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RESEARCH PAPER

Petal abscission in rose is associated with the differential expression of two ethylene-responsive xyloglucan endotransglucosylase/hydrolase genes, *RbXTH1,* and *RbXTH2*

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Abstract

Abscission is a process that involves shedding of plant organs from the main plant body. In this study it is shown that the process of petal separation in the fragrant rose, *Rosa bourboniana*, is accompanied by the expression of two xyloglucan endotransglucosylase/hydrolase genes, *RbXTH1,* and *RbXTH2*. The sequences of the two genes show 52% amino acid identity but are conserved at the catalytic site. The genes are up-regulated soon after the initiation of the abscission process and their transcription is associated with the progression of abscission, being faster in ethylene-treated flowers but slower during field abscission. Transcription is ethylene responsive, with the ethylene response being tissue-specific for *RbXTH1* but largely tissue-independent for *RbXTH2*. Expression is correlated with an increase in xyloglucan endotransglucosylase (XET) action in petal abscission zones of both ethylene-treated and field abscising flowers. Proximal promoters of both the genes drive β-glucuronidase expression in an ethylene-responsive and abscission-related manner in agrobacteria-infiltrated rose petals, indicating that *cis*-elements governing ethylene-responsive and abscission-related expression probably lie within the first 700 nucleotides upstream of the translational initiation codon. The results show that cell wall remodelling of the xyloglucan moieties through the XET action of XTHs may be important for cell separation during abscission.

Key words: Abscission zone, ethylene, petal abscission, RbXTH1, RbXTH2, rose, XET, xyloglucan endotransglucosylase/hydrolase.

Introduction

Organ abscission is a complex process wherein organs are shed in response to internal and external cues so as to reduce the burden of maintaining biosynthetic activities in the dispensable organ. In plants, abscission is commonly observed in leaves, petals, stamens, whole flowers, fruits, and fruitlets, particularly in dicotyledonous plants, and is usually responsive to ethylene (van Doorn, 2001). Organ separation is preceded by the action of cell wall hydrolases within the abscission zone—a 2–3 cell layer region that joins the organ to the main plant body. This region is the site of action for developmental changes and responses to stresses that lead to abscission (Taylor and Whitelaw, 2002). Detailed studies on wall hydrolysis in abscission, performed in well-established model systems such as bean, tomato, and *Arabidopsis*, have provided a lot of evidence for the role of polygalacturonases and endoglucanases in the abscission process of leaves and flowers (Tucker *et al.*, 1991; Kalaitzis *et al.*, 1995, 1997; del Campillo and Bennett, 1996; Burns *et al.*, 1998; Lashbrook *et al.*, 1998; Brummel *et al.*, 1999; Gonzalez-Carranza *et al.*, 2002, 2007; Jiang *et al.*, 2008).

However, the complexity of plant cell walls due to the presence of celluloses, hemicelluloses, cross-linked xyloglucans, rhamnogalacturonans, galactomannans, pectins, etc. would suggest that efficient wall disassembly should require the concerted action of not a few but several types of wall-modifying proteins and enzymes with specificity towards the different wall polymers and linkages. Recently, expansins have been shown to aid the process of leaf and petal abscission (Belfield *et al.*, 2005; Sane *et al.*, 2007) while pectate lyases have also been indicated in stamen and petal abscission (Cai and Lashbrook, 2008; Agusti *et al.*, 2008; Singh *et al.*, 2011).

Xyloglucans are the major hemicelluloses of primary cell walls particularly in dicotyledonous plants and non-graminacous monocots, and may account for up to 10–20% of the wall component (Hayashi, 1989; Fry, 1989). They tether cellulose microfibrils by cross-linking them through non-covalent linkages, thus providing strength to the walls during growth. Modification in the length of xyloglucans during cell expansion is brought about by the enzyme xyloglucan endotransglucosylase/hydrolase (XTH) primarily through endotransglycosylation, thus enabling the cell wall to expand without weakening (Smith and Fry, 1991; Fry *et al.*, 1992; Nishitani and Tominaga, 1992). XTHs belong to a multigene family (Xu *et al.*, 1996; Campbell and Braam 1999*a*; Rose *et al.*, 2002; Yokoyama *et al.*, 2004) where members play an important role during cell wall modification in several different processes. These include root hair initiation (Vissenberg *et al.*, 2000, 2001), hypocotyl elongation (Potter and Fry, 1994; Catala *et al.*, 1997, 2001), hydrolysis of seed storage carbohydrate (de Silva *et al.*, 1993), leaf growth and expansion (Schunmann *et al.*, 1997), aerenchyma formation (Saab and Sachs 1996), fruit softening (Schroder *et al.*, 1998; Ishimaru and Kobayashi, 2002; Saladié *et al.*, 2006), and tension wood (Nishikubo *et al.*, 2007, 2011).

In order to further an interest in the process of petal abscission, rose was chosen as a system for study. The genus *Rosa* provides an interesting material for petal abscission since the fragrant varieties of rose are highly sensitive to ethylene and undergo rapid petal abscission, while the hybrids (and non-fragrant varieties) are quite resistant to petal abscission. Given the importance of xyloglucans in providing strength to the cell wall for attachment of organs, it was speculated that XTHs have a role in mediating abscission. Here it is shown that the progression of abscission in rose petals is closely associated with the differential expression of two XTH genes, *RbXTH1* and *RbXTH2*, and an increase in xyloglucan endotransglucosylase (XET) action in abscission zones.

Material and methods

Plant material

Flowers of *Rosa bourboniana* (cv Gruss an Teplitz) and *Rosa hybrida* were chosen for study. Flowers of the same developmental stage were picked prior to sunrise, when only the outer whorl of petals of the bud had opened. This ensured that the flowers were unpollinated and unlikely to have undergone pollination-associated ethylene release. Flowers with a pedicel of ~5–7 cm were cut with a sharp blade and the stalks were immediately placed in water.

Ethylene and 1-MCP treatments

Flowers were kept in water in a closed air-tight chamber, and ethylene at a concentration of 0.5 µl l^{-1} was injected into the chamber. Ethylene treatment was carried out for ~18 h for *R. bourboniana* (time of abscission 16–18 h) and ~52 h for *R. hybrida* (time of abscission 50–52 h). Petal abscission zones (~2 mm^2 at the base of the petal in contact with the thalamus) were collected at 0 (ethylene untreated), 4, 8, and 12 h for *R. bourboniana*, as well as at 24, 36, and 48 h for *R. hybrida* as described (Sane *et al.*, 2007). For high ethylene dose treatment, flowers of *R. bourboniana* were treated with 15 µl l^{-1} ethylene for 3 h and abscission zones collected at 30, 60, and 120 min. For studies on natural abscission (time of abscission 38–45 h), flowers of *R. bourboniana* were marked at the time of opening of the outermost whorl and petal abscission zones were collected from these flowers without ethylene treatment at time intervals of 4, 8, 12, 24, and 36 h.

For 1-methylcyclopropene (1-MCP) treatment, flowers were kept in the chamber and treated with 0.5 µl l^{-1} 1-MCP (Ethyl Bloc from Biotechnologies for Horticulture Inc., Walterboro, SC, USA) for 12 h.

