Inhibition of fucosylation of cell wall components by 2-fluoro 2-deoxy-L-fucose induces defects in root cell elongation

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SUMMARY

Screening of commercially available fluoro monosaccharides as putative growth inhibitors in Arabidopsis thaliana revealed that 2-fluoro 2-L-fucose (2F-Fuc) reduces root growth at micromolar concentrations. The inability of 2F-Fuc to affect an Atfkgp mutant that is defective in the fucose salvage pathway indicates that 2F-Fuc must be converted to its cognate GDP nucleotide sugar in order to inhibit root growth. Chemical analysis of cell wall polysaccharides and glycoproteins demonstrated that fucosylation of xyloglucans and of N-linked glycans is fully inhibited by 10 μM 2F-Fuc in Arabidopsis seedling roots, but genetic evidence indicates that these alterations are not responsible for the inhibition of root development by 2F-Fuc. Inhibition of fucosylation of cell wall polysaccharides also affected pectic rhamnogalacturonan-II (RG-II). At low concentrations, 2F-Fuc induced a decrease in RG-II dimerization. Both RG-II dimerization and root growth were partially restored in 2F-Fuc-treated seedlings by addition of boric acid, suggesting that the growth phenotype caused by 2F-Fuc was due to a deficiency of RG-II dimerization. Closer investigation of the 2F-Fuc-induced growth phenotype demonstrated that cell division is not affected by 2F-Fuc treatments. In contrast, the inhibitor suppressed elongation of root cells and promoted the emergence of adventitious roots. This study further emphasizes the importance of RG-II in cell elongation and the utility of glycosyltransferase inhibitors as new tools for studying the functions of cell wall polysaccharides in plant development. Moreover, supplementation experiments with borate suggest that the function of boron in plants might not be restricted to RG-II cross-linking, but that it might also be a signal molecule in the cell wall integrity-sensing mechanism.

Keywords: Arabidopsis thaliana, 2-fluoro 2-deoxy-L-fucose, cell elongation, rhamnogalacturonan-II, boron, root.

INTRODUCTION

Analyses of a wide range of plant species have revealed that all plant cell walls contain three classes of structural polysaccharides: cellulose, hemicelluloses and pectins, which together form a complex extracellular matrix (Albersheim et al., 2010). The synthesis of cell wall polysaccharides is performed by membrane-localized polysaccharide synthetases and glycosyltransferases (GTs). Glycosyltransferases are enzymes that allow the transfer of single sugars from nucleotide sugar donors onto specific acceptors. Reverse genetic experiments targeting GT sequences have provided numerous data concerning the function of cell wall polysaccharides in plant development (Bouton et al., 2002; Vanzin et al., 2002). However, for some cell wall polymers such as rhamnogalacturonan II (RG-II), mutations
affecting GTs or enzymes involved in the biosynthesis of RG-II-specific monosaccharides are lethal or induce very strong developmental phenotypes (O'Neill et al., 2001; Ahn et al., 2006; Delmas et al., 2008; Deng et al., 2010; Dumont et al., 2014). The lack of information available from these knock-out phenotypes can be partially addressed by studying RNA interference (RNAi) lines (Voxeure et al., 2011). Rhamnogalacturonan II is a low-molecular-weight complex polysaccharide that is composed of at least 12 types of residues that are linked by at least 22 distinct glycosidic bonds (Bar-Peled et al., 2012). Rhamnogalacturonan II contains four side chains linked to a homogalacturonan backbone made of seven to nine α-1,4-galacturonic acid residues (Figure S1 in supporting information). Despite its complex structure, RG-II is evolutionarily conserved in the plant kingdom (Matsunaga et al., 2004). It is present in the primary cell walls of all higher plants, predominantly in the form of a dimer that is cross-linked by a borate di-ester bond made of seven to nine α-1,4-galacturonic acid residues (Figure S1) (Kobayashi et al., 1996; O'Neill et al., 1996). The lethality of knock-out lines affecting RG-II biosynthesis makes the study of its biosynthesis and function challenging. To date, only one α-1,3-xylosyltransferase, named RGXT, has been fully characterized. This GT is involved in the transfer of a xylose onto a fucose residue (Egelund et al., 2006; Delmas et al., 2008; Deng et al., 2010; Dumont et al., 2014). Nevertheless, despite numerous efforts to determine why plants need such a complex molecule to ensure efficient cell wall formation and cell elongation, the precise function of RG-II remains unclear.

The use of modified monosaccharides, such as deoxy or fluorinated sugars, has been reported as a powerful pharmacological approach for studying carbohydrate metabolism and physiology in prokaryotic and eukaryotic organisms (Som et al., 1980; Edmonds and Peddie, 2006). After uptake from the culture medium, the sugar analogue can either compete with endogenous metabolites or inhibit enzymes. The analogue 2-deoxy-D-glucose inhibits protein glycosylation (Schwarz and Datema, 1980) and, in plants, inhibits callose production and delays gravitropism (Jaffe and Leopold, 1984). In Prototexa zopfii, a unicellular alga, deoxy-glucose also inhibits β-1,4-glucan formation (Datema et al., 1983). A recent study reported the effects of 2β-deoxy-Kdo, a deoxy analogue of Kdo, which is found in RG-II (Figure S1) (Smyth et al., 2013). This deoxy sugar was demonstrated to inhibit cytosolic CMP-Kdo synthase and, as a consequence, to affect RG-II biosynthesis and cause severe root growth phenotypes (Smyth et al., 2013).

Replacement of hydroxy groups by fluorine atoms makes deoxy fluoro sugars useful probes for the study of enzyme mechanisms (Burkart et al., 2000). The fluorine atom has a small atomic radius with high electronegativity and ionization potential. After conversion into nucleotide sugars, fluorinated analogues can form stable complexes with enzyme-binding sites. These molecules have been used in several studies as probes to elucidate the mechanisms and functions of GT (Burkart et al., 2000; Brown et al., 2012). Recently, Rillahan et al. (2012) investigated two fucosyltransferase inhibitors [2-fluoro 2-deoxy-L-fucose (2F-Fuc) and 6-fluoro-L-fucose (6F-Fuc)] and one sialyltransferase inhibitor [3-fluoro 3-deoxy-N-acetyl-neuraminic acid (3F-Neu5Ac)], which hamper the fucosylation and sialylation of protein N-linked glycans, respectively, in mammalian cells. The 2F-Fuc inhibitor has been demonstrated to inhibit both fucosyltransferase enzymes and the de novo cytosolic synthesis of GDP-Fuc in mammalian cells (Okeley et al., 2013). In plants, the use of fluorinated sugars has received relatively little attention to date. 2[18F] fluoro 2-deoxy-D-glucose has been applied to study both the real-time translocation of photosynthetic products in living plants (Hattori et al., 2008) and plant defence (Ferrier et al., 2012), while UDP 2-fluoro 2-deoxy-D-glucuronic acid has also been used to study the enzyme mechanism of plant UDP-α-D-apiose/UDP-α-D-xyllose synthase (Choi et al., 2011).

