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In vitro activities of four xyloglucan endotransglycosylases from *Arabidopsis*

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Summary

Xyloglucan endotransglycosylases (XETs) are encoded by a gene family in *Arabidopsis thaliana*. These enzymes modify a major structural component of the plant cell wall, xyloglucan, and therefore may influence plant growth and development. We have produced four *Arabidopsis* XETs (TCH4, Meri-5, EXGT and XTR9) using the baculovirus/insect cell system and compared their biochemical activities. TCH4, as previously demonstrated, and the other three proteins are capable of carrying out transglycosylation of xyloglucans. The K_m for XLLGol acceptor oligosaccharide is in the range of 20–40 μM for all the XETs except XTR9, which has a K_m of 5 μM and is significantly inhibited by high levels of XLLGol. All four enzymes are most active between pH 6.0 and 6.5. TCH4 and XTR9 have temperature optima of 18°C, whereas Meri-5 and EXGT are most active at 28 and 37°C, respectively. Although the activity levels of three of the XETs are not influenced by the presence of fucose on the xyloglucan polymer, XTR9 has a clear preference for non-fucosylated xyloglucan polymer. The four XETs show a marked preference for XLLGol over either XXFGol or XXXGol as acceptor oligosaccharide. All four XETs are glycosylated; however, only the activities of TCH4 and Meri-5 are affected by the removal of the *N*-glycan with PNGase F. These four enzymes most likely function solely as transglycosylases because xyloglucan endoglucanase activity was not apparent. Subtle differences in biochemical activities may influence the physiological functions of the distinct XETs *in vivo*.

Introduction

Cell wall biogenesis and structure are likely to strongly influence plant cell shape and growth processes. Elucidation of how cell wall biogenesis occurs and is regulated and how structural alterations occur is therefore important for understanding plant morphogenesis. For example, plant cell expansion is controlled by regulated cell wall loosening which allows the turgor pressure of the protoplast to drive the cell to enlarge (Cosgrove, 1993). As the cell increases in size, new cell wall material is added to the existing wall to maintain wall thickness and strength (Cosgrove, 1997; Nishitani, 1995; Taiz, 1984). The wall alterations that occur during expansion are not yet fully elucidated, but this process most likely involves cell wall modifying enzymes (McCann and Roberts, 1994). Current models of the plant cell wall describe the primary wall as a series of intertwining networks of cellulose/hemicellulose, pectic polysaccharides, and proteins (reviewed in Carpita and Gibeaut, 1993; McCann and Roberts, 1994). One of the major dicot hemicelluloses, xyloglucan, is capable of creating molecular crosslinks between adjacent microfibrils through hydrogen bonds (Fry, 1989; Hayashi, 1989; McCann *et al.*, 1990; Passioura and Fry, 1992). Therefore, enzymes involved in xyloglucan modification may affect cell wall characteristics and, ultimately, cell shape and plant form.

Xyloglucan endotransglycosylases (XETs) split xyloglucan polymers endolytically and then rejoin one of the newly generated free ends with 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). XETs have been proposed to be involved in the process of cell expansion. In both maize roots and leaves, XET activity is highest in expanding regions; as the rate of expansion decreases, XET activity decreases but is still readily detectable (Palmer and Davies, 1996; Pritchard *et al.*, 1993). Application of the growth-promoting hormone gibberellic acid (GA3) induces the elongation of pea internodes, the hypocotyls of lettuce and cucumber, and barley leaves, with a concomitant increase in XET activity (Potter and Fry, 1993, 1994; Smith *et al.*, 1996). However, Xu *et al.* (1995) found a paradoxical pattern of expression for the *XET*-encoding gene *TCH4* in *Arabidopsis*. A 1 kb fragment of the *TCH4* genomic locus, including 958 bp 5′ to the putative transcription start site, is sufficient to drive expression of the *GUS* reporter gene in growing organs such as expanding leaves, emerging lateral root primordia, expanding trichomes, and in hypocotyls induced to elongate by low levels of light. Thus, *TCH4::GUS* expression correlates with cell expansion. However, *TCH4* mRNAs accumulate also in response to both mechanical perturbation and cold (Braam and Davis, 1990; Polisensky and Braam, 1996), treatments that result in reduced plant elongation. Based on these expression patterns, we have put forward a hypothesis that the TCH4 XET may function to incorporate nascent xyloglucan into walls and that this activity is recruited both in expanding walls and in walls

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being physically reinforced as a result of mechanical stress (Braam *et al.*, 1997; Xu *et al.*, 1996).

The genome of *Arabidopsis thaliana*, like that of many plants, encodes a family of XET and XET-related (XTR) proteins (Xu *et al.*, 1996). These include Meri-5, identified by its similarity to a gene expressed at high levels in cauliflower meristems (Medford *et al.*, 1991), and EXGT, isolated by its similarity to the azuki bean EXGT which exhibits XET activity (Okazawa *et al.*, 1993). XET-related genes isolated more recently in addition to sequences identified by the genome sequencing projects provide evidence of a XET family consisting of at least 16 members (Aubert and Herzog, 1996; Sato *et al.*, 1997; unpublished data from Bevan *et al.*, 1998; Nakamura, 1998; Okamoto *et al.*, 1995; Vysotskaia *et al.*, 1998). The high degree of sequence similarity of characterized members of this family, 34–87% identity at the amino acid level (Xu *et al.*, 1996), suggests that these proteins are likely to have related biochemical properties. However, the evolution of a large collection of related enzymes suggests the potential for diverse physiological functions. Small variations in primary amino acid sequence may alter specific properties of the XET activity encoded by each gene. For example, distinct XETs may function most efficiently under different physiological conditions, such as temperature extremes or cell wall pH. In addition, because xyloglucan polymers have heterogeneous patterns of specific sugar side-chain substitutions on the glucan backbone, distinct XETs may prefer particular xyloglucan substrates. Thus, the availability of specific substrates may determine which XET is functional. It is possible that some XETs may carry out hydrolysis of the xyloglucan, as has been demonstrated for nasturtium, azuki bean and kiwi fruit XETs (Edwards *et al.*, 1986; Fanutti *et al.*, 1993; Schröder *et al.*, 1998; Tabuchi *et al.*, 1997). In addition, independent regulation of expression of each gene by hormonal, developmental and environmental stimuli should allow the plant to tailor its response to a particular stimulus by recruiting or repressing specific XETs in limited regions of the plant (Xu *et al.*, 1996). Knowledge of the similarities and differences between these enzymes should shed light on the role of this gene family in plant growth and development. Therefore, we produced four XETs encoded by *Arabidopsis* using the baculovirus/insect cell expression system. The proteins, TCH4, Meri-5, EXGT and XTR9, were compared for K_m, pH optima, temperature optima, the need for glycosylation, and the ability to hydrolyse xyloglucan.

