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

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19
20 *Keywords:*
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22 Heterologous expression
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24 Cell elongation
25 *Equisetum*
26 Plant cell wall
27 Hetero-trans- β -glucanase
28

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

32 (homotransglucanase activity); MLG, mixed-linkage (1→3, 1→4)-β-D-glucan;
33 PCW, primary cell wall; GH, glycoside hydrolase; XXXGol, borohydride-reduced
34 heptasaccharide of xyloglucan (xylose₃ glucose₃ glucitol).

35

36 **ABSTRACT**

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

58

59 **1. Introduction**

60 Glycoside hydrolases (GHs) are a group of ‘carbohydrate-active enzymes’
61 (CAZy) that hydrolyse glycosidic linkages in both polysaccharides and low-
62 molecular-weight *O*-, *N*- and *S*-linked glycosides. More than 160 GH families
63 have been described, with enzymes grouped according to primary sequence
64 similarity. This has led to groupings that reflect common active-site topologies
65 and modes of catalysis, but interestingly usually not substrate specificity
66 (Barbeyron et al., 1998). High-resolution structural data have shown that
67 proteins within the same GH family have a conserved core, including active-site
68 residues, and major elements of secondary and tertiary structure, even when
69 primary structure similarity is low (Gebler et al., 1992). The GH16 family of
70 enzymes is most pertinent to the present work, a group with an extensive array
71 of substrate specificities leading to cleavage of β -1,3- or β -1,4-glycosidic bonds in
72 various glucans and galactans. The substrate specificity of GH16s is amongst the
73 most varied of any GH group, with enzyme activities including, but not limited
74 to, xyloglucan endotransglucosylase (XET; Rose et al., 2002; EC 2.4.1.207; also
75 known as xyloglucan:xyloglucosyl transferase), xyloglucan endohydrolase (XEH;
76 EC 3.2.1.151), endo-1,3- β -galactanase (EC3.2.1.-), endo-1,3- β -glucanase
77 (laminarinase, EC 3.2.1.39), lichenase (EC 3.2.1.73), and κ -carageenase (EC
78 3.2.1.83) (Viborg et al., 2019).

79 Xyloglucan endotransglucosylase/hydrolases (XTHs) are a subfamily of the
80 GH16 enzyme family (Cantarel et al., 2009) that catalyse xyloglucan
81 endotransglucosylase (XET) and/or xyloglucan endohydrolase (XEH) reactions
82 (de Silva et al., 1993; Fanutti et al., 1993; Tabuchi et al., 2001; Rose et al., 2002).
83 Although some XTHs catalyse both XET and XEH reactions (Bourquin et al.,
84 2002; Eklöf and Brumer, 2010; Shi et al., 2015), most XTHs for which detailed
85 kinetic data are available are strict XETs and display undetectable XEH activity
86 (Fry et al., 1992; Nishitani & Tominaga, 1992; Stratilová et al., 2010).
87 Phylogenetically, XTHs are divided into groups I/II (predominant XETs) and III
88 (predominant XEHs) (Fig. 1). Baumann et al. (2007) identified a unique
89 extension of the loop connecting strands β 8- β 9 in predominant XEHs as a major,
90 but not the only, contributor to defining XEH over XET activity. This loop lies

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

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

110 XET activity is ubiquitous throughout land plants and catalyses the
111 transfer of a xyloglucan glucosyl group, via the endolytic cleavage of the
112 xyloglucan backbone, to the *O*-4 of the non-reducing terminus of another
113 xyloglucan or a xyloglucan oligosaccharide (XGO), generating a new β -(1,4)-
114 glycosidic bond (Baydoun & Fry, 1989; Farkaš et al., 1992; Fry et al., 1992;
115 Nishitani & Tominaga, 1992; Bourquin et al., 2002). The activity of XET and
116 expression of XTHs has been detected at high levels in both growing tissues
117 (Pritchard, 1993; Palmer & Davies 1996; Vissenberg et al., 2000, 2001) and in
118 tissues where expansion has ceased (Arrowsmith & de Silva, 1995; Xu et al.,
119 1995; Palmer & Davies, 1996). Therefore, many roles have been proposed for
120 XET *in vivo* including restructuring of the PCW during secondary wall
121 deposition (Bourquin et al., 2002), cell-wall restructuring (Thompson & Fry,
122 2001), development of vascular tissues (Hernández-Nistal et al., 2010), PCW

