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Understanding How the Complex Molecular Architecture of Mannan-degrading Hydrolases Contributes to Plant Cell Wall Degradation*

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Background: The molecular architectures of cell wall degrading enzymes are complex.
Results: The activity of mannanases and esterases and CBM function against cell walls are driven by substrate context.
Conclusion: GH26 and GH5 mannanases target soluble and cell wall mannans, respectively, and CBM potentiation is cell wall-dependent.
Significance: The context of cell wall polysaccharides drives the molecular architectures of cell wall-degrading enzymes.

Microbial degradation of plant cell walls is a central component of the carbon cycle and is of increasing importance in environmentally significant industries. Plant cell wall-degrading enzymes have a complex molecular architecture consisting of catalytic modules and, frequently, multiple non-catalytic carbohydrate binding modules (CBMs). It is currently unclear whether the specificities of the CBMs or the topology of the catalytic modules are the primary drivers for the specificity of these enzymes against plant cell walls. Here, we have evaluated the relationship between CBM specificity and their capacity to enhance the activity of GH5 and GH26 mannanases and CE2 esterases against intact plant cell walls. The data show that cellulose and mannan binding CBMs have the greatest impact on the removal of mannan from tobacco and *Physcomitrella* cell walls, respectively. Although the action of the GH5 mannanase was independent of the context of mannan in tobacco cell walls, a significant proportion of the polysaccharide was inaccessible to the GH26 enzyme. The recalcitrant mannan, however, was fully accessible to the GH26 mannanase appended to a cellulose binding CBM. Although CE2 esterases display similar specificities against acetylated substrates *in vitro*, only *Cj*CE2C was active against acetylated mannan in *Physcomitrella*. Appending a mannan binding CBM27 to *Cj*CE2C potentiated its activity against *Physcomitrella* walls, whereas a xylan binding CBM reduced the capacity of esterases to deacetylate xylan in tobacco walls. This work provides insight into the biological significance for the complex array of hydrolytic enzymes expressed by plant cell wall-degrading microorganisms.

Microbial degradation of plant cell walls is an important biological process that is integral to the carbon cycle. This process is also of increasing industrial significance, as plant biomass is an abundant, renewable, and thus sustainable substrate for the environmentally relevant bioenergy and bioprocessing sectors (1, 2). Plant cell walls consist mainly of polysaccharides. Cellulose, the major component of the wall, is a β-1,4-linked glucose polymer that forms crystalline microfibrils. These microfibrils are embedded in and are cross-linked by matrix polysaccharides that consist of hemicelluloses and pectic polysaccharides (for review, see Ref. 3). Reflecting the diversity of glycans that comprise the substrate, microorganisms that degrade plant cell walls express an extensive repertoire of degradative enzymes that include glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases (4).

In addition to the chemical complexity of plant cell walls, their component polysaccharides make extensive interactions that greatly restrict the capacity of degradative enzymes to access their target substrates within these composite structures (4). In general, microbial plant cell wall degrading hydrolases are modular proteins in which the catalytic module is appended to one or more non-catalytic carbohydrate binding modules (CBMs)[2] that typically bind to the target substrate of the enzyme or crystalline cellulose, although both types of CBM can be present in the same protein (Refs. 5–7; for review, see Ref. 8). *In vitro* experiments have shown that CBMs, which bind to the internal regions of glycans (defined as *endo*-CBMs) potentiate the activity of glycoside hydrolases against insoluble polysaccharides, classically cellulose (9, 10). Furthermore, recent work has shown that CBMs, which bind to the terminal sugars of branched polysaccharides (defined as *exo*-CBMs), mediate a substantial increase in the activity of exo-acting glycosidases even though the glycan is soluble and thus fully accessible to enzymatic attack (11, 12).

The mechanism(s) by which CBMs enhance catalytic activity appears to vary. There is evidence that some *exo*-CBMs

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[2] The abbreviations used are: CBM, carbohydrate binding modules; CE2, carbohydrate esterase family 2; GH, glycoside hydrolase family.

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enhance enzyme binding to branched substrates through an avidity effect, and the resultant decrease in apparent K_m results in a substantial increase in catalytic activity (11). It has also been proposed that *exo*-CBMs, which target the degradation products generated by pectate lyases (7), or xylans in which the glucuronic acid is unmethylated (13) may direct enzymes to regions of the plant cell wall that are already undergoing degradation and are thus accessible to enzymatic attack. In contrast, *endo*-CBMs of plant cell wall hydrolases bring the appended enzyme into prolonged and intimate contact with its target substrate, allowing the catalytic module to access and cleave its target glycosidic bonds (14). Analysis of the functional significance of CBMs has to date predominantly utilized purified or highly processed substrates (9, 10, 14). The only report on the effect of CBMs on enzymes acting on complete cell walls showed that cellulose-specific CBMs potentiated the activity of xylanases and pectinases, whereas the activities of xylan degrading enzymes were enhanced by a CBM that binds to the xylose polymer (15). Although these data provide some insight into the juxtaposition of polysaccharides within plant cell walls and thus present a rationale for the modular structure of xylanases and some pectinases, further studies are required to provide a more complete understanding of the role of CBMs in enzyme action *in vivo*.

Xylans and mannans are the major hemicellulosic polysaccharides found in nature. Xylans are abundant in the primary cell walls of grasses such as *Miscanthus giganteus* and in the thickened secondary cell walls of dicotyledons such as tobacco. Heteromannan polysaccharides are relatively abundant in the walls of some non-angiosperm land plants such as the moss *Physcomitrella patens* (16) but are also found to some extent in the primary and, together with xylans, in the secondary cell walls of angiosperms. The backbone of this heterogeneous β-1,4-linked polysaccharide can consist exclusively of mannose (mannans) or a random sequence of glucose and mannose residues (glucomannan). Both mannans and glucomannans are often acetylated and can also be decorated with α-1,6-galactose residues (galactomannan and galactoglucomannan) (3). Heteromannans are degraded by mannanases that are located in the sequence-based glycoside hydrolase families (GHs) 5 and 26 in the CAZy database (17). Both GH5 and GH26 mannanases share the same-fold, catalytic apparatus and mechanism of action (acid/base-assisted double displacement mechanism), consistent with their location in clan GH-A (17, 18). There is significant variation in the specificity of mannanases, with some enzymes, typically those located in GH5, able to accommodate mannose and glucose residues at sugar binding subsites (see subsite nomenclature developed by Davies *et al.* (19)) distal to the active site (which by definition binds only mannose), whereas a cohort of GH26 enzymes display tight specificity for mannose at the critical −2 subsite, which dominates substrate binding (18). The molecular architecture of mannanases varies. Some mannanases consist of only a catalytic module, whereas others contain CBMs that bind to crystalline cellulose or mannan, with some enzymes containing both types of CBM. The influence of the GH origin, and the specificity of the individual subsites, on the *in vivo* activity of mannanases are unknown. Similarly, the contribution of cellulose- and mannan-specific CBMs to the activity of mannanases against intact cell walls is, currently, unclear. In this report we have analyzed the influence of subsite specificity and CBM composition on the activity of mannanases and esterases against intact cell walls. The data showed that although cellulose-specific CBMs potentiate mannanase action against tobacco stem secondary walls, mannose-specific CBMs play a more important role in the action of both mannanases and esterases against *Physcomitrella* cell walls. Although a significant proportion of the mannan in tobacco walls is recalcitrant to the model GH26 mannanase (*Cj*Man26A), the polysaccharide is accessible to *Cj*Man26A when the enzyme is appended to a cellulose binding CBM. These data indicate that the array of CBMs appended to mannanases confer context-dependent potentiation of enzymatic activity.

