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Monoclonal antibodies against methyl glucuronoxylan epitopes for understanding xylan structure and modification

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Abstract: Lignocellulosic biomass is an abundant and renewable source that could be utilized for production of many bioproducts. It mainly consists of cellulose, hemicellulose, and lignin, all of which come together to form a recalcitrant wall that impedes production of bioproducts. In order to overcome the recalcitrant nature of the biomass, it is important to fully understand how cell wall components are synthesized and assembled. Xylan is one of the hemicellulosic components of the wall that contributes to recalcitrance. Xylan has substitutions on the backbone that are formed by the orchestration of many enzymes. For example, glucuronoxylan methyltransferases are responsible for 4-O-methylation of glucuronic acid (GlcA) side chains on xylan. Mutant lines are valuable sources to understand the structure and importance of xylan. Here, we investigated the wild-type and *gxm1gxm2gxm3* triple mutant of Arabidopsis by employing a set of four xylan antibodies that specifically recognize methyl glucuronoxylan epitopes on xylan to prove their epitope. The triple mutant cell walls were observed with methyl glucuronoxylan specific monoclonal antibodies for the first time. Cell-wall-directed antibodies could help further our understanding on the synthesis and structure of plant cell walls.

Key words: Antibodies, Arabidopsis, cell wall, methyl glucuronoxylan, xylan

1. Introduction

Cellulosic biomass is a renewable source for production of many products such as ethanol that could be utilized in biofuels (Blanch et al., 2011). Polysaccharides in plant cell walls could be converted into monosaccharides that could be used to produce value-added bioproducts. However, the plant cell walls are recalcitrant in their nature, making them difficult to process for production, which increases the cost of final products (Liu et al., 2021). Therefore, it is important to understand how cell wall components are synthesized and distributed in the wall in order to design cheaper bioprocessing technologies.

Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin, all of which come together to form a recalcitrant wall. Xylan is a hemicellulosic component of plant cell walls that contributes to the recalcitrance (Himmel et al., 2007). Xylan has a backbone of a linear chain of β-1,4-linked xylosyl residues and the backbone may be substituted with glucuronic acid (GlcA), methylglucuronic acid (MeGlcA), and arabinose in dicotyledonous plants (Rennie and Scheller, 2014). Acetylation of xylan residues is also common and related to biomass recalcitrance (Pawar et al., 2017).

Xylan biosynthesis requires over a dozen of enzymes that work together to synthesize xylan in Golgi (Rennie and Scheller, 2014; Busse-Wicher et al., 2016). GlcA and MeGlcA substitutions on xylan backbone are accomplished by GUX1, GUX2, and GUX3 proteins (Lee et al., 2012). Gluruconoxylan Methyl Transferase (GXMT) proteins are responsible for 4-O-methylation of GlcA sidechains (Urbanowicz et al., 2012). DUF579 domain-containing proteins, namely GXM1 (At1g09610), GXM2 (At4g09990), GXM3 (At1g33800), are expressed in secondary cell-wall-forming cells in Arabidopsis. Mutations of *GXM* genes lead to a loss of methylation of GlcA side chains on xylan (Lee et al., 2012; Cornuault et al., 2015). The triple mutant *gxm1gxm2gxm3* has no detectable methylation of xylan GlcA (Cornuault et al., 2015). Acetylation on xylan could be carried out by Reduced Wall Acetylation (RWA) proteins, Trichome Birefringence-Like (TBL) proteins, the Altered XYloglucan 9 (AXY9) protein, and GDSL acetylesterases of xylan (Qaseem and Wu, 2020). Mutations in the genes that contribute to the side chain formation on xylan backbone could help us better understand how these proteins work together to accomplish xylan biosynthesis.

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It is also important to reveal how different cell types regulate the transcription of the genes.

Plant-cell-wall-directed monoclonal antibodies are powerful tools to understand the presence and localization of cell wall polysaccharides (Pattathil et al., 2010). There are over 200 such monoclonal antibodies produced by different groups over the years. Among them, there are four antibodies that specifically recognize methyl glucuronoxylan epitopes on xylan. These antibodies are CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 that recognize the methylation of GlcA substitutions on xylan side chains (Ruprecht et al., 2017). These antibodies could help us understand how side chain substitutions take place.

