fruits. Even the puncture in the fruit was not visible after a few hours (results no shown). **Figure 1** shows the infection progress in the external and in the internal tissues of Money Maker tomato fruits. After the initial period when we could observe the puncture place, the infection progress (30 h), and we observed a brown colour in the infection zone, internal and external, that indicates damage in the tomato tissues. After 60 h we could observe the grown of the micellium, still white and without spores, and evident damage in both external and internal tissues. As the infection progress (90 h), we observed a further fungal growth showing the characteristic blue

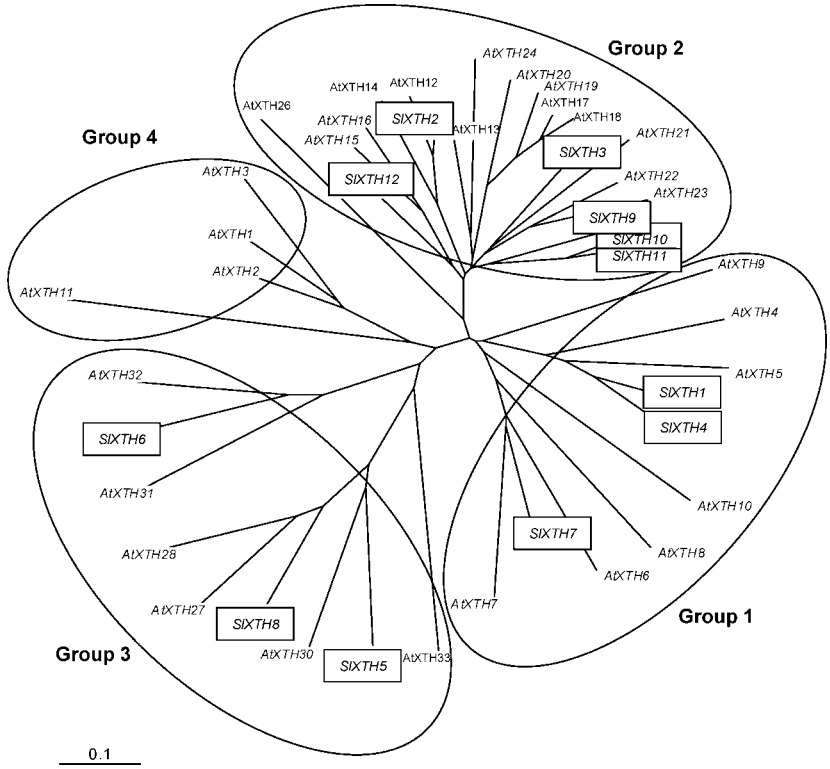

Figure 2. Unrooted phylogram of xyloglucan endotransglucosylase/hydrolase (XTH) full-length protein sequences from *Arabidopsis thaliana* L. (*AtXTHs*) and *Solanum lycopersicum* L. (*SlXTHs*), (boxed). The sequences grouped into four distinct Groups (1–4).

mould, with spores and extensive damage in the external and internal tissues. At 120 h, the fungus spread even more over the fruit, and in the internal tissues the necrotic area was even bigger. In Canario tomato fruits the progress of the infection was faster than in Money Maker.

Analysis "In Silico" of *SlXTH1-SlXTH12* Genes. *XTHs* genes belong to a multigenetic family and their sequences can be aligned within three or four distinct phylogenetic subgroups, depending on the parameters used by the aligment and species used for these analyses (*12*). We constructed a neigbor-joining tree using the aligned *AtXTHs* sequences of *Arabidopsis* and the *SlXTHs* sequences of tomato (**Figure 2**). The resulting phylogenetic tree showed the presence of four major groups for *AtXTHs* sequences of *Arabidopsis* whilst the *SlXTHs* sequences of tomato were aligned in only three groups, these results being in agreement with previous reports (*21*). Three *SlXTHs* were aligned within Group 1 (*SlXTH1, SlXTH4* and *SlXTH7*), six *SlXTHs* were associated with Group 2 (*SlXTH2, SlXTH3, SlXTH9, SlXTH10, SlXTH11* and *SlXTH12*), and *SlXTH5, SlXTH6* and *SlXTH8* were aligned within Group 3.