MATERIALS AND METHODS

Construction of Plasmids, Purification of Proteins, and the Source of Antibodies—The monoclonal antibodies LM21, which recognizes heteromannans (20), CCRC-M170, which binds exclusively to acetylated mannan,[3] and LM23, which binds to non-acetylated xylan (21), were used as unpurified hybridoma cell culture supernatants (LM antibodies are available from PlantProbes, and CCRC-M170 is available from CarboSource).[3] The plasmids encoding the catalytic modules of the *Cellvibrio japonicus* mannanases, *Cj*Man26A and *Cj*Man5A (5, 22), the GH10 (*Cj*Xyn10A) (24) and GH11 (*Np*Xyn11A) (25) xylanases, and the *C. japonicus* and *Clostridium thermocellum* family CE2 esterases (*Cj*CE2A, *Cj*CE2B, *Cj*CE2C, and *Ct*CE2) (26) were constructed as described previously. CBM2b-1-2, CBM2a, CBM3a, CBM35, and CBM27 were derived from *Cellulomonas fimi* xylanase Xyl11A (6), *C. japonicus* xylanase Xyn10A (24), *C. thermocellum* cellulosome-integrating protein CipA (27), *C. japonicus* mannanase Man5C (28), and *Thermotoga maritima* mannanase Man5 (29). The vector pFV1-PT (15), a derivative of pET22b, was used to construct the plasmids encoding the enzyme-CBM fusions. Briefly, pFV1-PT contains two multiple cloning regions that flank a sequence encoding a 15-residue Pro-Thr linker peptide. DNA sequences encoding the catalytic modules of the two mannanases (CjMan5A$_{CM}$ and CjMan26A$_{CM}$), two of the esterases, *Cj*CE2C and *Cj*CE2B, and the CBMs were amplified by PCR using thermostable KOD Hot start DNA polymerase (Novagen). The amplified DNAs encoding the catalytic modules were cloned into either BamHI-HindIII (*cjman5A*)- or BamHI (*cjman26A*)-digested pFV1-PT vector. The amplified DNA fragments encoding CBM3a, CBM2a, CBM35, CBM27, and CBM2b-1–2 were then cloned into appropriately restricted pFV1-PT that contained DNA sequences encoding the catalytic modules using various different restriction site combinations. The esterases and mannanases encoded by these recombinant plasmids contained N-terminal or C-terminal CBMs, respectively, attached through the Pro-Thr linker. A schematic of the enzymes used in this work is shown in Fig. 1. Soluble proteins were produced in *Escherichia coli* strain TUNER using standard growth regimes using LB medium supplemented with ampicillin at 50 μg/ml.

[3] M. G. Hahn, manuscript in preparation.

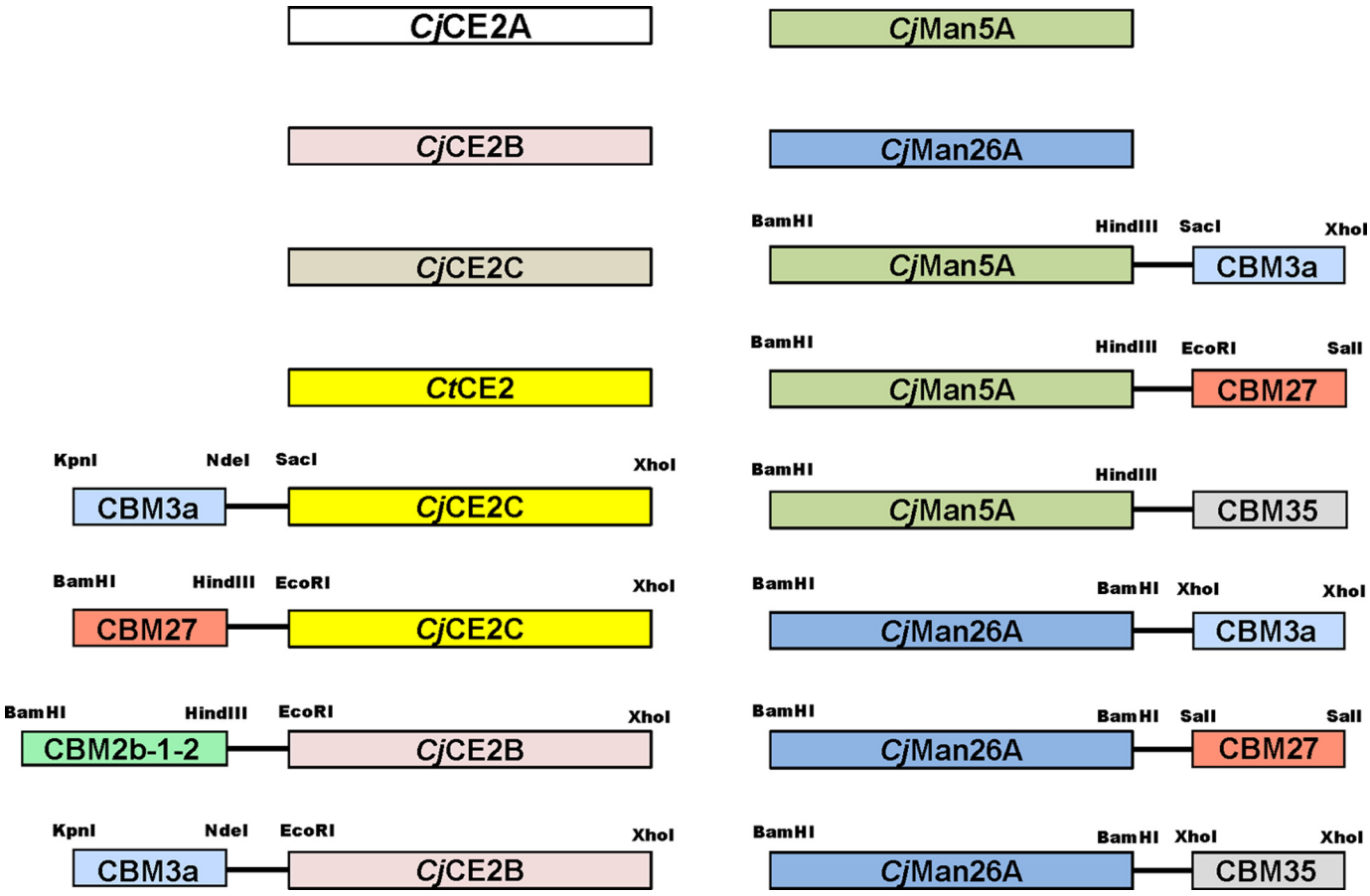


FIGURE 1. **Schematic of the mannanases and esterases used in this study.** The line joining the catalytic modules and CBMs represents a 15-residue Thr-Pro linker. The restriction sites used to clone the DNAs encoding the catalytic modules and CBMs are indicated.