In this study, four antibodies are localized in wild-type and *gxm1gxm2gxm3* triple mutant in Arabidopsis. The triple mutant cell walls are observed with methyl glucuronoxylan specific monoclonal antibodies for the first time. The triple mutant lacks methyl groups on GlcA substitutions and gives us further proof that CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 are indeed specific for methyl glucuronoxylan epitopes. Such use of cell-wall-directed antibodies may help further our understanding on the synthesis and structure of plant cell walls.

2. Materials and methods

2.1. Plant growth and tissue processing

Arabidopsis thaliana wild-type (WT, Columbia) and a xylan mutant line were used. The triple mutant, *gxm1gxm2gxm3* was previously generated by crossing single mutants (Cornuault et al., 2015) and obtained from the laboratory of Paul Dupree (University of Cambridge, Canbridge, UK). Seeds from WT and the triple mutant line were sown on soil containing compost with vermiculite and perlite. Germinated plants were grown for 5 weeks on at 20 °C (100μmol m^{-2}·s^{-1}, 16 h light/8 h dark, 60% humidity).

At 5 weeks, the basal part of the inflorescence stem was used for tissue processing. About 1 cm cuts from basal inflorescence stems were made with a razor blade and immediately fixed in 4% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.1 for 4 h on ice. Tissues were then washed with buffer twice for 10 min, washed with water once for 10 min, and dehydrated through a graded ethanol series (30%, 50%, 70%, 95%, 100%, and 100% [v/v] ethanol) for 30 min each step. The dehydrated tissues were gradually infiltrated with LR White embedding resin (Ted Pella, Redding, CA, USA) using 33% (v/v) and 66% (v/v) resin in 100% ethanol for 12 h each, followed by 100% resin for 12 h three times. The infiltrated tissues were put into gelatin capsules containing 100% resin and polymerized under 365-nm UV light at 4 °C for 48 h.

2.2. Sectioning and immunolabeling

Semithin cross sections (250 nm) from stems of both samples were cut by a Leica UC7 ultramicrotome (Leica, Vienna, Austria) and mounted on Frost Plus glass slides (Thermo Fisher Scientific, Waltham, MA, USA). A section from each sample were stained with toluidine blue (0.05% w/v in water) to observe stem anatomy.

Immunolocalization experiments were conducted as described in Avci et al. (2012). A base treatment, 1M KOH (w/v) was used to unmask the epitope of antibodies for 15 min at room temperature in some sections before proceeding to the next steps. All sections were blocked with 3% (w/v) nonfat dry milk in KPBS (0.01M potassium phosphate, pH 7.1, containing 0.5M NaCl) for 30 min followed by washing three times with KPBS for 5 min. Four monoclonal antibodies (undiluted) were applied on sections for 60 min. The sections were washed with KPBS three times for 5 min each. A goat antimouse IgG conjugated to Alexa-fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:100 in KPBS was applied and incubated for 60 min, followed by washing with KPBS for 5 min and distilled water for 5 min. A control experiment is run without a primary antibody with the same steps. Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA, USA) was applied and sections were observed under an Eclipse 80i microscope (Nikon, Melville, NY, USA) equipped with epifluorescence optics. Captured images were assembled together using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

3. Results

3.1. Epitope structure of methyl glucuronoxylan specific antibodies

Xylan backbone could be substituted with GlcA or MeGlcA and could be acetylated in Arabidopsis as shown in Figure 1. A synthetic glycan microarray study revealed that CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 bind exclusively to 4-O-methyl GlcA present on xylan sidechains (Ruprecht et al., 2017). Figure 1 illustrates the epitope for these antibodies as they specifically bind to methylated GlcA. They do not bind to unmethylated GlcA or they might not show binding if there are acetyl groups present in MeGlcA. Presumably, removal of acetyl groups by applying base treatment might help antibody to recognize the epitopes. In fact, xylan is heavily acetylated in Arabidopsis (Lee et al., 2014).

