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α-Fucosidases with different substrate specificities from two species of *Fusarium*

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Abstract Two fungal-secreted α-fucosidases and their genes were characterized. FoFCO1 was purified from culture filtrates of *Fusarium oxysporum* strain 0685 grown on L-fucose and its encoding gene identified in the sequenced genome of strain 4287. FoFCO1 was active on *p*-nitrophenyl-α-fucoside (pNP-Fuc), but did not defucosylate a nonasaccharide (XXFG) fragment of pea xyloglucan. A putative α-fucosidase gene (*FgFCO1*) from *Fusarium graminearum* was expressed in *Pichia pastoris*. FgFCO1 was ~1,800 times less active on pNP-Fuc than FoFCO1, but was able to defucosylate the XXFG nonasaccharide. Although FgFCO1 and FoFCO1 both belong to Glycosyl Hydrolase family 29, they share <25 % overall amino acid identity. Alignment of all available fungal orthologs of FoFCO1 and FgFCO1 indicated that these two proteins belong to two subfamilies of fungal GH29 α-fucosidases. Fungal orthologs of subfamily 1 (to which FoFCO1 belongs) are taxonomically more widely distributed than subfamily 2 (FgFCO1), but neither was universally present in the sequenced fungal genomes. *Trichoderma reesei* and most species of *Aspergillus* lack genes for either GH29 subfamily.

Keywords Fucose · Xyloglucan · *Fusarium oxysporum* · *Fusarium graminearum*

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Introduction

Fucose (6-deoxy-galactose; Fuc) is a common constituent of animal and plant glycoproteins and polysaccharides. In animals, *O*- and *N*-fucosylated proteins are involved in development, angiogenesis, fertilization, and cell adhesion (Ma et al. 2006). In plants, α-linked Fuc is present in glycoproteins and cell wall polymers including rhamnogalacturonans, arabinogalactan proteins (AGPs), and some xyloglucans (XGs), including XGs in grasses (McDougall and Fry 1994; Wu et al. 2010; Faik and Wiemels, unpublished results). XGs are a major hemicellulose in plants, comprising 10–30 % of the primary cell wall. XGs have different structures in different plant species, but all XGs have a backbone of β1,4-linked glucose (Glc) with α1,6-linked xylose (Xyl) sidechains, and the Xyl residues are themselves partially substituted with β1,2-linked galactose (Gal). In some plants, such as *Arabidopsis* and pea, the Gal residues are further substituted with α1,2-linked Fuc. In XGs that contain fucose, therefore, it is the terminal sugar on the side chains (Pauly and Keegstra 2008). In higher plants, specific

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glycosyl transferases have been identified that fucosylate XGs and AGPs (Perrin et al. 1999; Wu et al. 2010).

α-Fucosidases that hydrolyze Fuc from proteins and polysaccharides are widespread in animals, plants, bacteria, and fungi. Fucosidases are found mainly in CAZy Glycosyl Hydrolase (GH) families 29 and 95, with others reported to belong to families GH1 and GH30 (Cantarel et al. 2009; De La Torre et al. 2002; Eneyskaya et al. 2001; Günl et al. 2011; Leonard et al. 2008). Mutation of the α-fucosidase-encoding gene *FUCA1* in humans and other mammals results in fucosidosis, a lysosomal storage disease (Michalski and Klein 1999).

Because Fuc is the terminal sugar in the side chains of XGs that contain it, α-fucosidase must act before the other side-chain sugars, Gal and Xyl, can be removed by β-galactosidase and α-xylosidase, respectively. Removal of the side chains is necessary to release the Glc in the backbone of XG. Therefore, despite the paucity of Fuc in plant cell walls, α-fucosidase might be important for the complete deconstruction of XG during biotechnology applications in which maximizing the yield of fermentable sugars is critical.

Several bacterial and mammalian α-fucosidases have been biochemically characterized (Fukushima et al. 1991; Sulzenbacher et al. 2004). Among fungi, a secreted α-fucosidase was purified from the filamentous fungus *Fusarium oxysporum*, but the encoding gene was not identified (Yamamoto et al. 1986). A GH95 α-fucosidase from *Aspergillus nidulans*, prepared by expression in *Pichia pastoris* (AN_8149), has activity on cotton XG but not *p*-nitrophenyl-α-fucoside (pNP-Fuc; Bauer et al. 2006). Many fungal proteins in GenBank are annotated as GH29 α-fucosidases based on weak amino acid sequence identity to characterized bacterial and mammalian α-fucosidases, but there appears to be no supporting biochemical evidence for any of these annotations. Here, we report the biochemical and genetic characterization of two α-fucosidases from two different species of plant pathogenic filamentous fungi in the genus *Fusarium*.

Materials and methods

Fungal cultures

F. oxysporum strain 0685 was obtained from the Penn State University Fusarium Research Center (216 Buckhout Laboratory, University Park, PA) and grown on potato dextrose agar (Difco). For the induction of α-fucosidase, *F. oxysporum* started from agar plugs was grown for 7 days without shaking in 1-L flasks each containing 100 mL potato dextrose broth (Difco) plus 1 % L-Fuc. For DNA and RNA isolation, the resulting fungal mats were rinsed with sterile water, frozen in liquid nitrogen, and lyophilized. *Fusarium graminearum* (teleomorph *Gibberella zeae*) strain PH-1 (no. 9075, Fungal Genetics Stock Center, University of Missouri, Kansas City, MO) was grown as previously described (Paper et al. 2007).

Purification of α-fucosidase from *F. oxysporum*

Culture filtrates were concentrated by the addition of ammonium sulfate to 80 % saturation (51.6 g per 100 mL). After stirring for 30 min at 4 °C and centrifugation at 15,000×*g* for 15 min, the pellet was redissolved in 10 mL 25 mM sodium acetate (pH 5.0) and desalted with a 6-kDa exclusion column (BioRad 732-2010) prior to chromatographic purification.