At mid-exponential phase ($A_{600\ nm}$ = 0.6) recombinant protein expression was induced using 0.2 mM isopropyl β-D-1-thiogalactopyranoside and incubation for 16 h at 16 °C. The enzymes, which all contained a C-terminal His_6 tag, were purified by immobilized metal ion affinity chromatography using Talon™ resin and, typically, elution with 150 mM imidazole as described previously (30). All proteins were purified to electrophoretic homogeneity.

Enzyme Assays—To determine the specific activities of the enzymes against soluble substrates, the mannanases were assayed as described previously (18) in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mg/ml BSA (NaPB buffer) against the substrate carob galactomannan (Megazyme International) at 5 mg/ml, which was solubilized (homogenized) by autoclaving. Reducing sugar release was detected using 3,5-dinitrosalicylic acid (31). Esterase activity was assayed by the method of Montanier *et al.* (26) in NaPB buffer containing 1 mM 4-nitrophenyl acetate, and the product, 4-nitrophenolate, was monitored at 400 nm and quantified using a molar extinction coefficient of 10283 M^{-1} cm^{-1}. All assays were carried out at 37 °C.

Preparation of Plant Materials, Enzymatic Treatments, and Polysaccharide Immunodetection Procedures—Tobacco (*Nicotiana tabacum* L.), *M. giganteus*, and *P. patens* plants were grown as described (32, 33). Excised stem regions from tobacco and *Miscanthus* were fixed in 50 mM Pipes buffer, pH 6.9, containing 5 mM EGTA, 5 mM $MgSO_4$, and 4% (v/v) paraformaldehyde and were embedded in wax and sectioned as described previously for tobacco stems (34). *P. patens* gametophores were embedded in LR White resin as described (33). To avoid the oxidation of the acetyl group on mannans, *Physcomitrella* slides were used within 3 days of sectioning. As access to mannan can be masked by pectic homogalacturonan (20), tobacco stem sections were treated with pectate lyase (*Cj*Pel10A; 10 μg/ml) in 50 mM CAPS buffer, pH 10, containing 2 mM $CaCl_2$ for 2 h before experiments in which the action of the mannanases was investigated.

Mannanase and esterase treatments were carried out as described previously (35) at 100 and 130 nM, respectively, for 30 min in phosphate-buffered saline (PBS), although full enzyme titrations were also carried out in specific experiments. The xylanases *Cj*Xyn10A and *Np*Xyn11A were incubated with tobacco slides previously treated with the *Cj*CE2B esterase at a concentration of 1 μM for 1 h in PBS at 37 °C. Sections not treated with the enzymes were incubated for an equivalent time with the corresponding buffers. All sections were subsequently treated for 20 min with 5 μg/ml proteinase K (Sigma) in PBS before substrate detection to remove any enzymes still attached to the cell walls through their CBMs. The capacity of the proteinase to remove CBMs was confirmed by (*a*) the observation that these proteins were fully degraded in solution after incubation for 15 min with the proteinase at 5 μg/ml and (*b*) pre-

treatment of sections with untagged CBMs followed by proteinase K incubation did not affect the subsequent binding of His-tagged forms of these protein modules. Detection of mannan, acetylated mannan, and deacetylated xylan with LM21, CCRC-M170, and LM23 antibodies, respectively, deploying appropriate FITC-labeled secondary antibodies, was carried out as described (33, 34, 36).

Immunofluorescence Microscopy and Quantification of Enzyme Impact on Cell Wall Polysaccharides—Immunofluorescence analysis was carried out with an Olympus BX-61 microscope equipped with epifluorescence irradiation, and all micrographs were captured with an ORCA 285 camera (Hamamatsu) using Volocity software (PerkinElmer Life Sciences). The relative capacities of the enzymes to degrade their substrates within cell walls were determined by quantitative assessments of the immunofluorescence intensities, which were captured in equivalent micrographs using a protocol that has been described (35). Briefly, using Volocity quantitation software, the absolute level of fluorescence contained in the micrographs was determined. For cell wall deconstruction, the modulation of signal is the disappearance of epitopes after polysaccharide degradation in the case of LM21 and CCRC-M170 or the appearance of epitopes when using LM23 as the probe. Control micrographs obtained without enzymatic treatment were designated as 100% of initial fluorescence. In all cases the fluorescence quantification derives from the analysis of micrographs obtained from a minimum of three separate sections that were prepared from at least three separate plants. The fluorescence quantification values shown are the means of a minimum of four assessments.

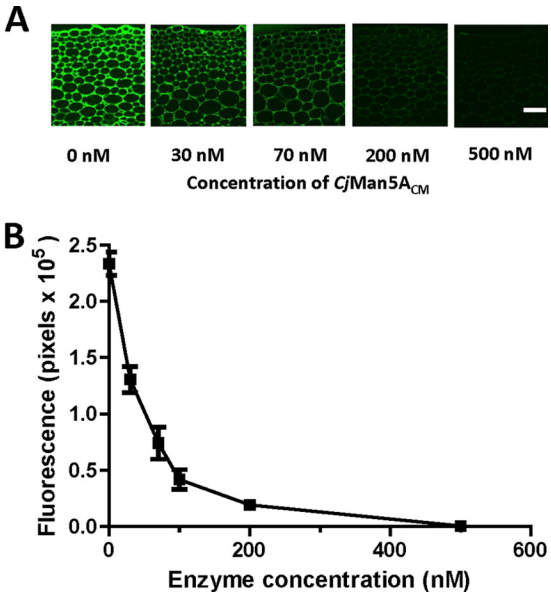


FIGURE 2. **The activity of *Cj*Man5A$_{CM}$ against tobacco cell walls.** Sections were incubated with *Cj*Man5A$_{CM}$ at the concentrations indicated for 20 min at 25 °C. The mannan remaining after enzymatic treatment was determined by probing with the mannan-specific antibody LM21. *Panel A* displays representative immunofluorescence micrographs of LM21 binding to enzyme-treated sections in which the *scale bar* is 50 μm. *Panel B* shows the fluorescence intensities of the enzyme treated sections. Each datum point presented in this figure and Figs. 3–7 is derived from three or four different sections. The *scale bar* is 50 mm.

RESULTS

Enzyme and CBM Selection

Mannanases—The two mannanases selected for this study were *C. japonicus* enzymes from GH26 (*Cj*Man26A) and GH5 (*Cj*Man5A) (18). The two enzymes display a similar ratio of activity (k_{cat}/K_m) against galactomannan and glucomannan, although the GH26 mannanase is ~10-fold more active against soluble forms of the two polysaccharides (18).

