Xyloglucan endotransglycosylases: diversity of genes, enzymes and potential wall-modifying functions

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Plant cells are enclosed by walls that define the shapes and sizes of cells and mediate cell-to-cell contact. The dynamics of plant growth, morphogenesis and differentiation require concomitant modifications of the walls. A class of enzymes known as xyloglucan endotransglycosylases have the potential to enzymatically modify wall components, but although their biochemical activity has been defined, the physiological roles of xyloglucan endotransglycosylases remain undefined. Xyloglucan endotransglycosylases are encoded by large gene families, and in an attempt to clarify their physiological role, the diverse regulation of the genes and properties of the proteins are being determined.

The plant cell wall plays a vital role in controlling the shape of individual cells and, therefore, in determining the morphology of the plant as a whole. The presence of a wall is one of the fundamental distinctions between plant and animal cells. This unique extracellular matrix is not simply a static framework that supports the plant, but a dynamic compartment that responds to the changing needs of the plant during growth and development. A change in wall extensibility allows turgor pressure to drive cell expansion[1] and the deposition of new wall materials maintains microfibril spacing and wall thickness[2]. Modifications to the wall are important for other processes as well. Leaf and flower abscission and fruit ripening involve the degradation of cell wall components within localized areas. Wall connections between adjacent cells are disrupted during air space formation between spongy mesophyll cells of leaves. Although the molecular basis of these wall modifications are in large part unknown, it is likely that they involve proteins that are capable of modifying wall constituents. Many potential wall-modifying proteins have been characterized, including pectinases (endogalacturonases, or Egases[3]), expansins[4] and, the subject of this review, xyloglucan endotransglycosylases (XETs).

Discovery of XETs

The plant cell wall is a complex network of cellulose, hemicelluloses, pectins and structural proteins[2,5]. The most abundant hemicellulose of the dicotyledenous wall is xyloglucan; this long polysaccharide can form hydrogen bonds with cellulose microfibrils and might provide a molecular tether between adjacent microfibrils[6,7]. Such xyloglucan crosslinks might restrain cell expansion and allow the generation of turgor pressure. Hydrolytic enzymes that break crosslinks in the cell wall could enable cells to expand, but at the risk of continually weakening the wall. Instead, it was proposed that transglycosylase activity could allow cells to expand without undermining wall structure[8]. Endolytic cleavage of the xyloglucan tethers would permit the cellulose microfibrils to separate; reattachment of the xyloglucan end to another xyloglucan chain present in the wall would restore a stable structure. The difficulty in finding an enzyme with transglycosylase activity lies in the nature of the reaction. Most enzyme assays follow the conversion of a substrate into a product, but for a transglycosylase, which hydrolyses and then recreates an identical bond at a novel site, the substrate and product are not readily distinguishable. However, transglycosylase activity would be expected to cause changes in the lengths of polysaccharide chains (Fig. 1).

XET activity was detected *in vitro* by mixing xyloglucans of two different molecular weights with a partially purified protein extract[9]. The xyloglucan reaction products showed molecular weight shifts towards the average weight of the two starting populations. Evidence for transglycosylation activity was also obtained by purifying an activity capable of creating both shorter and longer xyloglucan fragments from xyloglucan polymers of known molecular weight[10]. A simple *in vitro* protocol for the detection of XET activity by monitoring the incorporation of short, radiolabeled oligosaccharides into long xyloglucan polymers was then developed[11] (Fig. 1). This and other simple assays for transglycosylation activity facilitated the rapid identification of XETs from many species of higher plants and mosses[11] and their subsequent purification[12–14].

Proposed physiological functions of XETs

In vitro, XETs catalyse the intramolecular cleavage of a xyloglucan polymer and transfer the newly generated, potentially reducing, end to another xyloglucan chain. Although the detailed mechanism of the XET reaction is unknown, recent work has demonstrated that a covalent link probably exists between the XET and the xyloglucan polymer as an intermediate[15]. The XET is then released from the substrate upon completion of the reaction (the joining of two xyloglucan molecules). XETs clearly have a powerful potential to modify a major component of the plant cell wall; however, the physiological consequences of this XET activity remain unknown.