Results and discussion

Cloning and sequencing of XTR9

The *XTR9* cDNA was identified by screening the Genbank library of gene sequences with the coding region of the *TCH4* gene. Computer analysis of a partial cDNA sequence suggested that this gene had not been identified previously as a potential *XET*-related (*XTR*) gene. Sequencing of the full-length cDNA revealed that the encoded protein is significantly similar to the TCH4 XET and other XTRs (64% identical to TCH4, excluding the putative signal peptides; data not shown). For comparison, Meri-5 and EXGT share 75% and 50% amino acid identity with TCH4. Like other known *XTR*s, *XTR9* is predicted to encode a signal peptide, an *N*-linked glycosylation motif, four conserved cysteine residues (in addition to two non-conserved cysteines), and a region of similarity to β-glucanases of *Bacillus* spp., thought to be critical for catalytic activity (Campbell and Braam, 1998; de Silva *et al.*, 1993; Nishitani, 1995, 1997; Okazawa *et al.*, 1993; Xu *et al.*, 1996). The *XTR9* sequence has been allocated Genbank accession no. AF093672.

Heterologous expression of XTRs in Sf9 insect cells

To characterize the similarities and differences between the XTRs, we produced forms of TCH4, Meri-5, EXGT and XTR9 in insect cells. Recombinant baculoviruses harbouring each individual gene were generated by homologous recombination with appropriate transfer vectors (see Experimental procedures). The transfer vectors were designed to produce proteins with primary amino acid sequences identical to those encoded by the native genes in *Arabidopsis*; however, it is possible that there are species-specific differences in co- and/or post-translational modifications. Four days after inoculation of Sf9 insect cells with recombinant baculoviruses, the medium of each infected culture was examined for the presence of XTR protein by Western analysis with an anti-TCH4 antibody and XET activity assay. Western analysis verified the presence of a protein that reacts with the anti-TCH4 antibody in cultures expressing *TCH4*, *Meri*-5, *EXGT* and *XTR9* (data not shown). XET activity was readily detected in either the culture medium of infected cells or in partially purified enzyme preparations from the culture medium (see below); therefore each of these proteins is an XET and will be referred to as an XET, as opposed to XTR, for this report. XET activity and antigens capable of binding to the anti-TCH4 antibody are absent from insect cell cultures infected with a virus expressing β-glucuronidase (Campbell and Braam, 1998). XETs were extracted from the cell culture media by binding to ConA–Sepharose (see Experimental procedures). XET protein comprises approximately half of the total protein in the ConA eluate (data not shown). Although it is possible that other components in this eluate affect aspects of XET activity, we have found that activities of highly purified TCH4 and Meri-5 are indistinguishable from activities of these proteins in the ConA eluate (unpublished results).

Enzymatic properties of XET proteins

Determination of K_m values. We compared the activities of the four proteins towards a standard set of substrates, tamarind xyloglucan polymer and [^{3}H]-labelled xyloglucan oligosaccharide, XLLGol. The oligosaccharide, XLLGol, was composed of four β-1,4-linked glucose moieties, the first bearing a single xylose (X), the second and third bearing xylose–galactose disaccharides (L) and the fourth remaining unsubstituted (Gol) (see Figure 4 and Fry et al., 1993; for a fuller explanation of oligosaccharide nomenclature). Increasing amounts of unlabelled XLLGol were added to a constant amount of other reactants: [^{3}H]-XLLGol (1.5 KBq), tamarind xyloglucan polymer, and partially purified XET protein. After the data were corrected for changes in specific activity of the XLLGol, the K_m values were calculated from Lineweaver–Burke plots. The estimated K_m values for TCH4, Meri-5 and EXGT are all within the range 20–50 μM (Figure 1). Meri-5 and EXGT are slightly inhibited by high concentrations of acceptor XLLGol in the reaction, as reflected by the decrease in reaction rates at high XLLGol concentrations. XTR9, in comparison, shows significant substrate inhibition at high concentrations of XLLGol, in addition to a much higher affinity for XLLGol than the other XETs. The K_m for XTR9, estimated from assays performed at 1.5–20 μM XLLGol, is approximately 5 μM. This represents a lower limit for the value; the substrate inhibition prohibits a direct and accurate assessment of the K_m value. The K_m values of all four proteins for XLLGol are much lower than the estimated concentration of xyloglucan present in the cell walls (approximately 500 μM; Thompson and Fry, 1997). Thus, because the concentration of substrate greatly exceeds the K_m of the enzyme, transglycosylation would be expected to be controlled by regulation of the availability of active enzyme in the cell wall rather than restriction of substrate availability.

pH optima. The pH of the plant cell wall is estimated to be pH 5.5–6.0. Changes in wall pH can have profound effects on cell wall properties, possibly in part through pH regulation of cell wall enzymes. Therefore, we assayed the pH sensitivity of the activity levels of the four XETs in McIlvaine's citrate–phosphate buffers (Elving *et al.*, 1956) ranging from pH 3.5 to 8.0 (Figure 2). All four XETs show similar pH activity profiles, with highest activity at the physiological pH of the cell wall. XTR9 is most tolerant of low pH; it retains approximately 35% of its maximal activity at pH 4.5. TCH4 and Meri-5, in contrast, exhibit only 10–15% of maximal activity at this pH extreme. Meri-5 and TCH4, however, retain more activity at pH 8.0, demonstrating approximately 65% of maximal activity, whereas XTR9 is only 30% as active at pH 8.0 compared with pH 6.0. In contrast to the broad pH optima of the TCH4, Meri-5 and XTR9 enzymes, EXGT shows a very sharp pH sensitivity, with maximal activity at approximately pH 6.0. Studies of azuki bean EXGT reveal a similar pH profile (Nishitani *et al.*, US Patent 5 516 694, May 1996). This difference between EXGT and the other three enzymes may reflect the contribution of an amino acid substitution near the proposed active site; there is a strongly conserved sequence motif of XETs that is shared with the *Bacillus* β-glucanases: DEIDFEFLG. The first glutamate residue of this motif is essential for catalytic activity in both the β-glucanases and TCH4 (Campbell and Braam, 1998; Høj *et al.*, 1992; Keitel *et al.*, 1993; Planas *et al.*, 1992). X-ray crystallographic studies of one β-glucanase (Keitel *et al.*, 1993) identified hydrogen bonds between this residue (the equivalent of E97 of TCH4) and the indole nitrogen of a nearby tryptophan and a side-chain oxygen of the second aspartate of the conserved motif. TCH4, Meri-5 and XTR9 have all retained this tryptophan residue (W95 of TCH4), but in the EXGTs from *Arabidopsis* and azuki bean the tryptophan is replaced with a histidine (H). The nitrogen of the H side-chain may functionally replace the indole nitrogen of the tryptophan in hydrogen bonding to E97. As the pH rises above the pK_a for the H nitrogen (pK_a = 5.6, although this may be altered by the charges present in the local environment), it could disrupt this hydrogen bond and the proper positioning of amino acids in the proposed catalytic domain. This could account for the rapid loss of activity of both EXGT proteins when incubated in buffers higher than pH 6.0. The lack of acid-activation of XETs *in vitro* suggests that acid growth is not regulated through enhanced XET activity at low pH; instead, it would be predicted from these data that low pH would limit XET function *in vivo*.

