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Pollen tube cell walls of wild and domesticated tomatoes contain arabinosylated and fucosylated xyloglucan

Flavien Dardelle[1,†], François Le Mauff[1], Arnaud Lehner[1], Corinne Loutelier-Bourhis[2], Muriel Bardor[1], Christophe Rihouey[3], Mathilde Causse[4], Patrice Lerouge[1], Azeddine Driouich[1] and Jean-Claude Mollet[1,*]

[1]Laboratoire de Glycobiologie et Matrice Extracellulaire Végétale (Glyco-MEV), EA 4358, Normandy University, IRIB, VASI, 76821 Mont-Saint-Aignan Cedex, France, [2]COBRA, UMR6014 and FR3038, Normandy University, INSA Rouen, CNRS, IRCOF, 76821 Mont-Saint-Aignan Cedex, France, [3]Laboratoire Polymères, Biopolymères, Surfaces, UMR CNRS 6270, Normandy University, 76821 Mont-Saint-Aignan Cedex, France and [4]Génétique et Amélioration des Fruits et Légumes, INRA UR1052, 84143 Montfavet Cedex, France
** For correspondence. E-mail jean-claude.mollet@univ-rouen.fr*
[†]Present address: Fitoplancton Marino, S.L. 11500 El Puerto de Santa María, Cádiz, Spain.

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• **Background and Aims** In flowering plants, fertilization relies on the delivery of the sperm cells carried by the pollen tube to the ovule. During the tip growth of the pollen tube, proper assembly of the cell wall polymers is required to maintain the mechanical properties of the cell wall. Xyloglucan (XyG) is a cell wall polymer known for maintaining the wall integrity and thus allowing cell expansion. In most angiosperms, the XyG of somatic cells is fucosylated, except in the Asterid clade (including the Solanaceae), where the fucosyl residues are replaced by arabinose, presumably due to an adaptive and/or selective diversification. However, it has been shown recently that XyG of *Nicotiana alata* pollen tubes is mostly fucosylated. The objective of the present work was to determine whether such structural differences between somatic and gametophytic cells are a common feature of *Nicotiana* and *Solanum* (more precisely tomato) genera.
• **Methods** XyGs of pollen tubes of domesticated (*Solanum lycopersicum* var. *cerasiforme* and var. *Saint-Pierre*) and wild (*S. pimpinellifolium* and *S. peruvianum*) tomatoes and tobacco (*Nicotiana tabacum*) were analysed by immunolabelling, oligosaccharide mass profiling and GC-MS analyses.
• **Key Results** Pollen tubes from all the species were labelled with the mAb CCRC-M1, a monoclonal antibody that recognizes epitopes associated with fucosylated XyG motifs. Analyses of the cell wall did not highlight major structural differences between previously studied *N. alata* and *N. tabacum* XyG. In contrast, XyG of tomato pollen tubes contained fucosylated and arabinosylated motifs. The highest levels of fucosylated XyG were found in pollen tubes from the wild species.
• **Conclusions** The results clearly indicate that the male gametophyte (pollen tube) and the sporophyte have structurally different XyG. This suggests that fucosylated XyG may have an important role in the tip growth of pollen tubes, and that they must have a specific set of functional XyG fucosyltransferases, which are yet to be characterized.

Key words: plant cell wall, GC-MS, gametophyte, immunolabelling, oligosaccharide mass profiling, OLIMP, pollen tube, tomato, *Solanum lycopersicum*, *S. peruvianum*; *S. pimpinellifolium*, xyloglucan, XyG, *Nicotiana tabacum*.

INTRODUCTION

In flowering plants, sexual reproduction requires the mating of the sperm cells, carried by the pollen tube, with the ovule. After adhesion and rehydration on the stigma, the pollen grain germinates and produces a tube that grows within the stigma and style through the transmitting tract tissue. During the journey, interactions with the female tissues occur promoting the fast tip-polarized pollen tube growth and guidance (Johnson and Lord, 2006; Mollet *et al*., 2007; Cheung and Wu, 2008). Finally, attracted by the synergid cells, the pollen tube bursts and releases the two sperm cells, allowing the double fertilization and the formation of seeds (Franklin-Tong, 2010; Palanivelu and Tsukamoto, 2012).

The primary cell wall confers many functions such as cell adhesion, signal transduction as well as physical active barriers against micro-organisms (Durand *et al*., 2009; Fry, 2011). The cell wall of pollen tubes in most flowering species is mainly composed of three types of polysaccharides: β-glucans, hemicellulose and different pectin motifs including homogalacturonan, xylogalacturonan, rhamnogalacturonan-I (RG-I) and RG-II (Dardelle *et al*., 2010; Chebli *et al*., 2012; Mollet *et al*., 2013; Dumont *et al*., 2014). Cellulose, a β-(1→4)-linked glucan, is generally present in low amounts. In contrast, the main glucan found in the pollen tube cell wall is callose, a β-(1→3)-linked glucan (Qin *et al*., 2012; Mollet *et al*., 2013). In vegetative organs of eudicots, the main hemicellulose is xyloglucan (XyG) representing 20–25 % (dry weight) of the primary cell wall, whereas it accounts for only 2 % in certain monocots (Hsieh and Harris, 2009, Fry, 2011). XyG is linked to cellulose microfibrils by hydrogen bonds that regulate cell expansion,

avoid aggregation of cellulose microfibrils and increase the strength of the primary cell wall (Hayashi and Kaida, 2010; Scheller and Ulvskov, 2010; Popper *et al*., 2011). XyG is made of a β-(1→4)-glucan backbone that can be substituted by different side chains (Scheller and Ulvskov, 2010) named by one letter according to the nomenclature proposed by Fry *et al*. (1993) (Fig. 1). G is the non-substituted glucosyl (Glc) residue, X is the α-D-xylose (Xyl) linked to the C-6 of the β-D-Glc. Then, in most eudicots, the Xyl residue can be branched on the C-2 residue by a galactose (Gal) (L), itself substituted by a fucosyl (Fuc) residue (F) forming the fucogalactoXyG (Fig. 1A). Surprisingly, very little is known concerning the XyG composition of pollen tubes. Using the OLIMP (OLIgosaccharide Mass Profiling) method (Lerouxel *et al*., 2002), Dardelle *et al*. (2010) have shown that the XyG of *Arabidopsis thaliana* (Malvid, Brassicaceae) pollen tubes was highly fucosylated and *O*-acetylated as compared with the XyG found in vegetative organs. The main fragments detected were XXFG and XLFG, both being *O*-acetylated as indicated by the underlined letter (Dardelle *et al*., 2010) (Fig. 1A). These data suggested an important role for these two structural features during pollen tube growth and possibly in the cell wall interaction with the female tissues. Interestingly, in the lamiid clade (euasterids I) (including the Solanaceae with *Nicotiana* sp. and *Solanum* sp.) (APG III, 2009), XyG found in vegetative organs is not decorated with Fuc residues, and Gal was only detected in tomato (Sims *et al*., 1996; York *et al*., 1996; Jia *et al*., 2003, 2005, Hoffman *et al*., 2005). Instead, XyG displays abundant Ara*f*-(1→2)-Xyl*p* (S) and Ara*f*-(1→3)-Ara*f*-(1→2)-Xyl*p* (T) oligosaccharides linked to the glucan backbone and was named arabinoXyG (Fig. 1B) (York *et al*., 1996; Jia *et al*., 2003, 2005; Hoffman *et al*., 2005). In tomato and tobacco, the most abundant fragments were XXGG and XSGG (Fig. 1B) (York *et al*., 1996; Sims *et al*., 1996; Vincken *et al*., 1997; Jia *et al*., 2003, 2005; Hsieh and Harris, 2009). Recently, Lampugnani *et al*. (2013) showed clear structural differences in the composition of XyG in *Nicotiana alata* pollen tubes. They revealed that, in contrast to the vegetative organs, the cell wall was most exclusively a fucogalactoXyG type.