Esterases—The esterases selected for this study were four members of carbohydrate esterase family 2 (CE2) that were previously shown to deacetylate acetylated xylan and acetylated glucomannan and hydrolyze 4-nitrophenyl acetate (26). These enzymes were derived from either *C. japonicus* (*Cj*CE2A, *Cj*CE2B, and *Cj*CE2C) or *C. thermocellum* (*Ct*CE2). *Ct*CE2 and *Cj*CE2A were ~6-fold less active than *Cj*CE2B and *Cj*CE2C against 4-nitrophenyl acetate, and *Cj*CE2B was 20–40-fold more active against acetylated glucomannan than the other esterases, whereas *Ct*CE2 was 3–10-fold less active than the other enzymes against acetylated birchwood xylan (26).

CBMs—The CBMs deployed in this work targeted mannan (CBM27 and CBM35, which display high and low affinity for the polysaccharide) crystalline cellulose (CBM3a) or xylan (CBM2b-1-2).

Rationale for the Selection of the Protein Modules—As no member of GH5 and GH26 enzymes display the same CBM combination, the evaluation of the influence of non-catalytic carbohydrate binding modules on mannanase and esterase activity requires the generation of artificial enzymes. Thus, the basis for our enzyme selection strategy was to use highly characterized mannanases that display distinct specificities in the critical −2 subsite, where the GH5 enzyme can bind Glc or Man, whereas the GH26 mannanase only binds Man (see below). Again, CBM selection was based on highly characterized modules that displayed specificities relevant to mannan degradation in the cell wall.

The Activity of Mannanases and Esterases against Plant Cell Walls

Mannanases against Cell Walls—Sections of tobacco stem and *Physcomitrella* gametophores were incubated with a range of concentrations of the catalytic modules of *Cj*Man26A (*Cj*Man26A$_{CM}$) and *Cj*Man5A (*Cj*Man5A$_{CM}$), and the loss of mannan was monitored by measuring the fluorescence signal from the anti-mannan antibody, LM21. The data, Fig. 2, showed that there was a direct correlation between the amount of *Cj*Man5A$_{CM}$ incubated with the tobacco sections and the extent of mannan degradation. Thus, the activity of the GH5 enzyme against mannan was independent of the context of the polysaccharide within the cell walls. In contrast, ~60% of the mannan in tobacco was recalcitrant to degradation by the GH26 mannanase; 30 nM concentrations of the enzyme removed 40% of the polysaccharide in 20 min; however, the addition of 20-fold more enzyme (0.6 μM) did not result in substantial further degradation of the mannose polymer (Fig. 3). The titration profiles of the two mannanases against moss cell walls revealed a proportion of mannan that was recalcitrant to attack by both enzymes (Fig. 4). In the case of *Cj*Man5A$_{CM}$,

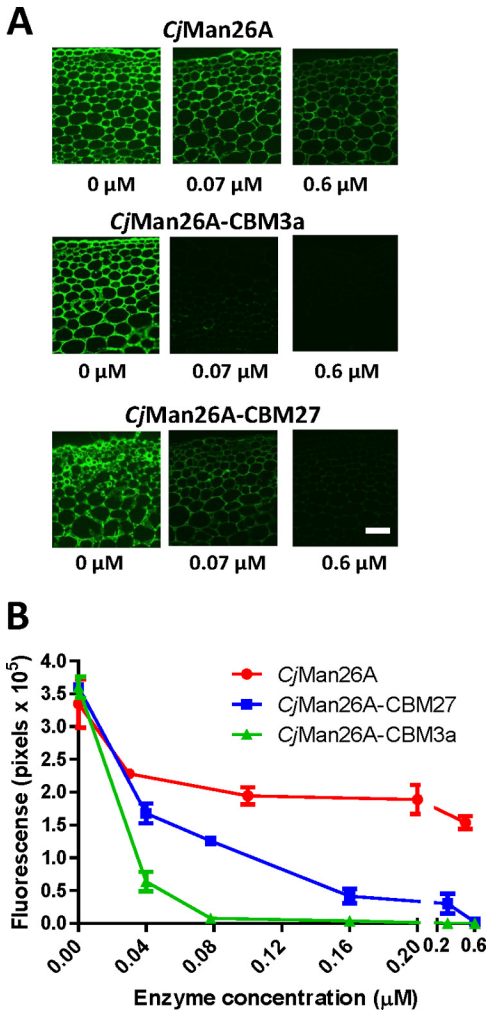


FIGURE 3. **The activity of *Cj*Man26A against tobacco cell walls.** Stem sections were incubated with wild type *Cj*Man26A (lacks a CBM) and the enzyme fused to cellulose (CBM3a) and mannan (CBM27) binding CBMs at the concentrations indicated for 20 min at 25 °C. The mannan remaining after enzymatic treatment was determined using the mannan-specific antibody LM21. *Panels A* and *B* display representative immunofluorescence micrographs and the quantified fluorescence intensities of all the sections, respectively. The *scale bar* is 50 μm.

the undigestible mannan corresponded to ~40% of the polysaccharide, whereas ~77% was resistant to CjMan26A$_{CM}$ attack at enzyme concentrations ≤2.5 μM.

The differential activity of CjMan5A$_{CM}$ and CjMan26A$_{CM}$, respectively, against, particularly, tobacco cell walls is surprising given that *Cj*Man26A is 6–10-fold more active against soluble mannans than *Cj*Man5A (18). A similar picture is presented by GH10 and GH11 xylanases. Although GH11 xylanases are more active than GH10 enzymes, the opposite is observed when the xylan is embedded in the cell wall (15, 35). The differential activity of the xylanases can be explained by the different topologies of the respective substrate binding clefts that are optimized to bind single chains in a deep cleft (GH11) or accommodate xylan in a more open cleft (GH10), allowing access to substrate that is in close association with other components of the wall. In contrast, the topology of GH5 and GH26 mannanases are very similar (18), suggesting that the differential activity of the two enzymes against the tobacco cell wall reflects the molecular details of their respective sugar binding subsites. In *Cj*Man26A the −2 subsite plays a pivotal role in the activity of the enzyme, reflecting the unusually high activity of the enzyme against small mannooligosaccharides (*Cj*Man26A is ~10^5-fold more active against mannotriose than GH5 mannanases (5, 18)), with the subsite displaying absolute specificity for mannose. In contrast the −2 subsite of *Cj*Man5A displays relaxed specificity binding with similar affinity to glucose and mannose. It is likely, therefore, that the intricate array of polar interactions between mannose and the −2 subsite of *Cj*Man26A requires the substrate to be highly exposed to solvent, which will be restricted in the context of the cell wall. The weaker and less specific hydrophobic interactions displayed by *Cj*Man5A and other GH5 mannanases may facilitate the capacity of these enzymes to attack mannans located in plant cell walls.

Esterases—The four CE2 esterases described above were incubated with sections of *Physcomitrella*, and the loss of the epitope recognized by CCRC-M170, an antibody that specifically binds to acetylated mannan, was monitored. The data (Fig. 5) showed that *Cj*CE2C, but none of the other CE2 esterases, was able to significantly deacetylate mannan in intact cell walls of *Physcomitrella*. To explore the capacity of the esterases to attack acetylated xylans within plant cell walls, tobacco stem sections were probed with LM23, an antibody that recognizes non-acetylated xylan but not acetylated xylan (21). The data showed that LM23 displays significantly increased binding to the secondary walls of xylem cells in tobacco stems after, but not before, treatment with the four CE2 esterases (Fig. 6). Incubation of the *Cj*CE2B-treated tobacco stem sections with either a GH10 (*Cj*Xyn10A) or GH11 (*Np*Xyn11A) xylanase removed the epitope recognized by LM23 (Fig. 6). These data indicate that the CE2 esterases deacetylate xylan in tobacco sections.

