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An extended set of monoclonal antibodies to pectic homogalacturonan

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ABSTRACT

Three novel rat monoclonal antibodies, designated LM18, LM19 and LM20, were isolated from screens for binding to *Arabidopsis thaliana* seed coat mucilage. The binding of these antibodies to mucilage subject to enzyme and high pH pre-treatments and to a series of model homogalacturonan-rich pectins with defined levels of methyl-esterification indicated their recognition of pectic homogalacturonan epitopes. The binding capacities of these monoclonal antibodies to cell walls in sections of tobacco stem pith parenchyma were also differentially sensitive to equivalent treatments with high pH buffers and pectate lyase. The epitopes bound by these antibodies display some similarities and some differences to the epitopes recognized by the previously isolated and established pectic homogalacturonan probes JIM5 and JIM7.

1. Introduction

Plant cell walls are a range of robust materials that are central to many aspects of plant biology and plant defence. Cell walls are largely polysaccharide based and the major sets of cell wall polysaccharides are broadly classed as cellulose, hemicelluloses and pectic polysaccharides. Cell wall polysaccharides are of considerable economic importance in terms of food, food ingredients, fibers, pharmaceuticals and biofuels. It is well established that pectic polysaccharides are the most structurally complex of cell wall polymers and that they are comprised of sets of galacturonic acid-rich domains that can have structural variants within plants. It is also clear that they are involved in a range of cell functions and plant processes although their involvement is not defined precisely.[1–4] Much of our considerable knowledge of pectic polysaccharide structure derives from the chemical characterization of samples isolated from bulk plant materials such as citrus or apple fruits. Complementary approaches and methodologies involving antibodies are required to place the undoubted complexity of pectic polysaccharide structures in cell and developmental contexts.[3,6]

The major polysaccharide domains of pectins include homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II).[2,4] HG polysaccharides are the major components of pectin and consist of 1,4-linked galacturonosyl residues that are synthesized in a largely methyl-esterified form. The complex, but structurally conserved, RG-II domain can be considered as a specific, elaborated form of HG that allows the boron-mediated cross-linking of HG chains.[4,7] RG-I is a structurally distinct and heterogeneous set of polymers based on rhamnogalacturonan (RG) backbones with diverse arabinan, galactan and arabinogalactan side chains.[4] RG-I is implicated in cell wall mechanical properties but this is poorly understood in mechanistic terms. The nature of the links between these polymers and their integration of these distinct polysaccharides into continuous networks of polymers that are abundant in primary cell walls also remains a matter of debate.[8–10]

HG is a multifunctional domain of pectin and has roles relating to primary cell wall assembly and cell extension, cell wall matrix porosity and plant defence responses.[3,4,11–14] Several sets of enzymes can act on HG in cell walls. Pectic HG can be de-esterified in muro by pectin methylesterases, and several lines of evidence have indicated that the methyl-esterification status of pectic HG is hypervariable and can reflect cell status, cell growth and development.[3,13,15] De-esterified HG can be cross-linked by calcium ions impacting on cell wall mechanical properties and on cell wall matrix porosity but can also be cleaved by polygalacturonases and pectate lyases—both of which arise from large gene families.[11,16,17] The regulation of HG structure in muro and hence cell wall properties is therefore complex.

Technologies and molecular probes capable of placing pectin chemistry and its variations in the context of biological functions at the level of tissues and cells are largely derived from the use of antibody probes.[3,5] Several antibodies to HG have been generated and these tools have indicated that the methyl-esterification of HG is highly varied in relation to cell development, but the significance and regulatory aspects of its control is not yet fully understood. The current set of HG-directed monoclonal antibodies include JIM5, JIM7, LM7, PAM1 and 2F4.[18–21] These antibodies have differing specificities in relation to HG methyl-esterification. Rat monoclonal antibodies JIM5 and JIM7 are widely used, and both bind

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optimally to partially methyl-esterified epitopes of HG and can be used to give a view of the overall methyl-esterification status of HG,[18] but can only provide partial imaging of this polymer and its variants within cell walls. Here we describe the characterization of three novel HG-directed rat monoclonal antibodies that were isolated from screens for antibody binding to *Arabidopsis thaliana* seed coat mucilage.