XETs were first proposed to function in wall loosening to enable turgor-driven expansion. Indeed, there is a good correlation between high levels of XET activity and cell expansion. In both maize roots and leaves, XET activity is highest in expanding regions. As the rate of expansion decreases XET activity decreases; however, activity is still readily detected in regions that have completed growth[16,17]. The application of gibberellic acid (GA) induces the elongation of pea internodes, the hypocotyls of lettuce and cucumber, and barley leaves, with a concomitant increase in XET levels[18–20]. XET-encoding genes have been shown to undergo upregulated expression in response to various growth promoting hormones, including GA, auxin and brassinolide[21–24]. In spite of the correlations between XET activity and gene expression with growth, evidence for a role for XETs in wall loosening is lacking. Indeed, application of XETs to heat-killed cucumber hypocotyls is not sufficient to restore acid-induced wall extension[25]. This is in contrast to the expansin proteins, which are

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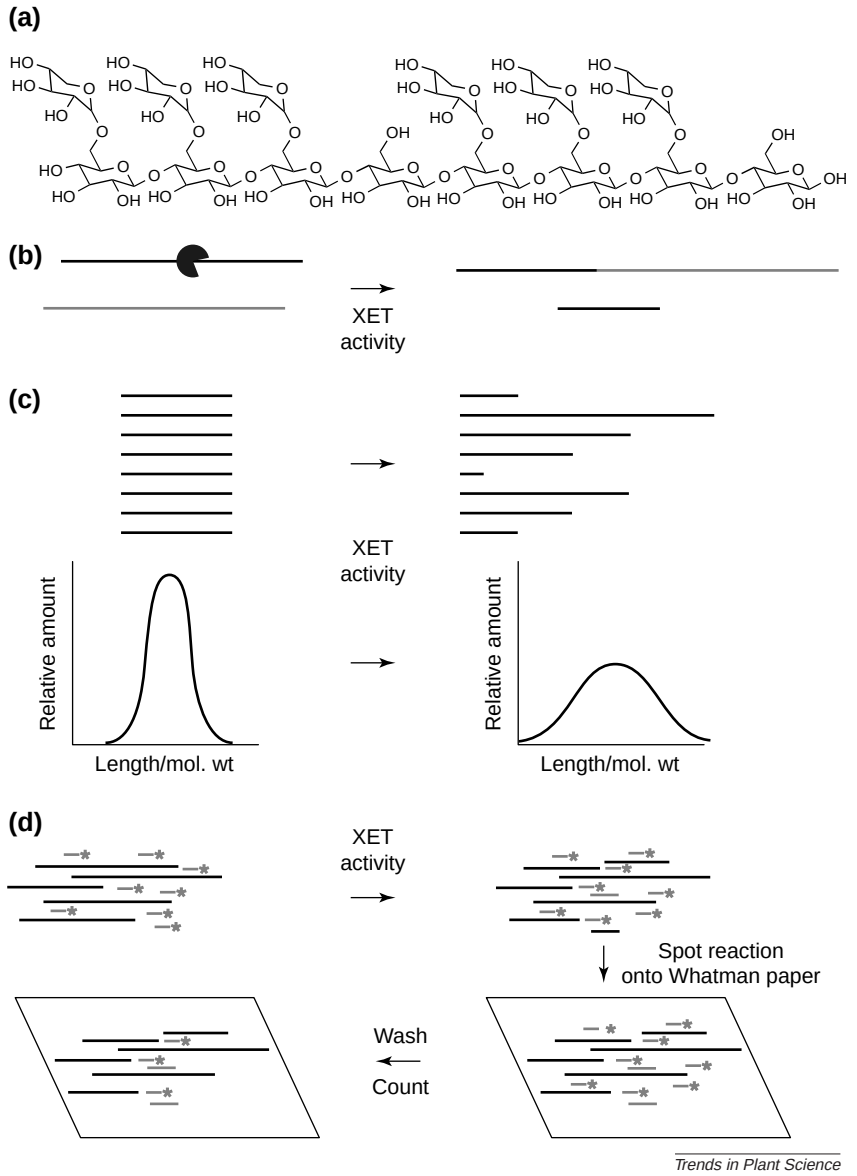

