Alteration of fruit characteristics in transgenic tomatoes with modified expression of a xyloglucan endotransglucosylase/hydrolase gene

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Abstract Some cell wall enzymes of the xyloglucan endotransglucosylase/hydrolase (XTH) family catalyze molecular grafting between xyloglucan molecules. In tomato, 25 genes for XTH proteins have been identified. We studied the tomato gene *SlXTH1*, which has highest homology to the cDNA of the XTH first purified from hypocotyls of *Vigna angularis*. *SlXTH1* mRNA was found to accumulate transiently at an early stage of fruit development, and the peak day of accumulation was about 12 days after pollination. The expression profile of *SlXTH1* mRNA was roughly associated with that of the reported enzyme activity, suggesting that the *SlXTH1* protein may be one of the main determinants of the enzyme activity. We then examined how alteration of expression of the *SlXTH1* gene could influence the characteristics of fruits. Several lines of transgenic tomatoes with different levels of *SlXTH1* transcript were produced by introducing a *SlXTH1* transgene in either the sense or antisense orientation downstream of a constitutive promoter. In these transgenic tomatoes, fruit size was positively correlated with the level of *SlXTH1* transcript, which was monitored at its peak stage. This is the first *in vivo* demonstration that *SlXTH1* can control the morphological character of plants through changes in its level of expression.

Key words: Cell wall, cell enlargement, fruit size, xyloglucan endotransglucosylase/hydrolase.

A marked increase in cell volume is one of the prominent features of plant cell growth and development. In dicotyledonous plants, the cell wall is mainly composed of cellulose microfibrils and interlaced xyloglucan chains which tether the microfibrils together (Carpita and Gibeaut 1993). When plant cells enlarge, rearrangement of the cell wall network has to occur. Because xyloglucan chains are amorphous polymers in contrast to the rigid crystal structure of cellulose microfibrils, xyloglucan chains fulfill a critical role in the rearrangement of the cell wall network.

The cleavage of xyloglucan molecules by the activity of hydrolases has been conceived as the key step for cell wall loosening, and hence cell expansion. This is based on the observation that inhibition of xyloglucan breakdown in epicotyls of azuki bean, *Vigna angularis*, by fucose-binding lectin or xyloglucan-specific antibodies resulted in blocking of auxin-induced cell wall extension (Hoson et al. 1991). However, the process of incorporation of newly synthesized xyloglucan molecules into the cell

wall matrix cannot be fully explained only by the cleavage of xyloglucans. Grafting of xyloglucan molecules into the expanding cell wall structure has to be taken into account. It was first hypothesized in 1976 that a transglycosylase should exist that would catalyze this molecular grafting (Albersheim 1976). This transglycosylase activity was subsequently detected in numerous plant species, and named endo-xyloglucan transferase (EXT) or xyloglucan endotransglycosylase (XET) (Fry et al. 1992; Nishitani and Tominaga 1992). cDNAs coding for this enzyme or family of genes have been isolated from a wide range of plants including monocots as well as dicots (Okazawa et al. 1993; Rose et al. 2002; Saladié et al. 2006; Yokoyama et al. 2004), with the gene name being replaced by XTH (xyloglucan endotransglucosylase/hydrolase) to reflect the XET and XEH activities of the protein (Rose et al. 2002). Recombinantly-expressed XTH proteins, including SIXTH2 (formerly LeEXT2) and SIXTH10 (formerly LeXET-B2), have been shown to possess XET activity in

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Abbreviations: DAP, day after pollination; EXT, endo-xyloglucan transferase; XET, xyloglucan endotransglucosylase; XEH, xyloglucan endotransglucosylase/hydrolase

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vitro (Arrowsmith and de Silva 1995; Catalá et al. 2001; Saladié et al. 2006). XET activity has been detected in the fruit of several species at various stages of development. In tomato (*Solanum lycopersicum* L.), xyloglucan-depolymerising enzyme activity was almost constant throughout tomato ripening (Maclachlan and Brady 1994) but was highest at the earlier stages of tomato fruit expansion (de Silva et al. 1994). In apple (*Malus domestica* Borkh. cv. Braeburn) and kiwifruit (*Actinidia deliciosa* [A. Chev.] C. F. Liang et A. R. Ferguson var. deliciosa cv. Hayward), a large peak and small peak of XET activity appeared at the stage of fruit expansion and softening, respectively (Percy et al. 1996).