Isolation of RNA

RNA was isolated from frozen petal abscission zones of *R. bourboniana* and *R. hybrida* as described by Asif *et al.* (2000). RNA was also isolated from different tissues such as petals, sepals, stamens, carpels, thalamus, pedicels, fruits, leaves, and stem before ethylene treatment (all samples) and after 0.5 µl l^{-1} ethylene treatment for 12 h (except fruit, stem, and leaf).

Isolation of XTH genes

DNA-free RNA from 8 h ethylene-treated abscission zone samples was reverse transcribed using the reverse transcriptase Superscript II from Invitrogen (Palo Alto, CA, USA) and primed with the 3′ RACE (rapid amplification of cDNA ends) adaptor primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′). For isolation of XTH homologues from rose, two primers, namely XTH-F 5′-GTATGCAGATAAAGATGGT TCCTGG3-′ and XTH-R 5′-ACKAGTKGCCCAGTCRTCYGCRTTCC-3′, were designed based on an alignment of XTH sequences from different plants. Amplification using these primers gave a PCR fragment of 343 nucleotides (nt). Based on the sequence of this fragment, primers RXF2 5′-CACGATGAGATCGACTTCGAGTT-3′, RXR2 5′-GTGGCCCARTCGTCGGCGTTCCA-3′ and RXR3 5′-GCTGGAGTARATCYTCATGGGTTGG-3′ were designed. 3′ RACE was performed with RXF2 and the 3′AP primer to obtain a fragment of 797 nt for

RbXTH1. For 5′ RACE, cDNA was prepared using the XTHR1 primer and the 5′ RACE adaptor primer (5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′) as described in the manufacturer's protocol (Invitrogen). 5′ RACE was performed using this cDNA and the RXR2 and RXR3 primers sequentially along with the 5′AP primer to obtain a fragment of 656 nt for *RbXTH1*. The amplified fragment was cloned and sequenced on an automated DNA sequencer (ABI 373A from Perkin Elmer). Based on these sequences, the complete open reading frame (ORF) of *RbXTH1* was amplified using the sequences RbXET1-BOF 5′-GCA CAG GAT CCA ATG GCT TCT TAC AAG-3′ and RbXET1-BOR 5′-GCC TCT TCT GGA TCC TCA TCC TCT TAA-3′ to obtain a product that contained the 885 nucleotide ORF. *RbXTH2* was obtained as an artefact during the amplification of the polygalacturonase gene using the primer RPG 5′-TTGGAAGTCTAGGCAAGGACCAGC-3′ with the 3′AP primer to obtain a fragment of 584 nt. Based on the partial sequence, gene-specific primers RNXR1 5′-ATC TAT ATT CAA GGG CAT TGA GTG C-3′ and RNXR2 5′-GTG CCT TCC CAC CAG TTA TTA GTG C-3′ were designed. 5′ RACE was performed using the SMART cDNA kit (Clontech Laboratories Inc., Palo Alto, CA, USA) using these primers in combination with the 5′SMART primer (5′-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GGG-3′) and the rose 8 h abscission zone SMART library to obtain a fragment of 859 nt. The complete 864 nucleotide ORF of *RbXTH2* was amplified using the primers RbXET2-BOF 5′-CAT GTA GGA TCC TAT GAG GAG TAG T 3′ and RbXET2-BOR 5′ ACT TGG ATC CTC ACG CCA AAC GTA CG-3′.

Northern blot analysis

Total RNA (30 μg) was resolved on a 1.2% denaturing formaldehyde–agarose gel using the method of Sambrook *et al.* (1989) and modified as given in the Qiagen Oligotex handbook (2002). RNA was transferred to nylon membranes (Hybond N, Amersham-Pharmacia Biotech, Uppsala, Sweden) by vacuum transfer using a vacugene apparatus (Pharmacia) and UV cross-linked. Radiolabelling of probes for northern blots was performed using [α-^{32}P]dCTP. Hybridization and washing of blots were performed as described (Sambrook *et al.*, 1989). Signals obtained on the blots were visualized on an X-ray film (Fujifilm SuperRX) or quantified on a phosphorimager (Molecular imager FX, BioRad) using the software QuantityOne-4.2.3 version.

Real-time PCR

For transcript analysis, real-time PCR was carried out using cDNA from the different petal abscission zone stages of *R. bourboniana* as well as *R. hybrida*. The reactions were run using the Power SyBr Green PCR master mix (Applied Biosystems Inc.) on an ABI Prism 7000 real-time PCR machine (Applied Biosystems Inc.). RNA was isolated from pooled abscission zone samples collected from several flowers at the specific stages mentioned above over a period of 1 month for cDNA preparation. Reactions were carried out using the primers RbXET1BOF/RX1PR1 and RbXET2BOF/XET2PR1 (primers given below). Rose β-actin, amplified using the primers RactF1 (5′-ATGAC-ATGGAGAAGATCTGGCATCA-3′) and RactR1 (5′-AGCCTGGATGGCAACATACATAGC-3′), was used as an internal control. Reactions were run in triplicate with cycling conditions as follows: 50 °C, 2 min, 95 °C 10 min followed by 40 cycles of 95 °C 15 s and 60 °C 1 min. Values were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. For calculating relative change in expression, the 0 h sample data were averaged and considered as 1, and averaged values of all other samples were plotted against the 0 h sample.

Isolation and analysis of the RbXTH1 *and* RbXTH2 *promoters*

To obtain the proximal promoters of *RbXTH1* and *RbXTH2*, a genome walker library of rose genomic DNA was prepared using the genome walker kit (Clontech). For *RbXTH1*pro, reverse primers RX1PR1 (5′-GGA GCT GCC ATT GTT GCA GAG ACC AT-3′) and RX1PR2 (5′-GTA AGC TCA AGA GCA GAG TCC ATTG C-3′) were used in combination with the genome walking adaptor primers GWAP1 (5′-GTA ATA CGA CTC ACT ATA GGG C-3′) and GWAP2 (5′-ACT ATA GGG CAC GCG TGG T-3′) to obtain a fragment of 787 nt. This fragment was used as template in combination with the GWAP2 primer and the primer Xet1ProRBam (5′-CTT GTA GGA TCC CAT TTT TCT CTG C-3′) to obtain a fragment of 764 nt. To obtain the proximal promoter of *RbXTH2*, the primers XET2PR1 (5′-AAAAGTGGCTGGTCGTGC-AAAGGCTGAG-3′) and XET2PR2 (5′-AAC ACG GAT CCT CAT AGA GGG ATA CAT G-′) were used in combination with the GWAP1 and GWAP2 primers to obtain a fragment of 743 nt. Both the fragments containing the initiation codon and the region upstream of it were cloned first in pTZ57R/T (Fermentas) and then introduced at the *Bam*HI site of a modified pBI101 (pBI101.1 for *RbXTH1* and pBI101.2 for *RbXTH2*) in translational fusion with an intron-containing β-glucuronidase (GUS) gene introduced from pCAMBIA1301. The plasmid was introduced into the *Agrobacterium* strain GV3101.