Fig. 1. Xyloglucan endotransglycosylase (XET) activity and assays. (a) Xyloglucan is a complex polysaccharide consisting of a β-1,4-linked glucan backbone substituted with side chains of xylose. Some of the xyloses can be further modified by the addition of galactose or galactose and fucose. The types of side chains vary between tissues and ages of cells, and affect the activities of different XET isozymes. (b) XETs sever xyloglucan molecules internally and then attach the newly generated, potentially reducing end to the non-reducing end of another xyloglucan molecule present in the cell wall. (c) Initially, this activity was detected by monitoring the gel filtration properties of xyloglucan in solution. A relatively homogeneous xyloglucan mixture will travel as a narrow peak during gel filtration chromatography. XET activity randomizes the size of this population, resulting in the generation of both shorter and longer polymers, detected as peak broadening. (d) XETs can incorporate short, radiolabeled, xyloglucan-derived oligosaccharides into much longer xyloglucan polymers via transglycosylation. Once the reaction mixture is terminated, it is spotted onto cellulose (Whatman paper). The xyloglucan hydrogen bonds to cellulose, whereas the short oligosaccharides can be removed simply by washing the paper in water. The amount of radiolabeled oligosaccharide incorporated during the reaction is determined by scintillation counting of the cellulose-bound xyloglucan.

capable of disrupting polymer hydrogen bonding and are proposed to release xyloglucan tethers from microfibrils to enable wall extension[4]. The observations that XET activity remains detectable in regions that have ceased growing[16,17], and that XET mRNA levels increase in *V. angularis* segments with sucrose-induced enhanced wall deposition[22], suggest an alternative function for XETs. Although it remains likely that XETs have a role in cell expansion, this function might not be involved in cell wall loosening but in wall biogenesis. When plant cells expand, the walls do not become thinner[2], therefore new cell wall material must be incorporated in the existing wall structure. This is supported by double labeling studies[26], which suggest that newly synthesized xyloglucan is covalently linked to pre-existing xyloglucan molecules in the cell wall. XETs might participate in this process. The idea that XETs are involved in cell wall biogenesis is consistent with a lack of extension in the cucumber hypocotyl extension assay and the residual XET activity in cells that have recently completed expansion[25]. It is likely that cells continue to reinforce their walls to lock them into place once expansion is complete.

This idea of XET function in wall biogenesis is also consistent with the otherwise paradoxical expression patterns of an *XET* gene, *TCH4*, from *Arabidopsis thaliana*[23]. Analysis of *TCH4::GUS* reporter gene expression in transgenic *Arabidopsis* indicates a strong correlation between gene expression and expanding tissues. Perhaps the clearest example is demonstrated by light-regulated hypocotyl extension: seedlings grown in high light have short hypocotyls and minimal *TCH4::GUS* activity staining, whereas *TCH4::GUS* is strongly expressed in elongated hypocotyls of seedlings grown under low light or in darkness[23]. However, in direct contrast, *TCH4* expression is also up regulated by mechanical stimulation, a treatment that leads to an overall decrease in elongation growth of *Arabidopsis*[27]. From expression correlations, one might hypothesize that TCH4 has roles in both cell expansion and the mechanical stress response. Wall biogenesis and, in particular, xyloglucan incorporation and modification of existing crosslinks, might occur in both expanding cells and in cells with walls under strain and undergoing reinforcement. In response to mechanical perturbation, such as touch or wind, the mechanical properties of plants are often altered[28]. These alterations might result from changes in cell wall composition or arrangement, perhaps including increased xyloglucan incorporation through the action of XETs.