Temperature optima. Plants are capable of continual growth even when subjected to temperature extremes. How growth is maintained under these conditions is not known. One possibility is that growth-controlling enzymes are unusually temperature-tolerant. We investigated the temperature sensitivities of the four XET proteins within the range of –5 to 45°C (Figure 3). TCH4 and XTR9 are active over a broad range of temperatures; optimal activity levels are detected at 18°C. In contrast, EXGT and Meri-5 show higher temperature preferences and are most active at 37 and 28°C, respectively. These two enzymes retain high activity (approximately 80% of maximum) at 45°C, whereas TCH4 and XTR9 activity levels are reduced by 55–60%. All four proteins are markedly cold-tolerant and retain activity at temperatures as low as –5°C, albeit at a significantly reduced level. The activity profile at different temperatures of baculovirus-produced TCH4 differs from that of recombinant TCH4 produced in bacterial cells (Purugganan *et al.*, 1997). Not only is the baculovirus-produced protein much more active (Campbell and Braam,

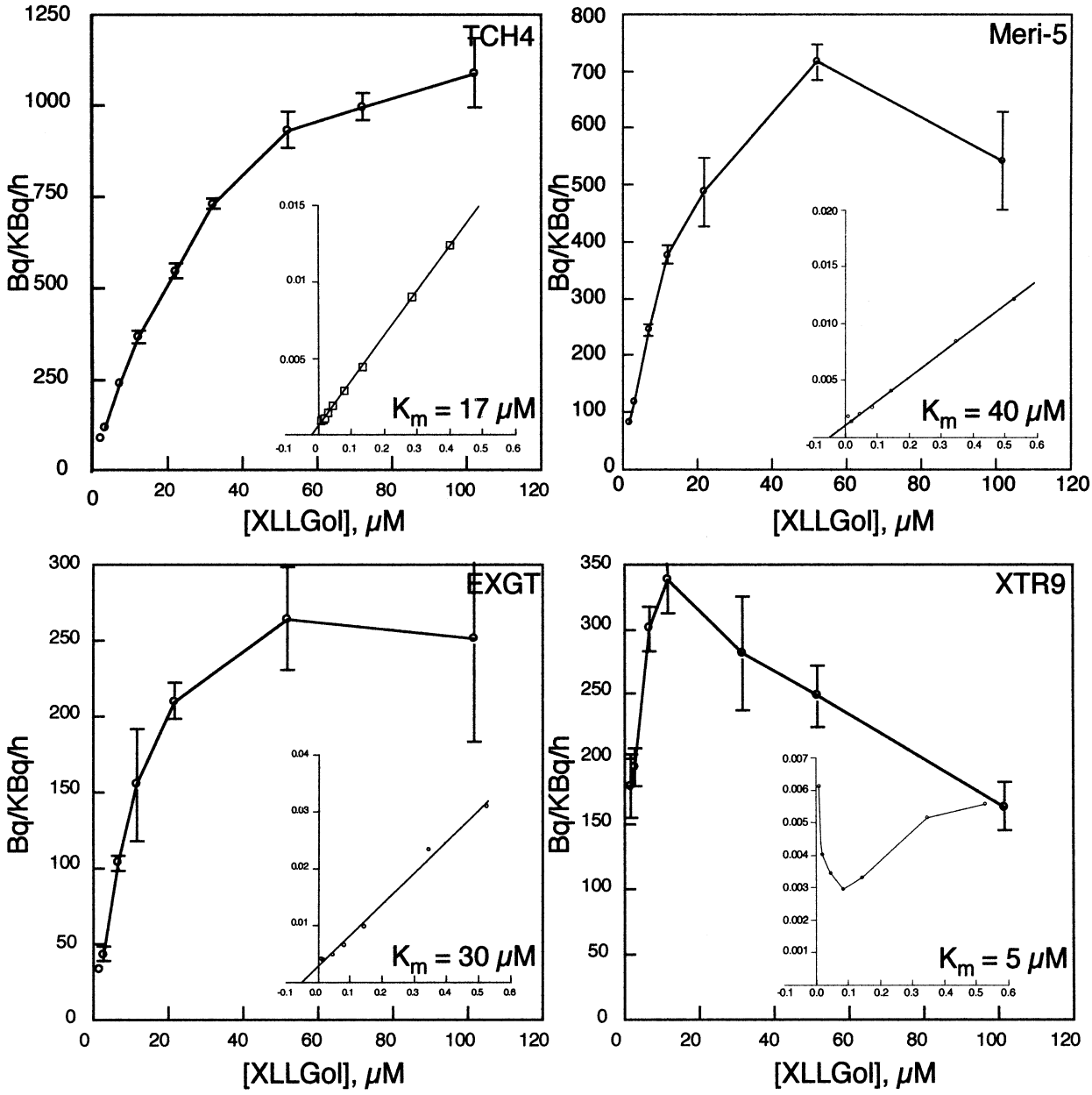

Figure 1. Determination of K_m for XLLGol.
Unlabelled XLLGol was added in increasing concentrations to the reaction with a fixed concentration of [^{3}H]XLLGol of 1.9 μM. XET activities were determined as detailed in Experimental procedures and are reported as Bq of [^{3}H]-xyloglucan produced per KBq of [^{3}H]XLLGol supplied per h of reaction time. The results were adjusted for the change in specific activity of the oligosaccharide. K_m values were estimated from Lineweaver–Burk plots (insets). The enzyme for each experiment is indicated in the upper right corner of each panel. All reactions were performed in triplicate and are reported as the mean ± standard deviation. Where an error bar is absent, the margin of error is smaller than the symbol.

