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Three highly acidic *Equisetum* XTHs differ from hetero-trans-β-glucanase in donor substrate specificity and are predominantly xyloglucan homo-transglucosylases

Claire Holland [a], Thomas J. Simmons [a,d], Frank Meulewaeter [b], Andrew Hudson [c], Stephen C. Fry [a]*

[a] The Edinburgh Cell Wall Group, Institute of Molecular Plant Sciences, The University of Edinburgh, Daniel Rutherford Building, The King's Buildings, Max Born Crescent, Edinburgh EH9 3BF, UK

[b] BASF Innovation Center Gent– Trait Research, Technologiepark-Zwijnaarde, 9052 Gent, Belgium

[c] Institute of Molecular Plant Sciences, The University of Edinburgh, Daniel Rutherford Building, The King's Buildings, Max Born Crescent, Edinburgh EH9 3BF, UK

[d] Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB1 2QW, UK

* To whom correspondence should be addressed (email s.fry@ed.ac.uk)

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Abbreviations: XTH, xyloglucan endotransglucosylase/hydrolase (protein); MXE, MLG:xyloglucan endotransglucosylase (activity); CXE, cellulose:xyloglucan endotransglucosylase (activity); XET, xyloglucan endotransglucosylase

(homotransglucanase activity); MLG, mixed-linkage (1→3, 1→4)-β-D-glucan; PCW, primary cell wall; GH, glycoside hydrolase; XXXGol, borohydride-reduced heptasaccharide of xyloglucan ($xylose_3$·$glucose_3$·glucitol).

ABSTRACT

Transglycanases are enzymes that remodel the primary cell wall in plants, potentially loosening and/or strengthening it. Xyloglucan endotransglucosylase (XET; EC 2.4.1.207), ubiquitous in land plants, is a homo-transglucanase activity (donor, xyloglucan; acceptor, xyloglucan) exhibited by XTH (xyloglucan endotransglucosylase/hydrolase) proteins. By contrast, hetero-trans-β-glucanase (HTG) is the only known enzyme that is preferentially a hetero-transglucanase. Its two main hetero-transglucanase activities are MLG : xyloglucan endotransglucosylase (MXE) and cellulose : xyloglucan endotransglucosylase (CXE). HTG is highly acidic and found only in the evolutionarily isolated genus of fern-allies, *Equisetum*. We now report genes for three new highly acidic HTG-related XTHs in *E. fluviatile* (EfXTH-A, EfXTH-H and EfXTH-I). We expressed them heterologously in *Pichia* and tested the encoded proteins' enzymic activities to determine whether their acidity and/or their *Equisetum*-specific sequences might confer high hetero-transglucanase activity. Untransformed *Pichia* was found to secrete MLG-degrading enzyme(s), which had to be removed for reliable MXE assays. All three acidic EfXTHs exhibited very predominantly XET activity, although low but measurable hetero-transglucanase activities (MXE and CXE) were also detected in EfXTH-H and EfXTH-I. We conclude that the extremely high hetero-transglucanase activities of *Equisetum* HTG are not emulated by similarly acidic *Equisetum* XTHs that share up to 55.5% sequence identity with HTG.

1. Introduction

Glycoside hydrolases (GHs) are a group of 'carbohydrate-active enzymes' (CAZy) that hydrolyse glycosidic linkages in both polysaccharides and low-molecular-weight *O*-, *N*- and *S*-linked glycosides. More than 160 GH families have been described, with enzymes grouped according to primary sequence similarity. This has led to groupings that reflect common active-site topologies and modes of catalysis, but interestingly usually not substrate specificity (Barbeyron et al., 1998). High-resolution structural data have shown that proteins within the same GH family have a conserved core, including active-site residues, and major elements of secondary and tertiary structure, even when primary structure similarity is low (Gebler et al., 1992). The GH16 family of enzymes is most pertinent to the present work, a group with an extensive array of substrate specificities leading to cleavage of β-1,3- or β-1,4-glycosidic bonds in various glucans and galactans. The substrate specificity of GH16s is amongst the most varied of any GH group, with enzyme activities including, but not limited to, xyloglucan endotransglucosylase (XET; Rose et al., 2002; EC 2.4.1.207; also known as xyloglucan:xyloglucosyl transferase), xyloglucan endohydrolase (XEH; EC 3.2.1.151), endo-1,3-β-galactanase (EC3.2.1.-), endo-1,3-β-glucanase (laminarinase, EC 3.2.1.39), lichenase (EC 3.2.1.73), and κ-carageenase (EC 3.2.1.83) (Viborg et al., 2019).

Xyloglucan endotransglucosylase/hydrolases (XTHs) are a subfamily of the GH16 enzyme family (Cantarel et al., 2009) that catalyse xyloglucan endotransglucosylase (XET) and/or xyloglucan endohydrolase (XEH) reactions (de Silva et al., 1993; Fanutti et al., 1993; Tabuchi et al., 2001; Rose et al., 2002). Although some XTHs catalyse both XET and XEH reactions (Bourquin et al., 2002; Eklöf and Brumer, 2010; Shi et al., 2015), most XTHs for which detailed kinetic data are available are strict XETs and display undetectable XEH activity (Fry et al., 1992; Nishitani & Tominaga, 1992; Stratilová et al., 2010). Phylogenetically, XTHs are divided into groups I/II (predominant XETs) and III (predominant XEHs) (Fig. 1). Baumann et al. (2007) identified a unique extension of the loop connecting strands β8-β9 in predominant XEHs as a major, but not the only, contributor to defining XEH over XET activity. This loop lies

adjacent to the active site in *Tropaeolum majus* (*Tm*)NXG1 – a GH16 protein with predominant XEH activity – and is capable of interacting with the substrate in the positive sub-sites of the binding cleft. Truncation of this loop results in diminished XEH activity and a significant increase in XET activity (Baumann et al., 2007).

XET is an example of a transglycanase activity. Transglycanases (also known as polysaccharide endotransglycosylases) are polysaccharide-remodelling enzymes that catalyse the transfer of a non-terminal glycosyl group from a donor polysaccharide to an acceptor substrate (typically another polysaccharide or an oligosaccharide molecule) and are thought to be involved in the construction and reversible loosening of the primary cell wall (PCW), allowing PCW reconstruction and elongation (Fry et al., 1992; Darley et al., 2001; Thompson & Fry, 2001). Known PCW-related transglycanase activities include XET (Baydoun & Fry, 1989; Farkaš et al., 1992; Fry et al., 1992; Nishitani & Tominaga, 1992), trans-β-mannanase (Schröder et al., 2004), and trans-β-xylanase (Franková and Fry, 2011; Derba-Maceluch et al., 2015). Transglycanase activities are predominately studied *in vitro* using a donor polysaccharide and an oligosaccharide acceptor labelled with a detectable moiety, typically fluorescent or radioactive.