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

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

142 Interestingly, the recently discovered *Equisetum fluviatile* enzyme, hetero-
143 trans- β -glucanase (HTG), which is also an XET-active GH16 enzyme, turned out
144 to be predominantly a *hetero*-transglucanase, i.e. the preferred donor substrate is
145 qualitatively different from the preferred acceptor substrate (Simmons et al.,
146 2015). This protein is responsible for the previously reported hetero-
147 transglucanase activity found in several *Equisetum* spp., and described as
148 mixed-linkage (1 \rightarrow 3, 1 \rightarrow 4)- β -D-glucan (MLG) : xyloglucan endotransglucosylase
149 (MXE) (Fry et al., 2008a). The same enzyme also possesses cellulose : xyloglucan
150 endotransglucosylase (CXE; Simmons et al., 2015), and lower XET activity.
151 Therefore, it is perhaps unsurprising given the conserved active site between
152 XTHs and HTG that MXE activity has also been reported as a side-reaction of
153 some XTHs. Hrmova et al. (2007) observed a barley XTH (HvXTH5) with MXE
154 activity of ~0.2% (of the XET activity) in the presence of MLG as donor and

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

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

168 *Equisetum* is a unique “living fossil”. Since it diverged from its closest
169 living relatives more than 370 million years ago (Pryer et al., 2001; Knie et al.,
170 2015), it has become evolutionarily isolated and is the only remaining genus of
171 the order Equisetales (or ‘class Sphenopsida’). Interestingly, *Equisetum* has been
172 shown to have a number of unusual biochemical features including the presence
173 of the unusual polysaccharide MLG (Fry et al., 2008b; Sørensen et al., 2008; Xue
174 & Fry, 2012), and the enzyme activities MXE and CXE (Fry et al., 2008a; Mohler
175 et al., 2013; Simmons et al., 2015).

176 Typically, the primary cell walls (PCWs) of plants are classified into Type
177 I (found in most seed-plants) and Type II (in commelinid monocots), but the
178 *Equisetum* PCW is distinctly different from either. In Type I PCWs, xyloglucan is
179 the predominant hemicellulose (Pauly et al., 1999; O’Neill & York, 2003),
180 comprising ~20% of the wall’s dry weight, whilst pectin contributes ~30% (Ridley
181 et al., 2001). By contrast, Type II PCWs are low in pectin and xyloglucan [e.g. 2–
182 5% xyloglucan in barley (Scheller & Ulvskov, 2010)]; the xyloglucan is replaced
183 by hetero-β-xylans and in some tissues also MLG as the principal
184 hemicellulose(s) (Carpita & Gibeaut, 1993). Whilst the *Equisetum* PCW contains
185 high levels of MLG (Fry et al., 2008b), characteristic of some Type II PCWs, it
186 has a low heteroxyylan content but a moderately high xyloglucan and pectin

187 content (Popper and Fry, 2004; Fry et al., 2008b; Silva et al., 2011; Xue & Fry,
188 2012), unlike conventional Type II PCWs. In addition, *Equisetum* PCWs, like
189 those of many ferns, have a high (gluco)mannan content (Popper and Fry, 2004;
190 Silva et al., 2011), distinguishing them from both Type I and Type II. Differences
191 in the fundamental structure of the PCW are compatible with there being
192 additional differences, in specific wall enzymes, other proteins and
193 developmental signals.

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

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

205 In addition to its unique specificity, HTG is also distinctive within known
206 XTH-like proteins in its unusual acidity [predicted pI 4.66 (Table S1); observed
207 pI 4.1 (Simmons et al., 2015)]. Known XTHs cover a wide range of isoelectric
208 points as judged by isoelectric focusing (Iannetta & Fry, 1999; Farkaš et al.,
209 2005) and predicted by gene sequences (Table S1). AtXTH3, which possesses
210 CXE as well as XET activity (Shinohara et al., 2017), is also moderately acidic
211 (predicted pI 5.99; Table S1). We hypothesised that low pI might be a
212 functionally significant feature of heterotransglycanase enzymes and therefore
213 focused this investigation on acidic *Equisetum* XTHs.

