Detecting the orientation of newly-deposited crystalline cellulose with fluorescent CBM3

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ABSTRACT

Cellulose microfibril patterning influences many of the mechanical attributes of plant cell walls. We developed a simple, fluorescence microscopy-based method to detect the orientation of newly-synthesized cellulose microfibrils in epidermal peels of onion and Arabidopsis. It is based on Alexa Fluor 488-tagged carbohydrate binding module 3a (CBM3a) from Clostridium thermocellum which displayed a nearly 4-fold greater binding to cell walls at pH 5.5 compared with pH 8. Binding to isolated cellulose did not display this pH dependence. At pH 7.5 fibrillar patterns at the surface of the epidermal peels were visible, corresponding to the directionality of surface cellulose microfibrils, as verified by atomic force microscopy. The fibrillar pattern was not visible as the labeling intensity increased at lower pH. The pH of greatest cell wall labeling corresponds to the isoelectric point of CBM3a, suggesting that electrostatic forces limit CBM3a penetration into the wall. Consistent with this, digestion of the wall with pectate lyase to remove homogalacturonan increased labeling intensity. We conclude that electrostatic interactions strongly influence labeling of cell walls with CBM3 and potentially other proteins, holding implications for any work that relies on penetration of protein probes such as CBMs, antibodies, or enzymes into charged polymeric substrates.

1. Introduction

Cellulose in growing plant cell walls is deposited as a polylamellate network that physically limits the expansive growth of plant cells (Zhang et al., 2021). This β-1,4-linked glucan is synthesized by cellulose synthase complexes which can track along microtubules (Paredes et al., 2006; Baskin and Gu, 2012). In many primary cell walls that have been studied, cellulose microfibrils are deposited anisotropically in each lamella, changing direction in successive layers (Chafe and Wardrop, 1972; Kutschera, 2008; Zhang et al., 2014; Zhang et al., 2019). The growth of plant cells is dependent on the controlled loosening of the cellulose microfibril network (Cosgrove, 2016; Chebli and Geitmann, 2017; Cosgrove, 2018). Changes in the crossed-lamellar structure affect cell wall mechanics (Cavalier et al., 2008; Park and Cosgrove, 2012; Xiao et al., 2016; Xin et al., 2020). The orientation of cellulose microfibrils in the most recently deposited cell wall lamella, adjacent to the plasma membrane, provides information about recent cell wall synthesis and its response to environmental and developmental stimuli. Epidermal cells are of particular importance for the development of plants as they function to both promote and restrict growth (Savaldi-Goldstein et al., 2007; Zuch et al., 2021; Gruel et al., 2016). Visualization of the most recently deposited cellulose microfibrils in epidermal cells serves to improve our understanding of the synthesis of cell walls and the mechanisms underlying plant growth.

A variety of microscopy techniques can be used to assess cellulose patterning in the epidermis of plant tissues. Microscopy methods that scan the surface of an epidermal peel can provide information about the most recently deposited cell wall lamella while transmission, spectroscopic methods can reveal the net or average orientation of cellulose. Electron microscopy has been used for many decades to observe the structure of plant cell walls (Emmons, 1988; McCann et al., 1990; Goodenough and Heuser, 1985; Nicolas et al., 2022). More specifically, field emission scanning electron microscopy can provide high-resolution images of surface cellulose features, from a variety of plant tissues.
following the enzymatic removal of pectin and dehydration of the sample (Sugimoto et al., 2000; Carpita et al., 2001; Himmelspach et al., 2003; Fujita and Wasteney, 2014; Zhu et al., 2015; Xiao et al., 2016; Zheng et al., 2017; Zheng et al., 2018). Atomic force microscopy (AFM) can reveal the most recently deposited layer of cellulose microfibrils in never-dried samples but is limited to scanning where the tip can make contact with the wall surface (Zhang et al., 2014; Zhang et al., 2016; Zhang et al., 2017; Zhang et al., 2019). Polarized light microscopy, fourier-transform infrared spectroscopy, and vibrational sum-frequency generation spectroscopy have each been used to quantify the average orientation of cellulose microfibrils in plant tissues, characterizing the mechanical anisotropy of cells and the effect of stretching on cellulose reorientation in epidermal peels (Green, 1962; McCann et al., 1992; Suslov and VerbeLEN, 2006; KafLE et al., 2014; KafLE et al., 2017). Alternatively, fluorescence microscopy has proven useful for imaging plant cell walls and can provide detailed information about the orientation of cellulose microfibrils that is dependent on the specificity and penetration of the fluorescent dye or probe being used (SautER et al., 1993; VeRbelen and SticKens, 1995; VeRbelen and KerStens, 2000; KerStens et al., 2001; Ding and Himmel, 2006; Suslov and VerbeLEN, 2006; Anderson et al., 2018; BIdhendi et al., 2019; Parrotta et al., 2019; DeVeere et al., 2021).