Promoter analysis of the RbXTH1 *and* RbXTH2 *promoters through agroinjection in rose buds*

Promoter analysis was carried out as described by Tripathi *et al.* (2009). Briefly, recombinant agrobacteria containing the proximal promoters of *RbXTH1* and *RbXTH2* were grown in Luria broth, harvested, and the pellet suspended in LB to an OD of 1. Acetosyringone (0.1 mg ml^{-1}) was added to the suspension and young buds were injected with 0.5 ml of suspension through a 2 ml syringe in the centre of the petals with a needle (size 23, dimensions 0.63×25 mm). The agrobacterial suspension was allowed to infiltrate all

through the petal up to the point of attachment of the petal with the thalamus. Agroinjection was carried out on three flowers per construct and three petals per flower. After wiping off the excess suspension, the buds were kept for 2 d on the plant and then excised and treated with ethylene for 8 h in a closed chamber. Following ethylene treatment, the petals were detached and stained for GUS expression for 16 h at 37 °C as described by Gattolin *et al.* (2006), and destained in 70% ethanol. Light microscopy was performed on a Leica Wild M3Z microscope (Leica, Germany).

In situ co-localization of XET action

Detection of XET action in abscission zone cells was carried out as described by Vissenberg *et al.* (2000). Sections of 0 h (ethylene untreated), 8 h ethylene-treated, and 24 h field abscising petal abscission zones of *R. bourboniana* flowers were cut from the petal to the thalamus through the abscission zone with a sharp stainless steel blade and immediately placed in distilled water. These fine sections were incubated with the substrate xyloglucan oligosaccharides conjugated with sulphorhodamine (XGO-SRs; a kind gift from Professor Stephen Fry, UK, and Dr Harry Brumer, Sweden) at a concentration of 6.5 μM XGO-SRs in 25 mM MES buffer (pH 5.5) for 1 h at 37 °C in the dark. After 1 h of incubation, tissues were washed three times with ethanol:formic acid:water (15:1:4) for 10 min to remove unreacted XGO-SRs. After washing, tissues were kept in 5% formic acid (Merck) for 12 h to remove non-wall-bound XGO-SRs and then washed with distilled water and mounted on a glass slide for microscopy. Sections were observed under a LSM510 META confocal microscope (Zeiss) using a helium ion laser at 543 nm excitation light. To confirm that the formation of the fluorescent product was the result of XET action rather than a physical artefact or the adsorption of the substrate, assays were also performed on heat-inactivated tissues (tissue heated at 80 °C for 10 min).

Results

Isolation of two abscission-related XTH genes

Amplification of the XTH gene fragments was carried out using cDNA prepared from 8 h ethylene-treated petal abscission zones as template. *RbXTH1* was isolated using degenerate primers based on an alignment of several XTH sequences, while *RbXTH*2 was obtained as an artefact while performing 3′ RACE using a rose polygalacturonase-specific primer. Full-length sequences of both genes were obtained by extension (5′ and 3′ RACE) with gene-specific primers. The genes differed slightly in size, with *RbXTH1* encoding a putative protein of 294 amino acids and *RbXTH2* encoding a putative protein with 287 amino acids. A difference of almost 200 nt could be seen in the transcript sizes of the two XTH genes, with *RbXTH*2 having longer 5'- and 3'-untranslated regions (UTRs) of 89 nt and 366 nt, respectively as compared with 41 nt and 188 nt for *RbXTH1*. The 5'-UTR of *RbXTH1* was interrupted by an intron of 99 nt lying 6 nt upstream of the ATG start codon. The complete cDNA sequence of *RbXTH1* consisted of 1114 nt (accession no. DQ279095) while that of *RbXTH2* had 1319 nt (accession no. DQ320658). The predicted polypeptide sequences revealed that the proposed catalytic site for cellulose binding and XTH action DEIDFEFLG as well as sites for *N*-glycosylation immediately after the active site were conserved in RbXTH1 and RbXTH2. Four conserved cysteine residues towards the C-terminal end were also present. In addition, both RbXTH1 and RbXTH2 showed the presence of secretory signal peptides of 22 and 23 amino acids, respectively, as analysed by the TargetP software (http://www.cbs.dtu.dk/services/TargetP; Emanuelsson *et al.*, 2000).

BLAST analysis of the two XTH polypeptides revealed similarity to other XTH sequences from plants that are expressed during cell elongation processes as well as those that are expressed during fruit ripening. The two genes showed 52% amino acid identity and 70% similarity to each other. An alignment of the two sequences was carried out with other transcribed XTH sequences that have been identified recently from microarray studies in abscission zones of *Arabidopsis* and tomato, together with those of TmNXG1 (which shows endoxyloglucanase activity) and PttXET16A (that shows endotransglucosylase activity). The analysis revealed strong similarity of the two XTHs with the *Arabidopsis* sequences, but they differed from the TmNXG1 sequence particularly regarding the absence of the DYNII motif that has been associated with XEH activity (Fig. 1).

Transcription of RbXTH1 *and* RbXTH2 *is ethylene induced and abscission related*

In order to study the expression patterns of the genes during the course of abscission in *R. bourboniana*, northern blots containing RNA from petal abscission zones 0 h (ethylene untreated), 4, 8, and 12 h after ethylene treatment were probed with the two genes. As shown in Fig. 2, transcripts of both *RbXTH1* and *RbXTH2* showed a prominent ethylene-induced and abscission-related accumulation, with a peak of transcript accumulation at 8 h after ethylene treatment. *RbXTH2* showed a slight delay in transcript accumulation, with lower transcript levels at the 4 h stage as compared with *RbXTH1*. Treatment with 1-MCP, an ethylene perception inhibitor that delays petal abscission, greatly reduced expression of both *RbXTH1* and *RbXTH2*, indicating repression by 1-MCP.

Ethylene induction is tissue-specific for RbXTH1 *but tissue-independent for* RbXTH2

In order to test if the expression of both the genes was specific to abscission zones, RNA from different floral organ tissues such as sepals, carpels, stamens, petals, thalamus, and pedicels, as well as from other tissues such as fruit, leaves, and stem was probed with the two genes. Expression was tested in both ethylene-untreated (all tissues) and 12 h

