

RESEARCH PAPER

Differences in enzymic properties of five recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis thaliana*

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Abstract

Xyloglucan endotransglucosylase/hydrolases (XTHs) are cell wall enzymes that are able to graft xyloglucan chains to oligosaccharides or to other available xyloglucan chains and/or to hydrolyse xyloglucan chains. As they are involved in the modification of the load-bearing cell-wall components, they are believed to be very important in the regulation of growth and development. Given the large number (33) of XTH genes in *Arabidopsis* and the overlapping expression patterns, specific enzymic properties may be expected. Five predominantly root-expressed *Arabidopsis thaliana* XTHs belonging to subgroup I/II were analysed here. These represent two sets of closely related genes: AtXTH12 and 13 on the one hand (trichoblast-enriched) and AtXTH17, 18, and 19 on the other (expressed in nearly all cell types in the root). They were all recombinantly produced in the yeast *Pichia pastoris* and partially purified by ammonium sulphate precipitation before they were subsequently all subjected to a series of identical *in vitro* tests. The kinetic properties of purified AtXTH13 were investigated in greater detail to rule out interference with the assays by contaminating yeast proteins. All five proteins were found to exhibit only the endotransglucosylase (XET; EC 2.4.1.207) activity towards xyloglucan and non-detectable endohydrolytic (XEH; EC 3.2.1.151) activity. Their endotransglucosylase activity was preferentially directed towards xyloglucan and, in some cases, water-soluble cellulose acetate, rather than to mixed-linkage β -glucan. Isoforms differed in optimum pH (5.0–7.5), in temperature dependence and in acceptor substrate preferences.

Key words: *Arabidopsis thaliana*, Brassicaceae, cell elongation, cell wall, heterologous protein production, xyloglucan endotransglucosylase/hydrolase (XTH).

Introduction

The plant cell wall is a dynamic entity that, throughout plant growth and development, continually undergoes changes in composition and structure affecting its load-bearing capacity. In the primary cell wall of non-gramineous plants the hemicellulose xyloglucan is a structurally impor-

tant glycan (Carpita and Gibeaut, 1993) that tethers cellulose microfibrils. It is this cellulose/xyloglucan network that forms the basic load-bearing framework of these walls (Hayashi, 1989; Fry, 1989). Consequently, there is considerable interest in understanding the biochemical basis of the

synthesis and modification of xyloglucan as it is regarded as a fundamental regulatory element in processes such as cell growth and differentiation. The xyloglucan endotransglucosylase/hydrolase (XTH) family is one of the classes of enzymes that specifically act on xyloglucan (Nishitani and Vissenberg, 2007). They catalyse the cleavage and rejoining of xyloglucan chains (Fry *et al.*, 1992; Nishitani and Tominaga, 1992) through xyloglucan endotransglucosylase (XET) activity (Thompson and Fry, 2001) or they catalyse a hydrolysis of xyloglucan, the xyloglucan endohydrolase (XEH) activity. Baumann *et al.* (2007) provided structural evidence that the latter activity may have evolved from XET activity. All XTH enzymes studied to date display XET, XEH (Tabuchi *et al.*, 2001; Rose *et al.*, 2002) or both activities (De Silva *et al.*, 1993; Fanutti *et al.*, 1993) and their activity can loosen plant cell walls (Van Sandt *et al.*, 2007a).

XTH enzymes are generally encoded by large multi-gene families, for example, 33 genes in *Arabidopsis* and 20–60 XTH genes in diverse land plants from bryophytes and lycophytes to monocots and dicots (Yokoyama and Nishitani, 2001; Strohmeier *et al.*, 2004; Yokoyama *et al.*, 2004; Geisler-Lee *et al.*, 2006; Saladié *et al.*, 2006; Liu *et al.*, 2007; Van Sandt *et al.*, 2007b; Eklöf and Brumer, 2010). The existence of large XTH gene families in all these plants evokes the hypothesis that a wide range of tissue and developmental specificities among these enzymes may exist, in addition to specific enzymic properties. Previous studies indeed confirmed that many XTH genes of several plant species exhibited different expression patterns, both in spatial terms and in response to developmental and environmental stimuli. During the last two decades, XTH enzymes have, therefore, been associated with aerenchyma formation (Saab and Sachs, 1996), the generation of tracheary elements (Matsui *et al.*, 2005), softening during fruit ripening (Redgwell and Fry, 1993; Rose and Bennett, 1999), tension wood fibre formation and modification (Nishikubo *et al.*, 2007), mobilization of storage xyloglucan in seeds (Fanutti *et al.*, 1993), response to mechanical perturbations such as wind or touch (Braam and Davis, 1990; Xu *et al.*, 1995), elongation of parenchyma cells in epicotyls (Jiménez *et al.*, 2006), defence reaction against a plant parasite (Albert *et al.*, 2004), and endosperm cap weakening during seed germination (Chen *et al.*, 2002), and they are prominent in rapidly expanding tissues such as the elongation zone of roots, sites of future root hair initiation, growing root hairs (Vissenberg *et al.*, 2000, 2001, 2003), growing internodes of adult stems (Potter and Fry, 1993; Uozu *et al.*, 2000; Nakamura *et al.*, 2003; Romo *et al.*, 2005) and hypocotyls (Catalá *et al.*, 1997; Yun *et al.*, 2005).

In a comprehensive expression analysis of the *Arabidopsis thaliana* XTH gene family the tissue specificity and distinct responses to hormonal stimuli of all 33 members were studied. Transcripts of at least ten XTH genes were found in the root (Yokoyama and Nishitani, 2001). Expression of specific XTHs was detected in the dividing and/or elongating and/or differentiating region of the root and this in several cell types including the vascular tissues and root

hairs (Vissenberg *et al.*, 2005; Becnel *et al.*, 2006). These observations suggest that the diversification of the XTH gene family is closely associated with specialization of organ-specific expression and responsiveness to developmental signals. Furthermore, the presence of multiple isozymes at the same location, due to overlapping expression patterns, suggests that they might have diverse and specific enzymic properties. This idea is strengthened by the finding that pH and temperature optima of several XTHs can indeed differ within one species (Steele and Fry, 2000; Campbell and Braam, 1999). Besides a differential response to pH and temperature, differences in substrate availability may also influence the physiological effect of any given XTH.