The variation in substrate specificity displayed by some glycoside hydrolases against cell walls, which does not reflect their activity against soluble substrates, described in this study and previously (15, 35), can now be extended to carbohydrate esterases. As discussed above, the capacity of only *Cj*CE2C to target acetylated mannans in cell walls, even though all the CE2 esterases analyzed can attack acetylated mannan in solution, reflects the accessibility of polysaccharides in composite structures. Inspection of the substrate binding cleft of the CE2 serine esterases shows that these enzymes contain a highly conserved aromatic residue (Tyr-206 in *Cj*CE2B) that stacks against the sugar that is deacetylated within the polysaccharide backbone (26). *Ct*CE2C, *Cj*CE2A, and *Cj*CE2B contain additional aromatic residues within the binding cleft that likely make hydrophobic interactions with the acetylated polysaccharide chain. Although no structure of *Cj*CE2C is available, sequence alignments show that the enzyme contains an aromatic residue that is equivalent to Tyr-206 in *Cj*CE2B but lacks the other aromatic residues that, in *Ct*CE2C, *Cj*CE2B, and *Cj*CE2A, line the substrate binding cleft while also displaying significant sequence variation to the other CE2 esterases in the regions that form the substrate binding cleft (26). The lack of these distal aromatic residues likely results in a much more open substrate binding cleft, possibly explaining why only *Cj*CE2C can access acetylated mannans within the *Physcomitrella* cell walls.

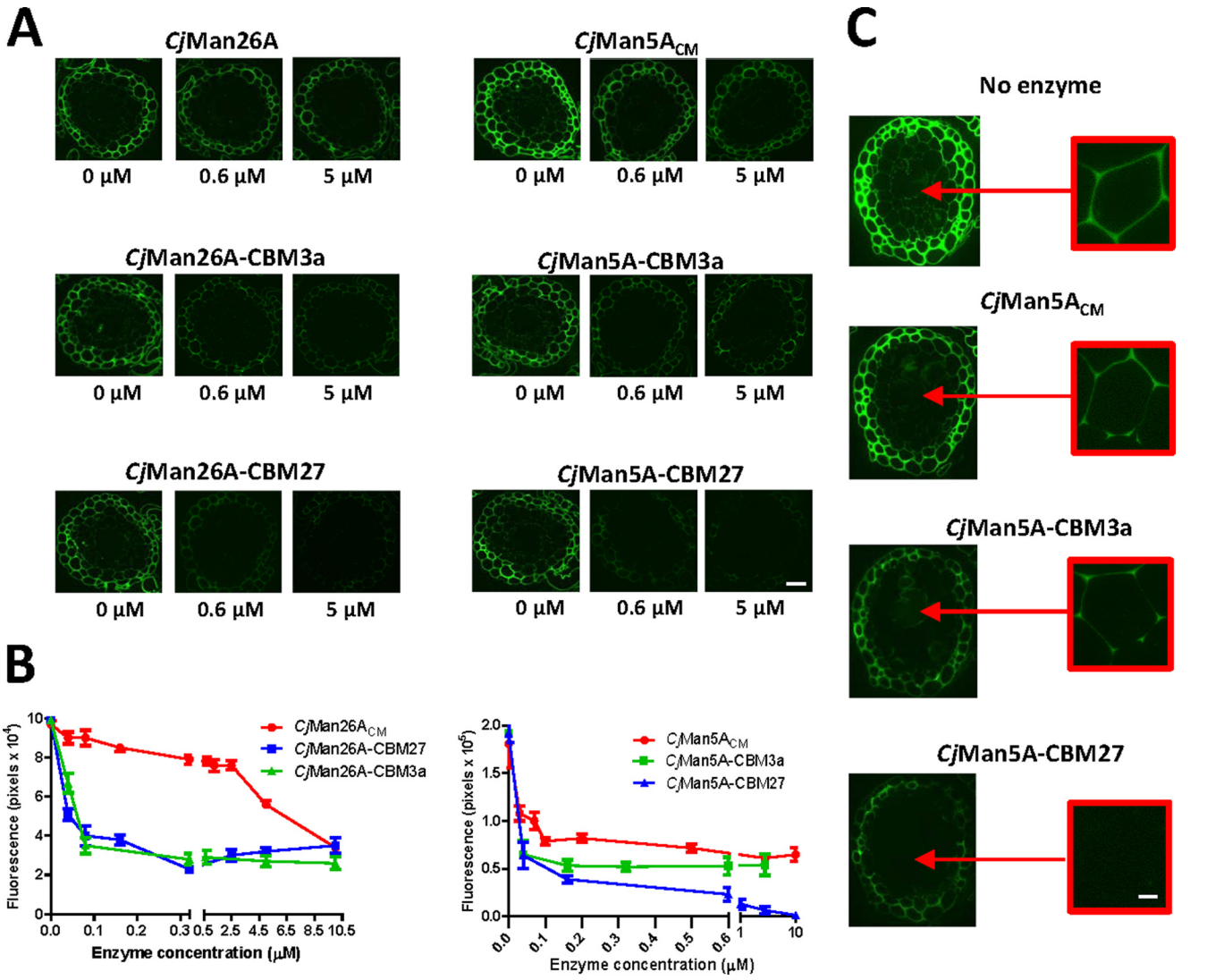


FIGURE 4. **The activity of *Cj*Man26A and *Cj*Man5A against *Physcomitrella* cell walls.** The experiments were performed as described in Fig. 3 with *panel A* showing representative immunofluorescence micrographs and *panel B* showing the quantified fluorescence intensities of the cells in the perimeter of all the sections. *Panel C* shows immunofluorescence micrographs in the *red boxes* of the representative examples of the inner cells (the pith area), magnified 5 times, of *Physcomitrella* treated with *Cj*Man5A and derivatives of the enzyme containing the various CBMs. The *scale bar* is 25 μm in *A* and 5 mm in the *red boxed images* in *C*.

The Influence of CBM on Enzyme Function

Soluble Substrates—To evaluate whether CBMs potentiate mannanases and hemicellulose esterases *in vivo*, *Cj*Man26A, *Cj*Man5A, *Cj*CE2C, and *Cj*CE2B were appended to crystalline cellulose (CBM3a (27))- and mannan (CBM35 and CBM27 (28, 29))-specific CBMs via a 15-residue Thr-Pro linker sequence (Fig. 1), typical of modular glycoside hydrolases (4, 24). The data presented in Table 1 show that the CBMs did not influence the activity of the mannanases against carob galactomannan or konjac glucomannan. Similarly, the activities of *Cj*CE2C and *Cj*CE2B against 4-nitrophenyl acetate and acetylated hemicellulosic polysaccharides (mannan and xylan) were not affected by appending the various CBMs to the two esterases. These data show that appending the CBMs to the mannanases and esterases through flexible linker sequences did not alter the conformation of the catalytic modules. The observation that the CBMs did not potentiate either esterase or mannanase activity against soluble substrates is consistent with the view that *endo* binding CBMs (used in this work) only play a role in the activity of enzymes against recalcitrant materials, where the target substrate is embedded in insoluble composite structures exemplified by plant cell walls (9, 14, 15).