1998), but it is also more active at high temperatures. As demonstrated previously (Campbell and Braam, 1998), co- and post-translational eukaryotic-specific modifications of the TCH4 XET are critical for optimal XET activity; these modifications may also be essential for structural stability at high temperatures.

Substrate preferences. Xyloglucan is a well-defined polysaccharide composed of a β-1,4-linked glucan backbone; typically, about 70% of the glucose residues bear side-chains of xylose (abbreviated X), xylose and galactose (abbreviated L), or xylose, galactose and fucose residues (abbreviated F). Individual molecules may be quite heterogeneous. To determine whether substrate structures differentially affect XET function, we assessed the effects of different substituent xyloglucan side-chains on the rates

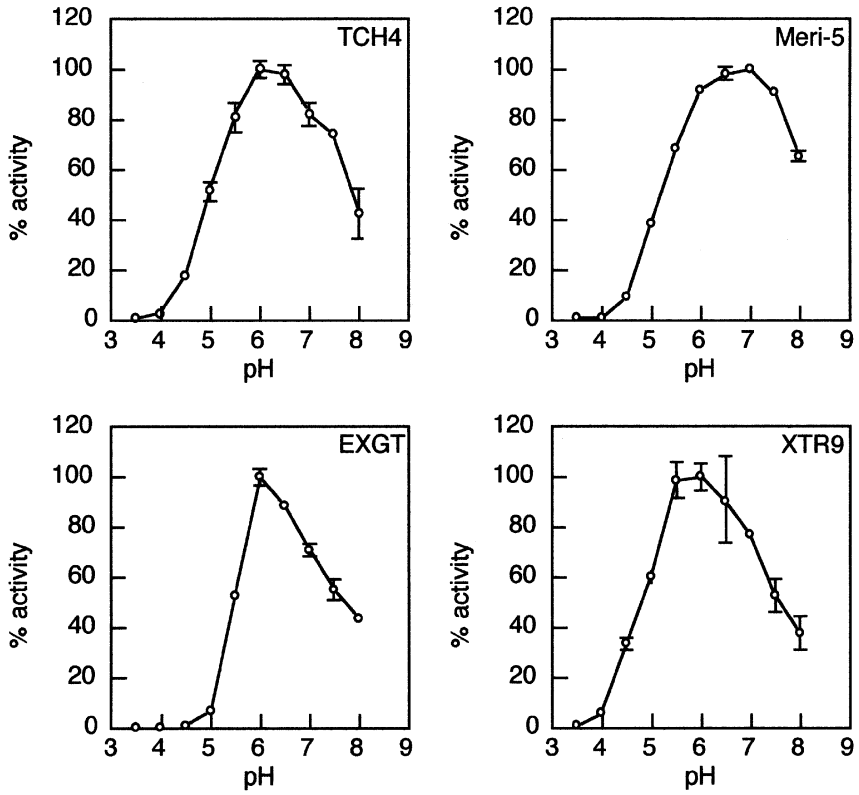

Figure 2. The pH dependence of XET activity on [^{3}H]XLLGol and tamarind xyloglucan.
XET assays were performed in McIlvaine's citrate–phosphate buffer adjusted for constant ionic strength (Elving *et al.*, 1956). The enzyme for each experiment is indicated in the upper right corner of each panel. Activity was measured as Bq of [^{3}H]-xyloglucan formed per KBq of [^{3}H]XLLGol supplied in a 1 h reaction. The results are expressed as a percentage of the maximum activity attained. All reactions were performed in triplicate and are reported as the mean ± standard deviation. Where an error bar is absent, the margin of error is smaller than the symbol.

at which the XETs catalyse transglycosylation. The reaction velocities of the enzymes were measured using two different donor xyloglucan polymers (without fucosyl residues, from tamarind seed; and with fucosyl residues, from pea) in combination with three different acceptor oligosaccharides with differing patterns of sugar side-chains: XXXGol, XLLGol and XXFGol (see bottom of Figure 4 and Fry *et al.*, 1993; for full explanation of nomenclature). For all four XETs, the highest reaction velocities are consistently achieved with tamarind xyloglucan polymer and XLLGol (Figure 4). However, both TCH4 and EXGT show only slight preferences between tamarind and pea xyloglucan polymers, indicating that for these two enzymes, variations in the donor polysaccharide do not strongly influence the enzyme reaction rate. For Meri-5, the preferred xyloglucan polymer substrate varies with the acceptor oligosaccharide. When XLLGol is used as an acceptor, Meri-5 prefers tamarind xyloglucan polymer. No xyloglucan polymer bias is detected with XXFGol as the substrate, whereas Meri-5 is somewhat more efficient at carrying out transglycosylation between XXXGol and pea xyloglucan polymer than between XXXGol and tamarind xyloglucan. In contrast to the three other XETs, XTR9 shows distinct substrate preferences. It is clearly more efficient at cleaving tamarind xyloglucan polymer, regardless of the acceptor oligosaccharide used in the assay. The fucosyl residues are predicted to alter the conformation of the xyloglucan polymer (Levy *et al.*, 1991, 1997); this altered conformation may reduce XTR9 binding. The other XETs may be less sensitive to this change in conformation.

The structure of the acceptor molecule, the oligosaccharide, greatly affects XET activity. All four enzymes achieve the highest reaction rates with XLLGol as an acceptor. The substitution of XXFGol reduces the activities of TCH4, Meri-5 and EXGT by 70–75%; XXXGol is more effective than XXFGol as a substrate for these three enzymes, but not as effective as XLLGol. Like the other three XETs, XTR9 exhibits its highest activities with tamarind xyloglucan and XLLGol as substrates. However, it is more effective at utilizing XXFGol as an acceptor molecule than XXXGol.