The analyses of the location of conserved sites and the location signal peptide in the protein sequences are indispensable to design the primers that are able to distinguish among all the *SlXTHs.* To analyse the *SlXTHs* predicted protein sequences, we used a predicted analysis *N*-terminal signal peptide (http://www.cbs.dtu.dk/services/SignalP and http://psort.nibb.ac.jp), and we identified the conserved DEIDFEFLG sequence, which is proposed to be the catalytic site for both hydrolase and transferase activity (*12*). Our results also indicated that all peptides showed a secretory pathway, although *SlXTH8* was predicted to be membrane anchored (data not shown). Following this, we used the phylogenetic tree to determine which members of *XTHs* had more homology sequences between them, and we searched for optimal primers to distinguish among the *XTHs* genes in real time RT-PCR assays. It was not possible to design specific primers to distinguish between *SlXTH10* and *SlXTH11*, since they have an extremely high degree of nucleotide sequence identity (90% across the full-length cDNAs), and also have short 3′ UTR regions. Hence, we designed a common couple of primers for both *XTHs* genes, based on *SlXTH10* sequence, although it would certainly cross hybridize with the *SlXTH11* paralogue. The designed primers to be used in Real Time RT-PCR assays are shown in **Table 1**.

Table 1. *SlXTHs* Gene-Specific Oligonucleotide Primers Pairs Used for Real Time RT- PCR

GenBank accession no.	name tomato genes	sequence of the 5′3′ oligonucleotides, forward/reverse
3950387	*RPL2*	ATTCACGATCCAGGGAGAGGTGC
		AGGCAACACGTTACCAACCATAAGAGTAG
D16456	*SlXTH1*	CTTGAGAAAACCAATTGGGCCAAC
		GAACCCAACGAAGTCTCCTATACTGTAATG
AF176776	*SlXTH2*	CCTACTCTATTCTTTGGAATCCTCGAAAT
		GGGATTCTTGGAAGTTTGGGCAT
AF205069	*SlXTH3*	CCATCACTTGGAATCCACAACGC
AY497476		GAAGTGGGAATGCAAGCATTAGCAC
AF186777	*SlXTH4*	GAGGGTTAGAGAAAACTAATTGGTCTGGG
		AACCCTATGAAGTTTTCTATATTCAGGGCC
AY497475	*SlXTH5*	CCCTAGTCTTTGTGATGAAAAAGATGTTGA
		CCAGTCTCTTTAAAATGTTGTTGCTCAACT
AY497477	*SlXTH6*	GGTTGCGCGGCCTACGAGAGT
		CTAACACTCAGGTGTGTGAGTATGGTCC
AY497478	*SlXTH7*	GACCAGCAAATTGTGCCTCCAAC
		CCGGCCCTACATTCTGGTGGG
AB036338	*SlXTH8*	TCCCAAGTGTGATATAGTCCTGGATTCTG
ETAG-A3		CCTCGGAGGCGATTAGCTTCCTTA
AY497479	*SlXTH9*	CATGGGAATTCCATTTCCCAAGAG
		CGCGTTGTTTGTTGAACCGATAGACTTA
X82684	*SlXTH10*	ACCGCTGTGTCATATAGACGCCG
LetXET-B2		ATCAGCGGAAACAGGGGATAGTAAGAC
X82685	*SlXTH11*	AAGTAGACGATGCAGCTGGAATTTCAA
LetXET-B1		GCCCAAACAGGACATAGCAAGAGAAG
AF215069	*SlXTH12*	CTTATCTTCTCAAGGACCCACTCATGATG
AF205069; LeBR1		CCAAATGATGGAGTAGGTGTGGAAGTTC