In this work, we report the effects of commercially available fluoro monosaccharides on seedling growth in Arabidopsis. Among the compounds tested, 2F-Fuc was the most effective growth inhibitor and arrested root elongation at micromolar concentrations. Biochemical analyses of cell wall polysaccharides showed that the fucosylation of xyloglucan and of N-glycosylated proteins was suppressed by 2F-Fuc. Moreover, defects in RG-II biosynthesis were found to be responsible for the growth phenotype observed in plants treated with 2F-Fuc.

RESULTS

Screening of fluoro sugar analogues for effects on cell elongation

Arabidopsis seeds were sown on solid growth medium containing commercially available fluorinated sugar analogues. Seedling root and shoot elongation were measured in the presence of fluoro deoxy-D-glucose isomers differing in the position of the fluorene atom (2F-Glc, 3F-Glc and 4F-Glc), 2-fluoro 2-deoxy-L-fucose (2F-Fuc) and 3-fluoro 3-deoxy-N-acetyl α-neuraminic acid (3F-Neu5Ac) at concentrations ranging from 2.5 to 200 μM (Figure S2). Considering the structural similarity between Neu5Ac and Kdo, 3F-Neu5Ac was tested as a possible Kdo transferase inhibitor. Peracetylated and non-acetylated fluoro sugars were tested, but effects were only observed with peracetylated sugars, indicating that the peracetylated molecules are likely to be entering the cell by passive diffusion through the plasma membrane. Four-day-old Arabidopsis
seedlings exhibited various susceptibilities to these fluorinated sugars, with growth defects being observed primarily in roots rather than shoots (Figure S2). Treatment with 3F-Neu5Ac did not induce any root growth inhibition even at a high concentration (200 µM). Growth inhibition was observed with fluoro glucose analogues in the 25–100 µM range, with the inhibitory effect depending on the position of the fluorine atom: the most effective glucose analogue was 2F-Glc, which inhibited root growth by 75% at a concentration of 25 µM (Figure S2). The strongest growth effect of all of the inhibitors was observed with 2F-Fuc, since the lowest concentration of this compound tested (2.5 µM) was sufficient to reduce root elongation by 70%. Moreover, this effect was observed in a dose-dependent manner (Figure 1a) and was not suppressed by supplementation with an excess amount of fucose (10 mM) (Figure S3). Considering the strong inhibition of root growth observed with 2F-Fuc treatment, we decided to focus on the biochemical and physiological effects induced by this sugar analogue.

2F-Fuc is a potent inhibitor of the fucosylation of cell wall polysaccharides

To investigate the effects of 2F-Fuc on the fucosylation of cell wall polysaccharides or glycoconjugates, we first carried out a click-mediated labelling experiment. Anderson et al. (2012) have recently shown that an alkynylated L-fucose analogue (Fuc-Al) is a useful probe for imaging fucose-containing cell wall polysaccharides in Arabidopsis. Figure 1(b)–(f) shows that the cell walls of 4-day-old seedlings were efficiently labelled after incorporation of Fuc-Al and coupling to an Alexa Fluor 488 azide probe via copper-catalysed azide–alkyne cycloaddition. In contrast, labelling was strongly reduced in seedlings treated with 25 µM 2F-Fuc, indicating that this fluoro analogue inhibits the transfer of Fuc-Al to cell wall polysaccharides (Figure 1e,f).

The fucose content in shoot and root cell walls of 10-day-old Arabidopsis seedlings treated with 2.5–25 µM 2F-Fuc were measured by gas chromatography (Figure 1g). In comparison with control seedlings (DMSO), the fucose content in root cell walls was reduced by about 50% in seedlings treated with 2.5 µM 2F-Fuc whereas only traces of fucose were detected in the root cell walls of seedlings treated with 10 or 25 µM 2F-Fuc. Beyond its effect on fucose content, 2F-Fuc treatment did not strongly affect the overall sugar composition of root cell walls in seedlings (Figure 2). For instance, the galacturonic acid (GalA) content in roots was about 25% of total cell wall sugars regardless of the 2F-Fuc concentration. Some limited [2F-Fuc]-dependent changes in sugar composition were observed, such as decreases in relative xylose content and increases in rela-

Figure 1. Effect of 2-fluoro 2-L-fucose (2F-Fuc) on plant growth and on fucose incorporation into root cell walls. (a) Four-day-old Arabidopsis seedlings grown on medium supplemented with peracetylated 2F-Fuc or DMSO (control). Scale bar = 0.3 cm. (b)–(e) Click-mediated labelling of Arabidopsis seedling cell walls using the alkynylated fucose analogue Fuc-Al. Four-day-old seedlings grown on MS were incubated in liquid MS medium ± Fuc-Al (b, c) for 16 h and 4-day-old seedlings grown on MS supplemented with 25 µM 2F-Fuc were incubated in liquid MS medium ± Fuc-Al + 25 µM 2F-Fuc (d, e) for 16 h and labelled. Scale bars = 100 µm. (f) Quantification of the root-tip fluorescence in (b)–(e). Data are the mean ± SEM (n > 6) from three experiments. Mann-Whitney test, ***P < 0.001. (g) Fucose contents (mol%) determined by gas phase chromatography analysis of cell walls isolated from 10-day-old Arabidopsis seedlings grown with 2.5–25 µM 2F-Fuc or DMSO (control). n > 300 seedlings per replicate. Data are the mean ± SEM of three biological replicates.
tive galactose content (Figure 2). In contrast to roots, the effect of 2F-Fuc on shoots was less severe. Twenty-five per cent of the fucose content in shoot cell walls was still detected in 25 μM 2F-Fuc-treated seedlings in comparison with the negative control (DMSO) (Figure 1g). Taken together, these data suggest that 2F-Fuc acts as a potent inhibitor of the fucosylation of cell wall polysaccharides at concentrations as low as 10 μM in root tissues.

To begin to probe the mechanism of 2F-Fuc inhibition, the effects of 2F-Fuc on a mutant defective in the fucose salvage pathway (Kotake et al., 2008) were investigated. This mutant, Atfkgp, is impaired in cytosolic L-fucokinase and GDP-L-fucose pyrophosphorylase activities that convert exogenously supplied L-fucose to its GDP-activated form (Kotake et al., 2008). Atfkgp seedlings were insensitive to 2F-Fuc (Figure S4), strongly suggesting that 2F-Fuc must be activated into its GDP nucleotide sugar in order to exert its inhibitory effects.