Anion exchange HPLC was performed on an 8.0-mm×7.5-cm TSK gel SP-5PW column (TOSOH Bioscience no. 08803) with a buffer system consisting of 25 mM Tris, pH 8.0 (buffer A), and buffer A plus 0.6 M NaCl (buffer B). The separation gradient was 5 min at 100 % A, a 40-min linear gradient to 100 % B, and 5 min at 100 % B, at a flow rate of 1 mL/min. Fractions showing activity with pNP-Fuc were pooled. Hydrophobic interaction chromatography was performed with a 7.5-mm×7.5-cm TSK gel Phenyl-5PW column (Tosoh Bioscience). Buffer A was 1.7 M $(NH_4)_2SO_4$+100 mM KH_2PO_4, pH 7.0, and buffer B was water. The gradient consisted of 5 min 100 % A followed by a 30-min linear gradient to 100 % B and then 10 min at 100 % B. The flow rate was 1 mL/min and 1-mL fractions were collected.

SDS-PAGE and proteomic analysis

Active fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 4–20 % acrylamide gradient (BioRad, no. 161-1159). The major band was cut from the gel and subjected to mass spectrometry-based proteomics as described (Paper et al. 2007; Nagendran et al. 2009). The results were initially analyzed using Scaffold (www.proteomesoftware.com) with the complete predicted proteome of *F. oxysporum* strain 4287 as the database (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). At the time of this analysis, strain 4287 was the only sequenced isolate of *F. oxysporum* (Ma et al. 2010). After the cDNA sequence of the orthologous gene from *F. oxysporum* strain 0685 was experimentally determined, the predicted protein was added to the proteomics database and the mass spectrometry data were reanalyzed.

Sequence comparisons were performed using the BLAST suite of programs at GenBank, with all available fungal genome sequences at GenBank, the Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/programs/fungi/index.jsf), and the Broad Institute (www.broadinstitute.org).

DNA and RNA isolation

DNA was isolated from lyophilized and ground mycelial mats using the Qiagen (Valencia, CA) Puregene Core Kit A and the mouse tail protocol. RNA was isolated from *F. graminearum* as described (Hallen et al. 2007). RNA was isolated from *F. oxysporum* mats using Trizol (Invitrogen, Grand Island, NY) and 2 % hexadecyltrimethyl ammonium bromide (Chang et al. 1993). The samples were treated with DNase (Roche no. 776785) and purified with the cleanup protocols and columns in the RNeasy kit (Qiagen no. 74904).

Sequencing and expression of FoFCO1 from *F. oxysporum*

Genomic and cDNA copies of FoFCO1 were sequenced by a combination of 5′ and 3′ RACE (Clontech SMARTer RACE cDNA amplification kit, Mountain View, CA). Initially, the PCR primers were based on the database sequence of FoFCO1 from *F. oxysporum* 4287. Once specific sequence data from strain 0685 were available, primers were designed based on this sequence (Electronic supplementary material (ESM) Table S1).

The coding regions of both genes including their native secretion signals were cloned into pPICZA and pPICZAα vectors (Invitrogen) with and without the C-terminal Myc/6xHis tags. The specific primers, restriction sites, and vector versions are given in ESM Table S2. *P. pastoris* was grown in 50-mL cultures as described (Banerjee et al. 2010). FgFCO1 was induced with 1 % methanol for 3 days, while FoFCO1 was induced for 2 days. After centrifugation at 8,000×*g* for 30 min, the culture supernatants were concentrated by tangential flow filtration using a 10-kDa cutoff membrane (Vivaflow, Sartorious, Concord, CA). Buffer exchange was performed during concentration with five volumes of 25 mM sodium acetate, pH 5.0. Further purification of the recombinant FoFCO1 was done by anion exchange chromatography as described above.

For protein immunoblotting of His-tagged proteins, the proteins were separated by SDS-PAGE as described above and blotted to nitrocellulose. For detection, a 1:20,000 dilution of 6× His antibody/HRP conjugate (Clontech no. 631210) and chemiluminescence (Pierce ECL Plus, Rockford, IL) were used.

Assay on pNP-Fuc

A 10-mM stock solution of *p*-nitrophenyl-α-L-fucopyranoside (pNP-Fuc; Sigma N3846) was made in water. Each assay (300 μL total volume) contained 10–30 μL enzyme, 100 μL of 100 mM sodium acetate (pH 5.0), and 100 μL of 10 mM pNP-Fuc. Initial assays were performed at 37 °C for 30 min, stopped by the addition of 600 μL of 1 M sodium carbonate, and the absorbance measured at 400 nm. The pH optimum was determined in buffers ranging from 2.0 to 8.0 in 0.5 U increments. The temperature dependence was determined at 25, 27, 42, 50, 58, 65, and 70 °C. Further experiments were done at pH 5 and 50 °C. Enzyme kinetics were analyzed using nonlinear curve fitting with GraphPad Prism (La Jolla, CA).

Assay on pea XGOs

Pea seeds were soaked in water for 24 h with bubbling air and grown in vermiculite in total darkness for 5–7 days. XG was purified using *trans*-1,2-cyclohexanediaminetetraacetic acid, pH 7.5, to remove pectins and extraction with 4 M KOH as described (Zablackis et al. 1995).

To prepare pea xyloglucan oligosaccharides (XGOs), lyophilized pea XG was dissolved in 25 mM sodium acetate, pH 5.0, at a concentration of 1 mg/mL. The undissolved material was pelleted in a microfuge (15,000×*g*) and discarded. The dissolved XG was digested with 2 U/mL endo-β1,4-glucanase (Megazyme, catalog E-CLTR) for 8 h at 37 °C to produce oligosaccharides. The XGOs relevant to this paper are shown in Fig. 1 and named using the structural nomenclature of Fry et al. (1993).

