The *reb1-1* Mutation of Arabidopsis. Effect on the Structure and Localization of Galactose-Containing Cell Wall Polysaccharides[1][W]

Eric Nguema-Ona, Christine Andème-Onzighi[2,3], Sophie Aboughe-Angone[2], Muriel Bardor, Tadashi Ishii, Patrice Lerouge, and Azeddine Driouich*

Unité Mixte de Recherche, Centre National de la Recherche Scientifique 6037, Federal Institute of Multidisciplinary Research on Peptides 23, Université de Rouen, 76 821 Mont Saint Aignan cedex, France (E.N.-O., C. A.-O., S. A.-A., M.B., P.L., A.D.); and Forestry and Forest Products Research Institute, Tsukuba Norin Kenkyu Danchi-nai, Ibaraki 305–8687, Japan (T.I.)

The Arabidopsis (*Arabidopsis thaliana*) *root epidermal bulger1-1* (*reb1-1*) mutant (allelic to *root hair defective1* [*rhd1*]) is characterized by a reduced root elongation rate and by bulging of trichoblast cells. The REB1/RHD1 gene belongs to a family of UDP-D-Glucose 4-epimerases involved in the synthesis of D-Galactose (Gal). Our previous study showed that certain arabinogalactan protein epitopes were not expressed in bulging trichoblasts of the mutant. In this study, using a combination of microscopical and biochemical methods, we have investigated the occurrence and the structure of three major Gal-containing polysaccharides, namely, xyloglucan (XyG), rhamnogalacturonan (RG)-I, and RG-II in the mutant root cell walls. Our immunocytochemical data show that swollen trichoblasts were not stained with the monoclonal antibody CCRC-M1 specific for α-L-Fucp-(1$\rightarrow$2)-β-D-Galp side chains of XyG, whereas they were stained with anti-XyG antibodies specific for XyG backbone. In addition, analysis of a hemicellulosic fraction from roots demonstrates the presence of two structurally different XyGs in *reb1-1*. One is structurally similar to wild-type XyG and the other is devoid of fuco-galactosylated side chains and has the characteristic of being insoluble. Similar to anti-XyG antibodies, anti-bupleuran 2IIC, a polyclonal antibody specific for galactosyl epitopes associated with pectins, stained all root epidermal cells of both wild type and *reb1-1*. Similarly, anti-RG-II antibodies also stained swollen trichoblasts in the mutant. In addition, structural analysis of pectic polymers revealed no change in the galactosylation of RG-I and RG-II isolated from *reb1-1* root cells. These findings demonstrate that the *reb1-1* mutation affects XyG structure, but not that of pectic polysaccharides, thus lending support to the hypothesis that biosynthesis of Gal as well as galactosylation of complex polysaccharides is regulated at the polymer level.

The plant cell wall plays a vital role in growth and development as well as in mediating interactions with the environment and other organisms. It is a dynamic and complex structure comprising cellulose microfibrils and a xyloglucan (XyG) network embedded within a matrix of polysaccharides and proteins (i.e. glycoproteins and proteoglycans). Four major types of noncellulosic polysaccharides are found in the primary walls of plant cells (in taxa outside the graminae), namely, the neutral hemicellulosic polysaccharide XyG, and three pectic polysaccharides, homogalacturonan (HG), rhamnogalacturonan (RG)-I, and RG-II (Carpita and Gibeaut, 1993).

XyG consists of a β-D-(1$\rightarrow$4)-glucan backbone to which are attached side chains containing either xylosyl, galactosyl-xylosyl, or fucosyl-galactosyl-xylosyl residues. In dicotyledonous and nongrass monocotyledonous plants, XyG is the principal polysaccharide that cross-links the cellulose microfibrils. XyG is able to bind cellulose tightly because its β-D-(1$\rightarrow$4)-glucan cellulose-like backbone can form many hydrogen bonds with the microfibrils, whereas the side chains give rise to regions where microfibril binding is interrupted and thus a single XyG molecule can interconnect separated cellulose microfibrils. This XyG-cellulose network forms a major load-bearing structure that contributes to the structural integrity of the wall and the control of cell expansion (Cosgrove, 1999).

In Arabidopsis (*Arabidopsis thaliana*) cell walls, generally six structurally distinct oligomers are released from XyG upon treatment with endo-β-(1$\rightarrow$4) endoglucanase. According to the nomenclature introduced by Fry et al. (1993), these fragments are XXXG, XLFG, XLLG, XLXG, XXLG, and XXFG (according to Lerouxel et al., 2002; Madson et al., 2003; Peña et al., 2004). It has been suggested that the terminal Fuc present in XyG

[1] This work was supported by the Centre National de la Recherche Scientifique and the University of Rouen (to A.D.) and by the "PROBRAIN" (to T. I.).

[2] These authors contributed equally to the paper.

[3] Present address: Department of Molecular and Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309–0347.

* Corresponding author; e-mail azeddine.driouich@univ-rouen.fr; fax 33–235146615.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Azeddine Driouich (azeddine.driouich@univ-rouen.fr).

[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.074997.

side chains is required for the stabilization of a planar conformation that facilitates binding to cellulose microfibrils (Levy et al., 1991). However, recent studies on *mur2* and *mur3* mutants have indicated that galactosylation rather than fucosylation of XyG is essential for maintaining the tensile strength of the cell wall during growth (Vanzin et al., 2002; Peña et al., 2004).

The pectic matrix is structurally complex and heterogeneous. HG domains consist of α-D-(1→4)-GalUA (GalA) residues, which can be methyl esterified, acetylated, and/or substituted with Xyl (Willats et al., 2001). Deesterified blocks of HG can be cross-linked by calcium, resulting in the formation of a gel that is believed to be essential for cell adhesion (Jarvis, 1984). Such an association is also important in controlling wall porosity (Carpita and Gibeaut, 1993). RG-I domains contain repeats of the disaccharide (→4-α-D-GalA-(1→2)-α-L-Rha-1→) in which rhamnosyl residues can carry oligosaccharide side chains consisting predominantly of β-D-(1→4)-galactosyl- and/or α-L-(1→5)-arabinosyl-linked residues (McNeil et al., 1982). Side chains of RG-I are believed to decrease the ability of pectic molecules to cross-link and form a stable gel network and are thereby able to influence the mechanical properties of the cell wall (Hwang and Kokini, 1991). In addition, the structure and tissue distribution of arabinan- or galactan-rich side chains of RG-I have been shown to be regulated during cell growth and differentiation of many species (for review, see Willats et al., 2001).

RG-II is the most structurally complex pectic polysaccharide discovered so far in plants (Ridley et al., 2001). It occurs in the cell walls of all higher plants as a dimer (dRG-II-B) that is cross-linked by a borate diester (Matoh et al., 1993; Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996). The backbone of RG-II is composed of a HG-like structure containing at least eight α-D-(1→4)-GalA-linked residues to which four structurally different oligosaccharide chains, denoted A, B, C, and D, are attached. The C and D side chains are attached to C-3 of the backbone, whereas A and B are attached to C-2 of the backbone (O'Neill et al., 2004). The C disaccharide contains Rha and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo), whereas the D disaccharide contains 2-keto-3-deoxy-D-lyxo-heptulosaric acid (Dha) and Ara (O'Neill et al., 2004). The A and B oligosaccharide chains are both composed of eight to 10 monosaccharides and are attached by a β-D-apiose residue to O-2 of the backbone. A D-galactosyl residue (D-Gal) occurs on the B chain.