XET activity is ubiquitous throughout land plants and catalyses the transfer of a xyloglucan glucosyl group, via the endolytic cleavage of the xyloglucan backbone, to the *O*-4 of the non-reducing terminus of another xyloglucan or a xyloglucan oligosaccharide (XGO), generating a new β-(1,4)-glycosidic bond (Baydoun & Fry, 1989; Farkaš et al., 1992; Fry et al., 1992; Nishitani & Tominaga, 1992; Bourquin et al., 2002). The activity of XET and expression of XTHs has been detected at high levels in both growing tissues (Pritchard, 1993; Palmer & Davies 1996; Vissenberg et al., 2000, 2001) and in tissues where expansion has ceased (Arrowsmith & de Silva, 1995; Xu et al., 1995; Palmer & Davies, 1996). Therefore, many roles have been proposed for XET *in vivo* including restructuring of the PCW during secondary wall deposition (Bourquin et al., 2002), cell-wall restructuring (Thompson & Fry, 2001), development of vascular tissues (Hernández-Nistal et al., 2010), PCW

assembly (Thompson et al., 1997), and the mobilisation of seed-storage xyloglucan (Reid et al., 2003; Farkaš et al., 1992).

Large multi-gene families containing 20–60 genes typically encode XET-active proteins (Eklöf & Brumer, 2010) — *Arabidopsis thaliana* has 33 *XTH* genes (Yokoyama & Nishitani, 2001) — giving rise to the functional, spatial and temporal differences observed between different isozymes, even within the same cell (Campbell & Braam, 1999a; Steele & Fry, 2000; Nishitani, 2005). Structural analysis of GH16 proteins has shown them to have a β-jelly-roll fold structure composed of two anti-parallel β-sheets which stack to form a β-sandwich consisting of one convex and one concave face (Johansson et al., 2004). Although variations in the primary structure of XTHs do not seem to significantly alter their conserved secondary structures, even small differences in primary structure can significantly alter their catalytic properties, including the XET : XEH activity ratio (Baumann et al., 2007). The variation possible between XTH isozymes is also exhibited by differences in their substrate specificities: while some XET-active XTHs are highly specific, others are more promiscuous with respect to their acceptor and donor substrate requirements (Kosík et al., 2010; Maris et al., 2011). Substrate specificity can be dependent on specific branching patterns or a requirement for a minimum length of donor/ acceptor substrate.

Interestingly, the recently discovered *Equisetum fluviatile* enzyme, hetero-trans-β-glucanase (HTG), which is also an XET-active GH16 enzyme, turned out to be predominantly a *hetero*-transglucanase, i.e. the preferred donor substrate is qualitatively different from the preferred acceptor substrate (Simmons et al., 2015). This protein is responsible for the previously reported hetero-transglucanase activity found in several *Equisetum* spp., and described as mixed-linkage (1→3, 1→4)-β-D-glucan (MLG) : xyloglucan endotransglucosylase (MXE) (Fry et al., 2008a). The same enzyme also possesses cellulose : xyloglucan endotransglucosylase (CXE; Simmons et al., 2015), and lower XET activity. Therefore, it is perhaps unsurprising given the conserved active site between XTHs and HTG that MXE activity has also been reported as a side-reaction of some XTHs. Hrmova et al. (2007) observed a barley XTH (HvXTH5) with MXE activity of ~0.2% (of the XET activity) in the presence of MLG as donor and

sulphorhodamine (SR)-tagged XGO as acceptor. Some specific arabidopsis XTHs (AtXTH13, 14 and 18) possess slight MXE side-activity (~2%, 2% and 3% of their respective XET activities), while AtXTH12, 17, 19 and 28 have virtually none (Maris et al. 2009; Maris et al. 2011). It has also been reported that AtXTH13, 14 and 18 possess CXE side-activity (~5%, 4% and 22% of their respective XET activities; Maris et al. 2009; Maris et al. 2011).

Most recently, Shinohara et al. (2017) observed a novel cellulose : cellulose endotransglucosylase (CET) activity catalysed as a side-reaction by AtXTH3. Unlike HTG, the predominant activity of AtXTH3 is still XET (specific activity for XET ~120 pmol mg^{-1} min^{-1}), but it presents significant CET (~35 pmol mg^{-1} min^{-1}, with cello-oligosaccharides as acceptor substrate) and CXE (~30–55 pmol mg^{-1} min^{-1}) in the presence of amorphous cellulose as donor substrate. In the case of crystalline cellulose as donor, this activity was very low.

Equisetum is a unique "living fossil". Since it diverged from its closest living relatives more than 370 million years ago (Pryer et al., 2001; Knie et al., 2015), it has become evolutionarily isolated and is the only remaining genus of the order Equisetales (or 'class Sphenopsida'). Interestingly, *Equisetum* has been shown to have a number of unusual biochemical features including the presence of the unusual polysaccharide MLG (Fry et al., 2008b: Sørensen et al., 2008; Xue & Fry, 2012), and the enzyme activities MXE and CXE (Fry et al., 2008a; Mohler et al., 2013; Simmons et al., 2015).

Typically, the primary cell walls (PCWs) of plants are classified into Type I (found in most seed-plants) and Type II (in commelinid monocots), but the *Equisetum* PCW is distinctly different from either. In Type I PCWs, xyloglucan is the predominant hemicellulose (Pauly et al., 1999; O'Neill & York, 2003), comprising ~20% of the wall's dry weight, whilst pectin contributes ~30% (Ridley et al., 2001). By contrast, Type II PCWs are low in pectin and xyloglucan [e.g. 2–5% xyloglucan in barley (Scheller & Ulvskov, 2010)]; the xyloglucan is replaced by hetero-β-xylans and in some tissues also MLG as the principal hemicellulose(s) (Carpita & Gibeaut, 1993). Whilst the *Equisetum* PCW contains high levels of MLG (Fry et al., 2008b), characteristic of some Type II PCWs, it has a low heteroxylan content but a moderately high xyloglucan and pectin

content (Popper and Fry, 2004; Fry et al., 2008b; Silva et al., 2011; Xue & Fry, 2012), unlike conventional Type II PCWs. In addition, *Equisetum* PCWs, like those of many ferns, have a high (gluco)mannan content (Popper and Fry, 2004; Silva et al., 2011), distinguishing them from both Type I and Type II. Differences in the fundamental structure of the PCW are compatible with there being additional differences, in specific wall enzymes, other proteins and developmental signals.

The discovery of HTG enzyme from *Equisetum fluviatile* – the first ever identified predominantly hetero-transglucanase – was an important advance from the discovery of XET activity itself. Although *HTG*-like genes occur in several *Equisetum* spp., they have not been detected in other land plants, supporting the finding that appreciable MXE activity is unique to *Equisetum* (Fry et al., 2008a; Mohler et al., 2013).

