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Identification of an Arabidopsis gene encoding a GH95 alpha1,2-fucosidase active on xyloglucan oligo- and polysaccharides

Renaud Léonard [a,*], Martin Pabst [a], Jayakumar Singh Bondili [a], Gérard Chambat [c], Christiane Veit [b], Richard Strasser [b], Friedrich Altmann [a]

[a] Department of Chemistry, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria
[b] Institute of Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria
[c] Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), BP53, 38041 Grenoble Cedex 9, France

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ABSTRACT

α1,2-linked fucose can be found on xyloglucans which are the main hemicellulose compounds of dicotyledons. The fucosylated nonasaccharide XXFG derived from xyloglucans plays a role in cell signaling and is active at nanomolar concentrations.

The plant enzyme acting on this α1,2-linked fucose residues has been previously called fucosidase II; here we report on the molecular identification of a gene from *Arabidopsis thaliana* (At4g34260 hereby designed *AtFuc95A*) encoding this enzyme. Analysis of the predicted protein composed of 843 amino acids shows that the enzyme belongs to the glycoside hydrolase family 95 and has homologous sequences in different monocotyledons and dicotyledons. The enzyme was expressed recombinantly in *Nicotiana bentamiana*, a band was visible by Coomassie blue staining and its identity with the α1,2-fucosidase was assessed by an antibody raised against a peptide from this enzyme as well as by peptide-mass mapping. The recombinant AtFuc95A is active towards 2-fucosyllactose with a Km of 0.65 mM, a specific activity of 110 mU/mg and a pH optimum of 5 but does not cleave α1,3, α1,4 or α1,6-fucose containing oligosaccharides and *p*-nitrophenyl-fucose. The recombinant enzyme is able to convert the xyloglucan fragment XXFG to XXLG, and is also active against xyloglucan polymers with a Km value for fucose residues of 1.5 mM and a specific activity of 36 mU/mg. It is proposed that the *AtFuc95A* gene has a role in xyloglucan metabolism.

1. Introduction

Fucose is often found as a peripheral residue on glycoconjugates in many organisms from bacteria to man and is therefore involved in numerous fundamental biological processes. More specifically, in plants, fucose is present on glycoproteins through asparagine-bound *N*-glycans, but also in arabinogalactan proteins or cell wall polysaccharides, i.e., type II rhamnogalacturonans and xyloglucans (Hayashi et al., 1984; Tsumuraya et al., 1988; Puhlmann et al., 1994).

Xyloglucans are the main hemicellulose compounds of dicotyledons. They are cell wall polysaccharides consisting of a cellulose-like β1,4-glucan backbone substituted by single units of α1,6-xylose residues. Some xylosyl residues are further substituted at O-2 by β-galactose residues which may themselves carry an α1,2-fucose residue (Hayashi et al., 1984). It has been thought that the presence of fucose facilitates the association of xyloglucans with cellulose but this hypothesis has recently been abandoned (Lima et al., 2004; Hanus and Mazeau 2006). The presence of fucose seems to make xyloglucans a better substrate for galactose acetylation (Perrin et al., 2003). The fucosylated nonasaccharide XXFG (Fig. 1) derived from xyloglucans plays a role in cell signaling and is active at nanomolar concentrations (York et al., 1984; Dunand et al., 2000). At a concentration of 1 nM, this oligosaccharin abolishes the auxin-stimulated growth (York et al., 1984). Even the disaccharide Fucα1-2Gal is an active molecule in bioassays (McDougall and Fry 1989; Vargas-Rechia et al., 1998) and a putative receptor for this molecule has been identified on the plasmalemma (Dunand et al., 2000).

Plants exhibit two kinds of fucosidase activities. Fucosidase I hydrolyzes α1,3- and α1,4-linkages of fucose to GlcNAc in Lewis-type oligosaccharides or to galactose whereas fucosidase II acts upon α1,2-linkages and is efficient against xyloglucan fragments (Ogata-Arakawa et al., 1977; Kobata 1982).