Each assay contained 125 μL 10 mg/mL pea XGOs, 2 μg enzyme in 25 μL enzyme (or buffer control), and 50 μL 50 mM sodium acetate, pH 5.0. The assays were performed at 37 °C for 12 h. The samples were subsequently desalted, filtered with Spin-X centrifuge tube filters (Costar, Sigma-Aldrich, no. 8169), and analyzed on a Shimadzu Axima MALDI-TOF in reflectron mode as described (Lerouxel et al. 2002).

Assay on [^{14}C]fucose-radiolabeled tamarind xyloglucan

GDP-[^{14}C]Fuc (8.369 GBq/mmol) was purchased from Perkin Elmer (Boston, MA). Pure tamarind XG was purchased from Megazyme International (Bray, Ireland). [^{14}C]Fucose-radiolabeled tamarind XG ([^{14}C]Fuc-tamarind XG) was produced using *Arabidopsis* fucosyltransferase (AtFUT1) expressed in *Drosophila melanogaster Sf2* cells (a gift from Dr. K. Keegstra, Michigan State University) as described (Faik et al. 2000). Reactions (100 μL total volume) were carried out in 50 mM sodium acetate (pH 5) by incubating ~10,000 cpm of [^{14}C]Fuc-tamarind XGs with 200–500 ng of FgFCO1 or FoFCO1 for 16 h at 45 °C. The reactions were terminated by adding 1 mL cold 70 % ethanol and the products precipitated for 1 h at −20 °C. After centrifugation (13,000×*g*, 10 min, room temperature), the pellets were washed three times with 1 mL cold 70 % ethanol. The pellets were dissolved in 300 μL water, 4 mL scintillation cocktail was added, and the radioactivity (in counts per minute) measured in a liquid scintillation counter.

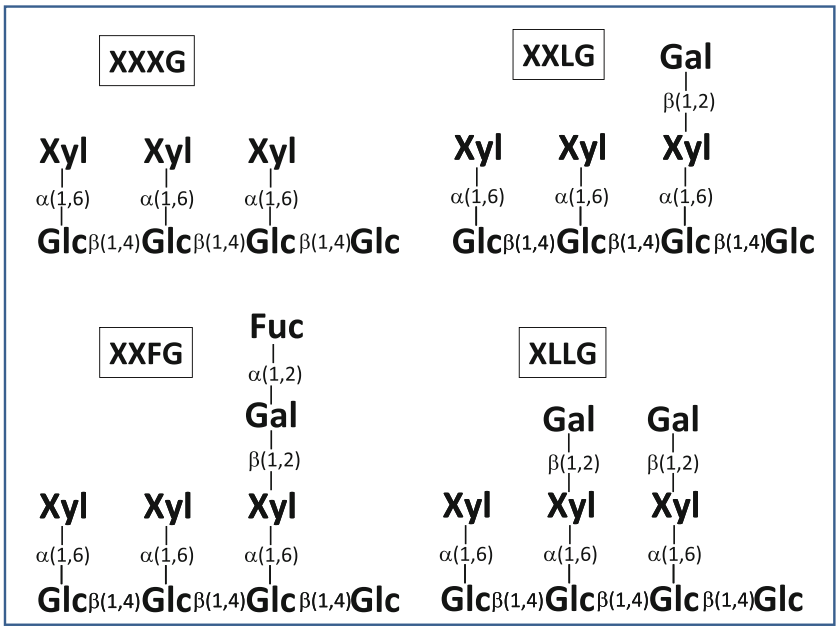

Fig. 1 Structures of xyloglucan oligosaccharides

Assay on [^{14}C]fucose-radiolabeled tamarind XGOs

Radiolabeled fucosylated tamarind XGOs ([^{14}C]Fuc-tamarind XGOs) were generated from [^{14}C]Fuc-tamarind XG by treatment with endo-β1,4-glucanase (Megazyme E-CELTR). The reactions (total volume, 100 μL) were performed in 50 mM acetate buffer (pH 5) by incubating ~10,000 cpm of [^{14}C]Fuc-tamarind XGOs with ~250 ng of FgFCO1 or FoFCO1 for 16 h at 45 °C. The reactions were terminated by boiling for 10 min and resuspended in 1 mL water. Free [^{14}C]Fuc was separated from [^{14}C]Fuc-tamarind XGOs by fractionation on a 90×1.5-cm Bio-gel P2 column (BioRad, Richmond, CA). Eluted radioactivity was monitored in 1 mL of each 2.25-mL fraction as described earlier (Zeng et al. 2008).

Assay on AGPs

Nonradioactive fucosylated AGPs were purified from 14-day-old transgenic *Nicotiana tabacum* BY2 cell suspension cultures expressing one of the *Arabidopsis* AGP-fucosyltransferases (*AtFUT4.1* or *AtFUT6*) using Yariv reagent (Schultz et al. 2000; Wu et al. 2010). The reactions (50 μL) were performed in 50 mM acetate buffer (pH 5) by incubating 50 μg nonradioactive fucosylated AGP's in the presence of 500 ng FgFCO1 or FoFCO1 for 16 h at 45 °C. The reactions were terminated by boiling for 10 min and the products precipitated with 1 mL cold 100 % ethanol for 4 h at −20 °C. Pellets were collected by centrifugation at 15,000×*g* for 25 min at 4 °C, and the ethanol in the supernatants (which should contain free Fuc) was removed by evaporation at 65 °C. The dried materials were resuspended in 50 μL, and 5 μL was analyzed for monosaccharide composition by fractionation by high pH anion exchange chromatography (HPAEC) using a CarboPac PA20 column (4×250 mm; Dionex, Thermo Scientific, Sunnyvale, CA). The column was eluted at a flow rate of 0.5 mL/min with 3 mM NaOH for 15 min. Pure Fuc standard was treated under the same conditions. To confirm that AGPs purified from cell cultures expressing *AtFUT4.1* and *AtFUT6* contained Fuc, they were subjected to total acid hydrolysis with 2 M trifluoroacetic acid as described (Faik et al. 2000) and analyzed by HPAEC. As expected, this analysis showed the presence of only Fuc, Ara, and Gal residues in these AGPs (data not shown).