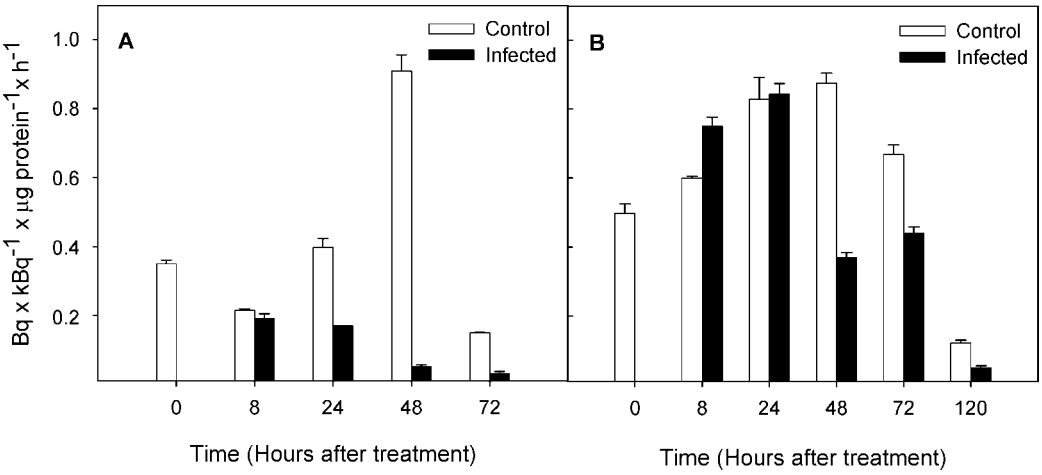

Figure 3. Specific activity of xyloglucan endotransglucosylase during tomato fruit infection. **A**: Tomato fruit cv. Canario. **B**: Tomato fruit cv. Money Maker. Dashed bars: control fruits. Filled bars: infected fruits. Values shown are means of three independent experiments, measuring in duplicate, using 24 fruits in each experiment ± SE.

Xyloglucan Endotransglucosylase Activity during Fruit Infection. To investigate the possible implication of the XTH enzyme in the host–pathogen interaction, we characterized the time course of XET specific activity during infection of two tomato fruit cultivars by *P. expansum*, and the results are plotted in **Figure 3**. The results showed that, as the infection progressed, there was an important decrease in XET activity in infected tomato fruits as compared with noninfected fruits after long infection periods, 24 and 48 h for Canario and Money Maker tomato fruits, respectively. The main differences in XET activity between both cultivars, were that in the Money Maker as compared with the Canario fruits, was a delay in the decrease in XET activity, that is, in the Canario fruits the differences in XET activity between infected and noninfected fruits was detected after 8 h of infection, meanwhile in the Money Maker it was noticeable after 48 h of infection. In addition, we observed a decrease in XET specific activity with time, this being especially evident after 72 h in Canario and after 120 h in Money Maker, indicating again a delay in Money Maker with respect to Canario.

Expression of Tomato *SlXTHs* Genes During Fruit Infection. We studied RealTime RT-PCR expression of 11 complete sequence *SlXTHs* genes in tomato fruit in two different cultivars, Canario and Money Maker. The expression results show the relative variation of mRNA amount with respect to a reference standard; however, the identification of such a variation can not be necessarily related to a decrease or enhancement in protein amount or in enzymatic activity. In any case, our results in **Figure 4** show the expression of the *SlXTHs* grouped into the three phylogenetic groups described above. We observed that all *SlXTHs* studied were expressed in both cultivars, with the higher expression found in *SlXTH4* (Group 1), *SlXTH9, SlXTH3* and *SlXTH12* (Group 2) and *SlXTH5* and *SlXTH8* (Group 3). The expression behaviour was similar in both cultivars, although higher expression was found in *SlXTH3, SlXTH9, SlXTH12* (belonging to the Group 2) in the Money Maker cultivar.