2. Results

The pectic mucilage secreted from the outer cell layer of seeds of *A. thaliana* is largely an unbranched rhamnogalacturonan although with HG elements.[22,23] This polysaccharide was isolated as described,[22] and Dionex HPAEC-PAD analysis of the isolated polymer indicated that rhamnose and galacturonic acid accounted for >95% (w/w) of the total sugars present with a GalA/Rha molar ratio of 1.40 and with other minor sugars being arabinose, galactose and glucose. Three rat monoclonal antibodies, designated LM18, LM19 and LM20, which bound to the isolated mucilage were assembled for characterization. LM18 and LM20 were isolated subsequent to immunization with the *Arabidopsis* seed mucilage, and LM19 was isolated subsequent to immunization with a galacturonan derived from apple fruit. In all assays, the binding of LM18, LM19 and LM20 was compared with the binding of established pectic HG-directed monoclonal antibodies JIM5 and JIM7.[18,24,25] The binding of these five rat monoclonal antibodies (with cell culture supernatant dilutions resulting in 90% of maximal binding) to *Arabidopsis* seed coat mucilage and to the mucilage subject to a range of pre-treatments as determined by ELISAs is shown in Figure 1. The binding of LM18 to the mucilage was unaffected by pre-incubation with CAPS buffer (pH 9.5) for pectate lyase (CAPS buffer) but abolished when pectate lyase was included in the buffer indicating recognition of HG. Pre-treatment of the coated wells with 0.1 M sodium carbonate (pH 11.4) led to some loss of binding in this assay. The binding of LM19 to seed mucilage was abolished by the pectate lyase pre-treatment but not by the high pH pre-treatments indicating recognition of HG, and that this antibody can bind to largely de-esterified HG. This pattern of binding is similar to that observed for JIM5 in these assays (Fig. 1). LM20 bound to the mucilage showed limited loss of binding in response to pectate lyase action, and its binding was more significantly reduced by the sodium carbonate pre-treatment. This binding reflected the recognition of these samples by JIM7—although the JIM7 antibody still retained a greater capacity to recognize the mucilage polysaccharide after sodium carbonate pre-treatment.

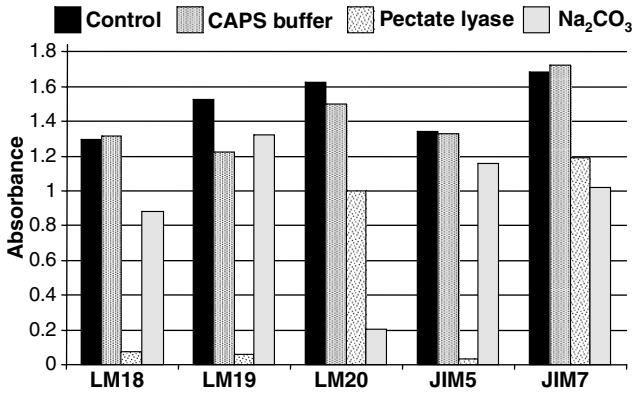

Figure 1. ELISA of rat monoclonal antibodies LM18, LM19, LM20, JIM5 and JIM7 binding to *Arabidopsis thaliana* seed coat mucilage. Binding of cell culture supernatants (at 90% of maximal binding) was assessed after no treatment, pre-treatment with either CAPs buffer, CAPS buffer with pectate lyase or with 0.1 M sodium carbonate for 1 h prior to antibody incubation. Results shown are representative of at least three separate experiments, and values given are means of three replicates with the standard deviations being <0.1 absorbance units.