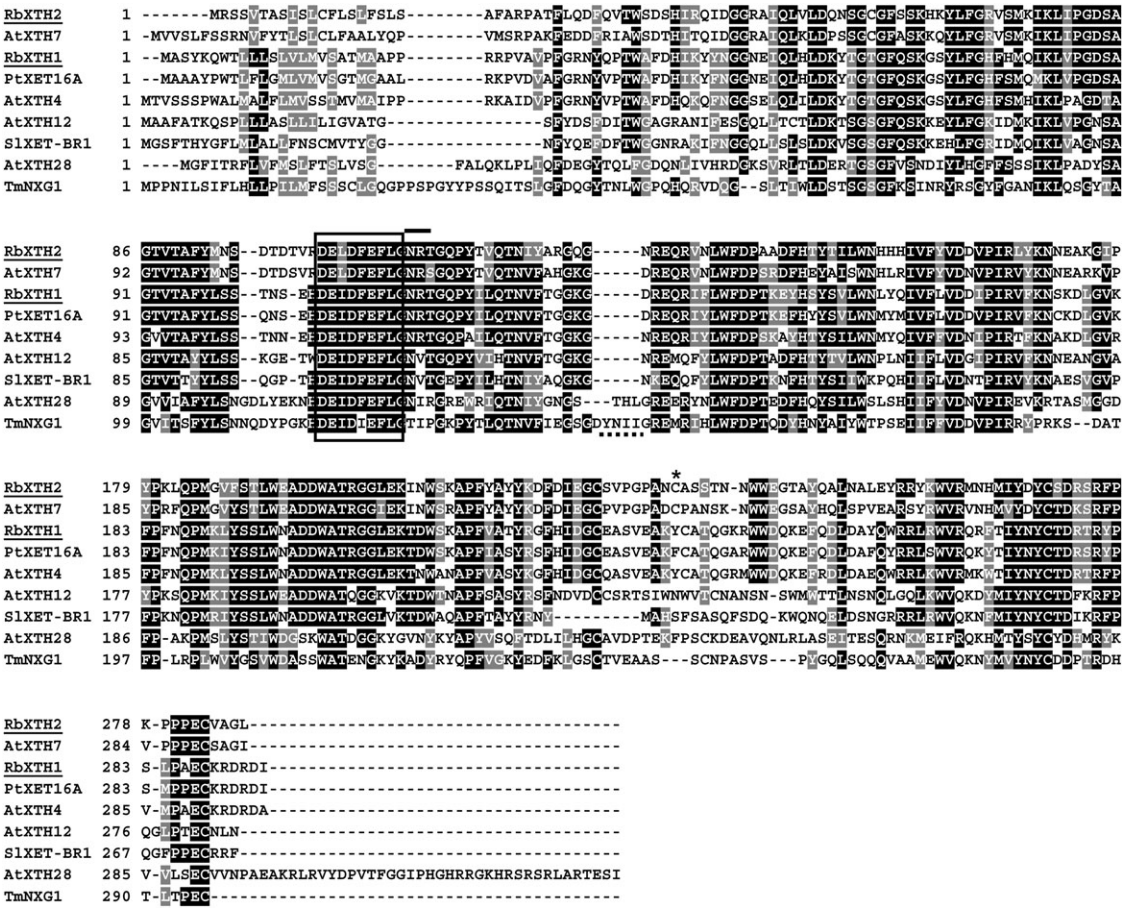

Fig. 1. Alignment of the amino acid sequences of RbXTH1 and RbXTH2 with putative abscission-related XTH polypeptides. The catalytic domain has been boxed while the NXT site for glycosylation has been overlined. The amino acid extension in loop2 of the TmNXG1 sequence that is associated with XEH activity (Baumann *et al.*, 2007) is underlined with dotted lines, while the second of the four conserved cysteine residues towards the C-terminal end has been marked by an asterisk. Full-length protein sequences were aligned using CLUSTALW and the conserved amino acids shaded using Boxshade. Sequences of *Populus* XET16A and nasturtium (*Tropaeolum majus*) TmNXG1 were used as references for XET and endoxyloglucanase activities respectively.

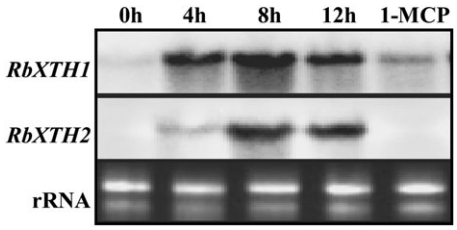

Fig. 2. Time course study of the transcript accumulation of *RbXTH1* and *RbXTH2* in petal abscission zones of ethylene-treated rose flowers on a northern blot. Blots were prepared with 30 μg of total RNA. Numbers indicate the time in hours for which flowers were exposed to 0.5 μl l^{-1} ethylene. M, flowers treated with 1 μl l^{-1} 1-MCP for 12 h.

ethylene-treated (sepal, petal, stamen, carpel, thalamus, and pedicel) tissues. As shown in Fig. 3A, the expression of *RbXTH1* was restricted to petals under ethylene-untreated conditions. Treatment with ethylene enhanced the expression of *RbXTH1* in petals and also induced expression in anthers to some extent. Transcript accumulation of *RbXTH2* could be seen in petals as well as in pedicels and to a lesser extent in sepals under ethylene-untreated conditions. Interestingly, treatment with ethylene greatly enhanced expression of the gene in many tissues such as sepals, petals, anthers, and thalamus. The results indicated the presence of strong ethylene-responsive elements in the promoters of the two genes that functioned in a tissue-specific manner in *RbXTH1* but were largely floral tissue-independent in *RbXTH2*. In order to determine the difference in relative transcript levels of *RbXTH1* and *RbXTH2* in abscission zones, petals, and thalamus, transcript levels were quantified using real-time PCR on cDNA from these tissues (both ethylene treated and untreated). As shown, transcript levels in abscission zones for both the genes were much higher than in ethylene-treated petals and thalamus.

Transcriptional increase of RbXTH1 *and* RbXTH2 *shows a temporal delay during field abscission in* R. bourboniana

The expression profiles of the two genes were also studied during the progression of natural abscission in *R. bourboniana*.