The structure of RG-II has been shown to be conserved throughout the primary cell walls of vascular plants (Ridley et al., 2001; Matsunaga et al., 2004). RG-II plays an important role in the structural organization of the cell wall and the control of cell growth. Cross-linking of RG-II via a borate ester and the formation of dimers in muro contribute significantly to the control of cell wall porosity (Fleischer et al., 1999) and tensile strength (Ryden et al., 2003). For instance, abnormally swollen cell walls have been shown to result from boron deficiency and decrease in RG-II dimer formation (Matoh, 1997; Ishii et al., 2001). In addition, it has been shown that plants carrying mutations that affect the boron-mediated cross-linking of RG-II have reduced intercellular attachment (Iwai et al., 2002) and growth (O'Neill et al., 2001).

In the past 10 years, screening and analysis of Arabidopsis mutants have become a widely used approach to unravel the mechanisms of cell wall biosynthesis and function. For instance, the identification and analysis of cellulose-deficient Arabidopsis mutants, such as *rsw* (root swelling) and *irx* (irregular xylem), have led to the finding that multiple cellulose synthase proteins are required for cellulose synthesis in the primary and secondary cell walls (Fagard et al., 2000; Peng et al., 2000; Taylor et al., 2000, 2003). The characterization of other mutants, such as *mur1*, *2*, *3*, and *4* (Reiter et al., 1997), with deficiencies in specific sugars of the noncellulosic fraction of the cell wall, is currently providing significant knowledge of the enzymatic machinery involved in complex polysaccharide biosynthesis. In particular, the *mur* mutants have allowed molecular cloning and characterization of genes encoding glycosyltransferases active in the synthesis of XyG as well as genes encoding sugar-synthesizing enzymes (Burget et al., 2003; Madson et al., 2003; Mølhøj et al., 2004).

The *root epidermal bulger1-1* (*reb1-1*) mutant of Arabidopsis, which is allelic to *root hair defective1* (*rhd1*) is characterized by a reduced elongation rate of the primary root and bulging of root trichoblast cells (Baskin et al., 1992; Andème-Onzighi et al., 2002). Bulging of trichoblasts has been observed mostly in the elongation and differentiation zones of the root. The *REB1/RHD1* gene has been cloned and shown to encode for a UDP-D-Glc 4-epimerase (UGE4) involved in the synthesis of D-Gal (Seifert et al., 2002). In our previous study using immunocytochemistry, it was shown that arabinogalactan proteins (AGPs) recognized by LM2 or JIM14 antibodies are not expressed in bulging trichoblasts of the mutant, although they are present in other cell types (Andème-Onzighi et al., 2002). Similarly, Seifert et al. (2002) have shown that the CCRC-M1-recognized epitope of XyG is absent from the epidermal and cortical cells of *rhd1*. Thus, it has been postulated that the UDP-D-Gal transfer to wall polymers can be regulated at the tissue and the cellular level (Seifert et al., 2002).

To expand upon these findings and gain further insight into the role of Gal-containing cell wall polysaccharides, we have investigated the localization and structure of RG-I and RG-II in addition to XyG in the mutant root cells by combining immunocytochemical and biochemical techniques. Our data show that swollen trichoblasts in the root elongation zone do express a galactosylated epitope associated with pectic polysaccharides recognized by anti-bupleuran 2IIC antibodies. In contrast, they do not express the α-L-Fucp-(1→2)-β-D-Galp side chain recognized by the monoclonal antibody (mAb), CCRC-M1, as previously described (Seifert et al., 2002). Structural analysis of XyG isolated from *reb1-1* mutant roots demonstrates

the presence of two structurally different types. One has a normal wild-type structure and the other a XyG that is devoid of α-L-Fucp-(1→2)-β-D-Galp and β-D-Galp side chains and insoluble. In contrast, no change in the structure of the pectic polysaccharides RG-I and RG-II isolated from *reb1-1* root cells is found. Based on these findings and those of Andème-Onzighi et al. (2002), we postulate that UGE4 might be part of a protein complex involved in the galactosylation of XyG and AGPs, but not in that of pectic polysaccharides in trichoblast cells.

RESULTS

Immunofluorescence Localization of XyG in Root Cells

To investigate the occurrence of XyG epitopes in the outer epidermal cell wall of roots, we used a whole-mount labeling technique, which is a rapid and reliable method for mapping polysaccharide epitopes (Willats et al., 2001; Vicré et al., 2005). We studied the distribution of epitopes recognized by the mAb, CCRC-M1, which is specific for α-L-Fucp-(1→2)-β-D-Galp side chains (Puhlmann et al., 1994) and those recognized by a polyclonal anti-XyG antiserum, which is specific for the β-D-(1→4)-glucan backbone (Lynch and Staehelin, 1992). The CCRC-M1-recognized epitope is detected in all cells of the wild type except in a small region around the quiescent center (Fig. 1, A and C). Root hairs are also labeled with the CCRC-M1 antibody (Fig. 1C). In the *reb1-1* mutant, an overall heterogeneous staining of the root is observed with some regions more stained than others (Fig. 1B). A close examination of swollen trichoblasts in elongation and differentiation zones shows either a patchy staining of these cells (Fig. 1D) or no staining at all (data not shown). The anti-XyG-recognized epitope is also detected in root cells of both the wild type and *reb1-1* mutant (Fig. 1, E and F). The intensity of the staining, however, appears much higher in the elongation zone of the *reb1-1* mutant (Fig. 1F). A close examination of swollen trichoblasts shows a uniform staining of their cell walls (Fig. 1H). Root hairs are also labeled (Fig. 1, G and H).

To confirm and extend these observations, we immunolabeled resin-embedded cross sections of wild-type and *reb1-1* roots using the same antibodies. We focused on the examination of the elongation zone where trichoblasts were shown to bulge (Andème-Onzighi et al., 2002). The anti-XyG antiserum stains all cell types equally in both the wild type and the *reb1-1* mutant (Fig. 2, A and B). The mAb CCRC-M1 labels all cell types in the wild type, although with different intensities (Fig. 2D). The antibody stains epidermal cells (i.e. trichoblasts and atrichoblasts) more strongly than other tissues. In contrast, in *reb1-1* mutant roots, the mAb CCRC-M1 does not stain swollen trichoblasts, although it does stain atrichoblasts and nonswollen trichoblasts (Fig. 2E; see also Seifert et al., 2002). Interestingly, CCRC-M1

