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ARTICLE

Overexpression of a Cell Wall Enzyme Reduces Xyloglucan Depolymerization and Softening of Transgenic Tomato Fruits

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Xyloglucan xyloglucosyltransferase/endohydrolase (*XTHs:* EC 2.4.1.207 and/or EC 3.2.1.151) has been proposed to have a dual role integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan and restructuring existing cell wall material by catalyzing transglucosylation between previously wall bound xyloglucan molecules. In this work we generated transgenic tomatoes with altered levels of an *XTH* gene. These transgenic fruits showed significant overexpression of the *XTH* proteins in comparison with the wild type. Specific XET activity was approximately 4.33 fold higher in the transgenic fruits compared with the wild type fruits, although in both cases the activity decreased during fruit development. Cell wall hemicelluloses extracted with 24% KOH showed a depolymerization of total sugar and xyloglucan during ripening, although this depolymerization was much lower in the transgenic than in the wild type tomatoes. These results suggest that the increased XET activity in the transgenic plants was responsible for the lower xyloglucan depolymerization. Fruit softening, during ripening, was lower in the transgenic tomatoes, indicating that the xyloglucan structure is related with the softening mechanism and that XET is one of the enzymes involved in the process. We suggest that the role of XET during fruit growth and ripening could be related to the maintenance of the structural integrity of the cell wall and the decrease in activity during ripening might contribute to the fruit softening.

KEYWORDS: Cell wall; fruit softening; xyloglucan; xyloglucan xyloglucosyltransferase

INTRODUCTION

Fleshy fruits soften during ripening mainly as a consequence of the catabolism of different cell wall components, the main changes being in pectic and hemicellulosic polysaccharides, two of the major cell wall components that undergo solubilization and depolymerization (*1*). 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 (PG), pectate lyase, pectin methylesterase, β-galactosidase, α-L-arabinofuranosidase, endo-(1,4)-β-D-glucanase, β-xyloxidase, expansin, xyloglucan xyloglucosyltransferase, and endomannanase (*1*). However, the real contribution of those enzymes to the softening process remains unclear, even after analyzing the role of an individual cell wall modifying enzyme in different fruits by using genetic engineering (*2*). For instance, in tomato, suppression of PG activity rendered fruit with an altered pectin metabolism but similar softening in relation to controls (*3*) and overexpression of the PG gene in *rin* (ripening inhibitor (*4*)) tomato fruit caused the solubilization of cell wall polyuronides but did not affect fruit softening (*5*). In the particular case of tomato fruit, in addition to changes in pectin structure, an important xyloglucan depolymerization occurs during fruit softening (*6*). The implication of the xyloglucan structure with fruit firmness was already suggested by Maclachlan and Brady (*6*) studying *rin* tomatoes, where they found a direct relationship between the maintenance of the molecular mass of xyloglucan and the fruit firmness. Those authors also suggested that the decrease in molecular weight of xyloglucan could have been due to the activities of *endo*-1,4-β-glucanases and/or xyloglucan xyloglucosyltransferase, both of which increased when tissue firmness declined most rapidly. However, the contribution of plant endoglucanases in xyloglucan depolymerization has been questioned in studies using transgenic plants, were it has been shown that plant endoglucanases have a minimal effect on the degree of xyloglucan polymerization (*7*, *8*). The other enzymes that could be involved in xyloglucan metabolism during fruit softening are xyloglucan xyloglucosyltransferase/endohydrolase (*9*, *10*), encoded by the *XTHs* family, enzymes that can act as xyloglucan xyloglucosyltransferase (XET), catalyzing the transfer of a xyloglucan molecule fragment to another xyloglucan molecule, and/or xyloglucan

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endohydrolase (XEH), which hydrolyzes one xyloglucan molecule (*11*−*13*).

Most of the reports have focused on the possible role of *XTH* genes in fruit growth or fruit softening in different fruits such as tomato, kiwi fruit, pears, litchi, and grape berry (*14* −*17*). In tomato fruits, six *SlXTHs* have been reported during different stages of fruit development, but only *SlXTH5* (*18*) and *SlXTH8* (*19*) expression has been reported to be associated with fruit ripening, although it seems that they could not be responsible for xyloglucan degradation during fruit ripening. In addition, a few reports also suggest that *XTH* plays an important role in cell wall turnover and maintenance rather than softening (*16*, *17*, *20*, *21*). Our previous studies with tomato fruit showed a decrease in *XTHs* expression and XET activity during ripening; therefore, we suggested that the role of XET during fruit development could be related to the maintenance of the structural integrity of the cell wall and the decrease in XET activity during ripening might contribute to the fruit softening (*19*).