Like other glycosidase activities necessary for the degradation of xyloglucan oligosaccharides, α1,2-fucosidase activity was found

Abbreviations: 2-FL, 2-fucosyllactose; GlcNAc, *N*-acetylglucosamine.

* Corresponding author. Tel.: +43 1 36006 6065; fax: +43 1 36006 6059.
E-mail address: renaud.leonard@boku.ac.at (R. Léonard).

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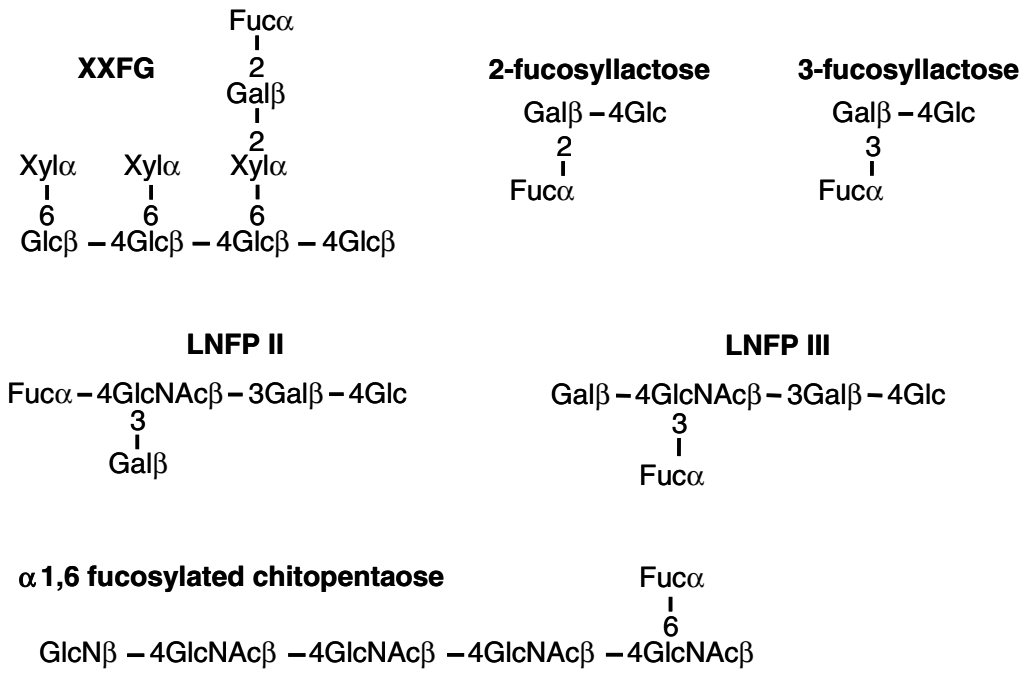

Fig. 1. Structures of oligosaccharides used in this study.

in the apoplastic fluid of *Arabidopsis thaliana* (Iglesias et al., 2006). Plant fucosidases have been purified and characterized in the earlier days of glycobiology (Yoshima et al., 1979; Imber et al., 1982; Scudder et al., 1990; Sano et al., 1992) but the proper identification of their genes is either extremely recent in the case of α1,3/4-fucosidase (Zeleny et al., 2006) or erroneous and incomplete as in the case of α1,2-fucosidase. Indeed, Augur et al. (1993) purified and characterized an α1,2-fucosidase from pea that is able to remove the fucosyl residue from xyloglucan oligosaccharides and published two years later what they supposed to be the corresponding cDNA sequence (Augur et al., 1995). Nevertheless, the corresponding protein was proven by another team to lack fucosidase activity and to be more likely a protease inhibitor (Tarrago et al., 2003). More recently de La Torre et al. published the sequence of an *A. thaliana* fucosidase, AtFuc1 (Swissprot entry Q8GW72), that they thought to be acting on α1,2 linkages (de La Torre et al., 2002) but which we showed to act exclusively on α1,3- and α1,4-linkages (Zeleny et al., 2006). This team also mentioned an additional sequence (Swissprot entry Q9FXE5) as an α1,2-fucosidase active against xyloglucan fragments (de La Torre et al., 2002) which was assigned to the family 29 of glycoside hydrolase in CAZy database (Carbohydrate Active Enzymes database, http://www.cazy.org/, (Coutinho and Henrissat 1999)).