Studies of zinnia leaves suggest that most of the fucosylated xyloglucan is associated with epidermal and vascular tissues; tissues which require high tensile strength. In contrast, fucosylated xyloglucan is reduced or absent in regions with weaker cell walls and cell–cell connections (McCann and Roberts, 1994). When zinnia mesophyll cells are cultured *in vitro*, they do not produce fucosylated xyloglucan until they have finished expanding (McCann and Roberts, 1994). Immunolocalization of XETs in developing leaves of *Arabidopsis* shows a strong accumulation of XET epitopes between rows of cells where it is predicted that airspace formation would soon occur (Antosiewicz *et al.*, 1997). This pattern of localization

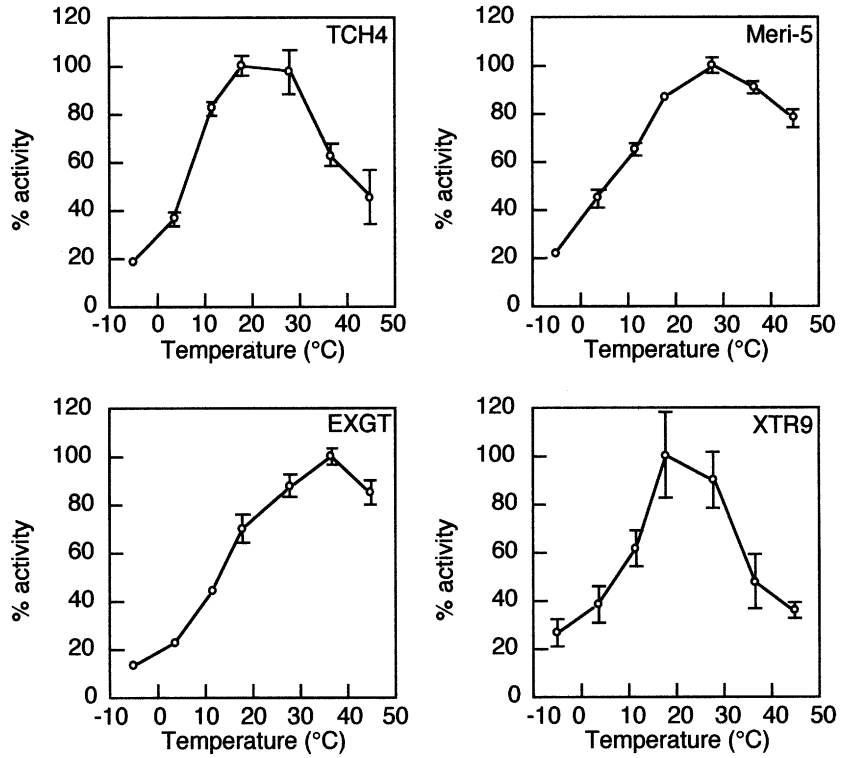

Figure 3. The temperature dependence of XET activity on [^{3}H]XLLGol and tamarind xyloglucan.
XET assays were performed in 100 mM sodium citrate/100 mM sodium phosphate buffer, adjusted to pH 6.0. The enzyme for each experiment is indicated in the upper right corner of each panel. Activity was measured as Bq of [^{3}H]-xyloglucan formed per KBq of [^{3}H]XLLGol supplied in a 1 h reaction. The results are expressed as a percentage of the maximum activity attained. All reactions were performed in triplicate and are reported as the mean ± standard deviation. Where an error bar is absent, the margin of error is smaller than the symbol.

combined with differential enzyme preferences for fucosylated substrates may reflect roles for XETs in the generation of intercellular spaces in mesophyll cell layers. The structure of the xyloglucan and the selective recruitment of XETs may be important for the limited cell wall degradation that must occur for this morphogenetic event in leaf development.

Effects of glycosylation. Asparagine-(*N*-)linked glycosylation serves many functions in glycoproteins, such as protection from proteolysis, targeting the protein to specific subcellular locations, protein–protein interactions, and cell–cell adhesion (Rayon *et al.*, 1998). The *N*-linked glycan can also affect the biological activity of the glycoprotein, possibly by influencing nascent protein folding, either during synthesis or post-translationally (Rayon *et al.*, 1998). To determine whether the presence of *N*-linked glycans is important for enzymatic activity of *Arabidopsis* XETs, we removed the glycan moieties and assayed activity levels. As previously demonstrated, removal of the *N*-linked glycan of TCH4 with the glycosidase PNGase F eliminates nearly all XET activity of TCH4 (Campbell and Braam, 1998). Meri-5, EXGT and XTR9 can be partially purified from infected cell culture medium using Con-A–Sepharose (data not shown), indicating that all three proteins are *N*-glycosylated during synthesis. We treated Meri-5, EXGT and XTR9 with the glycosidase PNGase F at 37°C for 2 h. Following treatment, the proteins were separated on a 12% acrylamide/SDS gel. Western analysis of the resulting gel shows that the mock-treated proteins (lanes marked '–') migrate at higher apparent molecular weights than PNGase F-treated proteins, verifying that all three proteins are glycosylated and the sugar moieties are efficiently removed by PNGase F (Figure 5a; data for TCH4 consistent with those shown in Campbell and Braam, 1998 are provided for comparison). Meri-5 is similar to TCH4 in that treatment of Meri-5 with PNGase F eliminates XET activity (Figure 5b). This loss of activity is probably the result of loss of the glycan; however, PNGase F also changes the linked asparagine residue to aspartate. This amino acid substitution could be responsible for the alteration in activity. This explanation, however, is unlikely to be true at least for the TCH4 protein because treatment with another glycosidase, Endo H, removes all but one sugar residue without affecting the polypeptide chain and significantly reduces XET activity (Campbell and Braam, 1998). The activities of XTR9 and EXGT, however, are not dependent on the presence of N-linked glycans; mock- and PNGase F-treated XTR9 have equivalent levels of XET activity, while glycosidase-treated EXGT retains 91% of its full activity. It is not clear why the activities of TCH4 and Meri-5 are drastically affected by glycosidase treatment and those of XTR9 and EXGT are not. Removal of the *N*-glycan may destabilize TCH4 and Meri-5, leading to unfolding, or the carbohydrate side-chain may be more directly involved in enzymatic function, for example by properly positioning the ligand in the active site of the enzyme (Campbell and Braam, 1998). These data indicate