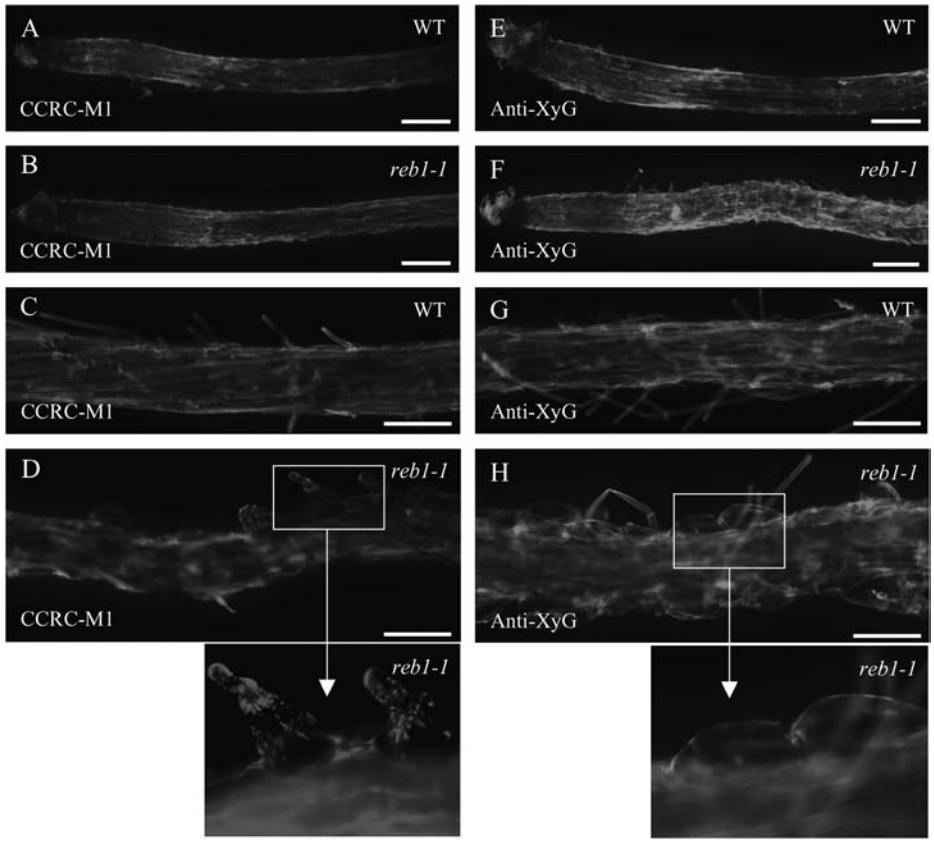

Figure 1. Immersion immunofluorescence staining of XyG in wild-type (A, C, E, and G) and *reb1-1* (B, D, F, and H) roots. A to D, Anti-XyG antiserum. E to H, mAb CCRC-M1 antibody. Bars = 140 μm (A, B, E, and F) and 100 μm (C, G, D, and H).

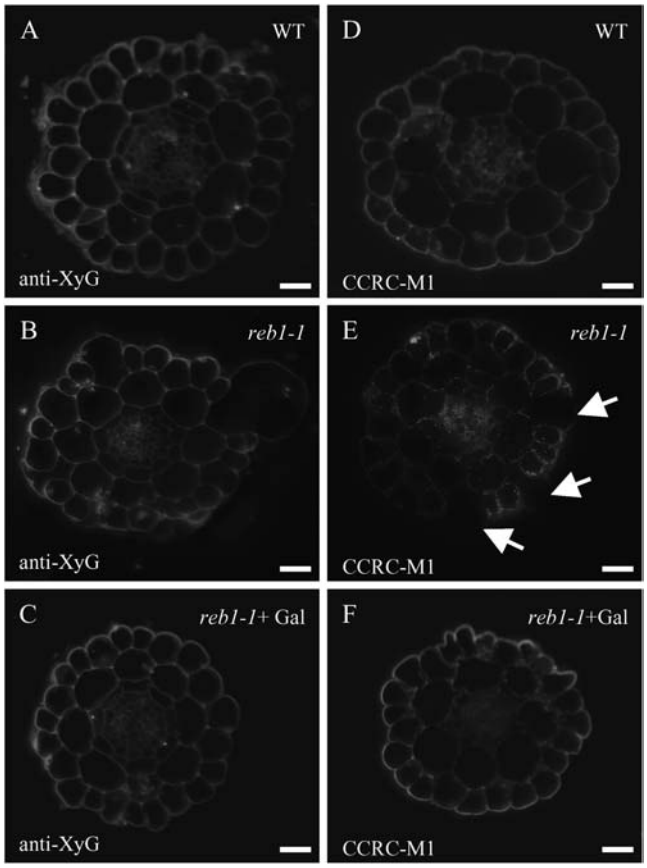

Figure 2. Fluorescence micrographs of cross sections in the root elongation zone showing staining with anti-XyG antiserum (A–C) and the mAb CCRC-M1 (D–F) for wild type (A and D), *reb1-1* (B and E), and *reb1-1* grown in the presence 10 mM D-Gal (C and F). Bars = 20 μm (A and D) and 25 μm (B, C, E, and F). Arrows indicate trichoblasts.

staining of trichoblasts is restored in mutant roots grown in the presence of 10 mM D-Gal (Fig. 2F). In this connection we also found that the addition of 10 mM D-Gal to the culture medium of *reb1-1* causes a complete recovery of the wild-type root length and morphology in addition to restoring AGP content (data not shown).

Taken together, these findings indicate that the structure of fuco-galactosylated side chains of XyG, recognized by CCRC-M1, might be modified in *reb1-1* trichoblasts.

Immunolocalization of the Pectic Polysaccharides RG-I and RG-II

We next examined distribution of epitopes associated with RG-I and RG-II using immunofluorescence microscopy and three antibodies. These are: (1) the polyclonal anti-bupleuran 2IIC, which recognizes an epitope consisting of β-D-galactosyl residues carrying glucuronic or 4-*O*-methylglucuronic acids found in RG-I (Sakurai et al., 1998; Andème-Onzighi et al., 2000); (2) the mAb, LM5, which is specific for β-D-(1→4)-galactan side chains of RG-I (Jones et al., 1997); and (3) a polyclonal anti-RG-II, which binds to an unknown epitope associated with the borate-RG-II complex (Matoh et al., 1998). Similar to XyG localization, we focused on the elongation zone of both wild-type and mutant roots and examined cross sections of resin-embedded roots.

The anti-RG-II antiserum stains all cell types in wild-type and mutant roots (Fig. 3, A and C). Epidermal cells appear to be strongly labeled as compared to other cell types. Similarly, the anti-bupleuran 2IIC antiserum stains all cell types equally, including trichoblasts, in both wild-type and mutant roots (Fig. 3, B and D). In contrast, the mAb LM5 labels epidermal cells in neither wild-type nor *reb1-1* mutants, whereas it labels other cell types, including the cortex, endodermis, and pericycle (data not shown). Evidently, LM5 is not a suitable probe to assess the occurrence of pectic Gal in epidermal cells of the elongation zone in Arabidopsis roots (see also Seifert et al., 2004).

Based on all these observations, we conclude that galactosylated pectins occur in the swollen trichoblasts of the mutant.