The aim of this work was to identify and characterise the activity of a number of *Equisetum* XTHs that were most closely related to HTG. By comparing relative XET, MXE and CXE activities, we aimed to determine their substrate specificities, and compare these to those of known XTHs from arabidopsis to determine the basis for any differences.

In addition to its unique specificity, HTG is also distinctive within known XTH-like proteins in its unusual acidity [predicted pI 4.66 (Table S1); observed pI 4.1 (Simmons et al., 2015)]. Known XTHs cover a wide range of isoelectric points as judged by isoelectric focusing (Iannetta & Fry, 1999; Farkaš et al., 2005) and predicted by gene sequences (Table S1). AtXTH3, which possesses CXE as well as XET activity (Shinohara et al., 2017), is also moderately acidic (predicted pI 5.99; Table S1). We hypothesised that low pI might be a functionally significant feature of heterotransglycanase enzymes and therefore focused this investigation on acidic *Equisetum* XTHs.

The work reported here required a heterologous expression system capable of synthesising adequate quantities of functional *Equisetum* proteins. *Escherichia coli* would be convenient but does not *N*-glycosylate eukaryotic proteins. Instead, we chose the methylotrophic yeast *Pichia pastoris*, which has successfully produced XTHs encoded by genes from cauliflower (Henriksson et

al., 2003), tomato (Catala et al., 2001; Chanliaud et al., 2004) and nasturtium (Baumann et al., 2007; Chanliaud et al., 2004) among others, and secretes only low levels of endogenous proteins (Daly & Hearn, 2005).

2. Materials and methods

2.1. Materials

Equisetum fluviatile was collected from Edinburgh, UK. Barley MLG (medium viscosity) was purchased from Megazyme (http://www.megazyme.co.uk) while tamarind seed xyloglucan was a generous gift from Dr K. Yamatoya, Dainippon Pharmaceutical Co. (http://www.ds-pharma.co.jp). [^{3}H]XXXGol was from EDIPOS (http://fry.bio.ed.ac.uk/edipos.html). Unless otherwise stated, MLG and xyloglucan were used at final concentrations of 0.5% (w/v) in 0.5% (w/v) chlorobutanol. Native HTG was purified from *Equisetum fluviatile* (Simmons et al., 2015).

2.2. Phylogenetic analysis

We estimated the evolutionary relationships of *E. fluviatile* XTHs to all known *A. thaliana* XTHs and *E. fluviatile* HTG by Maximum Likelihood in MEGA X (Kumar et al., 2018). A *Bacillus* glycoside hydrolase (WP_047947368.1) was included as an outgroup. Amino acid sequences were aligned with MUSCLE (Edgar, 2004). Sites corresponding to residues 39 to 294 of AtXTH1 (numbered without the predicted *N*-terminal leaders) that were represented in at least 75% of sequences were used to reconstruct a phylogeny under the LG model of substitution (Le & Gascuel, 2008), allowing for invariant sites and gamma-distributed rate differences between sites. Support was tested with 1,000 bootstrap replicates.

2.3. Cloning of putative XTH genes into Pichia pastoris

Putative *XTH* coding sequences (without their putative *N*-terminal leader sequence) were amplified from *E. fluviatile* cDNA by use of gene-specific primers designed from RNAseq data (courtesy of Dr I. Van Den Brande; BASF, Belgium) and Phusion® high-fidelity DNA polymerase (New England Biolabs,

USA). The primers, which included 5′-sequences complementary to the pPICZαA expression vector (underlined), were:

IB640 (5′-AGAGGCTGAAGCTGAATTCTCATTCGATCGTGACTTCTACATAAC-3′) and IB641 (5′-GAGATGAGTTTTTGTTCTAGACCGTTGAAGGCGCATTCTGGTGG-3′) for EfXTH-A;

IB664 (5′-AGAGGCTGAAGCTGAATTCGCAAACTTCAACCAAGACTTCAACATC-3′) and IB665 (5′-GAGATGAGTTTTTGTTCTAGACCGATATGCGAATTGGAACACTCAGGAG-3′) for EfXTH-H;

and IB666 (5′-AGAGGCTGAAGCTGAATTCTCTTCATCATTCGATCGTGACTTCTC-3′) and IB667 (5′-GAGATGAGTTTTTGTTCTAGACCGTTGAAGGCGCATTCTGGCGG-3′) for EfXTH-I.

GenBank nucleotide sequence accession numbers (BankIt2345959) are: EfXTH-A, MT495433; EfXTH-H, MT495434; EfXTH-I, MT495435.

For infusion cloning, the pPICZαA vector backbone was amplified with primers pPICH-L (5′-AGCTTCAGCCTCTCTTTTCTCGAG-3′) and pPICH-R (5′-GAACAAAAACTCATCTCAGAAGAGGATC-3′) and the methylated template DNA digested with *Dpn* I (EC 3.1.21.4; New England Biolabs, USA) and used for recombination with gel-purified *XTH* sequences according to the manufacturer's instructions (Invitrogen Life Technologies, 2010). The recombination products were used in the transformation via thermoporation of HC1061 *E. coli* cells (Life Technologies, CA, USA) and bacteria were selected on LB containing 0.1 mg/ml zeocin (Life Technologies, CA, USA). Colonies carrying the insert (thus inheriting zeocin resistance) were isolated and used in the transformation of TOP10 electrocompetent *E. coli* cells (Life Technologies, CA, USA) via electroporation, and then spread on LB + kanamycin A (Invitrogen Life Technologies) plates. Each clone was analysed for secreted *myc*-tagged protein by dot-blot (see 2.4) and then sequenced.

2.4. Transformation of Pichia

Recombinant plasmids were linearised with *Pme* I EC 3.1.21.-; New England Biolabs, USA) and before being used to transform *Pichia* strain SMD 1168H by electroporation. *Pichia* cells were selected on YPDS plates (Life Technologies, CA, USA) containing zeocin (1 mg ml^{-1}).

Cultures were grown in BMGY medium at 28°C overnight (12 clones per construct) prior to induction of expression in BMMY for at least 4 h (Invitrogen Life Technologies, 2010). The harvested culture supernatants were tested for expression by dot-blotting with a rabbit anti-*myc* primary antibody (ab9106, Abcam) and a goat anti-rabbit-HRP secondary antibody (ab97051, Abcam), detected by chemiluminescence.

2.5. Large-scale protein expression

The optimal *Pichia* clone for each construct was grown in 250 ml of BMGY overnight and resuspended in BMMY at A_{600} ~1. Expression proceeded for 16–24 h, after which the culture supernatant was stored at 4°C. Secreted proteins were concentrated on Amicon® UltraCel®-10K regenerated cellulose (MW cut-off = 10000; Merck Millipore Ltd., Ireland). Concentrated samples were stored at –20°C.