A variety of fluorescent dyes and probes are commonly used to label cellulose. Calcofluor White (SautER et al., 1993; BIdhendi et al., 2020), Congo red (in conjunction with polarized confocal microscopy) (VeRbelen and SticKens, 1995; VeRbelen and KerStens, 2000; KerStens et al., 2001; Ding and Himmel, 2006; Suslov and VerbeLEN, 2006), and Pontamine Fast Scarlet 4B (S4B) (Anderson et al., 2010; Liesche et al., 2013; BIdhendi et al., 2020) have been used to visualize cellulose in a variety of different plant species and cell types. Additionally, carbohydrate binding module 3 (CBM3; shown to bind the hydrophobic surface of crystalline cellulose) (Lehtio et al., 2003; Ding and Himmel, 2006; Hervé et al., 2010; Dagel et al., 2011; Georgelis et al., 2012; Ruel et al., 2012) has been used to label cellulose through fusion with a green fluorescent protein (GFP) reporter (Ding et al., 2006) or by binding of a fluorescent antibody specific to CBM3 (Parrotta et al., 2019; BIdhendi et al., 2020). Each of these studies reports a mean, net, or predominant orientation of cellulose fibers based on staining and imaging of the plant tissue of interest. These studies note inconsistencies in staining (higher or lower signal intensities) throughout, along, or between samples of the same plant tissues and frequently indicate that fibrillar patterning cannot be resolved in every cell or sample of the tissue of interest. The reason for labeling inconsistencies is often not examined, yet these findings suggest that resolution of fibrillar patterning relies on the specificity and penetration of the dye or probe.

In this study we developed a CBM3 Alexa Fluor conjugate and optimized its use to reveal cellulose fibrillar patterning in epidermal peels from onion and Arabidopsis. We explored the conditions which allowed for selective labeling of the most recently deposited cell wall lamella and propose a mechanism whereby this specificity is achieved. Beyond implementing the use of this novel cellulose tag, our results hold implications for other studies using proteins to label or modify a cell wall sample.

2. Methods

2.1. Carbohydrate conjugation and predictive modeling of CBM3-A488

Carbohydrate Binding Module 3a (Clostridium thermocellum; NZYTech CZ0057) was labeled with Alexa Fluor 488 C5 Maleimide (ThermoFisher Scientific A10254) and Alexa Fluor 488 was suspended in 300 µL of 20 mM HEPES pH 7.5 and added slowly to CBM3a in TCEP in 10-fold molar excess. The labeling reaction was incubated at 22 °C for 2 h and then at 4 °C for 16 h to ensure complete labeling. Unreacted Alexa Fluor 488 was removed using VIVASPIN 500 columns. Wash buffer was exchanged until the flow-through showed no residual Alexa Fluor 488 fluorescence, as detected with a NanaDrop 3300 (ThermoFisher Scientific ND-3300). The labeled CBM3 was pipetted off of the top of the filter column and the final concentration was determined with a Bradford protein assay. The conjugate is named CBM3-A488.

The primary sequence of CBM3a (PDB ID: 1NBC, full sequence with N-terminal hexa-histidine tag provided by the manufacturer, NZYTech) was submitted to the I-TASSER server (Zhang, 2008) for structural prediction. The top scoring model (by I-TASSER confidence score) was selected for further analysis. Most of the protein sequence was modeled using the published CBM3a crystal structure (1NBC) (Tormo et al., 1996); however, the 23-residue N-terminal extension added during cloning was modeled ab-initio using I-TASSER. The structure of Alexa Fluor 488 was downloaded from Chemspider (Pence and Williams, 2010) and the C5 maleimide linker was drawn by hand using MarvinSketch (Csizmadia, 1999). The Alexa Fluor 488 maleimide structure was imported to UCSF Chimera (v. 1.11.2) (Petterson et al., 2004), along with the CBM3a model from I-TASSER. The Alexa Fluor 488 maleimide was connected to the free cysteine (C78) and torsion angles were adjusted to avoid steric clashes.

2.2. Preparation of onion epidermal peels and labeling with cellulose dyes and probes

White onions (*Allium cepa*) ~8-10 cm in diameter were purchased from local grocery stores. The fifth onion scale, with the first scale being the outermost fleshy layer, was used for epidermal peel preparation. Abaxial epidermal cells were torn open by peeling as described previously (Zhang et al., 2016). Epidermal peels were floated cuticle side up for subsequent washing and staining steps. Peels were washed with 20 mM HEPES pH 7.5 with 0.01 % (w/v) Calcofluor White, 10 µg/mL GFP-CBM3 (NZYTech CZ0057; 500-fold dilution of stock; same CBM as CBM3-A488), or 10 µg/mL CBM3-A488. Peels were stained with shaking at 250 rpm at 22 °C for 15 min to eliminate cytoplasmic debris. Peels were then washed in 20 mM HEPES pH 7.5 for 1 h to remove residual Tween 20.