Decreased fucosylation of xyloglucan and glycoproteins in Arabidopsis seedlings after 2F-Fuc treatment is not responsible for defects in root growth

Plant cell walls possess several fucose-containing polysaccharides and glycoconjugates such as xyloglucan (Perrin et al., 2003), N-glycosylated proteins (Rayon et al., 1998), pectic rhamnogalacturonan-I (RG-I) (Nakamura et al., 2001) and RG-II (Glushka et al., 2003), as well as arabinogalactan proteins (AGPs) (Tryfona et al., 2012). To investigate whether the fucosylation of N-glycosylated proteins is affected by treatment with 2F-Fuc, we extracted proteins from Arabidopsis root seedlings treated with 25 μM 2F-Fuc. Protein N-linked glycan fucosylation was detected using a specific antibody directed against the core α-1,3-fucose epitope (Faye et al., 1993). A shown in Figure 3(a), glycoproteins were detected at much lower amounts in 2F-Fuc treated-roots compared with control roots. The specificity of the primary antibody was confirmed as glycoproteins were not detected in fucTA/fucTB Arabidopsis double mutant impaired in N-linked glycan fucosylation (Strasser et al., 2004). Moreover, the effect of 2F-Fuc on the growth of the cgl (complex glycan-deficient) mutant was investigated. This Arabidopsis mutant is impaired in its Golgi N-acetyl glucosaminyltransferase I, preventing the synthesis of fucosylated complex-type N-linked glycans, but does not exhibit obvious growth phenotypes when grown in standard conditions (von Schaewen et al., 1993). The root growth of cgl seedlings was affected by 2.5 μM 2F-Fuc treatment as observed for Col-0 seedlings (Figure 3b), indicating that the decrease in N-glycoprotein fucosylation induced by 2F-Fuc treatment is not responsible for the observed inhibition of root growth.

Arabidopsis xyloglucan consists of a β-1,4-glucan backbone substituted with α-1,6-xylosyl residues (side chain X; Fry et al., 1993). Terminal Gal (side chain L; Fry et al., 1993) or Fucα-1,2-Gal disaccharides (side chain F; Fry et al., 1993) can also be β-1,2-linked to xylosyl residues. Xyloglucan fingerprints from the cell walls of shoots and roots of

![Figure 2](image-url)  
Figure 2. Effect of 2-fluoro 2-L-fucose (2F-Fuc) on the sugar composition of root cell walls. Monosaccharide composition of root cell walls of control (0.25% DMSO) and seedlings treated with 2.5–25 μM 2F-Fuc. Data are mean ± SEM of three biological replicates. Unpaired t-test: ns, not significant; *P < 0.05, **P < 0.01; ***P < 0.001.

![Figure 3](image-url)  
Figure 3. Effect of 2-fluoro 2-L-fucose (2F-Fuc) on the fucosylation of N-glycoproteins. (a) Ponceau S staining (left) and Western blot (right) using antibody directed against the core α-1,3-fucose N-glycans. fucTA/fucTB, double fucTA/fucTB mutant impaired in N-linked glycan fucosylation. (b) Shoot and root lengths of 4-day-old cgl seedlings grown in the presence of 0.025% DMSO (control) or 2.5 μM 2F-Fuc (n > 20 seedlings). Mann-Whitney test.

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Arabidopsis seedlings treated with DMSO (control) or 2.5-25 μM 2F-Fuc were investigated according to Lerouxel et al. (2002). Cell walls were isolated and subjected to endoglucanase digestion, and the resulting fragments were analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry MALDI-TOF MS (Figure 4a). Comparison of fingerprints obtained from seedlings treated with 2.5-25 μM 2F-Fuc with the fingerprint of the negative control (DMSO) showed a dose-dependent decrease in the levels of fucosylated fragments (XXFG and XLFG) together with an increase in the levels of non-fucosylated fragments. No novel fragments were detected by comparison with control MS profiles. Quantification of xyloglucan fragments detected in the MALDI-TOF MS experiments indicated a 60% decrease in xyloglucan fucosylation for roots treated with 2.5 μM 2F-Fuc and only trace amounts of fucosylated fragments at higher 2F-Fuc concentrations (Figure 4b). In agreement with total fucose contents detected in shoot cell walls (Figure 1g), the fucosylation of xyloglucan in Arabidopsis shoots was only slightly affected by treatments with 2.5 and 10 μM 2F-Fuc. Only treatment with 25 μM 2F-Fuc was sufficient to induce a detectable decrease of xyloglucan fucosylation in cell walls derived from shoot tissues (Figure 4b).

AtFUT1 is a Golgi-localized fucosyltransferase that can add α-1,2-Fuc residues onto xyloglucan (Perrin et al., 1999). The effect of 2F-Fuc on Arabidopsis AtFUT1 was investigated in vitro using radiolabelled GDP-Fuc and tamarind xyloglucanase as an acceptor. The transferase capacity of recombinant AtFUT1 produced in insect cells was monitored in the presence of 1 mM 2F-Fuc. A weak inhibitory activity was observed (Figure S5), again suggesting that 2F-Fuc might need to be converted into a GDP nucleotide sugar to effectively inhibit the activity of AtFUT1 (Burkart et al., 2000; Okeley et al., 2013).

Next, Arabidopsis mur2 (murus2) and xxt1 xxt2 (xyloglucan xylosyltransferase) mutants, as well as tobacco seedlings, were grown in presence of 2.5 μM 2F-Fuc. Arabidopsis mur2 lacks functional AtFUT1 and its cell walls contain less than 2% of the wild-type amount of fucosylated xyloglucan without affecting root growth (Vanzin et al., 2002). The xxt1 xxt2 double mutant is impaired in xyloglucan xylosyltransferase activity and lacks detectable xyloglucan (Cavalier et al., 2008). Nicotiana benthamiana, like all Solanaceae, accumulates non-fucosylated xyloglucan in its cell walls (York et al., 1996; Hoffman et al., 2005), with the exception of pollen (Lampugnani et al., 2013; Dardelle et al., 2015). In both Arabidopsis mutants and tobacco seedlings, root elongation was strongly affected by 2F-Fuc treatment (Figure 4c). Again, this suggests that the inhibition of root growth by 2F-Fuc is not related to a deficiency in xyloglucan fucosylation. The same conclusion can be drawn for AGPs, since the root growth of Arabidopsis fut4 fut6 double mutants, which lack normal AGP fucosylation (Liang et al., 2013), was also inhibited by 2F-Fuc treatment (Figure 4c).

Arabidopsis seedlings treated with 2F-Fuc are impaired in RG-II biosynthesis

Boron is responsible for the RG-II dimerization that is required for plant growth (O’Neill et al., 2001). Thus, the
effect of boron on 2F-Fuc-treated seedlings was investigated. Supplementation of culture medium containing 2.5 or 25 μM 2F-Fuc with 0.75 mM boric acid was able to partially restore root growth in 4-day-old Arabidopsis seedlings, suggesting that the growth inhibition caused by 2F-Fuc is likely attributable to RG-II defects (Figure 5a).