2.6. XET and MXE activity assays

XET activity was assayed in a reaction mixture consisting of 10 µl *Pichia*-secreted enzyme extract, 1 kBq [^{3}H]XXXGol, 5 mg/ml xyloglucan and 50 mM MES (Na^+, pH 6.0), in a final volume of 20 µl, at 20°C; the reaction was stopped by addition of 10 µl of 50% (v/v) formic acid. Each sample was then loaded onto Whatman 3MM filter paper, dried and then washed thoroughly with free-flowing water, which removes unreacted [^{3}H]XXXGol. Each paper sample was dried, incubated with Goldstar Organic liquid scintillation cocktail (2 ml) and assayed for radioactivity (2 × 5 min). "Enzyme-free" controls involved the addition of formic acid before the enzyme. The MXE activity assay differed from the XET assay through the use of MLG as the donor polysaccharide instead of xyloglucan.

2.7. CXE activity assay

The cellulose used as donor substrate in CXE assays was Whatman No. 1 paper that had been incubated overnight at 37°C in 6.0 M NaOH and then washed in water repeatedly until neutral. The paper was then washed in

pyridine/acetic acid/water (33:1:300, by vol., pH 6.5) and then again with water. Finally, the paper was lyophilised, and aliquoted by mass.

Unless otherwise stated, 1 kBq [^{3}H]XXXGol in 33 µl enzyme extract [in 50 mM MES (Na^+); pH 6.0] was added to 10 mg of the pre-treated paper and incubated at 20°C. The reaction was stopped by the addition of 300 µl 10% (v/v) formic acid before repeated washing in water for 16 h to remove unreacted [^{3}H]XXXGol. Cellulose was then resuspended in 0.2 ml water and 2 ml ScintiSafe 3 liquid scintillant cocktail (Fisher Scientific, UK) and incubated for 24 h prior to assaying for radioactivity.

2.8. Effects of native Pichia secreted proteins on MLG and on Equisetum transglucanase activities

To determine whether native *Pichia*-secreted proteins degraded MLG, we conducted viscosity assays. The reaction mixture contained 3.64 mg/ml MLG and native protein secreted by *Pichia* expressing an empty pPICZαA plasmid (final concentration 9% v/v of crude culture medium) in 50 mM MES (Na^+, pH 6.0). The control received buffer in place of secreted proteins. The mixtures were incubated for 12 h at 20°C. Post-incubation, the MLG was drawn into a vertically clamped 1-ml glass pipette with its tip just submerged in the solution, and the time taken for the meniscus to fall by 200 µl was measured.

For the mixing experiments with native EfHTG, the reaction mixture contained 10 µl native EfHTG solution, 1 kBq [^{3}H]XXXGol (dried), 10 µl of culture supernatant from *Pichia* expressing an empty pPICZαA plasmid (final concentration 4.5% v/v of crude culture medium), and 5 mg/ml xyloglucan or MLG (for XET and MXE respectively), all in 50 mM MES (Na^+, pH 6.0); final reaction volume 40 µl. The enzyme-free control received buffer in place of EfHTG while the donor-free control received buffer in place of xyloglucan or MLG. The mixtures were incubated for 12 h at 20°C prior to loading onto Whatman 3MM filter paper as with the standard XET and MXE activity assays.

3. Results

3.1. Production of acidic Equisetum GH16 proteins in Pichia

Via a BLAST search of the NCBI non-redundant database and an *E. fluviatile* transcriptome database, using in-house licensed MASCOT software, we identified five sequences encoding acidic GH16 proteins with homology to known XTHs (named here *EfXTH-Ha*, *-Hb*, *-Hc*, *-A* and *-I*). The primary structures of EfXTH-Hb and EfXTH-Hc both differed from EfXTH-Ha by one amino acid. The amino acid substitutions from EfXTH-Ha were S → P at position 251 and V → A at position 86 for EfXTH-Hb and EfXTH-Hc respectively. Such small differences in primary structure mean that it is unlikely that EfXTH-Ha, -Hb and -Hc represent different genes. Indeed, EfXTH-Hb and EfXTH-Hc were not found in a second, independently generated, transcriptome. Therefore, EfXTH-Ha is here considered to be the consensus sequence and is referred to simply as EfXTH-H (Fig. 2).

Multiple sequence alignment of the identified acidic GH16 XTH homologues showed high conservation between their primary structures; their sequence identity ranged from 55.0 to 86.8% (Fig. 2). As expected, owing to the propensity for a conserved binding cleft and active site topology within a GH family, sequence homology both between the EfXTH proteins themselves and between them and the 33 arabidopsis XTHs is higher in the regions flanking the conserved active site (typically EL/IDFE), including the conserved *N*-glycosylation site. Unlike other GH16s, most XTHs studied have a conserved glycosylation site 5–15 residues towards the *C*-terminus from the active site (Johansson et al., 2004) thought to be vital for XET function as deglycosylation of this residue results in the loss of XET activity (Campbell & Braam, 1999; Henriksson et al., 2003). However, this may not be an absolute requirement in all cases as, for example, deglycosylation of this residue in *Ptt*XET16A resulted in retention of significant XET activity (Johansson et al., 2004). The percentage identity (evaluated by an EMBL-EBI FASTA protein similarity search) between the EfXTHs and any currently known, or predicted, XTHs from other species never exceeded 62%. This is perhaps unsurprising given the phylogenetic distance of *Equisetum* from all other genera (Des Marais et al., 2003). HTG, another XET-active transglucanase from *E. fluviatile*, shared only 49.6% (EfXTH-I) to 55.5% identity (EfXTH-H) with any of the acidic EfXTHs. Despite

this and their low predicted pI values, the total number of acidic amino acids, predicted molecular weight, and total number of *N*-glycosylation sites are consistent with other known XTHs and HTG (Table 1 and Table S1). However, the acidic EfXTHs have significantly fewer basic amino acids (ranging from 20 to 28 for EfXTH-H and EfXTH-I respectively) than the average for AtXTHs (36.6 basic amino acids; Table S1), and a lower basic : acidic amino acid ratio than AtXTHs. The average basic : acidic amino acid ratio for AtXTHs is 1.30, ranging from 0.86 for AtXTH23 to 2.05 for AtXTH32. By contrast, EfHTG with only 21 basic amino acids, has a basic : acidic residue ratio of 0.75, while the acidic EfXTHs have ratios of 0.71, 0.83 and 1.04 (EfXTH-H, EfXTH-A and EfXTH-I respectively).

The 33 AtXTHs have an average of 28.8 acidic amino acids, which falls within the very narrow range (27–29) for EfHTG and the acidic EfXTHs reported here, although the AtXTHs have a very wide range (21–47) of acidic amino acids. Thus, the acidic EfXTHs owe their low pI values to their small number of basic amino acids rather than numerous acidic ones (Table S1).

The native cDNA sequences from *E. fluviatile* (carrying an *N*-terminal *myc*-tag and *C*-terminal His-tag) were cloned into the pPICZαA vector, enabling protein production in *Pichia pastoris* SMD1168H. Successful production of recombinant proteins was determined via dot-blot analysis through detection of the *myc*-tag on the recombinant protein.