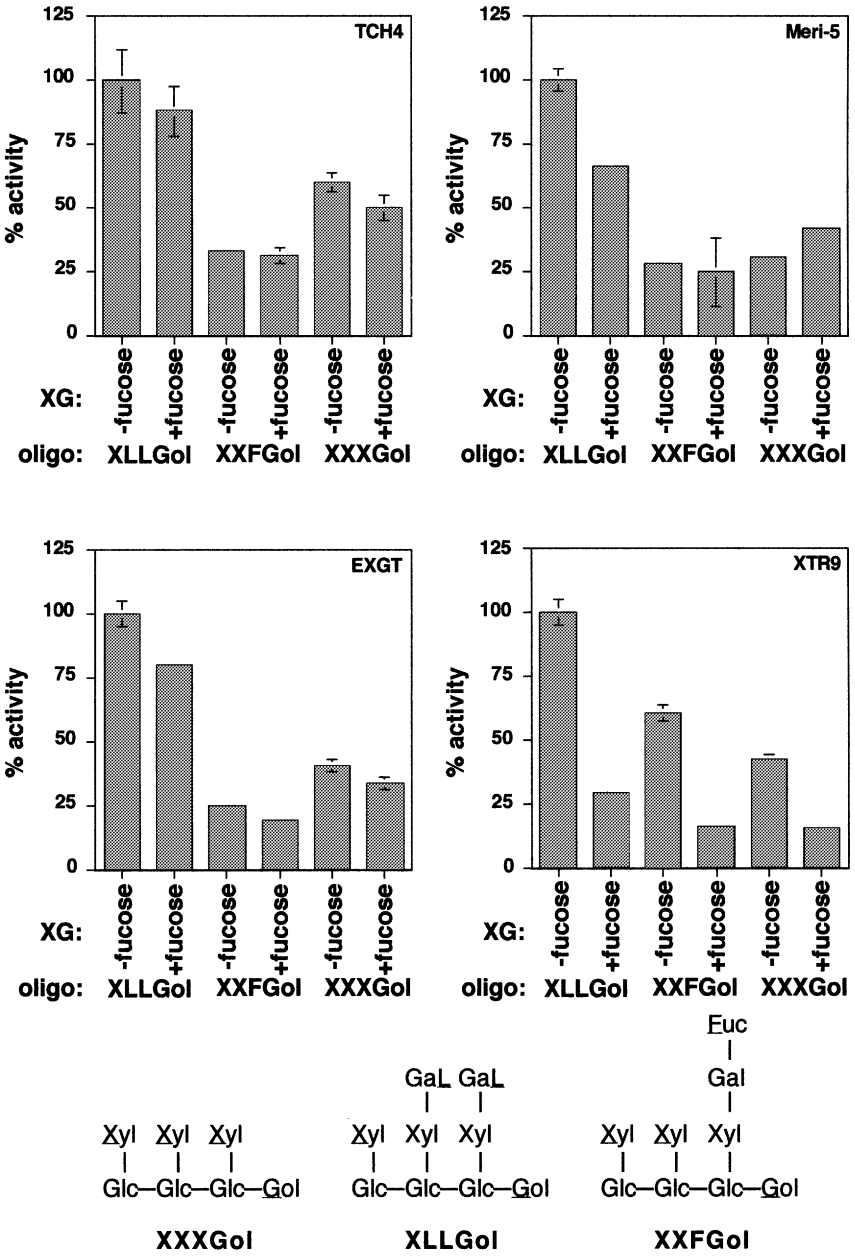

Figure 4. Substrate preferences.
XET assays were performed with either pea xyloglucan polymer (+ fucose) or tamarind xyloglucan polymer (– fucose) and [^{3}H]-labelled XLLGol, XXFGol or XXXGol, as indicated below each panel. The enzyme for each experiment is indicated in the upper right corner of the panel. Schematic structures of each oligosaccharide are shown at bottom (Glc, β-D-glucose; Xyl, α-D-xylose; Gal, β-D-galactose; Fuc, α-L-fucose). Rates of [^{3}H]-oligosaccharide incorporation into xyloglucan polymer were measured over a 1.5 h period. The results are expressed as a percentage of the maximum rate of incorporation attained. All reactions were performed in triplicate and are reported as the mean ± standard deviation. Where an error bar is absent, the margin of error is smaller than the symbol.

that the function of glycan moieties on TCH4 and Meri-5 is most likely not solely in aiding co-translational folding because mature, presumably fully folded proteins are dramatically altered in their activity by removal of the oligosaccharides. Amino acid sequence differences between the XTR9 and EXGT proteins and TCH4 and Meri-5 proteins must be responsible for this difference in the requirement of the presence of oligosaccharides for activity.

Endoglucanase activity. Endoglucanase activity, cleavage of a xyloglucan polymer endolytically without re-ligation, has been detected for XETs purified from nasturtium, azuki bean and kiwi fruit (Edwards *et al.*, 1986; Fanutti *et al.*, 1993; Schröder *et al.*, 1998; Tabuchi *et al.*, 1997). Although endoglucanase activity could be involved in cell wall loosening for cell expansion, the nasturtium and kiwi fruit XET endoglucanase activities are more likely involved in other physiological processes. The nasturtium XET is from seeds; this XET may therefore participate in degradation of stored xyloglucan polymer, enabling mobilization of carbohydrate reserves for seed germination (Edwards *et al.*, 1986). The kiwi fruit XET, purified from ripening fruit, may be involved in the cell wall changes that occur during fruit softening (Schröder *et al.*, 1998). Endoglucanase activity is expected to result in cell wall alterations