3.2. Immunolocalization of methyl glucuronoxylan specific antibodies

In order to show the epitopes that CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 recognize, WT and *gxm1gxm2gxm3* triple mutant were used for immunolocalization. First, sections from both stems were

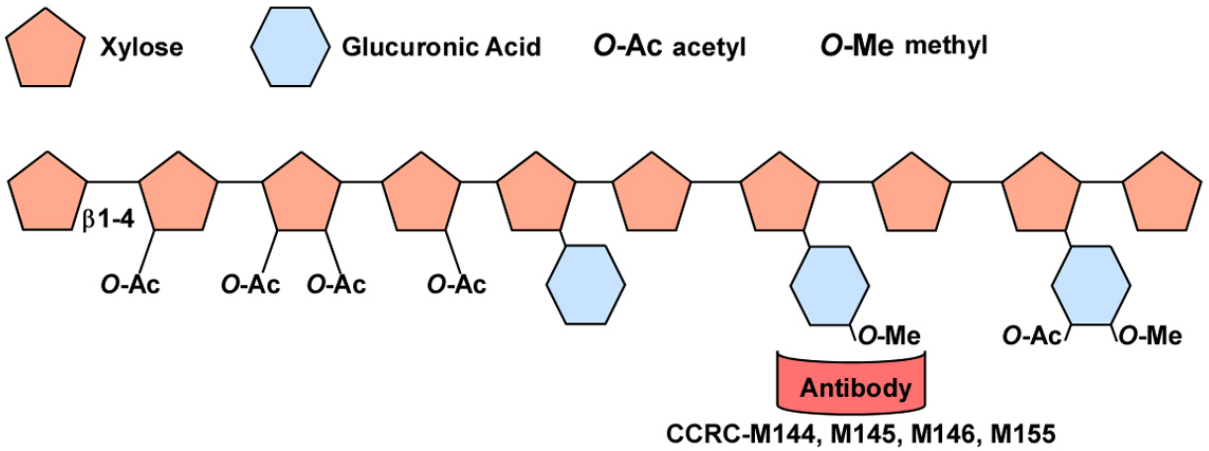

Figure 1. Illustration of xylan structure in Arabidopsis. CCRC-M144, 145, 146, and 155 can only recognize the MeGlcA side chain of xylan without any acetyl group. Antibodies do not recognize GlcA without methyl group.

stained with Toludine blue to show overall stem anatomy in Arabidopsis lines (Figures 2A and 2B). In Figure 2A, the name of the main cell types can be seen. In Arabidopsis stem, xylem (xy) and interfascicular fiber (iff) cells have secondary cell walls. Epidermis (ep), cortex (c), phloem (ph), and pith (p) cells have primary cell walls. Xylan is only present in the secondary cell walls of Arabidopsis. Immunolocalization experiments (Figures 2C–2K) were conducted on serial sections taken from the same area and represent very close proximity to the sections stained with Toluidine blue.

CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 did not bind to stem sections taken from WT (Figures 2C, 2E, 2G, 2J) and the triple mutant (Figures 2D, 2F, 2H, 2K). The epitope of antibodies was masked. Since the antibodies were created based on extractions with base (Pattathil et al., 2010), a base treatment (1M KOH) was applied to the sections. The base treatment presumably removes ester groups and acetyl substitutions on xylan (Marcus et al., 2010). After 1M KOH treatment, antibodies recognized the epitopes in xylem and interfascicular fiber cell walls (Figure 2, C_{base}, E_{base}, G_{base}, J_{base}). A control experiment was run by omitting primary antibodies and the results (data not shown) were the same as those of WT (Figures 2C, 2E, 2G, 2J) and the triple mutant (Figures 2D, 2F, 2H, 2K).

In order to prove that antibodies, in fact, recognize only MeGlcA, the same base treatment was applied to the stem sections from *gxm1gxm2gxm3* triple mutant (Figure 2 D_{base}, F_{base}, H_{base}, K_{base}). After the base treatment, there was no labeling of antibodies as expected since the triple mutant has no detectable MeGlcA (Cornuault et al., 2015), which further proved that the exact epitope of CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 antibodies is methyl glucuronoxylan.