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

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

222

223 **2. Materials and methods**

224 *2.1. Materials*

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

233

234 *2.2. Phylogenetic analysis*

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

245

246 *2.3. Cloning of putative XTH genes into Pichia pastoris*

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

251 USA). The primers, which included 5'-sequences complementary to the
252 pPICZ α A expression vector (underlined), were:
253 IB640 (5'-AGAGGCTGAAGCTGAATTCTCATTCGATCGTGACTTCTACATAAC-3') and
254 IB641 (5'-GAGATGAGTTTTTGTTCTAGACCGTTGAAGGCGCATTCTGGTGG-3')
255 for EfXTH-A;
256 IB664 (5'-AGAGGCTGAAGCTGAATTCGCAAACCTCAACCAAGACTTCAACATC-3') and
257 IB665 (5'-GAGATGAGTTTTTGTTCTAGACCGATATGCGAATTGGAACACTCAGGAG-3')
258 for EfXTH-H;
259 and IB666 (5'-AGAGGCTGAAGCTGAATTCTCTTCATCATTTCGATCGTGACTTCTC-3') and
260 IB667 (5'-GAGATGAGTTTTTGTTCTAGACCGTTGAAGGCGCATTCTGGCGG-3')
261 for EfXTH-I.

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

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

278

279 2.4. Transformation of *Pichia*

280 Recombinant plasmids were linearised with *Pme* I EC 3.1.21.-; New
281 England Biolabs, USA) and before being used to transform *Pichia* strain SMD
282 1168H by electroporation. *Pichia* cells were selected on YPDS plates (Life
283 Technologies, CA, USA) containing zeocin (1 mg ml⁻¹).

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

290

291 *2.5. Large-scale protein expression*

292 The optimal *Pichia* clone for each construct was grown in 250 ml of BMGY
293 overnight and resuspended in BMMY at $A_{600} \sim 1$. Expression proceeded for 16–24
294 h, after which the culture supernatant was stored at 4°C. Secreted proteins were
295 concentrated on Amicon® UltraCel®-10K regenerated cellulose (MW cut-off =
296 10000; Merck Millipore Ltd., Ireland). Concentrated samples were stored at
297 –20°C.

298

299 *2.6. XET and MXE activity assays*

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

310

311 *2.7. CXE activity assay*

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

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

317 Unless otherwise stated, 1 kBq [³H]XXXGol in 33 µl enzyme extract [in 50
318 mM MES (Na⁺); pH 6.0] was added to 10 mg of the pre-treated paper and
319 incubated at 20°C. The reaction was stopped by the addition of 300 µl 10% (v/v)
320 formic acid before repeated washing in water for 16 h to remove unreacted
321 [³H]XXXGol. Cellulose was then resuspended in 0.2 ml water and 2 ml ScintiSafe
322 3 liquid scintillant cocktail (Fisher Scientific, UK) and incubated for 24 h prior to
323 assaying for radioactivity.

324

325 *2.8. Effects of native Pichia secreted proteins on MLG and on Equisetum* 326 *transglucanase activities*

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

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

344

345 **3. Results**

346 *3.1. Production of acidic Equisetum GH16 proteins in Pichia*

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

359 Multiple sequence alignment of the identified acidic GH16 XTH
360 homologues showed high conservation between their primary structures; their
361 sequence identity ranged from 55.0 to 86.8% (Fig. 2). As expected, owing to the
362 propensity for a conserved binding cleft and active site topology within a GH
363 family, sequence homology both between the *EfXTH* proteins themselves and
364 between them and the 33 arabidopsis XTHs is higher in the regions flanking the
365 conserved active site (typically EL/IDFE), including the conserved *N*-
366 glycosylation site. Unlike other GH16s, most XTHs studied have a conserved
367 glycosylation site 5–15 residues towards the *C*-terminus from the active site
368 (Johansson et al., 2004) thought to be vital for XET function as deglycosylation of
369 this residue results in the loss of XET activity (Campbell & Braam, 1999;
370 Henriksson et al., 2003). However, this may not be an absolute requirement in
371 all cases as, for example, deglycosylation of this residue in *PttXET16A* resulted
372 in retention of significant XET activity (Johansson et al., 2004). The percentage
373 identity (evaluated by an EMBL-EBI FASTA protein similarity search) between
374 the *EfXTH*s and any currently known, or predicted, XTHs from other species
375 never exceeded 62%. This is perhaps unsurprising given the phylogenetic
376 distance of *Equisetum* from all other genera (Des Marais et al., 2003). HTG,
377 another XET-active transglucanase from *E. fluviatile*, shared only 49.6%
378 (*EfXTH-I*) to 55.5% identity (*EfXTH-H*) with any of the acidic *EfXTH*s. Despite