The present work attempted to determine by immunolabelling, OLIMP and gas chromatography–mass spectrometry (GC-MS) if XyG found in pollen tubes of other Solanaceae species shared a similar structural feature. Most of the investigated species were from the *Solanum* genus. They included domesticated tomatoes such as *S. lycopersicum* var. *Saint-Pierre* and *S. lycopersicum* var. *cerasiforme* 'wva106' (cherry tomato), and wild species *S. pimpinellifolium* and *S. peruvianum* that retraced the diversity, evolution and selection. In addition,

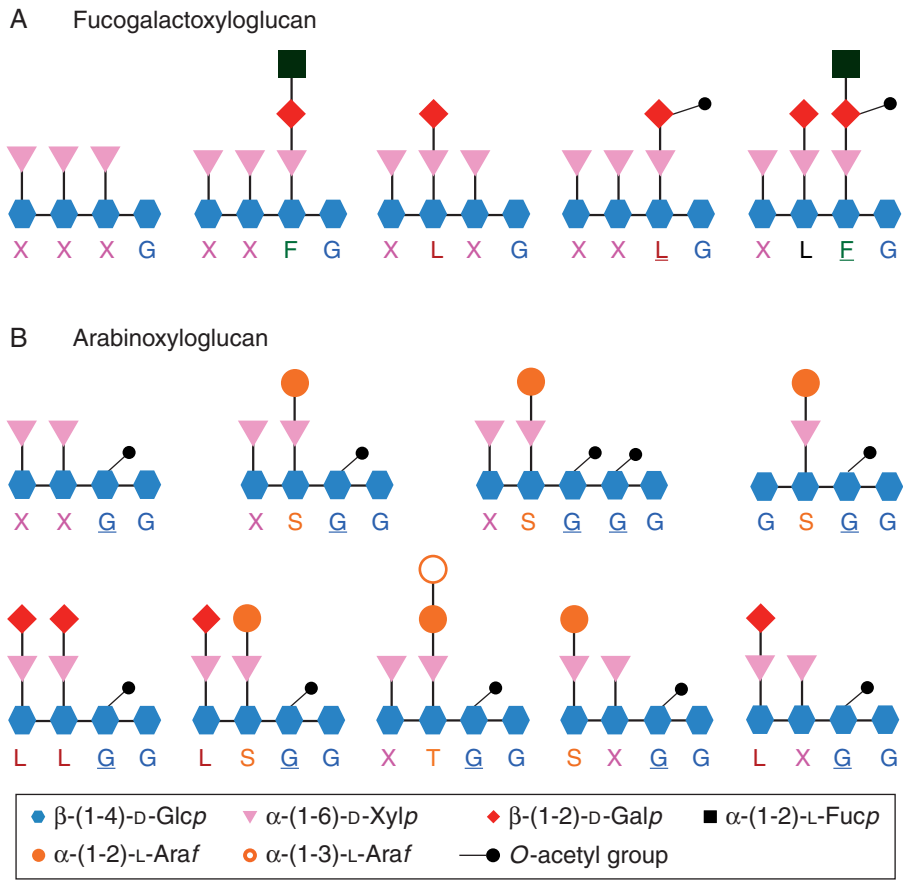

FIG. 1. Graphic representation of the main *endo*-glucanase-generated fragments released from (A) fucogalactoXyG and (B) arabinoXyG. The structures of XyG fragments are shown according to the one-letter code nomenclature proposed by Fry *et al*. (1993). Underlined and bold letters represent *O*-acetylated side chains. The main arabinoXyG fragments found in tobacco and tomato cultured cells and/or leaves are from Jia *et al*. (2003) and Hoffmann *et al*. (2005).

another species from the *Nicotiana* genus (*N. tabacum*) was also investigated.

Here, we show that XyG in the pollen tube cell wall of *N. tabacum* is structurally similar to that found in *N. alata* (Lampugnani *et al.*, 2013) and the relative level of fucosylated fragments was about 62 %. In contrast, XyG found in pollen tubes of tomato species is composed of arabinosylated and fucogalactosylated XyG. Finally, the relative abundance of fucosylated XyG was significantly lower in the domesticated than in the wild species, but, in all the cases, it did not exceed 20 %. Our data suggest that tomato pollen tubes have specific and functional XyG fucosyltransferases and that fucosylated XyG might be important for the tip growth of the male gametophyte within the female tissues.

MATERIALS AND METHODS

Plant growth

Wild tomatoes *Solanum pimpinellifolium* LA1589 and *S. peruvianum* CMV-INRA, and domesticated tomatoes *S. lycopersicum* var. *cerasiforme* 'West Virginia 106' (wva106) and *S. lycopersicum* var. *Saint-Pierre* (Truffaut, France) seeds were germinated in the dark for 5 d at 20 °C on wet cotton, transferred to sterilized soil and cultured in a growth chamber. All species were grown with a photoperiod of a 16 h light/8 h dark cycle at 25 and 22 °C during the light and dark period, respectively. Relative humidity was maintained at 60 %, and plants were watered every 2 d.

Cell suspension culture

The tomato (*S. lycopersicum* 'dombito') cell suspension was initiated from hypocotyl sections of seedlings (Muschitz *et al.*, 2009). Cells were harvested by filtration after 5 d of growth and chemically fixed with 4 % formaldehyde in PBS (phosphate-buffered saline: 8 g L^{-1} NaCl, 0·29 g L^{-1} KCl, 1·45 g L^{-1} Na_2HPO_4, 0·24 g L^{-1} KH_2PO_4) for immunolabelling or washed with and stored in 70 % ethanol (EtOH) at 4 °C for biochemical analyses.

Pollen tube culture

Pollen grains were collected from freshly dehisced anthers, and the stamens of three flowers (tobacco) or five flowers (tomato) were submerged in 5 mL of BK medium [1·62 mM H_3BO_3, 1·25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2·97 mM KNO_3 and 1·65 mM $MgSO_4{\cdot}7H_2O$] (Brewbaker and Kwack, 1963) containing 15 % sucrose. Pollen grains were suspended in the liquid medium by vortex, and the stamens were removed with tweezers. Pollen tubes were grown at 22 °C in the dark for 6 h under agitation. During this period, pollen tubes are actively growing. For immunolocalization experiments, pollen tubes were chemically fixed according to Dardelle *et al.* (2010). For XyG analysis, 3 vols of EtOH were added to the suspension and pollen tubes were separated from the medium and ungerminated pollen grains by filtration using a 50 µm nylon mesh filter. After several washes with 70 % EtOH, pollen tubes were stored at 4 °C in 70 % EtOH.

Immunolocalization of XyG epitopes

LM15 (Plant Probes, UK) and CCRC-M1 (CCRC, University of Georgia, USA) monoclonal antibodies (mAbs) were used to detect XXXG motifs (Marcus *et al.*, 2008), and α-Fuc-(1→2)-β-Gal (Puhlmann *et al.*, 1994) epitopes, respectively. Pollen tubes and the cell suspension were immunolabelled as described by Dardelle *et al.* (2010) and Follet-Gueye *et al.* (2012). Controls were performed by omitting the primary antibody. Samples were observed with a Leica DMLB microscope under bright field and fluorescence illumination with fluorescein isothiocyanate (FITC) filter sets (absorption, 485–520 nm; emission, 520–560 nm). Images were digitally acquired with a Leica DFC300FX camera at the same exposure time.