The objective of this work is to further investigate the implication of XET in fruit softening. To reach this objective, the work includes the generation of transgenic tomatoes with altered levels of a *XTH* gene, *SlXTH1*, to increase the XET activity in tomato fruits, and evaluate how the increased XET activity could affect the xyloglucan structure and the fruit ripening and softening process.

MATERIALS AND METHODS

Tomato Transformation. Transgenic tomato plants expressing a *Nicotiana tabacum* homologous *SlXTH1* under control of the constitutive 35S CaMV promoter (acc. no. D86730) were generated. Cloning and construction of the vector was described by Herbers et al. (*22*).

The construct was transformed into *Agrobacterium tumefaciens*, strain CV58C1 carrying the pGV2260 virulence plasmid. Transformation of tomato plants (cv. Moneymaker) was performed essentially as described by Ling et al. (*23*) using tomato cotyledons and a tobacco cell suspension culture as a feeder layer. Screening of approximately 50 kanamycin-resistant shoots revealed a number of transgenic shoots expressing variable amounts of *SlXTH1*.

Plant and Fruit Growth. Homozygotic tomato seeds (*Solanum lycopersicum* L. cv. Money Maker) were grown in soil in a greenhouse. Tomato fruits were harvested from 20−30 tomato plants, rinsed with distilled water, and surface-dried. The largest diameter of each fruit was measured and the color of the fruits determined by a colorimeter (Minolta CR-300, Madrid, Spain). Fruits were then selected into three categories at different stages of ripening: turning (T), pink (P), and red (R) (*19*).

Most of the experiments were performed using fruits (20−150) harvested from 3 independent harvests obtained in different years, and the replication strategy used was through seeds.

mRNA Extraction, cDNA Preparation, and Real-Time Quantitative PCR. 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). 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.

The PCR amplification was performed with gene-specific primers. The primer sequence (forward/reverse) for the homologous *SlXTH1* gene was CTTGAGAAAACCAATTGGGCCAAC/GAACCCAACGAAGTCTCCTATACTGTAATG and for RPL2 (a housekeeping gene, ribosomal protein large subunit 2), used as internal control for the gene, ATTCACGATCCAGGGAGAGGTGC/AGGCAACACGTTACCAACCATAAGAGTAG, as previously described (*19*).

Three replicates were performed for each sample in 25 μL final volume containing 1 μL of cDNA, 25 pmol of *SlXTH1* or RPL2 (18S) specific primer, 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 these were interpolated in a quantification standard curve (*24*). The Ct value for each sample was normalized to RPL2, 18S rRNA.

Protein Extraction and Xyloglucan Xyloglucosyltransferase (XET) Assay. Soluble and ionically bound proteins were extracted together by homogenization of partially thawed tomato fruit pericarp as previously described (*25*). The protein content of the extracts was assayed by the Coomassie Blue G dye-binding method (*26*).

Xyloglucan xyloglucosyltransferase 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 (*27*). [^{3}H]XXXGol was prepared by reduction of the reducing terminal glucose moiety of nonradioactive XXXG with NaB_3H_4. The solution of [3H]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 [3H]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, 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.

Extraction and Analysis of Cell Wall Polysaccharides. Cell walls were extracted from tomato fruits followed by sequential extraction of pectic and hemicellulosic polysaccharides as described by Cutillas-Iturralde et al. (*28*). The sugar content of hemicelluloses and xyloglucan (percent cell wall dry weight) extracted with 4 and 24% KOH was determined using the phenol−sulfuric acid method for total sugars (*29*) and the iodine staining method (*30*) for xyloglucan determination. Gel chromatography of the 24% KOH fraction was carried out on a Sepharose CL-4B column (115 × 1.4 cm) equilibrated with 1 M NaOH. The columns had been calibrated with authentic dextrans (5000, 510, 70, 9.4 kDa) purchased from Pharmacia and Sigma Chemical Co. The samples (ca. 3 mg) were dissolved in 2 mL of the respective buffer and eluted with the same buffer at a flow rate of 19 mL h^{-1}. Fractions with a sample size of 2 mL were collected, and an aliquot of each fraction was assayed for total and xyloglucan content. The molecular mass of the hemicellulosic fractions and the xyloglucan was estimated from the respective gel permeation chromatogram using the formula $m = S(M_i \times W_i)/SW_i$, where m is molecular mass, W_i is total sugar or xyloglucan content of the ith fraction, and M_i is the molecular mass of the ith fraction estimated from the calibration curves (*31*).