379 this and their low predicted pI values, the total number of acidic amino acids,
380 predicted molecular weight, and total number of *N*-glycosylation sites are
381 consistent with other known XTHs and HTG (Table 1 and Table S1). However,
382 the acidic EfXTHs have significantly fewer basic amino acids (ranging from 20 to
383 28 for EfXTH-H and EfXTH-I respectively) than the average for AtXTHs (36.6
384 basic amino acids; Table S1), and a lower basic : acidic amino acid ratio than
385 AtXTHs. The average basic : acidic amino acid ratio for AtXTHs is 1.30, ranging
386 from 0.86 for AtXTH23 to 2.05 for AtXTH32. By contrast, EfHTG with only 21
387 basic amino acids, has a basic : acidic residue ratio of 0.75, while the acidic
388 EfXTHs have ratios of 0.71, 0.83 and 1.04 (EfXTH-H, EfXTH-A and EfXTH-I
389 respectively).

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

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

400

401 *3.2. All acidic GH16 proteins from Equisetum exhibit XET activity and low levels* 402 *of MXE and CXE activity*

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

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

414

415 *3.3. Kinetics of the XET, MXE and CXE activities of EfXTH-H*

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

426 Suitable incubation times were selected to obtain approximately linear
427 initial rates, avoiding depletion of the acceptor substrate during the assays (Fig.
428 3). When crude *Pichia*-produced EfXTH-H was used (Fig. 3a), the yield of XET
429 reaction products was initially rapid (initial, approximately linear rate ~31
430 cpm/min), becoming non-linear after ~100 min (at ~3000 cpm) and plateauing at
431 ~8000 cpm after ~800 min. The theoretical maximum yield that would be
432 achieved if 100% of the [³H]XXXGol (acceptor substrate) were converted to
433 product was ~16000 cpm. The same enzyme preparation also exhibited
434 measurable MXE and CXE activities. Unexpectedly, however, in the MXE assay,
435 maximum incorporation of radioactivity was limited to ~300 cpm, achieved after
436 ~400 min, even though this represented the consumption of only ~2% of the
437 supplied [³H]XXXGol and the CXE products were still progressively
438 accumulating beyond 1400 min (Fig. 3a). The 'premature' cessation of MXE
439 product formation suggests degradation of the donor polysaccharide (MLG) by a
440 component of the native *Pichia* secretions.

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

443 plateauing, probably limited by acceptor availability (Fig. 3b). Interestingly, the
444 MXE activity of the purified EfXTH-H remained highly stable (at ~4.7 cpm/min)
445 over a 17-h period (Fig. 3b), demonstrating negligible denaturation of the
446 enzyme. Presumably during these assays no loss of MLG occurred because native
447 *Pichia* secretions were absent; this concept is addressed later in the mixing
448 experiment. The yield of MXE products greatly exceeded the ~300 cpm limit
449 previously observed for crude EfXTH-H, rising steadily to almost 5000 cpm over
450 1000 min. CXE activity observed was also greater with the purified enzyme,
451 although the difference from crude enzyme was not as dramatic as observed for
452 MXE, suggesting that MLG but not cellulose or xyloglucan is affected by native
453 *Pichia* secretions.

454

455 3.4. Viscosity assay and mixing experiments — *Pichia* secretions degrade MLG

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

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

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

476

477 **4. Discussion**

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

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

485 Given the unique ratio of activities observed for EfHTG (MXE > CXE >
486 XET; Fig. 3c), our core aim was to identify HTG's closest *Equisetum* XTH
487 relatives and compare their activity ratios (Table 2). This approach, looking at
488 natural variation, can usefully complement the approach of artificially
489 mutagenising XTHs at specific sites (Stratilová et al., 2019). All three
490 recombinant EfXTHs (-A, -H and -I), including all three minor variants of
491 EfXTH-H, were XET-active. However, although there was extensive homology
492 between their primary structures, and that of HTG, differences in activity ratios
493 were observed. EfXTH-A had the lowest relative MXE activity, only 0.2% of the
494 XET activity, which is similar to the value seen in a barley XTH (Hrmova et al.,
495 2007) and in crude extracts of numerous other land plants (Fry et al., 2008a). By
496 contrast, EfXTH-H had a somewhat higher proportion of MXE activity.
497 Therefore, EfXTH-A is a more specific XET than EfXTH-H. None of the EfXTHs
498 tested here exhibited relative MXE activities approaching that of HTG (whose
499 MXE was ~300% of its XET activity in the present work; Fig. 3c; Table 2) but the
500 values observed for EfXTH-H and -I were higher than those reported for most
501 other known land-plant XTHs and crude plant extracts. Extracts from
502 charophytic algae, on the other hand, often gave high relative MXE activities
503 (~20–250% of their XET activity; Fry et al., 2008a).