To directly assess RG-II dimerization in 2F-Fuc-treated seedlings, we used the polyacrylamide gel electrophoresis method described by Chormova et al. (2014) for the separation of monomeric RG-II (mRG-II) and dimeric RG-II (dRG-II). Cell walls were prepared from roots of 10-day-old seedlings grown with 0.25% DMSO or 2F-Fuc at concentrations of 2.5, 10 and 25 μM. Then RG-II was solubilized by treatment of cell walls with endo-polylacturonase and submitted to polyacrylamide gel electrophoresis (Figure 5b). The relative amount of cell wall sugars for each RG-II-enriched fraction was assessed by gas chromatography and identical cell wall quantities were loaded on the gel. Lemon mRG-II and dRG-II were used as standards. As reported previously (O’Neill et al., 2004), RG-II extracted from untreated Arabidopsis root seedlings existed mainly in a dimeric form. Treatment of seedlings with 2.5 μM 2F-Fuc induced a major decrease in dRG-II together with an increase in mRG-II and the appearance of a RG-II fragment. At 10 and 25 μM, 2F-Fuc greatly diminished in muro accumulation of RG-II (Figure 5b). Although the small RG-II fragment was still detectable by PAGE after treatment with 10 or 25 μM 2F-Fuc, these data suggest that inhibition of fucosylation results in defects in the biosynthesis of mRG-II and dRG-II. In the presence of 0.75 mM boric acid, PAGE of RG-II fractions isolated from seedlings treated with 2.5 μM 2F-Fuc showed an increase in the relative amount of dRG-II, suggesting that boron promoted RG-II dimerization even in the absence of normal fucosylation (Figure 5c). However, the abnormal small RG-II fragment was still detected under these conditions, indicating that its processing or dimerization was not restored in the presence of boric acid. In seedlings treated with high concentration of 2F-Fuc (25 μM), borate had no major effect on the PAGE RG-II profile, with the small RG-II fragment being the main band detected (Figure 5c).

Incubation of Arabidopsis light-grown seedlings with 8-azido 8-deoxy-Kdo (Kdo-N3) followed by click-mediated coupling to an alkyne-containing fluorescent probe through a copper-catalysed azide–alkyne cycloaddition provides an efficient method for imaging RG-II in cell walls without the need for RG-II-specific antibodies (Patrice Lerouge et al. Unpublished data). Figure 6(a) shows that incubation of seedlings with 50 μM Kdo-N3 induced a robust labelling of their cell walls by Alexa 488 alkyne. In contrast, this labelling was suppressed by treatment with 25 μM 2F-Fuc (Figure 6b,c), further confirming that high concentrations of 2F-Fuc inhibit biosynthesis of RG-II.

**Figure 5.** Effect of 2-fluoro 2-L-fucose (2F-Fuc) on rhamnogalacturonan-II (RG-II) dimerization.
(a) Root length of 4-day-old Arabidopsis seedlings treated with 2.5 or 25 μM 2F-Fuc, 0.75 mM boric acid or both. DMSO, negative control. Data are mean ± SEM (n > 18 seedlings). Mann–Whitney test.
(b) Polyacrylamide gel electrophoresis: RG-II was extracted from 10-day-old Arabidopsis roots grown on medium supplemented with 2.5, 10 or 25 μM 2F-Fuc. RG-II extracted from lemon was used as control (i), additional 16-h incubation with 0.1 M HCl treatment leads to a partial monomerization of RG-II (ii). RG-I, Rhamnogalacturonan I; mRG-II, monomeric RG-II; dRG-II, dimeric RG-II.
(c) The same experiment as in (b) with 2.5 and 25 μM 2F-Fuc-treated seedlings grown in a medium complemented with 0.75 mM boric acid.

**Analysis of root architecture in 2F-Fuc-treated Arabidopsis seedlings**

The Arabidopsis root is organized into an apical meristem where cells divide, an elongation zone where cells expand...
and a differentiation zone where cells acquire their determined sizes, shapes and functions. As reported in Figure 1(a), Arabidopsis seedlings treated with 2F-Fuc exhibit a strong root growth defect. Observation of the root tips of seedlings treated with 2.5 μM 2F-Fuc revealed that the entire elongation zone was strongly diminished, with differentiated cells closer to the root tip than in control seedlings (Figures 7a–e and S6). This resulted in a hairy root phenotype in 2F-Fuc-treated seedlings that is characterized by the appearance of densely packed root hairs (Figure 7f).

Staining of cell walls with the glucan-binding fluorescent dye calcifluor white revealed defects in cellulose organization and cell wall integrity in 2F-Fuc-treated root epidermal cells in the elongation zone. In contrast to control cells, where calcifluor staining was relatively evenly distributed along the cell wall, the cell wall network of 2F-Fuc-treated Arabidopsis roots appeared disorganized, as previously described for cellulose-deficient mutants (Anderson et al., 2010). Moreover the presence of gaps in the 2F-Fuc-treated cell walls suggests the existence of points of weakness in the wall (Figure 7g,h).

Closer investigation of the root tips of seedlings treated with 2.5 μM 2F-Fuc did not reveal disorganization in the meristematic zone of Arabidopsis roots in comparison with control roots with similar cell numbers, types and shapes (Figure 7c). Actively dividing cells were visualized in Arabidopsis seedlings expressing the GUS reporter gene under the control of the cyclin-dependent protein kinase 1 (CYCB1) promoter. Staining of seedlings expressing the pCYCB1::GUS fusion protein in 2F-Fuc-treated roots did not reveal any reduction in cell division activity in the apical meristem compared with control seedlings (Figure 7i,j), suggesting that 2F-Fuc does not dramatically affect cell division. For confirmation, the cell division rate of suspension-cultured BY-2 tobacco cells was monitored in the presence of 2F-Fuc. No significant effect was observed on BY-2 cell growth even at 100 μM 2F-Fuc (Figure S7).

After 2 weeks of growth in the presence of 2.5 μM 2F-Fuc, in addition to the densely packed root hair phenotype, Arabidopsis seedlings exhibited multiple adventitious roots, with an average of 2.2 adventitious roots per treated plant (Figure S8); these were not observed in control plants or in plants supplemented with 0.75 mM boric acid (Figure S8). These data suggest that 2F-Fuc induces important changes in Arabidopsis root architecture at micromolar concentrations.

**DISCUSSION**

2F-Fuc is a potent inhibitor of glycoconjugate and polysaccharide fucosylation and inhibits cell elongation

Screening of commercially available fluoro monosaccharide analogues as putative growth inhibitors revealed that 2F-Fuc is able to reduce root growth at micromolar concentrations. In contrast, 3F-Neu5Ac has no effects on root growth, whereas 2F-Glc, the most active fluorinated glucose molecule we tested, caused growth phenotypes at 25 μM. Growth effects were less severe on shoots for all of the analogues tested, possibly due to poor uptake of the fluoro monosaccharides into shoot tissues, which are surrounded by a waterproof cuticle, or due to differences in shoot cell walls compared with those of root tissues. The impacts of fluoro glucose analogues on either glucosyltransferase inhibition or glucose cytosolic metabolism were not investigated further. Several lines of evidence demonstrate that 2F-Fuc is an efficient inhibitor of glycoconjugate and polysaccharide fucosylation. First, a 50% decrease in the fucose content of root cell walls was observed after treatment with 2.5 μM 2F-Fuc, and only traces of fucose were detected after treatment with higher concentrations of 2F-Fuc. Analysis of N-glycoprotein and xyloglucan fucosylation (Figures 3 and 4) led to the same conclusions. This indicates that at 2F-Fuc...
concentrations as low as 10 \( \mu \text{M} \), the fucosylation of polysaccharides and glycoconjugates is almost entirely inhibited in Arabidopsis seedling roots. As observed for the growth phenotypes, less severe underfucosylation was found in shoots. In addition to the possibilities listed above, the less severe effects in shoots might also reflect minimal transport of the sugar analogue from roots to the aerial parts of the plant, possibly due to the diffusion barrier presented by the endodermis. Similar results were published by Villalobos et al. (2015) while this article was under review.