Washed epidermal peels were cut into 5 mm × 5 mm squares prior to labeling. Individual epidermal peel squares were incubated in 100 µL of staining solution. The concentrations of staining solutions mixed in 20 mM HEPES buffer pH 7.5 or 20 mM sodium acetate buffer pH 5.5 were as follows: 0.001 % (w/v) Calcofluor White (Fluka Analytical 18909), 0.01 % (w/v) Pontamine Fast Scarlet 4B (S4B) (Aldrich Rare Chemical Library #S479896), 10 µg/mL GFP-CBM3 (NZYTech CZ0057; 500-fold dilution of stock; same CBM as CBM3-A488), or 10 µg/mL CBM3-A488. Peels were stained with shaking at 250 rpm at 22 °C for 1 h. Following labeling, epidermal peel squares were washed twice in 20 mM HEPES buffer pH 7.5 for 1 h to remove residual dye or probe. As discussed below, washing in pH 7.5 buffer does not remove or alter peels initially labeled in other pH buffers. This protocol was adopted after preliminary experiments were conducted in order to streamline the process of labeling dozens of peels at a time.

2.3. Fluorescence microscope imaging and image analysis

Onion epidermal peels labeled with Calcofluor White, S4B, GFP-CBM3, and CBM3-A488 were imaged with an Olympus BX63 epifluorescence microscope using a 20x/0.75na objective. Calcofluor White was imaged with the DAPI channel (350 nm excitation), S4B with 561 nm excitation, and GFP-CBM3 and CBM3-A488 were imaged with the FITC channel (488 nm excitation). A Zeiss LSM780 laser scanning confocal microscope was used with a 63x/1.2na objective to capture z-stacks with 500 nm optical sections. Calcofluor White labeling was imaged with 405 nm excitation and 410–500 nm emission. S4B labeling was imaged with...
561 nm excitation and 570–650 nm emission. CBM3-A488 labeling was imaged with 488 nm excitation and 500–580 nm emission. Standard PMT channels were used and high gain was not used. The image brightness and contrast were adjusted in the microscope software to emphasize the fibrillar features.

Following fluorescence imaging, net fibril orientation and the degree of anisotropy of onion epidermal peel cells were analyzed with the ImageJ plugin FibrilTool (Boudaoud et al., 2014). The angle and the length of the red line generated by FibrilTool represent the net orientation and degree of anisotropy of detected fibrils within the region (cell) of interest, respectively.

2.4. Atomic force microscopy (AFM) scanning of onion epidermal peel cells

Onion epidermal cell walls were prepared and imaged by AFM as described previously (Zhang et al., 2014). Briefly, an epidermal strip pre-labeled with CBM3-A488 was fixed cuticle-side down to a glass microscope slide, with a layer of nail polish around the perimeter. The exposed cell wall surface was kept hydrated with a small volume of 20 mM HEPES pH 7.5 buffer. An optical camera on the AFM was used to identify the cells previously imaged with fluorescence microscopy based on cell outline morphology. AFM topography images were captured on a Dimension Icon AFM (Bruker, CA, USA) using a calibrated Scansasystem-Fluid + probe (spring constant of 0.7 N/m, nominal tip radius of 2 nm; Bruker, CA, USA). The Nanoscope scanning software was operated in the QNM PeakForce Tapping mode in fluid. Images were taken at 1 µm × 1 µm size at a resolution of 512 pixels/line. Scan speed, gain, and imaging force were optimized for each image. Images were processed with Nanoscope Analysis V 2.0 (Bruker).

2.5. pH dependence of CBM3-A488 labeling of onion epidermal cell walls

Washed onion epidermal peel walls were cut into 5 mm × 5 mm squares and labeled for 1 h in 100 µL of 10 µg/mL CBM3-A488 in nine different 20 mM buffers spanning the pH scale from pH 4 to pH 8, at half pH increments. The buffers used were as follows: 20 mM sodium acetate adjusted to pH 4, pH 4.5, pH 5, or pH 5.5; 20 mM sodium phosphate adjusted to pH 6, pH 6.5, or pH 7; 20 mM Tris-HCl adjusted to pH 7.5 or pH 8. Following labeling, the epidermal peel squares were washed twice (1 h and then overnight) in pH 7.5 buffer to remove residual CBM3. Images for fluorescence intensity comparison were collected with an Olympus BX63 microscope with the FITC channel, using identical exposure and gain settings. Z-stacks were collected and converted to maximum intensity projections to eliminate the effect of focal plane on the fluorescence intensity readings. Mean fluorescence intensity was calculated for each image using Olympus cellSens Dimension software and was averaged across all images of peels labeled at each pH increment for graphical interpretation.