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

506 4.1 (Simmons et al., 2015)], which might be suggested to be a feature
507 contributing to its unusually lax substrate specificity, or a feature reflecting the
508 functionality of hetero-transglycanase activities. Of the 33 arabidopsis XTHs,
509 only five are predicted to have pI values below 5.8, the lowest being 5.05
510 (AtXTH23; Table S1). However, in *Equisetum*, the low pI of HTG is not unique
511 among its GH16 proteins: in the present work, we found genes for three
512 *Equisetum* XTHs with predicted pI values lower than 5.8. These three acidic
513 EfXTHs, and *Equisetum* HTG, all have unexceptional numbers of Asp and Glu
514 residues (27–29 total acidic amino acid residues, very similar to the average
515 number, 28.8, of all arabidopsis XTHs; Table S1). On the other hand, the three
516 acidic EfXTHs and HTG have considerably fewer Lys, Arg and His residues (20–
517 28 total basic residues) than most arabidopsis XTHs (average 36.6, range 23–50).
518 Thus, few basic residues, rather than many acidic residues, accounts for the low
519 pI of the investigated EfXTHs. However, the most XET-specific example, EfXTH-
520 A, was not the least acidic, so there is no simple correlation between low pI and
521 high MXE or CXE activity in *Equisetum* XTHs broadly.

522 This characteristic is also discernible in other XTHs. For example, among
523 arabidopsis XTHs, AtXTH13, -14 and -18 all possess slight MXE side-activity
524 (~2%, 2% and 3% of their respective XET activities), while AtXTH12, -17, -19 and
525 -28 have virtually none (Maris et al. 2009; Maris et al. 2011). The former set —
526 AtXTH13, 14 and 18 — have predicted pI values of 5.1, 8.5 and 8.7 respectively
527 (Table S1), so again there is no simple relationship between MXE activity and
528 the proteins' acidity.

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

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

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

552

553 **Authors' contribution**

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

558

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565

566 **Appendix A. Supplementary data**

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

569

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759 wall construction in specific organs of *Arabidopsis*. *Plant Cell Physiol.* 42, 1025–1033.
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Tables

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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 Mr (kDa)	31.2	30.402	30.420	29.855	29.534

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*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 <i>Equisetum</i> 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 (pPICZ α A 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) \pm SE
MLG alone	95.0 \pm 2.0
MLG + <i>Pichia</i> secretion products	5.8 \pm 0.2
Water	<1

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Figure legends

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

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

804

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

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

814

815 Figure 3: Time courses for *in-vitro* transglucanase reactions of EfXTH-H and EfHTG
816 XET, MXE and CXE activities of *Pichia*-produced proteins: (a) unpurified EfXTH-H, (b)
817 His-tag-purified EfXTH-H, (c) EfHTG. Each was assayed with 10 μ l enzyme extract. In
818 (a), the MXE and CXE values have been increased 10-fold so that the trends can be
819 discerned.