Here we describe the recombinant expression in *Nicotiana benthamiana* and the biochemical characterization of an *A.thaliana* α1,2-fucosidase, whose gene was identified based on slight homology to the recently published sequence of an α1,2-fucosidase from *Bifidobacterium bifidum* (Katayama et al., 2004). We identified a homologue in *A. thaliana* (At4g34260). The enzyme's activity towards xyloglucan and xyloglucan fragments was investigated.

2. Results

2.1. Identification of the GH95 A. thaliana α1,2-fucosidase gene sequence

On the basis of the recently published sequence of the α1,2-fucosidase from *B. bifidum* (Katayama et al., 2004), the putative homologues in *A. thaliana* were searched using the tBLASTn program. In *A. thaliana*, only one sequence can be found with some significant homology to the bacterial gene. This gene, *At4g34260*, encodes a protein of 843 amino acids (Swiss prot entry Q8L7W8) (Fig. 2) with an uncleavable signal peptide ending at position 24 according to Psort (Nakai and Horton 1999) or a cleavable one ending at position 27 according to SignalP (http://www.cbs.dtu.dk/services/SignalP/).

No significant sequence homology can be found between At4g34260 and the *A. thaliana* gene encoding an α1,3/4-fucosidase previously published by our group (Zeleny et al., 2006). This α1,3/4-fucosidase belonged to the CAZy (Carbohydrate Active Enzymes database, http://www.cazy.org/) family 29 of glycoside hydrolase family (GH29) (Coutinho and Henrissat, 1999) that catalyses the hydrolysis using a retaining mechanism and is also present in mammals and several pathogenic bacteria. The protein encoded by At4g34260 belongs to the glycoside hydrolase family 95 (GH95) described as possessing an inverting mechanism (Intra et al., 2007) and being shared by plants and various bacteria but absent in animals.

A cDNA homologue of *At4g34260* can be found in *Oryza sativa* (NM_001070916) with an identity at the protein level of about 50.2% (Fig. 2). The gene structure is not conserved between *A. thaliana* and *O. sativa*, the first possessing 9 exons and 8 introns (3461 bases from ATG to TGA) whereas the second is encoded across 10 exons dispatched on ca. 10 kb of genomic DNA. Genomic sequences with significant level of homology are present in the databases for plant species like *Lotus japonicus* (AP006430), *Vitis vinifera* (AM438689), *Medicago sativa* (AC174354) and *Brassica rapa* (AC189325) (Fig. 2). During the revision of this manuscript, a homologous protein with α1,2-fucosidase activity toward xyloglucan fragments has been purified from lily flowers (Ishimizu et al., 2007). The sequence of this enzyme shares 55% of identity at the amino acid level with the Arabidopsis protein encoded by At4g34260 and was shown to co-purify with a vacuolar mannosidase (Ishimizu et al., 2007).

Five potential *N*-glycosylation sites are present on the sequence, the fourth of them being more unlikely to be used due to the