[^{14}C]Fucose-radiolabeled AGPs ([^{14}C]Fuc-AGPs) were generated using *Arabidopsis* fucosyltransferase (*AtFUT6*) expressed in tobacco BY2 cells and nonfucosylated AGPs purified using Yariv reagent from wild-type tobacco BY2 cells (Wu et al. 2010). α-Fucosidase assays were performed in a total volume of 100 μL in 50 mM acetate buffer (pH 5) by incubating ~10,000 cpm [^{14}C]Fuc-AGPs with 500 ng FgFCO1 or FoFCO1 for 16 h at 45 °C. The reactions were terminated by boiling for 10 min, 1 mL cold 100 % ethanol was added, and the products were precipitated for 16 h at −20 °C. Precipitated AGPs were collected by centrifugation at 15,000×*g* for 25 min at 4 °C. The pellets were resuspended and the radioactivity measured as described above.

Accession numbers

The sequences of *FoFCO1* and *FgFCO1* have been deposited in GenBank with accession numbers JX274678 and JX274679, respectively.

Results

Identification of the gene encoding secreted α-fucosidase from *F. oxysporum*

F. oxysporum strain 0685 was grown on L-fucose, which induces a protein with α-fucosidase activity on pNP-Fuc (Yamamoto et al. 1986). When analyzed by SDS-PAGE, the culture filtrates contained a major band at ~70 kDa (Fig. 2). Fractionation of the culture filtrate following activity on pNP-Fuc indicated that this major band was the α-fucosidase.

The purified protein was subjected to mass spectrometry-based proteomics analysis using the proteome of *F. oxysporum* strain 4287 as the queried database (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html). At the time of the analysis, this was the only available *F. oxysporum* genome sequence. The α-fucosidase was identified as an ortholog of protein FOXG_15218 with ten peptides and 21 % coverage (Fig. 3a). This protein is referred to herein as FoFCO1. BLASTP analysis against the NCBI nr database indicated that FoFCO1 has orthologs in other fungi. Many of these are annotated as α-fucosidases, but without supporting biochemical evidence. The most similar biochemically characterized protein is α-fucosidase from the bacterium *Thermotoga maritima*, with 31 % amino acid identity (Sulzenbacher et al. 2004).

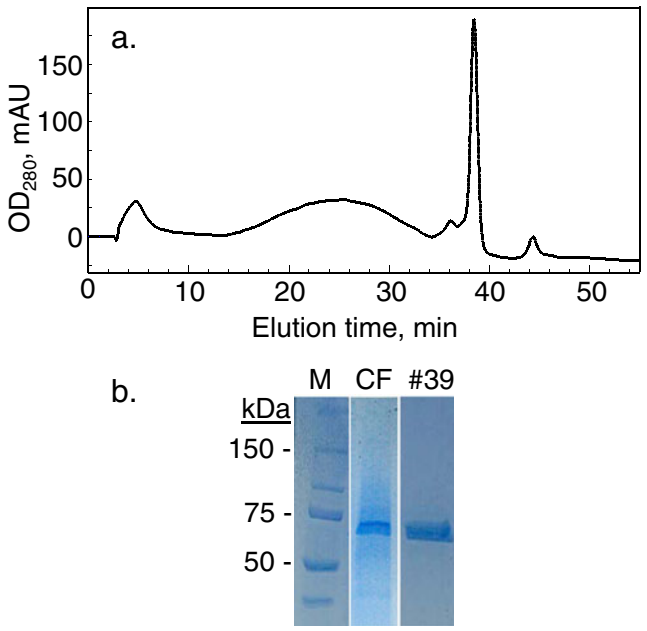

Fig. 2 Purification of FoFCO1. **a** HPLC trace of hydrophobic interaction chromatography of culture filtrates of *F. oxysporum*. **b** SDS-PAGE stained with Coomassie blue. *M* markers, *CF* crude culture filtrate, *#39* fraction eluting between 38 and 39 min from the chromatographic separation shown in (**a**)

In order to confirm the DNA and the predicted protein sequences of FoFCO1, genomic DNA and cDNA copies were obtained from strain 0685. There were 37 amino acid differences between the experimentally determined sequence of FoFCO1 from strain 0685 and the sequence of FOXG_15218 predicted from the genome of strain 4287 (ESM Fig. S1). It is not possible to know without sequencing a cDNA from strain 4287 whether these differences are natural or due to sequencing and annotation errors. There is a large apparent indel spanning the third intron; FoFCO1 from strain 0685 is therefore predicted to have an additional 22 amino acids (ESM Fig. S1). This difference is probably due to a misannotation of FOXG_15218 (see below). When the proteomics analysis of FoFCO1 was redone with the experimentally derived sequence of strain 0685 included in the query database, a much better alignment was obtained, which resulted in the identification of more peptides and an increase in the total coverage from 21 to 44 % (Fig. 3b).

Since the current work was completed, additional strains of *F. oxysporum* have been sequenced. The experimentally determined sequence of FoFCO1 is 98 % identical to FOXB_05755 (GenBank no. EGU83738.1) of *F. oxysporum* strain 5176. The two proteins differ in a single 11-amino acid region near the N terminus, which is possibly due to a sequencing artifact in FOXB_05755. The perfect alignment between FoFCO1 and FOXB_05755 around the third intron supports the conclusion that the 22-amino acid indel in FoFCO1 compared to FOXG_15218 is an error in the latter sequence (ESM Figs. S1 and S2).

Enzymological characterization of FoFCO1

The K_m of native FoFCO1 for pNP-Fuc was 0.85 mM (95 % confidence interval (CI)=0.6–1.1 mM) and the k_{cat} was 1,379 min^{-1} (95 % CI=1,248–1,517 min^{-1}). FoFCO1 had a broad temperature optimum between 42 and 55 °C and a pH optimum of 5.0, with >80 % of the activity retained between pH 4 and pH 6.

