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The *Arabidopsis XET*-related gene family: environmental and hormonal regulation of expression

Wei Xu†, Paul Campbell†, Ajay K. Vargheese and Janet Braam*
Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892, USA

Summary

Enzymes that modify cell wall components most likely play critical roles in altering size, shape, and physical properties of plant cells. Regulation of such modifying activity is expected to be important during morphogenesis and in eliciting developmental and physiological alterations that arise in response to environmental conditions. Previous work has shown that the *Arabidopsis TCH4* gene encodes a xyloglucan endotransglycosylase (XET) which acts on the major hemicellulose of the plant cell wall. The expression of *TCH4* is dramatically upregulated in response to several environmental stimuli (including touch, wind, darkness, heat shock, and cold shock) as well as the growth-enhancing hormones, auxin and brassinosteroids. This paper reports the presence of an extensive *XET*-related (*XTR*) gene family in *Arabidopsis*. In addition to *TCH4*, this family includes two previously identified genes, *EXT* and *Meri-5*, and at least five additional genes. The cDNAs of the *XTR* family share between 46 and 79% sequence identity and the predicted XTR proteins share from 37 to 84% identity. All eight proteins include potential N-terminal signal sequences and most have a conserved motif (DEIDFEFLG) that is also found in *Bacillus* β-glucanase and may be important for enzyme activity. The members of the *XTR* gene family are differentially sensitive to environmental and hormonal stimuli. Magnitude and kinetics of regulation are distinct for the different genes. Differential regulation of expression of this complex gene family suggests a recruitment of related, yet distinct, cell wall-modifying enzymes that may control the properties of cell walls and tissues during development and in response to environmental cues.

Introduction

Plants are remarkably sensitive to environmental conditions and have evolved the ability to undergo adaptive developmental and physiological changes in response to environmental stimuli. For example, in response to mechanical stimuli such as wind or touch, plants generally grow shorter and stockier and often become stronger or more flexible. This developmental response, called thigmomorphogenesis (reviewed in Biddington, 1986; Jaffe and Forbes, 1993; Mitchell and Myers, 1995), can enable plants to withstand further environmental challenges.

Alterations in morphogenesis are likely to require modifications in the plant cell wall. The wall is a fundamental determinant of cell shape, and therefore of tissue and organ form. Thus, it might be expected that in response to environmental cues, the availability of cell wall-modifying enzymes would be regulated. Previously, we have shown that the *TCH* genes of *Arabidopsis* are strongly and rapidly upregulated in expression following touch, wind, rain, darkness and heat shock (Braam, 1992; Braam and Davis, 1990; Sistrunk *et al.*, 1994). *TCH4* encodes a xyloglucan endotransglycosylase (XET) which can modify the major hemicellulose of the cell wall (Xu *et al.*, 1995). XETs (also called endoxyloglucan transferases (EXTs), Okazawa *et al.*, 1993) cleave xyloglucan polymers internally and ligate the newly generated reducing end to another xyloglucan chain (Fanutti *et al.*, 1993; Farkas *et al.*, 1992; Fry *et al.*, 1992; Nishitani, 1995; Nishitani and Tominaga, 1992; Smith and Fry, 1991). Because xyloglucans are thought to cross-link cellulose microfibrils in the plant cell wall (Fry, 1989; Hayashi, 1989; Hayashi *et al.*, 1994a, 1994b; McCann *et al.*, 1990; Passioura and Fry, 1992), XET activity may be critical in determining properties of the wall, such as extensibility, tensile strength and integrity (Fry, 1989; Fry *et al.*, 1992; McCann *et al.*, 1992; Redgwell and Fry, 1993; Smith and Fry, 1991; Talbott and Ray, 1992). Furthermore, XET activity may be important for incorporation of xyloglucan into the cell wall, perhaps ligating short xyloglucan subunits that may be secreted (McCann *et al.*, 1992). The identification of TCH4 as an XET provides evidence of the potential physiological relevance of the dramatic regulation of expression of the *Arabidopsis TCH* genes.

TCH4 is a member of a multigene family in *Arabidopsis*. Okazawa *et al.* (1993) isolated a cDNA, called *EXT*, by screening for cDNAs with sequence relatedness to an azuki bean XET-encoding gene. *Meri-5* was isolated as a gene expressed in meristems (Medford *et al.*, 1991) and was later found to share significant sequence similarity to XETs (Okazawa *et al.*, 1993; de Silva *et al.*, 1993, 1994).

Here we describe an investigation of the *XET*-related gene family of *Arabidopsis*. We have identified and sequenced five novel *XET*-related (*XTR*) cDNAs. Regulation of expression of the *XTR* genes by auxin, brassinosteroids,

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*For correspondence (fax +1713 285 5154; e-mail braam@bioc.rice.edu).
†These two authors contributed equally to this work.

and environmental stimuli is described, and the potential significance of these gene family members and their differential regulation of expression is discussed.

Results

An XET*-related gene family in* Arabidopsis

The independent identifications of *TCH4, EXT* and *Meri-5* provided the first evidence of an *XET*-related gene family in *Arabidopsis* (Medford *et al.*, 1991; Okazawa *et al.*, 1993; Xu *et al.*, 1995). As shown in Figure 1, under low-stringency hybridization conditions (right), a DNA blot probed with *TCH4* reveals multiple hybridizing bands, suggesting the presence of an extensive gene family. With high-stringency conditions, only the expected *TCH4* genomic fragments are recognized (Figure 1, left).

Comparing *TCH4* sequences to expressed sequence tags (EST) characterized by the *Arabidopsis* sequencing project (Höfte *et al.*, 1993; Newman *et al.*, 1994), additional *XET*-related (*XTR*) cDNAs were identified. We determined the complete sequences of *XTR*s *2, 3, 4, 6* and *7* cDNAs and a comparison of these sequences with previously identified *XET*-related genes is shown in Figure 2(a). The sequences shown begin with the proposed initiator codons (ATG) of the longest open reading frames, except for *XTR3* and *XTR4*, which, based on alignments with the other genes and the absence of an ATG codon at the start of the longest open reading frame, are likely missing 5′ end sequences.