3.2. All acidic GH16 proteins from Equisetum exhibit XET activity and low levels of MXE and CXE activity

All the acidic EfXTHs tested displayed measurable levels of XET activity during a 1-h incubation, but the observed MXE and CXE activities were markedly lower (Table 2). Interestingly, the ratio of XET : MXE : CXE activities varied between the different proteins. In contrast, MXE and CXE are the preferred activities of EfHTG (Simmons et al., 2015 and Table 2). EfXTH-H and EfXTH-I displayed comparable MXE and CXE activity. EfXTH-A had very low MXE and CXE activities (0.2–0.3% of the XET activity; Table 2). Thus, EfXTH-A is a more specific XET. Therefore, these XET-active acidic EfXTHs differ in

donor-substrate specificity, and their MXE activity tends to correlate with CXE, supporting the hypothesis that MXE and CXE activity are attributable to similar structural changes relative to other XTH proteins.

3.3. Kinetics of the XET, MXE and CXE activities of EfXTH-H

The donor polysaccharides for XET and MXE assays are water-soluble xyloglucan and MLG respectively. By contrast, paper was the (insoluble) cellulose used for the CXE assay. The difference in substrate solubility influences the concentration and availability of the donor polysaccharide to the enzyme and the detection of transglucanase products. The differences between the activity assays mean that no direct comparisons can be made between the CXE assay and the other two assays with respect to radioactivity incorporated per hour. However, by expressing activity per given volume of enzyme for each transglucanase activity, it is possible to calculate a meaningful relative ratio of all three activities.

Suitable incubation times were selected to obtain approximately linear initial rates, avoiding depletion of the acceptor substrate during the assays (Fig. 3). When crude *Pichia*-produced EfXTH-H was used (Fig. 3a), the yield of XET reaction products was initially rapid (initial, approximately linear rate ~31 cpm/min), becoming non-linear after ~100 min (at ~3000 cpm) and plateauing at ~8000 cpm after ~800 min. The theoretical maximum yield that would be achieved if 100% of the [^{3}H]XXXGol (acceptor substrate) were converted to product was ~16000 cpm. The same enzyme preparation also exhibited measurable MXE and CXE activities. Unexpectedly, however, in the MXE assay, maximum incorporation of radioactivity was limited to ~300 cpm, achieved after ~400 min, even though this represented the consumption of only ~2% of the supplied [^{3}H]XXXGol and the CXE products were still progressively accumulating beyond 1400 min (Fig. 3a). The 'premature' cessation of MXE product formation suggests degradation of the donor polysaccharide (MLG) by a component of the native *Pichia* secretions.

The His-tag-purified EfXTH-H was tested at a higher concentration, giving an initial approximately linear XET rate of ~580 cpm/min but quickly

plateauing, probably limited by acceptor availability (Fig. 3b). Interestingly, the MXE activity of the purified EfXTH-H remained highly stable (at ~4.7 cpm/min) over a 17-h period (Fig. 3b), demonstrating negligible denaturation of the enzyme. Presumably during these assays no loss of MLG occurred because native *Pichia* secretions were absent; this concept is addressed later in the mixing experiment. The yield of MXE products greatly exceeded the ~300 cpm limit previously observed for crude EfXTH-H, rising steadily to almost 5000 cpm over 1000 min. CXE activity observed was also greater with the purified enzyme, although the difference from crude enzyme was not as dramatic as observed for MXE, suggesting that MLG but not cellulose or xyloglucan is affected by native *Pichia* secretions.

3.4. Viscosity assay and mixing experiments — Pichia secretions degrade MLG

A large decrease in MLG's viscosity was observed during a 12-h incubation with native *Pichia* secretions (from a *Pichia* strain expressing an empty pPICZαA plasmid) (Table 3). The observed ~15-fold increase in specific fluidity of the MLG solution indicates appreciable but far from complete depolymerisation: the specific fluidity (which is related to the number of scission events; Fry, 1998) of 4 mg/ml MLG with and without pPICzαA was 0.008 and 0.13 respectively. Partial degradation of MLG suggests that the MXE activity of crude EfXTH-H previously observed (Fig. 3a) was limited because the MLG polysaccharide chains became too small for them to act as efficient donor substrates. It is less likely that the transglucanase products were too small to remain on the paper during the washing procedure, as even small MLG fragments (e.g. hexasaccharides and larger) have been found to remain bound when dried onto paper and to be practically immobile on paper chromatography.

Further to this, when native EfHTG purified from *E. fluviatile* plants (nEfHTG) was assayed for XET and MXE activity, MXE activity decreased by almost 50% when mixed with medium from *Pichia* (expressing the empty pPICZαA plasmid) secretions compared with the secretion-free control (Fig. 4). There was negligible effect on the XET activity, supporting the hypothesis that

MLG donor substrate itself was being degraded rather than the xyloglucan oligosaccharide acceptor substrate or the enzyme.

4. Discussion

We identified the first three *Equisetum* genes known to encode GH16 XTHs and heterologously produced the corresponding proteins in functionally active form in *Pichia*. These proteins catalyse transglycosylation (especially XET) reactions.

Untransformed *Pichia* was found to secrete MLG-degrading enzyme(s), which had to be removed for reliable MXE assays. This was successfully achieved by His-tag purification of the EfXTH.

Given the unique ratio of activities observed for EfHTG (MXE > CXE > XET; Fig. 3c), our core aim was to identify HTG's closest *Equisetum* XTH relatives and compare their activity ratios (Table 2). This approach, looking at natural variation, can usefully complement the approach of artificially mutagenising XTHs at specific sites (Stratilová et al., 2019). All three recombinant EfXTHs (-A, -H and -I), including all three minor variants of EfXTH-H, were XET-active. However, although there was extensive homology between their primary structures, and that of HTG, differences in activity ratios were observed. EfXTH-A had the lowest relative MXE activity, only 0.2% of the XET activity, which is similar to the value seen in a barley XTH (Hrmova et al., 2007) and in crude extracts of numerous other land plants (Fry et al., 2008a). By contrast, EfXTH-H had a somewhat higher proportion of MXE activity. Therefore, EfXTH-A is a more specific XET than EfXTH-H. None of the EfXTHs tested here exhibited relative MXE activities approaching that of HTG (whose MXE was ~300% of its XET activity in the present work; Fig. 3c; Table 2) but the values observed for EfXTH-H and -I were higher than those reported for most other known land-plant XTHs and crude plant extracts. Extracts from charophytic algae, on the other hand, often gave high relative MXE activities (~20–250% of their XET activity; Fry et al., 2008a).