CBM Impact on Mannanase against Cell Walls—With respect to mannan degradation in tobacco cell walls, the CBM3a mediated a 5-fold increase in activity of the GH5 mannanase (Fig. 7). In contrast, the mannan-specific CBMs, CBM27 and CBM35, had a less dramatic effect on the activity of *Cj*Man5A (~2-fold) against tobacco cell walls (Fig. 7). With respect to *Cj*Man26A, both CBM3a and CBM27 enabled the enzyme to hydrolyze the mannan that was inaccessible to the catalytic module alone Fig. 3. As with the GH5 mannanase, *Cj*Man26A-CBM3a was more active against tobacco cell walls than the enzyme fused to the mannan binding CBM (*Cj*Man26A-CBM27) (Fig. 3). The influence of the CBM27 and CBM3a on mannanase activity against *Physcomitrella* cell walls was essentially reversed with respect to *Cj*Man5A$_{CM}$. When the

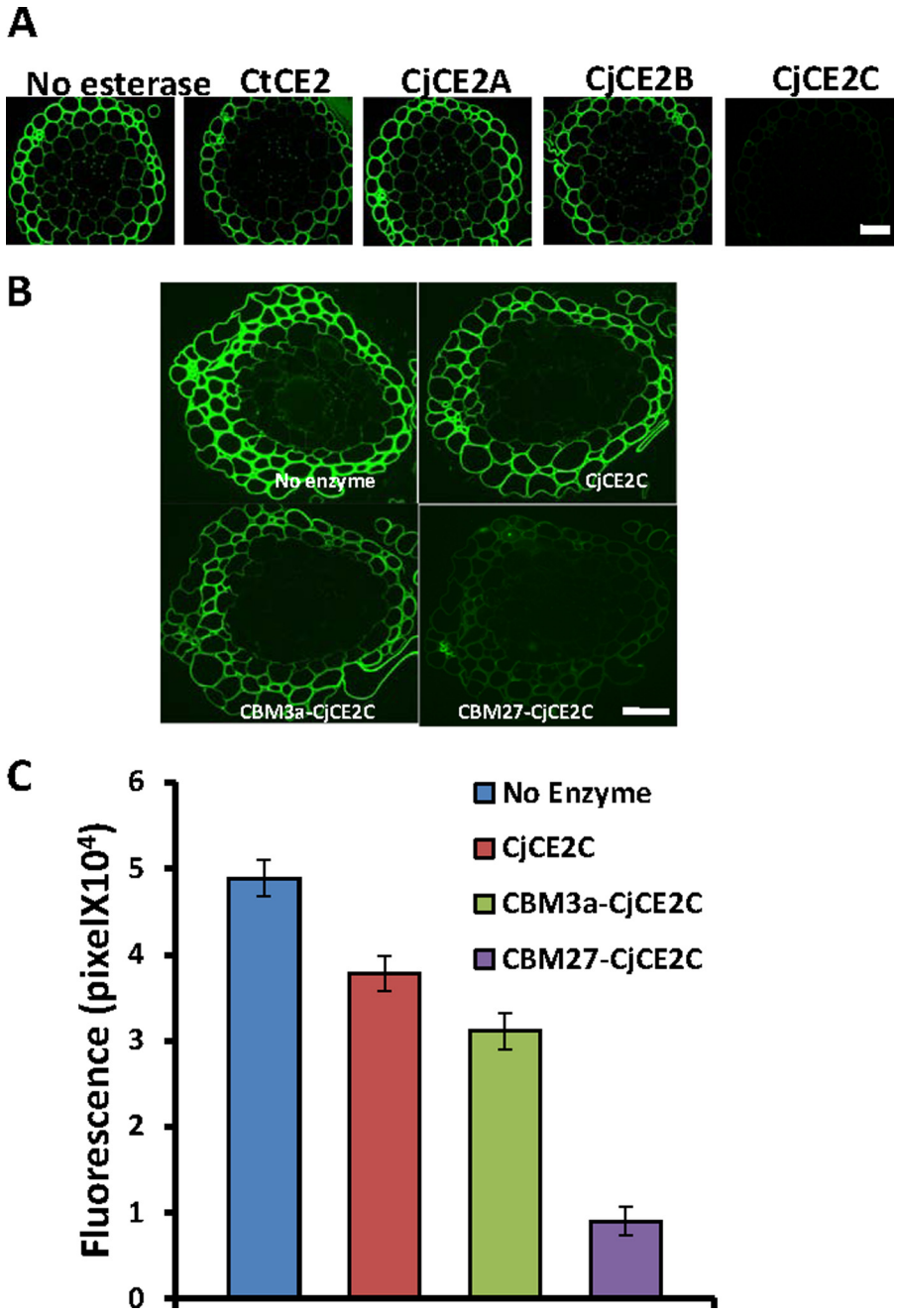


FIGURE 5. **The activity of the CE2 esterases against *Physcomitrella* cell walls.** In *panel A* the four CE2 esterases (lacking a CBM) at 6 μM were incubated with *Physcomitrella* sections for 1 h as described under "Materials and Methods." The presence of acetylated mannan was detected using the antibody CCRC-M170, which specifically binds to acetylated mannans, and immunofluorescence microscopy. In *panels B* and *C Physcomitrella* sections were treated with 0.13 μM *Cj*CE2C, *Cj*CE2C-CBM27, and *Cj*CE2C-CBM3a for 70 min. The presence of acetylated mannan was detected by immunofluorescence microscopy using CCRC-M170 antibody as the probe. *Panel B* shows the fluorescence images of the sections, and *Panel C* shows the quantified fluorescence intensities. The *scale bar* is 25 μm.

CBM27 was fused to *Cj*Man5A$_{CM}$, the enzyme was able to completely digest the mannan that was recalcitrant to degradation by just the catalytic module (Fig. 4). CBM3a mediated a small increase in the activity of the GH5 mannanase, but the cellulose-specific CBM did not enable the enzyme to remove the "recalcitrant" mannan from *Physcomitrella* cell walls (Fig. 4). It would appear, therefore, that the mannan, which is inaccessible to *Cj*Man5A$_{CM}$, is not in close contact with cellulose. When fused to CBM3a or CBM27, 100 nM GH26 mannanase was required to degrade 66% of the mannan; further removal of the mannose polymer, even when the concentration of the two derivatives of *Cj*Man26A, was increased 100-fold, was not observed (Fig. 4). In contrast, 10 μM *Cj*Man26A$_{CM}$ was required to remove 66% of the polysaccharide. It would appear, therefore, that the mannan available to the GH26 mannanase appears to be in close contact with cellulose, as both CBM27 and CBM3a mediated a similar increase in access to the polysaccharide.