Chemical Analyses of Gal-Containing Cell Wall Polysaccharides in *reb1-1* Root Cells

First, we determined the monosaccharide composition of cell walls isolated from roots and shoots using gas chromatography (GC; Fig. 4). As previously reported (Seifert et al., 2002), *reb1-1* root cell walls contain less Gal than the wild type (Fig. 4A), whereas there is no significant difference in monosaccharide composition

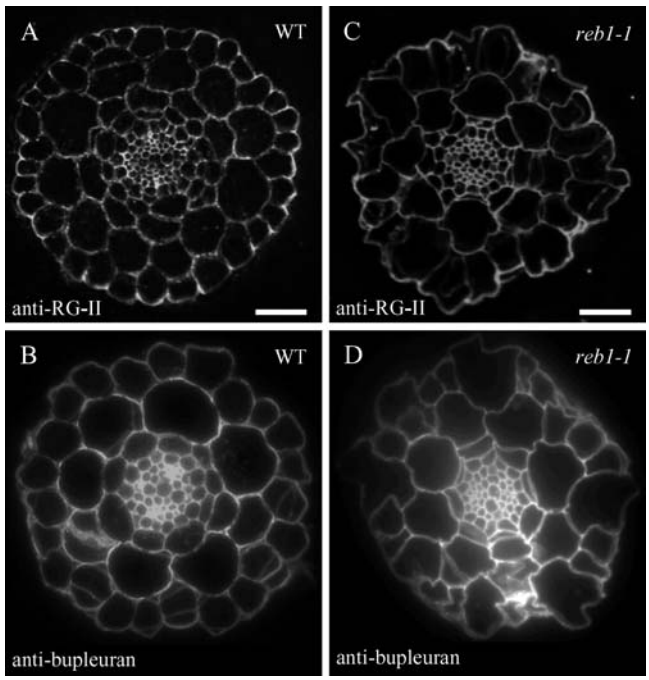

Figure 3. Fluorescence micrographs of cross sections in the root elongation zone showing staining with anti-pectin antibodies for wild type (A and B) and *reb1-1* (C and D). A and C, Anti-RG-II antiserum. B and D, Anti-bupleuran 2IIC antiserum. Bar = 30 μm.

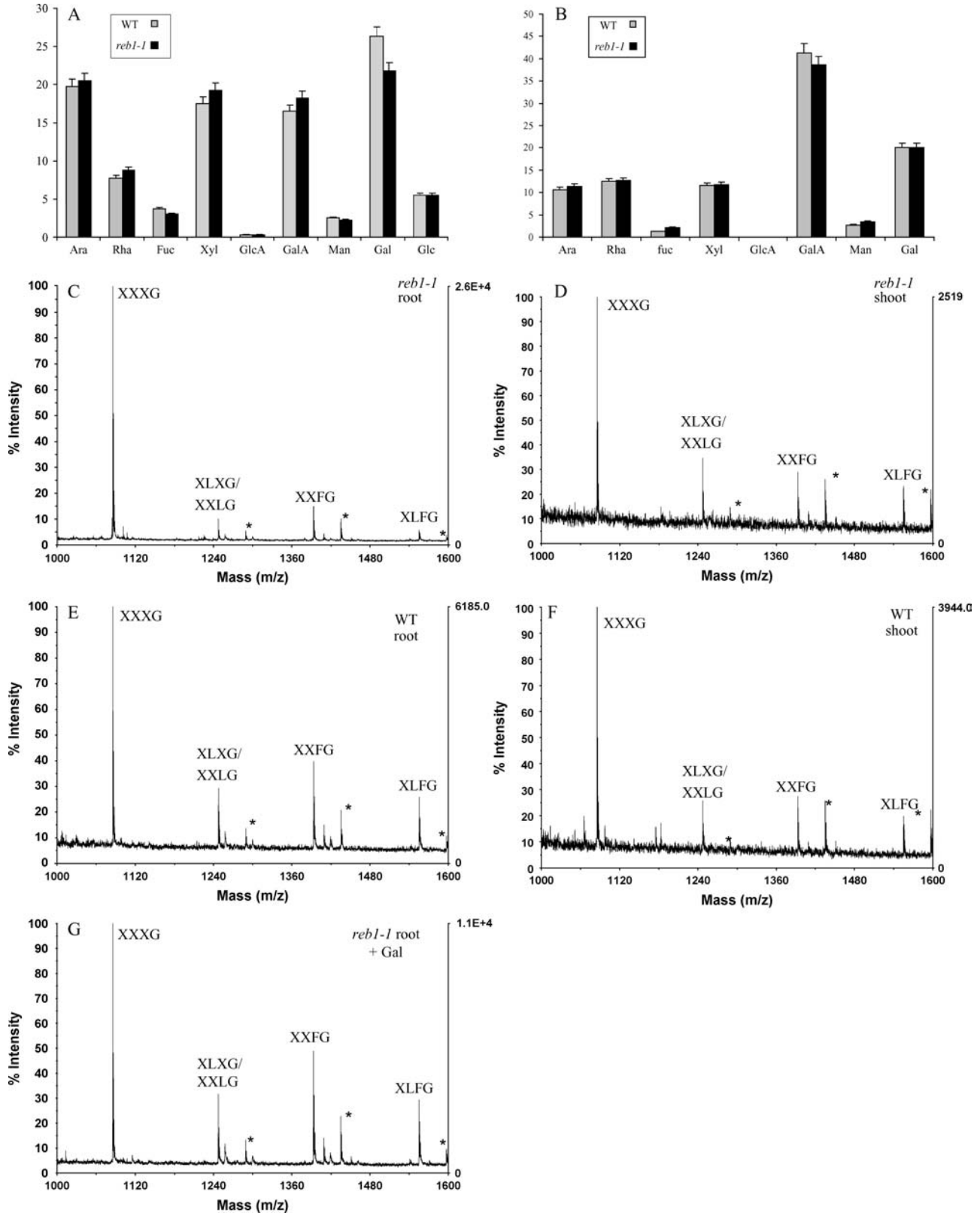

Figure 4. Monosaccharide composition and analysis of XyG structure released from the cell wall. A and B, Monosaccharide composition of crude cell wall material extracted from *reb1-1* and wild-type roots (A) and shoots (B). C to F, MALDI-TOF MS spectra of XyG oligosaccharides released from the wild-type and *reb1-1* cell wall isolated from root and shoot tissues. G, MALDI-TOF MS spectrum of XyG oligosaccharides released from the cell wall isolated from the root of *reb1-1* grown in the presence 10 mM D-Gal. Asterisks (*) indicate ions related to acetylated fragments.

between the wild-type and *reb1-1* shoot cell walls (Fig. 4B). Second, we investigated the structure of XyG, RG-I, and RG-II polysaccharides (see below).

Analysis of XyG Structure

To determine XyG structure, we used the enzymatic oligosaccharide fingerprinting strategy (Lerouxel et al., 2002; Ray et al., 2004) to determine the XyG structure. *reb1-1* and wild-type crude cell wall material from roots and shoots were treated separately with an endo-β-D-(1→4)-glucanase and the resulting fragments characterized using matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The ions corresponding to the fragments XXFG, XXLG/XLXG, and XLFG decrease dramatically in *reb1-1* mutant roots as compared to wild-type roots (Fig. 4, C and E; Table I). In contrast, no significant difference in the structure of XyG is found between wild-type and *reb1-1* shoots (Fig. 4, D and F). These results show that the *reb1-1* mutation specifically affects XyG structure in the root, but not in the shoot, consistent with the known alteration of morphology in the mutant specifically in the root. Interestingly, a complete recovery of a wild-type XyG structure occurs when *reb1-1* grows in the presence of 10 mM D-Gal (Fig. 4G).