We have performed double-stranded sequencing of nucleotides 220–770 of a cDNA (designated *Meri-5* in Figure 2a) derived from Columbia ecotype mRNA that is nearly identical to the *Meri-5* sequence previously reported (Medford *et al.*, 1991), except for nine distinct sequence differences. These sequence differences were also reported by Arrowsmith and de Silva (1995) for a cDNA derived from mRNA of the Landsberg *erecta* ecotype. Importantly, these sequence changes would result in a protein product with significantly increased relatedness to the proteins encoded by *TCH4* and the *XTR*s.

The members of the *Arabidopsis XTR* gene family share an unusual degree of similarity with up to 79% nucleotide sequence identity within the region composing the open reading frames (Figure 2b). As expected for non-coding regions, the sequences corresponding to the 3′ untranslated regions (UTRs) are more divergent.

Conservation of sequence among the XTR *gene products*

Figure 3(a) shows an alignment of the deduced amino acid sequences of the *XTR* gene family of *Arabidopsis*. The putative gene products share between 35 and 84% identity (Figure 3b).

Each protein contains a potential N-terminal signal sequence rich in hydrophobic amino acids. Using the predictions of von Heijne *et al.* (1986) and the SIGCLEAVE program (Rice, personal communication), cleavage would most likely occur after the last amino acid residues shown in Figure 3(c). The putative mature protein regions often show higher percentages of amino acid identity than the full-length primary sequences (Figure 3b).

Except for TCH4, for which enzymatic activity has been assessed (Xu *et al.*, 1995), it is uncertain whether the proteins encoded by this *Arabidopsis* gene family have XET activity. However, all the putative proteins, except XTR4, have the conserved DEIDFEFLG sequence (noted with asterisks in Figure 3a) which is very similar in sequence to a region in *Bacillus* β-glucanase that has been proposed to be within the active site (Borriss *et al.*, 1990). The strong sequence relatedness of this region between plant and bacterial proteins suggests that this region may be critical for the cleavage of (1-4)-β-glycosyl linkages (Okazawa *et al.*,

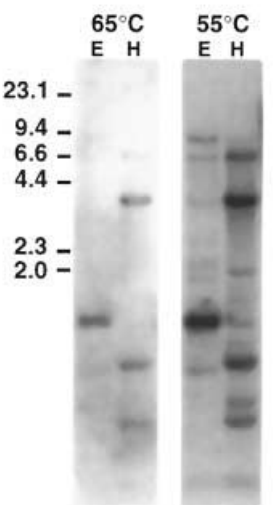
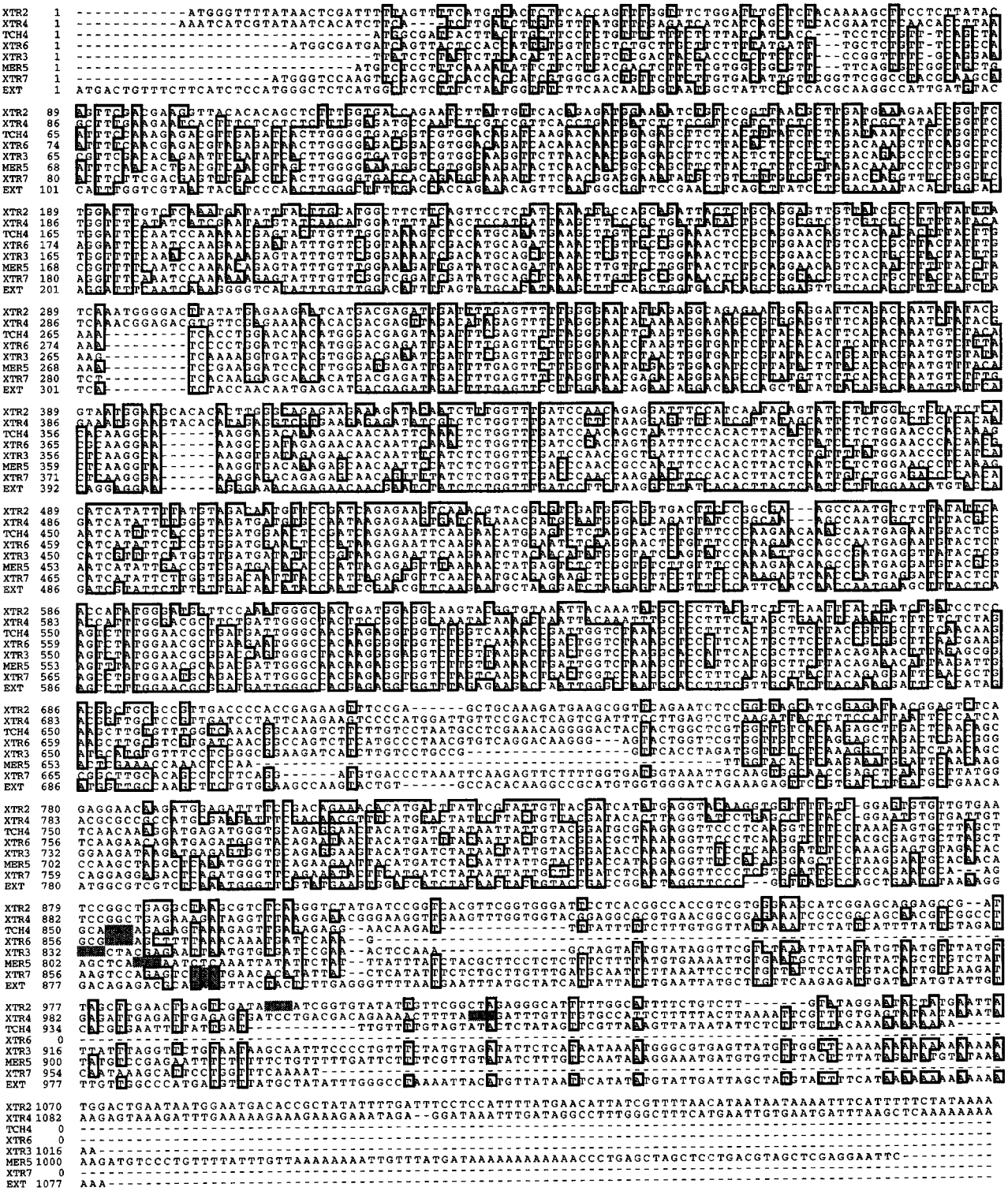


Figure 1. Presence of *TCH4*-related sequences in the *Arabidopsis* genome. Genomic DNA of *Arabidopsis* was digested with either *Eco*RI (E) or *Hin*dIII (H) as indicated, size separated on a gel, blotted to a membrane, and hybridized with a *TCH4* probe under high (65°C) or low (55°C) stringency. Molecular mass markers are indicated at left.