\[ \text{Atfkgp mutant seedlings impaired in L-fucokinase and GDP-L-fucose pyrophosphorylase activities of the salvage pathway (Kotake et al., 2008) grew normally in the presence of 2F-Fuc (Figure S4). This demonstrated that 2F-Fuc must probably be converted into its GDP-nucleotide form to inhibit root growth. Moreover, enzymatic assays performed on AtFUT1 also suggested that the 2F-Fuc must be converted into GDP-2F-Fuc to mimic the transition state and bind tightly to the active site of fucosyltransferases, leading to competitive inhibition (Burkart et al., 2000; Rillahan et al., 2012; Okeley et al., 2013). In mammals, the} \]

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Figure 7. Effect on Arabidopsis root architecture. (a)–(d) Four-day-old Arabidopsis seedlings grown with 0.025% DMSO (a, b) or 2.5 \( \mu \text{M} \) 2-fluoro 2-L-fucose (2F-Fuc) (c, d). The frames at the apex of the root (a, d) indicate the zone shown in (b, c), (b, c) Longitudinal sections showing the root cell organization in the division and the elongation zones. Arrows indicate the presence of root hairs (rh). (e) Distance measured between the apex and the early differentiation zone of 4-day-old Arabidopsis seedlings grown with 0.025% DMSO or 2.5 \( \mu \text{M} \) 2F-Fuc. Data are mean \( \pm \) SEM (n > 15 roots). Mann-Whitney test, \( *** P < 0.001 \). (f) Root hair density of Arabidopsis seedlings grown with 0.025% DMSO or 2.5 \( \mu \text{M} \) 2F-Fuc (n > 25 roots). Data are mean \( \pm \) SEM. Mann-Whitney test, \( *** P < 0.001 \). (g), (h) Calcofluor staining of Arabidopsis roots grown with 0.025% DMSO (g) or 2.5 \( \mu \text{M} \) 2F-Fuc (h). Arrowheads point out cell wall defects in the elongation zone. (i), (j) Analysis of root meristem activity of 4-day-old Arabidopsis \( \rho \text{CYCB1::GUS} \) seedlings grown with 0.025% DMSO (i) or 2.5 \( \mu \text{M} \) 2F-Fuc (j). \( \rho \text{CYCB1::GUS} \) line were used to visualize the division zone in non-treated and treated plants. Scale bars = 1 mm (a)–(d); 20 \( \mu \text{m} \) (b), (c); 25 \( \mu \text{m} \) (g), (h); 100 \( \mu \text{m} \) (i), (j).
GDP-activated 2F-Fuc inhibitor inhibits mammalian Golgi-resident fucosyltransferases. This inhibitor also depletes mammalian cells of GDP-Fuc through the feedback inhibition of its de novo synthesis (Okeley et al., 2013), thus depriving the cytosol of the activated form of fucose that is required by Golgi fucosyltransferases. In future work, experiments using recombinant enzymes and synthetic GDP-activated 2F-Fuc will be required to determine which inhibition mechanism occurs in plants treated with 2F-Fuc and is responsible for the underfucosylation of glycoconjugates and polysaccharides observed here.

Root growth is strongly affected by 2F-Fuc at micromolar concentrations. Using genetic and biochemical analyses, we investigated the relationship between the underfucosylation of cell wall fucose-containing polymers and the root growth phenotype. Arabidopsis xyloglucan and N-linked glycoproteins contain terminal fucose residues. However, these sugar motifs are not crucial for plant growth, since mutants impaired in xyloglucan and N-glycoprotein fucosylation do not exhibit visible phenotypes when grown in standard conditions (von Schaewen et al., 1993; Vanzin et al., 2002). The fut4 fut6 double mutant deficient in AGP fucosylation also appears to be healthy (Liang et al., 2013). Moreover, in the presence of 2F-Fuc, xyloglucan-, AGP- and N-glycan-deficient mutants (mur2, xxt1/xxt2, fut/fut6 and cgl) exhibited growth inhibition phenotypes similar to those observed in wild-type plants, further suggesting that defects in glycoprotein, AGP and xyloglucan fucosylation are not responsible for the observed root developmental phenotypes.

The inhibition of fucosylation of cell wall polysaccharides would also be expected to affect RG-II, which contains fucose residues on two of its side chains (Figure S1). At low concentrations, PAG experiments showed that 2F-Fuc induced a decrease in RG-II dimerization in root seedlings and the formation of a small RG-II fragment of unknown structure (Figure 5b). This fragment may result from the absence of the internal fucose residue in side chain A in 2F-Fuc-treated seedlings, as reported by Pabst et al. (2013) for the Arabidopsis mur1 mutant, which is defective in fucose biosynthesis. At higher concentrations of 2F-Fuc, dimers of RG-II were greatly diminished, but this was not counterbalanced by a relative increase in the level of monomeric RG-II (Figure 5b), suggesting that deposition of RG-II and/or its stability in plant cell walls is strongly affected by 2F-Fuc. Disruption of RG-II biosynthesis at 25 $\mu$M 2F-Fuc was confirmed by the inability of plants treated with 25 $\mu$M 2F-Fuc to incorporate Kdo-N$_2$ in a click-mediated labelling experiment (Figure 6). We postulate that the synthesis andimerization of RG-II within the Golgi apparatus involves enzyme complexes that cannot produce normal RG-II when side chain biosynthesis is affected. It is worth noting that the GalA content in root cell walls was not disturbed by 2F-Fuc treatment, indicating that impairment of RG-II biosynthesis does not affect the overall synthesis of pectins. Sugar composition analyses of 2F-Fuc-treated roots also indicated a decrease in relative xylose content and an increase in relative galactose content. However, it is highly speculative to conclude whether these changes in cell wall composition are a direct result of the absence of fucosylation of cell wall polysaccharides or are adaptive modifications in cell wall biosynthesis in response to 2F-Fuc-induced growth defects.