Although the bulk of the mannan is in the thicker cell walls of cells at the perimeter of *Physcomitrella* gametophores (which

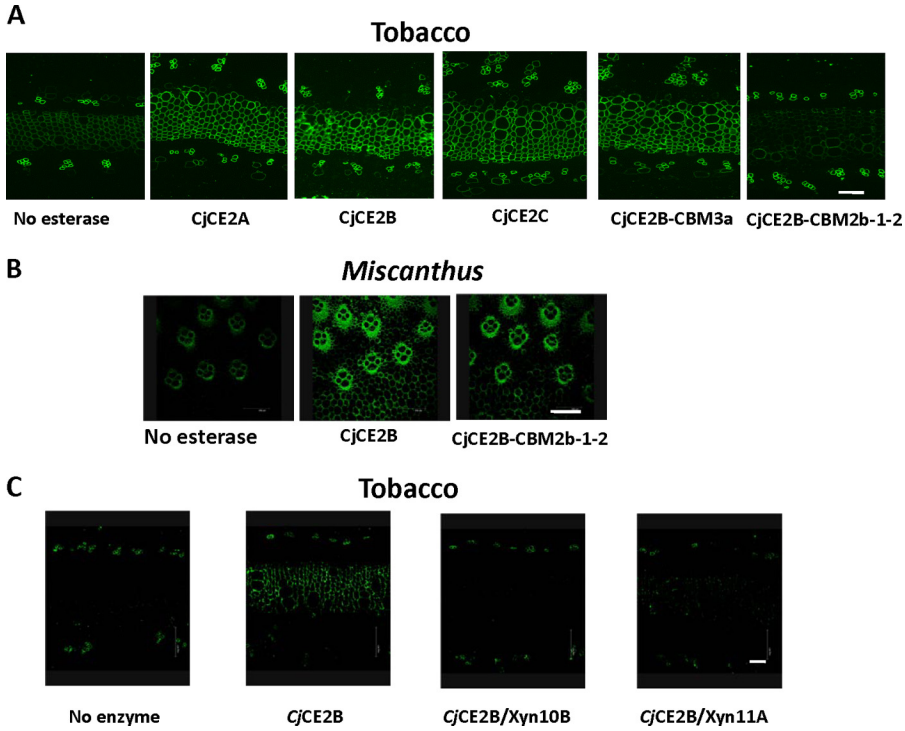


FIGURE 6. **The activity of the CE2 esterases against tobacco and *Miscanthus* cell walls.** The figure displays the immunofluorescence images of sections of tobacco (*panels A* and *C*) and *Miscanthus* (*panel B*) stems incubated with 7.6 μM CE2 esterases for 2 h and probed with LM23, which binds to undecorated xylans but not to acetylated xylan (21). In *panels A* and *B* the esterase-treated sections were probed directly with LM23, whereas in *panel C* the esterase-treated sections were incubated with the xylanases Xyn10A (*Cj*CE2B/Xyn10A) or Xyn11A (*Cj*CE2B/Xyn10B) before probing with the antibody. The *scale bar* is 25 μm in *A*, *B*, and *C*.

TABLE 1
The activity of esterases and mannanases against soluble substrates

Enzyme	Substrate	Relative activity[a]
CjMan5A	Carob galactomannan	1.00[a]
CjMan5A-CBM3a	Carob galactomannan	0.85
CjMan5A-CBM27	Carob galactomannan	0.80
CjMan5A-CBM35	Carob galactomannan	0.90
CjMan26A	Carob galactomannan	1.00[a]
CjMan26A-CBM3a	Carob galactomannan	1.00
CjMan26A-CBM27	Carob galactomannan	0.95
CjMan26A-CBM35	Carob galactomannan	0.98
CjCE2C	4-Nitrophenyl acetate	1.00[a]
CjCE2C-CBM3a	4-Nitrophenyl acetate	0.95
CjCE2C-CBM27	4-Nitrophenyl acetate	0.96
CjCE2C-CBM35	4-Nitrophenyl acetate	0.93
CjCE2B	4-Nitrophenyl acetate	1.00[a]
CjCE2B-CBM2b-1-2	4-Nitrophenyl acetate	0.96

[a] The activity of the enzymes containing CBMs is expressed relative to the constructs that comprise only the catalytic modules of the mannanases and esterases, which are thus defined as 1.0.

were used to quantify the activity of the two mannanases and the effect of the CBMs (Fig. 4)), the thinner cell walls in the central region of the sections also contain mannan, which are particularly abundant at the intercellular junctions. Mannanase treatment of the *Physcomitrella* sections degraded the mannan in these thinner cell walls; however, the mannan epitopes at the junctions was more recalcitrant to enzymatic attack (Fig. 4). Although the crystalline cellulose binding CBM3a did not enhance the capacity of the mannanases to degrade the mannan within the intercellular spaces, the CBM27 facilitated the complete removal of the polysaccharide from these regions (Fig. 4). These data have some resonance with pectin degradation in the primary cell walls of tobacco stem pith parenchyma, where the galacturonic acid polymer lining the intercellular space, particular at the corner of these structures, was recalcitrant to pectinase attack (15). The data presented here, in concert with the study of Hervé *et al.* (15), indicate that the polysaccharides present in the intercellular spaces, where cellulose is likely to be absent, can be shielded from enzymes and hence may be recalcitrant to biological attack.

CBM Impact on Esterases against Cell Walls—Appending CBM27 to *Cj*CE2C resulted in a 4-fold elevation in the activity of the esterase against *Physcomitrella* cell walls (Fig. 5). The cellulose binding CBM3a, however, did not cause a significant improvement in esterase activity against the acetylated mannan in *Physcomitrella* walls. These data are analogous with the observation that mannan-specific CBMs have a greater influence on GH5 mannanase activity against *Physcomitrella* cell walls than CBMs that bind to crystalline cellulose. The influence of CBMs on the activity of CE2 esterases against xylan in secondary cell walls of tobacco and in primary cell walls of *Miscanthus* stems was also explored. The data, presented in Fig. 6, show that CBM3a, which binds to crystalline cellulose, had no significant effect on the activity of the esterase against tobacco cell walls, as assessed using the antibody LM23 which binds to undecorated but not acetylated xylan. Surprisingly, however, CBM2b-1-2, a high affinity xylan-specific CBM (34), essentially abolished the capacity of *Cj*CE2B to deacetylate tobacco xylan and reduced the activity of *Cj*CE2B against *Miscanthus* cell wall xylan. It is possible that CBM2b-1-2 only binds to deacetylated xylan and thus directs the appended esterase to regions of xylan polymers or cell walls that lack the target substrate for the enzyme, such as, for example, the secondary cell walls in fibers surrounding the vascular bundles in *Miscanthus* stems. The