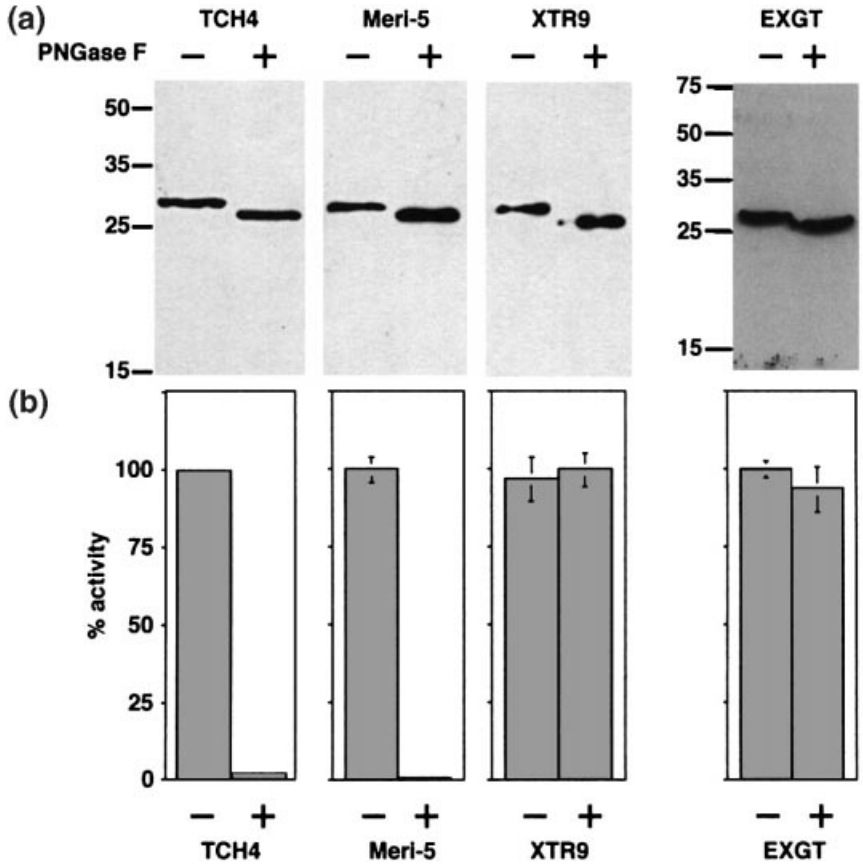

Figure 5. Effects of glycosylation on XET activity.
(a) Aliquots of each isozyme were incubated in the absence ('–') or presence ('+') of the glycosidase PNGase F. Samples were analysed by Western blot with anti-TCH4 antibody to verify the complete removal of carbohydrate side-chains. Molecular mass markers are indicated on the left.
(b) Aliquots of each mock- and glycosidase-treated sample were assayed for XET activity, measured as Bq of [^{3}H]-xyloglucan formed per KBq of [^{3}H]XLLGol supplied in a 1 h reaction. The results are expressed as a percentage of the maximum incorporation attained. All reactions were performed in triplicate and are reported as the mean ± standard deviation. Where an error bar is absent, the margin of error is smaller than the symbol.

distinct from those of transglycosylation; to gain insight into the possible effects of *Arabidopsis* XETs on cell walls, we tested each for the ability to hydrolyse xyloglucan polymer.

Individual xyloglucan molecules can be very long, up to 400 nm (McCann and Roberts, 1994). Purified xyloglucan in solution is therefore quite viscous. To detect endoglucanase activity, we monitored the decrease in viscosity of a xyloglucan solution in the presence or absence of XLLGol oligosaccharide (as described by Lorences and Fry, 1993). Transglycosylation of the very short oligosaccharide onto the long polymers preferentially shortens the average polysaccharide chain length in the xyloglucan solution, resulting in decreased viscosity. As shown in Figure 6, partially purified samples of all four *Arabidopsis* XETs rapidly catalyse the depolymerization of xyloglucan in the presence of XLLGol oligosaccharide, as expected for XET enzymes. Within 3 h, all four XETs have reduced the viscosity of the xyloglucan solution to 15–30% of the initial value. However, only a small decrease in viscosity, approximately 25%, is detected in the absence of oligosaccharide. A decrease in viscosity is also apparent using comparable extracts from insect cells infected with a baculovirus that does not produce an XET (data not shown). We conclude therefore that the *Arabidopsis* XETs do not have prominent endolytic activities but instead predominantly carry out transglycosylation of xyloglucan polymers. Endoglucanase activity may be restricted to XET isozymes involved in the specialized processes of seed germination and fruit ripening.

Conclusion

Meri-5, EXGT and XTR9 all exhibit xyloglucan endotransglycosylase activities and therefore most likely function as XETs *in vivo*. We demonstrated that the four XETs from *Arabidopsis* characterized to date are similar in their enzymatic properties; however, each enzyme does possess unique characteristics of pH, temperature and substrate preference. These four enzymes represent a subset of the known XETs and XET-related proteins in *Arabidopsis*. Our data indicate that the differences in amino acid sequence among these four XET isozymes do not greatly affect their enzymatic activities. We predict therefore that the evolution of closely related enzymes must reflect a critical role for this enzymatic activity in plant development or physiology. This characterization of the enzymatic activities combined with analyses of the expression patterns and phenotypic consequences of mutation of individual members of this protein family will continue to

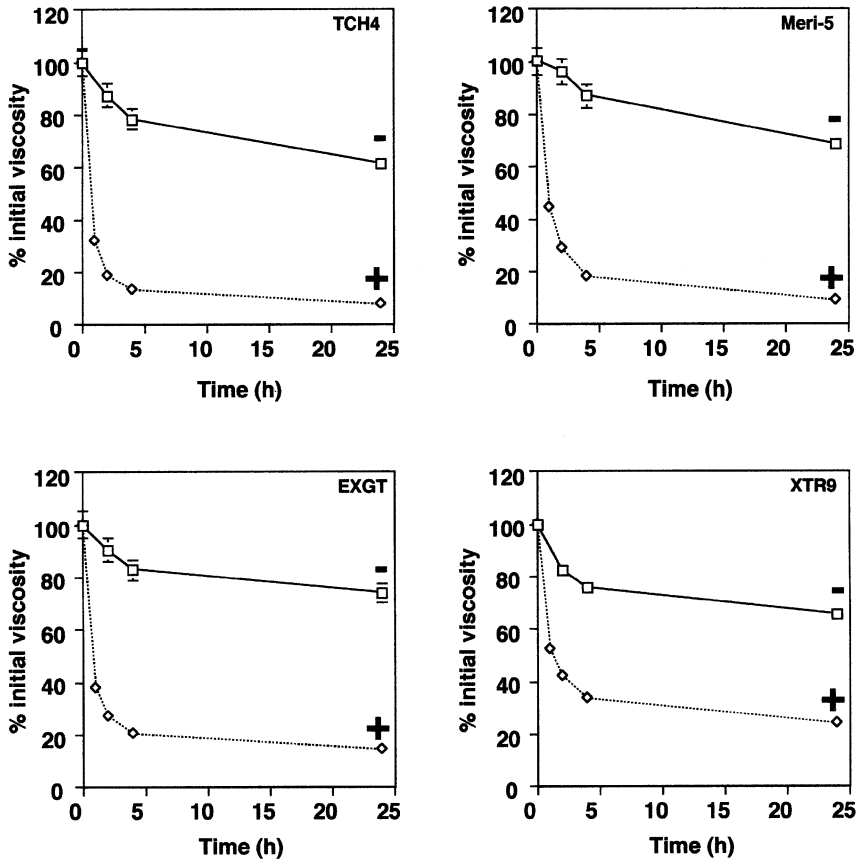

Figure 6. Assays for glucanase activity.
The loss of viscosity of a solution of tamarind xyloglucan was measured in the presence (dotted line with diamonds; '+') or absence (solid line with squares; '–') of 30 μM XLLGol. The enzyme for each experiment is indicated in the upper right corner of the panel. Results are reported as a percentage of the initial viscosity. All reactions are reported as the mean ± standard deviation. Where an error bar is absent, the margin of error is smaller than the symbol. Each experiment was replicated 3–5 times.

elucidate the role of XETs in plant growth and development.

Experimental procedures

Insect cell culture

Sf9 insect cells adapted to grow in SF900-II serum-free medium were obtained from Life Technologies (Bethesda, Maryland, USA). Cells were maintained as adherent monolayer cultures in tissue culture flasks. When cells reached confluency (approximately 2×10^6 cells ml^{-1}), the cultures were diluted and reseeded in new flasks at 5×10^5 cells ml^{-1}. All cultures were maintained at 28°C in SF900-II serum-free medium (Life Technologies).