Based on these experimental results, this article describes the differences and similarities between five predominantly root-expressed *Arabidopsis* XTHs. These represent two sets of closely related genes: AtXTH12 and 13 on the one hand (trichoblast-enriched) and AtXTH17, 18, and 19 on the other (expressed in nearly all cell types in the root; see Supplementary Fig. S1 at *JXB* online) for phylogenetic trees of XTH gene products, see Yokoyama and Nishitani, 2001; Baumann *et al.*, 2007). The corresponding cDNA molecules were heterologously expressed in the yeast *Pichia pastoris* and the resulting proteins were biochemically characterized. Enzymic characteristics such as pH and temperature optimum, donor and acceptor substrate preference, and their endo-transglycosylation versus hydrolytic activity capacities were tested *in vitro*. Their presumed physiological role during root development is discussed.

Materials and methods

Construction of the expression vector

AtXTH12 (At5g57530), *AtXTH13* (At5g57540), *AtXTH17* (At1g65310), *AtXTH18* (At4g30280), and *AtXTH19* (At4g30290) cDNAs were amplified without their predicted secretion signal sequence with primers including *XhoI* or *Clai* and *XbaI* restriction sites and ligated into a pGEM-T vector. The *XhoI/XbaI* or *Clai/XbaI* fragment from these plasmids was ligated into the pPICZ α B or pPICZ α C vector, respectively. As a result, the inserted fragment was in-frame with the sequence coding for the vector's alpha factor secretion signal peptide. In combination with the *P. pastoris* expression system (Invitrogen) this construct yields production of the XTH proteins with a *c-myc* epitope and a HIS tag at their C-terminus and secretion of these proteins into the yeast culture medium. To ensure that the expression vector contained the desired sequence, the plasmid with 5' and 3' AOX1 vector-specific primers was sequenced. This covered the whole alpha factor signal sequence, the gene's coding region and the C-terminal tags followed by the stop codon.

Recombinant protein production

After linearization of the pPICZ α -XTH expression vector with *PmeI* the *P. pastoris* strain GS115 was transformed by electroporation according to Invitrogen's instructions. Transformed yeast colonies were selected on YPDS plates (yeast/peptone/dextrose/sorbitol regeneration medium) containing zeocin concentrations up to 1000 $\mu\text{g ml}^{-1}$. The presence of the expression construct in the yeast genome was confirmed by colony-PCR on selected colonies.

A colony was selected, transferred to liquid buffered complex medium containing glycerol (BMGY) and $100 \mu\text{g ml}^{-1}$ zeocin and allowed to grow overnight at 28°C and 250 rpm. $80 \mu\text{l}$ of this culture was inoculated to 100 ml of fresh BMGY medium in a 500 ml culture flask and grown overnight at the same conditions until an $\text{OD}_{600}=4$ was reached. A 5 min centrifugation (2000 g) harvested the yeast cells and they were diluted to $\text{OD}_{600}=1$ in buffered complex medium containing methanol (BMMY). The production of proteins was induced in 1.0 l flasks containing 200 ml of culture at 28°C and 250 rpm for 3 d with the addition of methanol to a final concentration of 1% every 24 h. After these 3 d, yeast cells were discarded by centrifugation (5000 g, 10 min) and the proteins were recovered from the medium by a 40% (w/v) ammonium sulphate precipitation and a centrifugation (10 000 g, 45 min). Pellets containing the XTH proteins were redissolved in 50 mM MES (Na^+) buffer, pH 6.0 and dialysed overnight against 4.0 l of the same buffer +25 mM NaCl.

Large-scale production of AtXTH13 and subsequent affinity purification was performed according to the methods described by Baumann *et al.* (2007). Protein electrospray mass spectrometry was performed as described by Sundqvist *et al.* (2007).

XET dot-blot activity assay

A dot-blot assay (Fry, 1997) was used to test the xyloglucan endotransglucosylase (XET) activity of the recombinant proteins. Briefly, this assay visualizes the transfer of non-fluorescent, paper-bound xyloglucan with a high molecular weight to sulphorhodamine-labelled xyloglucan oligosaccharides. The enzyme solution was spotted on the test paper and incubated overnight at 28°C in aluminium foil. Afterwards, a wash in formic acid:ethanol:water (1:1:1 by vol.) for 1 h removed non-reacted fluorescent xyloglucan oligosaccharides. Upon UV illumination of the test paper the remaining fluorescence is an indication of XET activity.

Viscometric assay

MES buffer (100 μl , 50 mM, pH 6.0) plus 100 μl of either AtXTH extract in MES buffer, or as a control 100 μl *Trichoderma viride* cellulase (4 μg ; Sigma; EC 3.2.1.4) in MES buffer, were added to 200 μl of 1% tamarind xyloglucan. For all extracts, 10 \times the final enzyme concentration was used compared with the radioactive XET assay. The viscosity of the solutions was measured as their flow rate through 200 μl pipettes after 0, 2, 8, and 24 h of incubation. Data are only shown for 2 h treatment as no changes occurred in the XTH samples beyond this reaction time.