HTG, with its predominant hetero-transglycosylation activities, is an acidic protein, having an unusually low pI [predicted 4.66 (Table S1); observed

4.1 (Simmons et al., 2015)], which might be suggested to be a feature contributing to its unusually lax substrate specificity, or a feature reflecting the functionality of hetero-transglycanase activities. Of the 33 arabidopsis XTHs, only five are predicted to have pI values below 5.8, the lowest being 5.05 (AtXTH23; Table S1). However, in *Equisetum*, the low pI of HTG is not unique among its GH16 proteins: in the present work, we found genes for three *Equisetum* XTHs with predicted pI values lower than 5.8. These three acidic EfXTHs, and *Equisetum* HTG, all have unexceptional numbers of Asp and Glu residues (27–29 total acidic amino acid residues, very similar to the average number, 28.8, of all arabidopsis XTHs; Table S1). On the other hand, the three acidic EfXTHs and HTG have considerably fewer Lys, Arg and His residues (20–28 total basic residues) than most arabidopsis XTHs (average 36.6, range 23–50). Thus, few basic residues, rather than many acidic residues, accounts for the low pI of the investigated EfXTHs. However, the most XET-specific example, EfXTH-A, was not the least acidic, so there is no simple correlation between low pI and high MXE or CXE activity in *Equisetum* XTHs broadly.

This characteristic is also discernible in other XTHs. For example, among arabidopsis XTHs, AtXTH13, -14 and -18 all possess slight MXE side-activity (~2%, 2% and 3% of their respective XET activities), while AtXTH12, -17, -19 and -28 have virtually none (Maris et al. 2009; Maris et al. 2011). The former set — AtXTH13, 14 and 18 — have predicted pI values of 5.1, 8.5 and 8.7 respectively (Table S1), so again there is no simple relationship between MXE activity and the proteins' acidity.

Thus, it is unlikely that a low pI and low basic amino acid residue content are key requirements for MXE activity on a biochemical level. It may still, however, be the case that the proper functioning of MXE and CXE activities *in muro* is contingent on a low pI.

The same conclusion applies to XTHs that possess moderate CXE activity. EfXTH-H and EfXTH-A have the highest and lowest relative CXE activity (Table 2), but their predicted pI values are rather similar (~4.6 and 4.9 respectively; Table 1).

We have previously suggested that EfHTG's predominant heterotransglucanase activity is related to three specific amino acid residues — Pro-10, Ser-34 and Leu-245 — which participate in binding of the donor and/or acceptor substrates in the active site (Simmons et al., 2015). These three amino acid residues are replaced in the majority of arabidopsis XTHs, and in all three EfXTHs reported here, by Trp, Gly and Arg respectively. Besides HTG, the only other known GH16 protein able to catalyse transglycosylation at an appreciable rate with cellulose as the donor substrate is AtXTH3 (Shinohara et al., 2017): this, however, has the standard Trp and Gly at the first two of these three key positions, but it has Lys (instead of the conventional XTHs' Arg or HTG's Leu) at the third. This Lys in place of Arg may account for AtXTH3's high ability to tolerate cello-oligosaccharides as acceptor substrate, which other AtXTHs and EfHTG do not. These substitutions in HTG plausibly account for the unique activity range of HTG, not emulated by conventional XTHs, including the three acidic XTHs from *Equisetum* reported here.

Authors' contribution

SCF, FM, AH and CH designed the research, with the project conceived by SCF, FM and AH. CH performed most of the experiments. TJS purified the native *Equisetum* enzyme and identified the GH16 EfXTH sequences. CH and SCF wrote the manuscript. All authors commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at.....

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760

Tables

Table 1: Numbers of salient amino acid residues and predicted *N*-glycosylation sites in mature EfXTH proteins compared to mean of the 33 AtXTHs

Amino acid residue	**Mean of 33 AtXTHs**	EfXTH-H	EfXTH-A	EfXTH-I	EfHTG
Asp	**16.7**	20	18	18	19
Glu	**12.1**	8	11	9	9
Trp	**8.9**	9	8	8	9
Lys	**16.6**	9	11	12	8
Arg	**14.0**	7	9	10	8
His	**6.1**	4	4	6	5
Cys	**4.4**	4	4	4	4
Tyr	**14.2**	14	11	11	15
Total acidic AAs	**28.8**	28	29	27	28
Total basic AAs	**36.6**	20	24	28	21
Total N-glycosylation sites (NXS/T)	**1.79 (range 0–6)**	2	4	4	1
Predicted pI *	**7.76**	4.57	4.88	5.65	4.66
Predicted M_r (kDa)	**31.2**	30.402	30.420	29.855	29.534

*Neglecting glycosylation, phosphorylation etc.

773 **Table 2**: Relative XET, MXE and CXE activities of *Equisetum fluviatile* GH16
774 recombinant XTHs compared with native *Equisetum* HTG

775 Transglycanase activities of three heterologously produced EfXTHs, showing
776 radioactivity incorporation rate per 10 µl enzyme extract. Data are corrected for
777 enzyme-free controls.

Protein	**XET/ 10 µl/ min (cpm)**	**MXE/ 10 µl/ min (cpm)**	**CXE/ 10 µl/ min (cpm)**	**XET : MXE : CXE**
EfXTH-H †	58.6 ± 11.2	2.20 ± 0.20	4.77 ± 0.40	100 : 3.8 : 8.1
EfXTH-H crude (Fig. 3a)*	31.4 ± 0.9	0.59 ± 0.16	0.41 ± 0.02	100 : 1.9 : 1.3
EfXTH-H purified (Fig. 3b)*	582 ± 79	4.65 ± 0.11	2.72 ± 0.12	100 : 0.8 : 0.5
EfXTH-A †	97.3 ± 4.9	0.167 ± 0.001	0.32 ± 0.08	100 : 0.2 : 0.3
EfXTH-I †	10.5 ± 0.1	0.30 ± 0.02	0.22 ± 0.05	100 : 2.9 : 2.1
EfHTG, native, purified from *Equisetum* plants (Fig. 3c).*	0.050 ± 0.006	0.153 ± 0.006	0.080 ± 0.002	100 : 308 : 160

778

779 * These estimates are based on initial rates measured during the first (approximately linear) 4–
780 1440 min of incubation from the data in Fig. 3. Error shown is the SE of the fitted linear
781 regression.

782 † These assays are based on 60-min incubations with 10 µl of crude *Pichia*-produced protein. Errors
783 are SE of 3 replicates.

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788 **Table 3**: Viscosity assay to determine effects of native secreted *Pichia* proteins on MLG.

789 MLG (3.64 mg/ml) was incubated for 12 h with either endogenous *Pichia* secretion
790 products (pPICZαA empty vector) or an equal volume of buffer. Water acted as an
791 indicator of efflux time expected following complete polysaccharide degradation. SE
792 indicates standard error from 5 repeats.