Newly synthesized xyloglucans were much smaller than the bulk xyloglucans found in the wall (Talbott and Ray 1992). This finding supports the idea that newly synthesized xyloglucans are integrated into the cell wall matrix by the function of the XET activity of XTH, resulting in the elongation of xyloglucan chains (Cosgrove 1997; Nishitani 1995; Nishitani and Tominaga 1992).

In a separate line of study, McQueen-Mason et al. reported the isolation of expansin, which catalyzes the in vitro extension of cell walls held in tension at acid pH (McQueen-Mason and Cosgrove 1994; McQueen-Mason et al. 1992). In vitro experiments with XTH proteins failed to detect loosening or extension of the wall (McQueen-Mason et al. 1993). This suggests that XTH controls the yield threshold of cell enlargement by modulating the size of the polysaccharide cluster (Cosgrove 1997), but does not introduce loosening or extension of the wall. However, expression of XTH genes has been correlated with cell expansion in a variety of tissues (Cui et al. 2005; Feng et al. 2008; Hyodo et al 2003; Shin et al. 2006; Yun et al. 2005), and addition of active XET protein was shown to cause cell wall loosening in vivo in onion epidermal cells (van Sandt et al. 2007). This suggests that in certain tissues and under certain circumstances, XET activity may be involved in wall loosening.

We originally isolated a tomato gene of this family using the V. angularis EXT gene as a probe (Okazawa et al. 1993), resulting in a tomato gene registered as TOMEXT3 in GenBank (accession number D16456). This gene, which was also named as LeEXT (Catalá et al. 1997), has since been renamed as SlXTH1 (Saladié et al. 2006), and in tomato 25 XTH genes have now been identified (Saladié et al. 2006). The expression of 10 of these genes was examined during tomato fruit development, and mRNA of all of them was detected at all developmental stages (Miedes and Lorences 2009). Seven of the genes (SIXTH1, SIXTH2, SIXTH3, SIXTH5, SIXTH9, SIXTH10 and SIXTH11) showed mRNA accumulation during fruit growth or maturation, and three genes (SIXTH3, SIXTH5 and SIXTH9) showed a correlation of mRNA accumulation with ripening

(Saladié et al. 2006). Four genes (*SlXTH1*, *SlXTH2*, *SlXTH10* and *SlXTH11*) were expressed in both pre-ripe and ripe stage. *SlXTH1*, *SlXTH10* and *SlXTH11* were expressed at high levels in expanding fruit (Saladié et al. 2006). Overexpression of *SlXTH1* in tomato fruit has been found to have small effects on xyloglucan depolymerization and fruit softening, but effects on fruit growth were not examined (Miedes et al. 2010). The early growth of tomato fruits is largely the result of cell division, and all later growth is associated entirely with cell enlargement (Gillaspy et al. 1993). Therefore, we targeted growing tomato fruits to demonstrate the function of XTH *in vivo*.

In this study, we first determined in which organs and fruit developmental stages the XTH genes (*SlXTH1* and *SlXTH11*) were expressed and compared expression profiles between the two XTH genes. For the expression analysis of the genes, cDNA probes were designed based on the sequence of *SlXTH1* and *SlXTH11*. The probe for *SlXTH11* would certainly crosshybridize with the *SlXTH10* paralogue because *SlXTH10* and *SlXTH11* have 90% sequence identity across the full-length cDNA. Expression of *SlXTH1* was compared with that of *SlXTH10/11*. Then, we elucidated the physiological role of XTH using transgenic tomatoes that expressed either sense or antisense RNA of *SlXTH1* constitutively, focusing on the stage of fruit growth.