820

821 Figure 4: Effect of *Pichia pastoris* secretions on apparent MXE and XET activities of
822 native EfHTG protein

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

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

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830

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

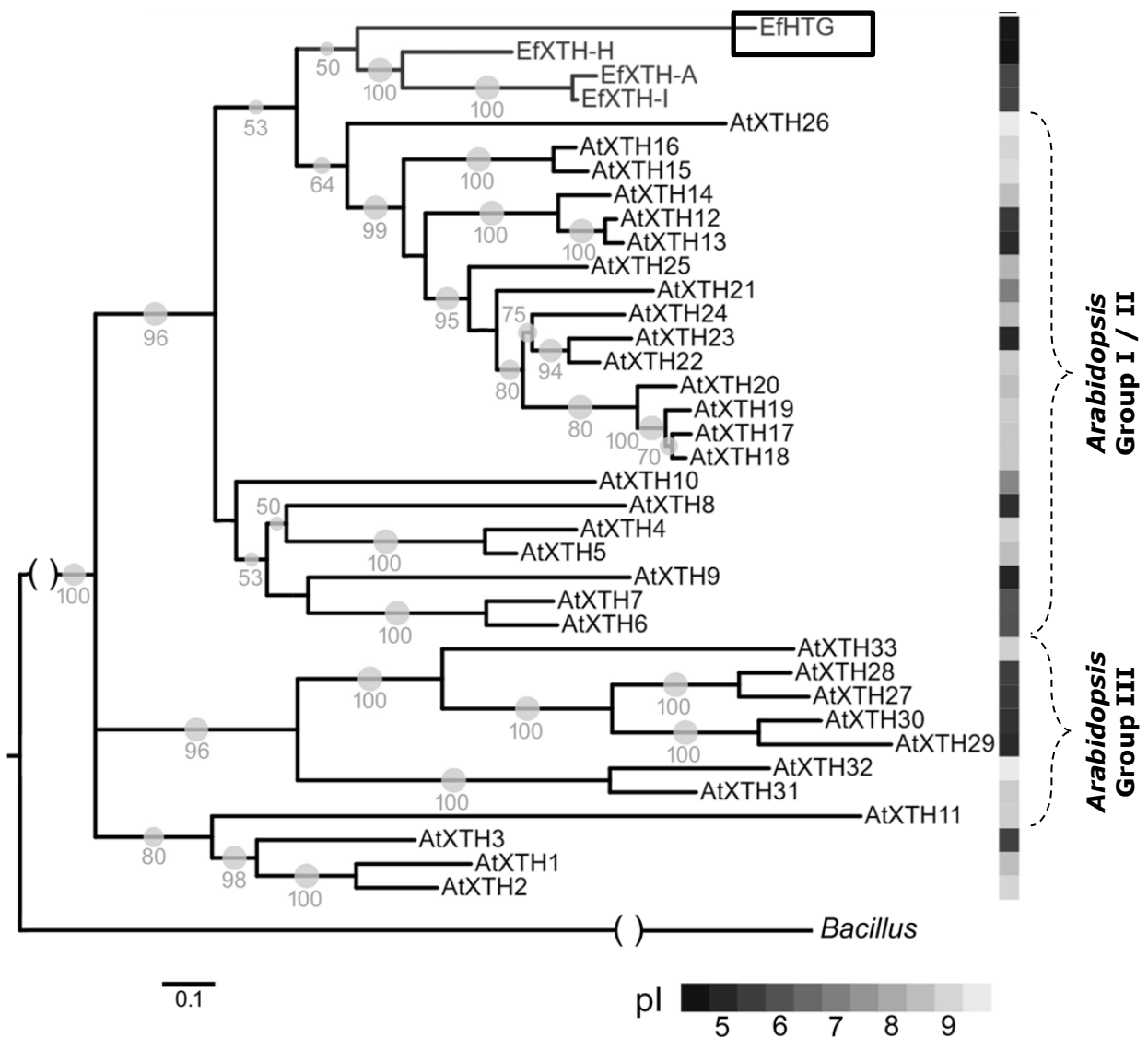


Figure 1: Relationship between XTH proteins from *A. thaliana* and *E. fluviatile* and HTG from *E. fluviatile*