To explore the specificities of these monoclonal antibodies further, their capacity to recognize a series of model HG-rich lime pectins (in which a high ester starter pectin had been de-esterified with a plant PME (acting in blockwise way) or a fungal PME (acting in a random way) resulting in pectins with differing extents of esterification[25,26]) was assessed by ELISAs. The binding of all five antibodies to F69 (pectin treated with an *Aspergillus niger* PME until 69% methyl-esterification remained), F43, F19, P66 (pectin treated with orange peel PME until 66% esterification remained), P48, P16 and polygalacturonic acid is shown in Figure 2. LM18 displayed a novel pattern of recognition with a preference for F series pectins. Of the P-series pectins there was some preference for P46, and recognition of PGA was less than that seen for any of the F or P series pectins. LM19 bound strongly to all pectins assayed including PGA, showing little discrimination in relation to the extent or pattern of methyl-esterification. This was in contrast to JIM5 which bound comparatively weakly to P16 and PGA. LM20 bound very weakly to PGA and at an equivalent level to that observed for JIM7. However, in contrast to JIM7, LM20 showed reduced recognition of F19.

Defining epitopes bound by polysaccharide-directed antibodies can be aided by hapten inhibition studies with defined oligosaccharides from target antigens.[18] The only HG/RG-related oligosaccharides available for this study were the dimer and trimer of α-1,4-linked galacturonic acid. The tri-galacturonide inhibited the binding of LM18 to lime pectin (F69) by ~20% when present at 1 mg/mL, but showed no capacity to inhibit the binding of LM19 and LM20 when present at 1 mg/mL. The dimer resulted in no inhibition of binding by any of the antibodies when present at 1 mg/mL.

The capacity of these antibodies to bind to pectin in the context of cell walls was studied with transverse sections of tobacco stem with a focus on the primary cell walls of the pith parenchyma. This system allows the preparation of large numbers of equivalent sections that are required for a range of pre-treatments as shown in Figure 3. All five HG-directed monoclonal antibodies were incubated with equivalent sections that were untreated or pre-treated with CAPS buffer (pH 9.5), CAPS buffer plus pectate lyase or with 0.1 M sodium carbonate. LM18 bound only extremely weakly to untreated pith parenchyma cell walls, more strongly to cell walls lining intercellular spaces (CWLIS) when treated with CAPS buffer, and this binding was sensitive to the pectate lyase action (Fig. 3). The higher pH treatment (sodium carbonate) led to an abundance of the LM18 epitope throughout all cell walls. This binding to sodium carbonate pre-treated cell walls was abolished by a pectate lyase pre-treatment (not shown). The binding of LM19 and JIM5 displayed a similar pattern of responses to these treatments with binding being sensitive to pectate lyase and the epitopes being abundant throughout all cell walls after sodium carbonate treatment. However, there was a distinction between these two antibodies in that in untreated and CAPS buffer pre-treated sections the binding of JIM5 was most abundant in regions of CWLIS, and LM19 showed some binding to the adhered primary cell walls of adjacent cells (Fig. 3). LM20 displayed a very distinct and interesting pattern of cell wall recognition. In an untreated section, LM20 bound most effectively to CWLIS and the pre-treatment with CAPS buffer (pH 9.5) led to the abundant recognition of all cell walls. Pre-treatment with pectate lyase in the presence of the CAPS buffer resulted in LM20 binding being restricted to CWLIS, that is, the LM20 epitopes in cell walls other than CWLIS were sensitive to pectate lyase action. The sodium carbonate pre-treatment led to loss of all binding. The binding of JIM7 was abolished by sodium carbonate but not by CAPs buffer or pectate lyase and in contrast to LM20 occurred in all cell wall regions.