793

Solution	Viscometer efflux time (s) ± SE
MLG alone	95.0 ± 2.0
MLG + *Pichia* secretion products	5.8 ± 0.2
Water	<1

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796

Figure legends

Figure 1: Relationship between XTH proteins from *A. thaliana* and *E. fluviatile* and HTG from *E. fluviatile*

Best Maximum Likelihood tree showing relationships between *A. thaliana* (At) and *E. fluviatile* (Ef) proteins. The tree is rooted on a *Bacillus* glycosyl hydrolase, on a branch that is not to scale. Percentage values are shown for nodes that were recovered in at least 50% of bootstrap replicates. The heat-map represents predicted isoelectric points.

Figure 2: Alignment of GH16 predicted protein sequences of acidic XTH-homologues and HTG from *Equisetum fluviatile*

ClustalW multiple sequence alignment by MUSCLE (3.8). Homology between constructs is indicated as (*) identical, (:) conserved substitutions, and (.) semi-conserved substitutions. The predicted signal peptide cleavage site (SignalP-4.1) is indicated by the end of the underlined section, and the active site and the conserved Asn *N*-glycosylation site are shaded. The sequences, alignment and tree will be available (on publication) on TreeBase, study accession number TBS2:S26112 or link http://purl.org/phylo/treebase/phylows/study/TB2:S26112

Figure 3: Time courses for *in-vitro* transglucanase reactions of EfXTH-H and EfHTG

XET, MXE and CXE activities of *Pichia*-produced proteins: (a) unpurified EfXTH-H, (b) His-tag-purified EfXTH-H, (c) EfHTG. Each was assayed with 10 μl enzyme extract. In (a), the MXE and CXE values have been increased 10-fold so that the trends can be discerned.

Figure 4: Effect of *Pichia pastoris* secretions on apparent MXE and XET activities of native *Ef*HTG protein

XET and MXE activities of native HTG purified from *Equisetum fluviatile* plants (*Ef* HTG), the secretion products of a pPICZαA-expressing *P. pastoris* culture (*Pichia*), and a mixture of these. A control with no deliberately added donor was included, revealing

any activity due to contaminating polysaccharides from either the *Equisetum* or the *Pichia*.

Supplementary Table 1: Summary of all *Arabidopsis thaliana* XTHs and the acidic *Equisetum fluviatile* XTHs, compared with EfHTG.

Figure 1 B&W

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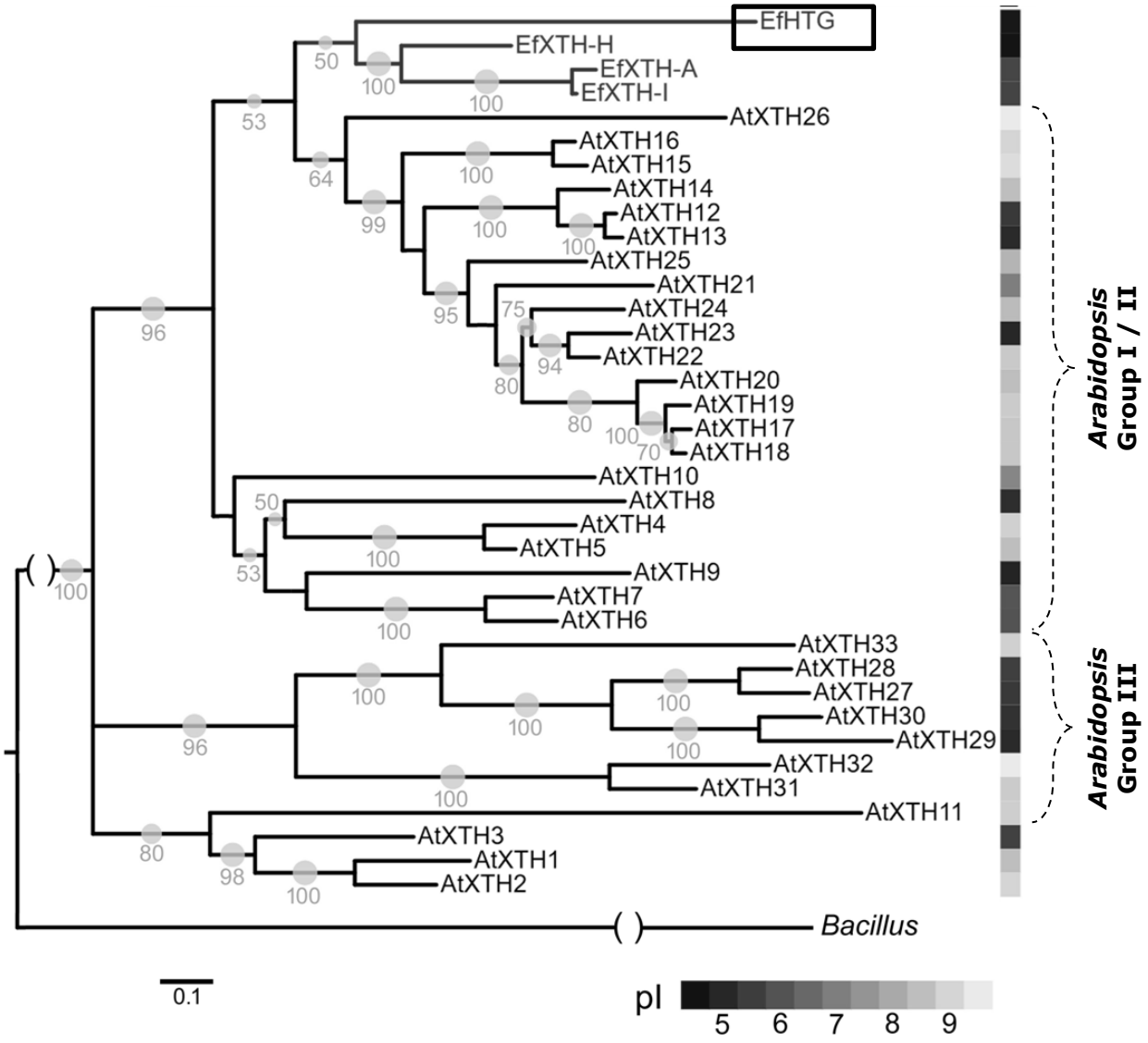

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Best Maximum Likelihood tree showing relationships between *A. thaliana* (At) and *E. fluviatile* (Ef) proteins. The tree is rooted on a *Bacillus* glycosyl hydrolase, on a branch that is not to scale. Percentage values are shown for nodes that were recovered in at least 50% of bootstrap replicates. The heat-map represents predicted isoelectric points.

Figure 2 B&W

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Figure 2: Alignment of GH16 predicted protein sequences of acidic XTH-homologues and HTG from *Equisetum fluviatile*
ClustalW multiple sequence alignment by MUSCLE (3.8). Homology between constructs is indicated as (*) identical, (:) conserved substitutions, and (.) semi-conserved substitutions. The predicted signal peptide cleavage site (SignalP-4.1) is indicated by the end of the underlined section, and the active site and the conserved Asn *N*-glycosylation site are shaded.