We analysed the *SlXTHs* expression during infection of Canario tomato fruit by *P. expansum* and the results are expressed in **Figure 5**. The results show that in noninfected fruits after 8 h of incubation were not much differences in *SlXTHs* expression with time, with the exception of *SlXTH2* that increased in both cultivars. However, we found a general decrease in expression, more so after long incubation periods, of the eleven *SlXTHs*, grouped into the three phylogenetic

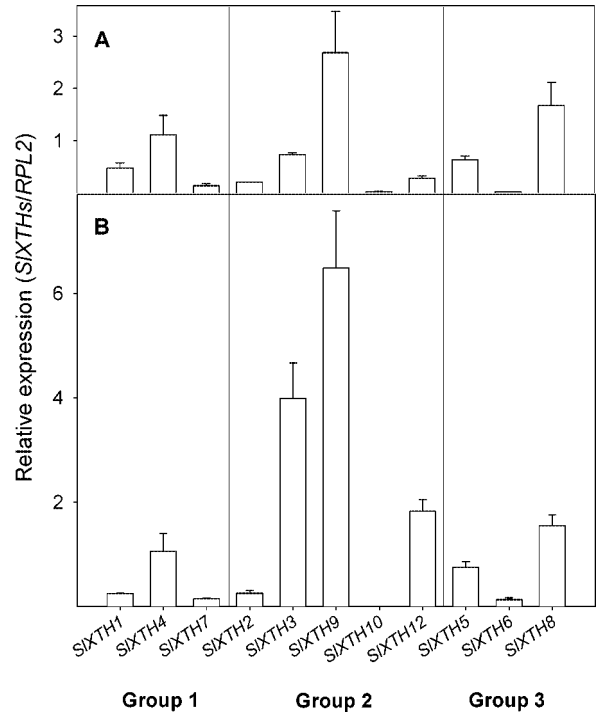

Figure 4. Expression analysis of *SlXTHs* tomato genes by Real Time RT-PCR. **A**: Tomato fruit cv. Canario. **B**: Tomato fruit cv. Money Maker. Values shown are means of three independent experiments, measuring in duplicate, using 24 fruits in each experiment ± SE.

groups, in the infected fruit as compared with the non infected ones. This decrease was more evident after 24 h of infection and remains as the infection progressed, these results being in agreement with the XET activity.

When we studied the *SlXTHs* expression in Money Maker tomato fruit during fungus infection, we also found a decrease in the expression of the *SlXTH5, SlXTH6, and SlXTH8*, belonging to the Group 3, in the infected fruits in comparison with the non infected ones, noticeable after 8 h of incubation and up until 120 h (**Figure 6**). *SlXTHs* expression of the members of the Groups 1 and 2 displayed a different behaviour as the infection progressed. *SlXTH2* (Group 2) showed a lower expression in the infected fruits as compared with the non infected ones, the same as in Group 3. Meanwhile *SlXTH1* and (Group 1) and *SlXTH3* (Group 2) showed a higher expression