To further assess XyG structure, we analyzed hemicellulosic fractions isolated from *reb1-1* and wild-type root cell wall material using 4 M KOH. It is worth noting that the 4 M KOH extract obtained from *reb1-1*, unlike from the wild type, contained two hemicellulosic subfractions: one soluble and the other insoluble. As shown in Figure 5, A, C, and D, soluble hemicellulosic fractions isolated from both wild-type and *reb1-1* roots share identical chemical features. Consistent with the previously reported structure of Arabidopsis XyG (Zablackis et al., 1995), the monosaccharide composition includes Fuc, Xyl, Gal, and Glc residues (Fig. 5A). Both high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) profiles (Fig. 5, C and D) and MALDI-TOF mass spectra (data not shown) of fragments generated by an endoglucanase treatment of soluble XyG fractions show the presence of XXXG, XXFG, XLXG, and XLFG as major oligosaccharides, with similar relative intensities in both wild-type and *reb1-1* preparations. As a consequence, we conclude that both wild-type and *reb1-1* soluble hemicelluloses consist of a XXXG-type XyG bearing α-D-Xylp (X), β-D-Galp-(1→2)-α-D-Xylp (L), or α-L-Fucp-(1→2)-β-D-Galp-(1→2)-α-D-Xylp (F) side chains in similar proportions.

The insoluble XyG fraction from *reb1-1*, which accounts for approximately 3% of the total XyG, strongly differs from soluble fractions. Sugar composition shows that the insoluble material consists mainly of Xyl and Glc in a 2.5:4 ratio (Fig. 5A). The HPAEC-PAD profile and the MALDI-TOF mass spectrum of the endoglucanase-generated fragments demonstrate that this XyG fraction is composed nearly exclusively of XXXG subunits (Fig. 5, B and E). The XXFG and XLFG fragments do not exceed 5% of the oligosaccharide population. As a consequence, we conclude that, in contrast to the wild type, *reb1-1* roots contain two types of XyG: a major species identical to the wild type and a minor species almost completely devoid of fucosylated and galactosylated side chains. Considering that trichoblast cells are not immunolabeled with the mAb CCRC-M1, specific for Fuc-containing XyG side chains, we propose that the XyG structure in *reb1-1* is cell type-specific with a wild-type structure in atrichoblasts and other inner tissues and a nongalactosylated (and thus a nonfucosylated) XyG in trichoblast cells.

Table I. *Comparison of relative abundance (percentage) of XyG oligosaccharides obtained by HPAEC-PAD from root cell walls of wild type, reb1-1, and reb1-1 grown in the presence of 10 mM D-Gal*

HPAEC-PAD signals representing the XyG fragments were integrated. Data represent the mean and SD of three independent experiments. HPAEC-PAD profiles are presented as supplemental data (Supplemental Fig. 1).

XyG Fragments	XXXG	XXFG	XLXG	XXLG/XLFG
Wild-type roots	42.5 ± 3	29.1 ± 1	17.2 ± 3	11.2 ± 3
reb1-1 roots	77.3 ± 3	12.6 ± 3	5.5 ± 1	4.6 ± 2
reb1-1 roots + Gal	44.0 ± 3	29.4 ± 1	16.6 ± 1	10.0 ± 3

Analysis of RG-I and RG-II

Pectic polysaccharides were extracted from alcohol-insoluble residue (AIR) following treatment by endo-polygalacturonase (EPG) as previously described (Ishii et al., 2001). The purified EPG-soluble fraction was then separated by size-exclusion chromatography (SEC) gel permeation into RG-I and RG-II polysaccharide fractions. In both chromatograms of wild-type and *reb1-1* root cell walls, RG-I is the main polysaccharide present, whereas RG-II occurs as a minor polysaccharide, mostly under its dimer form (dRG-II; Fig. 6). In addition, the ratio between RG-I and RG-II polysaccharides as well as dimerization levels of RG-II are similar in both wild type and *reb1-1* (Fig. 6).

The monosaccharide composition of RG-I and d-RG-II isolated from root cell walls is similar between the *reb1-1* mutant and the wild type (Tables II and III). In addition, no differences were found in a liquid chromatography-MS analysis of the B chains of RG-II released by mild acid treatment (data not shown). Together, these results indicate that the *reb1-1* mutation has no effect on the structure of the two major Gal-containing pectic polysaccharides of the cell wall, RG-I and RG-II.

DISCUSSION

The *reb1-1* plant is deficient in one of the five Glc epimerases that synthesize D-Gal and is known to produce altered AGPs (Andème-Onzighi et al., 2002; Seifert et al., 2002). The goal of this study was to