2.6. Pectate lyase digestion of onion epidermal cell walls

Washed onion epidermal peel walls were cut into 5 mm × 5 mm squares and were incubated individually in 100 µL of 10 µg/mL pectate lyase (Cellobiose japonicus; Megazyme E-PILY) in 50 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) pH 10 with 2 mM CaCl$_2$, for 5, 15, 45, or 120 min. These conditions were used for pectate lyase digestion because pH 10 chemically de-esterifies homogalacturonan, enabling more complete enzyme action as pectate lyase cannot act on methylesterified galacturonic acid residues. Onion epidermal peel squares were then washed three times for 10 min each in 20 mM HEPES pH 7.5. Treated onion peel squares were labeled with CBM3-A488 in pH 5.5 or pH 7.5 and washed in pH 7.5 buffer, as described above. Peels incubated in 50 mM CAPS pH 10 with 2 mM CaCl$_2$ without pectate lyase for 120 min did not show an increase in CBM3-A488 fluorescence labeling intensity at pH 5.5 or pH 7.5. Treated onion peels were imaged and the mean fluorescence intensity of each image was calculated as described above.

2.7. Zeta potential measurements of CBM3-A488 and onion epidermal peel fragments

The isoelectric point (pI) of the CBM3-A488 conjugate was experimentally determined as follows. CBM3-A488 solutions were prepared at a concentration of 1 mg/mL in 20 mM buffers at each pH of interest. Zeta potential measurements were acquired with a Zetasizer (Malvern Panalytical Ltd, Malvern, United Kingdom). Graphical analysis allowed for calculation of the experimental pI of CBM3-A488.

Epidermal peels of the fifth onion scale were isolated and washed as described above. Onion epidermal wall fragments, suitable for zeta potential measurements, were produced as described previously with some alterations (Wang et al., 2020). Washed epidermal peels were pulverized at 30 Hz for 10 min with a cryomill (Retsch GmbH, Haan, Germany). The resultant wall fragments were filtered through a 100 µm mesh sieve and settled for 15 min. The wall fragments that remained in solution after 15 min were collected and freeze-dried for future use. Freeze-dried wall fragments were rehydrated at a concentration of 1 mg/mL in 50 mM CAPS buffer pH 10, shaking at 500 rpm for 40 h at 26 °C. Rehydrated fragments were treated with 10 µg/mL pectate lyase in 50 mM CAPS buffer pH 10, with 2 mM CaCl$_2$, shaking at 500 rpm for 45 min at 26 °C. Control fragments were incubated in the same way with 10 µg/mL heat-killed pectate lyase (incubated at 95 °C for 10 min). Fragments were then washed three times by centrifugation at 10,000 g for 5 min in either 20 mM sodium acetate pH 5.5 or 20 mM HEPES pH 7.5 and finally re-suspended to a concentration of 1 mg/mL. Zeta potential measurements were obtained with a Zetasizer.

2.8. Preparation of Arabidopsis leaf epidermal peels and labeling with CBM3-A488

Arabidopsis leaf epidermal peels were prepared according to previously published methods, with some modifications (Li et al., 2013). Healthy and relatively flat leaves from 3 to 4 week old wild type Arabidopsis thaliana (Columbia) plants were excised from the rosette and floated abaxial side down in water. The leaves were removed from the petri dish and laid flat on a glass microscope slide with the abaxial side facing up. The right-half and the left-half of the leaf lobes were cut away from the midvein. A small tear was made at the top of the leaf lobe with ultra-fine forceps. The abaxial epidermal layer was then peeled from the mesophyll layer of the leaf by holding the leaf down while pulling the tear at a < 45° angle. Arabidopsis epidermal peels were then washed and labeled with CBM3-A488 as described for onion above.

3. Results

3.1. CBM3a conjugated with Alexa Fluor 488 binds to crystalline cellulose

Alexa Fluor 488 was conjugated with CBM3a to enable visualization of its binding to cellulose in cell walls. Protein modeling revealed an exposed cysteine on the surface of the CBM3a protein to which the Alexa Fluor 488 maleimide could be conjugated, via the free thiol group (Fig. S1). The model of CBM3-A488 is predicted to allow its binding to a single cellulose chain in silico (Fig. S1). This prediction was tested in vitro using crystalline cellulose (Avicel 101), which was indeed strongly labeled (Fig. S2D). Previous research confirmed the specificity of CBM3a as a selective probe for cellulose surfaces (with negligible binding to xylol glucan) in muro (Zheng et al., 2018). Therefore, the CBM3-A488 conjugate can be used to fluorescently label crystalline cellulose in cell wall samples.
3.2. CBM3-A488 reveals fibrillar orientations that vary cell by cell

The CBM3-A488 probe was used to visualize cellulose in onion epidermal peels, revealing distinct fibrillar patterns with both epifluorescence microscopy and scanning confocal fluorescence microscopy (Fig. 1). Bright CBM3-A488 labeling was seen around the outer edge of each cell where the anticlinal wall was torn during the peeling process (Fig. 1). The bright, striated signal observed around the edge of the cell came from the base of each of the anticlinal wall tears (Fig. 1B). An edge-to-edge fibrillar patterning was observed in every cell that was torn open, although intensities varied.