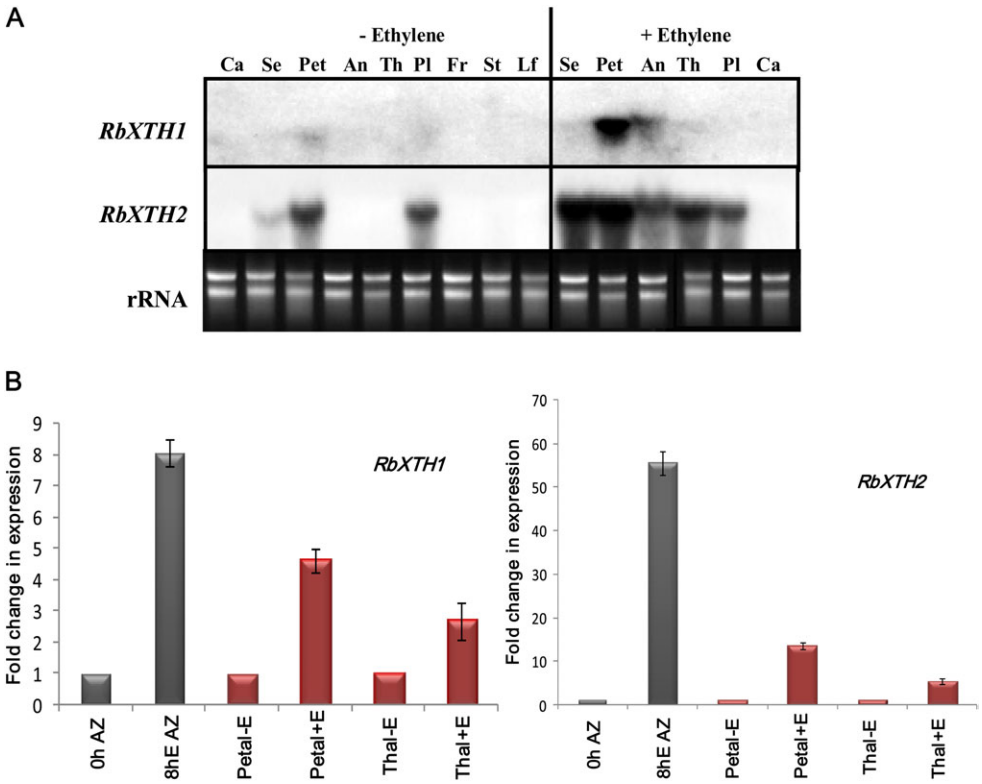

Fig. 3. (A) Transcript accumulation of *RbXTH1* and *RbXTH2* in different ethylene-untreated and treated tissues by northern blot. Ethylene treatment was performed for 12 h only for floral tissues. Ca, carpel; Se, sepal; Pet, petal; Ant, anther; Th, thalamus; Fr, fruit; Pl, pedicel; St, stem; Lf, leaf. (B) Comparative real-time PCR analysis of transcript accumulation of *RbXTH1* and *RbXTH2* in petal abscission zones, petals, and thalamus of *R. bourboniana* flowers before and after 8 h ethylene treatment. RNA was isolated from floral tissues (petals, petal abscission zone, and thalamus) that were excised prior to ethylene treatment from 0 h samples, as well from samples treated with ethylene for 8 h. Reactions were run in triplicate and the averaged values of the 0 h sample considered as 1. Averaged values of other samples were calculated against the 0 h sample. The rose β-actin gene was used as an internal control for normalization.

The process of abscission in the field takes a longer time than ethylene-induced abscission and is completed in 40–45 h after bud opening. Real-time analysis of the expression of the two genes using rose actin as control showed a gradual increase in transcription with progression of abscission. However, in comparison with ethylene-treated samples, there was a temporal delay in transcript accumulation of the two genes, with peak expression occurring at 24 h (Fig. 4).

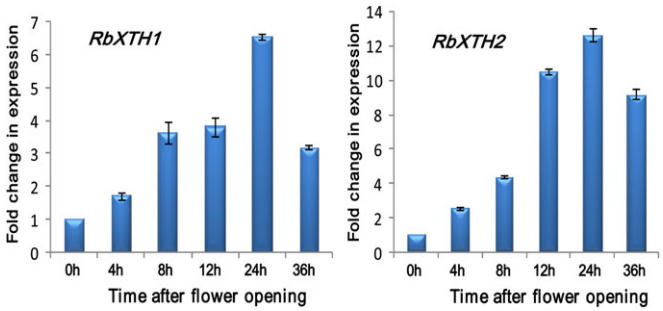

Fig. 4. Real-time PCR analysis of transcript accumulation of *RbXTH1* and *RbXTH2* in petal abscission zones of rose flowers under conditions of natural abscission. Numbers indicate the time (in hours) at which each sample was harvested. Analysis was carried out as described in Fig. 3B.

Transcript accumulation of RbXTH1 *and* RbXTH2 *is delayed and reduced in* R. hybrida

The expression of the two genes was also tested in abscission zones of ethylene-treated flowers of *R. hybrida* (Fig. 5A, B). These flowers do not normally undergo abscission in the field but senesce instead. However, prolonged treatment of excised flowers with exogenous ethylene does induce petal abscission except that it takes three times as long for abscission (50–52 h) as in *R. bourboniana* (16–18 h). When abscission zone cDNA from the hybrid rose was tested for transcript accumulation of *RbXTH1*, expression was found to be low during the early stages of abscission. Transcript levels increased at 36 h and 48 h, but were much lower (almost a third) than levels seen in *R. bourboniana*. Transcript levels of *RbXTH2* were not detectable during the early stages of abscission and could only be seen late, albeit at low levels, at 24 h and 48 h.

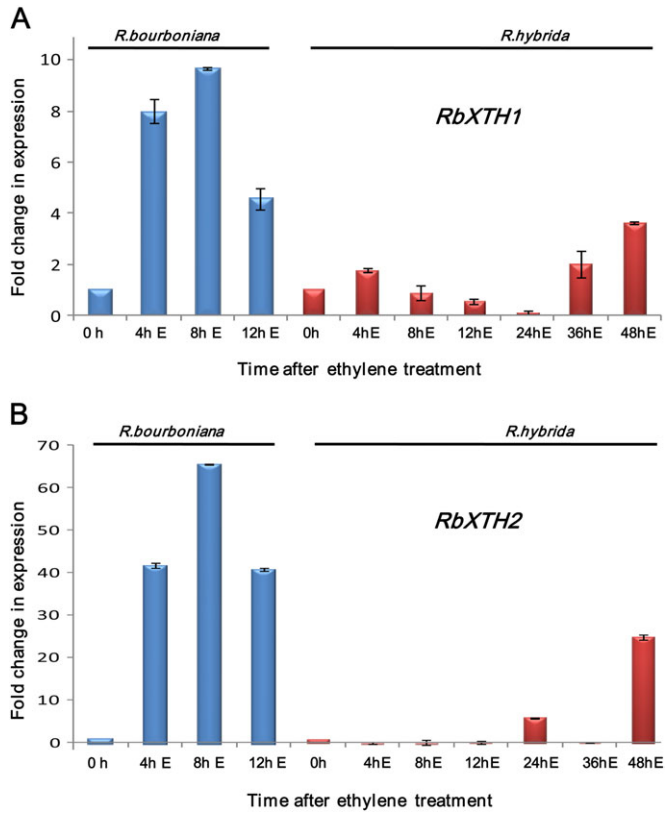

Fig. 5. Real time PCR analysis of transcript accumulation of RbXTH1 (A) and RbXTH2 (B) in petal abscission zones of ethylene treated flowers of *R. hybrida*. Petal abscission zone RNA from *R. bourboniana* was used for comparison. Numbers indicate the time (in hours) at which each sample was harvested. Analysis was carried out as described in Fig.3B

Transcription of RbXTH1 *and* RbXTH2 *is accelerated upon high ethylene dose treatment*

In rose, as in many other flowers, the speed of abscission is governed by the dose of ethylene. Unlike 0.5 µl l^{-1} ethylene, which brings about abscission in 16–18 h, treatment with 15 µl l^{-1} ethylene accelerates the process of abscission, causing petal fall within 3 h of ethylene treatment (Singh *et al.*, 2011). Experiments were carried out to test whether the rapid abscission in response to high ethylene dose was associated with the early expression of the two XTH genes. As shown in Fig. 6, the transcript levels of both *RbXTH1* and *RbXTH2* began increasing within 30 min of ethylene exposure. By 60 min, transcript levels of both *RbXTH1* and *RbXTH2* had increased by 5- to 6-fold. This indicated that the expression of the two genes was closely associated with the progression of abscission regardless of the duration required for its completion. Surprisingly, there was a decrease in *RbXTH2* transcripts at 2 h.