Radioactive XET assay

As described in Fry *et al.* (1992) radioactive XET activity assays were performed in reaction volumes of 30 μl that contained final concentrations of 100 mM acetate (Na^+), pH 5.7, 10 mM CaCl_2 , 0.3% (w/v) tamarind xyloglucan, 1 kBq [^3H]XLLGol (unless otherwise stated; for preparation see Steele and Fry, 2000) and 10 μl of AtXTH enzyme extract previously dissolved in 1% BSA. After incubation for 2 h at 20°C (unless otherwise stated) the reaction was stopped by the addition of 20 μl 50% formic acid. The reaction mixtures were then spotted on Whatman 3MM paper, air-dried, washed under running tap water overnight, dried again, and assayed for ^3H by scintillation-counting in 2 ml of Wallac 'OptiScint' scintillant. The measurements of blanks occurred in an identical way, but the XTH enzyme extract was omitted from the 1% BSA solution. All assays were performed three times and are presented as the difference between the mean of sample and blank \pm the sum of the standard deviations. Assays at different temperature and pH are presented as a percentage of the maximal activity measured in the assay. Activity is expressed as relative % activity. In substrate preference experiments, rates are reported relative to the total radioactivity of the acceptor substrate used, i.e. expressed as % ($\text{cpm}_{\text{product}}/\text{cpm}_{\text{substrate supplied}}$) per unit of reaction time.

For the measurement of the pH optimum, citrate buffer was adjusted to pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5. HEPES buffer was adjusted to pH 7.0, 7.5, and 8.0. In all cases the buffers (Na^+) were used at a final concentration of 100 mM.

For the measurement of the temperature optimum, the reactions were incubated on ice, in a cold room at 4°C and in water baths of 20, 28, and 37°C .

To investigate the donor-substrate preferences of the XTHs, tamarind xyloglucan was replaced by various water-soluble β -glucans: carboxymethylcellulose (CMC, Na^+ salt, low viscosity) and barley mixed-linkage (1 \rightarrow 3, 1 \rightarrow 4)- β -glucan (MLG) were from Sigma Chemical Co.; hydroxyethylcellulose (HEC) was from Fluka; and water-soluble cellulose acetate (WSCA) was prepared as described by Fry *et al.* (2008). Various ^3H -labelled oligosaccharides (prepared as described by Hetherington and Fry, 1993) were used instead of [^3H]XLLGol to define the acceptor-substrate preferences of the XTHs. When [^3H]cellohexaitol was tested as a potential acceptor substrate, the polymeric reaction products were isolated by washing free of unreacted oligosaccharide with 75% ethanol as described by Fry *et al.* (2008).

Reducing sugar assay for determining XEH activity

To quantify the XEH activity of purified AtXTH13, the Somogyi-Nelson reducing sugar assay (Nelson, 1944) was used. Specific assay conditions were: 200 mM buffer (sodium acetate pH 5.5 or sodium citrate pH 6), xyloglucan 1 g l^{-1} and $6.67 \mu\text{M}$ AtXTH13, with incubation for 24 h at room temperature (21°C). A standard curve for glucose was used, with the lowest concentration point of $55.5 \mu\text{M}$ Glc.

Initial-rate kinetics with a minimal xylogluco-oligosaccharide substrate

The absolute rates of hydrolysis and transglycosylation of a mixture of XXXGXXXG-based xylogluco-oligosaccharides bearing naturally variable xylose galactosylation was performed with the HPLC assay devised by Baumann *et al.* (2007). In the present study, a CarboPacTM PA200 column (Dionex) was used with the following elution gradient: 0–4 min, 100 mM NaOH, 60 mM NaOAc; 4–17 min, 100 mM NaOH, 60–250 mM NaOAc; 17–18 min, 500 mM NaOH, 500 mM NaOAc; 18–22 min, 100 mM NaOH, 60 mM NaOAc.

Results and discussion

Recombinant production of active XTH proteins

In an expression analysis study of all 33 members of the *Arabidopsis* XTH gene family, at least ten genes were found to exhibit predominantly root-specific expression (Yokoyama and Nishitani, 2001). To gain insight into the potential differential roles of these enzymes in *Arabidopsis* roots, two sets of XTH enzymes were produced that belong to very closely related clades (Baumann *et al.*, 2007) but with different expression patterns (see Supplementary Fig. S1 at JXB online), namely AtXTH12 and AtXTH13, and AtXTH17, AtXTH18, and AtXTH19, in the heterologous expression system of *P. pastoris*. To test whether the proteins exhibit XET activity, ammonium sulphate precipitates from the culture media of methanol-induced yeasts were assayed. They were subsequently all subjected to a series of identical *in vitro* tests. The kinetic properties of purified AtXTH13 were investigated in greater detail to rule out interference with the assays by contaminating yeast proteins. Fluorescent dot-blot

activity assays using the resuspended precipitates from yeasts containing the pPICZ α -AtXTH expression vectors, clearly revealed fluorescence on the test papers (Fig. 1). As this is indicative of XET activity, the observations prove that active enzymes had been produced. No XET activity was detected in the medium from induced empty vector-containing yeast, confirming the specificity of the assay.

To check the potential of the XTH proteins to catalyse xyloglucan hydrolysis, a viscometric test was performed on a 1% xyloglucan solution (Fig. 2). None of the enzymes was able to decrease the viscosity of the xyloglucan solution within 24 h of reaction time, whereas cellulase, a β (1-4) endoglucanase that is known to hydrolyse xyloglucan, decreased the viscosity over 80% within 2 h. So, none of the five XTH enzymes was able to cleave the xyloglucan polymers in the absence of the acceptor substrate oligosac-

charides. It can therefore be concluded that AtXTH12, 13, 17, 18, and 19 exhibit only XET and no detectable XEH activity under the conditions tested. Based on gene sequences, the XTH family is divided into three groups (Yokoyama and Nishitani, 2001; Rose *et al.*, 2002). So far, members of group I and II are found to exhibit only XET activity, whereas some members of group III have XEH activity. As all five XTHs studied in the present work belong to groups I/II, our findings fit well with the predicted activities based on their gene sequences. These data also confirm the enzyme activities hypothesized by Baumann *et al.* (2007) based on protein sequences. Mark *et al.* (2009) suggest that at least one loop of certain amino acids lining the active site helps to determine XEH over XET activity. This hypothesis is confirmed in this study since the absence of XEH activity in AtXTH12, 13, 17, 18, and 19 is correlated with the absence of this so-called loop 2 (data not shown).