CBM3-A488 labeling of onion epidermal peels was compared with other commonly used dyes and probes, including Calcofluor White, S4B, and GFP-CBM3. With epifluorescence microscope imaging, Calcofluor White stained the entire onion epidermal wall relatively evenly and did not reveal cellulose fibrils (Fig. 2A). Staining with S4B revealed some indication of patterned fluorescence yet fibrils were not clearly visible (Fig. 2B). Dark wrinkles were visible in the anticlinal wall with each of these stains. Variation in cell size and shape did not affect the observed labeling patterns. Labeling with GFP-CBM3 at pH 7.5 showed dim and poorly defined fibrils that varied in clarity from cell to cell (Fig. 2C).

Finally, CBM3-A488 labeling of cellulose was distinctly fibrillar (Fig. 2D). Calcofluor White and S4B staining of the abaxial epidermal wall and the anticlinal side walls (outlines of each cell) did not differ significantly in fluorescence intensity while labeling with the CBM3-based probes was considerably dimmer on the abaxial wall as compared to the anticlinal walls. The dark cell outlines visible with Calcofluor White and S4B staining (Fig. 2A, B) were not specific to these dyes. These dark cell outlines were obscured in the GFP-CBM3 and the CBM3-A488 labeled cell walls (Fig. 2C, D) due to the higher signal intensity in the anticlinal walls and the image contrasting required to visualize the fibrillar features in the abaxial walls. GFP-CBM3 labeling did show fibrils but they were not as distinct as those revealed by CBM3-A488. This could be due to a number of factors such as the probe’s ability to access cellulose binding sites (GFP-CBM3 is three times larger than CBM3-A488) and the difference in relative brightness (extinction coefficient × quantum yield; the brightness intensity of Alexa Fluor 488 is twice that of GFP). CBM3-A488 labeling of cell walls was further investigated to characterize the fibrillar patterns as well as the conditions that enable resolution of fibrils.

The angle of fiber orientation, revealed by CBM3-A488, was typically consistent across the whole face of an individual cell, but varied from cell to cell (Fig. 3A). The angle of fibrillar patterning and degree of anisotropy was quantified in individual cells (red lines in Fig. 3A-C). Fibril angle quantification revealed that the average angle of cellulose fibril orientation in the most recently deposited cell wall lamella was 57.8° ± 18.7° (mean ± SD, n = 202) from the long axis of the cell. In the majority of cells (75%), the fibrils were oriented between 45° and 90° (perpendicular) to the long axis of the cell (Fig. 3D). In a few cells (~2.5%) fibrils ran nearly parallel with the cell’s long axis (at an angle < 20° from the cell’s long axis, Fig. 3B). Occasionally, fibrils appeared more isotropic at the end of the cell which may represent altered cellulose deposition in these regions or could be signal from a lower lamella being visible due to incomplete synthesis/coverage by the most recently deposited lamella (Fig. 3C). These data show that CBM3-A488 reveals edge-to-edge fibrillar patterns in onion epidermal cells that is consistent with previously reported AFM imaging (Zhang et al., 2016), with the majority of cells displaying a cellulose orientation greater than 45° from the cell’s long axis.

3.3. Fibrillar patterns revealed by CBM3-A488 correspond to AFM-detected surface cellulose microfibrils

To determine if the fibrillar patterns revealed by CBM3-A488 labeling corresponded to surface cellulose microfibril patterning in onion cell walls, atomic force microscopy (AFM) imaging was employed. Onion epidermal peels were labeled with CBM3-A488 and imaged with fluorescence microscopy to capture the prominent fiber directionality. As fluorescence microscopy cannot resolve individual cellulose microfibrils (~3 nm wide), the fibrillar patterning revealed by CBM3-A488 labeling likely represents bundles of cellulose fibers. The same cells were then located under AFM and scanned. AFM height images showed that individual cellulose microfibrils on the surface were organized in bundles orientated in a common direction that matched the fluorescent fibrils observed with CBM3-A488 staining (Fig. 4, n = 9). Thus, AFM imaging of the surface cellulose microfibrils invariably corroborated the directionality of the fluorescently-detected fibrillar bundle patterning, which varied from cell to cell.