Materials and methods

Plant material and bacterial strain

Tomato (*S. lycopericum* cv. Shugyoku) seeds used for Ti plasmid-mediated transformation were obtained from the National Research Institute of Vegetables, Ornamental Plants and Tea, MAFF, Japan. *Agrobacterium tumefaciens* LBA4404 used as a host for the transformation of tomato was purchased from Clontech (Mountain View, CA, USA).

Enzymes and plasmids

Restriction and DNA-modifying enzymes used were products of Takara Bio Inc. (Otsu, Japan) or purchased from New England Biolabs (Ipswich, MA, USA). Binary vector pIG121-Hm (Ohta et al. 1990) modified from pBI121 was kindly supplied by Prof. Nakamura (Nagoya University, Japan).

RNA extraction, northern blotting and RT-PCR

Total RNA was extracted from fruits, axillary buds, leaves and stems using the guanidinium thiocyanate method according to Chomczynski and Sacchi (1987). Northern blotting was carried out with *SlXTH1* cDNA as a probe. Reverse transcription was performed using an RNA PCR Kit with AMV ver. 2 reverse transcriptase (Takara Bio Inc.) following the protocol recommended by the manufacturer. Polymerase chain reaction (PCR) was carried out with 25 cycles of 30 s denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C. Primer TOM-XSP (5'-TTTTCTAGACCATGGGTATCATAA-AAGGAG-3') and primer TOM-SAP (5'-TTGGAGCTCATT-

TTAAATATCTCTGTCCTT-3') corresponding to the SIXTH1 cDNA sequence (Okazawa et al. 1993) were used for the detection of SIXTH1 transcript. Primer XET-SP1 (5'-TCTT-CTCAAGGAGCAGGGCA-3') and primer XET-AP1 (5'-TGC-TGAACCCATCGAAGCCT-3') corresponding to the SIXTH11 cDNA sequence (Arrowsmith and de Silva 1995) were used for the detection of *SIXTH11* transcript. For the detection of β tubulin gene, a consensus sequence was designed from an assembly of multiple sequences: ATHTUBB (GenBank Accession No. M20405), GMTUBB (GenBank Accession No. X60216), PSGTUB1 (GenBank Accession No. X54844), RICBETAT (GenBank Accession No. D13224), SOYSB1TUB (GenBank Accession No. M21296) and ZMB1TUB (GenBank Accession No. X52878). PCR was carried out with 25 cycles of 30 s denaturation at 94°C, 2 min annealing at 55°C and 1 min elongation at 72°C with synthesized mix primer TUB-SP2 (5'-AAGATYMGGGAGGAGGAGTACCC-3') and mix primer TUB-AP1 (5'-TCGCTGACTCKSCGGAACAT-3') designed for the consensus sequence.

Quantitative RNA dot hybridization analysis

Slot dot blotting was carried out with EASY Titer ELISA SYSTEM (Pierce Co., Rockford, IL) as described by Sambrook et al. (Sambrook et al. 1989). After blotting, the membranes were stained with methylene blue and the total blotted RNA amount was measured as optical density (OD) from photographs of the blot using Molecular Analyst (Bio-Rad Labs, Hercules, CA). The total RNA amount was quantified within $3 \mu g$ from photographs of the blot membranes, where the amount of total RNA and OD were shown to be in a linear relationship. A SIXTH1 sense-specific riboprobe was synthesized from a plasmid template which has the coding sequence of the SIXTH1 gene fused to the T7 promoter in an inverted orientation, using a T7 RNA Synthesis Kit (Nippon Gene Co., Ltd., Tokyo, Japan) with $[\alpha^{-32}P]$ -UTP. Hybridization was performed at 42°C in 50% formamide, 6X SSC, 0.1% SDS and 5X Denhardt's reagent overnight. Membranes were washed for 20 min at room temperature in 2X SSC and 0.1% SDS, followed by a wash for 20 min at 65°C in 2X SSC and 0.1% SDS and three washes in 0.1X SSC at the same temperature. SIXTH1 mRNA level was measured as a value of photostimulated luminescence (PSL) from autoradiographs of the blot using BAS2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). It was quantified to within 20 ng from autoradiographs of the blot, where the amount of SIXTH1 mRNA and PSL are in linear relationship, and normalized by the total blotted RNA level. To determine the stage with the highest level of endogenous SIXTH1 gene expression in fruit development, total RNA was extracted from tomato fruits collected at 7, 8, 9, 12, 14, 18 and 43 days after pollination (DAP) and analyzed as described above.