Cell wall extraction

Pollen tubes, cells or leaves were homogenized with a glass potter and treated three times with 70 % EtOH at 70 °C. The insoluble residues were then incubated with a chloroform/methanol mixture (1:1, v/v) and acetone to yield the cell wall extracts.

Xyloglucan analysis

Preparation of xyloglucan fragments. A 1–5 mg aliquot of the cell wall extract was incubated overnight at 37 °C under agitation at 180 rpm in 500 µL of *endo*-(1–4)-β-D-glucanase (5 U, EC 3.2.1.4; Megazyme) prepared in 10 mM ammonium acetate buffer, pH 5. After the addition of 1·5 mL of 96 % EtOH and centrifugation (10 min at 8000 *g*), the supernatant was evaporated to concentrate the XyG oligomers. XyG from tamarind seeds (Megazyme) was treated similarly in parallel and used as a control for the digestion step.

Extraction of the hemicellulose-enriched fraction. The enzyme-resistant pellets or the crude cell wall extracts were treated with 0·5 % ammonium oxalate at 100 °C for 1 h to remove the pectins. After centrifugation, the pellet was treated overnight with 4 M KOH supplemented with 20 mM $NaBH_4$ to extract the hemicellulose-enriched fraction. The solution was neutralized with acetic acid to pH 5·5, centrifuged and the supernatant was dialysed against ddH_2O. The hemicellulose-enriched fraction was freeze-dried and submitted to a new *endo*-(1–4)-β-D-glucanase digestion or treated for monosaccharide composition and glycosyl-linkage analysis.

MALDI-TOF MS of oligosaccharides. A voyager DE-Pro matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Applied Biosystems) equipped with a 337 nm nitrogen laser was used to analyse XyG fragments. Mass spectra were collected in the reflector delayed extraction mode using 2,5-dihydroxybenzoic acid (Sigma-Aldrich) as matrix. The matrix, freshly dissolved at 5 mg mL^{-1} in a solution composed of 70:30 acetonitrile/0·1 % trifluoroacetic acid (TFA), was mixed with the water-solubilized oligosaccharides in a ratio 1:1 (v/v). The spectra were recorded in a positive mode, using an acceleration voltage of 20 000 V with a delay time of 100 ns and >50 % of the laser energy. They were

externally calibrated using commercially available mixtures of peptides and proteins (ProteoMass™ MALDI Calibration Kit, Sigma-Aldrich). Laser shots were accumulated for each spectrum in order to obtain an acceptable signal to noise ratio (sum of two spectra of 1000 shots per spectrum). Values are expressed as relative percentage and are means ± s.d. from MALDI-TOF spectra obtained after *endo*-glucanase digestion of the cell wall from three independent biological samples.

Structural characterization of oligosaccharides by MALDI-TOF/TOF MS. In order to confirm the structure and the presence of fucosyl residues, oligosaccharides were permethylated as described by Mathieu-Rivet *et al*. (2013). The eluates were spotted in DHB matrix 5 mg mL^{-1} onto a MALDI plate and analysed by the Autoflex III time-of-flight (TOF/TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency-tripled Nd:YAG laser emitting at 355 nm, a LIFT cell, a FlexControl (3.3) and FlexAnalysis (3.3) software package (Bruker Daltonics) in positive reflector mode.

Monosaccharide composition by gas chromatography–flame ionization detection (GC-FID). Hemicellulose-enriched fractions were hydrolysed by 2 M TFA for 2 h at 110 °C. Monosaccharides were then derivatized with 1 M methanol-HCl (Supelco) at 80 °C overnight followed by a mixture of hexamethyldisiloxan:trimethyldisiloxan:pyridine (3:1:9, Supelco) at 110 °C for 20 min. After drying, derivatives were dissolved in 1 mL of cyclohexane and injected in the 3800 GC system equipped with a CP-Sil5-CB column (Agilent Technologies). The gradient of temperature was from 120 to 160 °C at 10 °C min^{-1}, 160 to 220 °C at 1·5 °C min^{-1} and 220 to 280 °C at 20 °C min^{-1}. Quantification was based on the internal standard and response factors previously determined for each monosaccharide.

Glycosyl-linkage analysis by gas chromatography–electron ionization (GC-EI) mass spectrometry. Hemicellulose-enriched extracts were modified to their partially methylated alditol acetate monosaccharides as described by the glycotechnology core resource of San Diego (http://glycotech.ucsd.edu/protocols/07_Comp_Analysis_by_Alditol_Rev2.pdf) and Plancot *et al*. (2014). Derivatives were then injected in a GC-EI mass spectrometer composed of a Hewlett-Packard 6890 series gas chromatograph coupled with an Autospec mass spectrometer of EBE geometry (Micromass, Manchester, UK) equipped with an Opus 3.1 data system. Chromatographic separations were obtained using a Zebron Z5-MSi (30 m, 0·25 mm id, 0·25 µm film thickness, Phenomenex) capillary column. Helium was the carrier gas and the flow-rate was 0·8 mL min^{-1}. The temperature programming started at 100 °C for 1 min, ramped to 160 °C at 10 °C min^{-1}, then to 220 °C at 2 °C min^{-1} and finally to 270 °C at 15 °C min^{-1} (maintained at 270 °C for 1 min). The temperature of the injector, the interface and the lines was 250 °C. Injections of 0·5 µL of samples were performed in splitless mode. The EI mass spectra were recorded using an electron energy of 70 eV, an acceleration voltage of 8 kV and a resolving power of 1000. The trap current was 200 µA and the magnet scan rate was 1 s per decade over a *m/z* range 600–38. The temperature of the ion source was 250 °C.

Statistical analysis

Comparison of the relative abundance of fucosylated XyG fragments between the different species was determined by one-way analysis of variance (ANOVA; $P < 0{\cdot}05$) with GraphPad InStat version 3.1 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

RESULTS

Immunolocalization of XyG in tomato pollen tubes and cell suspension

Using a well-described germination medium (Brewbaker and Kwack, 1963), the pollen germination rate of *S. lycopersicum* var. *cerasiforme* 'wva106' and var. *Saint-Pierre* was consistently >80 % and pollen tubes were actively growing, reaching over 700 µm in length after 6 h of growth (Fig. 2D, G). Germination rates of *S. pimpinellifolium* and *S. peruvianum* reached 71 and 32 %, respectively. The means of the pollen tube length were 630 ± 310 and 260 ± 180 µm, with some pollen tubes reaching over 1 mm (Fig. 2J, M). In the cell walls of the tomato cell suspension (Fig. 2B) and pollen tubes (Fig. 2E, H, K, N), a signal was detected by probing with the LM15 mAb. On the other hand, the CCRC-M1 mAb did not label the cell wall of the tomato cell suspension (Fig. 2C). In contrast, pollen tube cell walls of all the tomato species were labelled in the tip region and in the shank of the tubes (Fig. 2F, I, L, O), suggesting the presence of fucosylated XyG.

MALDI-TOF MS analysis of XyG from tomato leaves and cell suspension

XyG from *S. lycopersicum* var. *cerasiforme* leaves contained predominantly the arabinosylated motifs with the main fragment at *m/z* 1127 (XSGG/SXGG) (31 ± 1·5 %) (Fig. 3; Supplementary Data Fig, S1B, Table S1). The four main fragments (*m/z* 995, 1127, 1157 and 1289) accounted for > 80 % of the total ion count (Fig. 3; Fig. S1B, Table S1). No fucosylated but several galactosylated motifs such as LLGG (*m/z* 1319) were detected (Fig. 3; Fig. S1B, Table S1). No significant differences were found between the domesticated species [i.e. *S. lycopersicum* var. *Saint-Pierre* (Fig. S1A) and *S. lycopersicum* var. *cerasiforme* (Fig. S1B)] and the wild species [i.e. *S. pimpinellifolium* (Fig. S1C) and *S. peruvianum* (Fig. 3; Fig. S1D)]. In *S. lycopersicum* 'dombito' suspension-cultured cells, the same motifs were detected with an additional ion at *m/z* 1169 in relatively high proportion (21·2 ± 0·4 %) that may correspond to TGGG (Fig. S2, Table S1).