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

RESULTS

Tomato Plant Transformation. Following *Agrobacterium*-mediated transformation, approximately 50 kanamycin-resistant tomato shoots were selected and screened for *SlXTH1* RNA and protein. Lines 1, 8, 10, and 13 showed a reasonable overexpression of tobacco *SlXTH1* RNA and protein as demonstrated by western and Northern blotting, and line 13 was selected for further experiments (data not shown).

Expression Analysis of *SlXTH1*. The results of the expression analysis of homologous *SlXTH1* gene by real-time quantitative PCR are shown in **Figure 1**. In the wild type tomato fruits, *SlXTH1* was expressed during fruit ripening, although its level of expression decreased as the fruit ripened. Although the constitutive 35S CaMV promoter was used to drive expression of the transgene, the same expression pattern was observed in the transgenic tomato fruits; however, the expression was much higher, between 0.6 and 4 times, as compared to the wild type, during the three ripening stages studied.

XET Activity during Fruit Growth and Ripening. Soluble XET activity was determined in the tomato fruit at different stages

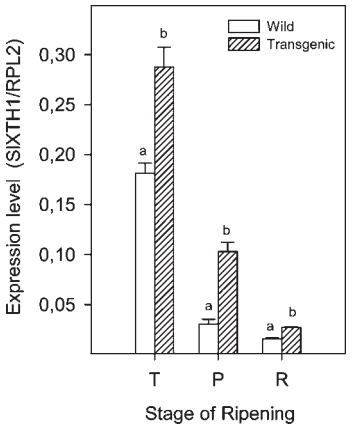

Figure 1. Expression analysis of *SlXTH1* tomato gene by real-time quantitative PCR from wild and transgenic tomato fruits at different stages of ripening. Ripening stages are expressed as turning (T), pink (P) and red (R), according to fruit color. Values shown are means of 2 independent experiments using 12 fruits from 2 different harvests in each determination ±SE. Different letters indicate significant differences between wild and transgenic tomato lines (*t* test, *p* value <0.05).

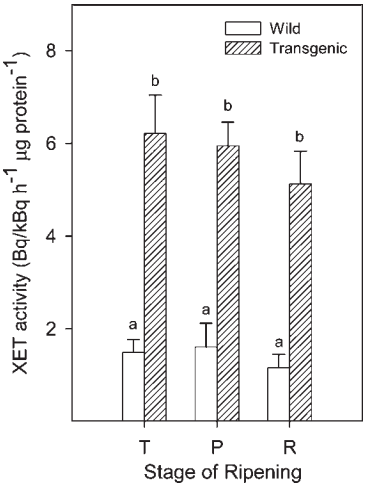

Figure 2. Specific activity of xyloglucan xyloglucosyltransferase (XET) from wild and transgenic tomato fruits at different stages of ripening. The XET assay was performed by measuring [^{3}H]XXXG incorporation into XG by the protein extract. Ripening stages are expressed as in **Figure 1**. Values shown are means of 3 independent experiments using 20 fruits from 3 different harvests in each determination ±SE. Different letters indicate significant differences between wild and transgenic tomato lines (*t* test, *p* value <0.01).

of ripening using [^{3}H]XXXGol as the acceptor for the endotransglucosylation reaction (**Figure 2**). Results showed that, in transgenic fruits, the XET specific activity was 3 times higher as compared to that in wild type and no significant differences were found as ripening progressed.