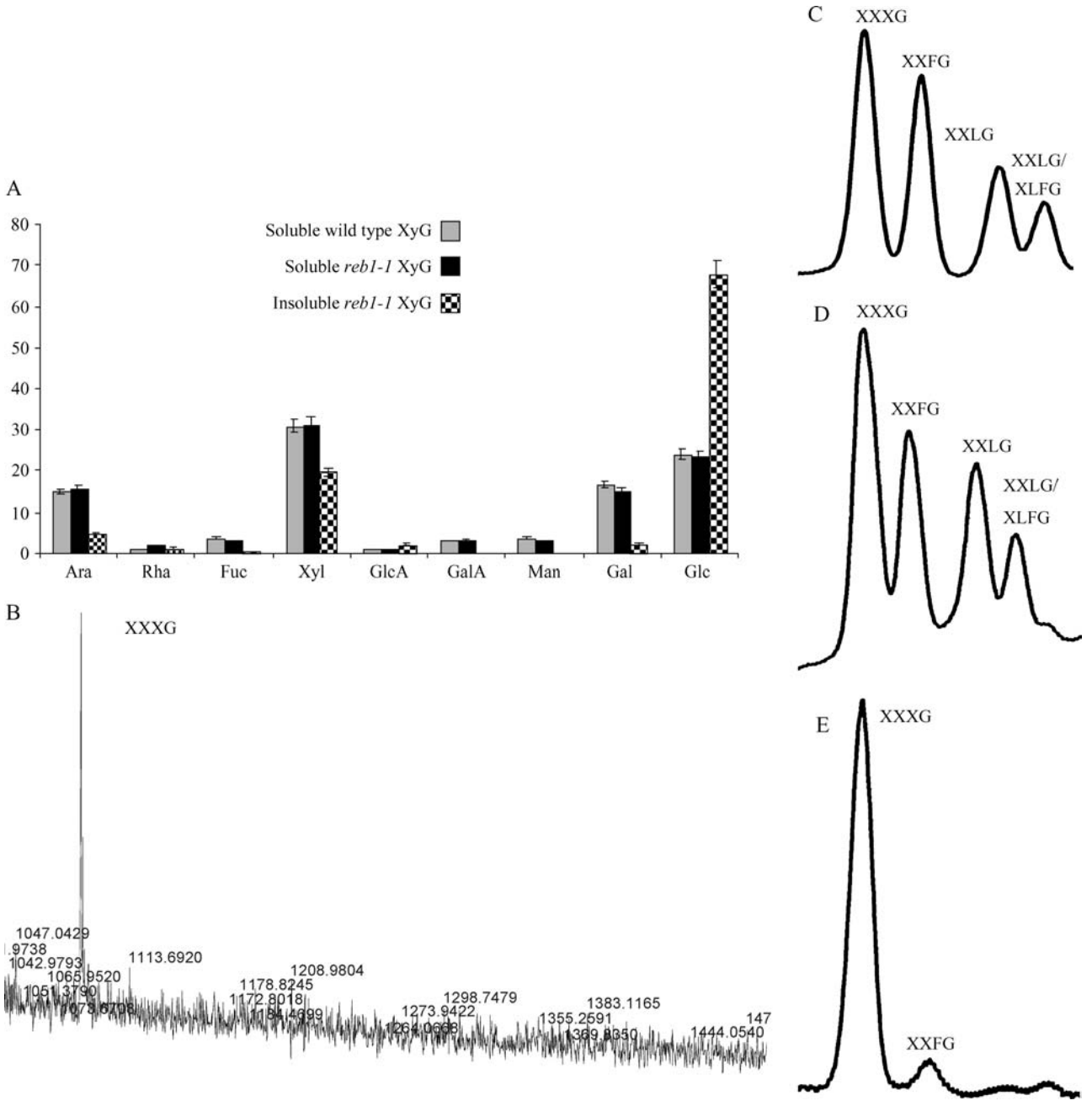

Figure 5. Monosaccharide composition and analysis of XyG structure released from 4 M KOH hemicellulosic fractions. A, Monosaccharide composition of soluble and insoluble hemicellulosic fractions isolated from the wild type and *reb1-1*. B, MALDI-TOF MS spectra of XyG oligosaccharides released from *reb1-1*-insoluble hemicellulosic fraction. C and E, HPAEC-PAD profiles of XyG oligosaccharides released from soluble hemicellulosic fractions of wild type (C) and *reb1-1* (D) and from insoluble hemicellulosic fraction of *reb1-1* (E).

determine the effect of the *reb1-1* mutation on the structure and cellular localization of three major Gal-containing cell wall polymers, XyG, RG-I, and RG-II. Our principal findings are: (1) *reb1-1* mutant root cells make two types of XyG that are structurally different: a typical, wild-type polymer and a polymer that is insoluble and devoid of Gal and Fuc residues; (2) swollen trichoblasts do express galactosylated epitopes associated with pectins, but not fuco-galactosylated epitopes associated with XyG; and (3) the structure of the pectic polysaccharides RG-I and RG-II is unchanged. Thus, the *reb1-1* mutation appears to affect only a certain type of cell wall polymer (i.e. XyG and AGP). These findings suggest that UGE4 might be involved specifically in the galactosylation of XyG and AGPs, but not in that of pectic polysaccharides, and link galactosylation of XyG, in addition to AGPs, to cell expansion and morphogenesis.

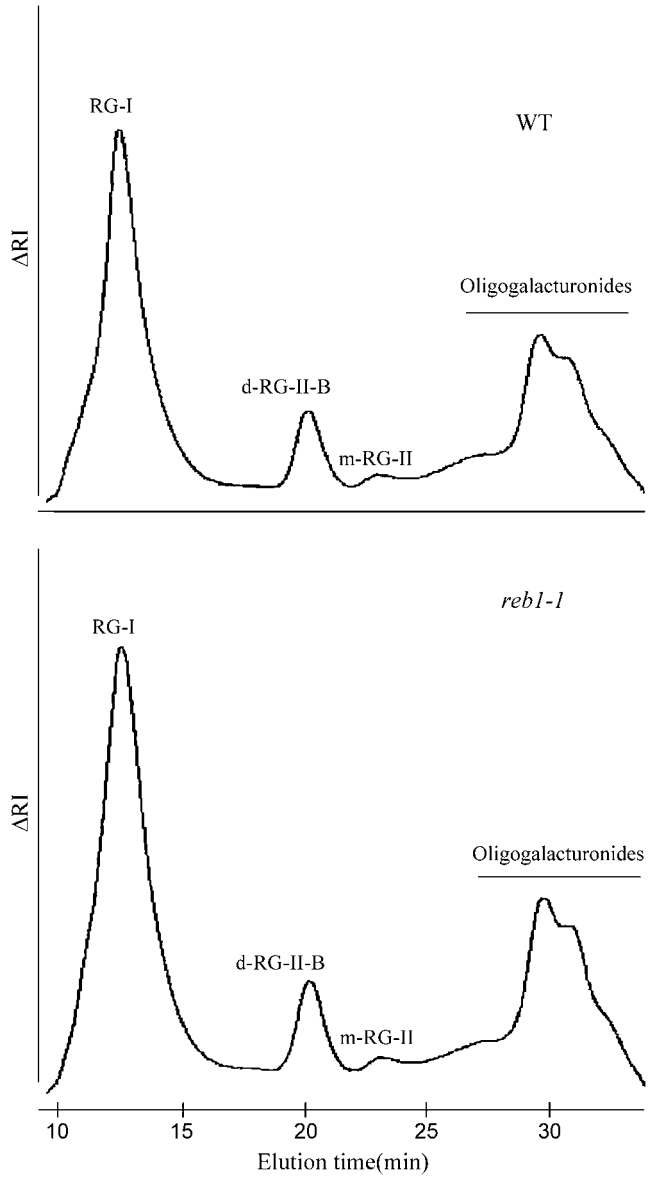

Figure 6. SEC-refractive index profiles of the material released following EPG treatment of wild-type and *reb1-1* root cell walls.

Galactosylation of XyG Is Possibly Required for Cell Expansion

Using an enzymatic fingerprinting strategy, we show that *reb1-1* root cells produce an abnormal XyG in addition to the normal one. The abnormal XyG lacks α-L-Fucp-(1→2)-β-D-Galp substitutions and has the feature of being water insoluble. Additionally, and as shown previously (Seifert et al., 2002), immunolabeling shows that swollen trichoblasts are not stained with CCRC-M1. Therefore, we conclude that the synthesis of the abnormal XyG may occur predominantly, if not exclusively, in swollen trichoblasts, although we cannot exclude a change in XyG structure in some other tissues in the mutant. XyG is known to tether cellulose microfibrils in the primary wall, resulting in an extensive cellulose-XyG network that acts as the major tension-bearing structure (Hayashi, 1989; Carpita and Gibeaut, 1993). In muro reorganization of XyG, cross-links are believed to be a key factor in controlling wall strength and extensibility and, therefore, the directionality of cell expansion (Fry, 1995; Cosgrove, 2001).