Xyloglucans are synthesized in the Golgi apparatus and delivered to the wall in secretory vesicles[29,30]. Newly synthesized xyloglucan might be a lower molecular weight than mature wall polymers[31]. Extracted xyloglucan from onion cell walls shows periodicity in length distributions, suggesting that xyloglucan might be built in the wall from subunit blocks[32]. These data suggest that xyloglucan polymers are assembled in the wall by the joining of nascent xyloglucan polymers to

each other or to polymers present in the wall. XETs have an appropriate enzymatic activity to carry out this role and are therefore good candidates for incorporating nascent xyloglucan into the wall.

There are additional potential roles for XET activity. XET activities and levels of *XET* mRNAs have been shown to increase during fruit ripening[33–35], leading to the proposal that XETs might function in cell wall degradation during fruit softening. A flooding-responsive *XET* gene has been identified in maize that could potentially participate in flooding-induced aerenchyma formation[36]. This process involves the degradation of cell walls to form air spaces in flooded roots. Antibodies to XET reveal significant accumulation of XETs between rows of mesophyll cells in developing leaves where it is predicted air spaces will be formed[37].

The specific wall modification by XET action, such as biogenesis or degradation, might be determined by the availability of substrate and the activity of other wall modifying proteins. For example, if XET activity increases as nascent xyloglucan is secreted, xyloglucan polymers are assembled and walls are built. However, if no new xyloglucan is supplied to the wall, XET activity could lead to rearrangements between, or degradation of, previously deposited xyloglucan. A reduction in the number of crosslinks would be predicted if XET activity were enhanced when walls are under tension during loosening and no nascent xyloglucan is available. Thus, specific wall modifications would be determined by a complex interplay between XET activity levels, concomitant secretion of nascent xyloglucan and the extent of wall loosening and tension.

XETs might also perform functions that are not related to cell wall structure. Xyloglucan is used as a storage polysaccharide in nasturtium seeds, where it accumulates to high levels. During germination, this xyloglucan reserve is hydrolysed and mobilized in the developing seedling[38]. The nasturtium seed XET can act as both a transglycosylase and, under some conditions, a xyloglucan hydrolase[13,38]. Therefore, this enzyme probably functions to release stored xyloglucan for seedling nutrition.