4. Discussion

Xylan is one of the major polysaccharides that contribute to the biomass recalcitrance, which impedes efficient and cost-effective processes such as biofuel production (Himmel et al., 2007; Qaseem and Wu, 2020). Therefore, in order to reduce biomass recalcitrance, it is imperative to know the structure and the synthesis of xylan. GXMs are glucuronoxylan methyltransferases that are responsible for the normal rate of GlcA methylation on xylan. There are three GXMs in Arabidopsis and each is functionally redundant glucuronoxylan methyltransferase (Lee et al., 2012). Therefore, a triple mutant is produced to have complete loss of methylation on glucuronoxylan side chains of xylan (Cornault et al., 2015). Mutating a GXM gene enhances xylan release during mild hydrothermal pretreatment (Urbanowicz et al., 2012), which indicates that loss of methylation on xylan could make plants less recalcitrant.

Acetylation of xylan is regulated and also linked to biomass recalcitrance. Therefore, proteins that are involved in xylan acetylation are targets to reduce biomass recalcitrance. Deacetylation of xylan in aspen and rice increases saccharification efficiency (Pawar et al., 2017, Zhang et al., 2017). Binding of CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 antibodies to their epitopes might be affected by the presence of acetyl groups. So far, there have been no antibodies that can recognize acetyl epitopes on xylan. However, the complete effects of xylan acetylation on biomass recalcitrance are not yet fully understood. Production of such antibodies