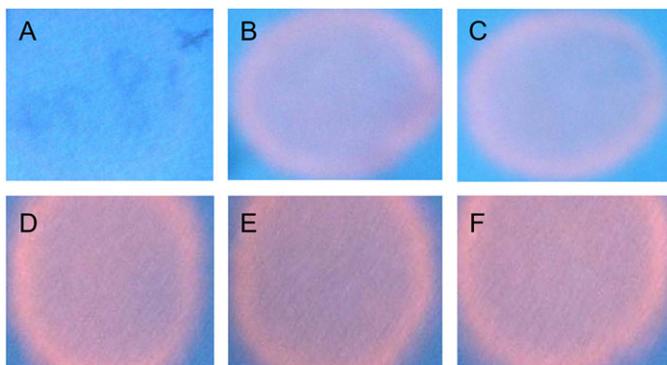


Fig. 1. Fluorescent XET dot-blot activity assay using recombinant AtXTH12, 13, 17, 18, and 19. XET activity of the 40% (w/v) ammonium sulphate-precipitated proteins from culture media of methanol-induced yeast containing a pPICZ α empty (A), pPICZ α -AtXTH12 (B), pPICZ α -AtXTH13 (C), pPICZ α -AtXTH17 (D), pPICZ α -AtXTH18 (E) or pPICZ α -AtXTH19 (F) vector was detected by use of the fluorescent dot-blot activity assay.

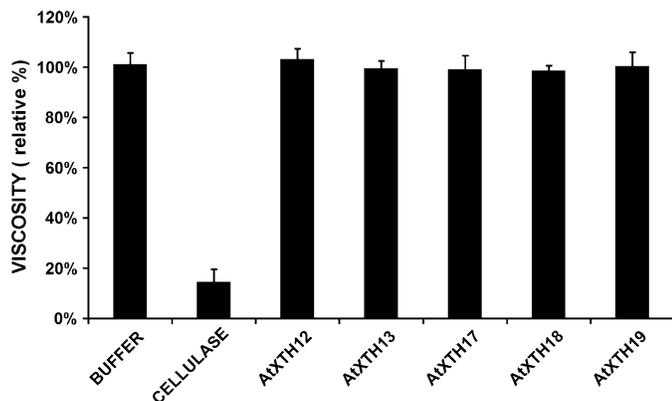


Fig. 2. XEH activity test on recombinant AtXTH12, 13, 17, 18, and 19. Viscometric assay on a 1% xyloglucan solution to test the enzymes' potential to hydrolyse xyloglucan. Results are compared with a negative control where only buffer was added and with a positive control where cellulase, a β (1-4)-endoglucanase hydrolysing xyloglucan, was used.

Detailed kinetic properties of AtXTH13

To provide further validation of the dot-blot and viscometric methods used to delineate XET versus XEH activity in crude ammonium sulphate precipitates of *Pichia pastoris* media, AtXTH13 was rigorously purified and subjected to an alternative kinetic analysis. AtXTH13 was purified to apparent homogeneity (SDS-PAGE) in one step from the crude culture supernatant on a xylogluco-oligosaccharide affinity column (see Supplementary Fig. S2 at *JXB* online). Mass spectrometry of the intact protein after purification revealed that the AtXTH13 cDNA construct was correctly expressed and post-translationally modified with variable N-linked glycans on one site (Table 1). The glycoform composition of AtXTH13 was similar to that of *PttXET16-34* expressed in *P. pastoris*, with GlcNAc₂Man₈ and GlcNAc₂Man₉ predominating (Kallas *et al.*, 2005). AtXTH13 was also produced in similar levels as *PttXET16-34* in shaking-flask cultures (data not shown).

The Somogyi–Nelson reducing-sugar assay revealed that AtXTH13 had undetectable hydrolytic activity toward tamarind seed xyloglucan in a 24 h assay, thus supporting the

Table 1. Mass spectrometry data for purified recombinant AtXTH13

| Polypeptide | N-linked glycan | Calculated mass | Observed mass ^b | % Base peak intensity |
|---|---------------------------------------|-----------------|----------------------------|-----------------------|
| EAE-AtXTH13-c-myc-His ₆ ^a | HexNAc | 33 023.7 | 33 022.7 | 20 |
| EAE-AtXTH13-c-myc-His ₆ | HexNAc ₂ Hex ₇ | 34 361.9 | 34 361.1 | 30.2 |
| EAE-AtXTH13-c-myc-His ₆ | HexNAc ₂ Hex ₈ | 34 524.0 | 34 523.6 | 100 |
| EAE-AtXTH13-c-myc-His ₆ | HexNAc ₂ Hex ₉ | 34 686.2 | 34 685.1 | 73.8 |
| EAE-AtXTH13-c-myc-His ₆ | HexNAc ₂ Hex ₁₀ | 34 848.3 | 34 848.5 | 25.2 |

^a The AtXTH13 construct starts at ^{23A}GS and ends with NLN²⁸⁴.

^b Versus horse heart myoglobin as an external standard.

similarly negative result obtained with the viscometric assay (*vide ut supra*). A conservative upper limit of the hydrolytic activity is $5.8 \times 10^{-3} \text{ mol min}^{-1} \text{ mol}^{-1}$ AtXTH13, based on the lowest concentration of glucose measured in the standard curve, the quantity of protein added and the assay time.