3.4. Binding of CBM3-A488 to onion cell walls is pH dependent

CBM3-A488 labeling of onion cell walls was tested at a range of pH values to determine optimal labeling conditions for fibril visualization. Focusing on the wall (below where the sidewalls are clearly resolved) allows for the visualization of fibrillar patterning with CBM3-A488. Fibrillar patterning was most distinct with labeling at pH 7.5 (Figs. 2-4). Labeling with CBM3-A488 at pH 5.5 resembled staining with Calcofluor White and S4B, characterized by a lack of distinct fibrillar patterning and dark cell outlines (Fig. S3). GFP-CBM3 also exhibited a pH-dependent difference in labeling, mirroring CBM3-A488. The pH-dependent visualization of fibrillar patterning with CBM3-A488 labeling, which is not seen with Calcofluor White or S4B, prompted further investigation into the factors which contribute to this phenomenon.

CBM3-A488 binding to the cell wall at each pH of labeling was quantified by the mean fluorescence intensity of maximum intensity projections to determine if pH affected the intensity of labeling. We acquired Z-stacks through the entire epidermal peel, with identical exposure and gain settings, for each pH of labeling. (Note: The low

![Fig. 1. CBM3-A488 labeling shows fibrils. Onion epidermal cell walls labeled with CBM3-A488 show fibrillar features with (A) epifluorescence and (B) laser scanning confocal microscopy. Scale bars = 20 µm.](image-url)
exposure settings required to capture representative images of the fluorescence intensities at each pH did not effectively resolve the fibrillar pattering that can be seen in the single-plane, contrasted images in Figs. 2-4). The pH at which fibrils are best observed (between pH 7 and 8) corresponded to the lower end of the range of labeling fluorescence intensity (Fig. 5A). Quantification of fluorescence intensities showed peak labeling at pH 5.5, followed by a rapid decline in intensity that progressed towards a plateau above pH 7 (Fig. 5B). The pH dependence experiment was repeated twice (n = 21), using different imaging conditions, with both sets displaying the same result shown by the representative data in Fig. 5. Pure crystalline cellulose (Avicel 101) labeled with CBM3-A488 at pH 5.5 and pH 7.5 did not show a significant

Fig. 2. CBM3-A488 labeling compared to other cellulose dyes and probes. Epifluorescence microscopy images of Calcofluor White (A), S4B (B), GFP-CBM3 (C), and CBM3-A488 (D) labeled onion epidermal peels show varying resolution of cellulose fibril patterning. Scale bars = 50 µm.

Fig. 3. Orientation of cellulose fibrils labeled with CBM3-A488. Net orientation and degree of anisotropy of cellulose fibrils recognized by CBM3-A488 were analyzed with FibrilTool. Yellow boxes are regions of interest where the fibrillar features are apparent. Red lines are the vector output by FibrilTool, showing the net orientation and degree of anisotropy (length of the line). Comparison of neighboring cells on the same epidermal peel shows variation in fibril angle (A). A representative cell with cellulose fibrils oriented parallel to the cell’s long-axis (B); some cell corners show a lower degree of anisotropy than the rest of the cell (C). A Raincloud plot illustrates the net orientation of fibrils with respect to long axis of analyzed cells, n = 202 (D). The half violin plot displays the distribution of fibril angles, the box and whiskers plot depicts the inter-quartile range and the maximum and minimum fibril angles, with the diamond data points representing outliers. Images captured with epifluorescence microscope. Scale bars = 20 µm.
difference in fluorescence intensity (Fig. S2E). Consistent with the Avicel results, the strong fluorescence of onion walls labeled with CBM3-A488 at pH 5.5 persisted after extensive washes with pH 7.5 buffer (Fig. S4). AFM scanning showed that the cell wall structure does not change when epidermal peels are incubated at pH 5.5 and then washed in pH 7.5 buffer (Fig. S5). These results collectively demonstrate that the strong pH dependence of CBM3-A488 labeling is a cell wall phenomenon and is not due to pH dependence of Alexa Fluor 488 fluorescence, CBM3-A488 binding to cellulose, or structural changes in the cell wall between these two pH.

We hypothesized that the pH-dependent variation in CBM3-A488 labeling strength arose from changes in the electrostatic potential of the CBM3a protein as a function of pH. To test this hypothesis, we measured the zeta potential of CBM3a (Fig. 5C). The effective isoelectric point (point of net neutral charge) was between pH 5 and pH 5.5, with a positive surface charge at more acidic pH and a negative charge at more basic pH. The isoelectric point of CBM3a coincided with the pH at which the highest fluorescence intensity was observed (pH 5.5). Because of its
high concentration of acidic pectins, the cell wall should have a negative potential (Wilson et al., 2021); hence electrostatic repulsion of CBM3a would vary with pH, being maximal at neutral pH and decreasing as pH approached the CBM3a isoelectric point.