Petal abscission is associated with an abscission-specific increase in XET action

Having observed high transcript levels of both *RbXTH1* and *RbXTH2* during abscission (both ethylene induced and natural), tests were carried out to determine if abscission was associated with an increase in XET action in the abscission zones. The XET assay was performed using sulphorhodamine-labelled conjugates of xyloglucan oligosaccahrides as described (Vissenberg *et al.*, 2000). Sections of 0 h (ethylene untreated), 8 h ethylene-treated, and 24 h naturally abscising abscission zones of *R. bourboniana* were incubated with the sulphorhodamine-labelled XET substrate for 1 h, washed, and photographed under a confocal microscope. Heat-inactivated samples of the abscission zones were used as controls. As shown in Fig. 7, no detectable activity was observed in the 0 h abscission zone sections. In contrast, samples from flowers treated with 0.5 µl l^{-1} ethylene for 8 h showed a strong orange fluorescence at the junction of the petal and the thalamus. This fluorescence was not observed (except for some non-specific binding of XGO-SRs at the edges of the petals) when petals were heat treated prior to the incubation. A similar increase in XET action was also observed in 24 h natural abscission zone samples. The increase was much higher during natural abscission than in the 8 h ethylene-treated samples and spread out into the thalamus. Heat inactivation led to loss of activity in this region, indicating that the action was enzymatic in nature. A close-up of the area (shown in the inset of Fig. 7) revealed that the orange fluorescence was localized at the periphery of the cells (seen clearly in the larger cells), indicating that it was associated with the cell walls. The results collectively showed that the progression of abscission was associated with rearrangements in cell wall xyloglucans that could be a feature of the abscission process.

The proximal promoters of RbXTH1 *and* RbXTH2 *drive expression in an ethylene-inducible and abscission-specific manner*

Since the activation of both *RbXTH1* and *RbXTH2* occurred in a differentially ethylene-responsive and abscission-related manner, the proximal promoters of the two genes were isolated for further study. A 713 nt region upstream of the initiation codon, containing the proximal promoter of *RbXTH1*, and a 728 nt region upstream of the initiation codon, containing the proximal promoter of *RbXTH2*, were isolated by genome walking. These promoters were fused with an intron-containing GUS gene in the pBI101 background and introduced into the *Agrobacterium* strain GV3101. Flower petals in the bud stage were agroinjected with these constructs as described, excised after 2 d, and tested for GUS expression in the presence and absence of ethylene (Fig. 8). As shown, agroinjection of the rose petals with the *RbXTH1pro-GUS* construct led to negligible GUS expression in the petal abscission zones. However, treatment of flowers with ethylene for 8 h prior to GUS assay led to a specific GUS increase in the base of the petals at the point of separation of the petal from the thalamus. Similar experiments performed with the *RbXTH2pro-GUS* fusion led to visualization of the GUS expression in the base of the petals in both the presence and

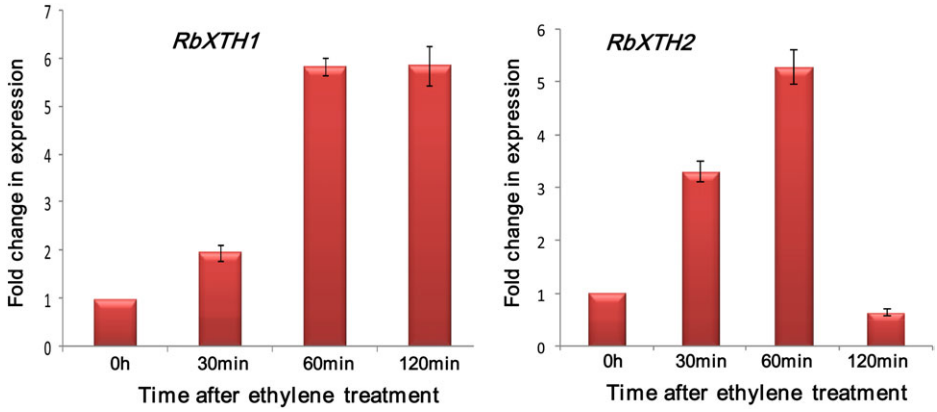

Fig. 6 Real-time PCR analysis of transcript accumulation of *RbXTH1* and *RbXTH2* in petal abscission zones after 15 µl l^{-1} ethylene treatment of *R. bourboniana* flowers. Numbers indicate the time (in minutes) at which each sample was harvested. Analysis was carried out as described in Fig. 3B.

absence of ethylene. However, in absence of ethylene, the GUS expression was localized only to a small portion of the abscission zone at one side. Treatment with ethylene increased GUS expression in the base all over the rim and to some extent adjacent to the rim in the 'neck'. In some flowers, expression in the petal was also observed in the presence of ethylene (data not shown). These results indicated that *cis*-elements in the proximal promoters of *RbXTH1* and *RbXTH2* were able to drive expression in an ethylene-responsive and abscission-related manner.

Discussion

Attachment of plant organs to the main plant body through the abscission zone requires strong cell–cell adhesion and close interactions between the wall components and middle lamella that include cellulose, hemicelluloses, pectins, etc. Since xyloglucans have been speculated to be the major load-bearing molecules in dicotyledonous plants, where abscission is most commonly observed, disruption of the xyloglucan chains by hydrolysis or through extensive endotransglucosylation through the action of XTHs could be a major factor in aiding abscission. Preliminary evidence for their involvement has come through recent microarray studies in soybean, *Arabidopsis*, citrus, and tomato (Tucker *et al.*, 2007; Cai and Lashbrook, 2008; Lashbrook and Cai, 2008; Agusti *et al.*, 2008, 2009; Meir *et al.*, 2010). In soybean, two genes, *XET1* and *XET2*, were shown to be expressed in leaf abscission zones and this expression was ethylene responsive, as seen in the present studies (Tucker *et al.*, 2007). In *Arabidopsis*, stamen abscission was associated with expression of several XTHs such as *AtXTH4*, *AtXTH6*, *AtXTH7*, *AtXTH12*, *AtXTH19*, and *AtXTH28* at different stages of abscission (Lashbrook and Cai, 2008). In citrus, ethylene treatment of laminar abscission zones resulted in expression of three XTHs, *CitXTH1*, *CitXTH2*, and *CitXTH3*, within 24 h (Agusti *et al.*, 2008, 2009), while in tomato a single XTH, *XET-BR1*, was up-regulated in pedicel abscission zones (Meir *et al.*, 2010). In banana finger drop, a form of abscission more related to the fruit ripening process, at least three XTHs, namely *MaXTH6*, *MaXTH8*, and *MaXTH9*, were found to be transcribed in the drop zone (Mbeguie-e-Mbeguie *et al.*, 2009). Two XTH genes, *RbXTH1* and *RbXTH2*, were isolated from the fragrant abscising rose, *R. bourboniana*, and it was shown that petal abscission in this rose is associated with the rapid abscission-related expression of the two genes. Although the two genes showed only 52% amino acid identity to each other, they were highly conserved with other reported XTH genes that are known to play a role in cell extension processes as well as in non-extensive processes such as fruit ripening. Amino acid residues that are critical for catalysis, such as the DEI/LDFEFLG motif, and others for *N*-glycosylation, etc. (Campbell and Braam, 1998) were conserved in both proteins.