Expression of FoFCO1 in *P. pastoris*

Both 6× His-tagged and untagged versions of FoFCO1 were expressed in *P. pastoris*. Three independent transformants without the His tag showed activity on pNP-Fuc, but three transformants with the tag showed no activity (data not shown). Possibly, the His tag interferes with expression or activity. SDS-PAGE of culture filtrates from the nontagged transformants 24, 48, or 72 h after induction showed multiple faint protein bands. After concentration, the culture filtrates were fractionated by anion exchange chromatography. A diffuse band between 75 and 100 kDa was visible only in the strain (transformant 16-8) expressing FoFCO1 and not in the empty vector control (Fig. 4a). The diffuse pattern may be due

a.
MHIRLLSQLG TVVCLGTASV GALSLKHDKR ATSPASLKIG SPVLTSKWLE GSDYEQIVEF
FITNSDGKNP LTWTDQLHVI VESSSLETTT PGTLLRLGPK QSAVVQVGVK NKAGVKAGTR
CDATAVVTWG PKQDPKKSSK AFSGQCGIGD YEASESSLEH HWNPDWFHEI KYGIFIHWGL
YSVPAFGNRP GPKQDYAEWY GYRMTQPDFP SQTYQHHRDT YGENFNYDDF VSNFTGANFD
AEDWMNLVAD AGAHKRSTVH YGPKRDFVKE LLDVAKAKHP EIRRGTYFSM PEWFNPAYVK
YAWDQHYKEI YWGRPPTNPY TNKSIEYTGY VEVDDFINDI QNPQIEALFY DYDIEMLWCD
IGGPNKAPDV LAPWLNWARD QGRQVTFNDR CGAAGDYSTP EYSGISFNPK KFESNRGLDP
FSFGYNYLTT DDEYLSGEEI VKTLVDNVVN NGNFLLNMGP KGDGTIPKQQ QLNLLDAGEW
IKDHGEGIFG TRYWPTAQTS GSLRFAMKPD AFYIHHVGQP SSPLVINEPV PWVEGDEVTA
VGGSAHGTVL QVARNDGSFS VQLPDNVVQG DKYIWTIKIA YSTGK

b.
MHIRFLSHLG TGLCLGTASV GALSLKHDKR ATSPASIVIG NPVLTSKWLE GSDYEQVVEF
FITNSDANNP LTWADQLQVT VESSSLETTT PGTLLRLGPN QSAVVQVGVK NKAGVKAGTQ
CGATAVLTWG PKENPKKSSK DFSGQCGIGD YDASASSLEH HWNPDWFHEI KYGIFIHWGL
YSVPAFGNRP GPNQDYAEWY GYRMTQPDFP SQTYQYHRAT YGENFNYDDF VSNFTGASFD
AEDWMNLVAD AGAQYVVPVT KHHDGWALFD FPESVSKRST VHYGPKRDFV KELLDVAKAK
HPEIRRGTYF SMPEWFNPAY AKYYWDQHYK EIYWGRPPTN PYTNKSIEYT GYVEVNDFIN
DIQNPQMEAL FYDYDIEMLW CDIGGPNKAP DVLAPWLNWA RDQGRQVTFN DRCGAAGDYS
TPEYAGISFN PRKFESNRGL DPFSYGYNYL TTDDEYLSGE EIVKTLVDNV VNNGNFLLNM
GPKGDGTIPK QQQLNLLDAG EWIKSHGEGI FGTRYWPTAQ TSGSLRFAMK PDAFYIHHVG
QPSSPLVISQ PVPWVEGDEV TAVGGSAHGT VLQVARNGGS FSVQLPDNVV QGDKYIWTIK
IAYSTGK

Fig. 3 Proteomics analysis of FoFCO1. **a** Sequence of FOXG_15218 from strain 4287 (Broad Institute). Peptides identified by proteomics analysis of FoFCO1 purified from strain 0685 are *underlined*. **b** Sequence of FoFCO1 from strain 0685 determined from the cDNA sequence. Peptides identified from the same mass spectrometric analysis are *underlined*; the coverage is much better for the corrected sequence (44 vs. 21 %). An alignment of the two sequences is shown in ESM Fig. S1

to heterogeneous glycosylation. Fractions containing this protein had activity on pNP-Fuc (Fig. 4b). Because of the heterogeneity, it was not possible to purify sufficient quantity of FoFCO1 to obtain accurate kinetic data.

Identification and expression of α-fucosidase (FgFCO1) from *F. graminearum*

Earlier, a putative GH29 α-fucosidase (designated FGSG_11254; alternate names FG11254 and fgd463-60) was found in the secretome of *F. graminearum* growing on media containing pectin, maize cell walls, carrot cell walls, oat bran, xylan, or dried distillers' grains, but not when grown on sucrose, collagen, or N- or C-limited media (see Table S1 in Paper et al. 2007). Although FGSG_11254 was not detected in the secretome of *F. graminearum* during infection of wheat (Paper et al. 2007), its mRNA is up-regulated 6.1-fold 144 h after infection of barley (Güldener et al. 2006). FoFCO1 and FGSG_11254 are both annotated as belonging to CAZy family GH29, but they are only weakly similar (<25 % amino acid identity, expect score 1.05E−23).

The sequencing of a corresponding cDNA confirmed the accuracy of the annotation of FGSG_11254 including the lack of any introns. Both His-tagged and untagged versions of the protein were expressed in *P. pastoris*, and in three of the four transformants tested (two untagged and one tagged), there was strong expression of a protein of the correct molecular weight (Fig. 5). The presence of the His tag was confirmed immunologically (Fig. 5).

pNP-Fuc was a poor substrate for recombinant FgFCO1. The K_m was 9.8 mM (95 % CI=8.1–11.4 mM) and the k_{cat} was 8.5 min^{-1} (CI=7.7–9.3 min^{-1}). That is, the k_{cat}/K_m of FgFCO1 with pNP-Fuc was 1,870 times lower than FoFCO1. The low level of activity of FgFCO1 was probably not an artifact of heterologous expression because pNP-Fuc activity was not detected in culture filtrates of *F. graminearum* grown on media that are known to induce the protein (Table S1 in Paper et al. 2007; data not shown).