In summary, light labeling and distinct fibrillar patterns were observed with CBM3-A488 labeling at pH 7.5, whereas the highest fluorescence intensity was observed at pH 5.5, coincident with the CBM3a isoelectric point. We hypothesized that this phenomenon depended on CBM3-A488 diffusion into the wall. Deeper penetration of CBM3-A488 at low pH would enable more lamellae to be labeled, thereby increasing the total fluorescence signal, but obscuring the surface fibrillar features. Confocal Z-stacks confirmed that at pH 5.5, CBM3-A488 labeling could be seen throughout the entire 6 µm depth of the onion epidermal cell wall (Fig. S6). At pH 7.5, CBM3-A488 fibrillar patterning was observed in the surface optical sections and fluorescence signal was not detected deeper in the wall (Fig. S6). No difference was observed in the penetration or labeling patterns of Calcofluor White and S4B at the two assayed pH, with both mirroring the fluorescence labeling depth of CBM3-A488 at pH 5.5 (Fig. S6). Ultimately, fibrillar patterns are only observed when the probe selectively labels the surface of the cell wall.

3.5. Removal of pectin enhances CBM3-A488 binding and reduces the negative charge of the wall

CBM3-A488 showed reduced penetration into the cell wall when the protein carried a negative charge (at pH 7.5). Homogalacturonan is the most abundant polysaccharide in onion epidermal walls (Wilson et al., 2021) and imparts a negative charge to the wall. To test homogalacturonan’s role in this phenomenon, we conducted the following experiments.

The labeling of onion cell walls with CBM3-A488 was examined following pectate lyase digestion to partially remove homogalacturonan. We hypothesized that removal of negatively-charged homogalacturonan would allow the CBM3-A488 probe to penetrate further into the wall. Increased labeling after pectate lyase digestion was visually evident at both pH 5.5 and pH 7.5 (Fig. 6A). (Note: The low exposure settings required to capture representative images of the fluorescence intensities did not resolve the fibrillar patterning that can be seen in Figs. 2-4). Quantification of fluorescence intensity extended this observation, showing a steep increase in labeling following five minutes of pectate lyase digestion, a further increase with 15 min of digestion, and a leveling of fluorescence intensity with subsequent digestion (Fig. 5B). This trend was consistent for CBM3-A488 labeling at both pH

![Fig. 6. Pectate lyase (PL) digestion of onion epidermal peels enhances CBM3-A488 labeling. Epifluorescence microscopy was used to capture representative maximum intensity projections of onion cell walls, digested with pectate lyase for different amounts of time and subsequently labeled with CBM3-A488 at two pHs (A). Mean fluorescent intensity measurements show an increase in CBM3-A488 labeling with pectate lyase digestion (B). Open circle markers represent labeling at pH 5.5 and dark square markers represent labeling at pH 7.5. (n = 12) Image scale bars = 20 µm. Error bars represent standard error of the mean.](https://example.com/fig6.png)
5.5 and pH 7.5. Confocal Z-stacks showed that pectate lyase digestion increased the penetration of CBM3-A488 into the cell wall and obscured fibrillar patterning at pH 7.5 (Fig. S6, bottom row). Removal of homogalacturonan from the onion cell wall likely increased CBM3-A488 labeling by allowing for increased penetration of the charged conjugate to deeper cell wall lamella in addition to unmasking cellulose for CBM3-A488 to bind.

To test our prediction that pectate lyase digestion reduces the electrostatic charge of the onion wall, we measured the zeta potential of onion epidermal fragments pretreated with buffer or pectate lyase for 45 min. The wall fragments were washed and re-suspended in buffers of pH 5.5 and pH 7.5, or the pectate lyase digested wall fragments re-suspended at both pH 5.5 and pH 7.5. pectate lyase treatment resulted in a significant reduction in the negative electrostatic potential at both pH (Fig. 7). The electrostatic potential of the cell wall at pH 5.5 and pH 7.5, both before and after pectate lyase digestion, were equivalent. Therefore, pH does not affect the electrostatic charge of the cell wall, in the assayed range. This data indicates that the pH-dependent difference in CBM3-A488 labeling is due to the charge carried by the probe (Fig. 5C), not the electrostatic potential of the cell wall (Fig. 7).

3.6. CBM3-A488 labeling of Arabidopsis leaf epidermal peels revealed fibrillar patterns

To further test the potential of this newly developed fluorescent cellulose probe, Arabidopsis leaf epidermal peels were labeled with CBM3-A488. The distinct lobed shape of Arabidopsis pavement cells could be clearly observed with CBM3-A488 labeling, which brightly labeled the torn anticlinal walls at the cell outlines (Fig. 8A). This phenomenon was also observed with CBM3-A488 labeling of onion epidermal peels (Figs. 1-4). Fibrillar patterns were observed in Arabidopsis pavement peels and were found to cross the cells, edge-to-edge, as is evident in Fig. 8B and C. These results demonstrate the efficacy of CBM3-A488 to fluorescently label the most-recently deposited layer of cellulose fibrils in epidermal peels of a variety of plant species.