The expression of both the genes was strongly governed by abscission cues and was correlated to the speed of abscission. Thus, up-regulation was seen within 4 h in abscission induced with low ethylene (0.5 µl l^{-1}; time of abscission 16–18 h) treatment but was accelerated within 30 min when abscission was triggered by a high ethylene dose (15 µl l^{-1}, time of abscission 3 h). In field abscising flowers, where progression of abscission is slower than in ethylene-treated flowers and under developmental control, peak transcript levels accumulated much later at 24 h. Expression of both *RbXTH1* and *RbXTH2* was also strongly ethylene responsive and repressed upon treatment with 1-MCP, an ethylene perception inhibitor. An interesting feature about the activation by ethylene was the differential tissue-specific response. Apart from abscission zones, activation of *RbXTH1* by ethylene was restricted only to petals and partly to anthers, while that of *RbXTH2* was largely tissue-independent, with transcription being observed in many other tissues such as sepals, anthers, thalamus, and pedicels post-ethylene treatment. Indeed, proximal promoters of both the genes (713 nt in *RbXTH1* and 728 nt in *RbXTH2*) conferred ethylene-responsive and abscission-specific expression in agroinjected rose petals similar to that observed previously for the *RbCP1* promoter

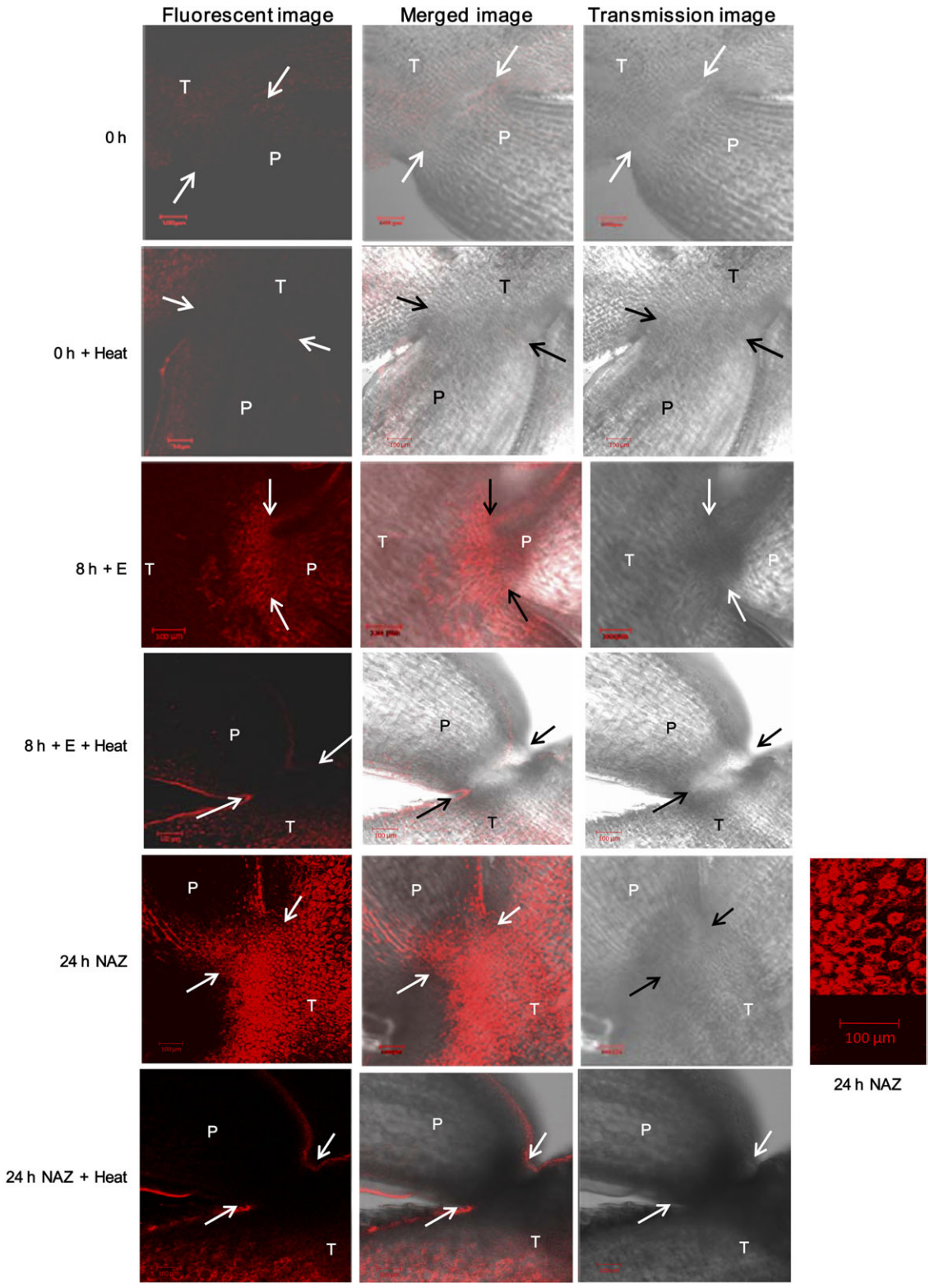

Fig. 7. *In situ* co-localization of XET action in petal abscission zones of *R. bourboniana* using sulphorhodamine conjugates of xyloglucan oligosaccharides (XGO-SRs). Thin sections of petal abscission zones were incubated with XGO-SRs for 1 h as described in the Materials and methods, washed, and sections observed under a confocal microscope at an excitation of 543 nm. Petal abscission samples of 0 h, 8 h ethylene-treated, and 24 h naturally abscising flowers (NAZ) were chosen for study. Heat-treated samples were chosen to demonstrate that the action was enzymatic in nature. The arrows mark the position of the abscission zone where the petal joins the thalamus, while the positions of the petal and thalamus have been marked with the letters 'P' and 'T', respectively. The inset shows localization of the XET action at the periphery of the cells in the 24 h NAZ samples.