Figure 2. Comparison of eight *XET*-related gene sequences of *Arabidopsis*.
(a) Sequence alignment. Nucleotide sequences are numbered beginning with the proposed ATG codon. *XTR3* and *XTR4* begin with the first nucleotides of the longest cDNAs available. Boxes indicate sequence identity and dashes indicate gaps inserted to maximize alignment. The alignment was generated using the PileUp program of the Wisconsin Package of the Genetics Computer Group (GCG version 8; Devereux *et al.*, 1984). Stop codons at the ends of the longest open reading frames are shaded. GenBank accession numbers are listed in Experimental procedures.
(b) Percent nucleic acid sequence identity. Nucleotide sequences corresponding to the coding regions of the *XTR* gene family were compared using the Gap program of GCG (Devereux *et al.*, 1984). The values indicate the percent sequence identity between pairs of genes.

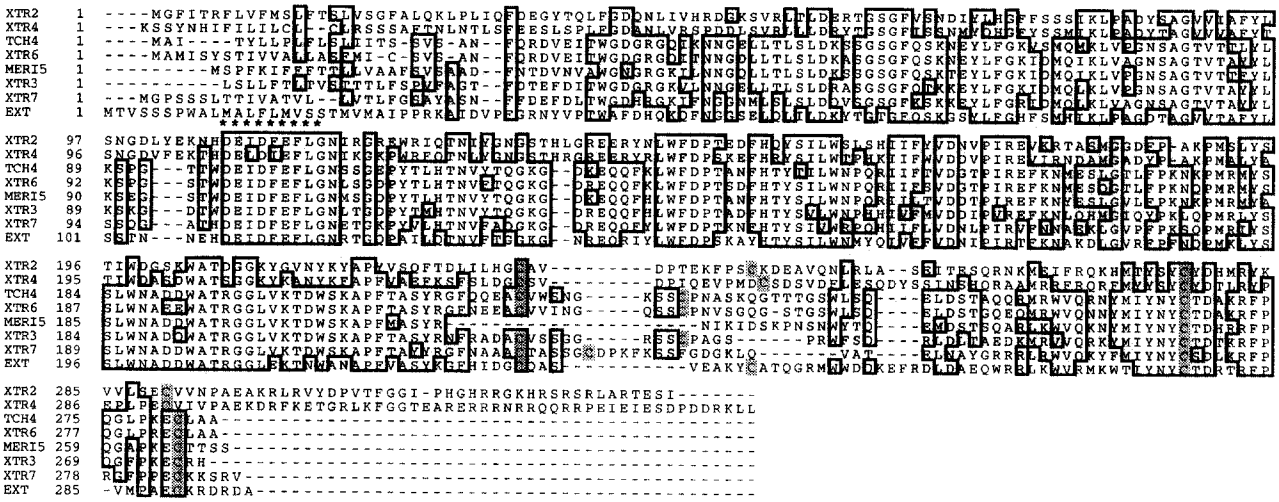
(a)



(b)

	TCH4	Meri5	EXT	XTR2	XTR3	XTR4	XTR6
Meri5	72						
EXT	56	58					
XTR2	50	48	48				
XTR3	69	69	55	46			
XTR4	49	48	52	57	47		
XTR6	79	69	55	48	69	48	
XTR7	61	65	56	47	66	50	64

(a)



(b)

	TCH4	Meri5	EXT	XTR2	XTR3	XTR4	XTR6
Meri5	70/75						
EXT	48/50	48/50					
XTR2	36/36	34/34	38/39				
XTR3	68/73	64/67	51/51	38/37			
XTR4	35/36	35/35	37/39	54/55	37/39		
XTR6	84/87	67/71	49/51	36/37	69/74	37/38	
XTR7	63/66	59/61	53/55	37/38	64/66	38/38	63/66

(c)

GENE	SIGNAL PEPTIDE
XTR2	MGFITRFLVFMSLFTSLVSG
XTR4	KSSYNHIFILILCLCLRSSSA
TCH4	MAITYLLPLFLSLIITSSVSA
XTR6	MAMISYSTIVVALLASFMICSVSA
XTR3	LSLLF
Meri-5	MSPFKIFFFTTLLVAAFSVSA
XTR7	MGPSSSLTTIVATVLLVTLFGSAYA
EXT	MTVSSSPWALMALFLMVSSTMVMA

(d)

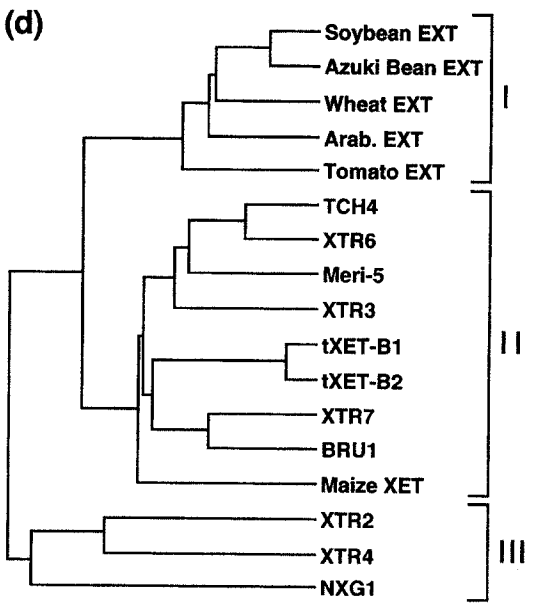


Figure 3. Alignment and comparison of deduced amino acid sequences.

(a) Amino acid alignment of the *Arabidopsis* XTR family. Amino acid residues are numbered from the proposed initiator methionines. For XTR3 and XTR4, numbering begins with the first amino acid of the longest identified open reading frame. The asterisks indicate a region of conservation shared with the *Bacillus* β-glucanase. Boxes indicate sequence identity; dashes indicate gaps inserted to maximize sequence alignment. The cysteines referred to in the text are shaded.