To date, several mutations affecting RG-II structure have been demonstrated to affect both RG-II dimerization and plant development. This has been mainly deduced from the study of mutants defective in the biosynthesis of monosaccharides contained in RG-II (O’Neill et al., 2001; Ahn et al., 2006; Delmas et al., 2008; Voezuer et al., 2012). For instance, depletion of UDP-D-apiose/UDP-D-xylose synthase, the enzyme responsible for the synthesis of the RG-II-specific apiose residue, results in cell wall abnormalities, including wall thickening and gaps. These phenotypes are probably due to the synthesis of abnormal RG-II in this mutant (Ahn et al., 2006). We observed similar altered cell wall patterns using calcofluor staining in 2.5 $\mu$M 2F-Fuc-treated seedlings (Figure 7g,h). Among other Arabidopsis mutants defective in RG-II biosynthesis, mur1 is impaired in fucose biosynthesis and is thus unable to properly fucosylate its cell wall polysaccharides and glycoproteins (Zablackis et al., 1996; Rayon et al., 1999; O’Neill et al., 2001). The fucosylation defects of cell wall polysaccharides and proteins observed in this study upon treatment with 2F-Fuc are similar to those observed in fucose-deficient mur1 mutants (Zablackis et al., 1996; Rayon et al., 1999; O’Neill et al., 2001). Both mur1 and 2F-Fuc-treated plants exhibit growth defects, cell wall weakness and decreased RG-II dimerization, demonstrating a relationship between RG-II biosynthesis and plant development. Although mur1 mutants and 2F-Fuc-treated seedlings exhibit similar underfucosylation and altered growth phenotypes, they differ in several aspects. In mur1, fucose is partially replaced in cell wall polysaccharides and glycoproteins by the structurally similar sugar L-Gal (Zablackis et al., 1996; Rayon et al., 1999; Reuhs et al., 2004). In MALDI-MS profiles of 2F-Fuc-treated plants, ions that could be assigned to L-Gal-containing xyloglucan fragments or to their acetylated forms were not detected. Thus, we conclude that L-Fuc is probably not replaced by L-Gal in the xyloglucan of Arabidopsis seedlings upon treatment with 2F-Fuc. Furthermore, the MUR1 gene encodes a GDP-D-mannose-4,6-dehydratase that catalyses the first step in the de novo synthesis of GDP-L-fucose (Bonin et al., 1997). This mutant can be rescued by supplying exogenous L-Fuc, which is converted into GDP-Fuc through the salvage pathway (Bonin et al., 1997; O’Neill et al., 2001). In contrast, addition of fucose to the culture medium did not reverse the growth phenotype induced by 2F-Fuc (Figure S3).
Together, this information suggests that one or more fucosyltransferases are likely to be inhibited by 2F-Fuc. However, we cannot rule out that GDP-Fuc biosynthesis is also affected by this inhibitor.

Root cell elongation is strongly affected by 2F-Fuc treatment (Figure S6), which results in a high density of root hairs and the presence of differentiated cells close to the root tip (Figure 7f). This growth phenotype was previously reported in plants treated with 2β-deoxy-Kdo, which inhibits CMP-Kdo synthesis that is required for RG-II biosynthesis (Smyth et al., 2013). Defects in cell elongation in RG-II mutants have previously been reported to be responsible for impairment of pollen tube growth and fertilization (Delmas et al., 2008; Deng et al., 2010; Liu et al., 2011; Dumont et al., 2014). In our study, close inspection of the root tip and monitoring of cell division activity suggest that cell division was not affected by 2F-Fuc (Figure 7i,j). This was confirmed by investigation of the rate of division of suspension-cultured BY-2 cells in the presence of the inhibitor (Figure S7). These data, together with previous studies showing that suspension-cultured plant cells are capable of continuous and long-term growth on a boron-deficient medium (Fleischer et al., 1999; Chormova et al., 2014), indicate that RG-II dimerization is required for cell elongation but not for cell division. In addition to the inhibition of cell elongation, 2F-Fuc induces the formation of multiple adventitious roots (Figure S7). These changes in root architecture caused by 2F-Fuc treatment are possibly due to a dysregulation of hormone-mediated signalling, since root growth is closely related to the synthesis, distribution and transport of auxin and ethylene (Biliou et al., 2005; Gutierrez et al., 2012; Abreu et al., 2014).

Boron is required for RG-II cross-linking in plant cell walls, but is likely to serve other functions in cell expansion

It is known that RG-II is the main boron-binding site in the cell walls of plants, enabling this pectic polymer to dimerize. Supplementation of RG-II-deficient mutants with boron has been shown to partially restore wild-type growth phenotypes and the wild-type accumulation of RG-II dimers, demonstrating that RG-II dimerization is required for proper cell elongation (O’Neill et al., 2001; Voxeur et al., 2012; Smyth et al., 2013). For instance, supplementation of mur1 plants with borate partially restores RG-II cross-linking and wild-type growth patterns (O’Neill et al., 2001). The ability of borate to partially rescue root growth that we observed in seedlings treated with 2.5 μM 2F-Fuc (Figure 5a) is comparable with observations previously reported for RG-II-deficient mutants. The PAGE analysis of RG-II of 2.5 μM 2F-Fuc-treated plants supplemented with boric acid indicated an increase in the relative amount of dRG-II, suggesting that boric acid was able to restore the dimerization of RG-II in inhibitor-treated roots. These data are in agreement with previous studies demonstrating a link between proper RG-II cross-linking and efficient cell elongation.

Boron was also shown to partially restore cell elongation in roots treated with high concentrations of 2F-Fuc (Figure 5a). At such concentrations, RG-II biosynthesis is strongly affected without modification of the overall pectin content of roots (Figure 2). In the PAGE experiment, the relative amounts of loaded cell wall fractions were identical. Thus, the disappearance of RG-II bands is probably a result of inhibition of RG-II biosynthesis rather than an overall decrease of pectic materials in the fractions isolated from the treated roots. Disruption of RG-II biosynthesis was confirmed by a metabolic click-labelling experiment using an azido Kdo analogue. As a consequence, partial rescue of cell elongation by borate supplementation in roots treated with 2F-Fuc might not be solely attributable to the restoration of appropriate RG-II dimerization. It is known that rapidly expanding cells respond dramatically to defects in cell wall integrity. Several studies have reported that this response is mediated by a cell wall integrity-sensing mechanism involving cell wall sensors and receptors, such as receptor kinases (Humphrey et al., 2007; Hematy and Hofte, 2008; Boisson-Dernier et al., 2011; Engelsdorf and Hamann, 2014). These membrane-bound proteins allow for the maintenance of cell wall integrity and the coordination of plant development. We postulate that, in response to 2F-Fuc treatments, these ‘sentinels’ act as sensors that detect impairment of RG-II and in turn induce arrest of elongation. Since borate is able to partially restore elongation in cells that lack normal levels of RG-II, it might also function as a signalling molecule in the cell wall integrity-sensing mechanism. Boron could either act as free borate or be linked to the cis-diol motifs of metabolites, as reported for the bacterial boron-containing quorum-sensing signal (Chen et al., 2002). Partial restoration of wild-type elongation by borate supplementation has previously been reported in mutants exhibiting growth phenotypes and structural cell wall defects that are not related to RG-II. For instance, structural alteration of cell wall xyloglucans and pectins in mur3 and mur4 mutants causes a hypersensitive sugar-responsive phenotype that is also rescued by supplementation with boric acid (Li et al., 2007). The mur3 and mur4 mutants are affected in the synthesis of xyloglucan and RG-I, respectively, and the growth restoration effects are probably not related to borate cross-linking of cell wall RG-II in these plant mutants. Some authors have postulated that borate might also be involved in cross-linking of monosaccharide residues in xyloglucan and RG-I, as recently reported for glycosylinositol phosphoceramides of lipid rafts (Wimmer et al., 2009; Voxeur and Fry, 2014). However, no biochemical evidence for RG-I and xyloglucan borate-mediated cross-linking in cell walls has been reported thus far. As a consequence, the data reported by Li et al. (2007), together
with data presented here, suggest that borate might be perceived as a positive signal for cell wall elongation. As recently speculated by Funakawa and Miwa (2015), the roles of boron in the primary cell wall might not be restricted to the physical cross-linking of the pectic network but might also include its activity as a direct regulator of cell elongation and/or cell wall integrity sensing. As a consequence, beyond their structural functions in cell walls, RG-II dimers may also play a crucial role as in muro sequestration sites for boron, as has been reported for deesterified homogalacturonan and calcium (Braccini and Pérez, 2001; Wolf et al., 2009; Leroux et al., 2015).