Hemicelluloses and Xyloglucan Structure. The total sugar content in soluble hemicelluloses and xyloglucan from wild-type and transgenic tomato fruits is shown in **Table 1**. Hemicelluloses were extracted sequentially with 4 and 24% KOH. In the hemicellulosic fraction extracted with 4% KOH, we found no differences in sugar content between the wild type and the transgenic tomato fruits. On the other hand, the relative amounts of total sugars and xyloglucan did not change during fruit ripening. Xyloglucan was present in both fractions but was mainly extracted with 24% KOH, the main hemicellulosic fraction, representing around 40%

Table 1. Sugar Content of Hemicelluloses and Xyloglucan (Percent Cell Wall Dry Weight) Extracted with 4 and 24% KOH from Wild and Transgenic Tomato Fruits at Different Stages of Ripening[a]

	stage of ripening		
	T	P	R
4% KOH Fraction			
total sugars			
wild	4.52 ± 0.80	4.57 ± 0.86	4.70 ± 1.00
transgenic	4.98 ± 0.54	6.64 ± 0.70	5.28 ± 1.40
xyloglucan			
wild	1.20 ± 0.32	1.23 ± 0.40	1.13 ± 0.40
transgenic	1.22 ± 0.31	1.68 ± 0.37	1.31 ± 0.45
24% KOH Fraction			
total sugars			
wild	6.25 ± 1.49	6.19 ± 1.25	5.33 ± 1.04
transgenic	7.35 ± 1.78	5.20 ± 1.00	5.20 ± 1.80
xyloglucan			
wild	2.21 ± 0.73	2.53 ± 0.68	2.43 ± 0.69
transgenic	2.19 ± 0.46	2.55 ± 0.72	2.31 ± 0.80

[a] Ripening stages are expressed as turning (T), pink (P), and red (R), according to fruit color. Values shown are means of 3 independent experiments using 20 fruits from 3 different harvests in each determination ±SE.

of the total sugars in this fraction. In any case there were no differences in sugar content between wild type and transgenic fruits, as has been previously observed for the 4% hemicelluloses.

Results of gel permeation chromatography on a Sepharose CL-4B column of the hemicellulosic polysaccharides extracted with 24% KOH are shown in **Figures 3** and **4**. Three peaks of total polysaccharides (**Figure 3**) appeared in the chromatograms, the first in the void volume region and the other two peaks in the fractionation range of the column. The amount of total sugars, in the void volume peak, was higher in the transgenic tomatoes in comparison to wild type. In both cases, as the fruit ripened, we observed that the void volume peak was reduced. Total sugars in the second peak, around 510 kDa, showed a shift toward a lower molecular mass region during ripening, especially at the pink and red stages in the wild type tomatoes. However, this shift was not observed in the transgenic tomatoes. The amount of total sugars in the third peak increased as the fruit ripened, indicating a lower degree of hemicellulose polymerization. In addition, we observed a shift of this peak toward the lower molecular mass region of the wild type tomato fruits as compared with the transgenic ones, this being noticeable along all stages of ripening but particularly at the red stage. The xyloglucan distribution is shown in **Figure 4**. Xyloglucan eluted in the same fractionation range as total sugars, although most of the xyloglucan eluted in a peak located in the 510 kDa region, with a shoulder in a lower molecular mass region. High molecular mass xyloglucan, eluted in the void volume region, was only measured at the growth stages in the transgenic tomatoes (data not shown). For xyloglucan eluted in the fractionation range of the column, an important shift was observed toward the lower molecular mass region as the fruit ripened, particularly at the red stage and in the wild type tomatoes.

The average molecular masses of total sugars and xyloglucan in the 24% hemicellulosic fraction are shown in **Table 2**. We observed a significant reduction in the molecular mass of total sugars during fruit ripening. The reduction was higher in the wild type tomato fruits (48%) as compared with the transgenic species (38%). The same was observed in the xyloglucan, since this is the main polysaccharide in this hemicellulosic fraction. The molecular mass of xyloglucan decreased with ripening, this decrease being higher in the wild tomatoes (44%) than in the transgenic