Activity of FoFCO1 and FgFCO1 on pea and tamarind XG

Pea XG was extracted from etiolated seedlings and the presence of the expected fucosylated and galactosylated oligosaccharides after digestion with β1,4-glucanase were confirmed by mass spectrometry (Fig. 6b). Tamarind XG is not fucosylated and therefore lacks a signal at a mass/charge (*m*/*z*) ratio of 1,394 (Fig. 6a). When pea XGOs were digested with FoFCO1, the signal corresponding to the fucosylated XGO (XXFG) did not change (Fig. 6d). However, when pea XGOs were digested with FgFCO1, XXFG disappeared (Fig. 6c) and the signal at *m*/*z* 1,248, which is XXLG or XLXG, increased relative to XXXG (*m*/*z*

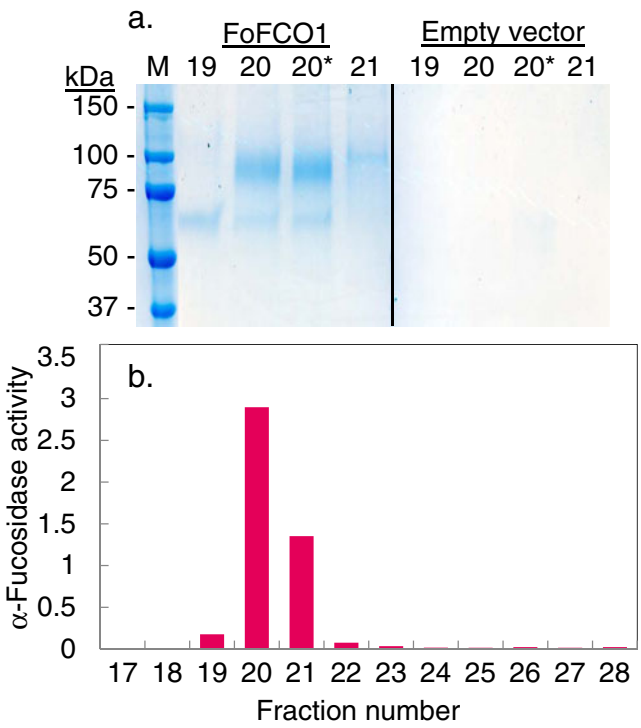

Fig. 4 Heterologous expression of FoFCO1. **a** SDS-PAGE of active anion exchange fractions of *P. pastoris* transformant 16-8 expressing FoFCO1 (*left*) or equivalent fractions expressing only empty vector (*right*). The *asterisk* indicates fraction 20 that had been concentrated 1.5-fold before SDS-PAGE. All lanes are from the same gel. *M* molecular mass markers. Gel was stained with Coomassie blue. **b** α-Fucosidase activity in the same anion exchange fractions. Activity is expressed as micromoles pNP released per milliliter fraction volume

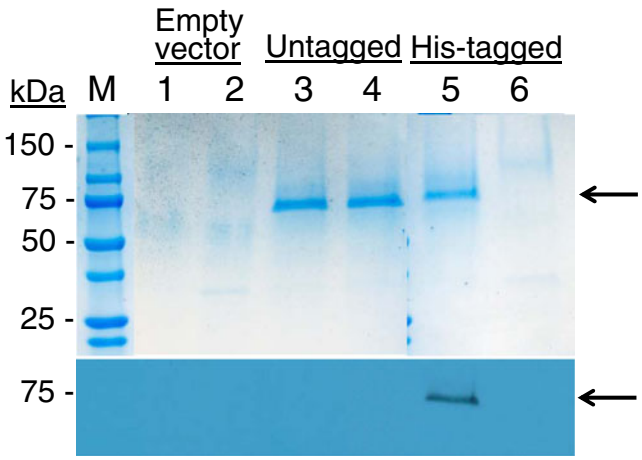

Fig. 5 Heterologous expression of FgFCO1. *Top panel*, SDS-PAGE analysis of FgFCO1 expressed in *P. pastoris*. *Lanes 1* and *2*, Two independent isolates of *P. pastoris* transformed with empty vector pPICZAα. *Lanes 3* and *4*, Two isolates expressing untagged FgFCO1 in pPICZAα. FgFCO1 is indicated by the *arrow*. *Lanes 5* and *6*, Two transformants with 6× His-tagged FgFCO1, one (*lane 5*) showing the expression of the protein (*arrow*) and the other showing lack of expression (*lane 6*). Gel was stained with Coomassie blue. *Bottom panel*, Immunological detection of the 6× His tag in the *P. pastoris* transformant expressing FgFCO1 (*lane 5*, indicated by the *arrow*). *M* molecular mass markers. All lanes are from the same gel

1,085). These results indicate that FgFCO1 but not FoFCO1 uses fucosylated pea XGOs as substrates and that the inability of FgFCO1 to use pNP-Fuc is probably not due to heterologous expression producing a nonfunctional enzyme.

[^{14}C]Fuc-labeled tamarind XG and tamarind XGOs were prepared in vitro. Neither was a substrate for either fucosidase (ESM Fig. S3 and Table S3).

Activity of FoFCO1 and FgFCO1 on AGPs

In order to test whether either of the fucosidases could remove Fuc residues from fucosylated AGPs, two complementary assays were performed. One assay measured the release of [^{14}C]Fuc from [^{14}C]Fuc-AGPs radiolabeled in vitro, whereas the other assay measured the release of Fuc from AGPs that were fucosylated in vivo by tobacco BY2 cells expressing one of the *Arabidopsis* fucosyl transferases. In both cases, fucosidase activity was monitored chromatographically by the release of free Fuc. There was no evidence of fucosidase activity by either enzyme in either assay (ESM Figs. S4 and S5).