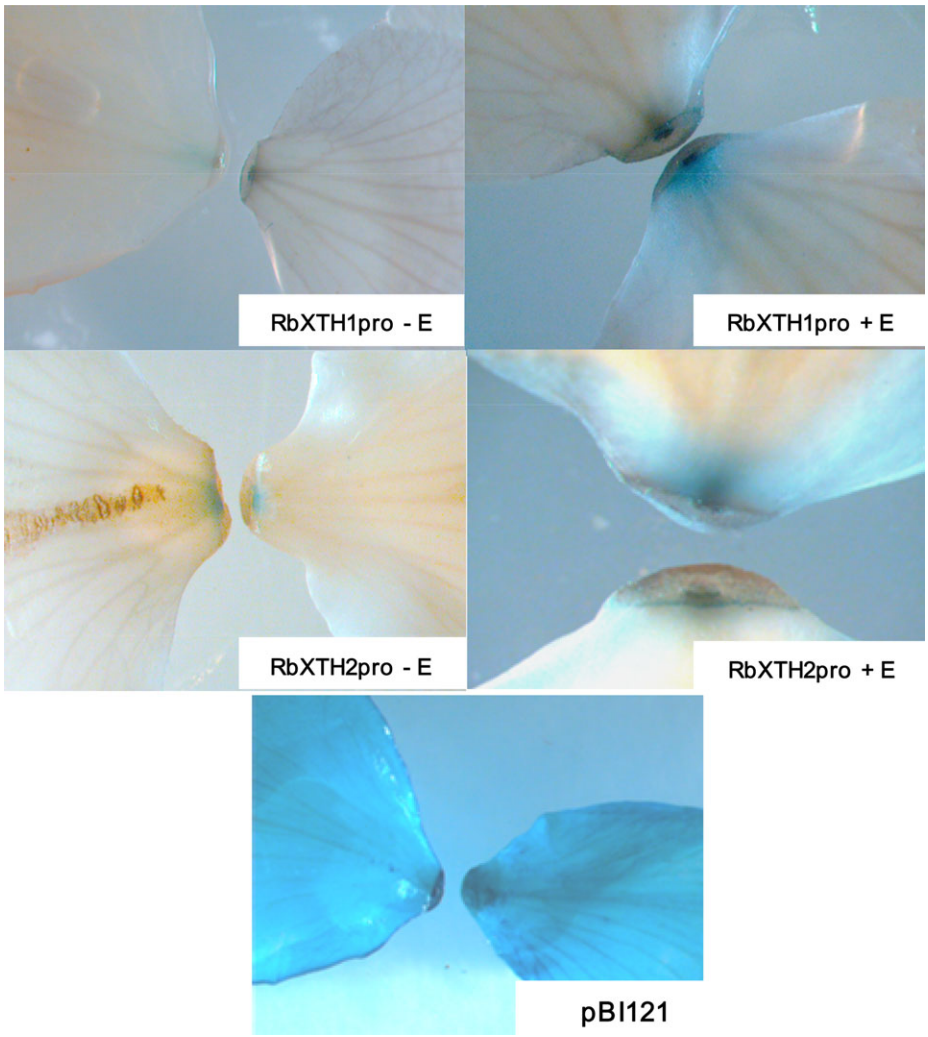

Fig. 8. Histochemical GUS staining to test the function of the *RbXTH1* and *RbXTH2* promoters using a translational promoter–GUS fusion construct. Petals in intact buds were infiltrated at the centre of the petal with agrobacteria containing different constructs (three flowers per construct and three petals per flower) using a syringe. Buds were kept for 2 d on the plant and then cut under water as described and used for GUS visualization in the absence of ethylene treatment (–E) or after ethylene treatment for 8 h (+E). GUS expression in the base of the petals (at the point of separation from the thalamus) is shown. Petals infiltrated with pBI121 (GUS driven by the constitutive CaMV35S promoter) are shown as a positive control.

(Tripathi *et al.*, 2009). Analysis of the promoter of *RbXTH1* revealed the presence of the *cis*-element ATTTCAAA that has been shown to be present in the tomato ethylene-responsive E4 gene, the carnation ethylene-responsive GST1 gene, and the rose cysteine protease promoter (Montgomery *et al.*, 1993; Itzhaki *et al.*, 1994; Tripathi *et al.*, 2009). Surprisingly, *RbXTH2*, which was strongly ethylene responsive, did not contain any known ethylene-responsive elements, although sequences related to ATTTCAAA with one to a few changes in the first 4 nt were present (data not shown). This indicates that the ethylene-responsive expression in *RbXTH2* might be conferred by *cis*-elements other than the GCC box and the ATTTCAAA elements or by the modified ATTTCAAA. The functionality of these elements in the promoter needs to be tested through detailed promoter deletion studies, and studies are currently in progress.

Unlike in the highly ethylene-responsive *R. bourboniana* petals, transcriptional up-regulation of the two XTH genes was delayed in abscission zones of ethylene-treated *Rosa hybrida* flowers, with levels increasing only from 24–48 h. Moreover, the levels were much lower than those in *R. bourboniana*. The hybrid flowers usually undergo senescence but not abscission (except after prolonged ethylene treatment). Since ethylene treatment leads to a delayed induction of transcription of the two XTH genes and to a much reduced level compared with the fragrant variety, it appears that the abscission machinery or the ethylene perception/signalling machinery might not be as active in *R. hybrida* abscission zones. Alternatively, there might be differences in promoter sequences of the two XTH genes in *R. hybrida* that might be responsible for the delayed and lower accumulation of transcript levels of the two XTH genes, although this needs to be tested. Incidentally,

RhXTH1, which is identical in sequence to *RbXTH1*, is transcribed during petal expansion of *R. hybrida* (Yamada *et al.*, 2009), although expression in abscission zones was not studied.

An important aspect of the studies presented here has been the correlation of the transcriptional increase in XTH genes with a substantial increase in XET action in the abscission zones of both ethylene-treated and field abscising flowers. XTHs can be dual functional enzymes with both endotransglucosylase and endohydrolase activities. At the beginning of the study, it had been speculated that cell wall loosening through the scission of the xyloglucan moieties by the xyloglucan endohydrolase (XEH) activity might be important for abscission. However, most XTHs studied so far have only shown the endotransglucosylase activity (Campbell and Braam, 1999*b*; Vissenberg *et al.*, 2000, 2001; Baumann *et al.*, 2007). Moreover, the lack of the amino acid extension in loop 2 (YNII, underlined in Fig. 1) in RbXTH1 and RbXTH2, which has been associated with the XEH activity of TmNXG1 (Baumann *et al.*, 2007), indicates that RbXTH1 and RbXTH2 might lack the XEH activity, although this has not been tested. Instead, the present studies showed an increase in XET action specifically at the site of abscission in ethylene-treated and naturally abscising flowers midway through the abscission process (Fig. 7). The XET action appeared to be localized to the cell walls which is the site of XET function and is in keeping with the presence of the signal peptide in sequences of both the XTHs. Nevertheless the contribution of other XTHs to the XET action observed cannot be ruled out. The increase in XET action indicates active cell wall remodelling of the xyloglucan moieties of the cells of the abscission zone. It is likely that changes brought about by the XET action may allow easier accessibility of the wall to other hydrolytic enzymes, thus accelerating abscission. Conversely, abscission has been shown to be associated with an increase in cell size in *Arabidopsis*, citrus, etc. (Bleecker and Patterson, 1997; Agusti *et al.*, 2009). The XET action could be required for rearrangement of the cross-linking wall hemicelluloses during cell enlargement. A major effect of this increase in cell size would be to reduce the cell surface area to volume ratio at later stages of abscission compared with early stages when the cells are small and densely packed. This in turn could lead to a decrease in wall strength, thus helping abscission. Interestingly, a lack of XET action was observed in ethylene-treated petals, in spite of the transcriptional increase in *RbXTH1* and *RbXTH2*. This was surprising and indicates that fine controls at the translational level might possibly operate to regulate expression in a tissue-specific manner.

To conclude, the present studies show that expression of XTH is a feature associated with abscission and governed in an abscission-specific and ethylene-responsive manner. Cell wall remodelling through rearrangement of the wall xyloglucans by the XET action of one to several XTHs might be a major determinant in the process of abscission.

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