(b) Percent amino acid identity between members of the *Arabidopsis* XTR family. Amino acid sequences, with and without the proposed signal peptides, were compared using the BestFit program of GCG (Devereux *et al.*, 1984). The values indicate the percent sequence identity between the entire available sequences of two proteins (before the slash) and between the two proteins lacking signal peptides (after the slash).

(c) Comparison of potential signal peptides. Sequences of the N-terminal portions of the *Arabidopsis* XTR family proteins were analyzed for signal peptides using the method of von Heijne *et al.* (1986) and the SigCleave program of EGCG (P. Rice, personal communication). Cleavage of the proposed signal peptides is predicted to occur following the last amino acid residue shown.

(d) Graphic representation of the amino acid percent sequence differences. Amino acid sequence relatedness of all known XET-related amino acid sequences is diagrammed using the PileUp program of GCG (Devereux *et al.*, 1984). Distance along the horizontal axis is directly proportional to the number of amino acid residue differences. The entire proteins (including proposed signal peptides) were used for this comparison. Numbers at right indicate classification into groups discussed in text. GenBank accession numbers are listed in Experimental procedures.

1993; de Silva *et al.*, 1993). XTR4 differs in that the isoleucine is replaced conservatively with a leucine and the first phenylalanine is replaced with an isoleucine. These two changes conserve the apolar, uncharged nature of the residues at these positions and most likely would not alter the tertiary structure drastically. Function also may not be significantly affected as evidenced by the fact that the nasturtium seed XET, for which enzymatic activity has been demonstrated, also contains the phenylalanine to isoleucine alteration (de Silva *et al.*, 1993). Each XTR protein has a potential N-linked glycosylation site (N–X–S/T) following this conserved region; however, for XTR2 and XTR4, this site is displaced by 15 residues toward the C terminus. Finally, except for Meri-5, each family member has four cysteine (C) residues in the C-terminal portion of the protein which may form disulfide bridges (Figure 3a). The positions of the second and fourth residues vary. The presence of all four cysteines is probably not required for activity, however, because a recombinant soybean BRU-1 protein, which has only two C-terminal cysteine residues (Zurek and Clouse, 1994), has been shown to have XET activity *in vitro* (Clouse, personal communication).

To visualize the sequence conservation of the *Arabidopsis* XTR family of proteins and similar proteins identified from other plant species, we generated the relationship tree (Devereux *et al.*, 1984) shown in Figure 3(d). For this analysis, we compared the complete amino acid sequences of the *Arabidopsis* XTR family members with the amino acid sequence determined for a nasturtium seed XET (NXG1, de Silva *et al.*, 1993) and deduced amino acid sequences for the soybean EXT, azuki bean EXT, wheat EXT, *Arabidopsis* EXT, tomato EXT (Okazawa *et al.*, 1993), tomato XETs B1 and B2 (de Silva *et al.*, 1994), soybean BRU1 (Zurek and Clouse, 1994), and maize XET (Peschke and Sachs, 1994; Saab and Sachs, 1995). Based on comparison analyses, the protein products can be classified into three groups; a similar interpretation of the relatedness of some of these proteins was presented previously by Nishitani (1995). Each group contains members found in more than one plant species, indicating that divergent genes encoding these proteins were likely present in a common ancestor. Group III (XTR2, XTR4, and NXG1) is the most diverged from the other family members. The members of Group III have three additional amino acid residues (residues 101–103 of XTR2) on the N-terminal side of the conserved DEIDFEFLG region, and either two (XTR2 and XTR4, residues 135 and 136) or five (NXG1, data not shown) additional amino acid residues following this conserved region. XTR2 and XTR4 can be further distinguished by their long C-terminal extensions of 30–40 amino acids. These long, hydrophilic extensions are absent from all other known *Arabidopsis* XTRs or XET and XET-related proteins from other species (Okazawa *et al.*, 1993; Saab and Sachs, 1995; de Silva *et al.*, 1993, 1994; Zurek and Clouse, 1994). The *XET*-related genes isolated by Okazawa *et al.* (1993), called *EXTs*, encode potential proteins comprising Group I. Group I members are distinct from those of Groups II and III in that they share a highly conserved region (i.e. region 227–255 of the *Arabidopsis* EXT). In the other groups, this is the most divergent region of the mature XET and XET-related proteins. The second group has the greatest number of members and includes pairs of proteins from the same species that are extremely similar. For example, tXET-B1 and tXET-B2 (de Silva *et al.*, 1994) from tomato are 92% identical, and XTR6 and TCH4 (Xu *et al.*, 1995) are 84% identical.

Table 1. Identification of the *XTR* family members in the *Arabidopsis* EST data base

Gene	ESTs	Library
TCH4	1	Mixed
	1	Green shoots
Meri-5	12	Mixed
	1	Siliques
	1	Etiolated seedlings
	2	Cultured leaf strips
EXT	8	Mixed
	2	Etiolated seedlings
XTR2	2	Mixed
	1	Siliques
	1	Green shoots
XTR3	2	Dry seeds
XTR4	2	Etiolated seedlings
XTR6	1	Etiolated seedlings
XTR7	7	Mixed
	2	Etiolated seedlings

Library refers to the cDNA library form which specific clones were isolated: mixed, λPRL2 library; green shoots, Ors-A library; siliques, Gif-seed A; etiolated seedlings, Versailles-VB; cultured leaf strips, Strasbourg-A. (For the λPRL2 library, refer to Newman *et al.*, 1994; the remaining libraries are from the *A. thaliana* transcribed genome: the GDR cDNA program.)

Differential expression of the XTRs

It is possible that the multiple XTRs have evolved so that specific subsets may function to alter cell wall properties during development and/or in response to particular hormonal and environmental stimuli. From the frequency of isolation of the *XTR* cDNAs and the sources of the template RNAs, information is gained on the abundance and tissue distribution of the *XTR* mRNAs. For example, as documented in Table 1, *EXT*, *Meri-5* and *XTR7* ESTs have been identified most frequently, indicating that expression levels of these gene family members are relatively high. *Meri-5* cDNAs were found in several different libraries generated

from different sources, such as etiolated seedlings, siliques and cultured leaf strips; whereas *TCH4, XTR3, XTR4* and *XTR6* appear to be more restricted in their expression patterns. However, because of the relatively low frequency of isolation of many of these ESTs, it is probable that these genes are expressed in more sites than identified to date. Overall, these data strongly suggest that the *XTR* genes are differentially expressed during development.