MALDI-TOF MS analysis of XyG from tomato pollen tubes

Four categories of XyG oligosaccharides (arabinosylated, xylosylated, galactosylated and fucosylated motifs) were found in tomato pollen tubes (Figs 4 and 5). The main motifs were XXGG/GSGG (*m/z* 995) (Figs 4 and 5; Supplementary Data Table S2). MALDI-TOF/TOF MS of the ion at *m/z* 1085 (corresponding to *m/z* 1365 after permethylation) confirmed the mixture of LSG and XXXG (Fig. S3A). Similarly, the ion at *m/z* 1289 (corresponding to *m/z* 1569 after permethylation) was

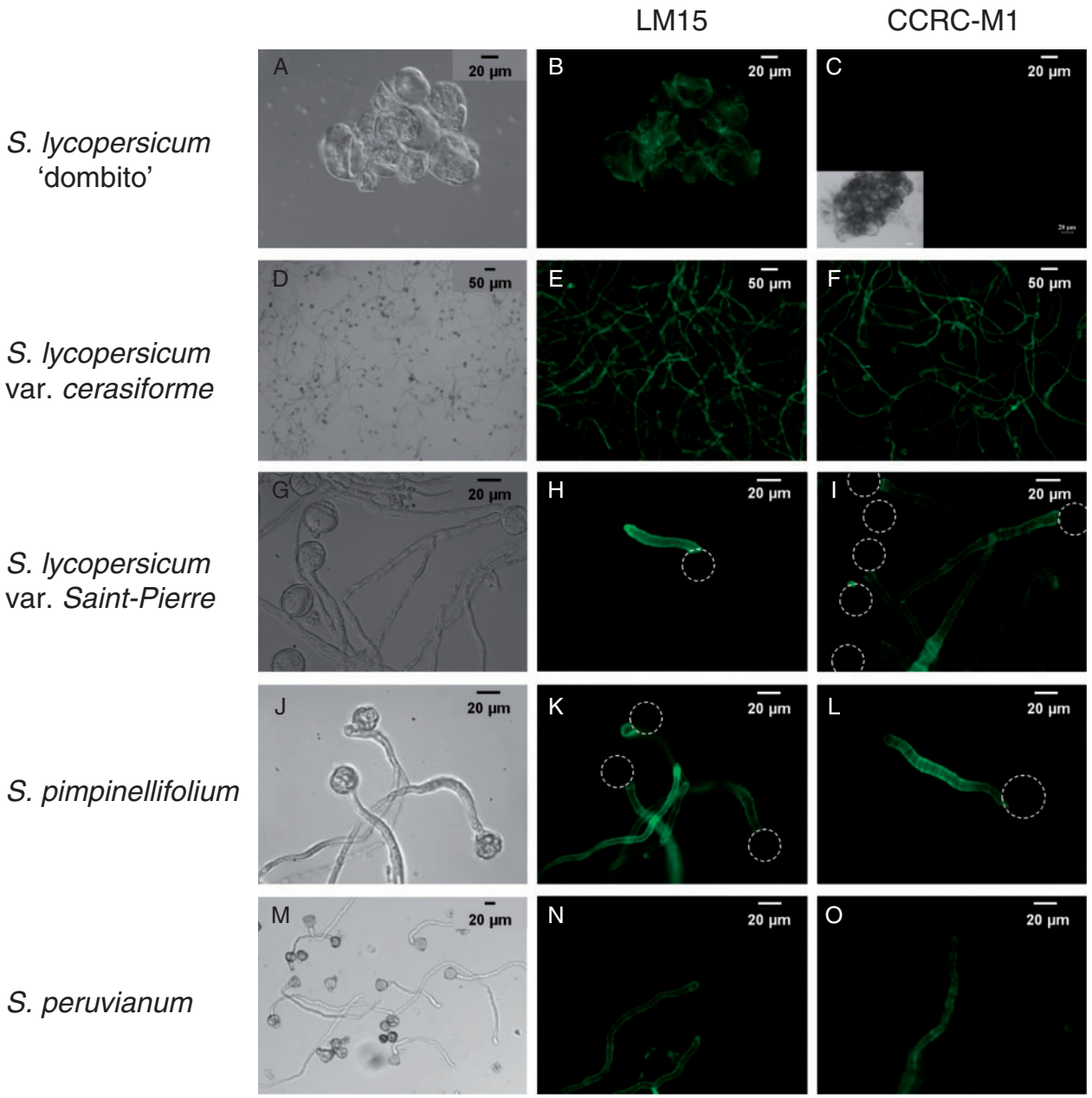

FIG. 2. Immunolocalization of xylosylated XyG with LM15 and fucosylated XyG with CCRC-M1 in tomato suspension-cultured cells and pollen tubes. Brightfield and fluorescent images of (A–C) *S. lycopersicum* 'dombito' cell suspension, (D–F) *S. lycopersicum* var. *cerasiforme* 'wva106', (G–I) *S. lycopersicum* var. *Saint-Pierre*, (J–L) *S. pimpinellifolium* and (M–O) *S. peruvianum* pollen tubes. Dotted circles indicate the location of the pollen grain.

composed of LSGG and XXLG structures in *S. peruvianum* pollen tubes (Fig. S3B). A new ion at *m*/*z* 1409 was observed in the hemicellulose-enriched fraction that may correspond to the bi-galactosylated motif (XLLG) (Fig. S4). Galactose residues were shown to be important for the interaction of XyG with cellulose microfibrils (Whitney *et al*., 2006), thus it is possible that galactosylated XyG was less accessible to the enzyme degradation in the cell wall residue. The main fucosylated fragments were XXFG (*m*/*z* 1435) and XLFG (*m*/*z* 1597) in all the investigated species (Figs 4 and 5; Table S2). An additional fucosylated motif XXFG (*m*/*z* 1393) was found in the XyG from wild species (Fig. 4C, D). The structure of the two oligosaccharides was confirmed by MALDI-TOF/TOF MS of the permethylated fragments (*m*/*z* 1743·9 and 1947·9) in *S. peruvianum* pollen tubes (Fig. 6). The sum of the relative abundance of fucosylated fragments ranged from $5{\cdot}3 \pm 1$ % in *S. lycopersicum* var. *Saint-Pierre* to $19{\cdot}4 \pm 2{\cdot}6$ % in *S. peruvianum* (Fig. 5; Table S2). *Solanum lycopersicum* var. *cerasiforme* and *S. pimpinellifolium* displayed intermediate abundance of $6{\cdot}5 \pm 1{\cdot}4$ and $12{\cdot}9 \pm 1{\cdot}7$ %, respectively (Fig. 5; Table S2). Statistical analyses indicated that the relative abundance of fucosylated XyG motifs was significantly higher in *S. peruvianum* and *S. pimpinellifolium* compared with *S. lycopersicum* sp. (Fig. 5; Table S2).