Taxonomic distribution of GH29 fungal α-fucosidases

Some species of *Fusarium* have orthologs of both FoFCO1 and FgFCO1 (e.g., *Fusarium verticillioides* and *F. oxysporum*), whereas others (e.g., *F. graminearum*) only have strong orthologs of FgFCO1 (ESM Table S2).

To determine the distribution of proteins related to FoFCO1 and FgFCO1 outside the genus *Fusarium*, FoFCO1 and FgFCO1 were used as queries to identify orthologs in all available fungal genomes at the Department of Energy Joint Genome Institute (JGI), the Broad Institute, and GenBank (as of January 2012). The sequences sort into two subfamilies, all of which are currently assigned to GH29. Subfamily 1, to which FoFCO1 belongs, contains 44 examples (Fig. 7; for simplicity, only a representative sample of these are shown). Subfamily 2, to which FgFCO1 belongs, is much smaller, containing only six sequences in all available fungal genomes. All six are shown in Fig. 7. Some fungi contain neither, both, or one or the other subfamily. Subfamily 1 is common among ascomycetes and basidiomycetes and is also present in the zygomycete *Mucor circinelloides* (Mucoromycotina). There are no members of subfamily 2 in any of the 45 basidiomycete genomes in the JGI database.

Trichoderma reesei, the source of many industrially important enzymes, especially for biomass conversion, lacks both GH29 subfamilies, although it does have at least one predicted α-fucosidase in GH95. Among nine species in the genus *Aspergillus*, only *Aspergillus niger* has an ortholog of

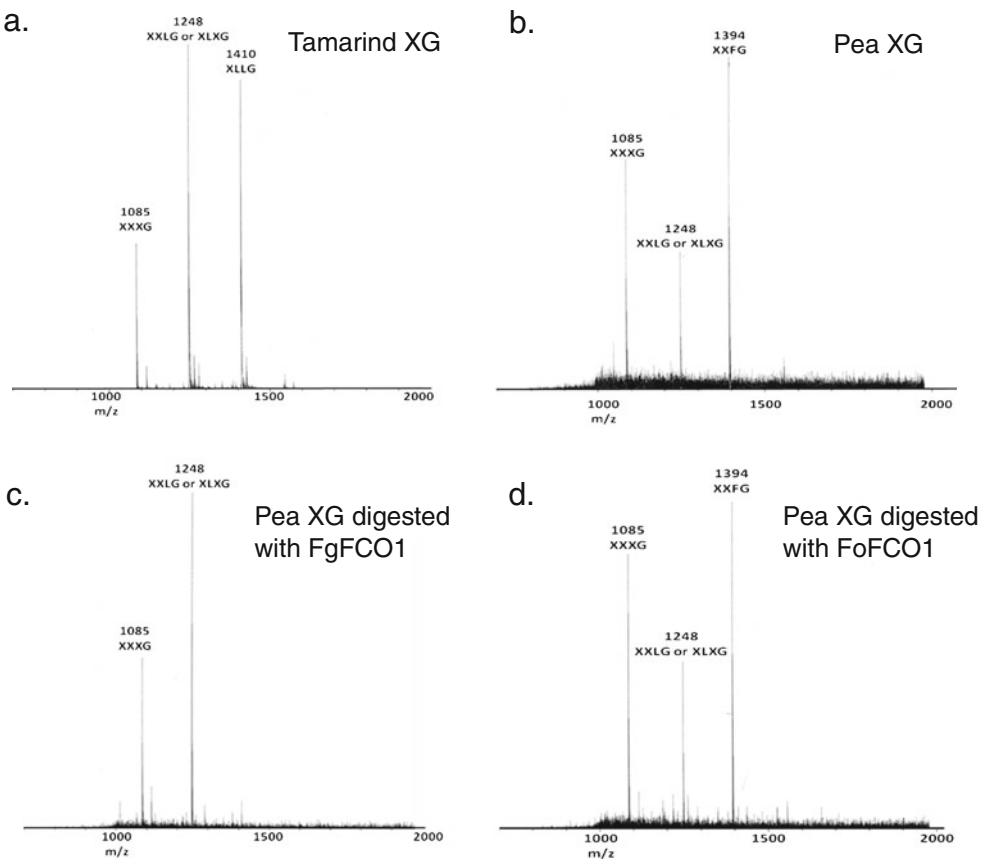

Fig. 6 α-Fucosidase activity on pea XG oligosaccharides. **a** Native tamarind XGOs. **b** Native pea XGOs. **c** Native pea XGOs treated with FgFCO1. **d** Native pea XGOs treated with FoFCO1. For the nomenclature of XG oligosaccharides, see Fry et al. (1993). For the structures, see Fig. 1. XLXG and XXLG have the same *m/z* ratio