Construction of chimeric genes

To construct pTX301 containing SenseSIXTH1, primer TOM-XSP (5'-TTTTCTAGACCATGGGTATCATAAAAGGAG-3') and primer TOM-SAP (5'-TTGGAGCTCATTTTAAATATCT-CTGTCCTT-3') corresponding to the *SIXTH1* cDNA sequence were used for the amplification of the *SIXTH1* coding sequence. Primers TOM-XSP and TOM-SAP have a linker sequence of *XbaI* (5'-TTTTCTAGA-3') and *SacI* (5'-TTGGAGCTC-3') at the 5'-end, respectively. pTX302 containing AntisenseSIXTH1 was constructed with the use of primer TOM-XAP (5'-TTGT-CTAGAATTTTAAATATCTCTGTCCTT-3') and primer TOM-SSP (5'-TTTGAGCTCCCATGGGTATCATAAAAGGAG-3') for PCR, which has a linker sequence *XbaI* (5'-TTTTCTAGA-3') and *SacI* (5'-TTGGAGCTC-3') at the 5'-end, respectively. Amplified fragments were inserted into the *XbaI-SacI* site of a binary vector pIG121-Hm, replacing the GUS-intron gene to complete pTX301 and pTX302.

Transformation of tomato

Tomato cotyledons were transformed by *Agrobacterium tumefaciens* LBA4404 with plasmid pTX301 or pTX302. Transformed plants were selected by evaluating growth on kanamycin and screened by inducing callus from leaf sections on kanamycin medium (Nagata et al. 1994).

Genomic Southern blot analysis

Genomic DNA was prepared from leaves of the T1 progeny of transformants according to the CTAB method (Rogers and Bendich 1994). *Aft*III-digested DNA (10 μ g) was separated by 0.7% agarose gel electrophoresis, blotted to nylon membrane and hybridized with a *SlXTH1* cDNA probe labeled with [α -³²P]-dCTP using a random prime labeling kit (Takara Bio Inc.).

Analysis of morphology of transgenic fruits

The diameter of tomato fruits harvested as complete red fruits (40–50 DAP) was measured, each fruit being measured three times and with three to nine fruit per line.

Results

XTH gene expression in tomato plants

Figure 1 shows that the *SIXTH1* probe hybridized strongly to mRNA expressed in elongating stems of 5cm-long axillary buds and mature green fruits and that the cDNA hybridized weakly to mRNA expressed in leaves and elongated stems. The level of expression of *SIXTH1* mRNA in mature green fruit was about one-half of that in elongating stem. *SIXTH11* showed a similar expression profile to *SIXTH1* in that both mRNAs were detected in mature green fruit as well as elongating stem. However, the signal intensity of the *SIXTH11* mRNA in mature green fruit relative to that in elongating stem was lower than that of *SIXTH1* mRNA. mRNA of neither *SIXTH1* nor *SIXTH11* was detected in mature red fruits.