Monosaccharide composition and glycosyl-linkage analysis of the hemicellulose-enriched fractions from tomato pollen tubes

The GC-FID and GC-EI MS analyses of the hemicellulose-enriched fractions from *S. peruvianum* pollen tubes showed the typical glycosyl-linkages found in fucogalactoXyG (Table 1). These included the 4-Glc*p*, 4,6-Glc*p*, t-Xyl*p*, 2-Xyl*p*, t-Gal*p*, 2-Gal*p* and t-Fuc*p* residues. In *S. pimpinellifolium*, we could detect 4-Glc*p*, 4,6-Glc*p*, t-Xyl*p*, 2-Xyl*p* and t-Ara*f*, but not t-Fuc*p*, 2-Gal*p* and t-Gal*p* (Table 1), probably due to the lower level of XyG fucosylation. Fucosyl residues accounted for 0·9 mol % in *S. peruvianum* and 0·6 mol % in *S. pimpinellifolium*. In addition, other polymers were also detected, such as the prominent β-glucan found in pollen tube cell walls (i.e. callose) composed of 3-Glc*p* and the pectic RG-I polymer made of Rha, GalA, Gal (t-Gal*p*, 4- and 6-linked) and Ara (t-Ara*f* and 5-Ara*f*) residues (Table 1).

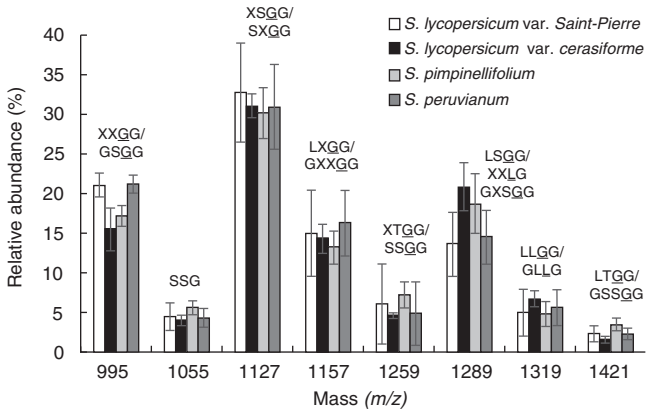

FIG. 3. Comparison of the oligosaccharide profile of the main *endo*-glucanase-sensitive XyG fragments between *S. lycopersicum* var. *Saint-Pierre*, *S. lycopersicum* var. *cerasiforme* 'wva106', *S. pimpinellifolium* and *S. peruvianum* leaves. The possible structures of XyG fragments are shown according to the nomenclature proposed by Fry *et al.* (1993) and shown in Fig. 1. The structures in bold were characterized in *S. lycopersicum* leaves and cell suspension by York *et al.* (1996), Jia *et al.* (2003) and Hoffman *et al.* (2005). Underlined structures represent *O*-acetylated side chains. The mean relative abundance is the average of three independent biological samples ($n = 3 \pm$ s.d.).

MALDI-TOF/MS, monosaccharide composition and glycosyl-linkage analyses of the XyG from N. tabacum *pollen tubes*

Spectra obtained from the cell wall residues of *N. tabacum* pollen tubes showed the dominance of fucosylated motifs (Supplementary Data Fig. S5A, Table S3) as reported in *N. alata* (Lampugnani *et al.*, 2013). The main fragments were the *O*-acetylated XXFG (*m/z* 1435) and XLFG (*m/z* 1639) (29·7 ± 1·9 and 23 ± 3 %, respectively) (Table S3). In the hemicellulose-enriched fraction, the same motifs were found, i.e. XXFG (*m/z* 1393) and XLFG (*m/z* 1555), but in their de-acetylated forms as expected with the KOH treatment (Fig. S5B, Table S3). Finally, all the glycosyl-linkages found in fucogalactoXyG were also detected, and the fucosyl residue accounted for 1·4 mol % (Table S4).

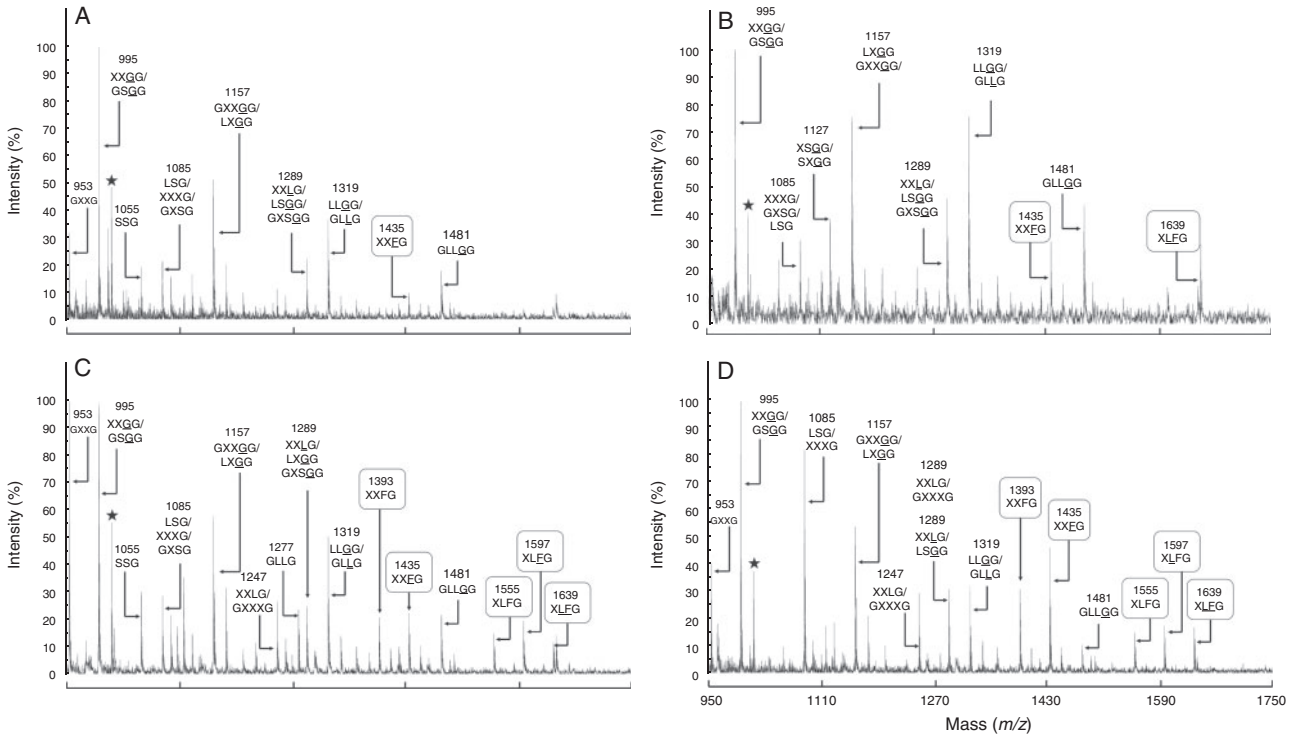

FIG. 4. MALDI-TOF MS of *endo*-glucanase-generated XyG fragments from the cell wall residues of (A) *S. lycopersicum* var. *Saint-Pierre*, (B) *S. lycopersicum* var. *cerasiforme* 'wva106', (C) *S. pimpinellifolium* and (D) *S. peruvianum* pollen tubes. The possible structures of XyG fragments are shown according to the nomenclature proposed by Fry *et al.* (1993) and shown in Fig. 1. In (D), the structures in bold were characterized in this study. Underlined structures represent *O*-acetylated side chains. The observed ions are mainly $[M + Na]^+$ adducts. Black stars represent unassigned fragments.