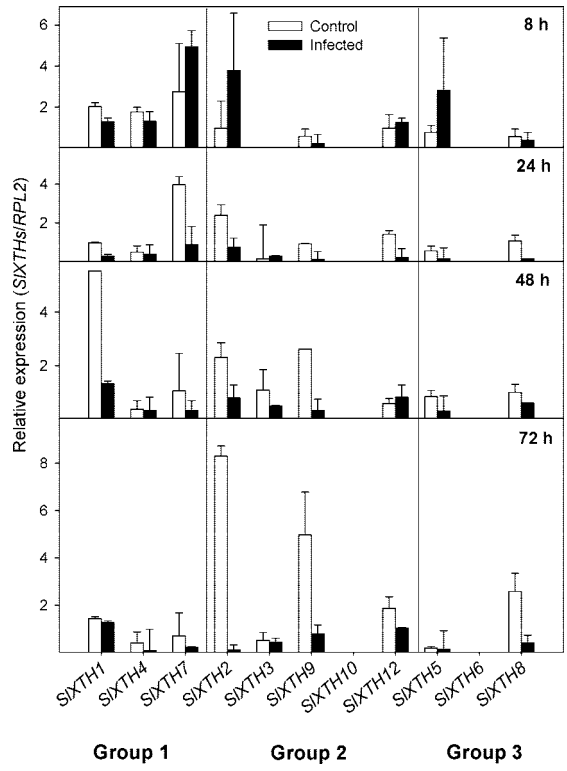

Figure 5. Expression analysis of *SlXTHs* genes during tomato fruit infection cv. Canario by Real Time RT-PCR. Dashed bars: control fruits. Filled bars: infected fruits. Values shown are means of three independent experiments, in measuring in duplicate, using 24 fruits in each experiment ± SE.

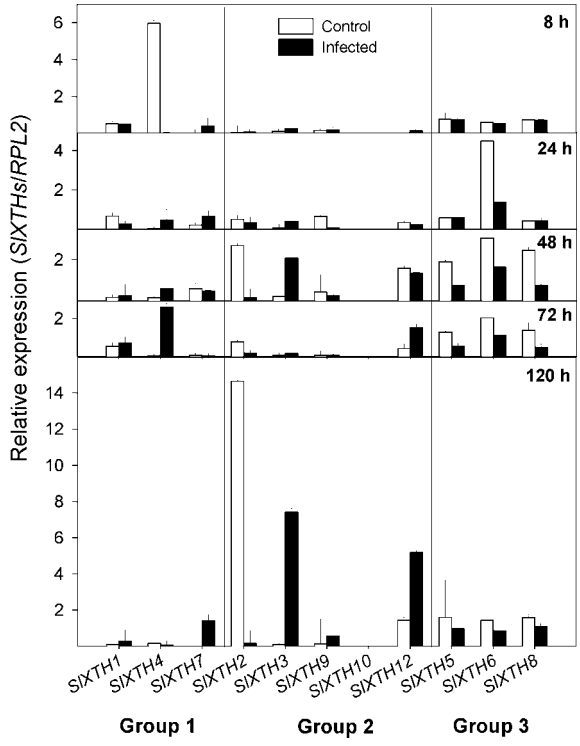

Figure 6. Expression analysis of *SlXTHs* genes during tomato fruit infection cv. Money Maker by Real Time RT-PCR. Dashed bars: control fruits. Filled bars: infected fruits. Values shown are means of three independent experiments, measuring in duplicate, using 24 fruits in each experiment ± SE.

in the infected fruits as compared with the noninfected ones. The rest of the *SlXTHs* showed a changeable expression pattern.

DISCUSSION

Xyloglucans are interlaced with cellulose microfibrills and act as a network providing tensile strength for the cell wall. In the context of the plant–pathogen interaction, the breakdown of the xyloglucan by fungal cellulases and xylosidases, spanning the space between cellulose microfibrills, could weaken the wall and provide increased access for fungal cellulases to degrade the cellulose microfibrills (*3, 8, 22*). Apart from the xyloglucan degrading enzymes, XTHs have been proposed to have a dual role integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan, or restructuring existing cell wall material by catalysing transglucosylation between previously wall bound xyloglucan molecules (*11, 12, 14*). Different reports support the idea that XTH indeed has a role in the maintenance of the cell wall structure, and expression and activity of XTHs have been reported to be correlated with cell elongation, differentiation of vascular systems and biotic and abiotic stimuli among other physiological processes (*12, 23–25*). In addition, a few reports showed a possible relationship between XTHs and the plant defence mechanism. In that sense, it has been reported that an induction of *MtXTH1* is involved in systemic modifications to the cell wall structure that facilitate development and proliferation of the fungus in the apoplast in mycorrhizal roots (*26*). Also, an increase in *SlXTH1* mRNA accumulation and XET activity during the defence reaction associated with the incompatible tomato-*Cuscuta* interaction has been reported (*23*). Also, *AgXTH1* and *AtXTH33* has been suggested to be involved in protecting plants against aphids, although altering *AtXTH33* expression in companion cells appears insufficient to increase protection against aphids (*27*). However in all cases, the observations do not predict whether the up-regulation benefits the plant defence mechanisms or the fungus/aphid settlement.