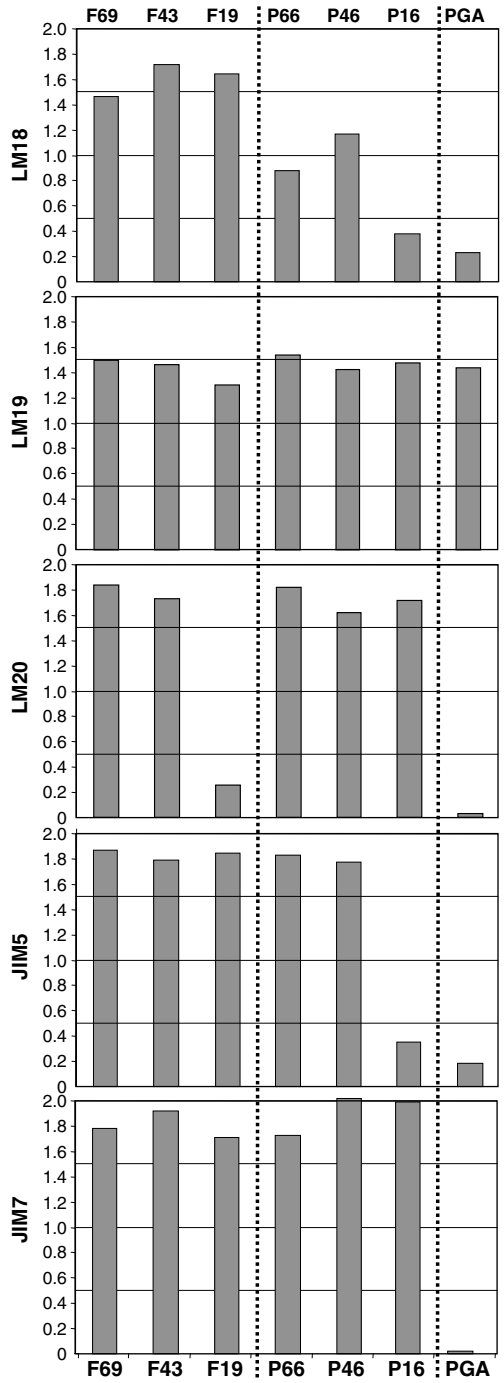

Figure 2. ELISA of monoclonal antibodies LM18, LM19, LM20, JIM5 and JIM7 binding to series of model lime pectins varying in extent of methylesterification. *Y*-axes indicate ELISA absorbance units. Results shown are representative of at least three separate experiments, and values given are means of three replicates with the standard deviations being <0.1 absorbance units.

3. Discussion

The binding of rat monoclonal antibodies LM18, LM19 and LM20 to *Arabidopsis* seed coat mucilage and to tobacco parenchyma cell walls is sensitive to pre-treatments with a recombinant pectate lyase indicating that these antibodies bind to HG. LM19 displayed some similar binding properties to JIM5. LM20 displayed some similar binding properties to JIM7. Monoclonal antibodies JIM5 and JIM7 have been extensively characterized,[18,24,25] and their binding to distinct patterns of methyl-esters on synthetic hexa-galacturonides determined.[18] JIM7 binding was effectively inhibited by methyl-esterified galacturonosyl residues flanked by unesterified residues, and JIM5 binding was inhibited by unesterified galacturonosyl residues flanked by methyl-esterified residues and also by unesterified HG oligogalacturonides.[18,25]

The binding of these five HG probes is likely to be complex with recognition of optimal and sub-optimal epitopes found in HG and related pectic polymers. It is perhaps surprising that LM18 and LM20 were isolated subsequent to immunization with a soluble component of *A. thaliana* seed coat mucilage that is largely rhamnogalacturonan. However, it is likely that the preparation contained some HG elements, and this appears to be immunodominant as indicated by the observation that JIM5 and JIM7 were also isolated subsequent to immunization with protoplasts and not HG-rich pectins.[24] It is formally possible that this set of antibodies may bind to rhamnosyl-containing elements of the rhamnogalacturonan backbone, but no direct evidence to support this has been obtained. Immunization of mice with *A. thaliana* seed coat mucilage has also recently resulted in the generation of anti-homogalacturonan monoclonal antibodies (CCRC-M34 and CCRC-M38), although in this case a mucilage-specific antibody (CCRC-M36) that may bind to rhamnogalacturonan backbone was also obtained.[27,28]