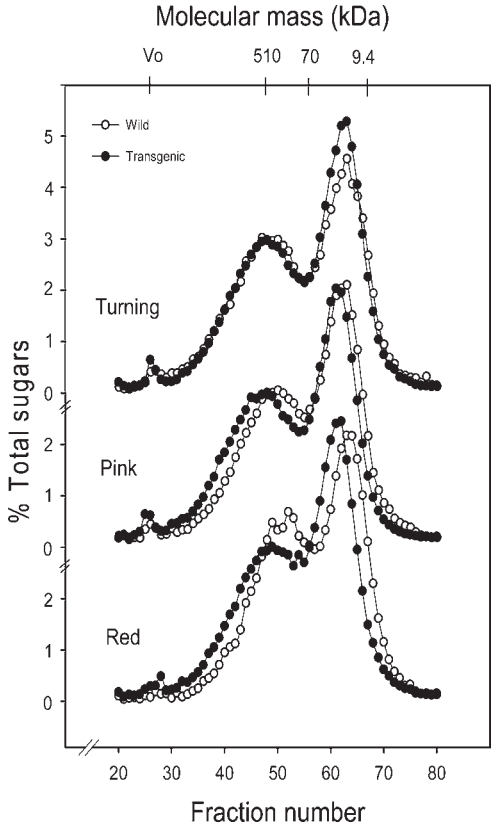

Figure 3. Distribution of molecular mass of total sugars of hemicelluloses extracted with 24% KOH and chromatographed on a Sepharose CL-4B column from wild (○) and transgenic (●) tomato fruits at different stages of ripening. The calibration scale, obtained using authentic dextrans (kDa), is shown at the top (Vo = void volume). Ripening stages are expressed as in **Figure 1**. Values shown are means of 3 independent experiments using 20 fruits from 3 different harvests in each determination ±SE.

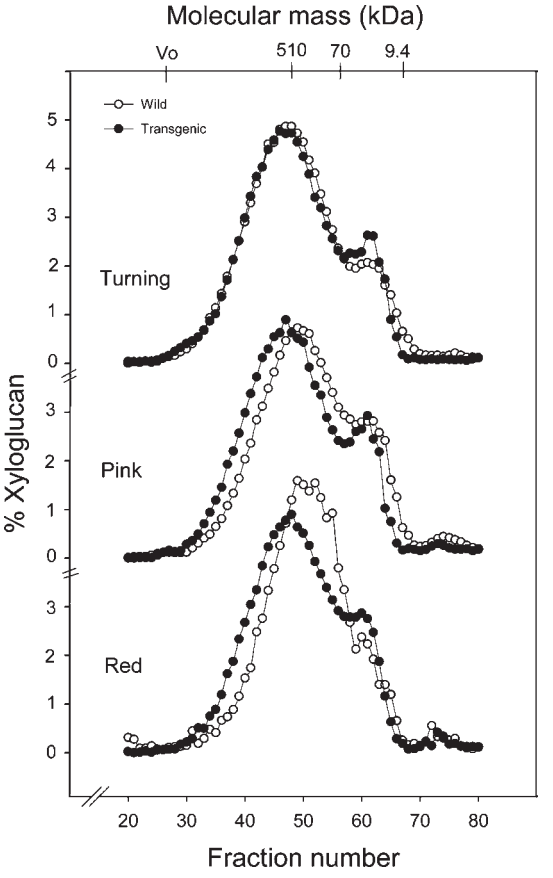

Figure 4. Distribution of molecular mass of xyloglucan of hemicelluloses extracted with 24% KOH and chromatographed on a Sepharose CL-4B column from wild (○) and transgenic (●) tomato fruits at different stages of ripening. The calibration scale, obtained using authentic dextrans (kDa), is shown at the top (Vo = void volume). Ripening stages are expressed as in **Figure 1**. Values shown are means of 3 independent experiments using 20 fruits from 3 different harvests in each determination ±SE.

tomatoes (30%). This means that the xyloglucan, in the transgenic tomatoes, has a higher degree of polymerization.

Changes in Fruit Firmness during Development. We evaluated the firmness of fruits using a fruit pressure tester (**Figure 5**) at the different stages of ripening. During fruit ripening an important decrease in fruit firmness occurs. This decrease was significantly lower in the transgenic tomato fruits at the pink and red stages of ripening, as compared to the case for wild type controls, indicating that softening of transgenic fruits remained lower compared to that in wild type fruits.

DISCUSSION

The implication of the hemicellulosic polysaccharides and the enzymes able to disassemble the cellulose−xyloglucan matrix as determinants of fruit softening has been reported by different authors (*1*, *2*). As we have previously mentioned, the enzymes encoded by the *XTH* genes have been considered as important candidates as wall-loosening enzymes (*9*, *10*). They can act as xyloglucan xyloglucosyltransferases (XETs), having thus a dual role, integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan or restructuring existing cell wall material by catalyzing transglucosylation between previously wall bound xyloglucan molecules, or act as xyloglucan endohydrolases (XEHs), hydrolyzing one xyloglucan molecule (*11*−*13*). Therefore, it is important to keep in mind that the only way to decrease xyloglucan molecular mass is by acting as XEH or as XET, but in this latter case using xyloglucan oligosaccharides as acceptors for the transglucosylation reaction and that would obviously depend in vivo on the availability of the substrates.