The electrostatic repulsion of CBM3-A488 from the cell wall at pH 7.5 is attributed to the presence of negatively-charged pectins, a major component of the cell wall. Removal of pectin from the onion cell wall increased CBM3-A488 labeling at both pH 5.5 and pH 7.5 and obscured fibrillar features. Aside from modulating the charge on the conjugate, changes in pH may affect pectin-cellulose interactions. A recent solid-state nuclear magnetic resonance study of Arabidopsis cell walls under acidic (pH 4) and neutral (pH 6.8) conditions revealed more dynamic pectin mobility and increased water-cellulose contacts at pH 4 (Phyo et al., 2019). Could a weakening of pectin-cellulose interactions at acidic pH increase the availability of cellulose for CBM3-A488 binding in our experiments? This seems doubtful because the electrostatic charge of the cell wall at pH 5.5 and 7.5 was equivalent (Fig. 7). This leaves the altered surface charge carried by CBM3-A488 as the dominant influence on wall labeling efficiency.

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The findings reported here hold broader implications for the use of protein probes (CBMs and/or antibodies) to label substrates of interest. Our results show a nearly 4-fold difference in CBM3-A488 labeling between the pH environments that exhibit the lowest (pH 8) and highest (pH 5.5) labeling (Fig. 8B). CBMs have been used in numerous reports to assess the correlation of cellulose crystallinity to the enzymatic and chemical degradability of recalcitrant biofuel feedstocks (Gourlay et al., 2015; Novy et al., 2019; Kawakubo et al., 2010). One study compared binding patterns of two CBMs to quantify crystalline (CBM3a) and amorphous (CBM28) cellulose content in Arabidopsis stem sections (Ruel et al., 2012). Labeling was conducted at pH 7.4 and the authors report a surprising lack of amorphous cellulose (CBM28 binding) in primary cell walls, while CBM28 binding was observed in secondary cell walls. CBM28 (pI 3.6) would carry a highly negative charge at pH 7.4.
which may account for the inability to detect amorphous cellulose in pectin-rich primary cell walls. While many other factors may have influenced the binding patterns observed in this report, future studies should account for the potential of CBMs, and even antibodies (pI ~ 8–9), to exhibit different pH-dependent labeling efficiencies.

Our results also hold implications for experiments involving enzymatic digestion of cell walls, reinforcing previous studies that reported pH-dependent changes in the digestion efficiency of multi-polymeric substrates. Saccharification of lignocellulosic material with cellulase was reported to be 10–20 % more efficient at a half pH unit higher than the enzyme’s optimum (pH 5.2 vs pH 4.8) (Lan et al., 2013; Lou et al., 2013). This phenomenon was attributed to the accumulation of negative charges on both the surface of lignin and the enzyme so that electrostatic repulsion prevented non-specific binding of the cellulase to the lignin-rich fraction (Lou et al., 2013; Rahikainen et al., 2013). These reports and our current work indicate that the electrostatic charge carried by a protein, modulated by the pH of the environment, may have a significant effect on its interactions with charged substrates.

A variety of potential experiments could make use of the specificity of CBM3-A488, to visualize the most recently deposited layer of cellulose microfibrils. As cell walls are deposited in a layered pattern, CBM3-A488 could be useful for tracking the synthesis of cell walls in developing tissues, with respect to changes in fibril orientation. Microtubules are responsible for guiding cellulose synthases in the plasma membrane as they deposit cellulose (Paredes et al., 2006) and have been observed in a variety of different orientations in actively growing cells (Duncombe et al., 2021). In our study, the cellulose fibril patterns revealed by CBM3-A488 in Arabidopsis pavement cells (Fig. 8) showed similarities to images of fluorescently-labeled microtubules in this cell type (Fu et al., 2005; Sampathkumar et al., 2014). It has been shown that microtubules rapidly reorient when plant cells are exposed to blue light (Saltini and Mulder, 2021) or experience mechanical stress (Bidhendi and Geitmann, 2019; Bidhendi et al., 2019; Colin et al., 2020). In future studies, CBM3-A488 could be used to visualize the cellulose orientation in the most recently deposited cell wall lamella following specific light or mechanical treatments, to quantify the effect that short-lived or sustained microtubule reorientation has on developing cell walls.

**Glossary**

**Onion scale**: Fleshy layers of onion bulb that are numbered by counting from the outermost fleshy layer.

**Abaxial**: The epidermal cells on the exterior surface of the onion scale (convex side) and/or the underside of an Arabidopsis leaf.

**Anticlinal**: The side (vertical) walls that surround each cell and are torn during the process of making an epidermal peel.

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**CRediT authorship contribution statement**

**Sarah A. Pfaff**: Conceptualization, Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Xuan Wang**: Conceptualization, Methodology, Investigation, Writing – original draft. **Edward R. Wagner**: Resources, Methodology, Investigation. **Liza A. Wilson**: Investigation. **Sarah N. Kiemle**: Project administration. **Daniel J. Cosgrove**: Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Appendix A. Supplementary data

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