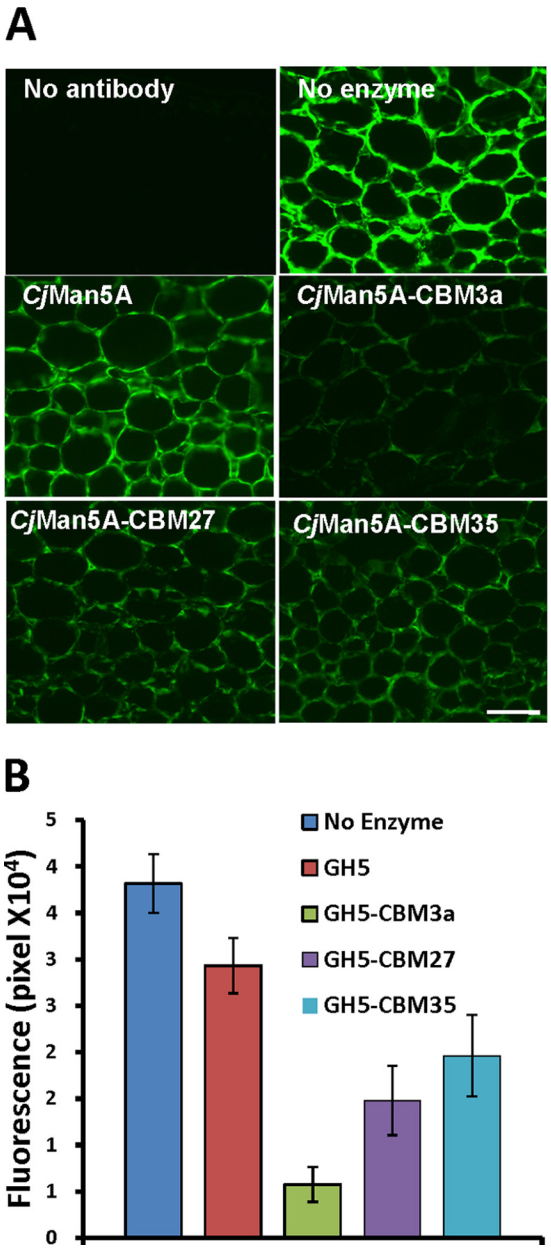


FIGURE 7. **The influence of CBMs on the activity of *Cj*Man5A against tobacco cell walls.** Sections of tobacco stems were incubated with 0.02 μM *Cj*Man5A and derivatives of the enzyme containing CBMs for 5 min. Representative fluorescence images of enzyme-treated tobacco sections labeled by the mannan-specific antibody LM21 are shown in *panel A*. Quantification of the fluorescence intensities of the equivalent images are shown in *panel B*. The *scale bar* is 50 μm.

lack of any effect by the cellulose binding CBM3a suggests that the acetylated xylan in tobacco cells is not in close contact with the cellulose microfibrils in tobacco walls.

DISCUSSION

The differential activity of GH5 and GH26 mannanases against intact cell walls provides a biological rationale for the cellular location and molecular architecture of these enzymes. *Cj*Man26A and the other GH26 enzymes expressed by *C. japonicus* are lipoproteins (37) and are thus cell-associated. The cellular location and biochemical properties of these enzymes are entirely consistent with their likely biological role; that is, the hydrolysis of mannooligosaccharides and mannan fragments released from plant cell walls (5). Indeed, other GH26 enzymes also display unusually high activity against small mannoligosaccharides and exhibit tight specificity for mannose at the −2 subsite (18, 38), suggesting that the targeting of mannan fragments released from cell walls may be a generic feature of this CAZy family. In contrast, the *C. japonicus* GH5 *endo*-mannanases contain CBMs and are secreted into the culture medium,[4] indicating that they attack cell wall mannans, consistent with the data presented in this report. The specificity of *Cj*Man5A for cell wall mannans requires that the individual subsites, distal to the active site, bind to substrate that is in close proximity with other polysaccharides. This likely imparts steric constraints, which limits the extent to which these subsites can interact with the mannose-containing polymers. The observation that *Cj*Man5A can bind both glucose and mannose at the −2 subsite suggests that the enzyme does not make interactions with O-2 (distinguishes glucose from mannose) and is consistent with the view that the mannanase makes limited contact with its substrate outside the active site. Indeed, the activity of *Cj*Man5A is ~10^5 and 10^4 less active than *Cj*Man26A against mannotriose and mannotetraose, respectively, confirming that the GH5 enzyme makes limited interactions with substrate compared with the GH26 mannanase at subsites proximal to the active site (subsites −2, +1, and +2 (18)). This feature is evident in a second GH5 *endo*-mannanase, *Ba*Man5A (18), hinting that GH5 mannanases are adapted to target cell walls and/or glucomannans.

A significant feature of this study is the observation that the crystalline cellulose binding CBM3a and the mannan-specific CBM27 have the largest impact on mannan degradation in tobacco and *Physcomitrella* cell walls, respectively. These data suggest that the context of the polysaccharides within the plant cell wall influences the function of the CBMs. In tobacco stem cell walls cellulose is the major polysaccharide and is thus the most significant receptor for CBMs. The prominent role of CBM3a in mannan degradation in tobacco also suggests that cellulose and mannan are in very close proximity within the wall, enabling the catalytic module of these enzymes, appended to cellulose through the CBM, to access their target substrates. By contrast, mannan is a much more abundant polysaccharide in *Physcomitrella* and, thus, mannanases that contain a CBM27, as opposed to enzymes with cellulose binding CBMs, will preferentially bind to these cell walls.

The observation that CBM specificity, required to achieve optimal mannanase action on cell walls, varies depending on the target configuration of cell wall polymers may provide insight into the complex molecular architecture displayed by mannanases and other hemicellulose-degrading enzymes (5, 6, 28). For example, *C. japonicus* expresses three GH5 mannanases that contain different combinations of crystalline cellulose binding CBMs from families CBM2a, CBM5, and CBM10, whereas one enzyme, *Cj*Man5C, contains both mannan- and cellulose-specific CBMs (5). It could be argued that introducing CBMs with different ligand specificities into a sin-

[4] H. J. Gilbert, unpublished data.

gle enzyme will increase its affinity for cell walls through avidity effects, which may result in a substantial increase in activity. Hervé *et al.* (15), however, showed that combining xylan and cellulose binding CBMs into a single xylanase did not enhance the activity of the enzyme against cell walls (compared with enzymes containing either a cellulose or xylan binding CBM), arguing against possible synergistic effects between the CBMs. A more likely explanation for the presence of CBMs with different specificities in some mannanases is that such a molecular architecture may enable these enzymes to target different cell walls. Thus, the presence of different combinations of cellulose binding CBMs is consistent with the observation that the context of the cellulose in different cell walls has a significant impact on the specificity of the "crystalline cellulose" binding CBMs (23). Similarly, the presence of mannan- and cellulose-specific CBMs enable *Cj*Man5C to target cell walls where cellulose or mannan is abundant.

In conclusion, this report demonstrates that both CBM specificity and the topology of the substrate binding cleft of the catalytic module dominate the substrate specificity of mannanases against plant cell walls. This report also offers the first insights into how plant cell wall architecture can have a profound influence on esterase specificity *in vivo*. It is apparent from the work described in this paper that the activity of glycoside hydrolases and esterases *in vitro* is a poor predictor of their activity on biologically significant substrates within cell walls. It is evident that the context of polysaccharides within cell walls greatly influences the specificity of degradative enzymes, providing an explanation for both their complex modular structures and the large number of isoforms of these biocatalysts expressed by single microbes.

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