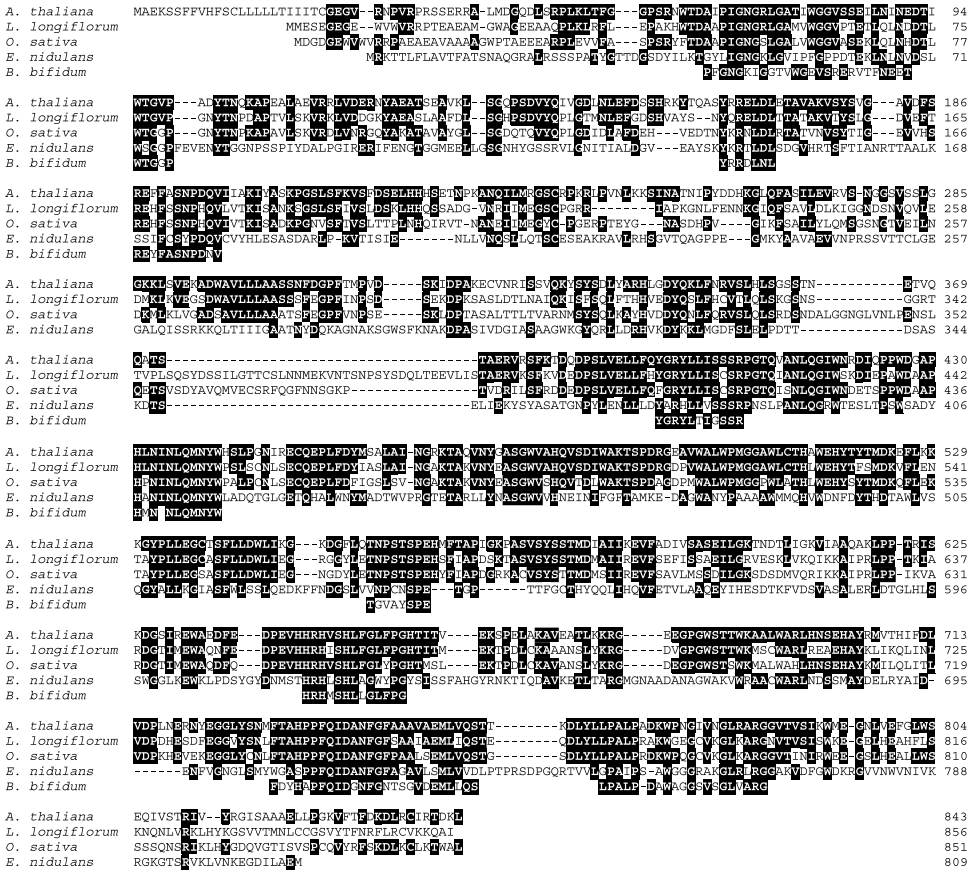

Fig. 2. Alignment of the amino acid sequences of AtFuc95A with homologous sequences from *O. sativa, Lilium longiflorum* and *E. nidulans* (*Emericella nidulans*, formerly known as *Aspergillus nidulans*). Peptide sequences from the α-1,2-fucosidase from *B. bifidum* with significant homology to AtFuc95A are shown. The glutamic acid and asparagine residues presumed to play roles in acid catalysis and nucleophilic attack, respectively (Nagae et al., 2007) are marked with an asterisk. Residues conserved between *A. thaliana* and at least one other specie are highlighted and gaps introduced to facilitate alignment are shown by dashes. The numbering shown commences from the start codon. No numbering is shown in the case of *B. bifidum* for which the sequence is only partially shown due to its strong size difference with other presented sequences.

presence of a proline immediately after the asparagine residue. The glutamic acid residue which was shown by crystallographic studies to be the general acid catalyst in the *B. bifidum* enzyme (Nagae et al., 2007) is present in the *A. thaliana* sequence as well as the asparagine residue involved in the nucleophilic attack. The subcellular localizations predicted by diverse programs are contradictory but give a small predominance to the extracellular compartment and the vacuole (according to WoLF PSORT, http://wolfpsort.org/) and the secretory pathway (according to Psort). For comparison, experimental data showed that 80% of the α1,2-fucosidase activity of *A. thaliana* was found in the apoplast (de La Torre et al., 2002). The α1,2-fucosidase purified from Lily flowers appeared to co-purify with a vacuolar mannosidase (Ishimizu et al., 2007).