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.

```

EfHTG      -----MLGLVFGMLVIMLASPKLAMAGFYGDFQVEPVPDHV-IIQSDSL
EfXTH-H    ----MVSILSSSSSFCGLRRCLVLLVFLV--QAAAAANFNQDFNITWAPDHVRVLDNSQL
EfXTH-A    MLLMATSTLSSNLAFLSMKAVLLLLLFFVSSSSATMSSFDRDFYITWAPERVKILDQGSQ
EfXTH-I    -----MASSTLNFNLVMLLLEFFVSSSSSSSSSFDRDFSI IWAPDRVKILDQGSQ
              : . : * . : . . : : * ** : . * : * : : . . . .

EfHTG      LQLTMDKNSGGSVVSKSNYLFGYFNMKMKLISGNSAGTVTTFYIFSDEA--NHDEIDDFEF
EfXTH-H    LQLTLDQASGSGFVSKNQYLFGNIDMQIKLVPGNSAGTVTAYYLYSTASPNSHDELDDFEF
EfXTH-A    LQLSLDNTSGSGFASKNKYLFGNIDMQIKLVPGNSAGTVTAYYLFSESE--QHDELDDFEF
EfXTH-I    LQLSLDNTSGSGFTSKNKYLFGNIDMQIKLVPGNSAGTVTAYYLYSESE--QHDELDDFEF
              *** : * : * . . . . * . : * * * : : * : * * : * * * * * : * : * * * * *

EfHTG      LGNYSGDPYLLHTNIFASGVGNREQQFFLWFDPTADFDHYTIIWNPQQILFLVDGRAVRS
EfXTH-H    LGNVSGQPYILQTNVFTSGKGEREERINLWFDPTADFHYSILWNPQIIIFSVDGTPIRV
EfXTH-A    LGNVSGQPYILQTNVFASGKGEREQLRFLWFDPTQDFHTYSVIWNPQHILFLVDGIPIRR
EfXTH-I    LGNVSGQPYILQTNVFASGKGEREQLRFLWFDPTQDFHTYSVSWNPQHILFLVDGIPIRR
              *** * : * * : * : * * : * * * * * * * * * * * * * * * * * * * * *

EfHTG      FPNNE-AIGVPYLKSQLMNVLHLSLWNGETWATLGGLRRIDWNSAPFVASYSTFVGDSC--
EfXTH-H    FPNNEKALGVPYLNKQSMSLYSTLWADGWATRGLDKIDWTQAPFVASYTNFKADACTN
EfXTH-A    YANKE-SLGVAFLNNQAMGVYSSLWNGDSWATRGLDKIDWTQAPFLASYRNFSASTACV
EfXTH-I    YANKE-SLGVAFPNNQAMGVYSTLWNGDSWATRGLDKIDWTQAPFLASYRNFSASSACV
              : . * : * : * . : : * * . : : * * * * * . * * * . * * * * * * * . . . .

EfHTG      FDSA---DSPCMAS--KWW-----NQAAAYQSLSTSDASSIQWVRENYLKYDYCYDTKL
EfXTH-H    YNTDYASAQACASSGDKWW-----NQPEYQTVASDDTSKLSWVRQNFIIYDYCSDTQR
EfXTH-A    VNTN---LNNCK----QLWAHSNFSNSDSKOLEQEQQLDQWVKNFIIYDYCTDAQR
EfXTH-I    VNRN---VHKCK----QLWAHS----KNSDQQLQEQHQLDQWVKNFMIYDYCTDAQR
              : * : * : * : : : : : . . . . * * : * * * * * : * : * : * :

EfHTG      YPNGFPRECSNRGF
EfXTH-H    NPVP-PPECSNSHI
EfXTH-A    NPTP-PPECAFN--
EfXTH-I    NPTP-PPECAFN--
              * * * * :

```

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.