Table 2. Changes in the Average Molecular Mass of Total Sugars and Xyloglucan of Hemicelluloses Extracted with 24% KOH from Wild and Transgenic Tomato Fruits at Different Stages of Ripening. [a]

	stage of ripening		
	T	P	R
molecular mass total sugar (kDa)			
wild	948.8 ± 139.8	779.5 ± 129.0	562.8 ± 5.0
transgenic	840.9 ± 84.2	1007.9 ± 137.8	776.8 ± 77.4
molecular mass xyloglucan (kDa)			
wild	1242.2 ± 127.7	938.3 ± 112.1	843.4 ± 112.1
transgenic	1271.8 ± 69.7	1216.7 ± 117.3	1108.6 ± 81.2

[a] Average masses were calculated from the values plotted in **Figures 3** and **4**. Ripening stages are expressed as turning (T), pink (P), and red (R), according to fruit color. Values shown are means of 3 independent experiments using 20 fruits from 3 different harvests in each determination ±SE.

The different *XTHs* genes have been grouped into four groups for *Arabidopsis thaliana* (*32*) and into three groups for *Solanum lycopersicum* (*18*, *19*). The *XTH* gene used for transforming the tomato plants was the *Nicotiana tabacum* homologous *SlXTH1*, which belongs to group 1, that has been previously associated with growth (*12*, *19*). When we analyzed the homology of the nucleotide

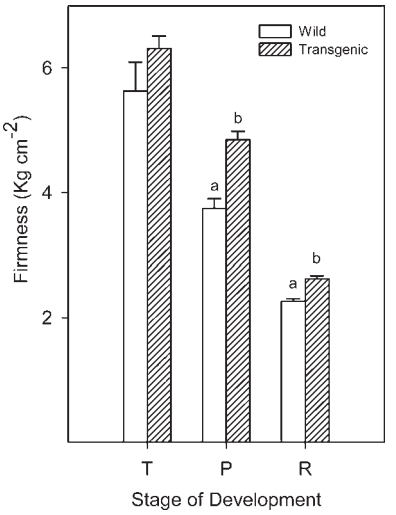

Figure 5. Changes in firmness of wild and transgenic tomato fruits at different stages of ripening. Firmness was determined in the pericarp tissue using a fruit pressure tester. Values shown are means of 3 independent experiments using 150 fruits from 3 different harvests in each determination ±SE. Different letters indicate significant differences between wild and transgenic tomato lines (*t* test, *p* value <0.05).

sequence between the components of group I, we found a 81% homology between *SlXTH1* and *SlXTH4* and 65% between *SlXTH1* and *SlXTH7* and thus assumed that the transgenic species altered the expression level of *SlXTH1* and not other *XTH* genes. Although *SlXTH1* has not been associated with fruit ripening, at the time this present work was initiated, no information regarding all the different *SlXTHs* was available; therefore, we transformed the tomato plants with the *SlXTH1* sequence available at that time. The original idea was to increase the XET activity to determine how the increase in xyloglucan transglucosylation could affect xyloglucan structure and then modulate growth and softening.

Our previous results concerning expression of *SlXTH1* in the wild type tomato fruits (*19*) showed a higher expression level during growth, which decreased during ripening. Also, our results with transgenic fruits showed a significant overexpression of *SlXTH1* in the transgenic fruits in comparison to the wild type, at the different stages of ripening (**Figure 1**). The increase in overexpression followed the same pattern as the wild type; therefore, it seems that the expression of the *SlXTH1* gene is developmentally regulated, even in the transgenic fruits.

When we measured the specific XET activity (**Figure 2**), we found a significant increase in activity in the transgenic fruits compared with the wild fruits (4.3 fold), although in both cases the activity slightly decreased during fruit ripening. It is important to mention that when we determined the XET activity, we were measuring the contribution of all the *XTHs* genes, not only *SlXTH1*; therefore, different isoforms could be contributing to the total activity. In any case, our results showed a total XET activity that was much higher in transgenic plants and that might be modifying in the same way the structure of the xyloglucan. We then determined the total sugar and xyloglucan content of the hemicelluloses (**Table 1**), but we did not find differences between wild and transgenic tomatoes. When we analyzed the molecular mass distribution of the hemicelluloses extracted with 4% KOH, we found that the degree of polymerization was higher in the transgenic tomatoes at the red stage of ripening, but the differences were not significantly different (data not shown). However, in the hemicelluloses extracted with 24% KOH (**Figures 3** and **4**), which represent the hemicellulosic polysaccharides more in contact with cellulose, we found a depolymerization of total sugar and xyloglucan happens during ripening, as has been previously reported by other authors (*6*, *33*). This depolymerization was much lower in the transgenic than in the wild tomatoes. Those results suggest that the increased XET activity in the transgenic plants was responsible for the lower xyloglucan depolymerization.