The kinetic properties of AtXTH13 were analysed further with an HPLC-based method (Baumann *et al.*, 2007), which measures the absolute rates of release of XGO₁ (Glc₄-based xylogluco-oligosaccharides) and XGO₃ (Glc₁₂-based xylogluco-oligosaccharides) from a mixture of minimal XET/XEH substrates, XGO₂ (Glc₈-based xylogluco-oligosaccharides), derived from tamarind seed xyloglucan and bearing naturally variable galactosylation (York *et al.*, 1990). Initial-rate kinetic analysis (Fig. 3) indicated that the rate of release of XGO₁ is identical to that of XGO₃ at all substrate concentrations across a wide range (8–2400 μM), as would be expected for a strict transglycosylase [hydrolytic activity would be manifested as a greater rate of XGO₁ release than XGO₃ release; a strict XEH would produce only XGO₁ from XGO₂ at all concentrations; Baumann *et al.* (2007)]. Fitting the Michaelis–Menten equation to the data shown in Fig. 3 indicated apparent k_{cat} and K_{m} values of $5.08 \pm 0.16 \text{ min}^{-1}$ and $64.1 \pm 9.9 \mu\text{M}$, respectively, for the rate of XGO₃ release. Apparent k_{cat} and K_{m} values for the rate of XGO₁ release were determined to be $5.18 \pm 0.16 \text{ min}^{-1}$ and $65.5 \pm 9.8 \mu\text{M}$, respectively (Fig. 3). k_{cat} values were consistent within 20% and K_{m} values were statistically identical for a replicate protein batch starting from a second *P. pastoris* cultivation.

Together, these analyses indicate that the AtXTH13 cDNA encodes a protein that has only XET activity. The

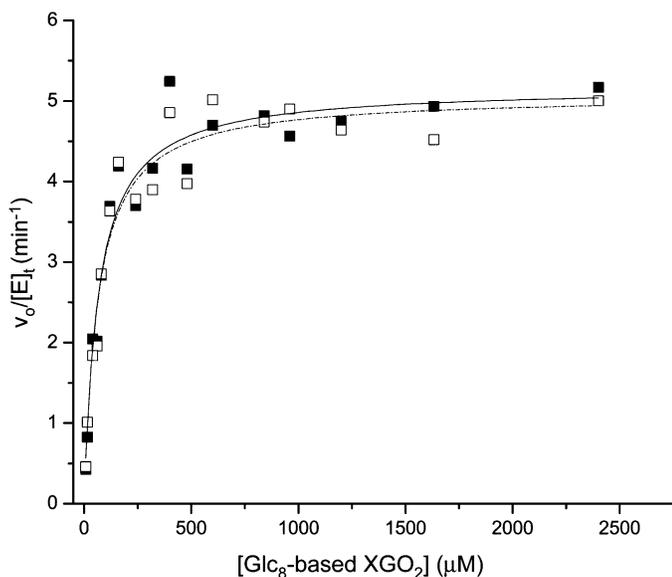


Fig. 3. Initial-rate kinetics of the release of XGO₁ and XGO₃ from XGO₂ by AtXTH13. (Black squares) Rate of XGO₁ release; (white squares) rate of XGO₃ release; (solid line) fit of Michaelis–Menten equation to XGO₁ data (k_{cat} $5.18 \pm 0.16 \text{ min}^{-1}$, K_{m} $65.5 \pm 9.8 \mu\text{M}$); (dotted line) fit of Michaelis–Menten equation to XGO₃ data (k_{cat} $5.08 \pm 0.16 \text{ min}^{-1}$, K_{m} $64.1 \pm 9.9 \mu\text{M}$).

results of assays on the purified enzyme using artificially low- M_{r} model donor and acceptor substrates, but with precisely known molar concentrations, support the results obtained from the analysis of ammonium sulphate-precipitated enzyme preparations using technically simpler assays with diverse low- M_{r} model acceptor substrates at radiotracer concentrations plus realistically high- M_{r} donor substrates. *Pichia pastoris* produces no endogenous XETs or XEHs, which would otherwise confound kinetic analysis of crude recombinant enzyme preparations. Thus, the viscometric and fluorescent dot-blot assays can be taken as reliable indicators of relative XEH versus XET activity. None of the AtXTH cDNAs encode proteins with measurable XEH activity. Furthermore, unpublished results comparable with those of Van Sandt *et al.* (2006) indicated no differences between assays performed with ammonium-sulphate precipitated recombinant *Selaginella* XTH versus the purified native form of the XTH. As the proteins produced in this study contain HIS and c-myc-tags, strictly speaking their structure is not identical to those acting in *Arabidopsis*. However, in the same study on *Selaginella* XTHs, it was shown that the presence of these tags did not influence the enzymic properties of the XTH proteins. Based on these findings, subsequent analysis of the biochemical properties of the XTH proteins in this study was performed using a quantitative radiometric assay of substrate transglycosylation, which is amenable to medium-throughput analyses.

Temperature and pH dependence

The XET activities of the different ammonium sulphate-precipitated enzyme preparations were tested with the radioisotope method, but at different temperature (Fig. 4) and pH conditions (Fig. 5) to define the operational range of the enzymes.

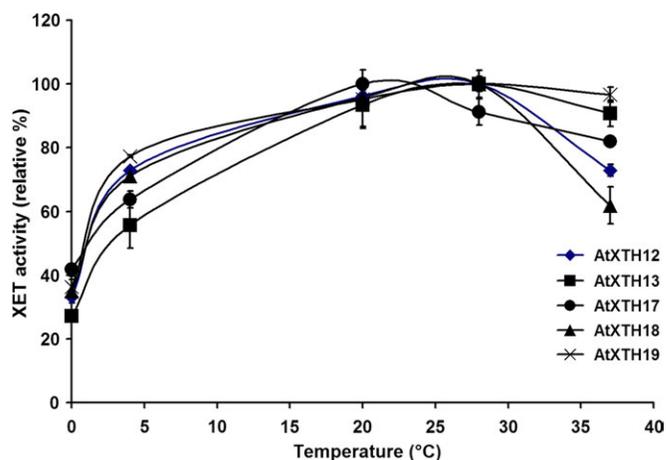


Fig. 4. Temperature–activity profile of XET activity of AtXTH12, 13, 17, 18, and 19 studied with the radioactive XET assay. Data are presented as a percentage of the maximal activity measured for the enzyme in question. The very slight background binding of the radioactive oligosaccharide to Whatman 3MM paper was subtracted. Each assay was performed in triplicate and standard deviations are shown.