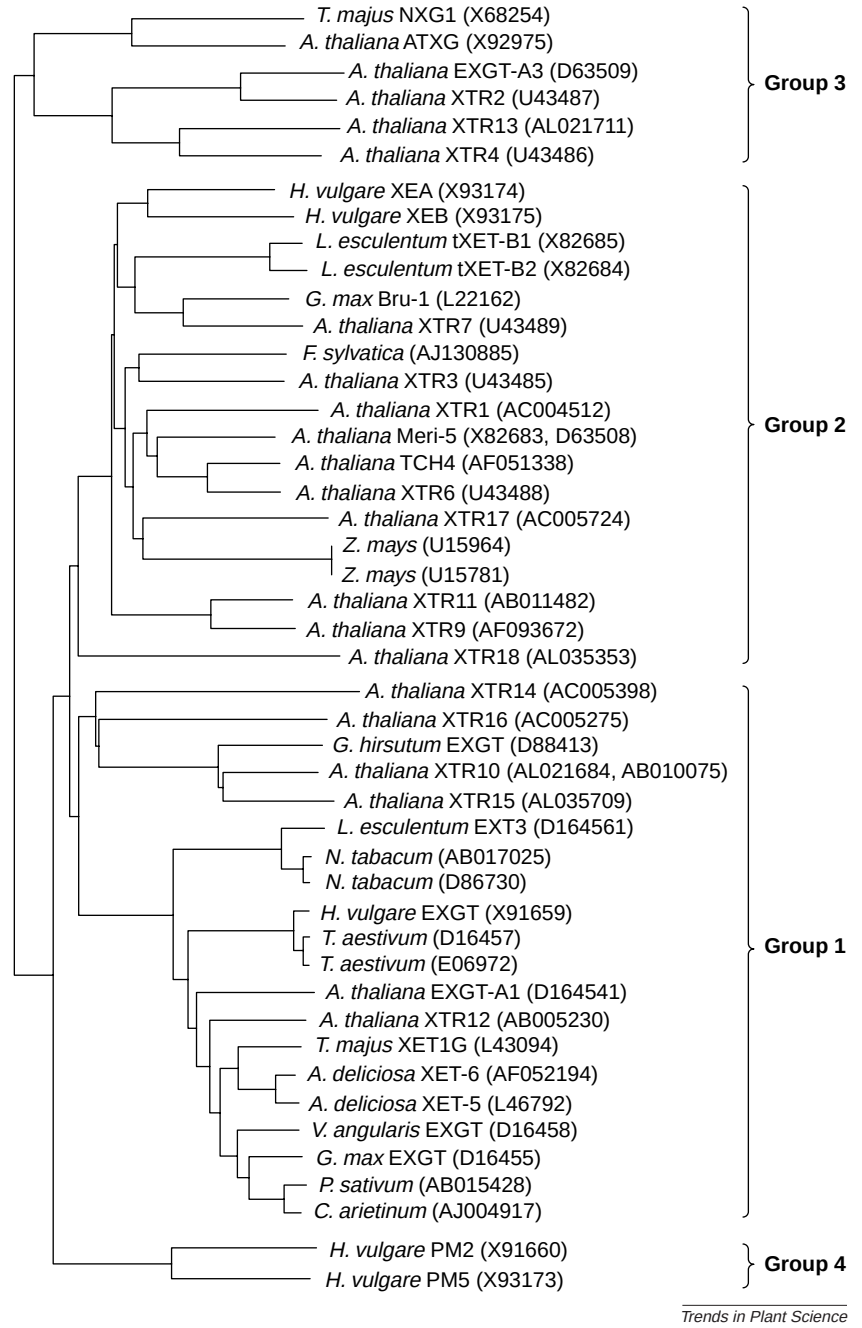

Fig. 2. The xyloglucan endotransglycosylase (XET) and *Arabidopsis* XET-related (XTR) family of proteins. This relationship tree was generated using the Clustal X and NJPLOT programs to align and analyse the proposed amino acid sequences (including putative signal peptides) encoded by the 46 identified *XET* and *XTR* genes. The 46 sequences can be loosely assembled into one minor and three major groups. The groups are numbered to maintain consistency with previous publications[22,24]. GenBank accession numbers are included with the names of the proteins.

XET gene families

The advent of the genome sequencing projects has revealed the presence of families of *XET*s and *XET*-related genes in plants. The high degree of sequence similarity between the predicted proteins suggests that they will have related biochemical functions. However, until enzyme activity is demonstrated, we refer to the *Arabidopsis XET*-related genes as *XTR*s (Ref. 24). The size of the family suggests that the *Arabidopsis* XETs and XTRs allow the plant to regulate xyloglucan modification in a complex fashion, either through differences in enzyme activities or gene regulation. A careful analysis of the individual genes and the encoded proteins will be necessary to understand the full significance of the large *XET* gene family.