Regulation of the XTRs *by auxin and brassinosteroids*

It has been proposed that XET-mediated modification of the cell wall may be important for cell expansion (Fry, 1989; Fry *et al.*, 1992; Smith and Fry, 1991). Auxin and brassinosteroids, growth promoting hormones, have been shown to upregulate expression of XET-encoding genes. *BRU-1* is post-translationally induced in expression by brassinosteroids (Zurek and Clouse, 1994), and *TCH4* is upregulated by auxin and brassinosteroids (Xu *et al.*, 1995). To test whether expression of other members of the *XTR* gene family are affected by auxin and brassinosteroids, we treated plants with 1 μM indole-3-acetic acid (IAA) or 1 μM 24-epibrassinolide (BR) and assayed *XTR* gene expression.

For this analysis, we generated probes primarily composed of sequences complementary to the 3′ UTRs (see Experimental procedures). The 3′ UTRs share very reduced sequence similarity (Figure 4a) and provide gene-specific probes that do not cross-react with other members of the gene family (Figure 4b). The 3′ UTR of the *XTR6* cDNA was insufficient in length for the generation of a gene-specific probe; therefore, the regulation of expression of *XTR6* has not been characterized.

As shown in Figure 5(a) and summarized in Table 2, in response to IAA treatment of plants, expression levels of *TCH4, EXT,* and, to a lesser extent, *XTR3* are increased; expression of the other family members does not change significantly. Furthermore, only TCH4 shows significant upregulation of expression in plants treated with BR (Figure 5b).

Regulation of the XTRs *by environmental stimuli*

We have shown previously that *TCH4* expression is rapidly and strongly upregulated in response to various environmental stimuli including touch, darkness, cold shock, and heat shock (Braam, 1992; Braam and Davis, 1990; Xu *et al.*, 1995). Therefore, we next examined whether other members of the gene family were regulated in expression similarly. As shown in Figure 6 and summarized in Table 2, the *XTR* gene family members are differentially expressed in response to various environmental stimuli. In response to touch (Figure 6a), *TCH4* shows the most rapid and dramatic response; *Meri-5* and *EXT* are upregulated mod-

(a)

	TCH4	Meri-5	EXT	XTR2	XTR3	XTR4
Meri-5	46					
EXT	46	43				
XTR2	38	35	40			
XTR3	41	41	42	40		
XTR4	42	35	41	42	40	
XTR7	47	44	45	42	42	50

(b)

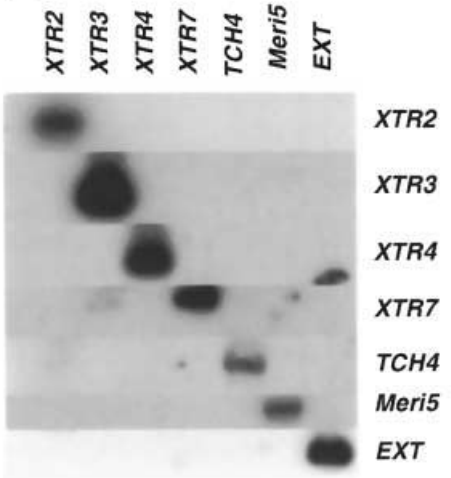


Figure 4. Comparison of 3′ untranslated regions and Southern analysis of hybridization probe specificity.
(a) Percent nucleotide identity of 3′ untranslated regions of members of the *XTR* gene family. Nucleotide sequences from the stop codon up to the poly-A tail were compared using the GAP program of GCG (Devereux *et al.*, 1984). The values indicate the percent sequence identity between a given pair of genes.
(b) Plasmid DNA of the cDNAs listed above the lanes were restriction digested and size separated on an agarose gel. The DNAs were then transferred to nylon membranes and hybridized with the probes indicated on the right.

estly. Only *XTR2* and *XTR3* mRNA levels are unchanged following darkness; interestingly, the magnitude and kinetics of darkness induction of expression of the other gene family members vary (Figure 6b). Consistent with this result, *XTR2* and *XTR3* have not been identified from etiolated tissue mRNAs by the EST sequencing project (Table 1). Following heat shock, *TCH4* and *Meri-5* are significantly upregulated in expression; *XTR3* may also be slightly upregulated. *EXT* mRNA levels, however, significantly decrease following heat shock. The apparent reduction in *XTR2* and *XTR4* mRNA levels is due to a slight overloading of lane 0 as indicated by hybridization to tubulin mRNA (Figure 6c). A significant response to cold shock is limited to *TCH4* (Figure 6d).

Discussion

Arabidopsis harbors an extensive family of genes whose members share significant sequence similarity with xylo-