LM18, LM19 and LM20 extend the current panel of available HG-directed antibodies. In terms of binding to tobacco pith parenchyma, primary cell walls LM18 and LM19 bind preferentially to de-esterified/saponified HG in a manner similar to JIM5. LM20 binds to a novel epitope that is similar to JIM7 and does not bind to de-esterified/saponified cell walls indicating that some esters are required for recognition. There are some complexities in the data presented here in terms of recognition profiles. For example, the differing behavior of the probes in response to sodium carbonate treatment prior to the in vitro assessment of antibody binding to seed coat mucilage and prior to binding to tobacco pith parenchyma cell walls. These differences in binding responses presumably relate to the context of the epitopes in the different and varied complex polymer sets of these assays. The mucilage is RG-rich, the lime pectins are HG-rich and the precise polymer context of the epitopes in tobacco pith parenchyma cell walls is unknown. Moreover, in the cell walls, other structural features and components are likely to influence antibody recognition and antigen modification in response to high pH treatments and enzyme action. In addition to the extent of methyl-esterification of galacturonosyl residues and the possible presence of rhamnosyl residues, other factors influencing antibody recognition could include the extent of HG acetylation and also substitution with xylosyl residues. Other, as yet unidentified ester links to pectins have also been reported[29], and these would be lost upon saponification revealing more HG epitopes.

The differential responses of antibody binding subsequent to the pre-treatment of mucilage/stem sections with CAPS buffer (pH 9.5) or with 0.1 M sodium carbonate (pH 11.4) are of interest. The higher pH treatment increased the binding of LM18, LM19 and JIM5 but abolished the binding of LM20 and JIM7. This suggests that LM20 and JIM7 require some HG methyl-esters for recognition, whereas LM18, LM19 and JIM5 can bind to HG with few or no esters. The pre-treatment of sections of plant material including tobacco stem with pectate lyase (in CAPS buffer) has recently been demonstrated to unmask xyloglucan epitopes by pectic HG removal.[30] The observations here indicate that a pre-treatment with sodium carbonate in combination with pectate lyase may lead to a more effective removal of HG from sections than the pH 9.5 treatment that is used for pectate lyase action. The pre-treatment with CAPS buffer led to increased binding of LM20 throughout all cell walls, and this was only partially abolished by pectate lyase treatment