2.2. Expression of a recombinant form of the putative A. thaliana α1,2-fucosidase

The ORF of the presumed *A. thaliana* fucosidase was cloned under control of the 35S promoter in the p21GT vector leading to the plasmid p21Fuc. This construct was transferred into agrobacteria and the putative fucosidase was transiently produced in *N. benthamiana* leaves by agroinfiltration. The supernatant obtained after homogenization of the leaves and centrifugation was analyzed by SDS-PAGE and western blotting. An antibody raised against a C-terminal peptide of the putative fucosidase specifically recognized a single band at a size of about 74 kDa (Fig. 3). Staining was observed with leaves transformed with p21Fuc but not with leaves agro-infiltrated with the negative control p21GFP. After SDS-PAGE and Coomassie blue staining (Fig. 3), a protein band was present at the same position as the band revealed by Western blotting in the leaves agro-infiltrated with p21Fuc. This protein was absent in the extract from leaves agro-infiltrated with p21GFP. The experimental mass is about 20 kDa lower than expected. To confirm that this band does correspond to the protein of interest, it was excised from the gel to proceed to a peptide mapping analysis. The identity of the Coomassie blue stained band with the putative fucosidase was assessed by MALDI peptide-mass mapping, which gave a sequence coverage of 13%. Peptides comprising amino acids 106 to 836 were found (complementary data Table 1).

2.3. Characterisation of recombinant A. thaliana α1,2-fucosidase

Extracts of leaves transformed either by p21Fuc or by p21GFP were investigated for their α1,2-fucosidase activity. As measured by MALDI, 2-fucosyllactose (2-FL) was readily defucosylated by the lysate of leaves expressing the recombinant enzyme but not by the lysate from the negative control (data not shown). No special requirements for divalent cations could be observed. The same results were obtained when the enzyme was recombinantly produced in the yeast *Pichia pastoris* (data not shown). Using initial substrate concentrations ranging from 0.125 to 2.5 mM and time and enzyme concentration allowing not more than 5% conversion, a Km value for 2-FL of 0.65 ± 0.04 mM was found using both Lineweaver-Burk and Hanes-Woolf plots (Table 1). This agrees well with the Km value of 0.67 mM for 2-FL as determined for α1,2-fucosidase purified from almond emulsin (Kobata 1982). The

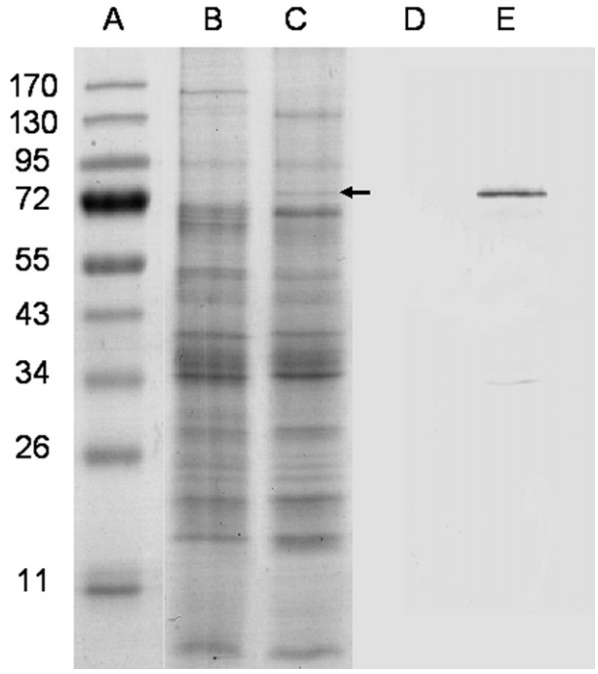

Fig. 3. Coomassie blue stained gel (A–C) and western blot analysis (D and E) of extracts from *Nicotiana benthamiana* leaves agro-infiltrated with p21GFP (B and D) and p21Fuc (C and E). The arrow shows the protein band determined by peptide mapping to be AtFuc95A.

Table 1
Kinetic parameters of AtFuc95A

	Maximal specific activity (mU/mg of enzyme)	Km (mM)
2-Fucosyllactose	110	0.65
Polymeric xyloglucan	36	1.5
XXFG	Active[a]	
p-Nitrophenyl-fucose	n.a.	
3-Fucosyllactose	n.a.	
Lacto-*N*-fucopentaose II	n.a.	
Lacto-*N*-fucopentaose III	n.a.	
α1,6-Fucosylated chitopentaose	n.a.	

n.a.: no activity.
[a] At a substrate concentration of 0.25 mM, AtFuc95A is 6.5-times more active with XXFG than with polymeric xyloglucans.

maximal specific activity of the *A. thaliana* fucosidase with 2-FL was estimated to be about 110 mU/mg based on an estimation by eye of the enzyme amount from Coomassie blue staining of the SDS-PAGE gel of the extract and comparison with BSA standards.