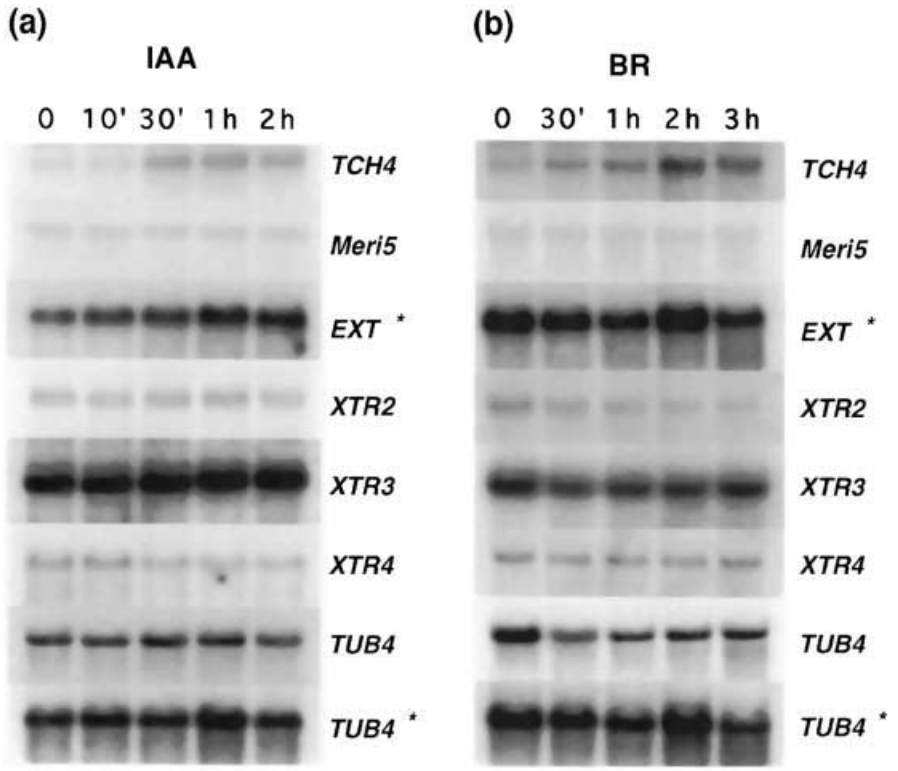


Figure 5. Differential induction of expression of the *XTR* gene family by auxin and brassinosteroids.
RLD plants, approximately 12 days old and grown in liquid culture, were treated with solvent alone (0.01% ethanol final concentration, lanes marked 0), or with (a) 1 μM indole-3-acetic acid (IAA) or (b) 1 μM 24-epibrassinolide (BR) in ethanol. Plants were harvested after treatment at the time intervals indicated above the lanes. Total RNA was prepared from the plants, size fractionated on gels, blotted to filters, and hybridized with the probes indicated at the right. Subsequent hybridization of the blots with a probe against tubulin mRNA was used to show the relative amounts of RNA present in each lane. Two different blots were used and are distinguished by the absence or presence of an asterisk following the probe name. *XTR7* mRNAs were undetectable and therefore are not shown. Prime (') stands for minutes, h stands for hours.

Table 2. Summary of the regulation of expression of the *XTR* gene family members

	IAA	BR	TOUCH	DARK	HS	CS
TCH4	↑	↑	↑↑↑↑	↑↑↑↑	↑↑	↑↑↑
Meri-5	–	–	↑	↑↑↑	↑↑	↓
EXT	↑	–	↑	↑	↓	↓
XTR2	–	–	–	–	↓	–
XTR3	↑	–	–	–	–	–
XTR4	↓	–	–	↑	–	↓
XTR7	–	–	–	↑	–	–

Up arrows (↑) indicate the relative strength of the upregulation of expression of a gene to a stimulus. A down arrow (↓) indicates that expression decreased in response to a stimulus. A dash (–) indicates that no significant change was detected.

glucan endotransglycosylases. The *Arabidopsis* XTR proteins can be classified into three groups based on relatedness. XETs identified from other species fall into each of these groups; therefore, the divergence of these proteins most likely occurred in a common evolutionary ancestor of these different plant species. Sequence conservation among the *Arabidopsis* XTRs ranges from between 35 to 84% amino acid identity. The putative signal sequences are generally more diverged in sequence and, therefore, likely have a relaxed requirement for conservation than sequences predicted to be within the mature proteins. Hence, the remarkable conservation of the sequences in the mature proteins is most likely due to evolutionary selection than to very recent gene duplications. Because of the strong conservation of primary sequence, we would predict that the encoded enzymes have related biochemical activities. It is possible, however, that one class of enzymes may prefer the presence of particular sugar sidechains on the donor and/or acceptor polymers. For example, recombinant TCH4 protein produced in *Escherichia coli* is more active against non-fucosylated xyloglucan than fucosylated xyloglucan (Purugganan *et al.*, unpublished results). Some of the XTRs may preferentially carry out hydrolysis of xyloglucan rather than transglycosylation or may have distinct pH or temperature optima. Alternatively, the enzymes may have identical enzymatic activities, but the consequences of the activities could be distinct. For example, some enzymes may contribute to cell wall degradation or loosening, whereas others may act to assemble nascent cell walls or reinforce established walls. Functional consequences could be determined spatially; that is, some of the *XTR*s may be expressed only in specific tissues or cells, or the XTR proteins may be specifically targeted to distinct regions of the cell wall. Characterization of *in vitro* enzymatic activities and determination of the localization of gene expression and XTR protein accumulation will lead to a fuller understanding of the precise functions of each *Arabidopsis XTR*.

There have been numerous correlations between XETs and cell expansion. It was first proposed that XETs may function in cell wall loosening (Fry, 1989; Fry *et al.*, 1992; Smith and Fry, 1991). During auxin-induced growth, changes in xyloglucan polymer lengths can be observed