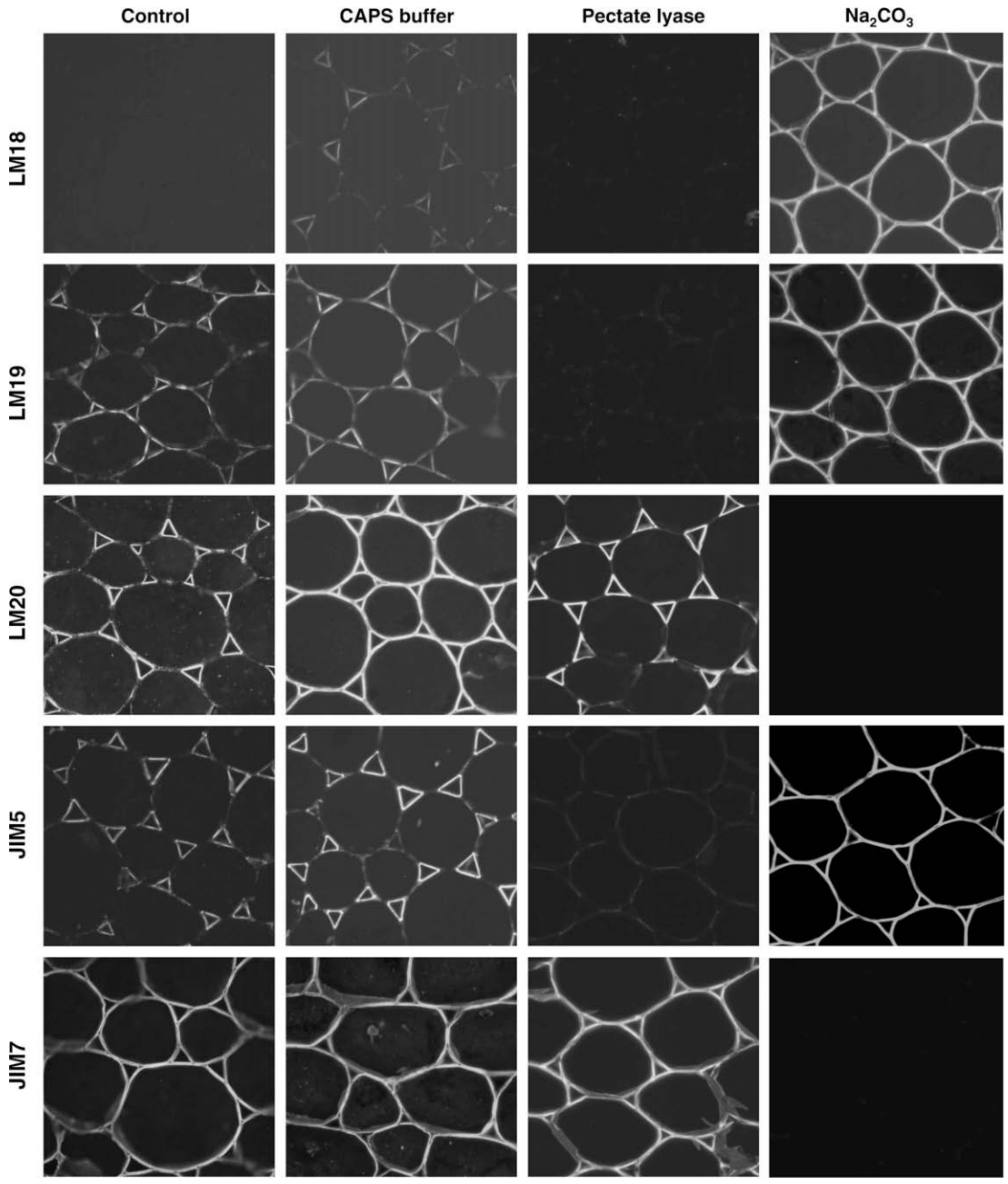

Figure 3. Indirect immunofluoresence detection of the binding of monoclonal antibodies LM18, LM19, LM20, JIM5 and JIM7 to cell walls of transverse sections of tobacco stem pith parenchyma. In some cases, equivalent sections were pretreated for 1 h with CAPs buffer, CAPs buffer plus pectate lyase or with 0.1 M sodium carbonate.

indicating distinct features of CWLIS—a facet of cell wall structure that is highlighted by several of the probes used here. It will be of interest to determine structures that are revealed by pH 9.5 treatment that are lost upon the pH 11.4 (sodium carbonate) pre-treatment. The calcium ions present in the CAPS buffer had no impact upon the LM20 binding pattern (data not shown).

4. Conclusion

Three new pectic HG-directed probes have been isolated that can be applied to the exploration of pectic HG heterogeneity within plant cell walls and pectin biology in general. These probes will be useful additions to the panels of probes that are being assembled for the high throughput epitope mapping of cell wall polysaccharides in relation to plant growth and plant diversity.[31] The novelty of LM18, LM19 and LM20 lies in their comparative binding patterns to HG in CWLIS and their differential sensitivities of binding to high pH and pectate lyase pre-treatments.