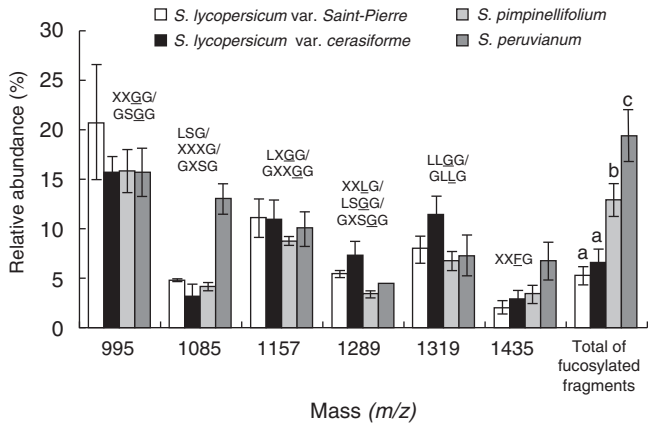

FIG. 5. Comparison of the oligosaccharide profile of the main *endo*-glucanase-sensitive XyG fragments between *S. lycopersicum* var. *Saint-Pierre*, *S. lycopersicum* var. *cerasiforme* 'wva106', *S. pimpinellifolium* and *S. peruvianum* pollen tubes. The possible structures of XyG fragments are shown according to the nomenclature proposed by Fry *et al.* (1993) and shown in Fig. 1. Underlined structures represent *O*-acetylated side chains. The mean relative abundance is the average of three independent biological samples ($n = 3 \pm$ s.d.). Different letters indicate significant differences ($P < 0{\cdot}05$).

DISCUSSION

In our previous work, we showed that the XyG in *A. thaliana* pollen tubes was highly fucosylated (Dardelle *et al.*, 2010) and hypothesized that this structural feature may have an important role in sexual plant reproduction. In the present study, using immunolocalization, OLIMP (Lerouxel *et al.*, 2002; Obel *et al.*, 2009; Günl *et al.*, 2011), GC and GC-MS analyses, we have investigated the structure of XyG of pollen tube cell walls of several *Solanaceae* species taking advantage of the fact that XyG of somatic cells/organs in this family was shown to lack fucosyl residues (Sims *et al.*, 1996; York *et al.*, 1996; Jia *et al.*, 2003, 2005; Hoffman *et al.*, 2005). The reason for and significance of this difference in the XyG structure between the lamiid clade and the other angiosperms are not known, but it has been suggested that it may be related to an adaptive and/or selective diversification (Del Bem and Vincentz, 2010).

XyG from the leaves of wild and domesticated tomato species is structurally similar

Analyses of XyG from the leaf and cell suspension of the domesticated species *S. lycopersicum* are consistent with previous works described in the literature. First, the cell wall of tomato cell suspension was labelled with the mAb LM15 that recognizes epitopes present in fucogalactoXyG and arabinoXyG. In contrast, no signal was observed with CCRC-M1, a mAb that binds to α-Fuc-(1→2)-β-Gal epitopes found in fucosylated XyG (Puhlmann *et al.*, 1994). Similar results were shown previously in tobacco suspension-cultured cells (Chevalier *et al.*, 2010) and organs including roots and leaves (Balestrini *et al.*, 1996; Brennan and Harris, 2011). Secondly, the relative abundance of the oligosaccharides analysed by MALDI-TOF MS from *S. lycopersicum* is in agreement with previous data obtained by chromatography and spectroscopy (York *et al.*, 1996; Jia *et al.*, 2003, 2005; Hoffman *et al.*, 2005). Altogether, the data indicate that XyG is not fucosylated, but it is mostly arabinosylated and can be galactosylated in the tomato species.

The data also indicate that the structures of XyG from the leaf of wild and domesticated tomato species are comparable. They suggest that the successive selections, primarily performed for improving the fruit traits, have not affected the XyG structure of the vegetative organs. In contrast, Zhang *et al.* (2012) reported that the culm cell walls of wild and domesticated rice species contain structural variations in the arabinoxylan structure with an increased level of xylan backbone in the domesticated species. The authors suggested that these structural changes of cell wall xylans may have helped the cultivated plants to increase their erect growth habit compared with the less upright growth habit of the wild species.

XyG of tomato pollen tubes is arabinosylated and fucosylated

Pollen tubes from all investigated species were significantly labelled with CCRC-M1, indicating the presence of epitopes associated with fucosylated XyG as shown in *A. thaliana* (Freshour *et al.*, 2003; Dardelle *et al.*, 2010; Chebli *et al.*, 2012) and *N. alata* (Lampugnani *et al.*, 2013) pollen tubes. The presence of fucosylated XyG in tomato pollen tubes was confirmed by MALDI-TOF MS, MALDI-TOF/TOF MS and GC-MS. The main XyG oligosaccharide (XXFG) found in tomato pollen tubes is also the principal motif detected in *A. thaliana* (Dardelle *et al.*, 2010), *N. tabacum* and *N. alata* (Lampugnani *et al.*, 2013) pollen tubes. The main difference compared with *A. thaliana* is the presence of the double *O*-acetylated XLFG fragment (*m*/*z* 1639), previously described in bilberry and peach fruits (Hilz *et al.*, 2007; Lahaye *et al.*, 2012). The relative abundance of fucosylated fragments was 52 % in *A. thaliana* (Dardelle *et al.*, 2010), 62 % in *N. tabacum* (Supplementary Data Table S3) and <20 % in tomato pollen tubes (Fig. 7). In addition, the typical glycosyl-linkages were characteristic of fucogalactoXyG. Despite slight differences in the relative abundance of the fucosylated motifs between *N. alata* (Lampugnani *et al.*, 2013) and *N. tabacum* pollen tubes, XyG is exclusively of the fucogalactoXyG type. In contrast, tomato pollen tube cell walls are composed of arabino- and fucogalactoXyGs. This uncommon mixture of arabinosylated and fucosylated XyG was reported once before in the leaf cell wall of *Nerium oleander* (lamiids, Gentianales), suggesting that this early divergent order may bridge an evolutionary gap between plants with Fuc–Gal and Ara substitution of the XyG in this clade (Hoffman *et al.*, 2005). Whether these two populations of XyG coexist in the cell wall or whether there is only one type of polymer that is branched with both Ara and Fuc residues is not known.

XyG of pollen tubes from wild tomatoes is more fucosylated than that from the domesticated species

The evolution of tomato species has been highly explored due to their agronomic and economic importance (Baudry *et al.*, 2001; Tanksley, 2004; Rodriguez *et al.*, 2009; Nakazato *et al.*, 2010; Bedinger *et al.*, 2011). To date, it has been