The recombinant enzyme, which we christen AtFuc95A according to Henrissat et al. (1998) is not able to hydrolyze 3-fucosyllactose, *p*-nitrophenyl-α-l-fucopyranoside, lacto-*N*-fucopentaose II or lacto-*N*-fucopentaose III (Table 1). Likewise, no activity could be found with an α1,6-fucosylated chitopentaose as determined by MALDI-TOF-MS (Table 1). Thus, AtFuc95A does not act on 3-, 4-, and 6-linked fucose residues, giving to AtFuc95A a close resemblance to the long known almond fucosidase II (Kobata 1982).

In order to assess that the recombinantly produced fucosidase was active against its presumed natural substrate, i.e., xyloglucan and xyloglucan oligosaccharides, the nonasaccharide XXFG was tested. As shown by MALDI-MS analysis, AtFuc95A removed the α1,2-fucose residue linked to the xyloglucan oligosaccharide to generate XXLG (Fig. 4). As for the other substrates, an extract from

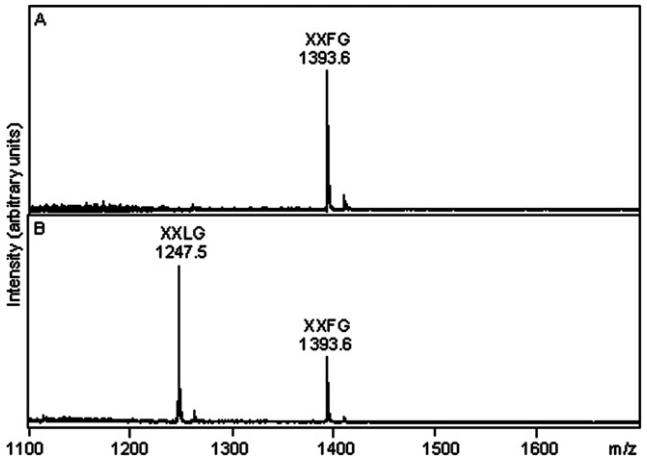

Fig. 4. MALDI-TOF analysis of the effect of AtFuc95A on the xyloglucan oligosaccharide XXFG. Results of incubations of XXFG with leaf extract agro-infiltrated with (A) p21GFP and (B) p21Fuc. Samples were incubated with extract for 10 min at 37 °C.

N. benthamiana leave agro-infiltrated with the vector carrying GFP instead of the fucosidase sequence did not show any activity. A range of pH values from 4.0 to 9.0 was tested showing a maximal activity of the enzyme at pH 5.0 (complementary data Fig. 1).

Finally we investigated whether the enzyme was active against xyloglucan polymer by quantifying the amount of fucose liberated during the reaction. After incubation, free fucose was labelled with anthranilic acid and quantitated by HPLC (Fig. 5). AtFuc95A was found to be active towards xyloglucans with a Km value of 1.5 mM (in terms of fucose) using both Lineweaver-Burk and Hanes-Woolf plots. The maximal specific activity of AtFuc95A toward polymeric xyloglucan was evaluated to be 36 mU/mg which is only about three times lower than what is obtained with 2-FL.