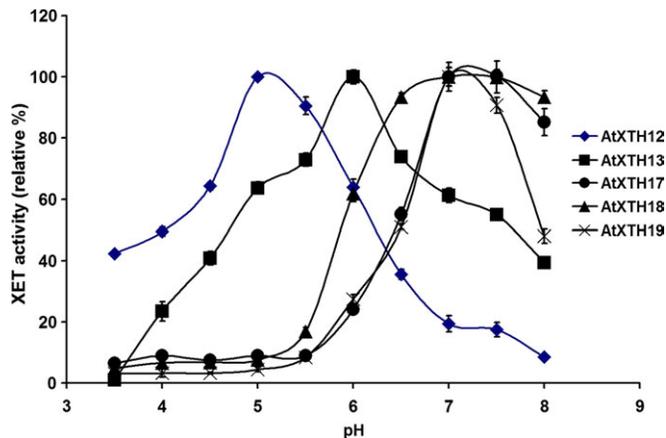


Fig. 5. pH profile of XET activity of AtXTH12, 13, 17, 18, and 19 studied with the radioactive XET assay. Other details as in Fig. 4.

AtXTH12, 13, 18, and 19 show the highest activity at 28 °C whereas AtXTH17 has its optimum at 20 °C (Fig. 4). AtXTH12, 17, and 19 have a very broad temperature range for activity, maintaining about 70% of their maximal activity between 4 °C and 37 °C. AtXTH13 is somewhat more cold-sensitive as its activity at 4 °C is between 50% and 60% of its maximal activity. AtXTH18, on the other hand, is more heat-sensitive as at 37 °C its activity drops to 60% of its activity at 28 °C. Despite their broad temperature ranges, the enzymes are not extremely cold-tolerant as AtXTH12, 13, 18, and 19 show an activity loss of 25–40% between 4 °C and 0 °C. A similar decline of 20% in activity was measured for AtXTH14 and AtXTH26 (Maris *et al.*, 2009). Only AtXTH17, which also has the lowest optimum at 20 °C, retains 50% of its maximal activity at 0 °C. Broad temperature–activity profiles have been seen in most XTH enzymes analysed so far. Taken together they show optimum temperatures ranging from 18 °C to 37 °C and extremes have been reported for heat-tolerant (55 °C) as well as cold-tolerant (–5 °C) enzymes (Purugganan *et al.*, 1997; Campbell and Braam, 1999; Steele and Fry, 2000; Kallas *et al.*, 2005; Van Sandt *et al.*, 2006). These broad-range temperature–activity profiles are rather uncommon as, in biological systems, the activity usually approximately doubles for every temperature rise of 10 °C until the optimum is reached.

The bell-shaped activity profiles of AtXTH12 and the highly-sequence similar AtXTH13 show optima at pH 5.0 and 6.0, respectively (Fig. 5). These optima are quite common for XTH enzymes (Purugganan *et al.*, 1997; Campbell and Braam, 1999; Steele and Fry, 2000). Less common is the acid-tolerance of AtXTH12, which still displays more than 40% of its maximal activity at pH 3.5. The activity drops below 20% of its maximal activity at pH 7.0 or higher. As seen by XTH::GUS reporter line analysis, AtXTH12 is produced in the root tip of *Arabidopsis* [Becnel *et al.*, 2006; see Supplementary Fig. S1 at *JXB* online (based on the Arex database; Birnbaum *et al.*, 2003; Brady *et al.*, 2007)]. One reason for the acid-tolerance of AtXTH12 might be the need to withstand the acidification of the

apoplast necessary for gravitropic reactions (Fasano *et al.*, 2001), a phenomenon that has been reported to induce XET expression, for example, in leaf sheath bases of rice seedlings (Cui *et al.*, 2005). On the other hand, a study that compared mRNA populations of *Arabidopsis* root hair cells and cells of an *Arabidopsis* root hair mutant, showed AtXTH12 to be 4-fold up-regulated in root hair cells (Dr Mark Jones, personal communication). The fact that AtXTH12 was not reported to be expressed in the root hairs by the promoter-XTH::GUS reporter line analysis study, could point to a role for AtXTH12 in root hair initiation as this was found to be coupled to a highly localized increase of XET action in *Arabidopsis* roots (Vissenberg *et al.*, 2001). This XET action was high at pH 4.5 and pH 5.5 but absent at pH 7.0, which matches perfectly with the pH–activity profile of AtXTH12. AtXTH13, on the other hand, is more tolerant to basic environments, retaining 40% of its maximal activity at pH 8.0, but displays only 20% of it at pH 4.0. This enzyme is inactive at pH 3.5 and could play a role later in root hair development.

By contrast with the bell-shaped curves of AtXTH12 and 13, the activity–pH profiles of AtXTH17, 18, and 19 totally shift towards the alkali side with their optima at pH 7.0 and with an unusual acid intolerance (Fig. 5). AtXTH18 shows the highest acid tolerance of these three enzymes. At pH 6.0 the enzyme still displays 60% of its activity at pH 7.0, whereas the activities of AtXTH17 and AtXTH19 drop below 30% of their maximal activity at pH 6.0. At or below pH 5.5, all three enzymes display less than 20% of their maximal activities. On the basic side, AtXTH17 and 18 show the highest tolerance with activities of more than 80% of their maximal activity at pH 8.0. On the other hand, AtXTH19 retains 90% of its maximal activity at pH 7.5, but shows a 40% activity drop between pH 7.5 and 8.0. The fact that all three enzymes have very similar pH–activity profiles is not surprising as they are phylogenetically very closely related to one another (Yokoyama and Nishitani, 2001; Baumann *et al.*, 2007). AtXTH17 and AtXTH18 are expressed in all cell types in the elongating and differentiating regions of the root, while AtXTH19 is expressed in the apical dividing and elongating regions, as well as in the differentiation zone (Vissenberg *et al.*, 2005). The slight difference in expression pattern when comparing the promoter::GUS analysis (Vissenberg *et al.*, 2005; and see Supplementary Fig. S1 at *JXB* online) can be due to drawbacks typical for both experimental strategies. Nevertheless, the three XTH isoforms are present in nearly all root cell types. These dispersed expression profiles of the enzymes may point to a rather unspecific role of the enzymes maintaining XET activity during alkalization of the apoplast due to stress or other signals (De Cnodder *et al.*, unpublished results). Furthermore, it cannot be excluded that these isoforms' main function is the modification of xyloglucan within the Golgi vesicles (Driouich *et al.*, 1993; Dupree and Sherrier, 1998) at presumably a somewhat higher pH before both XTHs and xyloglucan are secreted into the cell wall.