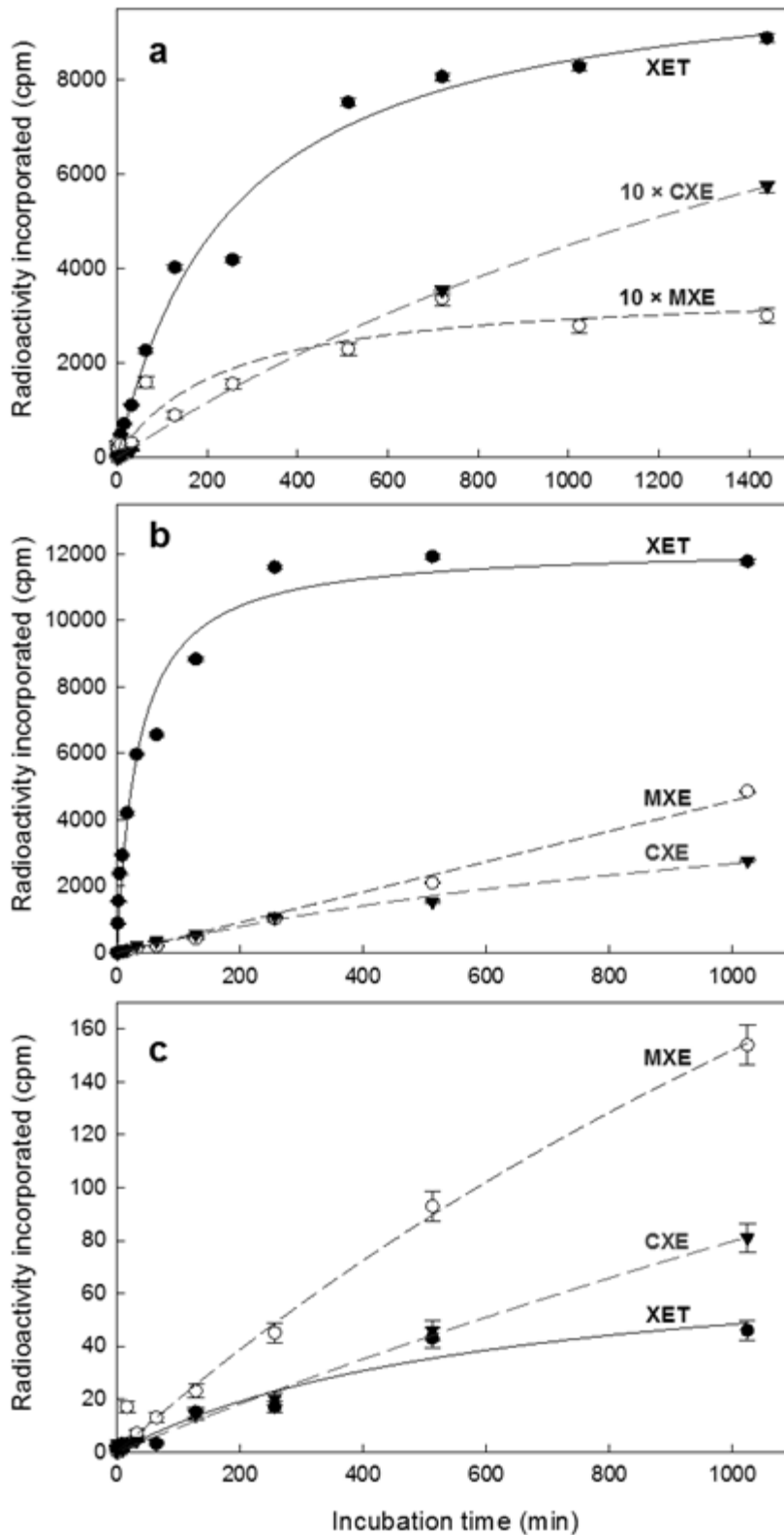


Figure 3: Time courses for *in-vitro* transglucanase reactions of EfXTH-H and EfHTG
XET, MXE and CXE activities of *Pichia*-produced proteins: (a) unpurified EfXTH-H, (b) His-tag-purified EfXTH-H, (c) EfHTG. Each was assayed with 10 μ l enzyme extract. In (a), the MXE and CXE values have been increased 10-fold so that the trends can be discerned.

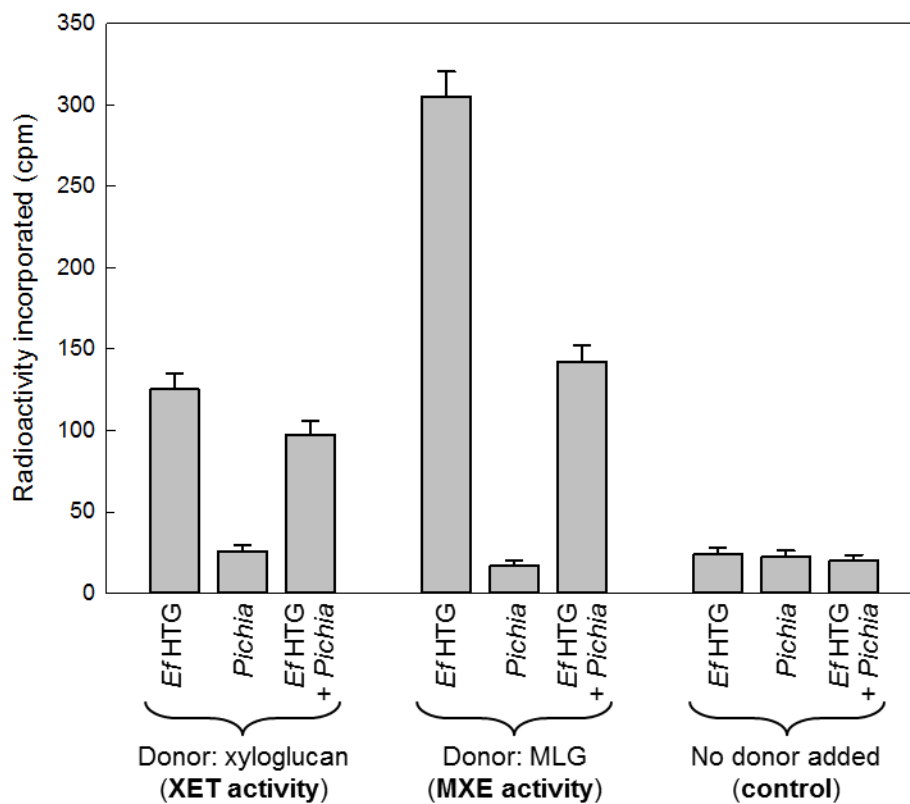


Figure 4: Effect of *Pichia pastoris* secretions on apparent MXE and XET activities of native *Ef*HTG protein

XET and MXE activities of native HTG purified from *Equisetum fluviatile* plants (*Ef*HTG), the secretion products of a pPICZ α A-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*.

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.

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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