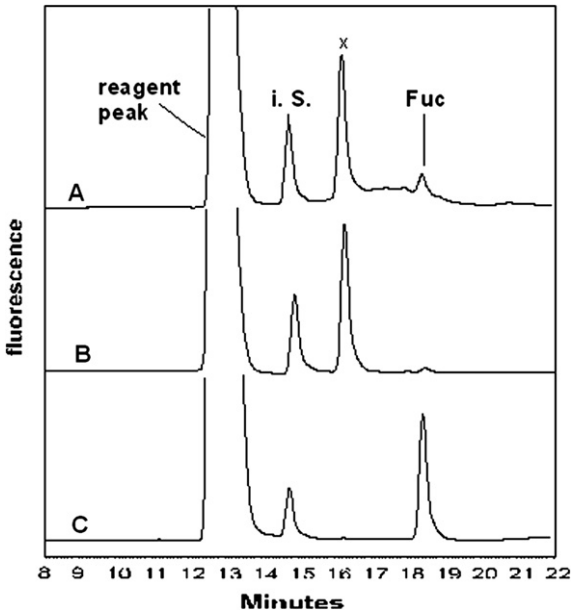

Fig. 5. Analysis of fucosidase activity on polymeric xyloglucan. Released fucose was determined by HPLC of anthranilic acid derivatized sugars. (A and B), assay performed at a xyloglucan concentration in terms of fucose of 1 mM and 0.125 mM, respectively. (C) Chromatogram with 2-deoxyglucose used as an internal calibration standard (IS) for all measurements and fucose. X is a contamination peak coming from the enzyme extract.

The hydrolysis rate of XXFG when measured at a substrate concentration of 0.25 mM was 6.5 times higher than that of xyloglucan at the same concentration of fucose, designating XXFG as an efficient substrate of AtFuc95A.

3. Discussion

We have cloned and expressed an *A. thaliana* gene, *AtFuc95A,* encoding an α1,2-fucosidase. In this work, the enzymatic activity of the recombinant protein was proven by HPLC and MALDI-TOF-MS with defined substrates in order to exclude an erroneous conclusion about the nature of the gene product. Of physiological relevance is the capacity of this enzyme to be active against fucosylated xyloglucan fragments as well as xyloglucan polymers. The substrate specificity of the enzyme, active toward 2-FL but neither toward *p*-nitrophenyl fucose nor toward 3- and 4-linked fucose in Lewis X and Lewis A motifs, respectively, as well as its Km value for 2-FL gives to AtFuc95A a close resemblance to the long known almond fucosidase II (Kobata 1982).

During the revision of this manuscript the purification of an α1,2-fucosidase from lily flowers belonging to the GH95 family was published (Ishimizu et al., 2007). The enzyme had an apparent size of 66 kDa and 46 kDa on gel even though the sequence of the corresponding ORF suggested an enzyme of 92 kDa. To recall, AtFuc95A expressed in tobacco likewise appeared 20 kDa smaller than expected. Even though Ishimizu et al. did not recombinantly express the enzyme, their finding corroborates our result about an α1,2-fucosidase of GH95 family in plants.

Fucosidase activities already have been assigned to several other genes. Two of these assignments have been proven by us and others to be erroneous as outlined in the introduction. However, another sequence, classified into the GH29 family, was recombinantly produced in *P. pastoris* and shown to have fucosidase activity towards 2-FL and xyloglucan fragments (de La Torre et al., 2002). A comparison of the specific activity observed for the GH29 fucosidase and the enzyme described in this paper shows that AtFuc95A is about 13 times more active against 2-FL (114 mU/mg compared to 146 pkatal/mg, i.e., 8.8 mU/mg). The difference seems to be even higher toward xyloglucans and xyloglucan oligosaccharide but in the absence of kinetic values for the enzyme characterized by de La Torre et al. (2002), a fair comparison with our data is not possible. The reason why plants would have two α1,2-fucosidases from two different GH families remains unclear and should be further investigated. However, the present work opens new possibilities to study the role of fucose in the plant cell wall with molecular biological methods.

4. Experimental

4.1. Identification and cloning of the fucosidase gene

RNA from *A. thaliana* 10 days old plantlets (cv. Colombia) was prepared using Trizol reagent (Invitrogen). cDNA was prepared using Superscript III (Invitrogen) reverse transcriptase with T18 as primer and Expand polymerase mix (Roche) was used for PCR.