In conclusion, this study further emphasizes the importance of RG-II and its borate-mediated dimerization in cell elongation. It also highlights the potential of glycosyltransferase inhibitors as new tools for studying the function of cell wall polysaccharides in plant development. The use of these inhibitors enables the control of timing and the dose-dependent inhibition of glycosyltransferases, two aspects that are not easily achievable by studying genetic mutants alone. By combining chemical and genetic tools, we can hope to better understand the complexity of cell wall synthesis, structure and dynamics.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana lines were derived from the Columbia ecotype (Col). Seeds were surface sterilized and sown onto Arabidopsis medium (Duchefa Biochemie no. DU00742.0025, https://www.duchefa-biochemie.com/) supplemented with 2 mM Ca (NO₃)₂ and 0.8% (w/v) Bacto Agar, pH 5.8. Plates with seeds were.Experimental procedures

Plates with seeds were placed for 2 days at 4°C in the dark, then submitted to 16-h day/8-h night cycles (120 °C m⁻² s⁻¹, 21°C) in a vertical position. For click chemistry experiments, sterilized seeds were sown on Murashige and Skoog (MS; 2.2 g L⁻¹) mineral medium containing 1% sucrose and 0.8% plant agar (see below).

Biochemicals

2-fluoro-2-deoxy-1-fucose was purchased from Calbiochem® (Merck Millipore, http://www.merckmillipore.com/). 2-Fluoro-2-deoxy-β-glucopyranose (2F-Glc), 3F-Glc and the 4F-Glc were from LC Scientific Inc. (http://www.lcsci.com/). Deoxy fluoroglucose analogues (5 mg) were peracetylated in 150 μl of 2:1 pyridine:acetic anhydride overnight at 20°C. Excess reagent was co-evaporated with toluene under a stream of air. Fluoro sugar analogues were then dissolved in DMSO (10 μl). To assess the effect of each sugar analogue on plant growth, the inhibitor was added directly to the growth medium at the desired concentration. To assess effects on cell division, peracetylated 2F-Fuc was added to the medium of tobacco BY-2 suspension-cultured cells 4 days after subculture. For boron experiments, 0.75 mM boric acid was used because higher concentrations of borate had a negative effect on Arabidopsis root length.

Metabolic click labelling of cell walls

Click chemistry experiments were performed as described in Anderson et al. (2012). Arabidopsis thaliana Col-0 sterilized seeds were sown on MS (2.2 g L⁻¹) mineral medium containing 1% sucrose and 0.8% plant agar. After 2 days at 4°C, the plates were placed vertically in a growth chamber for 4 days at 22°C under a 16-h light/8-h dark cycle. Four-day-old light-grown seedlings were transferred from MS mineral medium plates to 1 ml of liquid MS containing 2.5 μM Fuc-Al (Invitrogen, http://www.invitrogen.com/) for 16 h at 22°C in the growth chamber (16-h light/8-h dark). Seedlings were then washed three times with liquid MS and then transferred to 1 ml of 0.1 μM Alexa Fluor® 488 azide (Invitrogen) solution in liquid MS containing 1 mM CuSO₄ and freshly made 1 mM ascorbic acid. The solution was incubated for 1 h at room temperature (22°C) in the dark. Seedlings were then washed once with liquid MS, then for 5 min with liquid MS supplemented with 0.01% (v/v) Tween 20 and three additional times in liquid MS. Control experiments were carried out by omitting either the Fuc-Al or the fluorescent probe in click-mediated labelling assays. Inhibition of fucosyl transferases was carried out by adding 25 μM 2F-Fuc in the growth medium and then by supplementing the liquid MS with 25 μM 2F-Fuc during the Fuc-Al incorporation step. Fuc-Al labelling was examined using a confocal laser-scanning microscope (Leica TCS SP2 AOBS, excitation filter 488 nm, barrier filter 495-550 nm, http://www.leica.com/). For the click chemistry experiment using Kdo-N₃, the same protocol was carried out using 50 μM of the Kdo analogue and Alexa Fluor® 488 alkyne (Invitrogen). The fluorescence intensity of the root was calculated with ImageJ, by thresholding (pixel intensity > 16) to select the root boundary and measuring the mean of pixel intensity inside the area defined by the threshold.

Histochemical GUS staining

Six-day-old pCYCB1::GUS seedlings were incubated for 3 h in the dark, at 37°C, in the reaction solution containing 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 50 mM sodium phosphate pH 7, 0.2% Triton X100 and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). Arabidopsis plants were observed under bright field using a Leica M125 stereomicroscope equipped with a DFC295 camera. At least 10 seedlings per treatment were observed and photographed.

Histochemical calcofluor staining

Roots were incubated with 0.001% calcofluor white M2R (Sigma, http://www.sigmaaldrich.com/) for 5 min in the dark. Then, the roots were mounted on glass microscope slides in a drop of water and observed by spinning disk confocal microscopy (Zeiss Observer Z1, http://www.zeiss.com/) equipped with UV fluorescence (excitation filter 359 nm, barrier filter 461 nm). Root length and fluorescence were measured from images using the ImageJ program (Abramoff et al., 2004).

Plant cell wall preparation

The roots and shoots of 10-day-old seedlings grown on Arabidopsis medium supplemented or not with peracetylated 2F-Fuc were
manually separated and then heated for 15 min in 70% ethanol at 70°C. The tissues were then ground in a potter homogenizer and the homogenate was washed with 70% ethanol at 70°C and then with water. The remaining alcohol-insoluble residue was dried and was considered as representative of the cell walls.

**Sugar composition analysis by gas phase chromatography**

Alcohol-insoluble residues from shoot and root of about 400 seedlings were hydrolysed using trifluoroacetic acid (2 M, 2 h at 110°C) and then submitted to a methanolation for 16 h at 80°C with 500 μl of dried 1 M methanolic-HCl (Supelco, SigmaAldrich; http://www.sigmaaldrich.com/analytical-chromatography/supelco-analytical/about-supelco.html). After evaporation of the methanol, the methyl glycosides were converted into their trimethylsilyl derivatives at 110°C for 20 min with 200 μl of the silylation reagent (HMDS:TMCS:pyridine, 3:1:9; Supelco). After drying, derivatives were dissolved in 1 ml of cyclohexane and analysed using a 3800 GC system equipped with a CP-Sil5-CB column (Agilent Technologies, http://www.agilent.com/) and a flame ionization detector. The gradient temperature was from 120 to 160°C at 1°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 carried out using inositol as the internal standard and response factors previously determined for cell wall monosaccharides. Due to contamination with starch, the glucose contents were rejected from the sugar composition of root extracts.