5. Experimental procedures

5.1. Isolation of *A. thaliana* seed coat mucilage and sugar analysis

A. thaliana seed mucilage was isolated by ammonium oxalate extraction as described.[22] Monosaccharide composition of the isolated polymer was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). For analysis, duplicated samples were hydrolyzed in 4 N H_2SO_4 for 2 h at 120 °C and were then

neutralized with ammonium hydroxide. Duplicated standard solutions of L-Fuc, L-Rha, L-Ara, D-Gal, D-Glu, D-Xyl, D-Man, D-GalA and D-GluA were treated in the same way as the mucilage samples, and analyzed before the samples for verification of the response factors. HPAEC was performed using a Carbopac PA1 Analytical column (4 × 250 mm, Dionex) at 25 °C with a flow rate of 1 mL/min and gradient elution (0–1 min 20 mM NaOH; 1–45 min, 20 mM NaOH with linear gradient from 0 to 0.1 M NaOAc; 45–50 min 0.5 M NaOH (wash), 50–70 min 20 mM NaOH (equilibration)).

5.2. Immunization procedures and generation of rat monoclonal antibodies

Rat immunization, preparation of hybridomas and cell cloning were performed as described.[32] Briefly, a Wistar rat was injected with 100 μg mucilage polysaccharide in complete Freund's adjuvant administered sub-cutaneously on day 0, with the same amount administered with incomplete Freund's adjuvant on days 34 and 62. On day 194, the rat was given a prefusion boost of 100 μg mucilage in PBS by intraperitoneal injection. Spleen lymphocytes were isolated three days later and fused with rat myeloma cell line IR983F.[33] Antibodies were selected by ELISA using *Arabidopsis* seed mucilage as the immobilized antigen and this led to the isolation of rat monoclonal antibodies LM18 and LM20. A similar immunization procedure was followed with an apple fruit pectic galacturonan (47%GalA, 34%Xyl, 6% Ara, 5% Rha, 4% Gal) kindly provided by Henk Schols (Wageningen University), and this led to the isolation of rat monoclonal antibody LM19 that was found to bind to *Arabidopsis* seed mucilage and lime pectins. Imunoglobulin isotypes of the selected antibodies are as follows: LM18 is an IgG2c, and LM19 and LM20 are both IgM.

5.3. Immunochemical analysis of antibody binding to pectic polymers

In vitro immunochemistry and ELISAs with pectic polymers were carried out as described.[32] In all cases, immobilized antigens (*A. thaliana* seed coat mucilage and model lime pectins[26]) were coated on to microtitre plates at 50 μg/mL. Polymer pre-treatments were carried out in wells of the microtitre plates for 1 h, and plates were washed thoroughly prior to detection of antibody binding. Antigen pre-treatments included incubation with PL buffer alone (50 mM *N*-cyclohexyl-3-aminopropane sulfonic acid (CAPS), 2 mM $CaCl_2$, pH 9.5), PL buffer with pectate lyase (10 μg/mL, recombinant microbial pectate lyase 10A kindly provided by Harry Gilbert, Newcastle University) or 0.1 M sodium carbonate (pH 11.4). In addition to LM18, LM19 and LM20, two established pectic HG rat monoclonal antibodies JIM5 and JIM7[18] were used in the study.

5.4. Preparation of plant material and immunocytochemistry

Transverse sections of tobacco stems were used for immunocytochemistry as described.[30] Pre-treatments of stem sections (PL buffer, PL buffer + PL or 0.1 M sodium carbonate) were carried out for 1 h as described for in vitro immunochemistry above. Sections were then washed in PBS at least three times and incubated with a 10-fold dilution of appropriate rat monoclonal antibody followed by 100-fold dilution of anti-rat IgG linked to FITC. Samples were washed at least three times and then mounted in a glycerol-based anti-fade solution (Citifluor AF1, Agar Scientific, UK). Immunofluorescence was observed with an Olympus BX-61 microscope equipped with epifluorescence irradiation, and micrographs were captured with a Hamamatsu ORCA285 camera and IMPROVISION VOLOCITY software.

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