The expression profiles of *SlXTH1* and *SlXTH11* transcripts were then assayed in detail throughout fruit development using RT-PCR (Figure 2A). The development of tomato fruit was classified into the following five stages: immature green (IMG), mature green (MG), turning, pink and red. No liquid jelly material exists in any of the locules in IMG. Jelly material exists in all locules of MG, and color change is not visible on the fruit exterior, but is visible (a faint pink) on the inner radial pericarp wall of MG when cut open. Fruits are 10–30% red in "turning", 30–60% red in "pink", and



Figure 1. Northern blot analysis of *SlXTH1* and *SlXTH11* transcripts in different organs. The sources of the RNAs were: red fruits (lane 1), mature-green fruits (lane 2), growing stems (lane 3), non-growing stems (lane 4) and leaves (lane 5). Total RNA from each organ was subjected to electrophoresis and blotted to nitrocellulose membrane. Hybridizations were carried out with double-stranded cDNA probes of *SlXTH1* and *SlXTH11 (note that the SlXTH11 probe cross-hybridises with SlXTH10 and so will detect the sum of SlXTH10 and SlXTH11 transcripts).*



Figure 2. RT-PCR analysis of *SlXTH1*, *SlXTH11* and β -tubulin transcripts throughout fruit development. (A) shows the expression profile analyzed by RT-PCR throughout fruit development for *SlXTH1*, *SlXTH11* and β -tubulin. β -tubulin gene was used as a reference gene to standardize the quantity of template RNA. The sources of the RNAs were: immature-green fruits (IMG, lane 1 and 2), mature-green fruits (MG, lane 3 and 4), turning fruits (lane 5 and 6), pink fruits (lane 7 and 8) and red fruits (lane 9 and 10). (B) shows pictures of fruit in each of the odd number developmental stages at which RNA was prepared for RT-PCR. Upper panel, cross-section; lower panel, intact fruit.

100% red in "red" (Figure 2B). *SIXTH1* mRNA was detected at relatively high levels transiently in IMG and MG, the earliest stages of development, and again in turning fruit, the stage at which the fruit turns pink (Figure 2A). *SIXTH11* mRNA, in contrast, was detected at approximately constant amounts during fruit development (Figure 2A).

Transgenic tomatoes transformed to produce SIXTH1 sense or antisense RNA

To examine whether modified gene expression of SIXTH1 in transgenic plants could confer a change in tomato fruit characteristics, we produced transgenic tomatoes that exhibited either increased or reduced expression of *SlXTH1* compared with that of a wild-type plant. We made two DNA constructs, pTX301 and pTX302, for increased or reduced production of the enzyme through the expression of sense or antisense RNA, respectively (Figure 3). PTX301 has the complete coding sequence of the enzyme linked, in the sense orientation, downstream of the 35S promoter from cauliflower mosaic virus (35S-pro in Figure 3A). pTX302 has the coding sequence linked downstream of 35S-pro in an inverted orientation (Figure 3B). The nopaline synthase 3'-terminator sequence (NOS-ter) was linked downstream to the coding sequence of SIXTH1 in both constructs. The complete DNA construct spanning from the right border to the left border of T-DNA of pTX301 is referred to as SenseSIXTH1, and that of pTX302 is referred to as AntisenseSlXTH1. We introduced each of the two constructs into the genome of tomato (S. lycopericum cv. Shugyoku) using Ti plasmidmediated transformation and kanamycin selection. We obtained 24 lines of transgenic plants in total, which were propagated by cuttings and used for further study. Northern blots of axillary buds from most of the 24 transformed tomatoes showed different levels of SlXTH1 expression when probed with a radiolabeled RNA fragment specific for the sense strand of the SIXTH1 coding sequence (data not shown).

Genomic Southern blot analysis

Two lines from the plants transformed with pTX301 (SenseSIXTH1) and four lines from the plants transformed with pTX302 (AntisenseSIXTH1) were selected for further analysis. Genomic DNA from the transformed tomatoes was digested with the restriction enzyme AffIII and separated by gel electrophoresis. We then probed the Southern blots of the digested leaf DNA with a radiolabeled DNA fragment of the SIXTH1 coding sequence and analysed a 2.7 kb restriction fragment that spanned the region from the kanamycin resistance gene to NOS-ter, which is located at the 3' site of SenseSIXTH1 or AntisenseSIXTH1 (Figures 3A, B). In the lines 301-2 and 301-7 (SenseSIXTH1) and 302-9 and 302-12 (AntisenseSIXTH1), a 2.7 kb band was detected in common in addition to an endogeneous 5.0 kb band, which was detected in the untransformed line (Figure 3D, E). But in the lines of 302-11 and 302-15 (AntisenseSIXTH1), an additional 4.3 kb band was detected with the 5.0 kb band (Figure 3E).