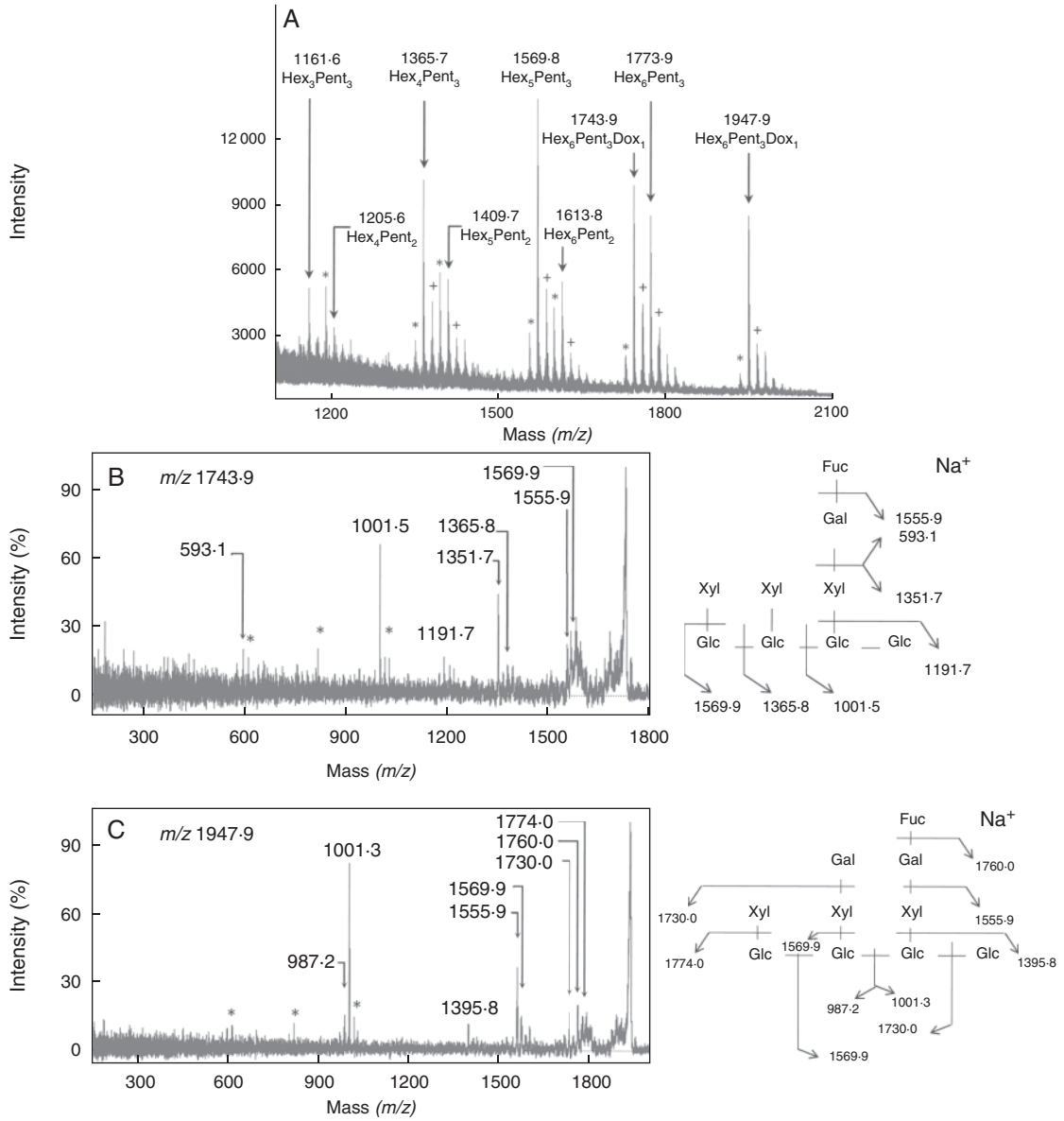

FIG. 6. Structural characterization of the fucosylated XyG oligosaccharides (XXFG and XLFG) from *S. peruvianum* pollen tubes. (A) MALDI-TOF MS spectrum of permethylated oligosaccharides. Ions are in the $[M+Na]^+$ adduct except for those annotated with + ($[M+K]^+$ adduct). Ions labelled by * correspond to underpermethylated ions. (B) MALDI-TOF/TOF MS of the precursor ion at $m/z = 1743{\cdot}9$ ($[M+Na]^+$ adduct) of the permethylated $Hex_5Pent_3Dox_1$ structure (XXFG) and the corresponding fragmentation pattern. (C) MALDI-TOF/TOF MS of the precursor ion at $m/z = 1947{\cdot}9$ ($[M+Na]^+$ adduct) of the permethylated $Hex_6Pent_3Dox_1$ structure (XLFG) and the corresponding fragmentation pattern. Hex, hexose; Pent, pentose; Dox, deoxyhexose; Glc, glucose; Gal, galactose; Xyl, xylose; Fuc, fucose; *, double fragmentation ion (in B and C).

assumed that *S. lycopersicum*, the domesticated species, shares a common ancestor with *S. pimpinellifolium* and *S. peruvianum*, both described as wild species. However, *S. pimpinellifolium* was proposed to be the closest wild ancestor of the domesticated *S. lycopersicum* var. *cerasiforme*, the cherry tomato (Fig. 7) (Nesbitt and Tanksley, 2002; Bai and Lindhout, 2007; Ranc *et al.*, 2008). It appears that there is a gradient in the abundance of fucosylated XyG motifs between the wild tomatoes (*S. peruvianum* and *S. pimpinellifolium*) and the domesticated *S. lycopersicum* (Fig. 7). The biological significance of the reduction in the abundance of fucosylated XyG in the cell wall of pollen tubes from the domesticated tomatoes is not known, but it may reflect an evolutionary trait and/or a selective pressure. Interestingly, differences in the structure and size of the stigma, style and transmitting tissue and in the composition of the exudates exist between the different species (Bedinger *et al.*, 2011). The different levels of XyG fucosylation may possibly contribute to adapt the pollen tube interaction and growth in the different pistil structures.

Tomato pollen tubes must have specific and functional XyG fucosyltransferases

The presence of fucosylated XyG in pollen tubes indicates that pollen must have specific and functional XyG

TABLE 1. *Monosaccharide composition and glycosyl-linkage analysis of* S. peruvianum *and* S. pimpinellifolium *hemicellulose-enriched pollen tube fractions*

	S. peruvianum	*S. pimpinellifolium*
Monosaccharide composition* (mol%)		
Glc	47·2	52·1
Xyl	15·8	13·2
Gal	14·1	13·1
Ara	13·9	13·8
Fuc	0·9	0·6
GalA	1·6	1·5
Rha	2·1	2
Man	4·4	3·8
Detected glycosyl-linkage†		
	3-Glc*p* (callose)	3-Glc*p* (callose)
	4-Glc*p* (XyG)	**4-Glc*p*** (XyG)
	4,6-Glc*p* (XyG)	**4,6-Glc*p*** (XyG)
	t-Xyl*p* (XyG)	**t-Xyl*p*** (XyG)
	2-Xyl*p* (XyG)	**2-Xyl*p*** (XyG)
	t-Gal*p* (XyG, RG-I, AGP)	ND
	2-Gal*p* (XyG)	ND
	4-Gal*p* (RG-I)	4-Gal*p* (RG-I)
	6-Gal*p* (RG-I, AGP)	6-Gal*p* (RG-I, AGP)
	t-Ara*f* (XyG, RG-I, AGP)	**t-Ara*f*** (XyG, RG-I, AGP)
	5-Ara*f* (RG-I)	5-Ara*f* (RG-I)
	t-Fuc*p* (XyG)	ND

*Determined by GC and expressed as mol%. Ara, arabinose; Fuc, fucose; Gal, galactose; Glc, glucose; GalA, galacturonic acid; Rha, rhamnose; Man, mannose; Xyl, xylose.

†Determined by GC-MS of partially methylated alditol acetates. t-Ara*f* denotes 1,4-di-*O*-acetyl-1-deuterio-2,3,5-tri-*O*-methyl-D-arabinitol, etc. Polymers that contain these glycosyl-linkages are indicated. XyG, xyloglucan; RG-I, rhamnogalacturonan-I; AGP, arabinogalactan proteins. In bold are the glycosyl-linkages found in XyG. ND, not detected.

fucosyltransferases. The transcriptome of *N. alata* pollen grains revealed the presence of most related genes involved in XyG biosynthesis such as the β-(1→4)-D-glucansynthase, α-(1→6)-D-xylosyltransferase and β-(1→2)-D-galactosyltransferase (Lampugnani *et al*., 2013). However, the RNA-Seq analysis of Lampugnani *et al*. (2013) failed to detect any RNA contigs that were predicted to encode α-(1→2)-L-fucosyltransferases, while they showed that the cell wall of *N. alata* pollen tubes contains most exclusively fucosylated XyG. In the *S. lycopersicum* 'Heinz' genome (Tomato Genome Consortium, 2012), three genes (*Solyc07g047920.1*, *Solyc06g061210.2* and *Solyc03g115830.1*) are predicted to encode XyG galactoside α-2-fucosyltransferases, and *Solyc07g047920.1* is expressed in flowers (SOL genomics network, Mueller *et al*., 2005; http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/search.cgi?ID=D004). This suggests that this gene may be a good candidate for the transfer of GDP-fucose onto the galactosyl residues in tomato pollen tube cell walls, but functional studies are required to confirm this hypothesis.