Substrate specificity

In a study of the substrate specificity of the enzymes, their ability to catalyse the transfer of different donor substrate polysaccharides to [³H]XXXGol, a xyloglucan-derived oligosaccharide, was tested (Fig. 6). All five enzymes showed low activity rates with all non-xyloglucan polymers tested in comparison to xyloglucan as donor substrate. For all enzymes the non-xyloglucan polymers were preferred in the order: water-soluble cellulose acetate (WSCA) >hydroxyethylcellulose (HEC) >mixed-linkage β-glucan (MLG) >carboxymethylcellulose (CMC), but with very low activity rates for MLG and CMC. A similar pattern of donor substrate preferences was observed for total XET activities extracted from *Equisetum* and grass species (Fry *et al.*, 2008) and may therefore be generally applicable. Thus AtXTH12, AtXTH13, AtXTH17, AtXTH18, and AtXTH19 are not absolutely specific for xyloglucan but do show a very high preference for xyloglucan as donor substrate.

All enzymes showed a much greater tolerance towards differences in the side-chains of the acceptor substrate oligosaccharides. The acceptor substrate preference was

studied on a selection of oligosaccharidyl-[³H]alditols with various side-chains and backbones differing in the number of glucose units (Fig. 7). With the exception of celohexaitol, all tested oligosaccharides were incorporated into the xyloglucan polymer, but to different extents. All enzymes show a clear preference for XXLGol, except AtXTH12, which prefers the fucosylated form XXFGol. XXFGol also proved to be a good substrate for AtXTH17, 18, and 19. Compared with the other XTH enzymes tested, AtXTH12 shows the highest tolerance of side-chain variation with good activities for five of the six tested acceptor substrates, including the pentasaccharide XXGol. The activity with this compound is very low for the four other enzymes. Although the characteristics of AtXTH17, 18, and 19 are quite similar, only plants with a reduced expression of AtXTH18 showed a root phenotype (Osato *et al.*, 2006). This could be because AtXTH18 exhibits the highest levels of mRNA expression of the three genes.

Overall, the enzymes are capable of attaching xyloglucan to xyloglucan-derived oligosaccharides with various side-chains, but they do show clear differences in their preferred

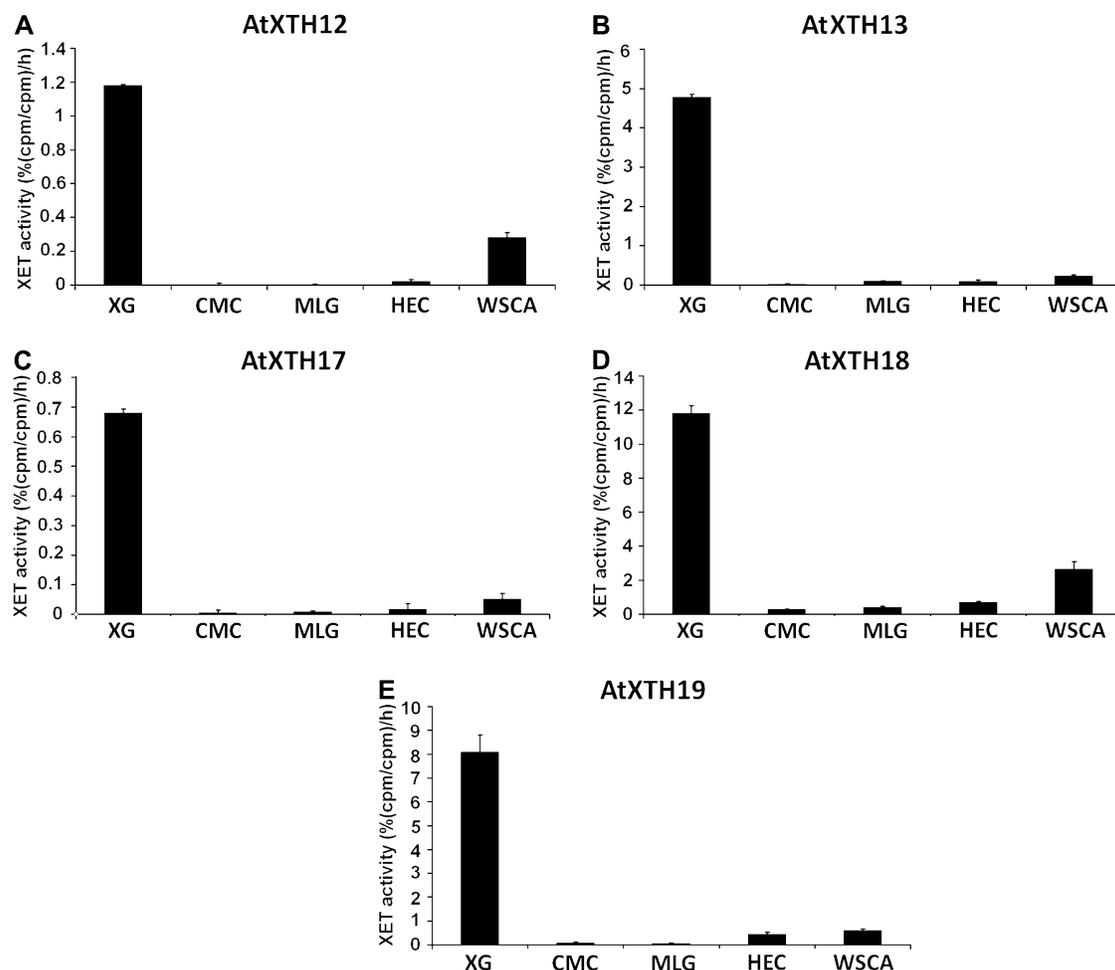


Fig. 6. Donor substrate preference of AtXTH12, 13, 17, 18, and 19 studied with the radioactive XET assay. Other details as in Fig. 4. Data are presented as radioactive polysaccharide produced, expressed as a percentage of the supplied radioactive acceptor substrate, per h of incubation time. The very slight background binding of the oligosaccharide to Whatman 3MM paper was subtracted. Each assay was performed in triplicate and standard deviations are shown.