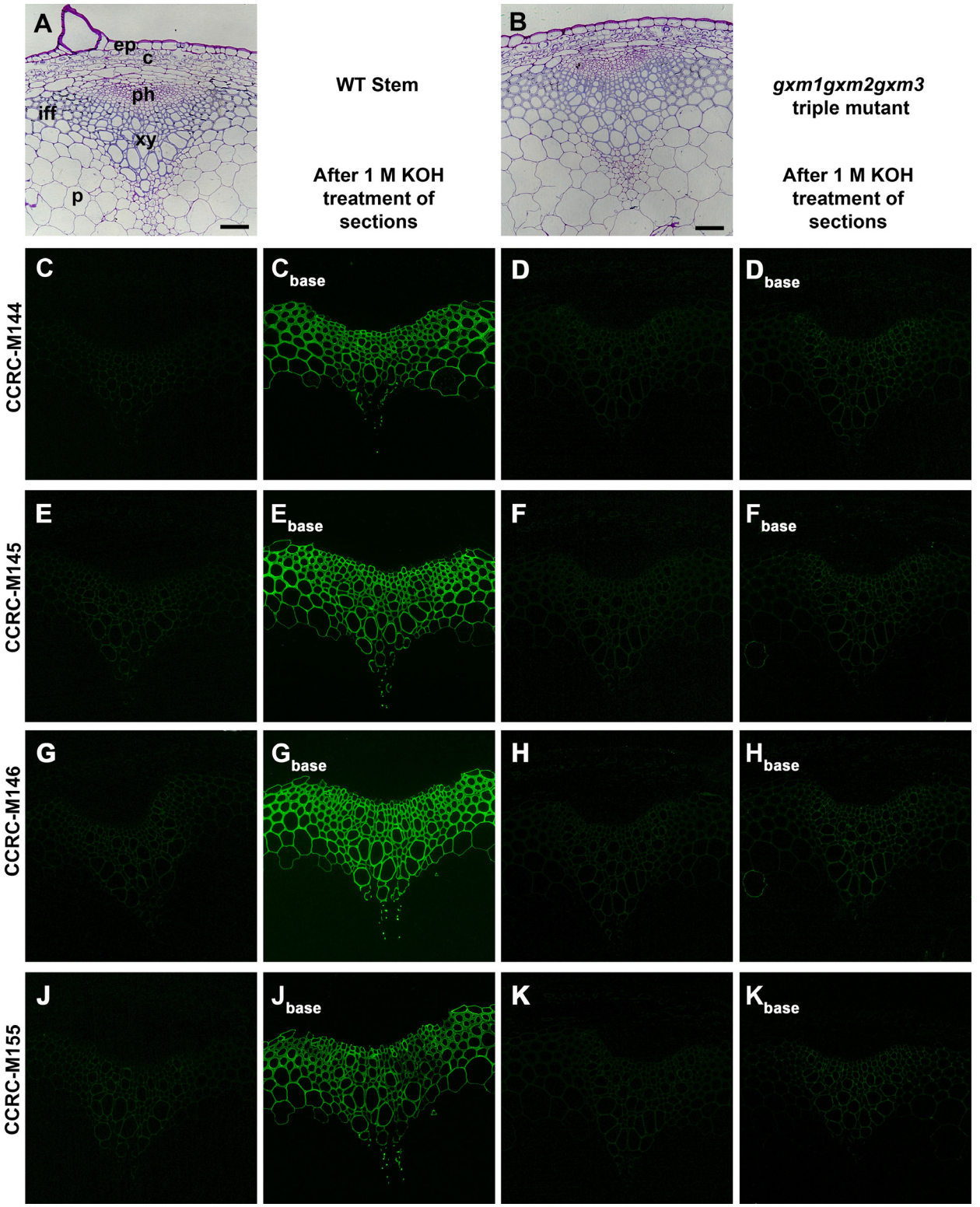

Figure 2. Immunolocalization of CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 in WT and the triple mutant stems. Toluidine-blue-stained images (A and B) show the overall anatomy of stems. Base-treated sections with 1M KOH are named as C-K_{base}. 1M KOH treatment is necessary to remove ester bonds for binding of the antibodies. Sections without any treatment are shown in C-K. Scale bars in A and B equal 50 μm and apply to correspondent images in WT and the triple mutant. Abbreviations in A stand for the following: ep: epidermis, c: cortex, iff: interfascicular fibers, ph: phloem, xy: xylem, p: pith cells.

in the future could give us important insights into various biomass sources.

Preparation of cell-wall-directed monoclonal antibodies was mostly dependent on base extracts from various plant cell wall preparations; therefore, developed antibodies recognize epitopes on base-treated cell wall glycans (Pattathil et al., 2010). This is important for some of the antibodies that have been produced so far. Therefore, in order to unmask the epitope of CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155, which is methyl glucuronoxylan, sections were subjected to a base treatment with 1M KOH. Similar base treatment was applied in previous studies to unmask epitopes of antibodies and presumably removes ester groups and acetyl substitutions on xylan (Marcus et al., 2010). Similarly, base treatment of sections with 0.1M KOH unmasked the epitopes of CCRC-M155 in various genotypes in Mischantus (Da Costa et al., 2017). Therefore, the results presented here are consistent with the literature.

This is the first study to combine all the available epitope data for CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 together. In order to further prove the epitope recognized by the antibodies, a xylan triple mutant that lacks methyl glucuronoxylan, is used for the first time. The epitope of CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 antibodies is methyl glucuronoxylan. The results are consistent with a synthetic glycan microarray study for these antibodies (Ruprecht et al., 2017). Other than slight intensity differences, there was no distinguishable binding difference of four antibodies, which implies that each antibody could be used in place of the other.

Antibodies can be used as probes to locate specific structural features of cell wall glycans and their various substituted forms in cells and tissues. They are also useful for the analysis of cell wall mutants as well as for the characterization of glycan isolated from the cell wall. Therefore, antibodies are powerful tools. Hemicellulose structures are more diverse than cellulose and differ across plant species and tissues (Mortimer et al., 2015). Therefore, using antibodies against different plant and tissue samples can reveal new xylan decorations on MeGlcA. For example, utilization of CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155 in mutants, where xylan is deacetylated (Pawar et al., 2017, Zhang et al., 2017), might provide valuable insights into MeGlcA substitutions. Hemicellulose composition of the secondary cell wall is also different among gymnosperms, monocots, and eudicots (Smith et al., 2017). Therefore, such antibodies could help identify native xylan decorations of different plants. In plant tissues in which MeGlcA epitopes are not masked as in Arabidopsis, base treatment may not be necessary to localize CCRC-M144, CCRC-M145, CCRC-M146, and CCRC-M155. Therefore, the results presented here could be helpful for understanding xylan structure and modification in future studies.

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Conflict of interest

The author declares no conflicts of interest.

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