Based on the homologous sequence of the α1,2-fucosidase from *B. bifidum* (Katayama et al., 2004), the primer combination used to clone the *A. thaliana* α1,2-fucosidase cDNA was 5′-GCTCTAGAG-CATGGCGGAGAAGTCGAGCTT-3′ and 5′-GACTAGTCCT ATAACTTGT-CGGTCCGAATGC-3′. PCR products were purified after gel electrophoresis using Illustra gel purification kit (GE Healthcare) and ligated into the pGEM-T vector (Promega). Positive clones were sequenced using the BigDye kit (Applied Biosystems). The expression vector p21Fuc was generated by digesting a pGEM-T clone with *Xba I* and *Spe I* and ligating the α1,2-fucosidase ORF into p21GT cut with the same enzymes. The vector p21GT is derived from pPT2 (Strasser et al., 2005) by insertion of an additional Spe I site into the multiple cloning region. Construct p21GFP was generated by insertion of the GFP ORF linked to the Golgi targeting sequence of the rat α2,6-sialyltransferase into the Xba I and Spe I site of p21GT.

4.2. Heterologous expression in Nicotiana benthamiana and Pichia pastoris

5–6 week old *N. benthamiana* plants were used for agroinfiltration experiments as described previously (Strasser et al., 2007). Briefly, p21Fuc and p21GFP were transfected into *A. tumefaciens* strain UIA143 by electroporation. A single colony arising from each transformation was inoculated into 5 ml LB medium supplemented with 50 μg/ml kanamycin and 25 μg/ml gentamycin and grown to stationary phase (20–24 h) at 29 °C with agitation. 300 μl of bacterial culture were centrifuged and washed twice with infiltration buffer (50 mM MES, pH 5.6, 2 mM sodium phosphate, 0.5% (w/v) D-glucose and 100 μM acetosyringone). For plant infiltration, bacteria were diluted to an OD600 of 0.3 for p21Fuc and p21GFP. Leaves were harvested 48 h after infiltration.

Transformation of *P. pastoris* was performed as previously described (Léonard et al., 2004) after insertion of the α1,2-fucosidase ORF in pGAP (invitrogen).

4.3. SDS-PAGE and western blot

Leaves of agro-infiltrated *N. bentamiana* were ground mass per volume with mortar and pestle in ammonium acetate 50 mM pH 5 and centrifuged 15 min at 14,000*g* at 4 °C. After dilution with 2 times Laemmli buffer and denaturation at 95 °C for 5 min, samples were loaded on a 12.5 percent acrylamide gel and either Coomassie blue stained or subjected to Western blotting. In this second case, the first antibody was a serum of a rabbit immunized with the peptide 822-AELLPGKVFTFDKDLRC-838 (Gramsh Laboratories, Schwabhausen, Germany) and the second antibody was the alkaline phosphatase anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Development was performed using Sigma Fast BCIP/NBT.

4.4. Fucosidase assays

For most assays, leaves of agro-infiltrated *N. bentamiana* were ground at 1 g per ml with mortar and pestle in 50 mM sodium acetate of pH 5 with the exception of the pH optimum experiments for which 5 mM buffer was used. The enzymatic assays were performed by adding 5 μl of substrate at a fucose concentration of 0.25 mM to 5 μl of leaf lysate diluted in ammonium acetate of pH 5. All enzymatic reactions were performed at 37 °C and stopped by heat inactivation at 95 °C for 5 min. The oligosaccharide XXFG as well as the polymeric xyloglucans used in this study were obtained from *Rubus fruticosus* cell culture medium as previously described (Kostlánová et al., 2005).

For MALDI-TOF-MS analysis, 1 μl of the reaction mixture was plated, followed by 1 μl of 2% (w/v) 2,5-dihydroxybenzoic acid in 30% acetonitrile and immediate drying *in vacuo*. Spectra were obtained on a Ultraflex MALDI-TOF/TOF (Bruker, Bremen, Germany) in positive reflectron mode. Percent of conversion was given by the numbers of counts obtained by mass analysis.

For the kinetic measurements, enzymatically released fucose was analyzed by reversed phase HPLC of anthranilic acid labelled monosaccharides as desribed (Anumula 1994).

The pH optimum of AtFuc95A was established by diluting the enzyme with 100 mM sodium acetate buffer of pH values from 4 to 6 or Tris/HCl buffer of pH 7–9.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.03.024.

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