The *reb1-1* mutant can now be added to the list of Arabidopsis mutants with altered XyG structure, even though this occurs only in a specific set of root cells. Two other mutants that make altered XyG are *mur2* and *mur3* (Vanzin et al., 2002; Madson et al., 2003). *MUR2* and *MUR3* genes encode for, respectively, fucosyl- and galactosyl-transferases that are specific for XyG (Vanzin et al., 2002; Madson et al., 2003). Mutation of the *MUR2* gene leads to the absence of XyG fucosylation (Vanzin et al., 2002), whereas mutation of *MUR3* leads to the absence of Fuc-Gal from the first Xyl residue and an increase in galactosylation at the middle Xyl in leaves, but not in etiolated hypocotyls (Madson et al., 2003). The only visible phenotype detected is in hypocotyls of dark-grown *mur3* seedlings, which exhibit a marked swelling of epidermal and cortical cells at the base along with a significant loss of tensile strength (<40% that of wild type). The XyG of these cells, unlike that in leaves, has no increased galactosylation of the middle Xyl residues. Our results (see above) indicate that the swollen trichoblasts lack galactosylated XyG as well. These observations on *reb1-1* roots and *mur3*-etiolated hypocotyls point to a possible link between the galactosylation of XyG and the control of cell expansion. Although tensile strength was not measured in *reb1-1*, it is reasonable to think that the lack of galactosylation of XyG, in addition to that of AGPs, induces a reduction in the mechanical strength of the cell wall in trichoblasts as it has been found for epidermal cells of *mur3*-etiolated hypocotyls. However, unlike XyG, AGPs have been shown to be required for anisotropic cell expansion (Willats and Knox, 1996). Therefore, the fact that bulging of trichoblasts in *reb1-1* is much more pronounced than swelling of the *mur3*-etiolated hypocotyl cells may be due more to the structural alteration of AGPs than to that of XyG.

Table II. *Composition of neutral sugars (mol %) of RG-I isolated from roots of wild type and reb1-1*

nd, Not detectable.

Glycosyl Residues	RG-I	
	Wild Type	*reb1-1*
Ara	34	31
Rha	10	12
Xyl	8	9
Fuc	8	8
Gal	40	40
Api	nd	nd
2-*O*-Me-Fuc	nd	nd
2-*O*-Me-Xyl	nd	nd

Table III. *Monosaccharide composition (mol %) of RG-II isolated from roots of wild type and reb1-1*

Glycosyl Residues	RG-II	
	Wild Type	*reb1-1*
Ara	18	16
Rha	9	9
Xyl	3	3
Fuc	3	3
Gal	18	19
Api	5	5
2-*O*-Me-Fuc	2	3
2-*O*-Me-Xyl	3	3
GalA	31	32
GlcA	3	2
Kdo	1	1
Dha	2	2
Aceric A	2	2

Changes in the mechanical properties of the cell wall during turgor-driven expansion is dependent on several loosening enzymes, including XyG-endotransglycosidase and expansins (Fry, 1995; Cosgrove, 2001), whose action could be modulated by the structure of the substrate (Nishitani and Tominaga, 1992; Catalá et al., 2001). Thus, the altered structure of XyG in *reb1-1* may well influence the activity of wall-loosening enzymes, which in turn would contribute to a decrease in tensile strength of trichoblasts. The abnormal XyG in *reb1-1* trichoblasts is ungalactosylated and is water insoluble, two features that have been shown to reduce strongly the accessibility of the XyG-endotransglycosidase to its substrate in the *mur3* hypocotyl (Peña et al., 2004). Thus, besides AGP, galactosylation of XyG could contribute indirectly to the regulation of anisotropic cell expansion and growth.

UGE4 Is Confined to a Protein Complex Specifically Involved in the Galactosylation of XyG and AGPs: A Hypothesis

Cell wall matrix polysaccharides and proteoglycans are synthesized in the Golgi apparatus and transported via secretory vesicles to the cell surface (Driouich et al., 1993). This requires the action of a set of Golgi glycosyltransferases, in addition to nucleotide sugar transporters and nucleotide sugar interconversion enzymes (Keegstra and Raikhel, 2001; Seifert, 2004). It has been postulated that these partners could interact physically to form complexes within Golgi membranes to coordinate sugar supply and polymer synthesis (Seifert, 2004). The *REB1/RHD1* gene encodes one of five UDP-D-Glc 4-epimerase isoforms, UGE4, which is responsible for the conversion of UDP-D-Glc into UDP-D-Gal (Seifert et al., 2002). The observation that the *reb1-1* mutation affects the galactosylation of AGPs (Andème-Onzighi et al., 2002) and XyG (this study; Seifert et al., 2002) specifically in trichoblasts indicates that the supply of UDP-D-Gal for the biosynthesis of these polymers is regulated at the cell-type level. Thus, it is postulated that, in *reb1-1*, ungalactosylated XyG and AGPs are associated with trichoblasts, but not with atrichoblasts. This could be explained by a possible compensation for the lack of UGE4 in atrichoblasts by the expression of a functional *UGE1* gene, an isoform shown to be strongly expressed in all root cells of the mutant, including the epidermis (Seifert et al., 2004), although it is not clear why such compensation might occur in atrichoblasts, but not in trichoblasts. An explanation is that the UGE1 enzyme in *reb1-1* trichoblasts could be specifically involved in channeling Gal to other polymers, such as pectins, rather than XyG and AGPs. Factors in support of this include (1) the observation that Gal-containing pectic epitopes revealed by the anti-bupleuran 2IIC antibodies are present in *reb1-1* trichoblasts and (2) the finding that Gal content of isolated RG-I is unchanged. Additionally, our chemical analysis of RG-II molecules demonstrates that neither Gal content nor dimerization is affected by the *reb1-1* mutation. Therefore, we hypothesize that UGE4 might be specifically involved in channeling of UDP-D-Gal to XyG and molecules of the arabinogalactan-II type (e.g. AGPs), whereas another isoform, possibly UGE1, would be responsible for providing Gal to pectic polysaccharides.

This hypothesis implies the occurrence of different biosynthetic complexes containing different UGE isoforms in addition to specific galactosyltransferases and UDP-D-Gal transporters. From our hypothesis, it follows that UGE4 is confined to complexes specifically involved in galactosylation of XyG and AGP. The UGE1 isoform would be confined to another complex involved in pectin galactosylation, including the addition of galactans to RG-I. Also, it is possible that such complexes might be associated with different Golgi cisternae, which would explain why some sugar epitopes of complex polysaccharides are added in some cisternae, but not in others (Zhang and Staehelin, 1992; Driouich et al., 1993). In mammalian cells, the formation of such complexes has been demonstrated for 2-*O*-sulfotransferase and uronosyl 5-epimerase, two enzymes involved in glycosaminoglycan biosynthesis (Pinhal et al., 2001).

Our hypothesis of a specific biosynthetic complex involved in XyG galactosylation is supported by transcript-profiling data comparing the expression of *UGE4* (At1g64440), *MUR2* (At2g03220), and *MUR3* (At2g20370) from which it emerges that these genes have identical patterns of transcription (http://jsp.weigelword.org/atgendev/atgen.jsp). Likewise, the three genes are coexpressed in various experiments related to root development, as revealed by the two-gene scatter-plot tool from NASCArray (http://affymetrix.arabidopsis.info/narrays/twogenescatter.pl). The coordinated expression of *UGE4* with two other genes involved in XyG synthesis is consistent with our hypothesis of a complex of the polymer- and sugar-synthesizing enzymes. In contrast, using the same tools, we found that the other *UGE* genes (*UGE1*: At1g1278; *UGE2*: At4g23920; and *UGE3*: At1g63180) have distinct expression profiles compared with *MUR2* and *MUR3*.

Clearly, this hypothesis could be confirmed or refuted by detailed work combining electron microscopy and immunocytochemistry of the Golgi polysaccharide biosynthetic complexes along with assays of protein-protein interaction among the putative components.