XyG is important in tip-polarized cells and can have unusual composition

Studies of mutants impaired in XyG xylosyltransferases (*xxt1*/*xxt2*), in *A. thaliana* that lacks detectable XyG (Cavalier *et al*., 2008) and in *Oryza sativa*, a grass species with low XyG content (Wang *et al*., 2014), showed no obvious growth defect

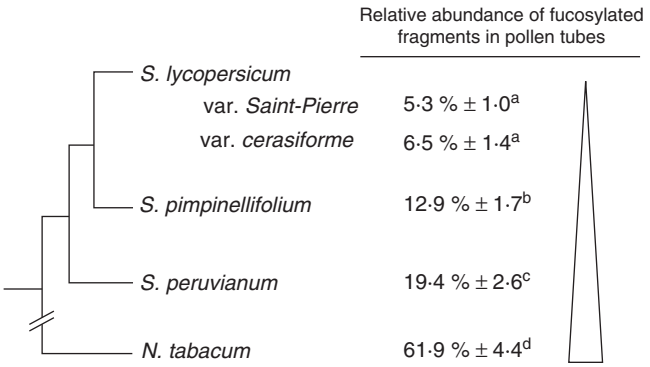

FIG. 7. Comparison of the relative abundance of fucosylated XyG oligosaccharides found in pollen tubes of *N. tabacum* and the tomato species. The schematic tree was based on the work of Rodriguez *et al*. (2009), Nakazato *et al*. (2010) and Bedinger *et al*. (2011). Different letters indicate significant differences ($P < 0·05$).

of the mutant plants except abnormal growth of root hairs, which are also tip-polarized cells. Interestingly, the pollen tube of the *xxt1*/*xxt2* double mutant was not labelled with the anti-XyG mAb, CCRC-M1 and LM15, and displayed severe phenotype and growth defects along with a slight decrease of the stiffness of the pollen tube (Draeger, 2012). These studies and others (Zabotina *et al*., 2008) clearly indicate that XyG, even in a low amount, is of prime importance for tip-growing cells and particularly pollen tubes.

The unexpected XyG structure in pollen tube cell walls found in *N. alata* (Lampugnani *et al*., 2013), *N. tabacum* and *Solanaceae* species indicates that tip-polarized cells have specific structural cell wall features. Peña *et al*. (2012) have also found an unusual type of XyG containing galacturonic acid residues in *A. thaliana* root hairs. Similarly, in the tip-growing moss *Physcomitrella patens*, XyG also contains galacturonic acid (Peña *et al*., 2008). This sugar residue has not been detected in *A. thaliana* (Dardelle *et al*., 2010) and *N. alata* (Lampugnani *et al*., 2013) pollen tubes nor in the *Solanaceae* species investigated in the present study but it reinforces the idea that tip-polarized cells have specific cell wall structural properties adapted to their fast intrusive growth, i.e. between the soil particles for the root hair or between the transmitting tract cells for the pollen tube.

Role of XyG in the mechanical properties of the cell wall

The impact of the structural differences (Ara-Xyl vs. Fuc-Gal-Xyl side chains) on the architecture of the cell wall, on the interaction between the polymers in the cell wall network and on the mechanical properties of the cell wall is not clear. However, by expressing tomato XyG arabinosyltransferases in the *A. thaliana* double mutant *mur3.1*/*xlt2* that lacks galactosylated XyG, Schultink *et al*. (2013) showed that the transgenic plants were able to produce the arabinosylated form of XyG, a structure not found in wild-type plants. This resulted in the partial restoration of the growth phenotype and mechanical properties of the cell wall in the double mutant, suggesting that XyG galactosylation and arabinosylation may fulfil similar functions (Schultink *et al*., 2013).

The role of fucosyl residues in XyG is still not clear. The fact that most eudicots have fucosylated XyG suggested that this structural feature may give a possible selective advantage for the plants (Hoffman *et al*., 2005). However, mutation in *AtFUT1* encoding a XyG fucosyltransferase or in *MUR3* encoding a XyG galactosyltransferase induced an absence of a fucosyl residue or a Fuc–Gal motif on the XyG, but the mutant plants showed normal growth habit and wall strength (Vanzin *et al*., 2002; Madson *et al*., 2003; Perrin *et al*., 2003), suggesting that the plants may compensate by modifying other cell wall structural features. In addition, it has been reported that fucosyl residues were not of prime importance for the interaction of XyG with cellulose microfibrils, in contrast to the galactosyl residues (Peña *et al*., 2004; Whitney *et al*., 2006). Park and Cosgrove (2012*a*, *b*) have shown by analysing the cell wall biochemical properties in the *xxt1*/*xxt2* mutant and cucumber hypocotyl treated with specific hydrolases that only a minor XyG component was important for the interaction with cellulose microfibrils and the cell wall mechanics. Thus, the presence of fucosyl residues and *O*-acetyl groups on the XyG may then modulate its interaction with cellulose microfibrils in the pollen tube cell wall, allowing the tube growth and elongation.

To conclude, the biological significance of the differences observed in the XyG structure between the male gametophyte and the sporophyte is unclear, but the finding suggests that fucosylation of XyG, even in low abundance, is an important feature allowing mechanical stability of the cell wall during the tip-polarized growth of the pollen tube and/or its interaction with the pistil. Finally, the completion of the detailed annotation of *S. pimpinellifolium* and other genomes and transcriptomic data should provide more insight into the evolution of the *Solanaceae* and in the discovery of new genes (Ranjan *et al*., 2012) that may allow the functional characterization of XyG fucosyltransferases specific to tomato pollen.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: MALDI-TOF MS of *endo*-glucanase-generated XyG fragments from *S. lycopersicum* var. *Saint-Pierre*, *S. lycopersicum* var. *cerasiforme*, *S. pimpinellifolium* and *S. peruvianum* leaves. Figure S2: MALDI-TOF MS of *endo*-glucanase-generated XyG fragments from *S. lycopersicum* 'dombito' cell suspension. Figure S3: structural characterization of LSG and LSGG/XXLG oligosaccharides from *S. peruvianum* pollen tubes by MALDI-TOF/TOF MS. Figure S4: MALDI-TOF MS of *endo*-glucanase-generated XyG fragments from the hemicellulose-enriched extract of *S. lycopersicum* var. *cerasiforme* pollen tubes. Figure S5: MALDI-TOF MS of *endo*-glucanase-generated XyG fragments from the cell wall extract and the hemicellulose-enriched extract of *N. tabacum* pollen tubes. Table S1: relative abundance of XyG oligosaccharides released after *endo*-glucanase digestion of the cell wall of *S. lycopersicum* 'dombito' cell suspension and *S. lycopersicum* var. *cerasiforme* leaves. Table S2: relative abundance of XyG oligosaccharides released after *endo*-glucanase digestion of the cell wall residue and the hemicellulose-enriched extracts from tomato pollen tubes. Table S3: relative abundance of XyG oligosaccharides released after *endo*-glucanase digestion of the cell wall residue and the hemicellulose-enriched extract from *N. tabacum* pollen tubes. Table S4: monosaccharide composition and glycosyl-linkage analysis of *N. tabacum* hemicellulose-enriched pollen tube extract.

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