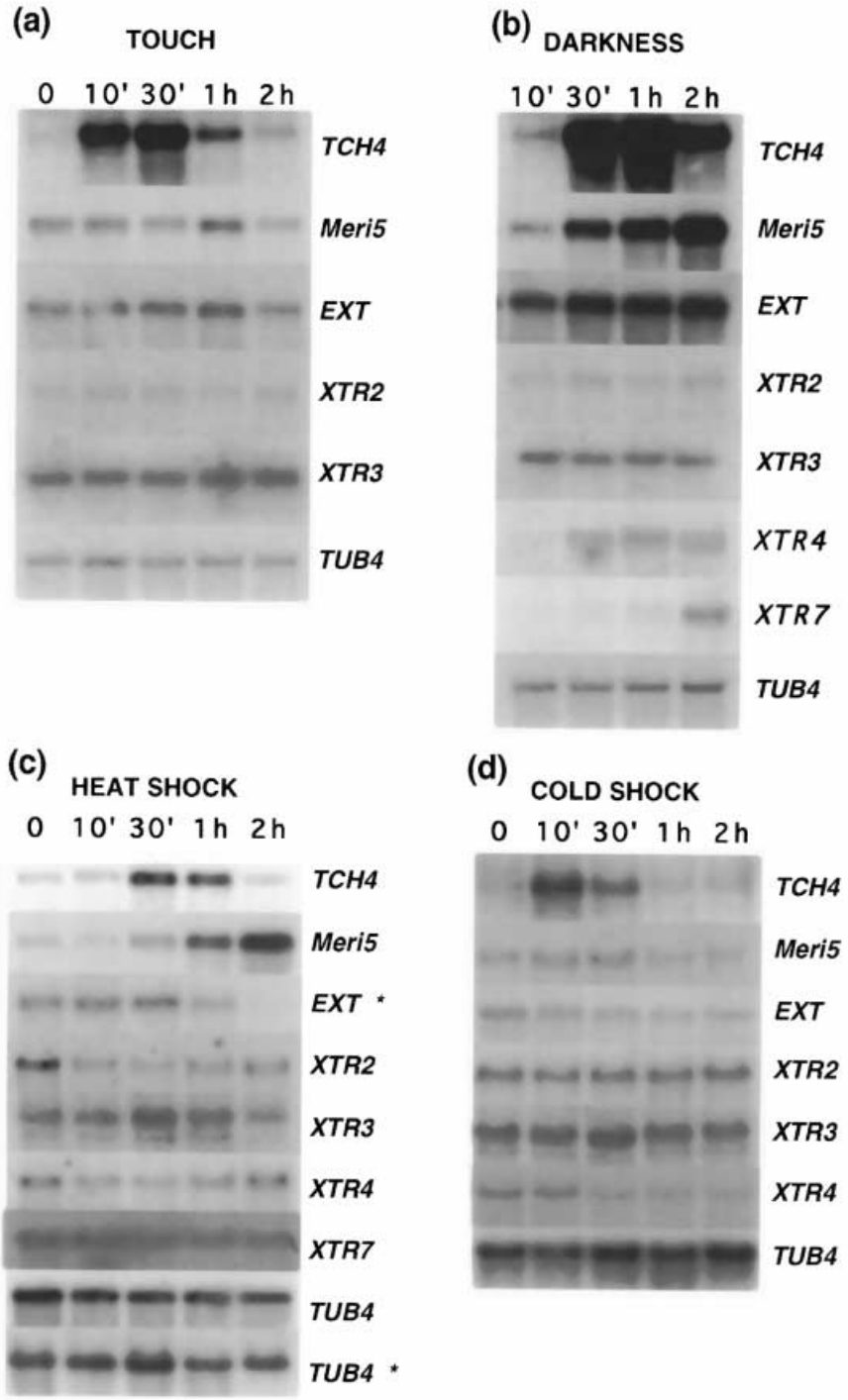


Figure 6. Differential induction of expression of the *XTR* gene family by environmental stimuli.

Approximately 15-day-old Columbia plants (for touch and darkness stimuli) or cultured cells (for heat and cold stimuli) were subjected to no treatment (lanes marked 0) or to (a) touch, (b) darkness, (c) heat shock, and (d) cold shock, as described in the Experimental procedures. Plants were harvested at the indicated times following treatment. Total RNA was prepared from the tissues, size fractionated on gels, blotted to filters, and hybridized with the probes indicated at the right. Subsequent hybridization of the blots with a probe against tubulin mRNA was used to show the relative amounts of RNA present in each lane. *XTR4* and *XTR7* mRNAs were undetectable in touch-stimulated plants, and *XTR7* mRNAs were undetectable in cold-treated cultured cells; therefore, these data are not shown. Prime (') stands for minutes, h stands for hours.

(Inouhe *et al.*, 1984; Labavitch and Ray, 1974; Lorences and Zarra, 1987; Nishitani and Masuda, 1983; Talbott and Ray, 1992; Wakabayashi *et al.*, 1991). Brassinosteroids have been shown to enhance expression of the *BRU-1* gene of soybean whose encoded XET could have a role in increasing the plastic extensibility of soybean epicotyl segments that occurs in response to brassinosteroids (Zurek *et al.*, 1994). The XET-encoding *TCH4* gene has been shown to be rapidly and specifically upregulated by auxin and brassinosteroids (Xu *et al.*, 1995). Here, we show that regulation by auxin and brassinosteroids is not a general property of the *XTR* genes. In addition to *TCH4, EXT,* an *XTR* family member with significance sequence divergence from *TCH4,* is upregulated in expression by IAA; no other *XTR*s tested are affected by BR. Thus, specific induction of expression of only a small subset of *XTR* genes probably

occurs in tissues that experience significant increases in endogenous auxin and or brassinosteroids. The physiological consequences of altered auxin and brassinosteroid levels are thus likely mediated in part by TCH4 and EXT.

Many of the *XTR* genes are regulated following environmental stress; however, the magnitude and kinetics of induction vary for the different genes and stimuli. It is expected that distinct physiological changes would occur in response to the different stimuli, and different sets of XTR isozymes may participate in generating the specific responses. For example, in response to heat shock, activities of TCH4 and Meri-5 may be required whereas EXT is not. Interestingly, *Meri-5* expression is most like that of *TCH4*; *Meri-5* expression is upregulated following touch, darkness and heat shock. However, the *Meri-5* mRNAs accumulate with delayed kinetics compared with *TCH4* mRNAs. The differential kinetics of upregulation suggest that the different XTRs may act sequentially during acclimation or adaptation to environmental conditions. *TCH4*, which is induced in the early stages of heat shock, may be needed in the initial response, while *Meri-5* would be required during the later stages. Hence, the complex regulation of the *XTR* gene family may provide a mechanism whereby appropriate cell wall alterations occur in response to environmental signals, thus eliciting adaptive morphological and physiological changes.

TCH4 expression levels are increased by all environmental and hormonal stimuli tested. These results suggest that there may be a common alteration of cell walls, perhaps mediated by TCH4, in plants subjected to various environmental stresses and during hormone-induced growth. However, the responses to these stimuli may be antithetical. That is, touch results in decreased shoot elongation; whereas, darkness or auxin causes increased elongation. To explain this apparent paradox, we propose that the *TCH4*-encoded XET activity is involved in incorporating xyloglucan into cell walls. This activity would lead to increased xyloglucan cross-links and hence cell wall reinforcement of non-growing cells stimulated by touch; however, in cells expanding in response to darkness or auxin, xyloglucan incorporation will lead to hemicellulose replacement to maintain wall thickness and integrity.

Interpretation of the physiological significance of the complex regulation of the *XTRs* is complicated by the fact that stimuli can have opposite effects on growth depending on the tissue responding. For example, exogenous auxin stimulates shoot growth but inhibits root growth, and light enhances leaf expansion but limits shoot elongation. Therefore, determining the organ and cell-type specificity of gene induction in response to different stimuli is an important next step to gaining insight into the roles of the different XTRs.