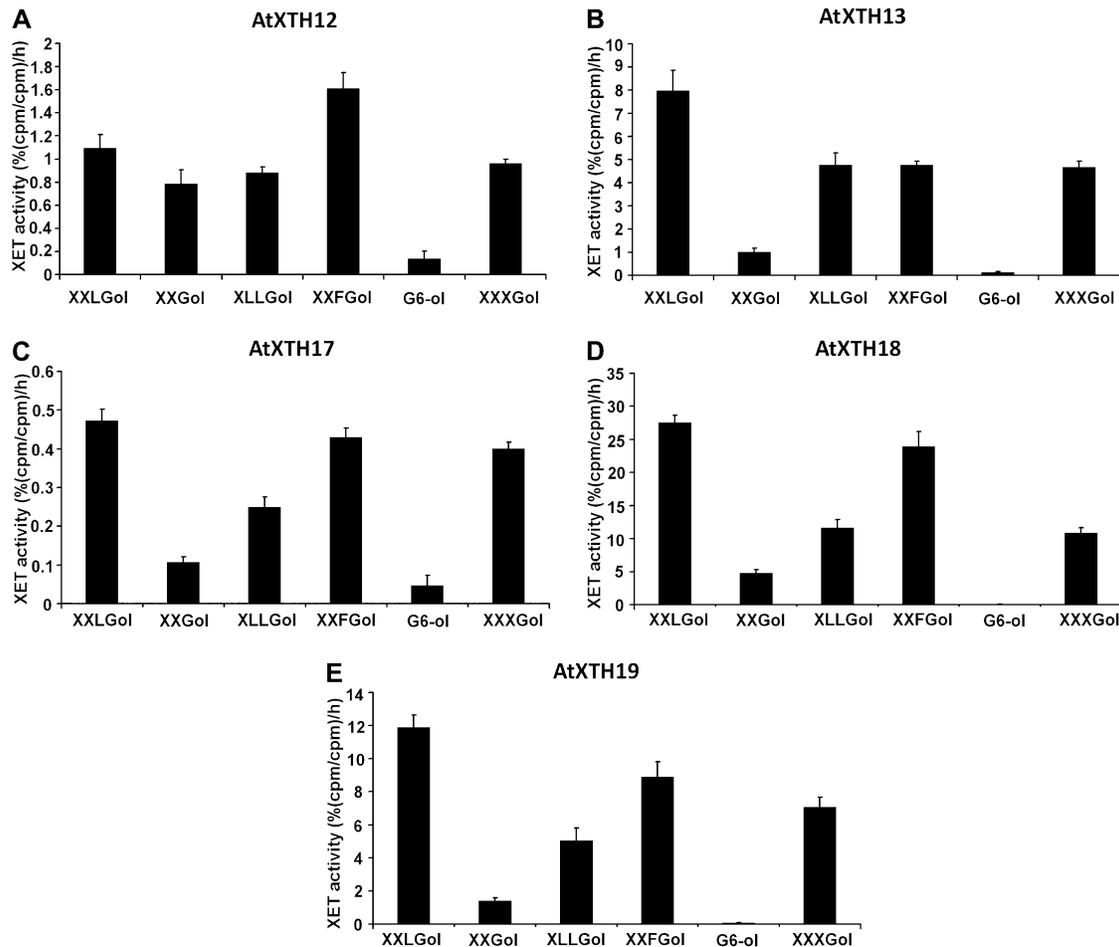


Fig. 7. Acceptor substrate preference of AtXTH12, 13, 17, 18, and 19 studied with the radioactive XET assay. In the case of [^3H]cellohexaitol, ethanol washing of precipitated products was used instead of water-washing of filter paper. Other details as in Fig. 6.

side-chain substitutions as well as in their tolerance of size variation. The physiological relevance of these characteristics is hard to elucidate as little is known yet about the variety of xyloglucan side-chains present within different parts of the root or at different developmental stages, and, crucially, at the presumed main natural acceptor substrate domain: the non-reducing terminus of the xyloglucan polysaccharide. It can, however, be stated as a general rule that the composition of the xyloglucan side-chains, leading to different donor and acceptor substrates, was indeed found to affect XET action differentially among isoforms (Purugganan *et al.*, 1997; Campbell and Braam, 1999; Steele and Fry, 2000; Saura-Valls *et al.*, 2008).

To conclude, the *AtXTH12*, *13*, *17*, *18*, and *19* genes all encode products which, under the conditions tested, lack XEH activity and have a clear preference for xyloglucan as the donor substrate. All enzymes showed specific characteristics in their activity profiles under different physiological conditions both between and within the two groups of related enzymes. As more and more evidence favours the hypothesis that plants regulate conditions such as pH and substrate availability very locally within different organ regions, and temporally, the knowledge of these character-

istics should give important insights into the physiological roles of the different enzyme isoforms during plant development.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Expression profiles of AtXTH12, 13, 17, 18, and 19 in the *Arabidopsis* root.

Supplementary Fig. S2. SDS-PAGE of AtXTH13 in the culture supernatant after 4 d cultivation and purification.

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