Many genes potentially encoding XETs have been partially or fully sequenced from at least 14 different species (Fig. 2). As *XET* and *XTR* genes continue to be isolated, it is becoming clear that all plant species probably contain large families of genes related to *XET*s. Independent researchers and genome sequencing

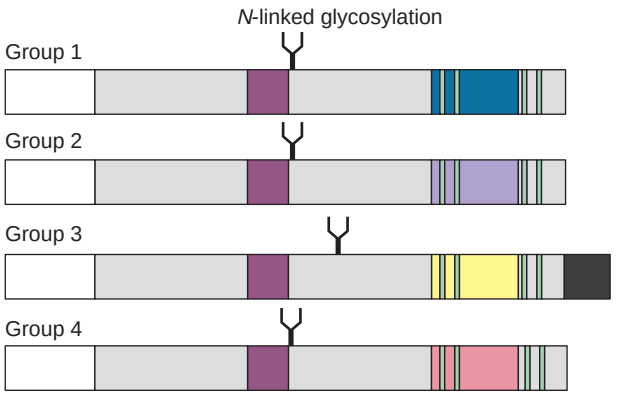

Fig. 3. The conserved features of the major groups of xyloglucan endotransglycosylase (XET) and *Arabidopsis* XET-related (XTR) proteins. All known XETs have (i) a hydrophobic amino terminus which probably functions as a signal peptide (white); (ii) the DEIDFEFLG region (purple) related to *Bacillus* β-glucanases; (iii) an *N*-linked glycosylation consensus site, and (iv) pairs of cysteines (green) in the carboxyl-terminal region. The carboxyl terminal portions show more divergence and permit the classification of an XET or XTR as a member of a particular group. The carboxyl-terminal extension (black) is unique to several members of the Group 3 XTRs, all from *Arabidopsis* (XTR2, XTR4, XTR13 and EXGT-A3). The importance of this carboxyl-terminal extension has not yet been determined. The divergent domains are indicated in yellow, violet, blue and purple.

projects have identified at least 21 *XET* and *XTR* sequences in the genome of *Arabidopsis* – and the genome has yet to be completely sequenced.

The 46 XETs and XTRs reported for 14 plant species (Fig. 2) share between 34–90% amino acid identity. Most sequence differences are found in the carboxyl terminal portion of the proteins, and based on the relatedness of these regions, the XETs and XTRs from many plant species can be loosely organized into four groups. Most strikingly, members of Group I [many called endoxyloglucan transferases, or EXGTs (Ref. 22; Fig. 2)] share high levels of sequence identity, from 70–89%, between different species of plants. This high sequence conservation among proteins from different species is not seen in the other groups, although it is found between pairs of proteins from the same species. Group I contains proteins from both monocots and dicots, which suggests that the precursor gene existed in the ancestor that gave rise to both monocots and dicots. This sequence conservation suggests that the proteins might carry out a similar function in different species, although xyloglucan makes up a substantially smaller fraction of the cell wall of grasses[5]. Interestingly, the greatest sequence divergences are found, at least to date, not between different species, but between pairs of proteins from a single species; for example, EXGT-A3 and XTR7, both from *Arabidopsis*, share only 34% identity. This suggests that two such divergent proteins have evolved distinct functions within a species.

The XET proteins are predicted to have several structural features in common: a putative signal peptide, a highly conserved domain similar to the active site of β-glucanases from *Bacillus* spp., the potential for *N*-linked glycosylation, and several cysteines that might form disulfide bridges (Fig. 3).

Although the amino termini of the XETs differ significantly in primary amino acid sequence, all are hydrophobic and potentially contain signal peptide leader sequences. When produced using recombinant baculovirus-infected insect cells, four *Arabidopsis* XETs (TCH4, Meri-5, EXGT and XTR9) are secreted into the cell culture medium, demonstrating that the proteins have functional signal peptides in a heterologous system[39,40]. Consistent with these results, TCH4-related antigens are detected in the cell wall of *Arabidopsis* (K. Vanden Bosch and J. Braam, unpublished). It is highly likely that these signal peptides will function in plants as well, and route the nascent proteins to the cell wall. A comparison of the *Arabidopsis* XETs indicates that the proposed mature proteins (without the signal peptides) show greater similarity compared with the amino acid sequences of the putative signal peptides[24]. It is likely, therefore, that evolutionary pressures, rather than recent gene duplications, have led to the conservation of sequences of the mature proteins.