Figure 3. Genomic Southern blot analysis. (A, B) Schematic representation of T-DNAs for tomato transformation. The cDNA of the SIXTH1 transgene was inserted between the 35S-pro and NOS-ter into the vector pIG121-Hm in the same orientation for SenseSIXTH1 as shown in (A), or in reverse orientation for AntisenseSIXTH1 as shown in (B). RB, Right border; LB, left border; Kmr, kanamycin resistant; Hmr, hygromycin resistant. Vertical bars show restriction enzyme AffIII site. The DNA fragment containing the SIXTH1 transgene was about 2.7 kbp long. (C, D) Schematic representation of genomic DNA around the T-DNA integration locus in the transformants 302-11 and 302-15 (C) and that around endogeneous SIXTH1 gene locus (D). The dotted line shows genome DNA and vertical bars show AffIII restriction site. (E) Autoradiogram of genomic Southern blot analysis. Total DNA from untransformed and transformed plants was digested with AffIII. Each sample was subjected to electrophoresis on a 0.7% agarose gel, blotted to nylon membrane and hybridized with a labelled cDNA of the SIXTH1 gene as a probe. Cont., untransformed control plant; 301-2 and 301-7, sense transgenic plants; 302-9, 302-11, 302-12 and 302-15, antisense transgenic plants. The number on the left shows the size of the DNA fragment as estimated from the size markers. The arrow on the right shows the 5.0 kbp band expected for the SIXTH1 endogenous gene.

Quantitative RNA dot hybridization analysis of the sense, antisense and control tomato fruits

To strictly compare the accumulation of *SlXTH1* mRNA between different lines of transgenic plants, we measured the amount of its mRNA at the same developmental time of fruiting, *i.e.* at the point when the amount of the



Figure 4. Quantitative RNA dot hybridization analysis throughout fruit development. RNA was isolated from fruits at 7, 8, 9, 12, 14, 18 and 43 DAP and hybridized with a labelled *SlXTH1* probe. For full details, see Materials and methods. The upper panel shows the image of the result of dot hybridization obtained by BAS2000 (Fuji Film). The graph shows amounts of *SlXTH1* mRNA relative to the amount on the seventh day, which is set as 100.

mRNA was maximum. This is because the expression level of SIXTH1 mRNA changes several-fold during fruit development, peaking at 12 days after pollination (DAP) as shown in Figure 4. The diameter of tomato fruit on this day was about 20 mm in untransformed plants. Total RNA was extracted from sample tomatoes harvested at 12 DAP and quantitative RNA dot hybridization was carried out using stringent conditions with a sense strand-specific probe to differentiate from transgenederived SIXTH1 antisense RNA. Figure 5A shows that the SIXTH1 gene was, relative to the control, 1.3–1.5 times overexpressed in lines 301-2 and 301-7 (SenseSIXTH1 lines), suppressed in 302-11 and 302-15 to the level of about 70% of wild-type (two lines derived from a single AntisenseSIXTH1 transgenic event, see Discussion) and expressed similarly to wild-type in 302-11 and 302-15 (AntisenseSlXTH1 lines).

Fruit size of transgenic tomatoes

We measured the diameter of transgenic tomato fruits to see the effect of alterations in *SlXTH1* mRNA accumulation on phenotype. Figure 5B shows that, in the SenseSlXTH1 lines, the diameter of tomato fruits was slightly higher than control (45.2 ± 5.5 mm in control untransformed fruit, 50.4 ± 3.1 and 49.9 ± 4.5 mm in 301-2 and 301-7 lines, respectively; *P*=0.08 and 0.18, respectively). In the AntisenseSlXTH1 lines, the diameters of 302-9 and 302-12 tomato fruit were significantly lower than control (29.4 ± 5.9 and 30.1 ± 6.5 mm, respectively; *p*<0.001), while those of 302-11 and 302-15 tomato fruit exhibited no significant difference to control (45.5 ± 5.8 and 43.0 ± 3.6 mm, respectively).