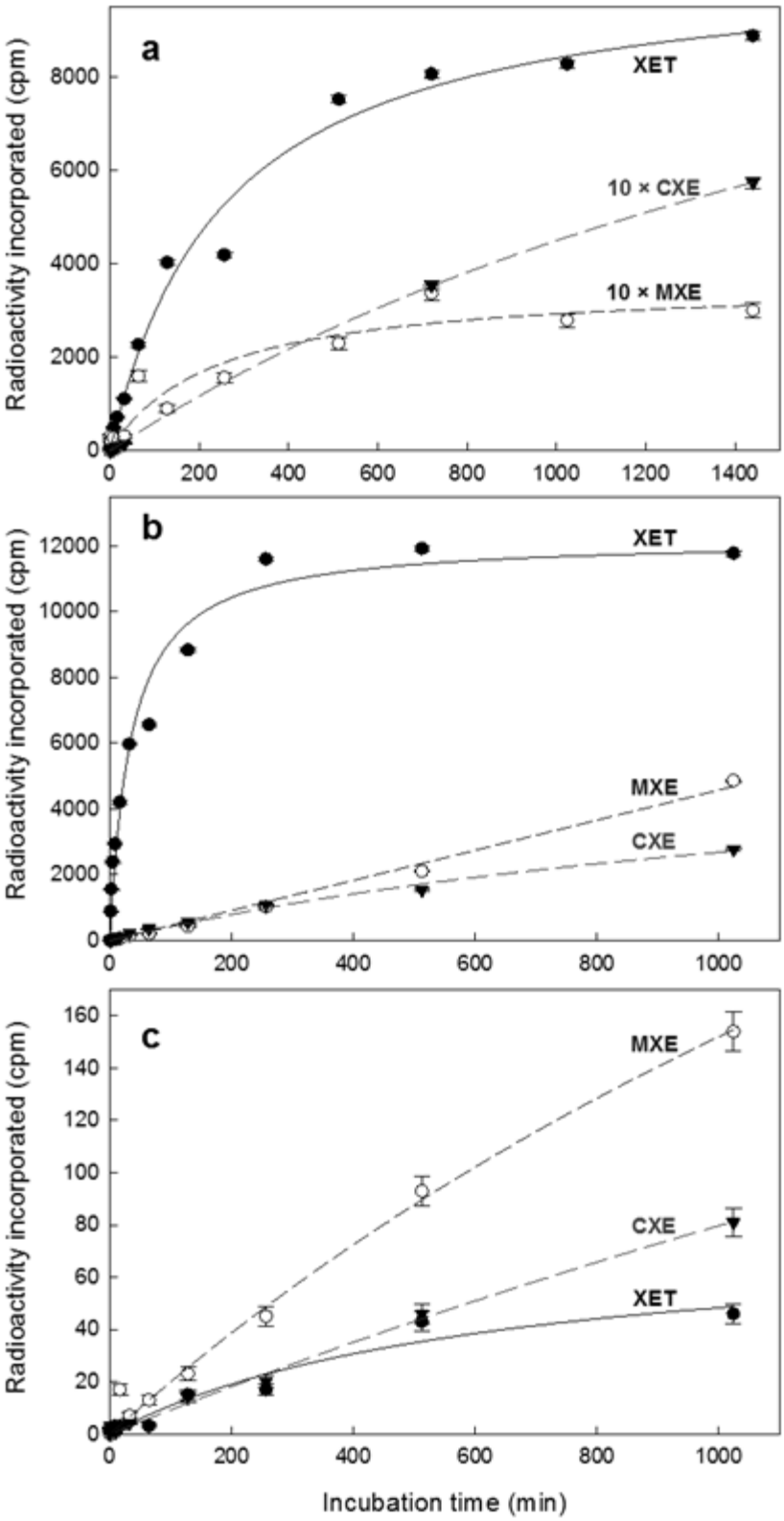

Figure 3: Time courses for *in-vitro* transglucanase reactions of EfXTH-H and EfHTG
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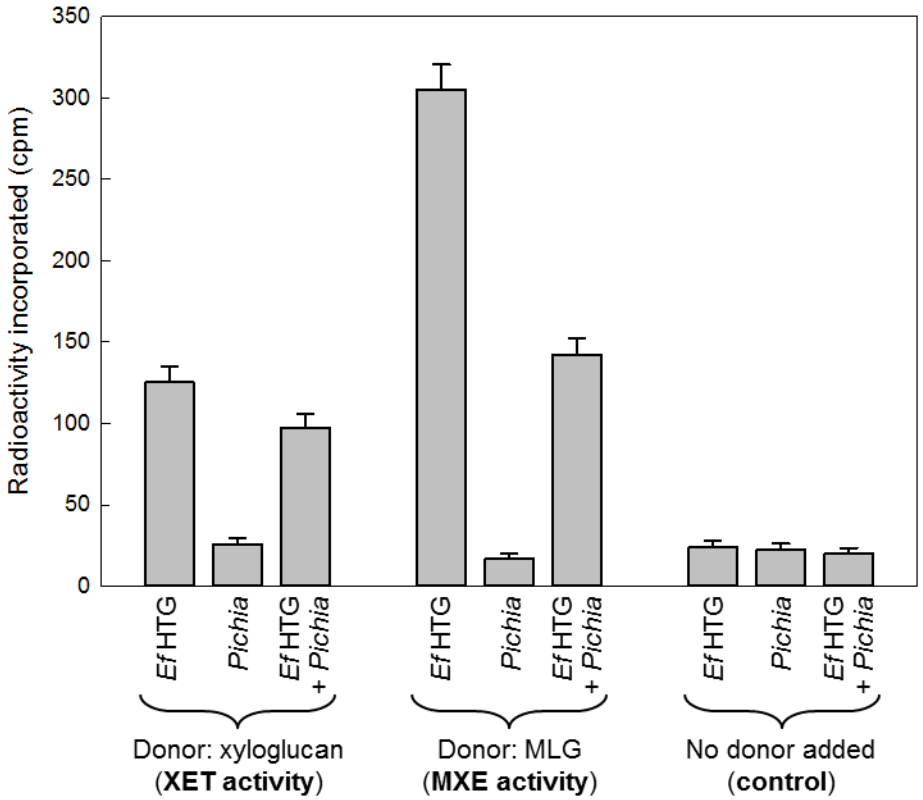

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***Declaration of Interest Statement**

Competing interests

A patent application (WO2015044209) has been filed by BASF Agricultural Solutions Belgium NV and The University of Edinburgh for the use of hetero-transglycosylase. F.M., A.H., S.C.F., T.S. and C.H. are inventors.

Credit Author Statement

SCF, FM, AH and CH designed the research, with the project conceived by SCF, FM and AH. CH performed most of the experiments. TJS purified the native *Equisetum* enzyme and identified the GH16 EfXTH sequences. CH and SCF wrote the manuscript. All authors commented on the manuscript.

e-component Table S1 Excel file

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