Since XET has been considered as a wall-loosening enzyme, as we mentioned earlier, the first reports after XET was discovered related XET with fruit ripening and, consequently, with fruit softening, particularly in fruits where an important xyloglucan depolymerization occurs, such as persimmon, kiwi, and tomato fruits (*6*, *28*). However, recent molecular studies were questioning the role of *XTHs* contributing to fruit softening. It has been reported that group 1, 2, and 3 *XTHs* predominantly exhibit XET activity, suggesting that *XTHs* do not represent primary cell wall loosening agents (*18*). Those authors also found that although expression of *SlXTH5* was abundantly expressed through fruit ripening, recombinant *SlXTH5* was not able to alter the mechanical properties of the cell wall and make the wall more extensible, suggesting that *SlXTH5* could not be responsible for xyloglucan degradation during fruit ripening. A few more reports also suggested that *XTH* plays an important role in cell wall turnover and maintenance rather than a role in softening. The expression of a pear *XTH* was constitutive, and this *PcXTH1* gene induction was associated with cell wall maintenance during development and ripening (*16*, *20*). Also, in litchi fruits, differential expression of *LcXTH1* was fruit-specific, playing a role in reducing litchi fruit cracking, while *LcXTH2* and *LcXTH3* showed a constitutive expression during fruit development (*17*). An interesting work with transgenic melons showed a correlation between xyloglucan depolymerization and fruit softening and that the process was ethylene-dependent. However, the expression of two *XTH* genes (grouped in group 2) was ripening-associated but only partially ethylene-dependent, suggesting that they do not play a significant role in fruit softening (*21*).

Our results on the fruit firmness (**Figure 5**) showed that fruit softening, which occurs when fruit ripens, was lower in the transgenic tomatoes, indicating that the xyloglucan structure is related to the softening mechanism and that XET is one of the enzymes involved in the process. The implication of the xyloglucan structure with fruit firmness was already suggested by Maclachlan and Brady (*6*) studying *rin* tomatoes (ripening inhibitor (*4*)), where they found a direct relationship between the maintenance of the molecular mass of xyloglucan and the fruit firmness.

It is possible that the way XET might be involved in the fruit softening mechanism could be completely different from that for other cell wall enzymes involved in fruit softening. That is, cell wall hydrolases such as pectinases and glucanases are responsible for cell wall degradation and fruit softening during fruit ripening. Meanwhile, XET would be responsible for the maintenance of xyloglucan and the cell wall structure. Since the XET decreases during ripening, the potential reconstructing role of the enzyme is also decreased and the fruit softens. If the XET activity is increased, as we did in the transgenic plants, the depolymerization of xyloglucan decreases and the softening process is delayed.

One open question is whether the results would have been the same if we have overexpressed a different *XTH* gene. However, most of the *XTHs* related with fruit growth or ripening seem to have XET activity instead of XEH activity (*18*), and there is the fact that we had measured an increase in XET activity and changes in xyloglucan structure associated with that increase. During fruit development, XET could be having a dual temporal role. On the one hand, during growth, its higher activity contributes to the maintenance of the fruit texture and, on the other hand, during

ripening its the decrease in activity also decreases the capacity for maintaining the cell wall structure, and so the fruit softens. This suggestion is in agreement with our previous results, where we found that both total XET and XEH activities were higher during fruit growth and decreased during fruit ripening, as well as the different *SlXTHs* expression (*19*). We suggest that the role of XET during fruit growth and ripening could be related to the maintenance of the structural integrity of the cell wall and that the decrease in XET activity during ripening might contribute to the fruit softening.

ABBREVIATIONS USED

MW, molecular mass; XEH, xyloglucan endohydrolase; XET, xyloglucan xyloglucosyltransferase; XTH, xyloglucan xyloglucosyltransferase/endohydrolase family genes.

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