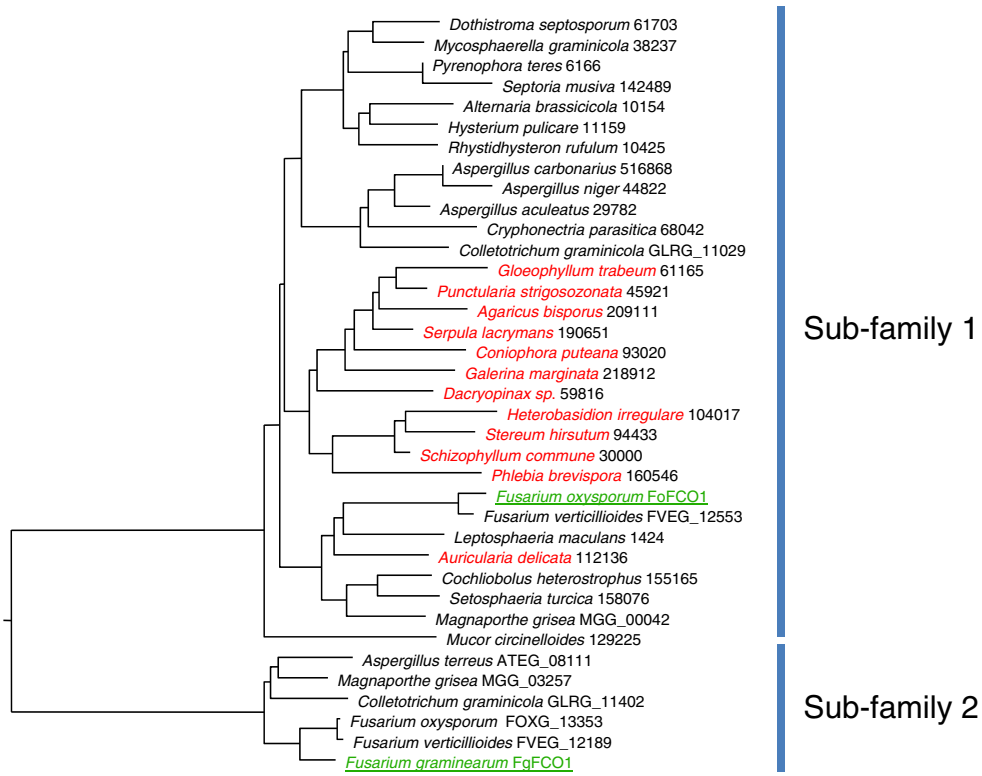

Fig. 7 Sequence alignment of FoFCO1, FgFCO1, and putative GH29 α-fucosidases from other fungi. All available sequences from subfamily 2 are included, but only a representative sample of those in subfamily 1. Sequences from Basidiomycota are shown in *red and bold*; all others are from Ascomycota, except *M. circinelloides* (Mucoromycotina). The two α-fucosidases from this study are shown in *green* and *underlined*. All sequences identified by *number* alone are from the DOE Joint Genome Institute. Other sequences are from the Broad Institute (FOXG_13352, FVEG_12189, FVEG_12553, GLRG_11029, GLRG_11042, MGG_00042, MGG_03257, and ATEG_08111). The alignment was performed with ClustalW (DNAStar, Madison, WI)

FoFCO1 and only *Aspergillus terreus* has an ortholog of FgFCO1. *Magnaporthe grisea* has two members of subfamily 1 (MG_00042 and MG_00316) and one of subfamily 2 (MG_03257; Fig. 7).

Discussion

Two extracellular α-fucosidases from two species of *Fusarium* were characterized. Although both proteins have been classified in Glycosyl Hydrolase family 29, they had only 23 % amino acid identity. The enzyme from *F. oxysporum* (FoFCO1) was active on the model substrate pNP-Fuc, but was not active on XGOs derived from plant cell walls. The enzyme from *F. graminearum* (FgFCO1) was active on XGOs, but not pNP-Fuc. When all available orthologs from all sequenced fungi were aligned, the two α-fucosidases defined two subfamilies of GH29 α-fucosidases. The subfamily containing FgFCO1 has many fewer members than the subfamily that contains FoFCO1. Whether the other members of each subfamily have the same spectrum of substrate preference as FoFCO1 and FgFCO1 remains to be established. Intriguingly, all of the fungi that contain an α-fucosidase of subfamily 2 are plant pathogens, except *A. terreus*, and FgFCO1 is upregulated during infection of barley (Güldener et al. 2006). It is therefore possible that this class of α-fucosidase has a role in plant pathogenesis.

FgFCO1 had very weak activity on pNP-Fuc. Some other fucosidases are also known to lack activity on pNP-Fuc (Bauer et al. 2006; De La Torre et al. 2002; Leonard et al. 2008). FgFCO1 did have activity on pea XG oligosaccharides, so a potential native substrate for FgFCO1 would be the fucosylated form of XG, which represents up to 30 % of the primary cell wall of many dicotyledonous plants. In light of this, it was surprising that FgFCO1 was not active on fucosylated tamarind XG. This might be because tamarind XG is not naturally fucosylated and was produced by artificial fucosylation of the tamarind XG polymer, which differs from native pea XG by being more heavily galactosylated. Because the XLLG subunit comprises more than 50 % of tamarind XG, a higher percentage of XLFG compared to XXFG would be produced after in vitro fucosylation (Fig. 6). On the other hand, pea XG is made almost entirely of XXXG and XXFG (Fig. 6). Thus, the Gal-Xyl side chain of XLFG might inhibit FgFCO1 by hindering the accessibility to Fuc. Alternatively, an excess of XLLG and XXLG arising from incompletely fucosylated tamarind XG might act as competitive inhibitors that prevent FgFCO1 from acting on the minor amount of XX[^{14}C]FG formed by in vitro fucosylation.

The natural substrate of FoFCO1 is apparently not pea XGOs, fucosylated tamarind XG or tamarind XGOs, or tobacco AGPs. Possible natural substrates include fucosylated glycoproteins, rhamnogalacturonan II, or an alternate form of fucosylated plant XG or AGPs (Lerouge et al. 1998; Wu et al. 2010; Zablackis et al. 1995). Although cereals are generally considered to have less XG than dicotyledons, fucosylated XG has been detected in *Festuca arundinacea* and in the roots of wheat (McDougall and Fry 1994; Faik and Wiemels, unpublished results). Therefore, one or both of the α-fucosidases characterized by this work could act on the cell walls of dicotyledons, non-commelinoid monocotyledons, or commelinoids.

Although Fuc is overall a minor component of most plant cell walls, α-fucosidase might nevertheless be important for the efficient breakdown of plant cell walls into their component monosaccharides. This is because Fuc needs to be removed prior to the other side-chain sugars in the course of complete depolymerization of fucosylated XG. In the absence of α-fucosidase, the tetrasaccharide sidechain of XG (Fuc-Gal-Xyl-Glc) would accumulate during the otherwise complete depolymerization of XG. Therefore, the inclusion of α-fucosidase in enzyme mixtures for biomass deconstruction might improve the yields of Xyl and Glc from both dicotyledonous plants and from cereals.

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