A major feature of plant XETs and XTRs is an amino acid motif, DEIDFEFLG, which is shared with a region of the *Bacillus* β-glucanases. XETs, like β-glucanases, cleave β-1,4 linkages of polysaccharides with glucan backbones. The distinction between the two is that XETs specifically recognize xyloglucans and can also re-ligate the newly generated end. Several lines of evidence, including X-ray crystallography and site-directed mutagenesis studies, suggest that this sequence motif is within the active site of the β-glucanases[41–44]. The strong sequence similarity between the XETs and β-glucanases has led several groups to propose that this sequence might be critical for cleavage of β-1,4 linkages of the xyloglucan backbone by XETs. To test this possibility, site-directed mutagenesis was used to produce recombinant TCH4 XET in which the proposed catalytic glutamate residue was altered to glutamine (E97Q)[40]. As with the unaltered form of recombinant TCH4, the E97Q mutant was soluble and glycosylated, suggesting that it had been processed correctly. However, the E97Q mutation eliminated >98% of the enzymatic activity, demonstrating that this residue is essential for catalysis. This result is consistent with the proposal that this conserved domain might comprise part of the XET active site.

Several proteins [NXG1 (Ref. 45), XTR4 (Ref. 24), ATXG (Ref. 46), XTR10 (Ref. 47) and XTR13 (Ref. 48)] do not contain the highly conserved DEIDFEFLG sequence (Fig. 3). In all four proteins the isoleucine (I) residue is replaced by another hydrophobic residue, either leucine (L) or valine (V). Three of the four proteins substitute the first phenylalanine (F) with I. Although the substitutions are not strictly conservative, they maintain the apolar, uncharged nature of the residues. These changes might have no effect on the cleavage of β-1,4 linkages; the nasturtium seed XET, NXG1, in which the F to I substitution occurs, is an enzymatically active XET (Ref. 45).

Most of the *Arabidopsis* XET and XTR proteins and many XET-related proteins from other plant species contain at least one potential *N*-linked glycosylation signal (N-X-S/T) immediately following the conserved DEIDFEFLG motif (Fig. 3). For EXGT-A3, XTR2 (Ref. 24), XTR4 (Ref. 24) and XTR13 (Ref. 48), the consensus signal is shifted 15 residues towards the carboxyl terminus. ATXG lacks glycosylation consensus motifs[46], whereas TCH4 and XTR3 possess additional potential *N*-linked glycosylation sites in the carboxyl-terminal portions of the proteins[25]. The potential sites are probably recognized by the glycosylation machinery of the plant cell; several XETs have been purified from whole plant extracts using, as a key step, lectin-affinity chromatography that is specific for *N*-glycosylated proteins[10,14,45]. However, the importance of *N*-glycan moieties for XET activity is not clear. The state of glycosylation of TCH4 and Meri-5 produced in insect cells significantly influences the XET activity of these two enzymes: treatment of either protein with PNGase F, a glycosidase that removes *N*-glycans under mild conditions, eliminates enzyme activity[39,40]. Although the lack of activity of glycosidase-treated TCH4 or Meri-5 might reflect the PNGase F-mediated conversion of the glycosylated

asparagine residue to an aspartate, the decrease is more likely to be a result of the removal of the carbohydrate side chain. Treatment of TCH4 with Endo H, another bacterial glycosidase that removes all but one *N*-acetylglucosamine residue from the protein, reduces TCH4 activity by 50%, but without altering any of the amino acids[39]. By contrast, glycosidase treatment of EXGT and XTR9 produced in insect cells does not affect enzymatic activity[40].