In summary, there is a family of closely related genes that encode XET-related proteins in *Arabidopsis*. These XTR proteins likely have related enzymatic activities; further characterization of these enzymes will help to define functional significance of the sequence divergences. Differential regulation of expression of the gene family members suggests that the abundance of a subset of the XTR proteins is controlled in response to hormonal and/or different environmental cues and that the physiological consequences may be mediated in part by XTR action on the plant cell wall.

Experimental procedures

Plant material and treatments

Arabidopsis thaliana plants, ecotypes Columbia and RLD, were cultivated under continuous light at 24°C in 65–75% humidity. Touch, darkness, heat shock and cold shock treatments were conducted as described previously (Braam, 1992; Braam and Davis, 1990; Xu *et al.*, 1995). Cultured cells were generated as described previously (Braam, 1992).

Plants to be treated with indole-3-acetic acid (IAA) or 24-epibrassinolide (BR, a generous gift of Steven D. Clouse, North Carolina State University) were grown for approximately 12 days in liquid culture containing 0.5× MS salts (Sigma, St Louis, MO), 1× Gamborg's vitamins (Sigma) and 1% sucrose, pH 5.7, on a rotary shaker at 120 r.p.m. in 24 h light at 24°C. Before the experiment (12–16 h), the medium was replaced with 20 ml fresh medium. Hormones were added to the media without disturbing the flasks. Controls consisted of addition of solvents only. Collection involved quickly decanting the media and submerging the plants in liquid nitrogen. Samples were stored at –80°C prior to RNA purification.

RNA manipulations

For RNA gel blots, total RNA was purified (Verwoerd *et al.*, 1989), subjected to electrophoresis on formaldehyde gels, and transferred to filters. Filters were probed with either hexamer-labeled DNAs (Feinberg and Vogelstein, 1983) or single-stranded DNAs generated using gene-specific antisense oligonucleotides and the Prime-a-Probe kit (Ambion, Austin, TX). Template DNAs for gene-specific probes were generated by polymerase chain reaction (PCR). The oligonucleotides used to generate the PCR products are as follows: *XTR2*, 5′-AAGCGTCTCAGGGTCTATGA-3′ and 5′-GTTCATAAAATGGAGGAAATC-3′; *XTR3*, 5′-TAGCTACGAGAATTAATGTG-3′ and 5′-AACCAACATAACTCACGCCC-3′; *XTR4*, 5′-AAAGATAGGTTTAAGGAAACG-3′ and 5′-CAATTCATGAAACGCCAAAGG-3′; *XTR7*, 5′-AAGAAGTCCAGAGTCTGATG-3′ and 5′-CGCCAGTGTGAGTAATTTAG-3′; *TCH4*, 5′-TATCTGCAGCTGCATAGAGAGA-3′ and 5′-GCGAAGCTTGTAACAAAGAGAA-3′; and *Meri-5*, 5′-CAACAAGCTCATAGAATCTC-3′ and 5′-TTTAACAAATAAAACAGGGAC-3′. The resultant products were ligated into the pCRII cloning vector following the manufacturer's instructions (Invitrogen, San Diego) and sequenced to verify that the correct product was amplified. For an *EXT*-specific probe, EST clone 104M7T7 (obtained from the *Arabidopsis* Biological Resource Center) was digested to completion with *Pvu*II and then labeled using the Prime-a-Probe kit and the oligonucleotide (5′-ATGAAAATACATAGCTAATCAAT-3′). A *Bam*HI/*Kpn*I fragment of an *Arabidopsis* β-tubulin (Marks *et al.*, 1987) was used as a probe to verify that similar amounts of RNA were present in all lanes.

DNA manipulations

For Southern analysis, genomic DNA was isolated from *Arabidopsis* as described (Cone, 1989). Approximately, 10 μg of genomic DNA were digested, size separated on an agarose gel, transferred to nylon membrane, and hybridized with a *TCH4* probe corresponding to nucleotides 27–362. Prehybridization and hybridization solutions were 5× SSPE, 5× Denhardt's reagent, 0.5% SDS, and 100 μg ml^{-1} sheared salmon sperm DNA (Sambrook *et al.*, 1989). For high-stringency conditions, the filter was hybridized overnight at 65°C, rinsed twice at room temperature in 2× SSC, 0.1% SDS, and washed at 48°C for 1 h in the same solution and 1 h at 65°C in 0.2× SSC, 0.1% SDS. For low-stringency conditions, the filter was hybridized overnight at 55°C, rinsed twice with 2× SSC, 0.1% SDS at room temperature, followed by a 1 h wash in the same solution at 55°C.

Southern analysis to verify the specificity of the hybridization probes was performed using established protocols (Sambrook *et al.*, 1989). cDNAs for each *XTR* family member were excised from the respective plasmid vectors with the restriction endonucleases used to construct the cDNA libraries (*XTR2, TCH4,* and *Meri-5, Eco*RI; *XTR3, Eco*RI and *Xho*I; *XTR4* and *XTR7, Not*I; *EXT, Sal*I and *Not*I). The digested DNAs were size separated on an agarose gel and transferred to nylon membrane. The Southern blots were hybridized with probes under the same conditions as for the Northern hybridizations.

Sequencing of the *XTR*s was performed on double-stranded plasmid using Sequenase version 2.0 and [^{35}S]dATP, following the manufacturer's protocols (U.S. Biochemicals, Cleveland, OH). Sequence analysis was performed using the PileUp, Gap, and BestFit programs of the Wisconsin Package of the Genetics Computer Group (Version 8; Devereux *et al.*, 1984).

Accession numbers

The *XTR*s were originally isolated as ESTs. Partial and complete sequences have the following GenBank accession numbers: *XTR2* = z17666, U43487; *XTR3* = z27022 and z32593, U43485; *XTR4* = z25684, U43486; *XTR6* = z30849, U43488; *XTR7* = z37612, U43489 (Höfte *et al.*, 1993). The GenBank accession numbers of gene sequences used to generate Figure 3(a) include: D16454, *Arabidopsis EXT*; D16455, soybean *EXT*; D16456, tomato *EXT*; D16457, wheat *EXT*; D16458, azuki bean *EXT*; U27609, *TCH4*; X82683, *Meri-5*; X82684, tomato *XET-B2*; X82685, tomato *XET-B1*; L22162, *BRU1*; U15781, maize *XET*; and X68254, *NXG1*.

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See Experimental procedures for the GenBank Data Library accession numbers of the gene sequences described in this paper.