MATERIALS AND METHODS

Plant Growth Conditions

Two lines of Arabidopsis (*Arabidopsis thaliana*) L. (Heynh.) were used: the wild-type Columbia and the *reb1-1* mutant (Baskin et al., 1992). Growth conditions were identical to those described by Andème-Onzighi et al. (2002). For phenotype recovery experiments, the culture medium of the *reb1 -1* mutant was supplemented with 10 mM D-Gal.

Cell Wall Extraction, Isolation of Hemicellulosic Fractions, and Digestion with Endoglucanase

Frozen root material (5 g) was heated at 70°C for 15 min in 70% (v/v) ethanol to inactivate enzymes. The roots were ground in a potter homogenizer, and the homogenate was washed twice with hot 70% (v/v) ethanol and once with water. The remaining AIR pellet was then freeze dried. The lyophilized AIR was used for the determination of monosaccharide composition by using GC and for the extraction of hemicellulosic wall polymers. To extract a hemicellulosic fraction, AIR was treated with boiled ammonium oxalate at 0.5% (2 × 1 h) followed by incubation in 4 M KOH overnight at room temperature as described by Ray et al. (2004). Two fractions were distinctly obtained from each sample (wild type, *reb1-1*, and *reb1-1* + 10 mM Gal): One is a soluble fraction (4 M KOH-soluble hemicellulosic fraction) and one is an insoluble fraction (4 M KOH-insoluble hemicellulosic fraction). It is worth noting that, unlike in the wild type and *reb1-1* + 10 mM Gal, only the biochemical analysis of the 4 M KOH-insoluble hemicellulosic fraction from *reb1-1* roots revealed the presence of XyG. The amount of the insoluble XyG in *reb1-1* roots was determined to be 3% of the total root XyG by quantification of monosaccharides from GC of the soluble and insoluble fractions.

XyG oligomers were generated either from AIR extract or from 4 M KOH hemicellulosic fractions after enzymatic digestion with endo-β-D-(1→4)-glucanase (EC 3.2.1.4; catalog no. E–CELTR; Megazyme) as described previously (Lerouxel et al., 2002; Ray et al., 2004).

Extraction and Analysis of RG-I and RG-II

Extraction of pectic material and further purification of RG-I and RG-II were performed as described by Ishii et al. (2001). Briefly, 3 g of frozen roots were suspended in aqueous 80% (v/v) heated ethanol and centrifuged at 2,500 rpm for 5 min. The insoluble residue was washed with 80% to 95% to 100% (v/v) ethanol, chloroform:methanol (1:1, v/v), acetone, and then air dried. The AIR was treated for 4 h at 4°C with 0.1 N NaOH to saponify the methyl and acetyl esters. The suspensions were adjusted to pH 5 with 10% (v/v) glacial acetic acid and then treated for 16 h at 30°C with a homogeneous preparation of EPG from *Aspergillus niger* (2.5 units; Megazyme). The suspensions were centrifuged and the insoluble residues washed with water. The EPG-soluble fractions were dialyzed (1-kD cutoff dialysis tubing) against deionized water and freeze dried. RG-I and RG-II polysaccharides were purified from the EPG-solubilized material by elution from SEC on a Sephadex G-75 (2.5 × 90 cm) and then a Superdex Prep 75 (Amersham-Pharmacia Biotech; 1.6 × 38 cm) column. SEC-refractive index was performed with a Shimadzu LC-10A system with a refractive index detector (model RID-10A; Shimadzu) connected to a Sephadex G-75 column or a Superdex-75 HR 10/30 column eluted at 0.6 mL min^{-1} with 50 mM ammonium formate, pH 5.3, as previously described (Ishii and Matsunaga, 1996). The presence of mRG-II and dRG-II-B in the EPG digests was determined by comparing their retention times with those of the authentic mRG-II and dRG-II-B from sugar beet (*Beta vulgaris*) and red wine.

Monosaccharide Composition Analysis

Monosaccharide composition of crude cell wall (AIR) and hemicelulosic fractions was determined as previously described by Ray et al. (2004). Neutral glycosyl composition of RG-I was determined by GC of their alditol acetate derivatives (York et al., 1986). Combined neutral and acidic glycosyl composition of RG-II was determined by GC of their trimethylsilyl methyl ester methyl glycoside derivatives (York et al., 1986).

HPAEC-PAD

Endoglucanase-generated XyG fragments were analyzed by HPAEC (DX 500 system; Dionex) equipped with a CarboPac PA-1 column and a GP 50 gradient pump. XyG fragments were separated using a gradient from 100 mM NaOH (solvent A) to 1 M NaOAc in 100 mM NaOH (solvent B) at 1 mL min^{-1} using the following conditions: 0 min, 100% A; 5 min, 95% A; and 30 min, 92% A. Assignments of peaks to XyG fragments were carried out according to published data (Vincken et al., 1996) and by comparison of their retention times with major XyG oligosaccharides prepared by digestion of XyG from tamarind with endoglucanase (see Lerouxel et al., 2002).

MALDI-TOF MS Analysis

MALDI-TOF mass spectra of the XyG fragments solubilized by endoglucanase were acquired on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems) equipped with a 337-nm nitrogen laser. Mass spectra were performed 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 30%:70% acetonitrile/0.1% trifluoroacetic acid, was mixed with the solubilized oligosaccharides in a ratio of 1:1 (v/v). These spectra were recorded in a positive mode, using an acceleration voltage of 20,000 V with a delay time of 100 ns. They were smoothed once and externally calibrated using commercially available mixtures of peptides and proteins (Applied Biosystems). In this study, the MALDI-TOF mass spectra of XyG oligosaccharides were calibrated using des-Arg-1-bradykinin (904.4681 D), angiotensin I (1296.6853), Glu-1-fibrinopeptide B (1570.6774 D), ACTH clip 18 to 39 (2465.1989), and bovine insulin (5730.6087). Laser shots were accumulated for each spectrum to obtain an acceptable signal-to-noise ratio.

Immunofluorescence Microscopy

Fixation, embedding, immunolabeling, and microscopy were performed as previously described (Andème-Onzighi et al., 2002). The antibodies were used at 1:10 for CCRC-M1 and anti-XyG (originally designated as anti-XG; see Moore and Staehelin, 1988), at 1:5 for anti-RG-II and LM5, and at 1:200 for anti-bupleuran 2IIC.

Immersion immunofluorescence staining of XyG epitopes was done on roots after a brief fixation with 4% paraformaldehyde according to the procedure of Willats et al. (2001).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At1g64440 for the *REB1/RHD1* gene.

ACKNOWLEDGMENTS

We wish to give special thanks to T. Baskin (University of Massachusetts) for his valuable comments and helpful suggestions on the manuscript as well as for the provision of the *reb1-1* seeds. We also acknowledge M. Hahn (University of Georgia), A. Staehelin (University of Colorado), and H. Yamada (Kitasato Institute at Tokyo) for gifts of antibodies. We are grateful to O. Lerouxel, M. Seveno, and C. Rihouey (University of Rouen) for help and advice throughout this work, as well as A. Faik (University of Ohio) for critical reading of the first version of the manuscript.

Received December 2, 2005; revised February 14, 2006; accepted February 14, 2006; published February 24, 2006.

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