We next measured the diameter of fruits of T1 plants derived from SenseSIXTH1 line 301-2, because the mRNA abundance of *SIXTH1* in the 301-2 line was



Figure 5. Alteration in fruit characteristics of transgenic tomatoes. (A) Quantitative RNA dot hybridization analysis of the sense (301-2, 301-7), antisense (302-9, 302-11, 302-12, 302-15) and untransformed control (cont.) tomato fruits. RNA was extracted 12 DAP, at which *SIXTH1* gene expression reached a maximum level as shown in Figure 4. Data are given as the mean (n=4 experiments). Results are indicated as percentages of *SIXTH1* mRNA abundance relative to that of the control untransformed fruit. (B) The size of tomatoes in the sense, antisense and control tomato plants. The size of tomatoes was determined from the fruit diameter, which was measured three times per fruit. Data are the mean±SEM (n=3 to 9 fruit per line). Asterisk indicates significant difference from the control (cont.) by t-test at p < 0.05(*), and 0.001(***).

significantly higher than in an untransformed line (Figure 5A). Figure 6A shows that, in two out of five heterozygous T1 plants $(+/-; 45.6 \pm 3.1 \text{ and } 45.7 \pm 2.2 \text{ mm})$ and one homozygous T1 plants $(+/+; 46.5 \pm 3.8 \text{ mm})$, the diameter of tomato fruits was significantly higher than in the control $(-/-; 33.6 \pm 4.1 \text{ mm})$. The diameter of untransformed tomato fruits was $39.0 \pm 4.6 \text{ mm}$ in this experiment. We examined cell size in pericarp tissue at the stage of immature/mature green fruit of homozygous 301-2-16 T1 plants. A higher number of "large cells" existed in the homozygous lines compared with untransformed control fruit (Figures 6B–E).

Discussion

In the expression analysis of XTH family genes, we hybridized mRNA with a probe under stringent conditions, which generally allows the detection of identical sequences or close to it. Among the 25 tomato



Figure 6. Alteration in fruit characteristics of T1 transgenic tomatoes. (A) The size of tomato fruit in 301-2 T1 plants. The black bar shows kanamycin sensitive T1 plant (301-2-2; -/-) and untransformed plants (from W-1 to W-7). The gray bar shows heterozygous T1 plants (301-2-3, 8, 10, 13, 15; +/-), which segregated in the ratio of 2:1 to 4:1 for kanamycin resistant:sensitive in the T2 generation. The open bar shows the homozygous T1 plant (301-2-16; +/+). T2 plants of the 301-2-16 line were all kanamycin resistant. Asterisks indicates significant difference from 301-2-2 by ttest at P<0.05(*), and 0.001(***). The fruit diameter was measured three times per fruit. Data are the mean \pm SEM (n=4 to 9 fruit per line). (B-E) Cross sections of control untransformed tomato fruits (B, C) and 301-2-16 transformant (D, E) were prepared from the midsection of pericarp at the immature/mature-green stage. The diameter of fruits was 11.9 mm (B), 13.0 mm (C), 11.8 mm (D) and 13.7 mm (E). The scale bar indicates 1 mm.

genes of XTH family, *SlXTH4* (Saladié et al. 2006) is the most homologous with *SlXTH1*, having homology of 82% and the longest sequence with consecutive identity of 25 bases long. It is unlikely that the *SlXTH1* coding sequence, which was used as a probe for the expression analysis of its mRNA, strongly hybridized with other mRNAs under stringent conditions. This is supported by the absence of cross-reacting bands in Southern analysis (Figure 3).

Additional hybridizing fragments (of 2.7 kbp and 4.3 kbp, Figure 3E) present in transformed but not in untransformed tomato DNA shows that at least one copy of the *SlXTH1* transgene was introduced into the genome of each transformed line. A 4.3 kbp fragment (Figure 3E) shows that transgenic lines 302-11 and 302-15 were derived from one transgenic event of AntisenseSlXTH1.