Generation of transfer vectors and recombinant baculoviruses

TCH4 protein was produced as previously described (Campbell and Braam, 1998). A cDNA encoding *Meri*-5 was isolated from the I-YES *Arabidopsis* cDNA library (Elledge *et al.*, 1991) using a hybridization probe against the *Meri-5* coding region and standard phage library manipulations (Sambrook *et al.*, 1989). The hybridization probe was generated by PCR amplification of a genomic fragment of *Meri*-5 using the primers 5′-TCCGAAG-GATCCACTTGGGATGAG-3′ and 5′-GAGTCTAGCTTGGCTTGTT-GATCC-3′. The phage containing a *Meri*-5 cDNA was converted into a plasmid (Elledge *et al.*, 1991) to generate plasmid pAK1. To create a transfer vector for the production of recombinant protein, the *Meri*-5 cDNA was cloned as an *Eco*RI fragment into the same site of pVL1392 (pBAC-Meri5). To produce an *EXGT* transfer vector, the *Sal*I/*Not*I fragment of EST clone 114A14T7 (containing the entire *EXGT* coding region) was cloned into the same sites of pBACgus-1 (pBAC-EXGT).

A partial sequence of *XTR9* was identified as the *Arabidopsis* EST 165P23T7 (Höfte *et al.*, 1993) by its similarity to *TCH4*. The approximately 1 kb *Eco*RI/*Not*I fragment of EST 165P23T7, containing the *XTR9* cDNA, was ligated into the same sites of Bluescript II KS (+) (Stratagene) to generate pBS-XTR9 and into pBACgus-1 (Novagen, Madison, Wisconsin, USA) to create pBAC-XTR9. Both strands of pBS-XTR9 were sequenced using an ABI Prism Sequencer with universal T3 and T7 primers and two internal primers, 5′-CGTCTCTGGTTCGATCC-3′ and 5′-CTTCTG-TAGCCCAGTCATCG-3′.

Recombinant protein production and purification

Recombinant baculoviruses were produced by co-transfection of BacVector-2000 linearized baculoviral genomic DNA (Novagen) with the transfer vectors pBAC-Meri5, pBAC-EXGT and pBAC-XTR9 by co-transfection (following the manufacturer's protocol). After 4 days, the insect cell culture medium was recovered as a source of recombinant virus. Sf9 insect cells were seeded at a density of 5×10^5 cells ml^{-1}. After allowing the cells to adhere to the flask for 1 h, the appropriate recombinant virus was added at approximately a 1:1000 dilution. Three days after infection, the Sf9 cells and culture medium were separated by low-speed

centrifugation (500 *g*) in a table-top centrifuge. The culture medium was used both as a source of amplified virus for subsequent infections and as starting material for protein purification and XET assays.

To increase the yield of EXGT protein, the recombinant *EXGT* baculovirus was subjected to three rounds of plaque purification (Lalumière and Richardson, 1995). X-Gluc, at a concentration of 25 μg ml^{-1}, was included in the agarose overlays to facilitate the identification of individual plaques. The isolated virus was amplified to produce a high-titre master stock following the BacVector-2000 protocols (Novagen). This high-titre stock was used at a 1:1000 dilution for the production of recombinant protein.

For partial purification of recombinant proteins, 100 ml of culture medium from infected cells were dialysed three times for 4 h against 4 litres of Con A column buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 mM Tris–HCl, pH 7.5) at 4°C. The dialysed culture medium was passed over a 500 μl concanavalin A–Sepharose CL-4B column (Sigma, St Louis, Missouri, USA) equilibrated in Con A buffer. After washing the column with 5 ml of Con A buffer, all bound proteins, including the XETs, were eluted with 0.5 α-methyl-D-mannoside (Sigma) in Con A buffer.

XET activity assays

Assays were performed as described previously (Campbell and Braam, 1998). Briefly, XET isozymes were incubated with xyloglucan polymer and tritiated acceptor oligosaccharide at room temperature (except where noted) for 1–2 h. The reactions were stopped by the addition of 70% formic acid, spotted onto 3 MM Whatman paper, and washed in running water to remove unincorporated oligosaccharides. The paper was then dried and scintillation counted. All results are the mean ± standard deviation of at least three reactions. Each experiment was reproduced at least twice with similar results.

Glycosidase treatment

For treatment with PNGase F (New England Biolabs), approximately 500 ng of partially purified XET protein was diluted into 50 mM sodium phosphate, pH 7.5, and incubated with or without PNGase F at 37°C for 2 h. For PNGase F treatment of EXGT, the protein was diluted into 100 mM sodium succinate, pH 6.0, and incubated with or without the glycosidase at 37°C for 2 h. After digestion, 100 ng aliquots of each XET from each treatment were analysed by Western blotting with anti-TCH4 IgG as previously described (Antosiewicz *et al.*, 1997) and enzyme activity assays.

Determination of pH optima

XET assays were performed with McIlvaine's citrate–phosphate buffer adjusted for constant ionic strength (Elving *et al.*, 1956). This allowed enzyme activity to be monitored from pH 3.5 to 8.0 using a single buffer system.

Determination of temperature optima

XET assays were performed using 200 mM sodium acetate, 200 mM sodium phosphate buffer, pH 6.0, supplemented with 25% glycerol. This buffer system deviates by less than 0.1 pH unit from 0 to 80°C (Swint and Robertson, 1993).

Analysis of substrate specificities

XET assays were performed using either tamarind or pea xyloglucan polymer (0.1% xyloglucan final concentration) with 1.5 KBq of ^{3}H-oligosaccharide (XLLGol, XXFGol or XXXGol; see bottom of Figure 4 and Fry *et al.*, 1993 for explanation of nomenclature). The rate of ^{3}H incorporation into xyloglucan was determined by incubating XETs with each substrate combination for 30, 60 and 90 min.

Viscometry

XET and possible glucanase activities were assessed by monitoring the change in viscosity of a xyloglucan polymer solution with respect to time. Reaction mixtures (300 μl) containing 0.7% tamarind xyloglucan polymer, 100 mM sodium succinate, pH 6.0, ± 3 μM XLLGol, and approximately 5 μg partially purified enzyme were aspirated into 200 μl glass pipettes. Relative viscosity for a sample was determined as the length of time required for the discharge of 100 μl of the reaction mixture under gravity flow. This time of flow was measured three times for each sample at each time point. Results are reported as the mean of three time measurements ± standard deviation.

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