**Extraction of proteins**

Two hundred to 800 roots from 10-day-old Arabidopsis seedlings grown on Arabidopsis medium supplemented with 0.25% DMSO or 25 μM 2F-Fuc were ground on ice in a 1 ml potter homogenizer in 2-aminooxyhydroxymethyl-1,3-propanediol (TRIS) 0.1 M pH 8 buffer containing 0.1% of SDS. The extract was then freeze-dried. Proteins extracts were then resuspended in 300 μl of TRIS-SDS buffer.

**Immunoblotting**

Protein extracts were separated by SDS-PAGE using a 12% polyacrylamide gel. The proteins were transferred onto nitrocellulose membrane and stained with Ponceau Red to control the transfer efficiency and to ensure that the same protein quantities were loaded for the two conditions. Before the immunoblotting, the free binding sites on nitrocellulose were blocked overnight with TRIS-binding sites on nitrocellulose were blocked overnight with TRIS-SDS 0.1% PBS (200–400 mesh) at 25% (p/v) in water. After centrifugation, 2 min at 5000 g, the supernatant was collected, mixed with 3.8 ml of scintillation liquid and then the radioactivity was counted for 1 min using a Packard Bell instrument. Apparent Kᵦ values of AfTFT1 towards xyloglucan were read on the double-reciprocal plots and thereafter referred to as Kᵦ for data in absence of 2F-Fuc (triangle plot) and Kᵦ in the presence of 2F-Fuc (square plot). Kᵦ was calculated as follows: Kᵦ = Kᵦ/[2F-Fuc]/(Kᵦ − Kᵦ).

**Isolation of RG-II**

Roots from 200 to 1000 seedlings were ground in a 1 ml potter homogenizer in 70% ethanol. The homogenate was centrifuged at 14 000 g for 10 min and the pellet was washed with 70% ethanol until the supernatant was clear. Additional washing was performed in 100% acetone. The resulting alcohol-insoluble residue was dried and was treated with 1 M Na₂CO₃ at 4°C for 16 h, then rinsed with water until neutral pH was achieved. The pellet was suspended in 500 μl ammonium acetate buffer (0.05 M, pH 4.8) with 5 U endo-polygalacturonase from Aspergillus niger (Megazyme) and incubated at 37°C for 16 h. Solubilized material was mass spectra were acquired on a Voyager DE-Pro MALDI-TOF (Ab Sciex, http://sciex.com/) instrument equipped with a 337-nm nitrogen laser. Mass spectra were performed in the reflector-delayed extraction mode using 2,5-dihydroxybenzoic acid as the matrix (Sigma-Aldrich). This matrix, dissolved at 5 mg ml⁻¹ in a 70/30 acetonitrile/0.1% trifluoroacetic acid solution, was mixed onto the target with the digests in a 1:1 (v/v) ratio. Spectra were recorded in a positive mode, using an acceleration voltage of 20 000 V with a delay time of 100 ns. They were externally calibrated using commercially available mixtures of peptides (ProteoMass™ Peptide MALDI-MS Calibration Kit, Sigma-Aldrich). A thousand laser shots were accumulated for each spectrum. The main ions detected were assigned to [M+Na]⁺ adducts from XXXG to XLFG on the basis of their molecular weight and literature data (Zabackis et al., 1995; Lerouxel et al., 2002). Relative quantification of the different oligosaccharide species was performed using their m/z ion intensity in MALDI-TOF spectra.

**Fucosyltransferase activity assay**

Fucosyltransferase activity assays were carried out using Δ68-AtFuT1 (deletion of 68 amino acids at the N-terminus for protein solubility) construct amplified from the U10760 clone (TAIR, https://www.arabidopsis.org/) and cloned into the pVT-Bac-His vector for expression in insect cells. Spodoptera frugiperda (Sf9, Invitrogen) cells used for the transfection were cultured in Grace medium (Invitrogen) and was considered as representative of the cell walls.

**Xyloglucan fingerprinting**

Xyloglucan fragments were generated by treating the alcohol-insoluble residues isolated from 200 to 500 seedlings with 4 U of endogluccanase (endo-1,4-β-D-glucanase from Trichoderma longibrachiatum, E-CELTR; Megazyme, https://www.megazyme.com/) in 100 μl of 10 mM sodium acetate buffer, pH 5, for 18 h at 37°C.
concentrated in a centrifugal vacuum concentrator. The RG-II was suspended in 25 μl of ammonium acetate buffer. The relative amount of cell wall sugars for each RG-II-enriched fraction was assessed by quantification of monosaccharides by gas chromatography analysis before running on PAGE.

Polyacrylamide gel electrophoresis of RG-II

Polyacrylamide gel electrophoresis was performed according to Chornova et al. (2014) with few changes. A 10% polyacrylamide stacking gel was added to a 26% polyacrylamide gel. Eight-microlitre RG-II samples were mixed with 2 μl of sample buffer [0.63 m TRIS-HCl containing 0.25% (w/v) bromophenol blue and 50% (v/v) glycerol, pH 8.8]. Eighty micrograms of RG-II extracted from lemon was used as control. For monomerization, lemon RG-II was incubated for 16 h at room temperature in 0.1 M NaOH (v/v). Before loading, the sample was neutralized with 0.1 M NaOH (v/v). Electrophoresis was conducted at 220 V for 130 min. The gel was then fixed in ethanol/acetic acid/water (4:1:5) for 30 min and washed three times in 30% ethanol for 10 min and three times in water for 10 min each. Silver staining was performed as described by Chornova et al. (2014).

Statistical analysis

The data were analysed statistically by using GraphPad software (http://www.graphpad.com/). Differences were considered statistically significant when $P \leq 0.05$ (†), highly significant when $P < 0.01$ (**), and very highly significant when $P < 0.001$ (**).

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SupportING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Structure of rhamnogalacturonan II.

Figure S2. Effects of fluoro monosaccharide analogues on plant growth.

Figure S3. Excess of fucose did not restore the inhibitory effect of 2-fluoro 2-L-fucose.

Figure S4. Cytosolic L-fucokinase and GDP-L-fucose pyrophospho-

rlyase activities are not affected by 2-fluoro 2-L-fucose.

Figure S5. Inhibition of AIFUT1 fucosyltransferase activity by 2-fluoro 2-L-fucose.

Figure S6. Effect of 2-fluoro 2-l-fucose on cell elongation.

Figure S7. Effect of 2-fluoro 2-L-fucose on BY-2 cell division.

Figure S8. Emergence of adventitious roots in 14-day-old treated Arabidopsis seedlings.

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Inhibition of plant cell wall fucosylation and root growth