Bearing in mind that for a successful infection of a plant by a fungus, the fungus has to weaken the plant's natural barriers, such as the cell wall, by degrading the cell wall polysaccharides, and since the induction of the cell wall degrading enzymes by the fungus is considered one of the important pathogenicity factors (*2, 3*), it seems reasonable to predict that any potential cell wall repairing enzyme could be a target for fungus inhibition. Our results showed an important decrease in XET activity after long infection periods, 24 and 48 h for Canario and Money Maker tomato fruits, respectively (**Figure 3**). Since the XTH protein belongs to a multigenic family, we wanted to determine which members of the family had altered expression in response to *P. expansum* infection. Tomato *SlXTHs* were previously grouped into three of the four existing groups described for the *Arabidopsis thaliana AtXTHs* family (**Figure 2**), confirming previous phylogenetic studies (*21*). In mature tomato fruit all the *SlXTHs* determined were expressed in a similar way in both fruit cultivars, although in Money Maker higher expression was found in *SlXTH3, SlXTH9, SlXTH12*, all of them belonging to the Group 2 (**Figure 4**). It is important to mention that this is the first time that the expression of 11 different *SlXTHs* in tomato fruit at the red ripe stage has been described. The *XTHs*, that have been reported to be expressed in fruits, were associated with the fruit growth stages or the preripe stages, all previous to the red ripe stage, and only *SlXTH5* expression (measured as total mRNA) have been reported (*21*). As the infection progressed, we found a generalised decrease in *SlXTHs* expression in both fruit cultivars (**Figures 5** and **6**), so we can not associate a particular *SlXTHs* group to the infection response to *P. expansum*. These results also indicate that the decrease en XET activity during infection

cannot be attributed to the decrease in expression of any particular *SlXTH* gene, nor to any phylogenetic *SlXTHs* group. Obviously, other *XTHs* could be contributing to the total XET activity, since, up to now, 25 tomato *XTHs* have been identified (11 partial length sequences) and that we have used 12 for our studies. However, since we have found a generalized decrease in *SlXTHs* expression in both fruit cultivars, it is possible to suggest a transcriptional control of the *SlXTHs* expression by the fungus that decrease *SlXTHs* expression. It could be that the posttranscriptional control may target a highly conserved region of the gene sequence.

All the above mentioned results support the suggestion that inhibition of *SlXTHs* expression, and consequently XET activity, could represent a novel pathogenicity factor that complement the others based on the activation of the cell wall degrading enzymes. As we previously suggested (*8*), if the XET activity is inhibited, the potential reconstructing role of the enzyme is also decreased, and the hemicellulose degradation by fungal β-glucanases continues, changing the cell wall structure. These changes might increase the fruit softening and wall disassembly, facilitating the fungus colonization and the progress of the infection.

ABBREVIATIONS USED

XEH, xyloglucan endohydrolase; XET, xyloglucan endotransglucosylase; XTH, Xyloglucan endotransglucosylase/hydrolase.

ACKNOWLEDGMENT

We wish to thank M.J. Renart for technical assistance and Mr D. A. Lindsay for correcting the English version of the manuscript.

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Received for review June 20, 2007. Revised manuscript received August 23, 2007. Accepted August 31, 2007. This work was funded by MCYT BFU2005-08770-C02 and GVA CTBPRB/2002/377.

JF0718244