A 5.0 kbp band (Figure 3D, E) was assigned to the SIXTH1 endogenous gene, because it was detected in the nontransgenic line (Figure 3E, lane Cont.). Transgenic 302-11 and 302-15 transformed with line the AntisenseSIXTH1 construct did not suppress the expression of SIXTH1 in spite of the introduction of an antisense SIXTH1 transgene, which would produce antisense RNA. Southern blot analysis (Figure 3E) and PCR analysis (data not shown) of the genomic DNA for the integrated AntisenseSIXTH1 DNA showed that the 3' region of SlXTH1 transgene was missing in the two lines (302-11 and 302-15), unlike in the two other suppression-effective lines (302-9 and 302-12). This may be the cause of the failure of suppression. In a Ti plasmid-mediated transformation of tobacco (Nicotiana tabacum), it was reported that the left border of T-DNA and its flanking T-DNA region were sometimes missing (Ohba et al. 1995). Thus lines 302-11 and 302-15 can be considered as transformation controls, with SlXTH1 mRNA abundances similar to wild-type.

In the transformation of tomato with pTX302 (AntisenseSIXTH1), there were more "multiple shoots", which grew into abnormal forms, than with pBI121 (control) or with the pTX301 (SenseSIXTH1) construct. We were able to induce root formation from the multiple shoots, but *SIXTH1* gene expression was not detected in this abnormal plant. This result suggests that a tomato plant in which *SIXTH1* gene expression is completely suppressed would not grow. If we use a stage-specific promoter for the suppression, the chance of obtaining "normal" transgenic tomatoes with altered fruit characteristics may increase.

In this study we showed a positive correlation between SIXTH1 mRNA abundance and fruit diameter (Figures 5A, 5B, 6A). "Large cells" appeared in a higher ratio in tomato fruits transformed to overproduce SlXTH1 than those of untransformed control tomato fruit (Figure 6B-E). Correspondingly, fruit with suppressed SlXTH1 gene expression grew to a smaller size than controls. These results suggest that alteration of fruit characteristics in transgenic tomatoes was due to the increase or reduction of cell enlargement by the alteration of SIXTH1 gene expression, since during the period before flowering, the cells stop undergoing cell division and all later growth of fruits is associated entirely with cell enlargement. The data thus provide further evidence for the in vivo role of XTH proteins in causing cell wall loosening and hence cell expansion (van Sandt et al. 2007).

Our study is the first *in vivo* demonstration that the level of *SlXTH1* gene expression during fruit development controls fruit size. Our results indicate that the suppression of endogenous *SlXTH1* expression causes an inhibition of the integration of newly synthesized xyloglucans into the wall and therefore of

cell enlargement, leading to decreased fruit expansion. The integration of high molecular weight xyloglucan or small xyloglucan oligosaccharides into the wall by the action of XET has been shown to reduce or promote the growth of pea stem cells, respectively (Takeda et al. 2002). An additional action of XTH proteins during fruit softening can also be inferred, with the activity being involved in the net depolymerization of xyloglucans during wall disassembly. This conclusion is based on our data that SIXTH1 gene expression increased again transiently during the turning stage when tissue firmness declined most rapidly (Figure 2), and it was also reported that the activity of buffer-soluble XG transglycosylation increased when tissue firmness declined most rapidly (Malachlan and Brady 1994). However, recently Miedes et al. (2010) showed that fruit softening, which occurs when fruit ripens, was reduced in transgenic tomatoes overexpressing a SlXTH1 transgene. This result indicates that XET activity might be acting to maintain wall strength during ripening, and that declining XET activity allows softening to occur. 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. Our data, and the higher expression of the endogenous SIXTH1 gene during fruit growth rather than during ripening (Figure 3; Miedes and Lorences 2009) indicate that the primary role of SlXTH1 during fruit development may be in cell expansion in the growing fruit. Further studies need to be done to clarify other roles of XTH in vivo.

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