There are four highly conserved cysteine residues in the carboxyl termini of the XETs and XTRs that have the potential to form two disulfide bonds, either inter- or intramolecularly (Fig. 3). Meri-5 and Bru-1 have only two cysteines with the potential to form one disulfide bond, whereas XTR9 and XTR11 have two additional cysteine residues (giving a total of six). However, in XTR9, the two additional cysteine residues are present in the amino terminal portion of the protein, and cleavage of the signal peptide would remove one of these residues: therefore, it is unlikely that this cysteine contributes to disulfide bond formation in the mature protein. XTR11 does have the potential to contain three disulfide bonds; the two additional cysteine residues are separated between the amino- and carboxyl-terminal portions of the protein. The TCH4 XET appears to contain at least one disulfide bond and reduction of this bond results in a significant decrease in activity[39].

SDS-PAGE analysis[39] and gel permeation chromatography indicate that the TCH4 XET behaves as a monomer in solution. Because of its relatively small size as a monomer, TCH4 and related XETs should be capable of diffusing through the small pores of the cell wall matrix that have been estimated to be between 6.5–10.0 nm (Refs 32,49). We would predict, therefore, that XETs have access to both the face of the wall in contact with the plasma membrane and the more distal regions of the wall. This localization would give the enzyme the opportunity to modify both newly deposited and older pre-existing wall components.

Sequence divergence among the XETs might be an indication of differences in function. Indeed, a comparison of *in vitro* activities among four *Arabidopsis* XETs revealed subtle differences in enzymatic properties[40]. The isozymes were found to have slightly different temperature and pH optima. In addition, the XETs differ in their abilities to utilize xyloglucan substrates that differ in sidechain substitution. Transglycosylation of xyloglucan by TCH4, for example, is not greatly affected by the presence of fucose side chains. By contrast, XTR9 is approximately four times more active against non-fucosylated xyloglucan. Such differential activities might have significant effects *in vivo*. Studies of *Zinnia* leaves suggest that most of the fucosylated xyloglucan is associated with epidermal and vascular tissues, which require high tensile strength, and are reduced or absent in regions with weaker cell-to-cell connections[2]. The fucose residues might be responsible, in part, for the increased tensile strength. Computer modeling[50], supported by *in vitro* binding assays[51], predicts that the fucosyl groups of xyloglucan might stabilize a xyloglucan conformation that can bind more tightly to cellulose. Plants might have distinct XETs that specialize in cell wall biogenesis during cell expansion, others that function subsequently to lock the wall in place, and some that help to disrupt cell-to-cell connections.

In addition, the evolution of a large *XET* gene family might reflect delegation to specific organs, tissues or cells. Different *XET*s or *XTR*s might be precisely regulated by developmental, hormonal or environmental cues. Subsets of the *Arabidopsis XET*s and *XTR*s show significant differences in total shoot mRNA accumulation by mechanical stimulation, darkness, temperature extremes, and the hormones auxin and brassinosteroids[24]. *TCH4* is unusual in that it is strongly upregulated by all the stimuli tested and therefore might have a specific role in responding to changing environmental conditions[23,24]. Regulation of this family can be complex; for example, in response to heat shock, distinct gene family members are either increased or decreased in expression levels with different response kinetics[24].

Analysis of the regulation of two of the *Arabidopsis* gene family members, *TCH4* and *Meri-5* with reporter gene fusions indicate that there is little overlap in the developmental expression of these genes. However, both *TCH4::GUS* and *Meri5::GUS* transgenes are expressed in lateral root primordia[24,52]. *Meri-5::GUS* expression occurs only at branch points above and below the emerging primordium, whereas *TCH4::GUS* expression is also detected within the primordium itself. Similarly, mutually exclusive, tissue-specific expression patterns have been reported for two XET-encoding genes of nasturtium[53].

Conclusion

*XET*s have evolved and diverged into a large gene family and are therefore likely to play an important role in modifying cell walls during plant growth and development. Over the next few years, several important steps need to be taken to elucidate the cellular and physiological functions of the enzymes. This includes an assessment of the enzymatic properties of the proteins, a determination of the differential regulatory properties of the genes, and the identification and characterization of mutants defective in distinct gene family members.

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